Biology of Reproduction

CCR2 mediates the adverse effects of LPS in the pregnant mouse

--Manuscript Draft--

Manuscript Number:	BIOLRE-2019-0209R1
Full Title:	CCR2 mediates the adverse effects of LPS in the pregnant mouse
Short Title:	CCR2/CCL2 in LPS induced preterm labour
Article Type:	Research Article
Section/Category:	Pregnancy
Keywords:	inflammation, chemokines, CCR2, myometrium, lungs, leucocytes
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Abstract:	In our earlier work, we found that intrauterine (i.u.) and intraperitoneal (i.p.) injection of LPS (10μg serotype 0111:B4) induced preterm labour (PTL) with high pup mortality, marked systemic inflammatory response and hypotension. Here, we used both i.u. and i.p. LPS models in pregnant wild type (wt) and CCR2 knockout (CCR2-/-) mice on E16 to investigate the role played by the CCL2/CCR2 system in the response to LPS. At untreated mice on E16, there were significant reductions in numbers of monocytes and macrophages and increases in neutrophils in myometrium, placenta and blood in CCR2-/- vs. wt mice. After i.u. LPS, parturition occurred at 14 hours in both groups of mice, in CCR2-/- mice monocytes and macrophages were generally lower in the myometrium and placenta, but this was less consistent in the circulation, lung and liver. Myometrial ERK activation was greater and JNK and p65 lower and the mRNA levels of chemokines were higher and of inflammatory cytokines lower in CCR2-/- vs. wt mice. At 7 hours post injection, 70% of wt pups were dead vs. 10% of CCR2-/- pups,

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	but at delivery 100% of CD1 and 90% of CCR2-/- pups were dead. Placental inflammation was less in CCR2-/- mice. Using the IP model, we found that all measures of arterial pressure increased in CCR2-/-, but declined in CD1 mice post-LPS. These data suggest that the CCL2/CCR2 system plays a critical role in the cardiovascular response to LPS and contributes to pup death, but does not influence the onset of inflammation-induced PTL.
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Question	**Response**

Dear Drs Wei Yan and Hugh Clarke,

Thank you for the opportunity to revise our manuscript. We have considered each of the points raised by the associate editor and each of the referees and addressed each in the revised manuscript as follows:

Associate Editor Comments:
Your manuscript has now been reviewed by two reviewers with expertise in the field. Both reviewers and the associate editor agree that your manuscript reports some interesting findings on the potential role of CCR2 in LSP-induced inflammation during pregnancy. However, the reviewers raised some specific questions and concerns that need to be addressed before the manuscript would be acceptable for publication in BOR.

> Key problems include- the correlation of cytokine levels in fetal brain with the rate of fetal death at different stages;
>
> the association of cytokines with higher activation of ERK, and lower activation of JNK and NF-KB in the myometrium of CCR2-/- mice;
>
> the clarification for decreased blood pressure is in LPS treated CD1 mice,
>
> as well as the need to clearly state the genetic background of the control and null mice used in this investigation, etc.

Thank you for these comments and the opportunity to completely revise the manuscript, which we have done taking into account the comments of the referees and the errors highlighted by the referees.

Reviewer Comments to the Author:
Reviewer 1: My most concerns

1. How do authors separate myometrial tissues?

Are there any references utilizing the whole uterine horn in myometrial functional study?

Thank you, we are not aware of any references.

Authors should exclude the effects of endometrial cells and implanted placental cells in uterus on your data.

Thank you for the opportunity to clarify this point, in this paper we used the same methods as our group has previously described [1]: the decidua basalis was cut away from the myometrial tissue and the decidua parietalis carefully removed from the myometrial tissue by mechanical scraping on ice. This removed the entire luminal and glandular epithelium. We have added this to the paper (129-131).

2. All legends or texts showed untreated CCR2-/- and wild type mice were used, however, in the figures, CD1 mouse was used as control. It is difficult for reviewers to identify which strains of mice (wt, wt cd1, cd1 etc) were exactly used in each specific experiments through the whole manuscript. In

reviewer's opinion, wt should be the CD1. So authors need to use an unanimous name of wt and CD1. Also, authors need to clearly state CCR2-/- and CD1/CCR2-/- when used.

We have checked the manuscript and changed all of the CD1 to wt.

3. What data can be used to explain CCL2/CCR2 system plays a critical role in the cardiovascular response to LPS and contributes to pup death in the abstract and discussion?

The involvement of chemokines in blood pressure regulation predominantly comes from control of inflammation in the vessels and heart, where inflammatory cell infiltration and oxidative stress in the vascular walls contributes to an increase in blood pressure[2]. Shen et al., were able to show that CCL2 deficiency was able to reduce CCL2-depandent macrophage recruitment and consequently reduce tissue inflammation and hypertension in a model of deoxycorticosterone and salt-induced hypertension cardiac fibrosis and hypertension. Previous work has demonstrated that a lack of functional CCR2 receptors may be associated with the emergence of hypertension in human[3].

minor concerns

1.There are many spelling errors in the text like um, ug, 4oC, Mgcl2 and some of confusing descriptions in line 54- 58 and other lines.

We've checked the spelling errors.

2. what does the p= 0.05-0.01 in line 223 indicate to? * or ** . Is there any significance between groups? Authors must consult to a statistician in calculating all the data.

We have previously discussed the analysis of these data with a statistician [4]. We followed their advice in the analysis of these data.

3. What is the aim of adding genetic variation to these strains in this study? Please state it in a relevant text.

CCR2 homozygous knockout mouse was originally crossed to C57BL/6 mice. The strain was backcrossed to a CD1 background as CD1 mice are a particularly fecund strain and typically have much larger litters than C57BL/6. The greater robustness and reproduction of the outbred strain was considered to be sufficiently advantageous to offset the heterogeneity of the background and allow comparison with our inflammation-induced preterm labour model in CD1 mice. This has been included in the text (100-105)

4. Please should the same pixel be used for all the pictures?

We've checked and made changes to pixels.

Reviewer 2: In the present manuscript, Hua et al. investigated the role of CCR2 in mediating LPS-induced preterm labour and pup death. They found that LPS administration induced parturition within

14 hours in both wt and CCR2-/- mice. Although fetal loss was delayed in CCR2-/- pups, few CCR2-/- pups were alive at delivery. Moreover, CCR2-/-mice demonstrated reduction of monocytes and macrophages in placenta and myometrium, along with lower inflammatory cytokines and higher mRNA levels of chemokines. Placental inflammatory signalings were less activated by LPS in CCR2-/- mice. In addition, LPS increased blood pressure in CCR2-/-, but induced hypotension in CD1 mice.

Overall, the manuscript is not without interest, but it also suffers from insufficient mechanistic study and lacks experimental support for some of the claims, their findings are mainly descriptive and somewhat over-interpreted. Below are my specific comments:

Major concerns:

1. The death of pups was attributed to brain inflammation; however, the fluctuation of cytokines in fetal brain wasn't consistent at different stages and therefore failed to reveal a clear correlation between these two events. Ideally, fetal autopsy should be firstly performed to determine the most-affected fetal organ by LPS that causing death.

We are sorry if our description of the results suggested that fetal brain inflammation was responsible for pup demise. We have altered our discussion to explain that the minimal change in fetal brain inflammation suggested that there was another cause of pup demise. We stated that the LPS serotype that we used has not been shown to cross the placenta and that other authors have shown that reduced placental perfusion was the cause of pup demise. The text has been revised to make this clear at multiple points.

2. It is not clear that whether the change in cytokines is the cause of higher activation of ERK, and lower activation of JNK and NF-KB in the myometrium of CCR2-/- mice.

In a previous study in wt mice, our group found that ERK1/2 increased slightly and non significantly at 3 hours and then declined and was significantly lower in labour than the 3-hour peak. The increased ERK activation in CCR2-/- suggested that CCL2/CCR2 might play an important role in LPS induced activation of MAPK pathway.

We are not sure of why there should be such differences in the response to LPS between the wt and CCR2-/- mice but given the time points at which we studied myometrial tissues, the different patterns may reflect difference in the timing of inflammatory cell infiltration. We aim to clarify the reasons in future work.

3. LPS administration is known to induce hypertension in rodent, and therefore, it has been used to induce preeclampsia-like symptoms. However, in this study, blood pressure is decreased in LPS treated CD1 mice, which should be explained.

We recognised that pregnant mice had a different response to LPS than observed in non-pregnant mice in our earlier work. We agree that LPS can produce variable responses dependent on the physiological situation, the dosage and the type used. In this study, we reproduced our findings of LPS-induced hypotension in pregnant CD1 mice and have discussed this extensively. Future work will investigate how LPS induces hypotension in pregnant mice.

LPS (1.0 μg/kg) tail vein injection on gestational day 14 induced preeclampsia-like symptoms in rats. Yamashita et al. showed that 80 mg/kg intraperitoneal (i.p.) LPS caused a greater than 90% mortality in

WT (C57bl/6) male mice and triggered a severe and progressive drop in mean arterial pressure (MAP) of about 60 mm Hg, a 40% reduction from pretreatment values (mean 97 mm Hg)[5]. In our study, mice received intraperitoneal LPS (10 μg) on E16 induced a greater fall in blood pressure in pregnant mice. This was consistent with our previous study [4]. This has been included in the text (400-405).

Reference:

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3. Shen JZ, Morgan J, Tesch GH, Fuller PJ, Young MJ. CCL2-dependent macrophage recruitment is critical for mineralocorticoid receptor-mediated cardiac fibrosis, inflammation, and blood pressure responses in male mice. Endocrinology 2014; 155:1057-1066.
4. Zollner J, Howe LG, Edey LF, O'Dea KP, Takata M, Gordon F, Leiper J, Johnson MR. The response of the innate immune and cardiovascular systems to LPS in pregnant and nonpregnant mice. Biol Reprod 2017; 97:258-272.
5. Yamashita T, Kawashima S, Ohashi Y, Ozaki M, Ueyama T, Ishida T, Inoue N, Hirata K, Akita H, Yokoyama M. Resistance to endotoxin shock in transgenic mice overexpressing endothelial nitric oxide synthase. Circulation 2000; 101:931-937.

CCR2 mediates the adverse effects of LPS in the pregnant mouse.

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Key words: inflammation, chemokines, CCR2, myometrium, lungs, leucocytes.

Conflict of interests: The authors confirm that they have no conflict of interest.

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Abstract

In our earlier work, we found that intrauterine (i.u.) and intraperitoneal (i.p.) injection of LPS (10μg serotype 0111:B4) induced preterm ~~labour~~labor (PTL) with high pup mortality, marked systemic inflammatory response and hypotension. Here, we used both i.u. and i.p. LPS models in pregnant wild type (wt) and CCR2 knockout (CCR2-/-) mice on E16 to investigate the role played by the CCL2/CCR2 system in the response to LPS.

~~At untreated mice on E16, there were significant reductions in~~ Basally, lower numbers of monocytes and macrophages and ~~increases in~~ higher numbers of neutrophils were found in the myometrium, placenta and blood ~~in~~of CCR2-/- vs. wt mice. After i.u. LPS, parturition occurred at 14 hours in both groups of mice~~, in~~. At 7 hours post injection, 70% of wt pups were dead vs. 10% of CCR2-/- ~~mice~~pups, but at delivery 100% of wt and 90% of CCR2-/- pups were dead. Myometrial and placental monocytes and macrophages were generally lower in ~~the myometrium and placenta,~~ CCR2-/- mice, but this was less consistent in the circulation, lung and liver. ~~Myometrial~~At 7 hours post LPS, myometrial ERK activation was greater and JNK and p65 lower and the mRNA levels of chemokines were higher and of inflammatory cytokines lower in CCR2-/- vs. wt mice. ~~At 7 hours post injection, 70% of wt pups were dead vs. 10% of CCR2 -/- pups, but at delivery 100% of CD1~~Pup brain and ~~90% of CCR2 -/- pups were dead. Placental~~placental inflammation was ~~less in CCR2 -/- mice.~~similar. Using the IP LPS model, we found that all measures of arterial pressure increased in CCR2~~-/-~~ -/- but declined in ~~CD1~~wt mice ~~post-LPS~~.

These data suggest that the CCL2/CCR2 system plays a critical role in the cardiovascular response to LPS and contributes to pup death~~,~~ but does not influence the onset of inflammation-induced PTL.

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Introduction

Every year, an estimated 15 million babies are born preterm worldwide with rates ranging from 5% in several European countries to 18% in some African countries [1]. Globally, preterm birth complications are the leading cause of childhood mortality, responsible for over 1 million deaths in 2013 [2]. Preterm ~~labour~~labor (PTL) is the most important cause of preterm delivery.

