

Progesterone, the maternal immune system and the onset of parturition in the mouse.

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Abstract

The role of progesterone (P4) in the regulation of the local (uterine) and systemic innate immune system, myometrial expression of connexin 43 (Cx-43) and cyclooxygenase 2 (COX-2) and the onset of parturition was examined in: 1) naïve mice delivering at term; 2) E16 mice treated with RU486 (P4-antagonist) to induce preterm parturition; and 3) in mice treated with P4 to prevent term parturition.

In naïve mice, myometrial neutrophil and monocyte numbers peaked at E18 and declined with the onset of parturition. In contrast, circulating monocytes did not change and although neutrophils were increased with pregnancy, they did not change across gestation. The myometrial mRNA and protein levels of most chemokines/cytokines, Cx-43 and COX-2 increased with, but not before, parturition.

With RU486-induced parturition, myometrial and systemic neutrophil numbers increased before and myometrial monocyte numbers increased with parturition only. Myometrial chemokine/cytokine mRNA abundance increased with parturition, but protein levels peaked earlier at between 4.5 and 9h post RU486. Cx-43, but not COX-2, mRNA expression and protein levels increased prior to the onset of parturition.

In mice treated with P4, the gestation-linked increase in myometrial monocyte, but not neutrophil, numbers was prevented and expression of Cx-43 and COX-2 was reduced. On E20 of P4 supplementation, myometrial chemokine/cytokine and leukocyte numbers, but not Cx-43 and COX-2 expression, increased.

These data show that during pregnancy P4 controls myometrial monocyte infiltration, cytokine and prolabor factor synthesis via mRNA dependent and independent mechanisms and, with prolonged P4 supplementation, P4 action is repressed resulting in increased myometrial inflammation.

Introduction

The uterus is a key site of immune activity during human pregnancy and labour [1-3]. As pregnancy advances the myometrium is invaded by increasing numbers of monocytes, dendritic cells and neutrophils. These cells, in addition to their immune roles, may modulate pregnancy outcome by influencing myometrial function [4].

Murine monocytes exist as, at least, two phenotypically and functionally distinct subsets; these are distinguished by levels of the cell surface marker Ly-6C, which is higher on monocytes in the bone marrow and immediately after they have migrated into the circulation. Sunderkötter et al. showed that the Ly-6C^{HI} bone marrow derived blood monocyte matures over the course of 3 days, with Ly-6C expression declining until the mature monocyte becomes part of the Ly-6C^{LO} monocyte subset [5] [6, 7]. The Ly-6C^{HI} monocyte subset is preferentially recruited to sites of active inflammation [5, 8, 9] by the production of chemokine ligand 2 (CCL2), which is also known as monocyte chemoattractant protein 1, and which stimulates Ly-6C^{HI} monocyte egress from the bone marrow and extravasation into tissue [8]. Tagliani et al. demonstrated that the uterus actively recruits the Ly-6C^{HI} monocyte subset rather than the Ly-6C^{LO} subset during gestational development [10]. Nevertheless, the timing and pattern of recruitment of monocyte subsets into the uterus in late gestation and prior to labour has not yet been investigated. The uterine macrophage population has been reported to increase in

density during pregnancy until labour [11-13], suggesting that macrophages may play a key role in parturition [2]. Recent evidence indicates that blood monocyte trafficking to the uterus is central to expansion of the macrophage population during pregnancy [13]. CCL2 production may be influenced by progesterone (P4) and uterine stretch [14-16]; since P4 withdrawal initiates a rise in CCL2 expression while continued P4 administration prevented the increase in CCL2 expression in the gravid horn of unilateral pregnant rats [16]. A role for colony-stimulating factor 1 (CSF-1, is also known as macrophage-CSF), which enhances cell differentiation into macrophages, has also been suggested as in ovariectomised mice, low levels of CSF-1 correlated with low uterine macrophage numbers suggesting that sex steroids tightly regulate CSF-1 production, indirectly controlling uterine macrophage density [17].

The regulation and function of the increase in the myometrial macrophage, monocyte and neutrophil numbers around the time of labour is still unclear and the subject of on-going research. The studies by Norman et al, in the human, highlighted the association between labour and inflammation, demonstrating an intense myometrial leukocyte infiltration in association with increased cytokine expression [1-3]. Further, inflammation has been found to play a central role in virtually all myometrial gene array studies of labouring myometrium [18-24]. Inflammatory cells can exert a direct or indirect effect on myometrial contractility, the former by enhancing ROCK activity and the latter via increased cytokine and prostaglandin synthesis [25, 26]. Importantly, P4 can reverse the effect of monocytes on myometrial contractility [26], consistent with a mutually antagonist relationship between P4 and inflammation, which forms the basis of several theories for the onset of human labour [27-29].

Many studies have considered the uterus in isolation, not considering that immune changes in the uterus may reflect changes in the systemic immune system. Further, while P4 has been shown to regulate uterine monocyte trafficking, it could exert effects on the systemic immune system,

potentially controlling both the local (myometrial) and systemic immune system. In this study, we initially investigated the behaviour of the innate immune system in CD-1 mouse pregnancy, studying the changes in myometrial immune cell trafficking in the context of the changes in other reproductive tissues (placenta and decidua) and the systemic immune system (blood, lung and liver); we then compared these to local and circulating cytokine levels and related them all to the changes in myometrial prolabour factor levels. The role of P4 in regulating these local and systemic responses was then evaluated by administration of RU486 to produce acute P4 withdrawal and P4 supplementation to reverse late gestation withdrawal.

Materials and Methods

Animal study ethical statement and husbandry conditions

CD1 outbred mice were purchased from Charles River (Margate, UK) at 6-8 weeks of age and subsequently housed in open cages at $21 \pm 1^\circ\text{C}$, on a 12:12 light/dark cycle, and given Harlan Teklad 2018 Global Diet rodent food and water *ad libitum*. Female mice were mated overnight with males and the day a copulatory plug was observed was designated embryonic day 0 (E0). Stud males were singly housed, whilst females were group housed with up to 5 others until E18 at which time they were singly housed to litter down.

The health status of all animals was monitored daily and all mice due to enter a treatment arm were inspected to assure good health prior to initiation of the protocol. The daily subcutaneous injection sites were varied to minimise discomfort to the animals and monitored for any abnormal response. Animals were monitored by infra-red CCTV (RF Concepts, Ireland) from E18 (or E16 in the RU86 treatment arm) and physical inspection in the lead up to labour, in case of labouring complications.

All experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986, under Home Office Licenses 70/6909 & 70/7518, with approval from the Animal Welfare & Ethical

Review Boards of Imperial College and University College London. The 3Rs and ARRIVE guidelines were consulted and implemented in completion of this study and in the production of this manuscript.

Leukocyte trafficking dynamics during late gestation and labour

The leukocyte populations and subsets were analysed in late gestation and labour in the uterus and systemically to gain a global understanding of the response to pregnancy and labour. The lungs, liver, blood and uterus of CD-1 mice were collected from naïve virgin non-pregnant mice (n=3 mice) and from time-mated mice. In the pregnant mice (E16, E17 and E18 and after the spontaneous delivery of at least one pup for the labouring samples, n=6 dams per group), myometrium, decidua and placentas were also collected (gating strategies described previously [30]). Postnatal samples were obtained within 24 hours post delivery.

Progesterone and RU486 treatment

Animals were selected for each treatment arm randomly, with control and P4 treated animals injected alongside each other to avoid bias i.e. E16 control group treated alongside E16 P4 group (although housed in different cages). Injections and tissue collections were performed at the same time each day and the animals returned to their home cage unless they were due to labour that day, at which point they were singly housed with suitable nest boxes and nesting materials. Due to pregnancy rates and to again avoid collection bias, multiple RU486 time point collections were performed on the same day, whilst labouring samples were collected over various days. We mated enough animals to ensure that we had a minimum of 6 animals at each time point. This sample size was chosen based on previous *in vivo* work [31], the requirements for statistical normality testing and on initial pilot study results. When the mating proved to be more successful than expected, we used the excess animals to increase numbers at different time points. For tissue collection in all treatment groups, animals were briefly anaesthetised using 5% isoflurane (in 1.5L/min oxygen) and a

cardiac puncture performed using a heparinised syringe, followed by immediate cervical dislocation. The excised tissues immediately processed for flow cytometry or snap frozen on dry ice prior for later molecular analysis.

Control Group: Mice were administered daily subcutaneous peanut oil (P4 vehicle control) injections from gestational day 14 (E14) until gestational day 18 (E18) and an additional subcutaneous injection of DMSO (RU486 vehicle control) on gestational day 16 (E16).

For tissue collection, animals were briefly anaesthetised using 5% isoflurane (in 1.5L/min oxygen) and a cardiac puncture performed, followed by immediate cervical dislocation on gestational days 16 (n=6 dams), 17 (n=8 dams), 18 (n=6 dams) and in labour (n=6 dams), which was identified following delivery of at least the first pup. Tissues were immediately processed for flow cytometry or snap frozen on dry ice prior to molecular analysis.

RU486 Model of Preterm Labour: Mice were administered daily subcutaneous peanut oil (P4 vehicle control) injections from E14 until E16 and a subcutaneous injection of 150µg of RU486 dissolved in 20µL DMSO on E16 at 10am. Tissues were collected at 4.5h (n=8 dams), 9h (n=8 dams), 13.5h (n=6 dams) post-RU486 administration and upon delivery of at least the first pup (labour) which took place approximately 18h post-RU486 administration (n=7 dams). Tissues were immediately processed for flow cytometry or snap frozen on dry ice prior to molecular analysis.

P4 Model of Prolonged Gestation: Mice were administered a daily subcutaneous injection of 2mg P4 dissolved in 40µL peanut oil from E14 until gestational day 20 (E20) and an additional subcutaneous injection of DMSO (RU486 vehicle control) on gestational day 16 (E16). Tissues were collected on days 16 (n=7 dams), 17 (n=8 dams), 18 (n=7 dams), 19 (n=9 dams) and 20 (n=7 dams). They were immediately processed for flow cytometry or snap frozen on dry ice prior to molecular analysis. Circulating P4 levels were maintained on this regimen until E20 (supplementary figure 1B).

Leukocytes were differentiated between vascular and tissue populations by staining with CD45. In the myometrium, the greater majority of leukocytes were within the tissue, conversely, in the lung and liver, the majority were shown to be a marginalised intravascular (supplementary figure 2).