~~Current concepts of PTL implicate infection~~Infection and inflammation are implicated as the main causative factors of PTL. The inflammatory process in human parturition is associated with activation of NFkB resulting in an increase in myometrial cytokine and chemokine levels [3, 4]. Inflammation is the first response to tissue injury and is driven by a chemokine-dependent infiltration of immune cells into the area of injury. The chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemotactic protein-1 (MCP-1), is a CC chemokine that mainly recruits monocytes, memory T cells and dendritic cells to the sites of inflammation [5, 6]. CCL2 has also been implicated in the pathogenesis of other diseases that involve monocyte infiltration. Its effects are mediated through the cell surface chemokine receptors CCR2 and CCR4, but of the two, CCR2, is the prime mediator of the effects of CCL2 and as such has been suggested to be an important therapeutic target [7].

In human term ~~labour~~labor, the mRNA and protein levels of CCL2 are increased in the choriodecidua, myometrium and cervix [8, 9]; while increased amniotic fluid CCL2 levels are present in samples from women in both preterm and term ~~labour~~labor [10, 11]. Similarly, in pregnant mice, serum and amniotic fluid CCL2 levels also increase with gestation [12]. While in rat studies, progesterone suppressed and RU486, an anti-progestin, increased myometrial CCL2 mRNA levels [13]. These data suggest that CCL2 may have a role in the onset of ~~labour~~labor in different species, but although RS504393, an efficient CCR2 antagonist, reduced myometrial monocyte numbers in response to RU486, the onset of ~~labour~~labor was not delayed [14], questioning the role of the CCL2/CCR2 system in the onset of ~~labour~~labor. Indeed, Nelson's group found that CCR2 knockout mice had a normal pregnancy duration

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with similar litter sizes and pup birthweight [15]. Consistent with CCR2 knockout, on E18 myometrial F4/80 transcripts did not increase, confirming defective monocyte trafficking, but Gr-1 transcript levels were normal suggesting granulocyte migration was normal [15].

The CCR2 knockout mouse model has been used mainly in studies of respiratory system, where the CCR2 defect markedly impaired monocyte recruitment to the site of inflammation. In CCR2-/- mice, after a *mycobacterium bovin* challenge, IFN-γ producing cells in draining lymph node were 70% lower [16]. Knockout of CCR2 also impaired dendritic cell trafficking, activation and maturation, again after *mycobacterium bovine* antigen challenge with reduced macrophage infiltration into the injected site. In the CLP induced mouse model of sepsis, TLRs regulated the CCR2 responsiveness in neutrophils via NF-kB and the absence of CCR2 or its inhibition improved survival ~~in experimental sepsis~~ [17], indicating direct or indirect effects on the CCL2/CCR2 axis on neutrophil as well as monocyte trafficking and function.

In this study, the response of the pregnant CCR2-/- mouse on E16 to LPS (10µg serotype 0111:B4) administered via intrauterine (i.u.) and intraperitoneal (i.p.) injection, which mimicked direct local myometrial inflammation and acute systemic inflammation respectively, is investigated. Specifically testing the hypothesis that CCL2/CCR2 system is critical to the onset of PTL, pup death and the systemic inflammatory and cardiovascular reaction to LPS.

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Materials and methods

Animals

Animal studies were performed under UK Home Office License 70/6906 (University College London) or 70/7372 (Imperial College London) and in accordance with the UK Animals (Scientific Procedures) Act of 1986. Virgin female and stud male CD1 outbred mice were purchased from Charles River (Margate, UK) at 6-8 weeks of age. Female CCR2 knockout (CCR2-/-) on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor Maine, USA). All mice were maintained in open cages at 21 ± 1°C, with *ad libitum* access to food (RM1 diet, Lillico Biotechnology, UK) and water, and a 12:12 light/dark cycle regimen. All mice were ~~acclimatised~~acclimatized for a minimum of 72 hours before undergoing any regulated procedure or mating.

Outbreeding of transgenic strains

The CCR2 homozygous knockout mice were originally crossed to C57BL/6 mice. The strain was backcrossed to a CD1 background as CD1 mice are a particularly fecund strain and typically have much larger litters than C57BL/6. In addition, the greater robustness and reproduction of the outbred strain was considered to be sufficiently advantageous to offset the heterogeneity of the background and allow comparison with our inflammation-induced preterm labour model in CD1 mice. ~~To add genetic variation to these strains and allow comparison with our inflammation-induced preterm labour model in CD1 mice,~~ CCR2-/- female mice (classed as the F0 generation) were mated with CD1 outbred males. Heterozygous pups (F1 generation) from different litters were mated together, producing a heterogeneous F2 generation (25% CCR2-/-, 50% heterozygous and 25% wild type litters). Genotyping was performed to determine between CCR2-/-, heterozygous and wild type animals. The resulting CCR2-/- F2 generation (males and females) were interbred to expand the colony for experimentation.

Timed-mating

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Timed mating was employed to enable accurate timing of injections (i.u. or i.p.). Female mice (~~CD1~~wt; CCR2-/-) were mated with a stud ~~CD1~~wt male overnight. The presence of a copulatory plug in the vagina of the female indicated successful mating and was classed as E0 (day 0) of gestation.

Preterm ~~labour~~labor mouse model

Mouse model of localized intrauterine inflammation and preterm delivery and tissue collection

Intrauterine administration of LPS to rodents has been described by Elovitz [18]. An adapted version of this model has subsequently been established in our group and further optimized. A laparotomy followed by intrauterine LPS (0111:B4, Sigma) injection was performed on day 16 (E16) of gestation as previously described [19]. The number of live fetuses per horn was determined before injection. Uterus of both wild type and CCR2 knockout mice were collected from naïve virgin non-pregnant mice and from time-mated mice (mated overnight with the following day termed E0). For pregnant mice, samples were collected during the morning of E6, E16, E18 and during labor (defined after the spontaneous delivery of at least one pup ~~for the labouring samples~~) and snap frozen for molecular analysis. For LPS mouse model, tissue collections were performed at 3h, 7h and the onset of ~~labour~~labor after intrauterine injection of PBS or LPS. Dams were culled via exsanguination by cardiac puncture, followed by immediate cervical dislocation. For plasma collection, whole blood was centrifuged immediately at 1000x g for 10 minutes and snap frozen on dry ice. Maternal lungs, livers, myometrium, placenta and pup brain were collected. Tissue was processed as previously described [19].

Cell preparation and Flow Cytometry

Myometrial samples were weighed immediately after collection. The decidua basalis was cut away from the myometrial tissue and the decidua parietalis carefully removed from the myometrial tissue

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by mechanical scraping on ice. This removed the entire luminal and glandular epithelium. Approximately 0.2g of tissue was processed for FACS using an adapted method previously published by our group [20]. Tissue samples were finely minced with scissors and added to a gentle MACS M tube (Miltenyi Biotec Ltd, Cologne, Germany) containing 1ml of IC fixation buffer (eBioscience, San Diego, USA) and homogenized for 1 minute. 5ml of FACS Wash Buffer (FWB) (1x PBS, FCS 2%, Sodium Azide 0.1% and 2mM EDTA) was then added to the tube to stop fixation and the homogenate was then passed through a 40µm nylon cell strainer (BD Falcon, Bedford, USA), and the cell suspension was then centrifuged at 400xg for 5 minutes. Cells were then washed in PBS-FCS 2% and resuspended in permeabilising wash buffer (PWB) (1x PBS, 0.1% Sodium Azide, 0.5% BSA and 0.2% Saponin) and then incubated with antibodies at 4°C in the dark for 30 min. After staining, cells were washed in either FWB (unfixed samples) or PWB (fixed samples).

The fluorophore-conjugated rat ~~antimouse~~anti-mouse MAbs antibodies used included: Ly6C (HK1.4), Gr1 (RB6-8C5), NK1.1 (PK136), F4/80 (CI:A3-1), CD45 (30- F11) (BioLegend), COX-2 (M-19) (Santa Cruz Biotechnology), CD86 (B7-2), CD11b (M1/70), MHCII (AMS-32.1), and F4/80 (BM8) (eBioscience). Levels of CD86 and COX-2 were calculated after subtraction of an isotype-matched control antibody to calculate MFI levels (IgG2a K Isotype Control (eBR2a, eBioscience)). FACS analysis was performed as previously described [19].

Mouse model of systematic inflammation and telemetry

Telemetry device implantation, recording, acquisition and data analysis

Telemetry probes (PA-C10, Data Sciences International) were surgically implanted as previously described [21]. On day 8 after surgery, continuous 24 hour recording (data collection) began using the Dataquest ART Acquisition System (Data Sciences International, v4.1) for at least 48 hours to collect data in non-pregnant animals (non-pregnant baseline). These data allowed animals to act as their own

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controls. Radiotelemeters were magnetically activated and deactivated. Whilst recording, animals were kept in their home cages with access to food and water ad libitum; however these cages were placed in a separate room for recording. This environment was chosen to cause fewer disturbances from other facility users and staff. Collected parameters included mean arterial pressure (MAP), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), heart rate (HR), and activity. Haemodynamic data were collected for 10 seconds every 30 seconds. ~~Labour~~Labor time and litter sizes were also recorded. Accurate ~~labour~~labor time was enabled by the use of infrared IP cameras in conjunction with the AVerDiGi (NV3000 Series) HYBRID Surveillance Platform (RF Concepts, Dundonald, UK).

Intraperitoneal (i.p.) injection of LPS

After telemetry implantation surgery, the intrauterine administration of LPS, which requires another laparotomy, was not possible under the current Procedure Project ~~Licence~~License (PPL). Therefore we aimed to establish a systemic endotoxaemic model using the intraperitoneal (i.p.) route.

Dams received a single i.p. injection of 10µg LPS (serotype O111:B4; Sigma-Aldrich, Poole, UK) per dam (in 100µL PBS) which resulted in no maternal mortality and gave the most consistent outcomes in latency to ~~labour~~labor and pup survival or vehicle control (PBS) were administered systemically (i.p.). Pregnant ~~CD1/~~wt and CCR2-/- mice were ~~anaesthetised~~anaesthetized under isoflurane for i.p. injection to ~~minimise~~minimize potential harm to fetuses and were carried out between 8am and 10am.

Model reproducibly and effect sizes

For i.u and i.p LPS models, animals were randomly selected for LPS or vehicle control administration. Both groups injected alongside each other during every surgical session to avoid bias. Surgeries were performed at the same time during each experimental session (between 1000 and 1200 h) to ensure that the gestational stage was as similar as possible between mice, thus reducing variation in the model. Intermediate collection time points were determined once the latency to labor post-LPS

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administration was calculated. Due to pregnancy rates and to again avoid collection bias, multiple time point collections were performed on the same day, while laboring samples were collected over various days. We mated enough animals to ensure that we had a minimum of six animals at each time point. The sample size was chosen based on previous *in vivo* work [22] and the requirements for statistical normality testing. When the mating proved to be more successful than expected, we used the excess animals to increase numbers at the different time points. A single pregnant dam represents a single experimental unit throughout this study.

Genomic DNA extraction and genotyping PCR

The ear clips from tested mice were taken and put in 75µl of solution 1 (NaOH 25mM, EDTA Na2 0.2mM), then vortexed. The sample was heated to 95°C for 10min and cooled down to 4°C. Solution 2 (Tris-HCl, pH8.0 40mM) was added and, the whole solution was vortexed and spun down. 4µl of final solution was used and mixed with PCR reaction mix which containing 2 µl PCR Buffer (×10), 2 µl dNTP's (2mM), 0.6 µl Mgcl2 (50mM), 1 µl of primer F (10µM), 1 µl of primer R (10µM), 4 µl of DNA, 9.3 µl dH2O and 0.1 µl Taq (5U/ µl). Genotyping PCR thermocycler condition for CCR2-/- was 95°C for 5min, 95°C for 30sec, 65°C for 1min, 72°C for 2min and, after 35 cycles heated 75°C for 10min and 4°C for 10min.

Quantitative RT-PCR

Total RNA was extracted and purified from cell or tissue samples using RNeasy mini kit (Qiagen Ltd, UK). After quantification, 1.0 µg was reverse transcribed with oligo dT random primers using MuLV reverse transcriptase (Applied Biosystems Ltd, UK). 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) that continually measures fluorescence caused by the binding of the dye to double-stranded DNA. In order to correct the variations, data were expressed relative to the amount of constitutively expressed housekeeping gene.

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Western blot analysis

Denatured protein samples (40-60ug) were loaded in 10-15 well gels (Invitrogen, UK) and run on 80-130 volts. After that, an electrophoretic transfer onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) was performed. Membranes were blocked in washing buffer (5M NaCL, Tris-HCL and Tween) containing 5% milk for 1 h at room temperature, washed in washing buffer and hybridized with the primary antibody over night at 4°C. Membranes were washed again and then incubated with secondary antibody at a dilution of 1:2000 for 2 h at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) was used for detection. Protein band size was determined using Novex Sharp Pre-stained Protein Standard (Invitrogen, UK).

ELISA

ELISA assay procedure was followed according to manufacturer's instructions (R&D Systems, Abingdon, UK). Plasma samples were diluted 1:2 in Assay Diluent RD1-14 and mixed well. The calculation of concentrations was performed using standard concentrations for comparison fit with a 4-parameter logistic curve.

Statistical analysis

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

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Results

Myometrial chemokine mRNA levels before and during pregnancy.

~~We~~In myometrial samples obtained before and during pregnancy, we measured ~~myometrial chemokine~~the mRNA levels of chemokines that ~~showed~~we had found previously to be elevated in myometrial samples taken at the time of human ~~labour before and during pregnancy (~~labor [3](supplementary figure 1A-E). We found that the myometrial mRNA levels of CCL2, CCL5 and CXCL1 were initially low in wt mice on E6, but increased to a peak on E18, in contrast, CXCL5 mRNA levels remained static throughout gestation and those of CCL20 declined. In CCR2-/- mice, myometrial chemokine mRNA levels were higher at baseline ~~(p=0.05-0.01),~~ through pregnancy and with the onset of parturition than in wt mice ~~and remained unchanged over gestation, however, with~~ (p=0.05-0.01). With advancing gestation and the increase in wt myometrial chemokine mRNA levels, the ~~difference was no longer significant on E18 in some cases~~differences were less consistent (supplementary figure 1A-E).

Inflammatory cell distribution on E16

We determined myometrial ~~inflammatory~~ myeloid cell numbers in different compartments (expressed per g of tissue or per ml of blood) on E16 (Fig.1) in CCR2-/- and wt ~~CD1~~ mice. ~~We then assessed basal leukocyte numbers (per g of tissue or ml of~~ In the myometrium, placenta and blood~~) on E16 and found that~~, the pattern was similar, with higher neutrophils and lower Ly-6C^{high} and Ly-6C^{low} monocytes ~~were significantly lower~~ in ~~all compartments~~the CCR2-/- mice (p~~=0.05-0.01),~~<0.05-0.001; Fig.1A-C); in the myometrium and placenta, macrophage counts were also lower in the CCR2-/- mice (p<0.05 and p<0.001 respectively; Fig.1A&B). In the lung and liver, neutrophil counts were similar, but Ly-6C^{high} and Ly-6C^{low} monocytes were ~~also significantly~~again lower in ~~all compartments except in~~ the ~~circulation (p=0.05-0.001) and neutrophils were significantly increased in the myometrium, circulation and placenta (p=0.01) of~~ CCR2-/- ~~vs. wt~~ mice (p<0.05-0.01; Fig.~~1a-e) but not in the lungs or liver.~~1D&E). In the myometrium, ~~resident macrophage numbers were lower (p=0.05), but~~ the expression of cell

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activation markers, COX-2 and CD86~~,~~ was greater on CCR2-/- macrophages in CCR2-/- vs. wt mice (p=0.01 and 0.001 respectively; supplementary figure 2A&B).

Pregnancy outcomes after LPS

To investigate the significance of the modified myometrial response in CCR2-/-, we used the localized septic insult model of sepsis i.u. injection of 10µg serotype 0111:B4. LPS ~~labour~~labor started after 14 hours in both groups (Fig.~~2a~~2A). However, at ~~the two time points (~~7 hours ~~and labour), the numbers of live or dead pups was dramatically different. At 7 hours~~post LPS, in wt mice, 70% of pups were dead vs. 10% in the CCR2-/- mice (p<0.001), but ~~at the time of labour~~with parturition, 100% of wt and 90% of CCR2-/- pups were dead (Fig.~~2b~~2B).

Inflammatory cell responses to LPS on E16

Next, we compared numbers of inflammatory cells in different ~~organs and in the circulation~~compartments in both wt and CCR2-/- mice after i.u. LPS. Intriguingly, in the circulation, inflammatory cells numbers were comparable in the two groups at both 7 hours post LPS and in ~~labour~~labor (Fig.~~3A~~3C). In contrast, in the myometrium ~~in CCR2 -/- vs. wt mice,~~ Ly-6C^{low} monocytes were consistently lower at both time points (both p<0.05), neutrophil numbers were higher and macrophage numbers were lower at 7h (both p<0.05~~)~~), both were similar in ~~labour~~labor, and Ly-6C^{high} monocytes were comparable at both time points ~~(Fig.3B). In the placenta~~ in CCR2-/- vs. wt mice (Fig.3A). In the placenta, Ly-6C^{low} monocytes at 7h and Ly-6C^{high} monocytes at ~~labour~~labor were lower (p<0.001 and p<0.05 respectively), macrophages were lower at both time points (both p<0.01) and neutrophils similar at both time points (Fig.~~3C).~~3B) in CCR2-/- vs. wt mice. In the lung, like the circulation, cell numbers were similar with the exception of Ly-6C^{low} monocytes, which were lower at 7 hours ~~in CCR2 -/- vs. wt mice~~ (p<0.001; Fig.3D~~). The same was true~~); similarly in the liver, except that the Ly-6C^{high} monocytes were lower in ~~labour~~labor in CCR2-/- vs. wt mice (p<0.05; Fig.3E).

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Myometrial cytokine mRNA and kinase responses to LPS on E16

We compared the effect of LPS on myometrial cytokine mRNA levels in CCR2-/- compared to wt mice. At 7h post i.u. LPS, the myometrial mRNA levels of CCL2 ($p<0.01$) and CCL5 ($p<0.05$) were higher and of IL-1β ($p<0.01$) and TNFα ($p<0.05$) were lower (Fig. 4A). In ~~labour~~labor, the myometrial mRNA levels of CCL5 ($p<0.01$) were higher and of CCL2 ($p<0.05$) and IL-1β ($p<0.01$) were lower in CCR2-/- mice (Fig. ~~4b). 4~~4B).

These changes in cytokine mRNA levels were associated with greater phosphorylation of ERK1/2 ($p<0.05$-0.01) in CCR2-/- myometrial samples at 7h and ~~labour~~labor and reduced phosphorylation of p65 at 7h ($p<0.05$) and of JNK ($p<0.01$) in ~~labouring~~laboring samples; COX-2 levels were lower at 7h only ($p<0.05$; Fig.4C&D). In basal, non-pregnant and E16 myometrium from wt and CCR2-/- mice, ERK1/2 activity was greater in the CCR2-/- mice~~. In myometrial samples from E16 pregnant mice, JNK phosphorylation was reduced and COX-2 levels were increased in the CCR2-/- mice (Supplementary figure 3A&B).~~ (data not shown).

Pup brain and placental and cytokine mRNA response to LPS on E16

To understand whether differences in pup inflammation were responsible for the more acute demise of the wt pups, we studied the mRNA levels of cytokines in pup brain and placenta obtained at 3h and 7h post LPS and during ~~labour~~labor (Fig5A-F).

In the pup brains, at 3 hours, the mRNA levels of CXCL1 were higher ($p<0.01$) and IL-1β lower ($p<0.05$) in CCR2-/- pup brains (Fig.5A); at 7h the mRNA levels of CCL2, CCL5 and IL-1β were higher in CCR2-/- pup brains ($p<0.05$-0.001; Fig.5B); and in ~~labour~~labor, the mRNA levels of CCL2, CCL20, CXCL1 and IL-1β were higher and ~~level~~ of ~~CCL6~~IL-6 and were lower in CCR2-/- pup brains ($p<0.05$-0.001; Fig.5C).

In the placenta at 3 hours, cytokine mRNA levels were similar in both groups, at 7h they tended to be lower in CCR2-/- mice~~,~~ but were only significantly lower in the case of CXCL1 ($p<0.01$; Fig.~~6E~~5E). In

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~~labouring~~laboring samples, placental mRNA levels of CCL2, CCL5, CCL20 and CXCL1 were lower in CCR2-/- mice (Fig.~~6F~~5F; p<0.05-0.01). These changes in gene expression were associated with reduced placental phosphorylation of ERK1, JNK and p65, COX-2 levels ~~were also reduced~~ at 7h in CCR2-/- vs. wt mice (all p<0.05; Supplementary figure ~~4A~~3A). In ~~labouring~~laboring samples of placenta, the findings were similar with reduced phosphorylation of ERK1/2 and p65~~, with reduced~~ and lower COX-2 levels ~~also,~~ in CCR2-/- vs. wt mice (p<0.05-0.001; Supplementary figure ~~4B~~3B).

Circulating IL-6 levels in response to i.u. LPS on E16

Plasma IL-6 level in both ~~CD1~~wt and CCR2-/- mice were measured before and during pregnancy, no differences were observed (Supplementary figure ~~5A~~4). In mice injected with i.u. LPS (Supplementary figure 4A) and in sham mice injected with i.u. PBS ~~and in mice injected with i.u. LPS,~~(Supplementary figure 4B), the levels of IL-6 were increased at 3 hours, they remained elevated in both wt and CCR2-/- mice injected with LPS at 7 hours and in ~~labour~~labor, but declined in those injected with PBS (Supplementary figure ~~5B~~4A&B). There were no differences between the 2 groups.

Haemodynamic response to LPS

We used the i.p. LPS PTL model to assess the effect of a direct systemic septic challenge on the haemodynamic response in pregnant wt and CCR2-/- mice. We found that, in contrast to the fall in blood pressure in wt mice, there was a transient increase in all blood pressure parameters in response to LPS in CCR2-/- mice, with a peak elevation in MAP of Δ28.24 ± 8.5 mmHg at 5 hours post-LPS administration (p<0.05-0.001; Fig.~~7A~~6A-C). Blood pressure parameters were then maintained at baseline levels, which were higher than in wt mice (Fig.~~7A~~6A-C). The nadir in MAP of Δ-31.1 ± 7.06 mmHg in wt mice was seen at 12 hours post-LPS, after which, this was maintained and did not resolve (Fig.~~7A~~6A-C). The HR fell acutely in both wt and CCR2-/- mice, reaching a nadir at 1-3 hours post LPS. The decline in HR and recovery to baseline was less marked and quicker respectively in CCR2-/- mice than in wt mice (p<0.05; Fig.~~7D~~6D). The ~~pups~~onset of labor and pup survival post IP LPS were ~~all~~

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~~dead~~similar for both ~~strains during labour.~~groups.

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Discussion

In this study, we have confirmed previous data showing a normal pregnancy outcome in CCR2-/- mice [15]. We demonstrated marked differences in basal myometrial chemokine mRNA levels and inflammatory cell numbers with increased neutrophils and reduced monocyte/macrophage numbers. Similar changes were observed in the placenta, blood, lung and liver. ~~These~~Despite these basal differences ~~did not result in any~~, there was no delay in the onset of LPS-induced preterm birth, despite a reduced myometrial inflammatory response, ~~but did delay~~and although pup death~~,~~ was delayed, it was ultimately similar in ~~association with reduced placental inflammation.~~both wt and CCR2-/- mice. The marked hypotensive response to LPS we previously reported was apparent in wt mice ~~was~~[21] but prevented in CCR2-/- mice. These data suggest that the CCL2/CCR2 system mediates some of the inflammatory response to LPS, but that although pup demise is delayed, ultimately ~~both~~neither is the onset of ~~PTL and~~labor delayed nor pup survival ~~are not~~ improved by inhibition of CCL2/CCR2 system. In contrast, the hypotensive response to LPS was blocked, suggesting a potential approach to the management of septic shock.