To label dividing cells *in vivo*, 100µl 10mg/ml BrdU in sterile 1x DPBS was administered by intraperitoneal injection (BD Pharmingen, New Jersey, USA) 6 hours before a subcutaneous injection of 150µg of RU486 (n=3 mice) or DMSO vehicle control (n=3 mice). Blood and myometrial samples were collected at labour or 24 hours post BrdU injection and processed according to the manufacturer's instructions (as described previously [30]).

CCR2 antagonist administration: Pregnant mice at E16 were given 10mg of the selective CCR2 antagonist RS504393 (n=6 dams) (Abcam, Cambridge, UK) by oral gavage 8 hours after subcutaneous injection of 150µg RU486. RS504393 was dissolved in DMSO at a concentration of 4mg/ml, and diluted 1:20 with PBS so that DMSO concentration was reduced to 5% thereby obtaining a final concentration of 2mg/kg for *in vivo* administration. 5% DMSO in PBS was used as the vehicle control (n=6 dams). This dose was selected based on CCR2 blockade experiments by Furuichi *et al.* and Kitagawa *et al.* [32, 33]. Blood, lung and myometrial samples were collected at labour or 12 hours after RS504393 oral gavage.

Cell preparation and flow cytometry

Blood was immediately mixed with 5mM EDTA and stored at 4°C until analysis within 12h. The lungs, sections of liver, myometrium, decidua and placentas were weighed and then immediately homogenised using a gentleMACS M tube (Miltenyi Biotec Ltd, Surrey, UK) containing 1ml of IC fixation buffer (eBioscience, Hatfield, UK) for 1min, diluted with 5ml of PBS, 2% FBS, sodium azide 0.1% 2mM EDTA and washed twice by centrifugation (400xg for 5min) prior storage on ice. The homogenate was then strained through a 40µm nylon cell strainer (BD Falcon, Oxford, UK) and the cell suspension was then centrifuged at 400xg for 5min. Cells were then resuspended in permeabilizing medium PBS, 0.1% sodium azide, 0.5% BSA and 0.2% saponin and then incubated

with antibodies at 4°C for 30min. The following fluorophore-conjugated rat anti-mouse MAbs antibodies were used: Ly6C (HK1.4), Gr1 (RB6-8C5), NK1.1 (PK136), F4/80 (Cl:A3-1), CD45 (30-F11) (BioLegend Cambridge UK) COX-2 (M-19) (Santa Cruz Biotechnology, Santa Cruz, CA) CD86 (B7-2), CD11b (M1/70), MHCII (AMS-32.1), F4/80 (BM8) (eBioscience). After staining, lysis of red blood cells was performed using FACS lysing solution (BD). Cell counts were determined using AccuCheck counting beads (Invitrogen, Paisley UK). Samples were run using CyAn ADP Beckman Coulter flow cytometer and Cell Quest 3.3 software. Data were analysed using FlowJo version 7.6.5.

Multiplex cytokine assays

Tissue lysates were prepared from snap frozen myometrium and lung samples in Bio-Plex® Cell Lysis buffer (BioRad, Hertfordshire UK) and a Precellys®24 (Stretton Scientific Ltd, UK) homogeniser following the manufacturer's instructions. Total protein concentrations were quantified using a DC™ Protein Assay (BioRad, Hertfordshire UK). Whole blood was mixed with 5mM EDTA immediately and centrifuged at 1,000g for 15 minutes and aliquots of plasma were stored at -70°C until assayed. 25µl of plasma and 250µg of total protein from tissue was added to a commercially available Bio-Plex Pro™ Mouse Cytokine 23-plex (normal gestation and parturition samples – lung, myometrium and serum) or a custom-made Bio-Plex Pro™ Mouse Cytokine assay (P4 and RU486 study – myometrium) (analytes measured include: IL-1α, IL-1β, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, G-CSF, IFN-γ, KC/CXCL1, MCP-1/CCL2, RANTES/CCL5, TNF-α and MIP-2/CCL20) and performed according to the manufacturer's instructions.

Quantitative RT-PCR analysis

Total RNA was extracted from tissue using an RNeasy kit and Qias shredder (Qiagen Ltd, UK). The concentration of RNA in each sample was measured by NanoDrop ND1000 spectrophotometer (ThermoFisher Scientific, UK). 1.0µg RNA was converted to cDNA using the MuLV reverse transcriptase system (Applied Biosystems Ltd., Warrington, UK), according to manufacturer's

protocols. Primer sets were designed using Primer3 software (NCBI) and obtained from Invitrogen (Supplementary Table 1). Quantitative PCR was performed in the presence of SYBR Green (Roche Diagnostics Ltd, UK), and amplicon yield was monitored by Rotor Gene R-G 3000 (Corbett Research, Australia).

Statistical analysis

All data were tested for normality using a Kolmogorov-Smirnoff test. Normally distributed data were analysed using a Student t test for 2 groups and an analysis of variance (ANOVA) followed by a Bonferroni *post hoc* test for 3 groups or more. Data that were not normally distributed were analysed using a Mann Whitney test for unpaired data and when comparing 3 groups or more a Kruskal-Wallis test, with a Dunn's Multiple Comparisons *post hoc* test. $p < 0.05$ was considered statistically significant.

Results

Initially, in naïve mice, we determined the changes in the local and systemic innate immune system, myometrial cytokine levels and prolabour gene expression in late mouse pregnancy. Then, we investigated the role of P4 in these changes by (i) provoking P4-withdrawal by administering RU486 (a P4 antagonist) on E16 and (ii) preventing the usual systemic withdrawal of P4 by giving 2mg of P4 daily from E14.

Naïve pregnant CD-1 mouse: Immune cell trafficking in normal pregnancy

To evaluate innate cell trafficking by flow cytometry, we focused on neutrophils, Ly-6C^{HI} monocytes and macrophages as the principle myeloid subpopulations implicated in previous studies. Data for Ly6c^{LO} monocytes were obtained, but only for blood and lungs, where these populations can be identified clearly and distinguished from resident macrophages [34]. The non-pregnant mice were

sacrificed at different stages of the estrous cycle and this may account for the variation in the cell numbers.

(i) *Myometrium*: Myometrial neutrophil density peaked on E18 (vs. NP, E16 and E17 $p < 0.0001$, Fig.1A), but decreased with the onset of labour (vs. E18, $p < 0.05$ Fig.1A). Similarly, Ly-6C^{HI} monocyte subset cell density increased (E18 vs. E16, $p < 0.05$ and E18 vs. NP, $p < 0.001$, Fig.1B) and decreased with labour ($p < 0.01$, Fig.1B). (ii) *Decidua*: Cell numbers did not change significantly in the decidua (supplementary Figure 3A&B). (iii) *Placenta*: Cell numbers did not change significantly in the placenta (supplementary Figure 3C&D). (iv) *Blood*: Neutrophils increased (NP vs. E16, $p < 0.05$, NP vs. E17 & E18, $p < 0.01$, Fig.1C) and tended to decline with the onset of labour (Fig.1C). The Ly-6C^{HI} monocyte population did not change significantly (Fig.1D). (v) *Lungs*: Neutrophil numbers peaked on E17 (vs. NP, $p < 0.05$, supplementary Figure 3E) and remained stable thereafter. The Ly-6C^{HI} monocyte population peaked on day 18 ($p < 0.05$) and tended to decline with the onset of labour (supplementary Figure 3F). (vi) *Liver*: Neutrophils peaked on E17 (vs. E16, $p < 0.05$) and remained stable thereafter (supplementary Figure 3G). The Ly-6C^{HI} monocyte population remained unchanged (supplementary Figure 3H). The Ly-6C^{LO} monocyte populations in blood and lungs remained unchanged and were not studied further (supplementary figure 4A&B).

Myometrial macrophage numbers were greater during pregnancy (E16, E17, E18 vs. NP, $p < 0.05$, 0.01 and 0.001 respectively, supplementary figure 5A), but declined with the onset of labour ($p < 0.05$, supplementary figure 5A). Placental and decidual macrophage numbers did not change through pregnancy (supplementary figure 5B&C). And the markers of macrophage activation remained stable (COX-2) or declined (CD-86) with advancing gestation in myometrium and decidua (supplementary figure 6A-D). The reduction in myometrial macrophage numbers was not related to increased rates of apoptosis (supplementary figure 7A). In post-natal studies, both neutrophils and Ly-6C^{HI} monocyte populations increased in the myometrium and decidua (all $p < 0.05$, supplementary figure 8A-D). In

contrast, macrophage numbers declined further in the myometrium ($p < 0.01$, supplementary figure 9A) and tended to decline in the decidua too (supplementary figure 9B).

Subsequent studies focussed on the trafficking of neutrophil and Ly-6C^{Hi} monocytes in the myometrium, placenta, blood and lungs only.

Naïve pregnant mouse: Myometrial and systemic cytokine levels

We investigated whether the leukocyte migration into the myometrium was due to a chemokine gradient and associated with increased inflammatory cytokine levels by measuring chemokine and cytokine mRNA and protein levels in the myometrium. *IL-1 β* , *IL-6*, *CCL2* and *CXCL2* mRNA expression increased in labouring myometrium ($p < 0.05$ - 0.01 Fig. 2A-D). Myometrial CCL2 protein levels ($p = 0.06$, E16 vs. E18), CXCL1 and IL-6 tended to increase with gestation, but were not significantly increased until the onset of labour (Fig. 2E-H). Interestingly, the mRNA and protein levels of IL-1 α and, to a lesser extent, IFN γ declined from day 17 to labour (supplementary figure 10A&E and C&G), in parallel with circulating P4 levels (supplementary figure 1A). CCL5 mRNA increased with labour but protein levels remained unchanged, GM-CSF levels of both mRNA and protein remained unchanged (supplementary figure 10B&F and D&H).

Circulating levels of IL-1 β , IL-6 and TNF α remained unchanged with advancing gestation and parturition (supplementary figure 11B-D). IL-1 α levels were higher on day 17 than 16 and during parturition than on day 18 (both $p < 0.05$, supplementary figure 11A). CCL2 levels increased significantly with the onset of labour compared to E18 ($p < 0.05$, supplementary figure 11E). Levels of CXCL1 were greater on day 17 of pregnancy compared to levels on days 16 and 18 (both $p < 0.05$, supplementary figure 11G). Interestingly, G-CSF (essential for neutrophil mobilisation) levels behaved similarly (day 17 vs. day 16, $p < 0.01$ and vs. day 18 < 0.001 , supplementary figure 11J).