Myometrial chemokine mRNA levels were higher in non-pregnant CCR2-/- mice as has been reported in other tissues previously for CCL2, CCL10, CCL23 (MIP-3) and CXCL2 (MIP-2α) ~~were also reported to be greater~~ in CCR2-/- mice [23-25]. ~~In our preliminary data, using a different FACs strategy, we found more monocytes in CCR2 -/- mice in both none pregnant~~With pregnancy and ~~E18. With~~ advancing gestation, chemokine levels remained static in CCR2-/- mice and rose in wt animals to similar levels by E18 in most cases. On E16, myometrial inflammatory cells showed marked differences~~, consistent~~ with ~~a failure~~lower numbers of ~~monocyte infiltration~~monocytes and an increase in neutrophils, the latter presumably driven by the increase in chemokines. The lower levels of monocytes are consistent with previous observations [25-28]~~. Similarly, intact polymorphonuclear neutrophils (PMN)~~ and neutrophil and lymphocyte infiltration has been reported to be intact in CCR2-/- mice [23, 29], with a relatively increased ~~PMN~~neutrophil tissue infiltration in some circumstances [25, 28] including in an influenza mouse model [30, 31]. The greater neutrophil numbers were suggested to be the result of

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increased neutrophil CXCR2 expression or perhaps the increased levels of eotaxin [30, 31], although the exact effect ~~on~~of eotaxin on neutrophils is debated [32]. The macrophage population did show greater expression of activation markers, suggesting that although numbers were reduced they may be more functionally active. The differences in cell numbers and activity was associated with higher ERK1/2 and lower JNK phosphorylation and increased in COX-2 protein levels. How changes in CCR2-/- status might result in increased ERK1/2 and reduced JNK phosphorylation is difficult to explain and the importance of the differential activation of MAPK subtypes unclear since there seems to have been lower inflammatory cytokine mRNA levels in the myometrium of CCR2-/- compared to wt mice.

Despite the difference in basal myometrial inflammatory cell numbers, the onset of LPS-induced PTL was not delayed in CCR2-/- vs. wt mice, with the onset of ~~labour~~labor occurring some 14 hours later in both groups similar to previous reports [33-35]. This is intriguing as previously, the administration of a broad-spectrum chemokine antagonist was able to inhibit LPS-induced PTL [36]. ~~This may imply~~It is possible that although the monocyte and macrophage counts were lower in CCR2-/- mice after LPS, the greater numbers of neutrophils may have been able to compensate. Equally, inflammatory cytokine mRNA levels were lower and, although the greater phosphorylation of ERK1/2 persisted post LPS, both p65 phosphorylation and COX-2 protein levels were lower. The greater activation of ERK may have contributed to the increase in neutrophil numbers as ERK is necessary for both neutrophil extracellular trap formation [37] and adhesion of neutrophils [38]. In terms of the lower COX-2, CCL2 has been shown to enhance COX-2 expression in endothelial cells [39] and the loss of the CCL2 effect may have contributed to the lower levels of myometrial COX-2. These data suggest that the myometrial inflammatory response was reduced in CCR2-/- mice, however the time of onset of parturition was similar in both wt and CCR2-/- mice. ~~This implies~~These data imply that LPS does not act via cells of the monocyte-macrophage lineage to induce ~~labour, alternatives include a direct effect on neutrophils or on myometrial cells to enhance their contractility. Previous work~~labor, as suggested by the finding that LPS acts via monocytes to enhance myometrial contractility [40]~~, but~~. Our observations are supported by the fact that we ~~showed~~found that reducing the RU486-induced

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monocyte infiltration of the myometrium ~~induced by RU486 had no effect on the timing of labour[14],~~ ~~supporting the idea that monocytes are~~ did not ~~important in~~delay the onset of ~~labour.~~labor [14].

Interestingly, pup survival was improved at 7 hours post LPS in CCR2-/- mice. This proved to be only a temporary phenomenon as by the onset of ~~labour~~labor virtually all pups were dead in both wt and CCR2-/- mice. Inflammatory cytokine mRNA levels were ~~lower~~only reduced for IL-1ß in CCR2-/- pup brains at 3 hours, suggesting that pup inflammation was not significantly lower in CCR2-/- ~~mice, which may have contributed to the improved pup outcomes in the short term. In the placenta,~~pups. Placental LY6C^{low} monocytes and macrophages numbers were reduced vs. wt mice at 7 hours~~,~~ but cytokine mRNA ~~was similar, although ERK1, JNK and p65 phosphorylation and COX-2~~ levels were ~~lower at 7h. Collectively, these data suggest~~similar, again suggesting that ~~placental~~ inflammation was ~~probably overall~~not reduced in ~~CCR2 -/- mice and have also contributed to~~ the ~~early improvement in pup survival.~~ placenta either. Previous studies on the impact of LPS in rat pregnancy suggest that fetal demise was a result of reduced placental perfusion [41], indeed this was our impression when we studied the effect of LPS in CD1 mice [19]. Others have reported that LPS can induce preeclampsia-like symptoms in rats, but Yamashita et al. showed that 80 mg/kg intraperitoneal (i.p.) LPS caused a greater than 90% mortality in WT (C57bl/6) male mice and triggered a severe and progressive drop in mean arterial pressure (MAP) of about 60 mm Hg, a 40% reduction from pretreatment values (mean 97 mm Hg) [42]. In our study, mice received intraperitoneal LPS (10 µg) on E16 induced a greater fall in blood pressure in pregnant mice. This was consistent with our previous study [21] ~~Here, we found that the hypotensive response to LPS, that we previously reported in pregnant CD-1 mice [21],~~ and was reproduced here in the wt mice. Remarkably, we found that this~~,~~ was reversed in the CCR2-/- mice. Indeed, rather than a hypotensive response, we recorded a transient increase in all blood pressure parameters. Previously, we suggested that the LPS-induced hypotensive response may be mediated through the local activation of inflammatory cells and their release of nitric oxide or another vasoactive compound. This suggestion was based on the observation of a greater lung margination by

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inflammatory cells in pregnant compared to non-pregnant mice [21]. Here, in the CCR2-/- mice, other than a reduced lung margination of Ly6C^{low} monocytes at 7h and lower Ly6C^{high} monocytes numbers in ~~labouring~~laboring liver samples, we observed a surprisingly similar inflammatory cell response to LPS in terms of circulating, lung and liver inflammatory cell changes and the response of circulating IL-6 was similar, again suggesting that the systemic inflammatory response was ~~similar~~equivalent in both groups implying that there must be another explanation for the difference in cardiovascular response. It is possible that there was a differential activation of the inflammatory cells or that the CCL2/CCR2 system has direct effects on vascular function. Chemokines are thought to regulate blood pressure via the control of inflammation in the vessels and in the heart. Inflammatory cell infiltration and oxidative stress in vessel walls contributes to an increase in blood pressure[43]. ~~Indeed,~~However, the lack of functional CCR2 receptors has been associated with hypertension in humans [44, 45], also CCL2 modulates pulmonary vascular endothelial function [46], suggesting that the CCL2/CCR2-/- system does have an effect on vascular function. Other studies investigated the impact of CCR2 inhibition in the response to sepsis and found improved survival in the caecal ligation and puncture model [17]; the benefits were suggested to be through reduced neutrophil organ infiltration [17], but the cardiovascular response was not assessed in these studies. The improvement in sepsis outcomes mediated by IL-10 is thought to be mediated at least in part through the repression of neutrophil CCR2 expression [47]. Once the mechanism of action of CCR2 antagonism in sepsis is understood, this approach may prove to be helpful in the management of sepsis particularly as an alternative to vasopressors in the maintenance of blood pressure. In the LPS-PTL model, pup demise may still be because of reduced utero-placental perfusion, which may have contributed to the increase in systemic blood pressure, but this possibility awaits further study.

This study shows that CCR2-/- does not prevent LPS-induced preterm delivery~~,~~ but reverses its hypotensive effect. Basally, and in response to LPS, ~~broadly,~~ monocyte and macrophage numbers are reduced and neutrophil numbers increased in most, but not all compartments~~. These data suggest~~, suggesting that monocytes do not play an essential role in the LPS-induced onset of PTL, but are

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important in the cardiovascular response to LPS during pregnancy. As such CCR2 antagonism may be useful adjunct to the management of sepsis in pregnancy.

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Legends

Figure 1 Inflammatory cell numbers on E16 in wild type and CCR2-/- mice.

Leukocyte cell count and densities in the myometrium (A), placenta (B), circulation (C), lung (D) and liver (E) from untreated CCR2-/- and wild type mice on E16. ~~The raw data~~Data were expressed as median, interquartile range and range and were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when the data were not. ~~(~~* indicates a difference of p<0.05, ** of p<0.01, *** of p<0.001). n=5-6.

Figure ~~2~~A2 Effect of iu LPS on interval to parturition and pup survival on E16 in wild type and CCR2-/- mice. ~~After operation, the time of delivery of CD1~~

Wild type and CCR2-/- mice ~~was recorded. Preterm delivery occurred in both CD1 and CCR2 -/- after~~on E16 were administered intrauterine LPS ~~injection. However, there was no difference between the two genotypes. n=6 for this experiment.~~(10µg serotype 0111:B4) or PBS. **A:** Interval to delivery (hours). **B:** ~~Figure 2B Pup survivals of CD1 and CCR2KO mice after intrauterine LPS injuction~~ The percentages of live pups ~~were recorded.~~ Data were ~~analyzed~~expressed as median, interquartile range and range and were analysed using ~~multiple~~an unpaired t-test~~. ***~~ when normally distributed and a Mann Whitney test when the data were not. ** indicates a significant difference ~~of~~ of p<0.~~001~~01, n=6.

Figure 3 Cell density of leukocytes in the myometrium, circulation, placenta, lung and liver of wild type and CCR2 KO mice following intrauterine injection of LPS

Leukocyte cell count and densities in the ~~circulation (A),~~ myometrium (~~B~~A), placenta (B), circulation (C), lung (D) and liver (E) from CCR2-/- and wild type mice on E16 after intrauterine LPS (10µg serotype 0111:B4). Data were expressed as median, interquartile range and range~~. Cell densities~~ and were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when the data

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were not. Statistical significance ~~were expressed with * indicates~~was indicated by * indicating a difference of p<0.05, ** of p<0.01, *** of p<0.001, n=5-6.

Figure 4 Effect of LPS on myometrial cytokine mRNA, COX-2 protein, phosphorylated ERK1/2, c-Jun and p65 levels.

~~A-B: At 7 hours in LPS treated mice, the myometrial expression of CCL2 and CCL5 was higher and of IL-1β and TNFα was lower in the CCR2-/- mice than in CD1 mice. In labouring samples from LPS treated mice, the myometrial expression of CCL5 was higher and of CCL2 and IL-1β was lower in CCR2-/- compared with CD1 mice.~~

~~C-D: 7 hours after LPS administration, COX-2 levels and the phosphorylation of p65 were lower and ERK1/2 phosphorylation was higher in the CCR2-/- myometrial samples. In labouring samples, the phosphorylation of ERK1/2 was increased and of c-jun was reduced.~~

~~Data were analyzed using unpaired t-test or Mann-Whitney test if data were not normally distributed. n=6 for this experiment. * indicates a significant difference of p<0.05, ** indicates p<0.01, *** indicates p<0.001.~~

Myometrium was obtained at 7 hours and in labor after the administration of LPS (10µg serotype 0111:B4) from wild type and CCR2-/- mice. **A-B:** Myometrial cytokine mRNA levels were measured using rtPCR. Data were expressed as median, interquartile range and range. **C-D:** Myometrial COX-2 and phosphorylated ERK1/2, c-Jun and p65 levels measured by western analysis and densitometry. Data were expressed as mean+SEM. The data were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when the data were not.* indicates a significant difference of p<0.05, ** indicates p<0.01, *** indicates p<0.001.

Figure 5 The effect of LPS on ~~CD1~~wild type and CCR2-/- pup brain and ~~placentae~~placental cytokine mRNA levels

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~~**A-C:**~~ Pup brain **(A-C)** and placenta **(D-F)** were obtained from the left horn ~~was harvested~~ at 3 hours, 7 hours and during ~~labour~~labor after intrauterine LPS (10µg serotype 0111:B4) injection. ~~At 3 hours post injection, the pup brains taken from the LPS treated CCR2 -/- mice showed higher CXCL1~~Cytokine mRNA levels were measure by rtPCR. Data were expressed as median, interquartile range and ~~lower IL-1β. At 7 hours, LPS exposed CCR2 -/- pups expressed higher levels of CCL2, CXCL1~~range and ~~IL-1β. In labouring samples, LPS exposed CCR2 -/- pups expressed higher levels of CCL2, CCL20, CXCL1 and IL-1β, but lower levels of IL-6. Data were analyzed using~~ were analysed using an unpaired t-test ~~or~~ when normally distributed and a Mann Whitney test ~~if data were not normally distributed. n=6 for this experiment. Data were presented as median with interquartile range.~~when the data were not. * indicates a significant difference of p<0.05, ** indicates p<0.01.

~~**D-F:** At 3 hours after LPS treatment there was no difference in chemokine or cytokine mRNA expression in placentae taken from CCR2 -/- and CD1 mice. At 7 hours, the expression of CXCL1 was less in CCR2 -/- when treated with LPS. In placentae obtained from labouring mice after LPS injection, the expression of CCL2, CCL5, CCL20 and CXCL1 was lower in the CCR2-/- samples. Data were analyzed using unpaired t test or Mann Whitney test if data were not normally distributed. n=6 for this experiment. Data were presented as median with interquartile range. * indicates a significant difference of p<0.05, ** indicates p<0.01~~

Figure 6 The haemodynamic response to LPS-induced inflammation in CCR2-/- mice.

The haemodynamic response in CCR2-/- and ~~CD1~~wild type mice was recorded for 24-hours following LPS administration (10µg serotype 0111:B4; intraperitoneal) on E16. (A) ΔMAP (B) ΔSAP (C) ΔDAP (D) HR. *p<0.5, **p<0.01, ***p<0.001, trends were compared using 2-way ANOVA and individual time points were compared using 2-way ANOVA and Bonferroni post-hoc test. Data are expressed as a mean change from pre-LPS baseline (pre) compared to PBS controls ± SEM (~~CD1~~wt, n=7; CCR2-/-, n=4).

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Supplementary figures

Figure 1 ~~CD1~~Wild type and CCR2KO mouse myometrial chemokine expression.

~~Myometrial~~Myometrial samples were obtained from non-pregnant and pregnant wild type and CCR2KO mice and chemokine, CCL2 (A), CCL5 (B), CCL20 (C), CXCL1 (D) and CXCL5 (E) mRNA levels measured by rt-PCR. Data are expressed as median, interquartile range and range~~. Data~~ and were compared ~~between CD1 and CCR2KO mice in different gestations. The raw data were analysed~~ using an unpaired t-test when normally distributed and a Mann Whitney test when the data were not~~.~~ * indicates a difference of p<0.05, ** of p<0.01, *** of p<0.001, n=6 at each time point.

Figure 2 Wild type and CCR2KO mouse myometrial ~~maxcrophage~~macrophage activation markers.

The activation status of myometrial macrophages as assessed by COX-2 and CD86 expression in the myometrium from untreated wild type mice and CCR2 KO mice on E16. Data are expressed as mean +SEM and were compared using an unpaired t-test ** indicates a difference of p<0.01 and *** of p<0.001, n=6.

Figure 3 The ~~**phosphorylation of myometrial NF-□B (p65), c-jun and ERK1/2 and COX-2 levels in CCR2-/- and CD1 mice.**~~

~~A-B: In NP myometrial samples, phosphorylation of ERK1/2 was increased in samples from CCR2-/- compared with samples from CD1 mice. In E16 myometrial samples, ERK1/2 phosphorylation and COX levels were increased and c-jun phosphorylation reduced in samples from CCR2-/- compared with samples from CD1 mice. Data were analysed using unpaired t tests, n=6 for this experiment. Data were presented as mean with SEM. * indicates a significant difference of p<0.05, ** indicates p<0.01, *** indicates p<0.001.~~

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~~Figure 4 The~~ effect of LPS on ~~CD1~~wild type and CCR2-/- mice placenta

~~a b: At~~Placenta were obtained from the left uterine horn at 3 hours and in labor after the administration of LPS ~~injection,~~(10µg serotype 0111:B4) from wild type and CCR2-/- mice. COX-2~~,~~ and phosphorylated ~~NF κB (p65), c jun and~~ ERK1 ~~were lower in CCR2 -/- placenta. At the time of labour after LPS injection, the phosphorylation of NF κB (p65) and ERK1 and ERK2~~/2, c-Jun and ~~the~~p65 levels ~~of COX2 were lower in CCR2 -/- placenta.~~measured by western analysis and densitometry. Data were ~~analyzed~~expressed as mean+SEM and were analysed using an unpaired t-~~-~~test ~~or~~when normally distributed and a Mann Whitney test ~~if~~when the data were not. * indicates a significant difference of p<0.05, ** indicates p<0.01, *** indicates p<0.001.

~~normally distributed. n=6 for this experiment. Data were presented as median with interquartile range. * indicates a significant difference of p<0.05, ** indicates p<0.01, *** indicates p<0.001.~~

Figure ~~5~~4 The effect of LPS/PBS on ~~CD1~~wild type and CCR2-/- plasma IL-6 level

~~A B : Throughout the gestation, labour and 3 hours, there is no change of plasma~~Plasma samples were obtained from wild type and CCR2-/- mice before conception, at E16 and during labor. Further samples were obtained from pregnant wild type and CCR2-/- mice after intrauterine PBS or LPS (10µg serotype 0111:B4). Plasma IL-6 ~~in both PBS~~ levels were measured by ELISA, expressed as median, interquartile range and range and analysed using an ANOVA and Bonferroni post-hoc test. ** indicates a significant difference of p<0.01.

Figure ~~LPS treated CD1 or~~ 5 Effect of intraperitoneal LPS or PBS on the interval to parturition and of intraperitoneal LPS on pup survival on E16 in wild type and CCR2-/- mice. ~~However, plasma IL-6 level in both PBS treated CD1~~

Wild type and CCR2-/- mice ~~at 3 hours is much higher than the level in~~on E16 were administered intraperitoneal LPS (10µg serotype 0111:B4) or PBS ~~treated mice at 7 hours, the time point that I~~

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~~consider~~. **A:** Interval to delivery (hours). **B:** The percentages of live pups. Data were expressed as ~~basal. At 7 hours and the time of labour, IL-6 in LPS treated CD1 and CCR2-/- mice is significantly higher in PBS treated ones. There is no difference between the two genotypes. Unpaired t test were used when data are~~mean+SEM and were analysed using an unpaired t-test when normally distributed~~,~~ and a Mann Whitney test ~~were used for non-parametric~~when the data~~. Data~~ were ~~presented as median with interquartile range. * Indicates~~ not. *** indicates a significant difference of p<0.~~05, ** indicates p<0.01.~~ 001, n=6.

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Figure 1

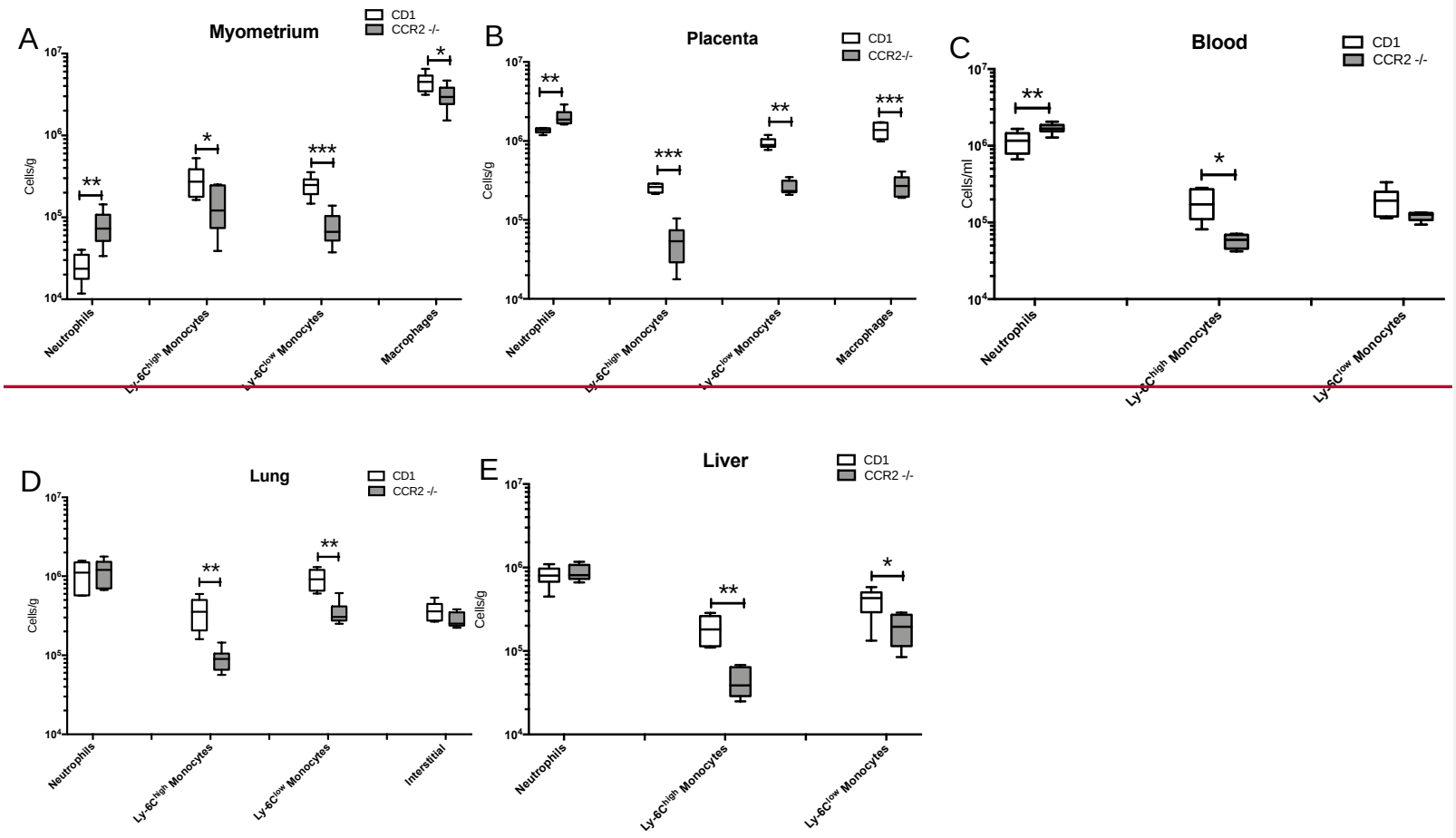

A
Myometrium
CD1
CCR2 -/-
Cells/g
Neutrophils
Ly-6C^high Monocytes
Ly-6C^low Monocytes
Macrophages
B
Placenta
CD1
CCR2-/-
Cells/g
Neutrophils
Ly-6C^high Monocytes
Ly-6C^low Monocytes
Macrophages
C
Blood
CD1
CCR2 -/-
Cells/ml
Neutrophils
Ly-6C^high Monocytes
Ly-6C^low Monocytes
D
Lung
CD1
CCR2 -/-
Cells/g
Neutrophils
Ly-6C^high Monocytes
Ly-6C^low Monocytes
Interstitial
E
Liver
CD1
CCR2 -/-
Cells/g
Neutrophils
Ly-6C^high Monocytes
Ly-6C^low Monocytes