Since the changes in circulating cytokines were slight, subsequent studies focussed on the myometrial cytokine levels.

Naïve pregnant mouse: Myometrial pro-labour factor levels

The expression of *Cx-43* and *COX-2* mRNA peaked in labour ($p < 0.05$ - 0.01 vs. days 16-18 and $p < 0.05$ - 0.001 vs. days 16-18 respectively, Fig.3A&B). Protein levels of *COX-2* and *Cx-43* also peaked in labour ($p < 0.05$ vs. day 18 and $p < 0.001$ vs. day 18 respectively, Fig.3C&D).

Naïve pregnant mouse: systemic P4 levels

P4 levels in the serum were measured throughout late gestation and in non-pregnant animals (supplementary figure 1A). Circulating P4 levels decline from gestation day 14 to labour, which correlates with an increase in infiltrating leukocytes to the myometrium. To investigate the relationship between P4 and leukocyte dynamics further, we studied the impact of RU486 (a P4 and glucocorticoid receptor antagonist) administration and P4 supplementation (supplementary figure 1B) on leukocyte trafficking, myometrial cytokine and prolabour levels.

The role of P4: RU486

Administration of 150 μ g of RU486 on E16 resulted in preterm labour within 17.3 ± 1.0 h (mean \pm SEM, supplementary figure 12). Samples were obtained before treatment, at 3 intermediate time points (4.5, 9 and 13.5h post RU486) and in labour.

RU486: Immune cell trafficking

(i) *Myometrial* neutrophil cell density increased with time after RU486 administration, and was significantly raised at 9h and during parturition vs. E16 values ($p < 0.05$ and $p < 0.001$ respectively, Fig.

4A). Myometrial Ly-6C^{HI} monocyte density increased with the onset of parturition ($p < 0.001$ vs. all time points, Fig. 4B). (ii) Placental Ly-6C^{HI} monocyte density declined with time (E16 vs. 4.5, 9 and 13.5 hours, all $p < 0.01$, supplementary figure 13B), although levels increased again in labour (vs. 9h, $p < 0.05$, supplementary figure 13B); there was no effect on neutrophil numbers. (iii) Blood neutrophil numbers peaked at 9h (vs. E16, $p < 0.05$, Fig. 4C); Ly-6C^{HI} monocytes increased to a peak at 9h then declined before rising again in parturition (both $p < 0.05$, Fig. 4D). (iv) Lung neutrophil (labour vs. E16 and vs. 13.5h, $p < 0.01$ and 0.05 respectively, supplementary figure 13C) and Ly-6C^{HI} monocyte (labour vs. E16, $p < 0.001$, supplementary figure 13D) numbers peaked with parturition. (v) Macrophage numbers increased in the myometrium, but not placenta, with parturition ($p < 0.05$ vs. E16 and 9 hours, supplementary figure 14A&B).

RU486: Myometrial cytokine levels

Cytokine mRNA expression only increased with parturition (*IL-1 β* , *IL-6*, *CCL2* and *CXCL1*, Fig. 5A-D); however, protein levels of IL-6, CCL2 and CXCL1 all rose earlier, peaking at 9h ($p < 0.05$ - 0.01 vs. E16, Fig. 5F-H). A similar phenomenon was observed for IL-1 α and TNF α (supplementary figure 15A&B, E&F). CXCL2 mRNA levels did not change, but protein levels peaked at 9h post RU486 (supplementary figure 15D&H). The mRNA levels of IL-4, IL-10 and IFN all remained stable, while their protein levels peaked at 4.5h and declined thereafter (supplementary figure 16A-C and E-G). Like IL-1 β (Fig. 5A&E), CCL5 mRNA levels peaked in labour, but protein levels remained unchanged (supplementary figure 16C&G).

RU486: Myometrial pro-labour factor levels

The expression of *Cx-43* mRNA peaked in labour ($p < 0.01$ vs. E16 and E17 and $p < 0.05$ vs. 4.5h, Fig. 6A) and of *COX-2* mRNA tended to increase to labour; protein levels of Cx-43 peaked at 13.5h ($p < 0.0001$

vs. day E16, Fig.6D) and of COX-2 declined to a nadir in labour ($p<0.05$ vs. E16, Fig.6E). OTR mRNA peaked at 9 hours (supplementary figure 17A), despite multiple attempts, the western analysis for OTR was inconsistent (data not shown).

RU486: Myometrial MAPK and NF κ B

The phosphorylation of p38 peaked at 9h (<0.05 vs. E16 and labour, Fig.7A) and was higher at 4.5h than in labour ($p<0.05$, Fig.7B). JNK phosphorylation was greater at 4.5h vs. labour ($p<0.01$) and at 9h vs. labour ($p<0.05$, Fig.7B). The phosphorylation of c-jun peaked at 4.5h ($p<0.05$ vs. E16, Fig.7C). Both ERK1/2 and p65 phosphorylation tended to increase, peaking at 9h (supplementary figure 18A&B).

RU486: CCR2 antagonist

The delivery time after PTL induced by RU486 in vehicle controls and mice given the CCR2 antagonist RS504393 was similar (supplementary figure 19). Although circulating Ly-6C^{HI} monocytes ($p<0.05$, supplementary figure 20F) and myometrial neutrophils ($p<0.05$, supplementary figure 20A), but not Ly-6C^{HI} monocytes ($p=0.062$, supplementary figure 20B), were significantly reduced.

The role of P4: P4 supplementation

Mice were given a daily subcutaneous injection of 2mg P4 dissolved in 40 μ L peanut oil from E14 until E20 when the animals were culled. On E20, the pups were dead and appeared to be wedged into the pelvis. Pair-wise comparisons were made with vehicle treated mice to E19; samples from P4 supplemented mice on E18, E19 and E20 were then compared longitudinally.

Initial studies were carried out in non-pregnant CD-1 mice given 2mg of P4 in 40µl peanut oil via a subcutaneous injection daily for 5 days. Only the cell density (cells/g) of myometrial neutrophils declined with P4 treatment ($p < 0.05$, supplementary figure 21A).

P4 supplementation: Immune cell trafficking

(i) Myometrium: P4 supplementation had no effect on neutrophil numbers (they were actually higher on E19 in the P4 treated group $p < 0.01$, Fig.8A), but did reduce the prelabour infiltration of Ly-6C^{HI} monocytes (E17 $p < 0.01$ and E18 $p < 0.001$, Fig.8B). From day 18 in P4-supplemented mice, neutrophils (E18 vs. E19, $p < 0.05$, Fig.8C) and monocytes (E18 vs. E19 and E20, $p < 0.01$, Fig.8D) increased on E19 and remained high on E20. *(ii) Placenta:* On E19, neutrophils ($p < 0.001$, supplementary figure 22A) and Ly-6C^{HI} monocytes ($p < 0.0001$, supplementary figure 22B) were lower in the P4-supplemented group. Neutrophils (E20 vs. E18, $p < 0.0001$ and E20 vs. E19, $p < 0.001$ supplementary figure 22C) and monocytes (E20 vs. E19 and E18, both $p < 0.0001$, supplementary figure 22D) were higher on E20 in the P4 supplemented mice. *(iii) Peripheral blood* neutrophils ($p < 0.01$, Fig.8E) and Ly-6C^{HI} monocytes ($p < 0.01$, Fig.8F) were lower in P4 supplemented pregnant mice on E19. On E20 in P4-supplemented mice, neutrophils (E20 vs. E18, $p < 0.01$ and E20 vs. E19, $p < 0.001$ Fig.8G) and monocytes (E20 vs. E18, $p < 0.05$ and E20 vs. E19 $p < 0.001$, Fig.8H) were increased. *(iv) Lungs:* On E19, Ly-6C^{HI} monocytes were lower in the P4-supplemented animals ($p < 0.01$, supplementary figure 22F). Again, on E20 in P4-supplemented mice, neutrophils (E20 vs. E18, $p < 0.05$, supplementary figure 22G) and monocytes (E20 vs. E19 and E18, $p < 0.01$ and $p < 0.001$ respectively, supplementary figure 22H) were higher. *(v) Macrophages:* Myometrial ($p < 0.05$) and placental ($p < 0.01$) macrophages were lower in P4 treated mice on E19, (supplementary figure 23 A&B). However, in contrast to other cell types, macrophage numbers did not rise from E18, but actually declined in the myometrium ($p < 0.05$, supplementary figure 23C) and remained static in the placenta (supplementary figure 23D).

P4 supplementation: Myometrial cytokine levels

Cytokine mRNA expression was greater with parturition in vehicle treated mice compared to E19 P4 treated mice (*IL-6*, $p < 0.05$, Fig.9B; *IL-1 α* and *TNF α* , $p < 0.05$ and $p < 0.01$ respectively, supplementary figure 24A&B and IL-10 supplementary figure 25C). However, protein levels were unchanged in all cases (Fig.9E-H and supplementary figures 24E-H and 25D-F) except for CCL2, where they were greater in vehicle treated mice on E18 ($p < 0.05$, Fig.9G).

With prolonged P4 supplementation (E18-E20), although mRNA levels for inflammatory cytokines and chemokines remained stable, the respective protein levels rose in all cases except CCL5 and GCSF (supplementary figures 26 and 37).

P4 supplementation: Myometrial pro-labour factor levels

Surprisingly, on E17, *Cx-43* and *OTR* mRNA expression was greater in P4-supplemented mice ($p < 0.05$ and $p < 0.001$ respectively, Fig.11A and supplementary figures 17B), on E18 mRNA expression was similar and on E19, the expression of *Cx-43*, *COX-2* and *OTR* mRNA was greater in labouring vehicle treated mice (all $p < 0.01$ Fig.11A&B and supplementary figures 17B). *Cx-43* and *COX-2* mRNA expression did not change with prolonged P4 treatment (Fig.11C&D). In all cases, there were no differences in the protein levels (Fig.11E-H).

P4 supplementation: Myometrial MAPK and NF κ B

P4 treatment had no consistent effect of MAPK/NF κ B activation. The phosphorylation of p38 was greater in vehicle treated mice on E17 only ($p < 0.05$, Fig.12B), JNK in P4-supplemented mice on E19

($p < 0.05$, Fig.12C) and c-jun on E16 in vehicle treated mice (Fig.12H). There were no differences in ERK1/2 and p65 phosphorylation (supplementary figure 28).

The role of P4: myometrial levels of inflammatory cytokines in labour

Myometrial cytokine levels were compared in mice after a spontaneous and RU486 induced onset of parturition and on day 19 of P4 treatment. Interestingly, only CCL2 levels were greater in the RU486-induced labour than in the spontaneous labour ($p < 0.05$, supplementary figure 29E).

The results of the studies are summarised in Table 1.

Discussion

This study shows that during pregnancy (i) there is a P4-regulated monocyte infiltration into the myometrium, (ii) P4 regulates cytokine and prolabor factor synthesis in the myometrium via mRNA dependent and independent mechanisms and, (iii) with prolonged P4 supplementation, P4 repression of prolabor gene expression was maintained, but its ability to control the local and systemic innate immune system was lost, consistent with the development of a limited functional withdrawal of P4 action in mouse parturition.

In terms of immune function, we found increased immune cell infiltration of the myometrium but not in the decidua or placenta prior to parturition. However, despite the marked increase in leukocyte extravasation, there was no co-incident increase in inflammatory cytokines, either at the mRNA or protein level, raising the possibility that inflammation occurs only after the onset of labour and questioning the role of leukocytes and inflammatory cytokines in the onset of labour. Indeed, in RU486-induced parturition, the CCL-2-mediated increase in myometrial leukocyte infiltration was reduced by treatment with a CCR2 antagonist, but the interval to parturition was not affected.

These data are important as they show that P4 prolongs gestation through the repression of myometrial pro-labour and inflammatory gene expression. The former seems to be mediated through repression of mRNA expression, but the latter seems to be mediated through both an mRNA dependent and independent mechanism, perhaps involving MAPK-regulation of TTP as we have recently reported to occur in human labour (Lei et al, 2017 unpublished observation). Finally, they show that a functional withdrawal of P4-action does occur, albeit limited to myometrial cytokine levels, but importantly, this occurs without any change in mRNA expression, hinting that our failure to identify a consistent mRNA signature of parturition in the human may be because the important changes occur at the protein level, perhaps induced through a change in kinase activity. Equally, the myometrium is not a homogeneous tissue, it contains many different cell types and P4 dependent effects will be modulated by changes in myometrial cell content and their relative P4-responsiveness. Similarly, the mismatch between mRNA and protein may be due to the heterogeneity of the tissue or possibly differences in protein and mRNA assay sensitivity.

Naïve mouse studies:

Myometrium-leukocyte changes: We found that leukocytes increased in the myometrium only, intriguingly peaking on E18 and declining with parturition, only to increase again post delivery. These changes occurred in the absence of changes in the leukocyte numbers in the decidua or placenta, which implies the existence of a specific myometrial immune regulation. Our monocyte and neutrophil data are similar to those of Shynlova et al, but differ in that the postnatal increase was less marked, perhaps because our samples were obtained up to 24 hours post delivery, rather than by 6 hours [13].

We found no change in macrophage (CD11b+, F4/80high cells) densities in any reproductive tissues or in their activation markers in contrast to the myometrial data of Shynlova et al, who found that myometrial macrophage numbers were higher on E18 compared to E15 [13] and the decidual data from Hamilton et al, who found that in the rat, macrophage numbers increased immediately before

parturition [11]. Both Shynlova et al and we found that macrophage numbers declined in the myometrium post delivery, at a time when macrophages might have been expected to be playing an active role in tissue repair/remodelling.

The increase in myometrial leukocyte density was associated with a trend for an increase in the levels of CXCL1, CCL2 and CCL5 between E17 and E18, but with no change in gene expression. Shynlova et al, also related myometrial cytokine levels to leukocyte numbers, on E15, E18, the day of parturition and post delivery of mouse pregnancy; they found that macrophages were highest on E18, but neutrophils and monocytes peaked in postnatal samples, with no significant increase on E18 compared to E15, similar to the changes in myometrial chemokines, which were only measured at 3 time points, E15, after the onset of parturition and post delivery, missing E18 [13]. In an earlier paper, Shynlova et al studied myometrial CCL2 levels in rat pregnancy in more detail and found that the levels of both mRNA and protein increased before the onset of labour on day 22 of gestation, consistent with the trends we observed [16]. The authors related the increase in CCL2 to a pre-parturition increase in macrophage numbers as assessed by immunohistochemistry [16] and suggested that the increase in myometrial CCL2 was stretch induced and triggered by the decline in systemic P4 levels.

Systemic leukocyte changes: Circulating neutrophils were higher during pregnancy than in non-pregnant mice, but did not change in late pregnancy and actually tended to decline with parturition. In the lung and liver, marginated (i.e. adherent to the vessel endothelium) neutrophils peaked on E17, while monocytes interestingly peaked on day 18. As shown in supplementary Figure 4, most of these cells were intravascular, and were termed marginated, rather than infiltrating as seen for virtually all myometrial leukocyte populations. The increase in marginated neutrophils may simply reflect the increase in circulating numbers, whereas the increase in monocytes occurred on the background of stable circulating numbers and implies a systemic activation with increased trapping of cells within the pulmonary microcirculation. Whether the increase in circulating leukocytes

contributed to the increase in myometrial leukocytes is uncertain. In the naïve mice, the similarity in the patterns suggests that they may be related, but the absence of any change in placenta or decidua implies either that immune suppression restricted the influx of leukocytes in these sites or that local factors contributed to the increase in the myometrium.

Pro-labour factors: *Cx-43* and *COX-2* mRNA and protein each increased with the onset of parturition. Shynlova et al in the mouse and Helguera et al in the rat, reported similar changes, but both only used 1 time point prior to labour, E15 (mouse) and E21 (rat) respectively, making direct comparisons with the current study difficult [16]^[35], Arthur et al, in a more detailed study of myometrial gene expression in the rat, found no pre-labour increase in either *Cx-43* or *COX-2* mRNA [36]. Similarly, previously, we showed no difference in *Cx-43* or *COX-2* mouse myometrial protein levels between E16 and E18 samples [31], supporting the idea that the increase in prolabour protein levels is tightly temporally regulated, with the increase occurring very close to or simultaneously with the onset of labour.

The role of P4:

Giving RU486 on E16 triggered parturition 17 hours later and, conversely, supplementing P4 from E14 prevented the onset of term labour. At 4.5 hours after RU486, neutrophil numbers increased in the myometrium, blood and lung; the myometrial MAPK system was activated and the myometrial levels of several cytokines and *Cx-43* increased. These data suggest that the increase in inflammation and prolabour factors in spontaneously labouring mice may take place within hours of the onset of labour and, consequently, defining whether inflammation occurs prior to the onset of parturition, with the implication of a possible causative role, will be difficult. In humans, although there is evidence of an increase in transcription factor activation in early labour, there is no increase in cytokine levels until labour is established suggesting that inflammation is a consequence rather than a cause of labour (see above for comments about differential assay sensitivity and myometrial composition,[37]). This may well be true in the mouse as well as, but this awaits further studies.

RU486 is both a PR and GR antagonist, some its effects may be related to inhibition of GR. Also, in contrast to the gradual decline in P4 levels in normal pregnancy, RU486 acutely blocks P4 action, completely changing the time-scale of the decline in P4 action.

RU486, myometrium leukocyte changes: After RU486 treatment, myometrial neutrophil numbers increased significantly at 9h and in labour, and Ly-6C^{HI} monocyte numbers increased at the time of labour. Myometrial chemokine levels, including CCL2, CXCL1 and CXCL2, increased from 4.5-9h, intriguingly without any increase in mRNA abundance myometrium total RNA, and probably accounted for the increase in myometrial leukocyte numbers. The apparent differential effect on neutrophils and monocytes, although typical of the classical kinetics of these two cell types, are difficult to explain given the similar changes in chemotactic factors. An increase in inflammatory cytokine mRNA abundance was only detectable in labour, but again the actual protein levels of all but IL-1 β , increased at between 4.5 and 9h (more striking changes were seen for IL-4, IL-10 and IFN γ , supplementary data). Similar to myometrial Ly-6C^{HI} monocyte numbers, myometrial macrophage numbers increased after the onset of RU486 induced parturition, suggesting that the change may be a consequence rather than a cause of parturition. Interestingly, Shynlova et al did not find any difference in myometrial macrophage, neutrophil or monocyte numbers in RU486-induced parturition compared to E15 levels and although they did report increases in IL-6 at mRNA and protein levels, chemokine levels were also not increased [13]. The absence of any significant change in myometrial cytokine levels in Shynlova et al's study may be because the analysis included postnatal samples in which cytokine levels were markedly raised, possibly obscuring the significance of the changes seen at the time of RU486-induced parturition [13].

RU486, Systemic leukocyte changes: In the present study, RU486 administration increased circulating neutrophils before the onset of labour, but lung margination for both neutrophils and Ly-6C^{HI} monocytes only increased after the onset of labour. These data show that RU486 regulates the systemic as well as the local maternal immune system, affecting neutrophil bone marrow release

and to a lesser extent neutrophil and monocyte activation. RU486 restores adaptive humoral and cellular immune function in LPS immunosuppressed mice, supporting a systemic effect [38]. RU486 acts as both a glucocorticoid and P4 receptor antagonist, meaning either pathway may be responsible for these effects, but since we observed increased lung margination for neutrophils (E17) and Ly-6C^{HI} monocytes (E18) in the naïve mouse coincident with the decline in circulating P4, it suggests that this is a progesterone-mediated effect and consequently that P4 regulates both the systemic and local innate immune system.

RU486, pro-labour factors: As reported by Shynlova et al [13], Cx-43 mRNA and protein levels appeared to increase in tandem, although a non-significant increase in Cx-43 protein levels appeared to occur before changes in mRNA abundance. Interestingly, the MAPK system was also activated between 4.5 and 9h and, in another study, myocardial p38 activation has been associated with enhanced translation of COX-2 mRNA, whereby COX-2 protein levels were increased without any change in mRNA expression [39]. This may explain the increase in protein levels without any apparent change in mRNA abundance. Although, in the present study after RU486, COX-2 mRNA did not change significantly and protein levels actually declined. We found a similar increase in cytokine levels in human myometrium in established labour with out any apparent increase in mRNA levels (Lei et al, unpublished data). These changes were associated with p38 activation and a decline in tristetraprolin (TTP), which is a key member of TIS11 family, which has been implicated in mRNA transcription, splicing, polyadenylation, translation and degradation [40].