Figure 2

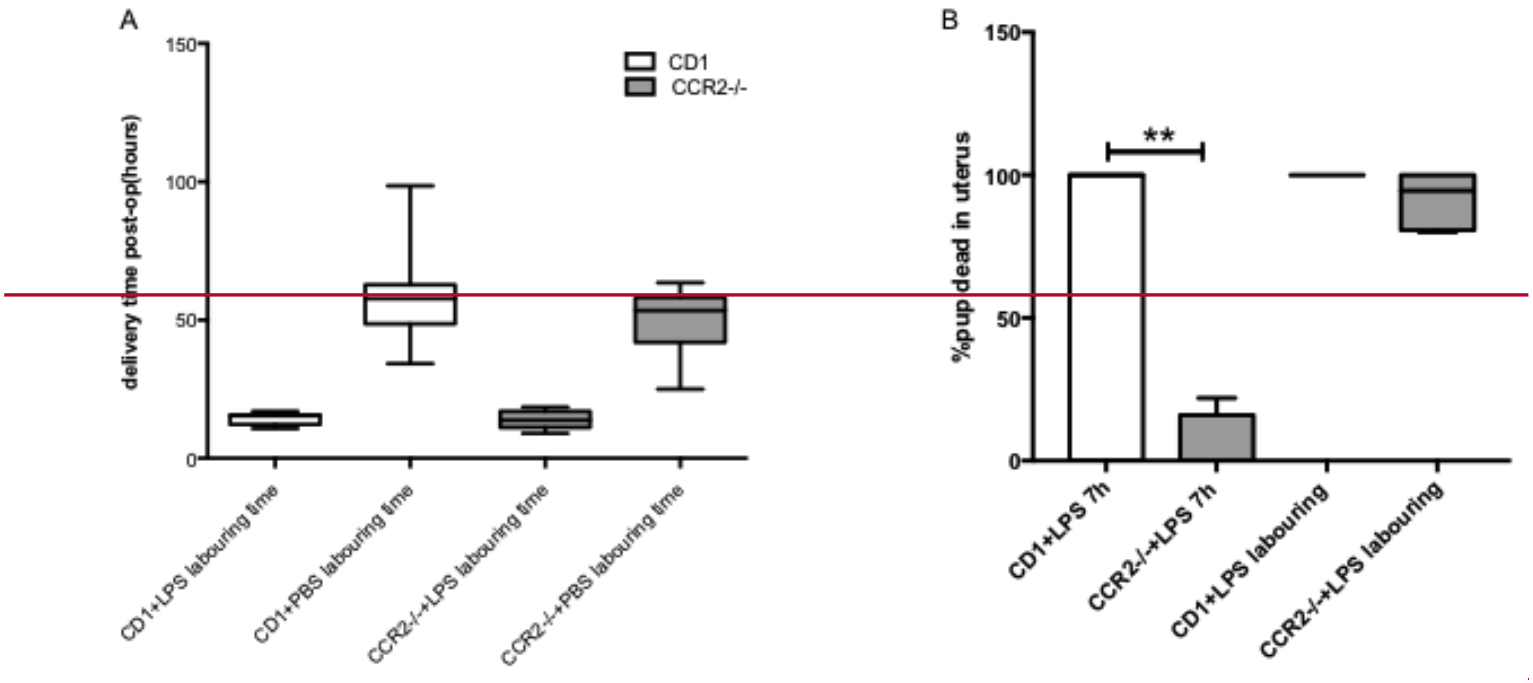

A
CD1
CCR2-/-
delivery time post-op(hours)
150
100
50
0
CD1+LPS labouring time
CD1+PBS labouring time
CCR2-/-+LPS labouring time
CCR2-/-+PBS labouring time
B
%pup dead in uterus
150
100
50
0
**
CD1+LPS 7h
CCR2-/-+LPS 7h
CD1+LPS labouring
CCR2-/-+LPS labouring

Figure 3

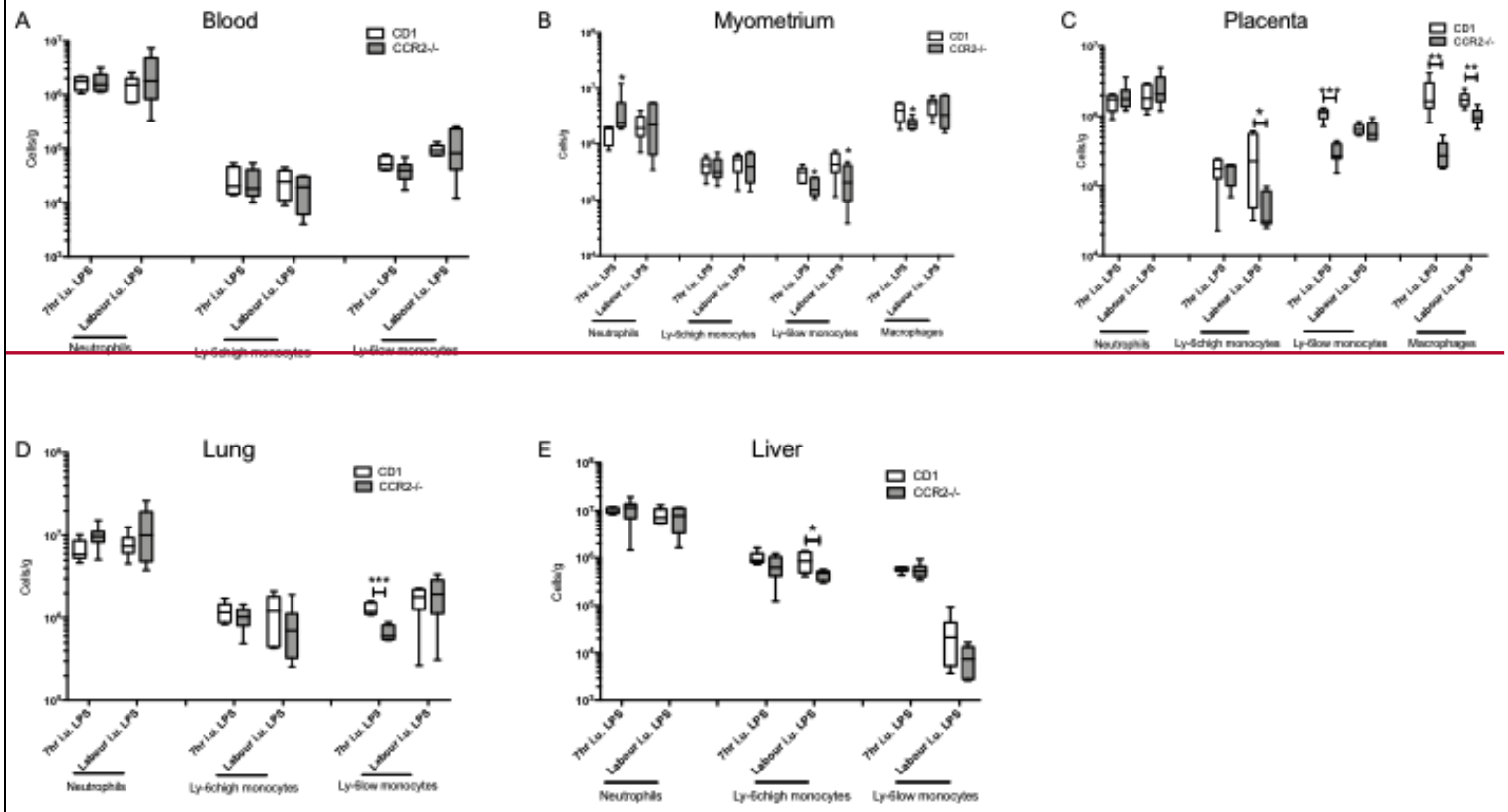
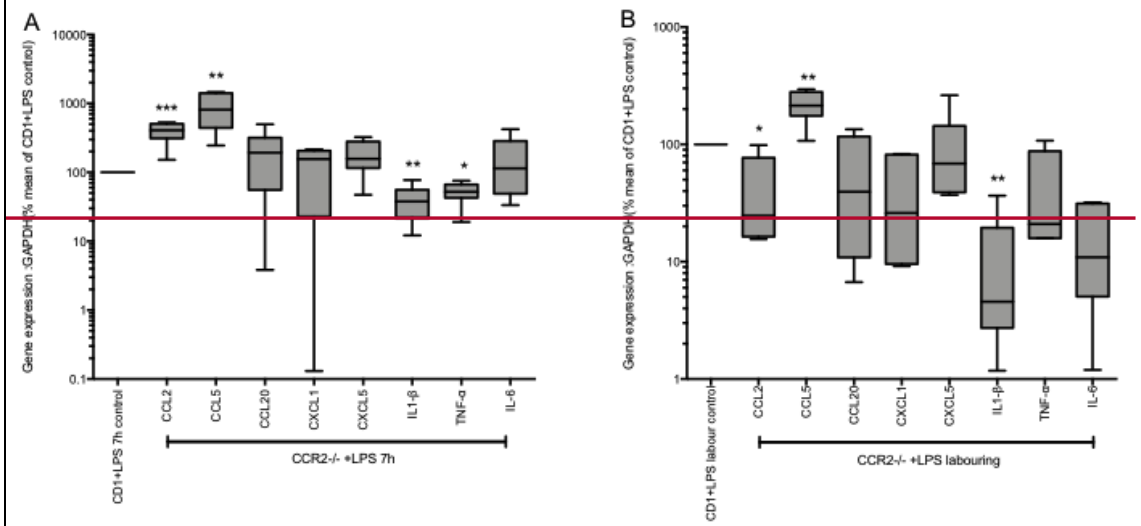

A
Blood
CD1
CCR2-/-
Cells/g
7hr i.u. LPS
Labour i.u. LPS
Neutrophils
Ly-6chigh monocytes
Ly-6low monocytes
B
Myometrium
CD1
CCR2-/-
Cells/g
7hr i.u. LPS
Labour i.u. LPS
Neutrophils
Ly-6chigh monocytes
Ly-6low monocytes
Macrophages
C
Placenta
CD1
CCR2-/-
Cells/g
7hr i.u. LPS
Labour i.u. LPS
Neutrophils
Ly-6chigh monocytes
Ly-6low monocytes
Macrophages
D
Lung
CD1
CCR2-/-
Cells/g
7hr i.u. LPS
Labour i.u. LPS
Neutrophils
Ly-6chigh monocytes
Ly-6low monocytes
E
Liver
CD1
CCR2-/-
Cells/g
7hr i.u. LPS
Labour i.u. LPS
Neutrophils
Ly-6chigh monocytes
Ly-6low monocytes

Figure 4


A
Gene expression :GAPDH(% mean of CD1+LPS control)
10000
1000
100
10
1
0.1
***
**
**
*
CD1+LPS 7h control
CCL2
CCL5
CCL20
CXCL1
CXCL5
IL1-β
TNF-α
IL-6
CCR2-/- +LPS 7h
B
Gene expression :GAPDH(% mean of CD1+LPS control)
1000
100
10
1
*
**
**
CD1+LPS labour control
CCL2
CCL5
CCL20
CXCL1
CXCL5
IL1-β
TNF-α
IL-6
CCR2-/- +LPS labouring