P4 supplementation, myometrium leukocyte changes: Unlike the repressive effect of P4 supplementation on myometrial CCL2 levels and consequently the Ly-6C^{HI} monocyte population, P4 supplementation had no effect on CXCL1/2 levels or neutrophil migration into the myometrium, despite the fact that P4 represses CXCL1 and CXCL2 in vascular endothelial cultures [41]. These data suggest that P4 supplementation has differential effects on myometrial chemokine expression and that an increase in neutrophils alone is not sufficient to induce parturition. RU486 increased both

monocyte and neutrophil myometrial infiltration, in association with an increase in CCL2, CXCL1 and CXCL2, suggesting that P4 may repress both monocyte and neutrophil chemotactic factors.

Myometrial cytokine levels, were similar in both P4-supplemented and naïve mice with the exception of CCL2 on E18, which were higher in naïve mice, perhaps accounting for the difference in myometrial Ly-6C^{Hi} monocytes numbers. Indeed, Tagliani et al. found that Ly-6C^{Hi} monocyte infiltration into the myometrium at E9/10 was CCR2 mediated [10]. In terms of pro-labour factors, intriguingly, both *Cx-43* and *OTR* mRNA abundance was greater in the P4-supplemented mice on E17, but at the onset of labour the abundance of *Cx-43*, *OTR* and *COX-2* mRNA was consistently greater in the myometrium of the control, vehicle treated mice. However, no difference could be seen at the protein level at any point.

The changes in the RU486-treated and P4-supplemented mice suggest that P4 regulates CCL2 levels and consequently myometrial Ly-6C^{Hi} monocyte numbers. To test this hypothesis, we administered the CCR2 antagonist RS 504393 8h after RU486 administration during the phase of monocyte mobilization from the bone marrow into the blood and before myometrial infiltration. The reduction in circulating Ly-6C^{Hi} monocyte numbers by RS 504393 confirmed effective blockade of the CCR2 receptor, however Ly-6C^{Hi} monocytes were not significantly reduced in the myometrium, although there was a trend ($p=0.0628$). It is possible that Ly-6C^{Hi} monocyte infiltration had started prior to RS 504393 administration or that other factors, such as CCL5 or CCL20 [42, 43], also contributed to myometrial Ly-6C^{Hi} monocyte recruitment. Interestingly, the neutrophil cell density in the myometrium was significantly reduced by CCR2 antagonism. Neutrophil CCR2 expression is induced by chronic inflammation and found in a mouse model of polymicrobial sepsis [44, 45]. It is possible that the administration of RU486 induced neutrophil CCR2 expression, or that pregnancy itself induces such a change. However, P4 supplementation did not inhibit the increase in myometrial neutrophil numbers, suggesting that RU486 makes neutrophils sensitive to CCL2 rather than any pregnancy induced effect. Further, in a study using broad-spectrum chemokine inhibitors (BSCI) in an inflammation-induced mouse model of preterm labour, parturition was delayed to term in 36% of

BSCI treated mice and this coincided with a significant reduction in myometrial neutrophil numbers [46]. However, BSCI treatment alone did not delay the spontaneous onset of parturition at term, suggesting that different pathways mediate the spontaneous onset of labour.

P4 supplementation and systemic leukocyte changes: P4 supplementation prevented the labour-associated increase in both neutrophil or Ly-6C^{HI} monocyte numbers in the blood and, for Ly-6C^{HI} monocytes, in the lungs. This may be a direct effect of P4 on bone marrow release or leukocyte activation, but it could also be mediated through P4-inhibition of parturition.

Effect of P4 supplementation on pro-labour factors: In terms of pro-labour factors, both *Cx-43* and *OTR* mRNA (supplementary figure 17) were greater in the P4-supplemented mice on E17, but the onset of labour the expression *Cx-43*, *OTR* and *COX-2* mRNA was consistently greater in the myometrium of the control, vehicle treated mice. However, no difference could be seen at the protein level at any point, perhaps because of the lack of sensitivity of the western blotting, although *Cx-43* tended to be higher in the control mice. Not surprisingly, we failed to see any consistent changes in MAPK/c-Jun activation with P4 supplementation, since, as we saw in the RU486 study, these pathways are tightly temporally regulated close to the onset of labour, further, activation of the MAPK/AP-1 system is followed by de-activation and a persisting repression which will make it appear that activity is reduced [47].

Functional P4 withdrawal: With advancing gestation in the naïve mice, we observed a trend for chemokine levels to increase with no evidence of an increase in mRNA expression. We observed a similar, but more marked occurrence after RU486 and with prolonged P4-supplementation. The effect of RU486 implies that inhibition of P4 action results in an increase in chemokine levels independent of mRNA expression. The similar pattern after prolonged P4-supplementation suggests the development of a functional withdrawal of P4 action, a phenomenon suggested to underpin the onset of human labour. The trends in chemokine levels at the end of naïve mouse pregnancy suggest

that this process may also be active during naïve mouse pregnancy. Interestingly, the disconnect between mRNA abundance and protein levels was not observed for prolabor factors and while their expression was increased at the end of naïve mouse pregnancy and after RU486, there was no increase with prolonged P4 gestation. Leukocyte numbers were increased in all compartments with prolonged P4 supplementation, this may be secondary to the change in chemokine levels, but raises the possibility of a systemic withdrawal of P4 effects. Alternatively, the prolonged pregnancy may have resulted in an inflammatory response and secondary increase in leukocyte numbers, particularly since the at the time of culling the pups were dead and appeared to wedged into the pelvis.

Functional significance of the myometrial leukocyte infiltration: In naïve mice, myometrial neutrophil and Ly-6C^{HI} monocyte numbers increased before the onset of parturition, suggesting that they may have a role in its onset. However, there was no evidence of any increase inflammatory cytokine levels until labour was established, suggesting that although leukocyte numbers were increased that the onset of labour was not mediated through an increase in inflammatory cytokine levels. It is possible that the increase in inflammatory cytokine levels occurred later, closer to the onset of parturition and we explored this possibility with RU486 and found that neutrophil numbers increased rapidly after RU486 administration co-incident with the increase in inflammatory cytokine levels raising the possibility that P4 controls the onset of parturition through regulating leukocyte numbers and their release of cytokines. However, although P4 supplementation prevented the onset of labour, it only repressed the increase in myometrial Ly-6C^{HI} monocyte, but not neutrophil, numbers, suggesting that neutrophils are not sufficient on their own to drive the onset of labour. Indeed, depletion of neutrophils did not prevent inflammation induced preterm labour in the mouse [48, 49], questioning the importance of neutrophils in the process of mouse parturition. Given these data, we gave a specific CCL-2 antagonist, RS504393, after RU486 administration. The CCL-2 antagonist reduced the increase in myometrial neutrophils and, to a lesser extent, Ly-6C^{HI} monocytes, but did not prevent or even delay RU486-induced parturition. Together, these data

suggest that the ability of RU486 to induce parturition is independent of its ability to increase myometrial leukocyte numbers and, conversely, that the ability of P4 to prolong pregnancy is independent of its ability to delay myometrial Ly-6C^{Hi} monocyte infiltration. Overall, these data add more weight to the suggestion that despite being present in abundant numbers, leukocytes have no role in the process of mouse parturition.

The anti-inflammatory properties of P4: P4 has been shown to repress inflammation in a number of experimental situations. Originally, in cell line experiments, P4 acting via the progesterone receptor B isoform has been shown to inhibit NFκB activation [50]. Similarly, in primary human myometrial cells, myometrial cell lines and human myometrial explants, P4 has been shown to exert a similar effect [27, 51, 52]. However, latterly, P4 has been shown to inhibit the AP-1 system through the induction of MKP-1 expression [47]. On the basis of these data, we chose to study the MAPK/AP-1 and p65/NFκB systems in this study. The data here, particularly with RU486, support the idea that P4 acts via inhibition of the MAPK-AP-1 system to inhibit pro-inflammatory gene expression and limit the inflammatory cell myometrial infiltration.

Conclusion: These data show that P4 represses myometrial pro-labour and inflammatory gene expression through mRNA dependent and independent mechanisms, the latter possibly involving p38 inhibition and the prevention of mRNA translation. They cast further doubt on the importance of inflammation in the onset of labour, showing that inhibition of the RU486-induced leukocyte infiltration does not delay the onset of parturition. Further, they demonstrate that a functional withdrawal of P4-action does occur and that this is associated with increases in inflammatory cytokines in the absence of any change in mRNA levels, suggesting that assessing the myometrial proteome may hold promise in our search to understand the onset of human labour.

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Figure 1 (A-D) Inflammatory cells

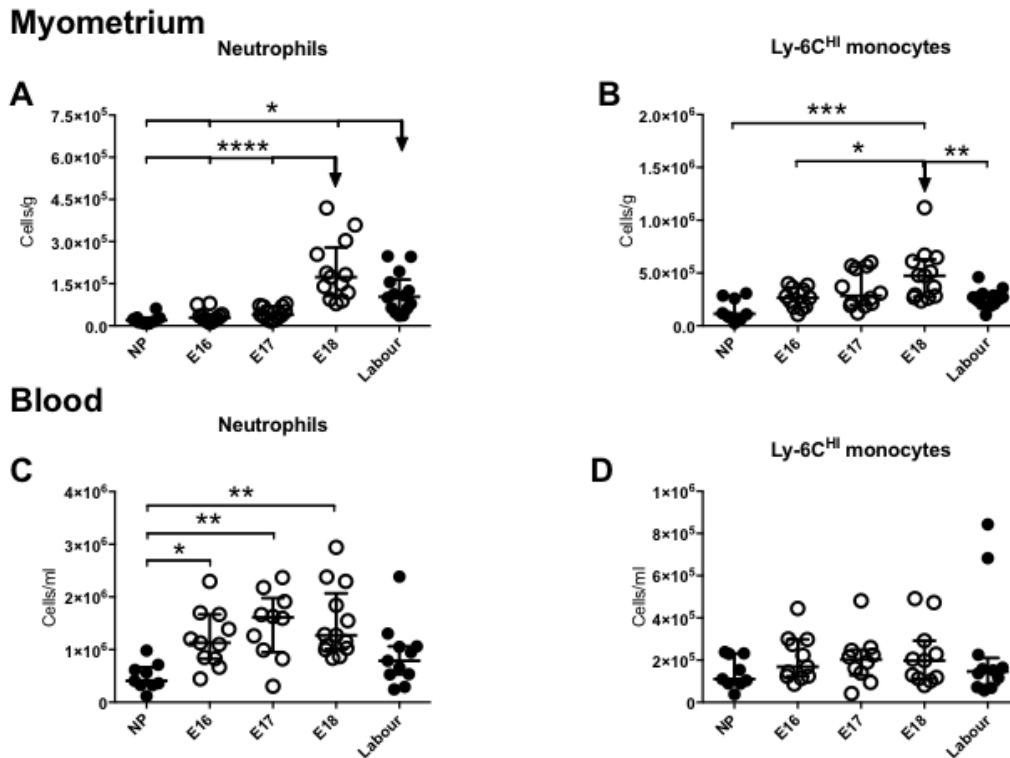


Figure 1 A-D. Neutrophil and Ly-6C^{HI} monocyte cell density in the **A&B** myometrium and **C&D** blood from non-pregnant and pregnant on gestation days 16, 17, 18 and labouring CD1 mice was quantified using Flow Cytometry (cells/g or ml). Data are expressed as individual data points with median and interquartile range superimposed. Non-pregnant, E16, E17 and E18 cell densities were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (*= $p < 0.05$, ** = $p < 0.01$, ***= $p < 0.001$ and ****= $p < 0.0001$, $n = 6-14$).

Figure 2 A-L Myometrial cytokine mRNA expression and protein levels

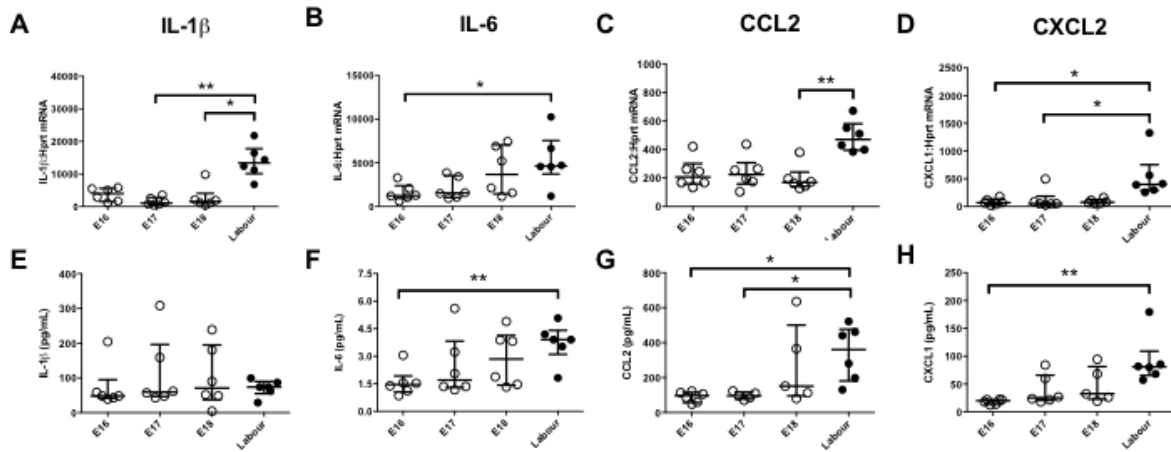


Figure 2 A-H. Myometrial cytokine (IL-1 β , IL-6, CCL2 and CXCL1) mRNA expression (housekeeping gene Hprt) and levels (pg/ml) measured by quantitative rtPCR and multiplex respectively. Data are expressed as individual data points with median and interquartile range superimposed and were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (*= $p < 0.05$ and ** = $p < 0.01$, $n = 6$).

Figure 3 A-D Prolabour factor mRNA expression and protein levels

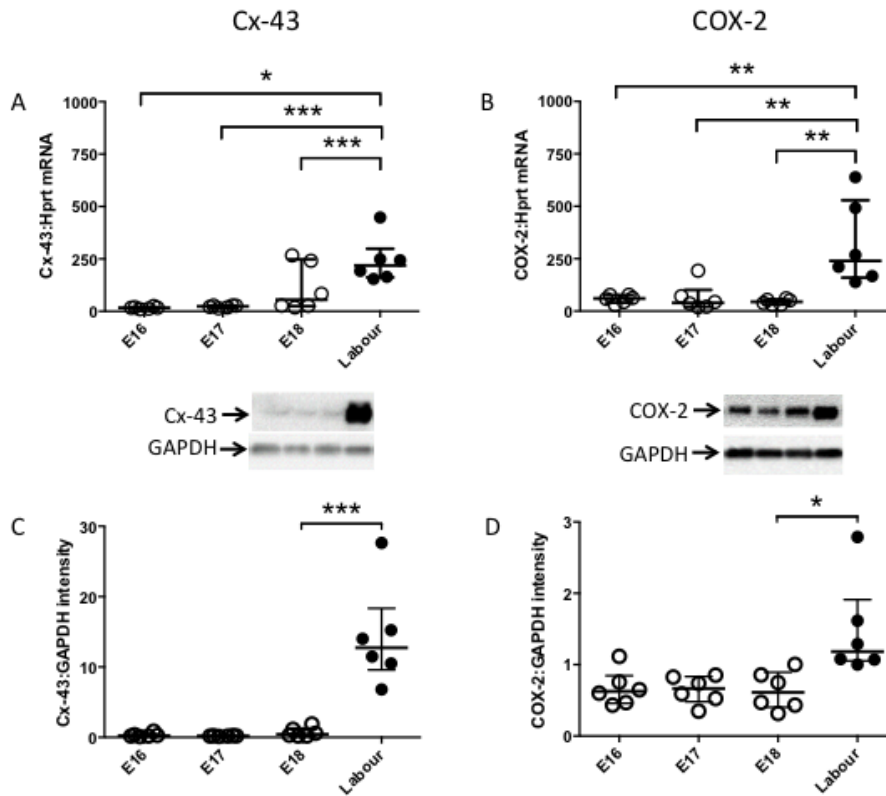
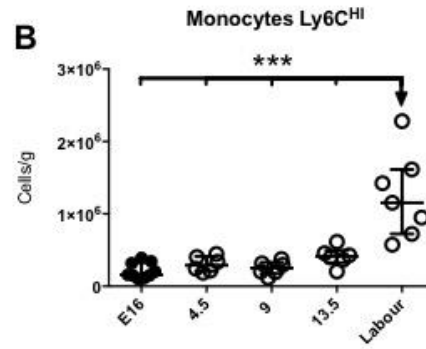
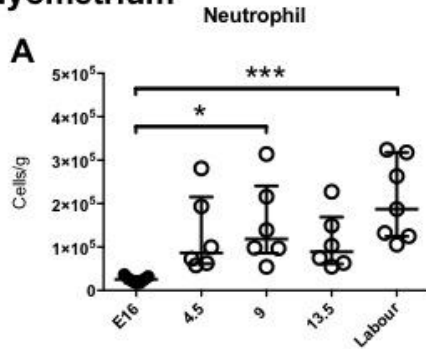


Figure 3 A-D. Myometrial connexin 43 (Cx-43, **A&C**) and cyclo-oxygenase 2 (COX-2, **B&D**) mRNA expression (housekeeping gene Hprt) and protein levels measured by quantitative rtPCR and western analysis respectively. Data are expressed as individual data points with median and interquartile range superimposed and were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (*= $p < 0.05$, **= $p < 0.01$ and ***= $p < 0.001$, $n=6$).

Figure 4 A-D RU486 flow data

Myometrium



Blood

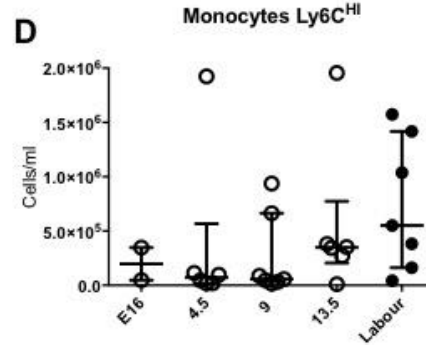
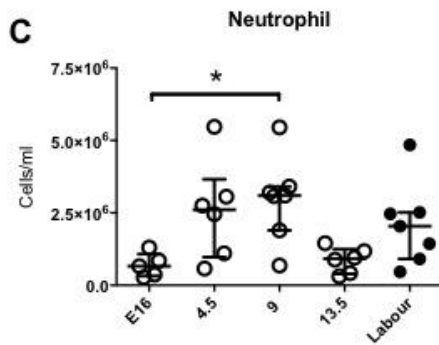


Figure 4 A-D. Neutrophil and Ly-6C^{HI} monocyte cell density in the **A&B** myometrium and **C&D** blood from pregnant CD-1 mice given 150µg of RU486 dissolved in 20µL DMSO on E16 at 10am. Samples were obtained at 4.5h intervals thereafter until the onset of parturition at approximately 17-18h post RU486 administration. Cell density (cells/g or cells/ml) was quantified using Flow Cytometry. Data are expressed as individual data points with median and interquartile range superimposed and were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (*= $p < 0.05$ and ***= $p < 0.001$, $n = 6-10$).

Figure 5 A-P RU486 myometrial cytokine mRNA/protein levels

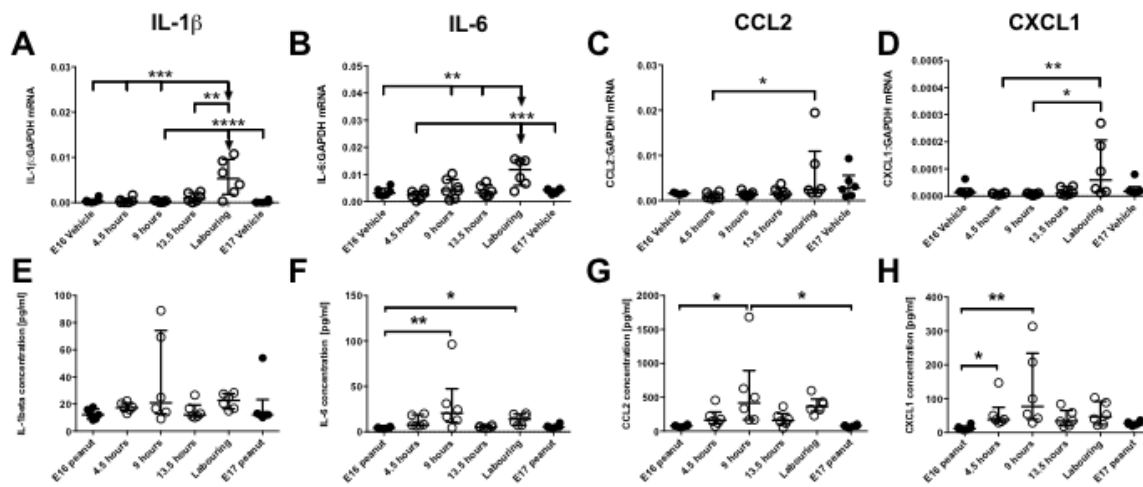


Figure 5 A-H. Myometrial cytokine (IL-1 β , IL-6, CCL2 and CXCL1) mRNA expression (housekeeping gene Hprt) and levels (pg/ml) measured by quantitative rtPCR and multiplex respectively. In myometrial samples from pregnant CD-1 mice given 150 μ g of RU486 dissolved in 20 μ L DMSO on E16 at 10am. Samples were obtained at 4.5h intervals thereafter until the onset of parturition at approximately 17-18h post RU486 administration. Data are expressed as individual data points with median and interquartile range superimposed and were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (* = p < 0.05, ** = p < 0.01, *** = p < 0.001 and **** = p < 0.0001, n = 6).