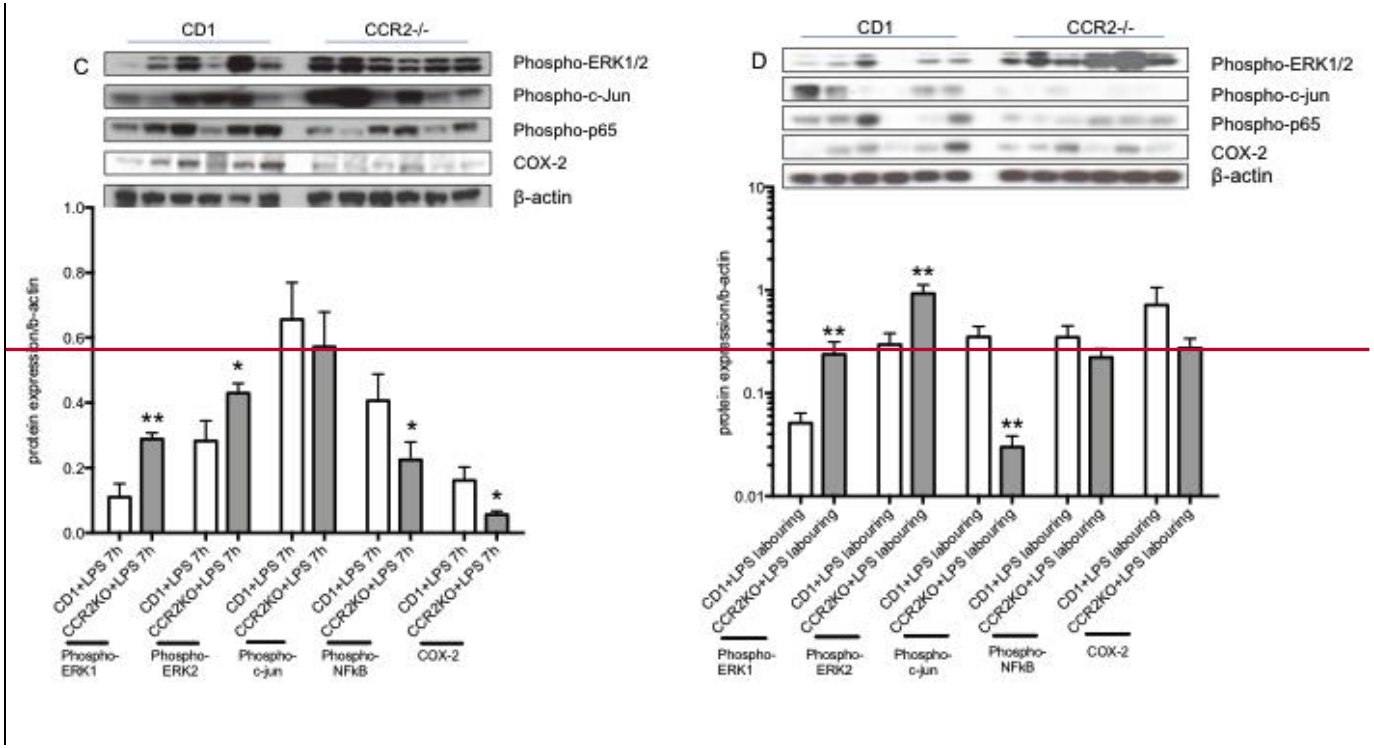
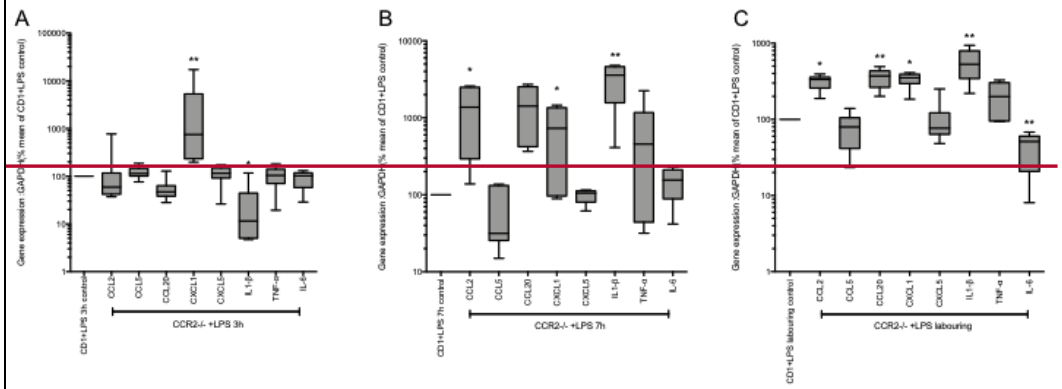
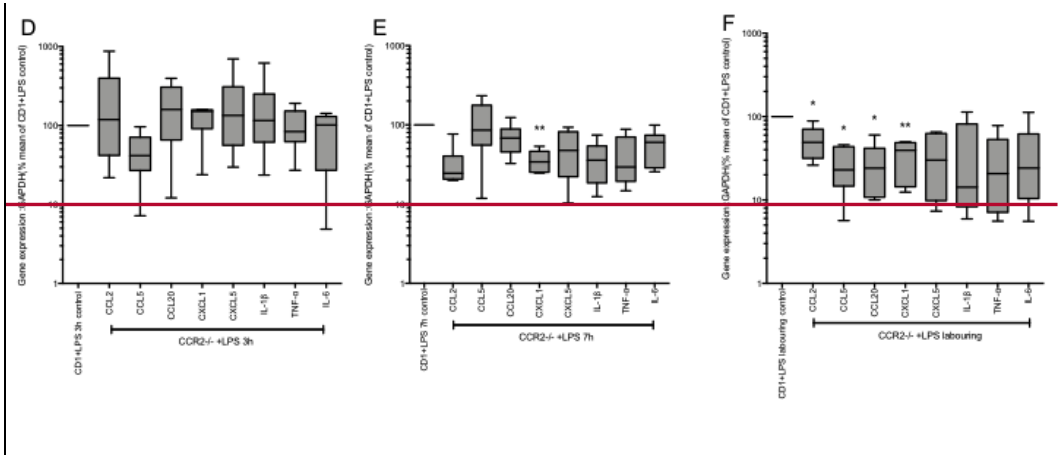
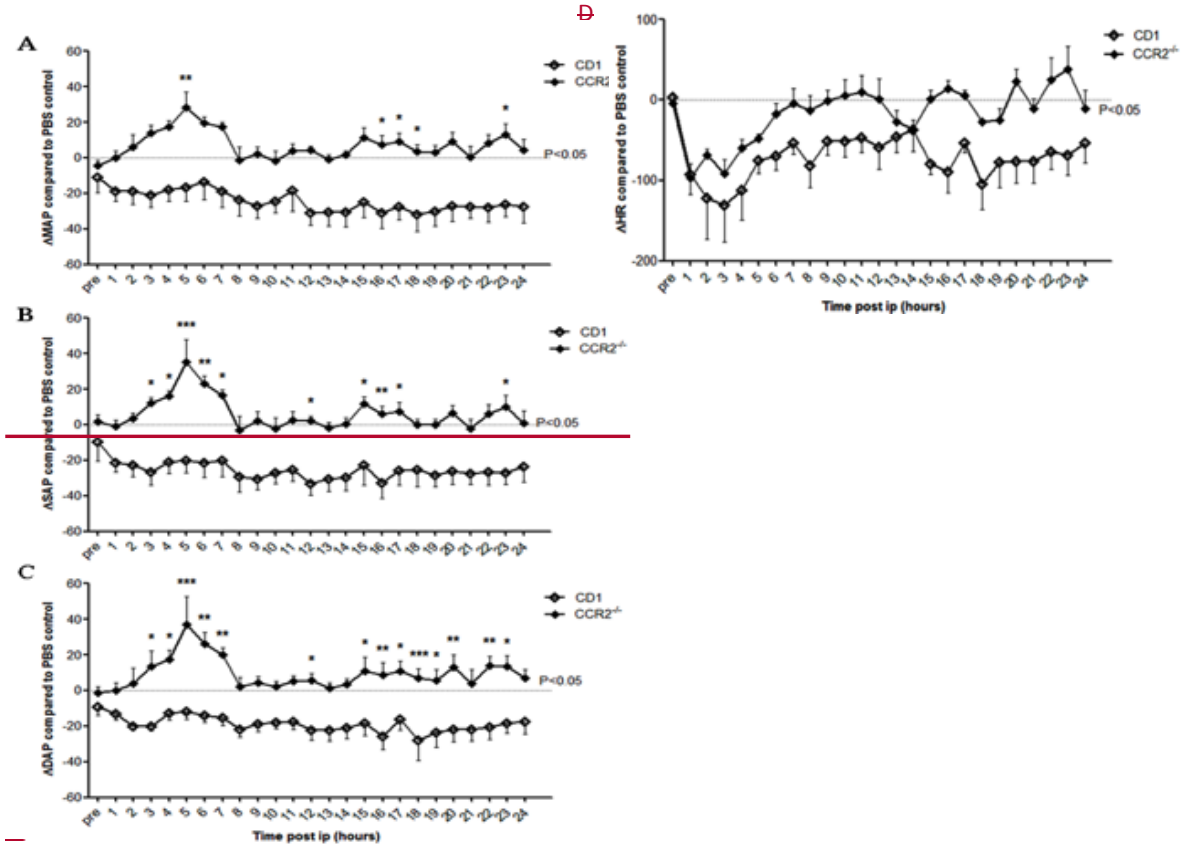

C
CD1
CCR2-/-
Phospho-ERK1/2
Phospho-c-Jun
Phospho-p65
COX-2
β-actin
protein expression/b-actin
CD1+LPS 7h
CCR2KO+LPS 7h
Phospho-ERK1
Phospho-ERK2
Phospho-c-jun
Phospho-NFkB
COX-2
D
CD1
CCR2-/-
Phospho-ERK1/2
Phospho-c-jun
Phospho-p65
COX-2
β-actin
protein expression/b-actin
CD1+LPS labouring
CCR2KO+LPS labouring
Phospho-ERK1
Phospho-ERK2
Phospho-c-jun
Phospho-NFkB
COX-2

Figure 5


A
B
C
Gene expression GAPDH-% mean of CD1+LPS control)
CD1+LPS 3h control
CCL2
CCL5
CCL20
CXCL1
CXCL5
IL-1β
TNF-α
IL-6
CCR2-/- +LPS 3h
CD1+LPS 7h control
CCR2-/- +LPS 7h
CD1+LPS labouring control
CCR2-/- +LPS labouring


D
E
F
Gene expression (GAPDH(% mean of CD1+LPS control)
1000
100
10
1
CD1+LPS 3h control
CCL2
CCL5
CCL20
CXCL1
CXCL5
IL-1β
TNF-α
IL-6
CCR2-/- +LPS 3h
CD1+LPS 7h control
CCR2-/- +LPS 7h
CD1+LPS labouring control
CCR2-/- +LPS labouring
*
**

~~Figure 6~~


A
B
C
D
CD1
CCR2-/-
ΔMAP compared to PBS control
ΔSAP compared to PBS control
ΔDAP compared to PBS control
ΔHR compared to PBS control
P<0.05
Time post ip (hours)

~~**Supplementary**~~

Figure 1

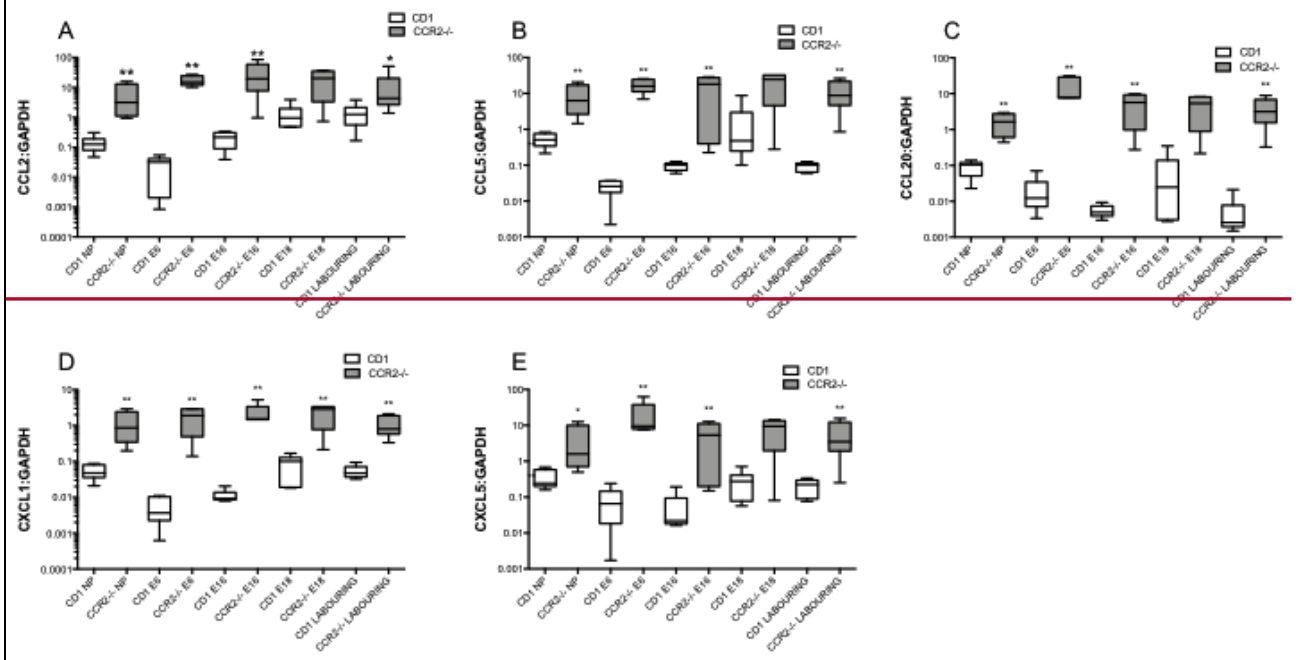

A
B
C
D
E
CD1
CCR2-/-
CCL2:GAPDH
CCL5:GAPDH
CCL20:GAPDH
CXCL1:GAPDH
CXCL5:GAPDH
CD1 NP
CCR2-/- NP
CD1 E5
CCR2-/- E5
CD1 E16
CCR2-/- E16
CD1 E18
CCR2-/- E18
CD1 LABOURING
CCR2-/- LABOURING

Figure 2

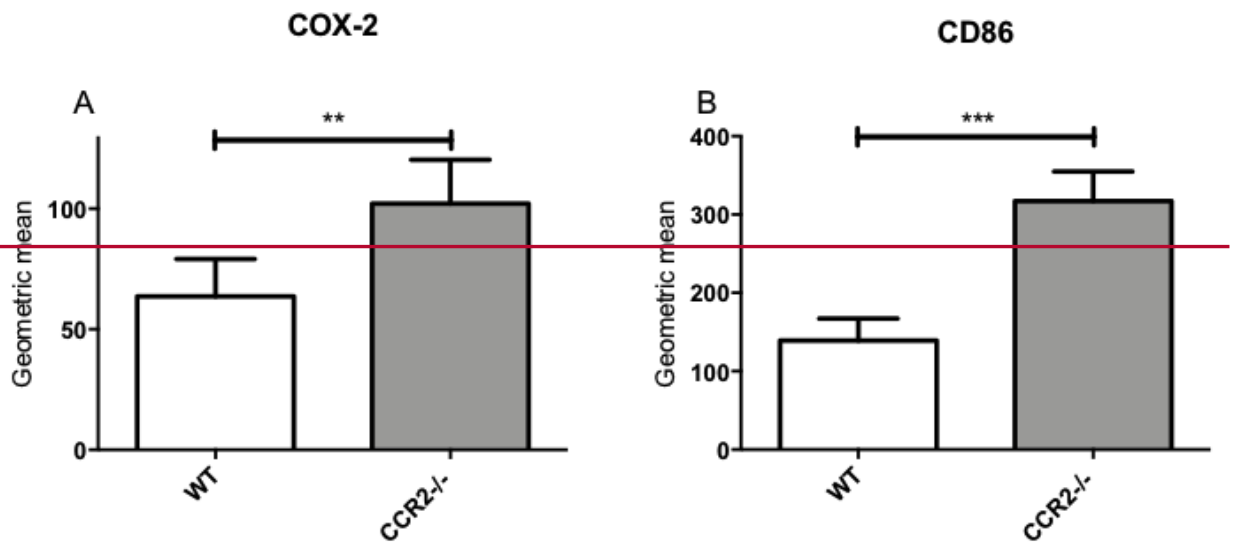

COX-2
A
**
Geometric mean
100
50
0
WT
CCR2-/-
CD86
B
***
Geometric mean
400
300
200
100
0
WT
CCR2-/-