Figure 6 A-F RU486 prolabour factor mRNA and protein levels

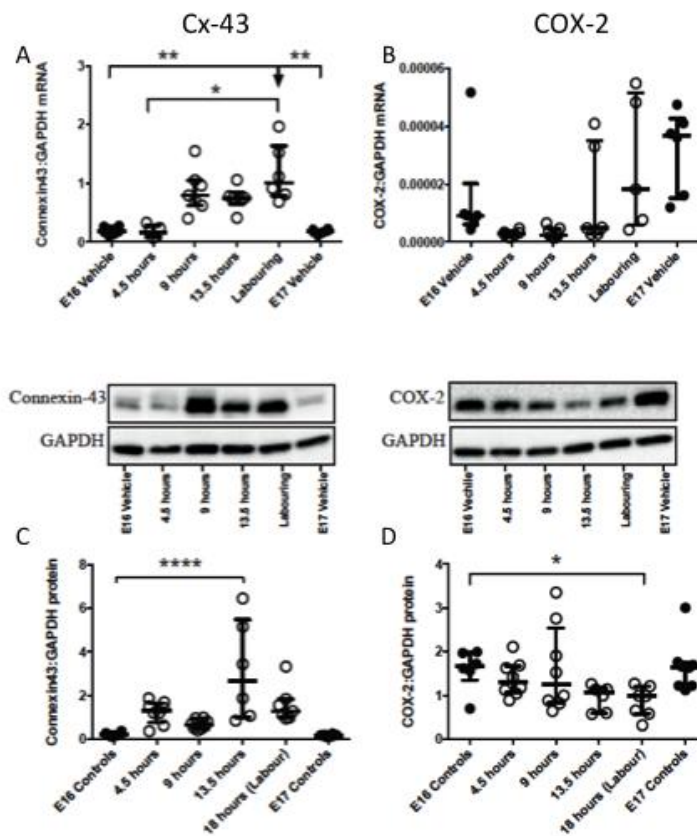


Figure 6 A-D. Myometrial connexin 43 (Cx-43, **A&C**) and cyclo-oxygenase 2 (COX-2, **B&D**) mRNA expression (housekeeping gene GAPDH) and protein levels measured by quantitative rtPCR and western analysis respectively. In myometrial samples from pregnant CD-1 mice given 150 μ g of RU486 dissolved in 20 μ L DMSO on E16 at 10am. Samples were obtained at 4.5h intervals thereafter until the onset of parturition at approximately 18h post RU486 administration. Data are expressed as individual data points with median and interquartile range superimposed and were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (*= $p < 0.05$, ** = $p < 0.01$ and ****= $p < 0.0001$, $n = 5-8$).

Figure 7 A-C RU486 MAPK phosphorylation

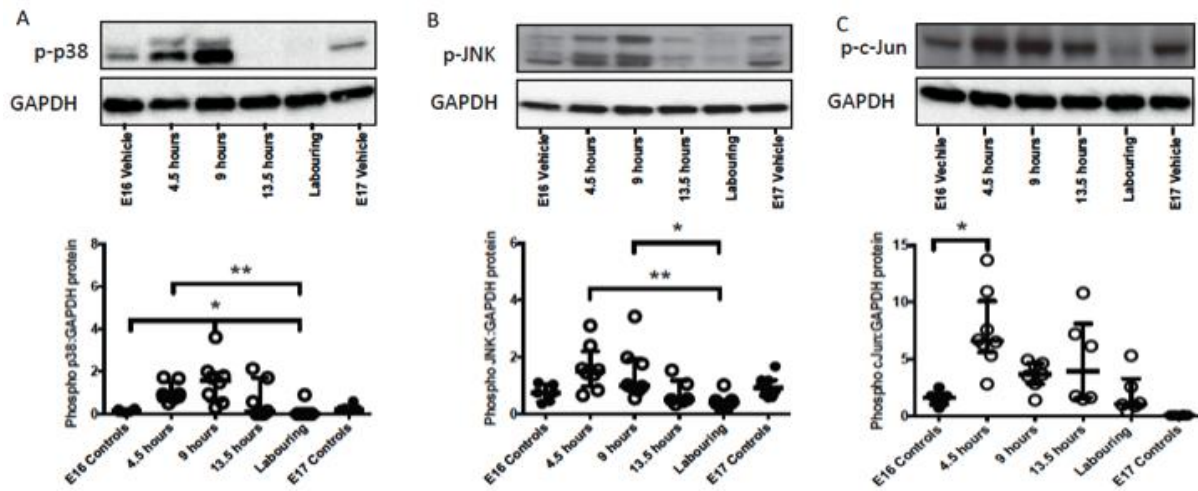
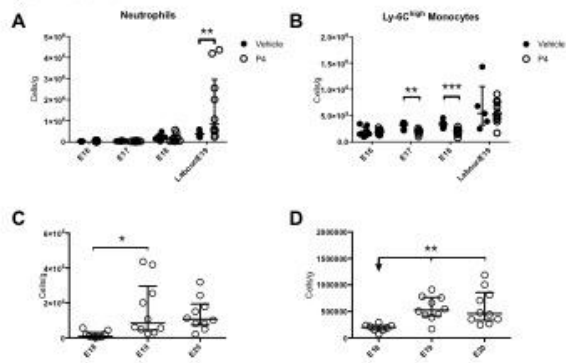


Figure 7 A-C. Myometrial (A) phospho-p38 (p38, (B) phospho-JNK (pJNK) and (C) phospho-cjun (p-c-jun) measured by western analysis in myometrial samples from pregnant CD-1 mice given 150 μ g of RU486 dissolved in 20 μ L DMSO on E16 at 10am. Samples were obtained at 4.5h intervals thereafter until the onset of parturition at approximately 18h post RU486 administration. Data are expressed as individual data points with median and interquartile range superimposed and were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (*= p<0.05 and ** =p<0.01, n=5-8).

Figure 8 A-H P4 supplementation flow data

Myometrium



Blood

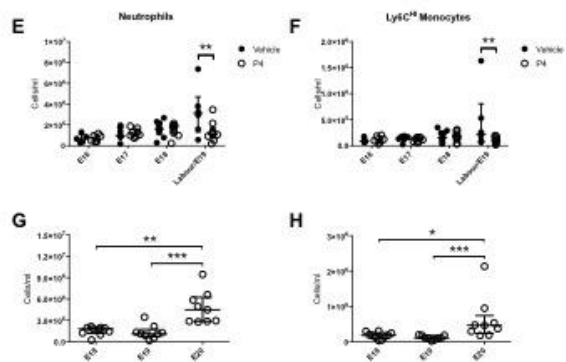


Figure 8 A-H. Neutrophil and Ly-6C^{HI} monocyte cell density in the myometrium (**A-D**) and blood (**E-H**)

from pregnant control mice and CD-1 mice given 2mg progesterone daily dissolved in 40µL peanut oil from E14 until E20. Cell density (cells/g) was quantified using Flow Cytometry. Comparative data (P4 supplemented vs. vehicle) shown until E19 (**A&B, E&F**), when vehicle treated mice delivered.

Thereafter, the data are shown between E18-E20 for P4 supplemented mice alone (**C&D, G&H**). The data are expressed as individual data points with median and interquartile range superimposed.

Paired data were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when data was not. Longitudinal data were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post-test when it was not. (** = $p < 0.01$ and *** = $p < 0.001$, $n = 6-10$).

Figure 9 A-H P4 supplementation cytokine mRNA/protein levels

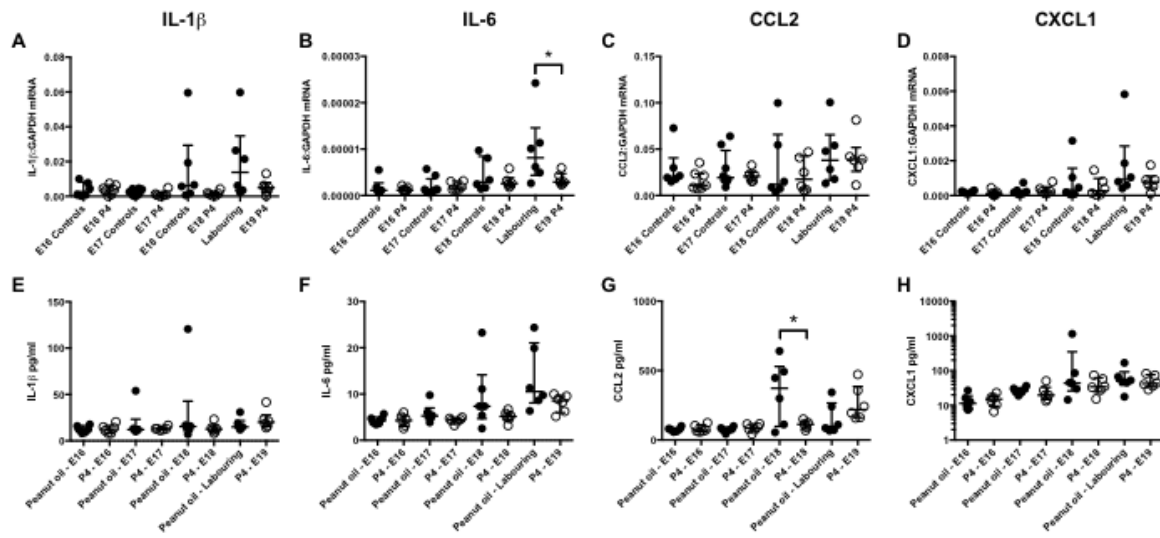


Figure 9 A-H. Myometrial cytokine (IL-1 β , IL-6, CCL2 and CXCL1) mRNA expression (housekeeping gene GAPDH) and levels (pg/ml) measured by quantitative rtPCR and multiplex respectively. In myometrial samples from pregnant control mice and CD-1 mice given 2mg progesterone dissolved in 40 μ L peanut oil from E14 until E20. Comparative data (P4 supplemented vs. vehicle) shown until E19 (**A&B, E&F**), when vehicle treated mice delivered. Thereafter, the data are shown between E18-E20 for P4 supplemented mice alone (**C&D, G&H**). The data are expressed as individual data points with median and interquartile range superimposed and were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when data was not. (*= $p < 0.05$, $n = 6$).

Figure 10 A-H P4 supplementation cytokine mRNA/protein levels E18-20

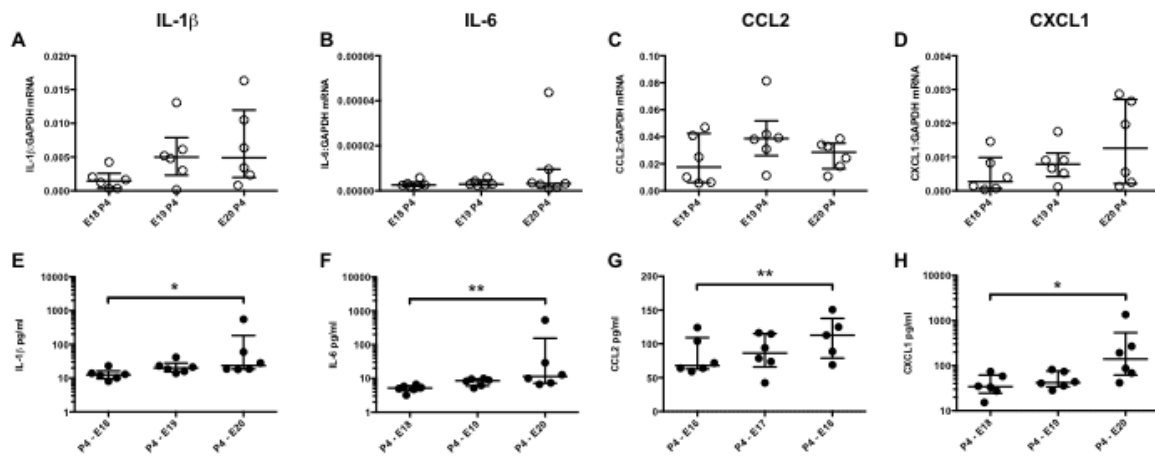


Figure 10 A-H. Myometrial cytokine (IL-1 β , IL-6, CCL2 and CXCL1) mRNA expression (housekeeping gene GAPDH) and levels (pg/ml) measured by quantitative rtPCR and multiplex respectively. In myometrial samples on E18, 19 and 20 from pregnant CD-1 mice given 2mg progesterone dissolved in 40 μ L peanut oil from E14 until E20. Data are expressed as individual data points with median and interquartile range superimposed and were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (* = p < 0.05 and ** = p < 0.01, n = 6).

Figure 11 A-H P4 supplementation Prolabour factor mRNA/protein levels.

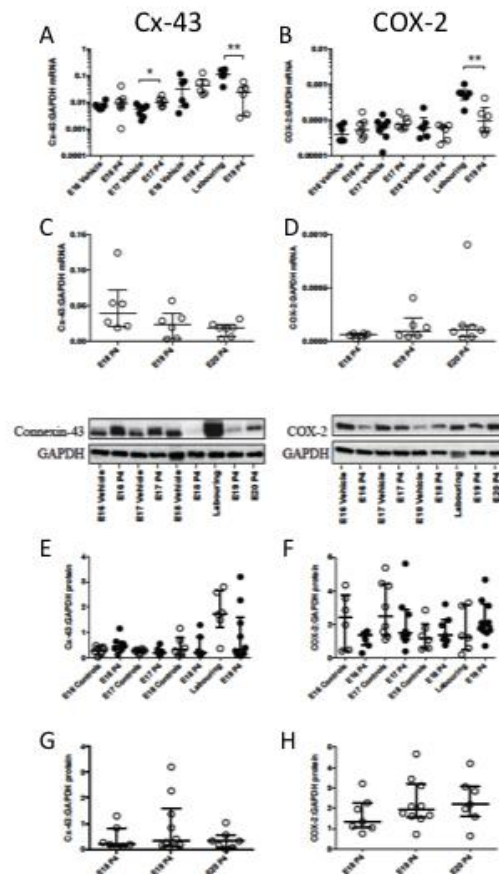


Figure 11 A-H. Myometrial connexin 43 (Cx-43, A,C,E&G) and cyclo-oxygenase 2 (COX-2, B,D,F&H) mRNA expression (housekeeping gene GAPDH) and protein levels measured by quantitative rtPCR and western analysis respectively. In myometrial samples obtained from pregnant control mice and CD-1 mice given 2mg progesterone dissolved in 40 μ L peanut oil from E14 until gestational E20. Comparative data shown until E19 and longitudinal data E18-E20. Data are expressed as individual data points with median and interquartile range superimposed. Paired data were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when data was not. Longitudinal data were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post test when it was not. (*= p<0.05 and ** =p<0.01, n=5-8).

Figure 12 A-F P4 supplementation MAPK activity.

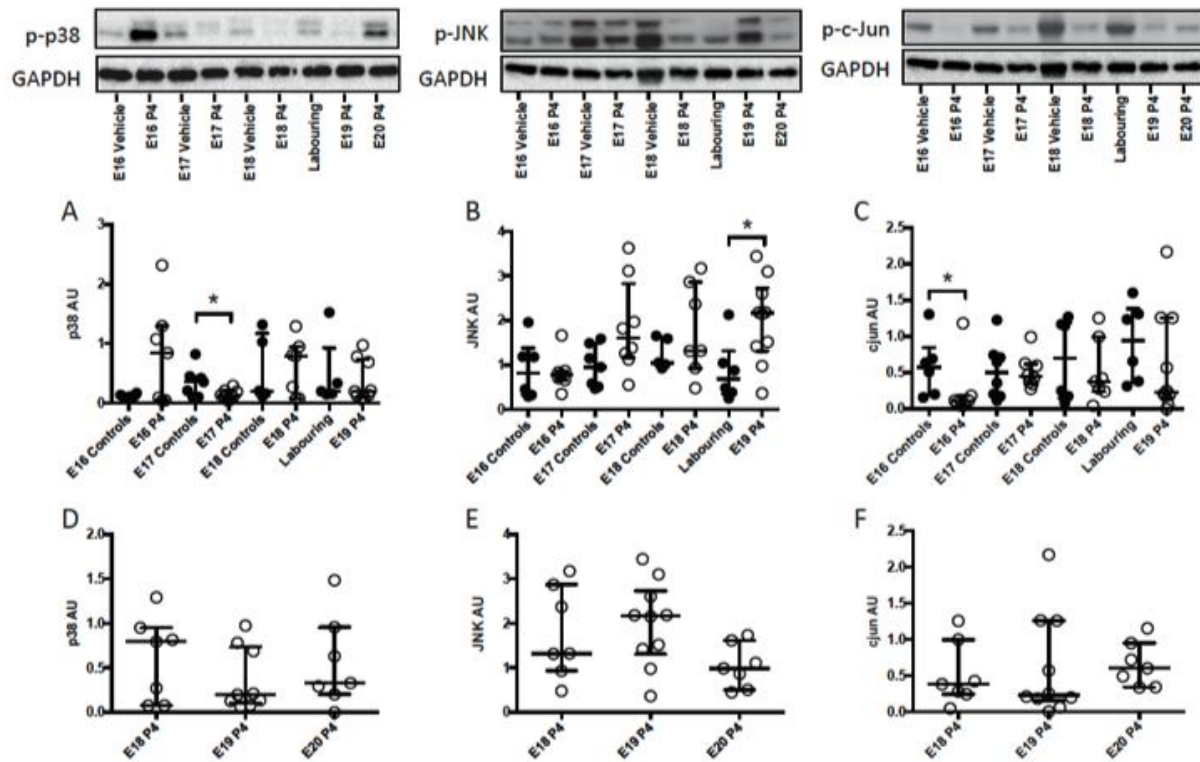


Figure 12 A-F. Myometrial phospho-p38 (p38, A&D), phospho-JNK (pJNK, B&E) and phospho-cjun (p-c-jun, C&F) measured by western analysis in myometrial samples obtained from pregnant control mice and CD-1 mice given 2mg progesterone dissolved in 40 μ L peanut oil from E14 until gestational E20. Comparative data shown until E19 and longitudinal data from E18-E20. Data are expressed as individual data points with median and interquartile range superimposed. Paired data were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when data was not. Longitudinal data were compared using a One-way ANOVA with Bonferroni post-test when normally distributed and a Kruskal-Wallis with Dunns post-test when it was not. (*= $p < 0.05$, $n = 5-8$).

Table 1. A summary of the results in the naïve, RU486 treated and P4 supplemented mice.

Feature	Naïve mouse	RU486	P4 supplementation	Prolonged P4 E18-E20
Myometrial leukocyte infiltration	Increased E16-E18	Increased 4.5h to parturition	Reduced Ly-6C ^{HI} monocyte	Increased neutrophil and Ly-6C ^{HI} monocyte
Placental leukocyte infiltration	None	Reduced Ly-6C ^{HI} monocyte	Reduced neutrophil and Ly-6C ^{HI} monocyte	Increased neutrophil and Ly-6C ^{HI} monocyte
Systemic leukocyte numbers	Increased neutrophil and Ly-6C ^{HI} monocyte	Increased neutrophil and Ly-6C ^{HI} monocyte	Reduced neutrophil and Ly-6C ^{HI} monocyte	Increased neutrophil and Ly-6C ^{HI} monocyte
Myometrial chemokine levels	Significantly increased with parturition only	Significantly increased before parturition	Reduced CCL2 levels	Increase in all
Myometrial inflammatory cytokine levels	Significantly increased with parturition only	Significantly increased before parturition	No significant difference	Increase in all
Prolabour factors	Significantly increased with parturition only	Significantly increased before parturition	No significant difference	No increase in levels