Figure 3

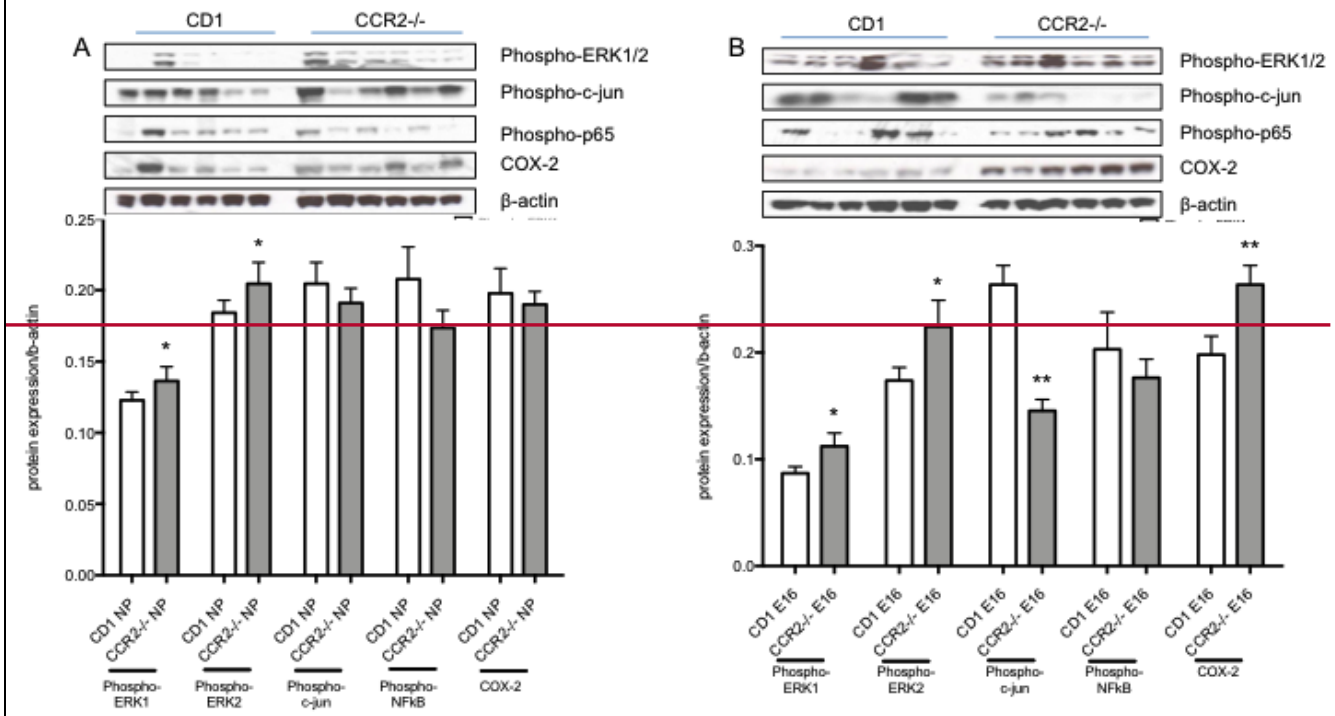

A
CD1
CCR2-/-
Phospho-ERK1/2
Phospho-c-jun
Phospho-p65
COX-2
β-actin
protein expression/b-actin
0.25
0.20
0.15
0.10
0.05
0.00
CD1 NP
CCR2-/- NP
Phospho-ERK1
Phospho-ERK2
Phospho-c-jun
Phospho-NFkB
COX-2
B
CD1
CCR2-/-
Phospho-ERK1/2
Phospho-c-jun
Phospho-p65
COX-2
β-actin
protein expression/b-actin
0.3
0.2
0.1
0.0
CD1 E16
CCR2-/- E16
Phospho-ERK1
Phospho-ERK2
Phospho-c-jun
Phospho-NFkB
COX-2

Figure 4

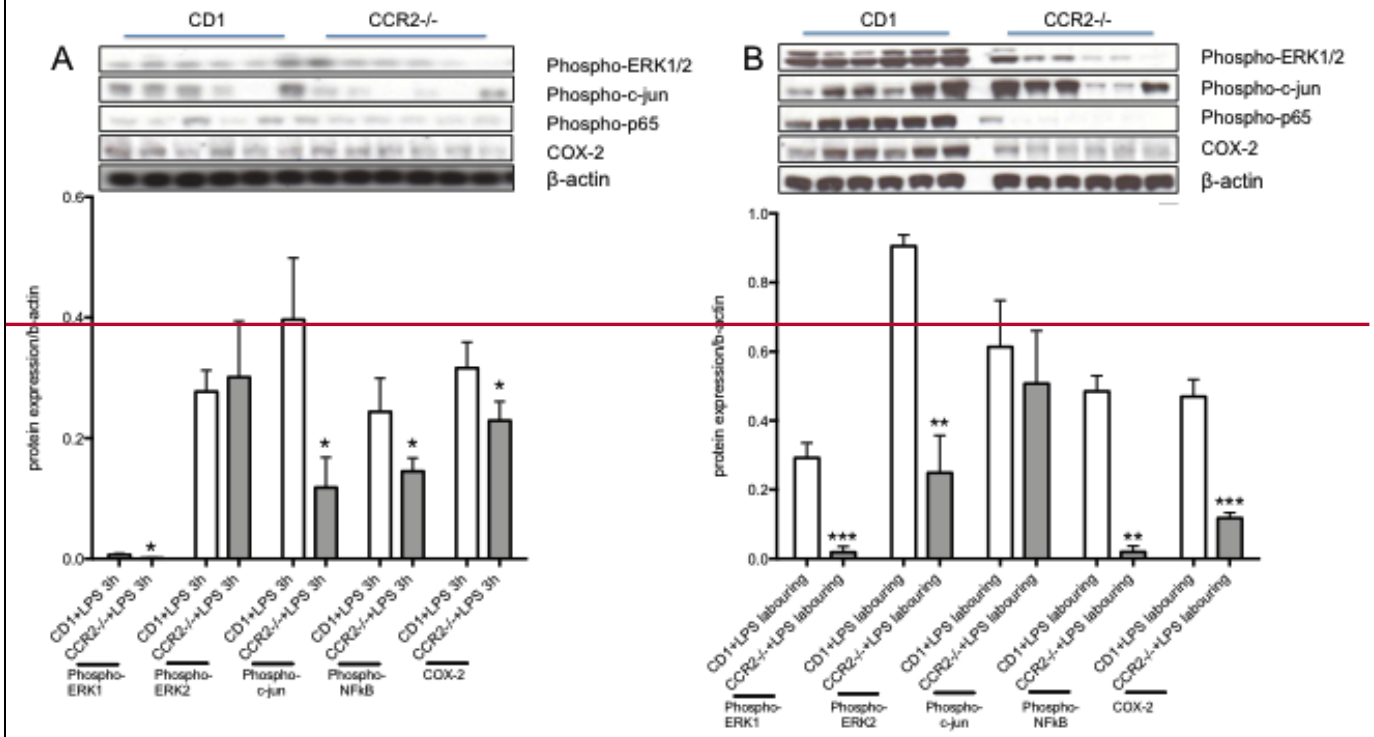
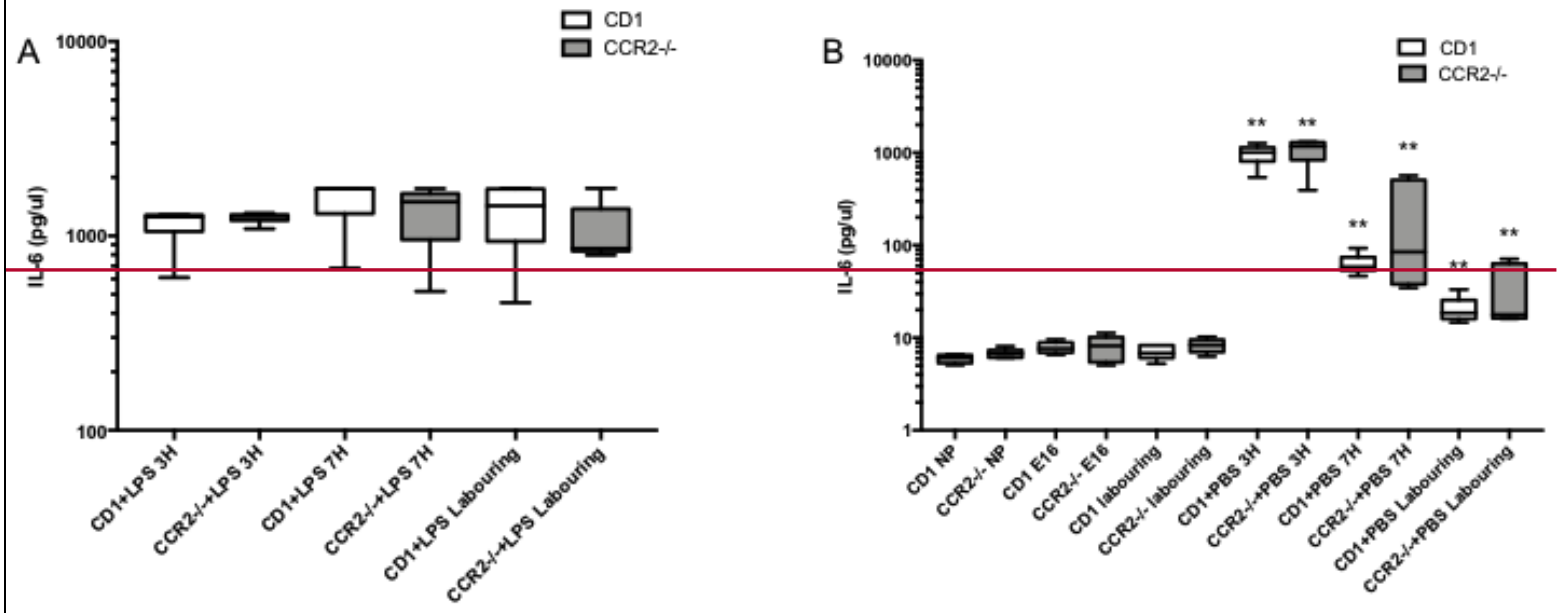

A
CD1
CCR2-/-
Phospho-ERK1/2
Phospho-c-jun
Phospho-p65
COX-2
β-actin
protein expression/b-actin
CD1+LPS 3h
CCR2-/-+LPS 3h
Phospho-ERK1
Phospho-ERK2
Phospho-c-jun
Phospho-NFkB
COX-2
B
CD1
CCR2-/-
Phospho-ERK1/2
Phospho-c-jun
Phospho-p65
COX-2
β-actin
protein expression/b-actin
CD1+LPS labouring
CCR2-/-+LPS labouring
Phospho-ERK1
Phospho-ERK2
Phospho-c-jun
Phospho-NFkB
COX-2

Figure 5



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CCR2 mediates the adverse effects of LPS in the pregnant mouse.

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Key words: inflammation, chemokines, CCR2, myometrium, lungs, leucocytes.

Conflict of interests: The authors confirm that they have no conflict of interest.

Abstract

In our earlier work, we found that intrauterine (i.u.) and intraperitoneal (i.p.) injection of LPS (10µg serotype 0111:B4) induced preterm labor (PTL) with high pup mortality, marked systemic inflammatory response and hypotension. Here, we used both i.u. and i.p. LPS models in pregnant wild type (wt) and CCR2 knockout (CCR2-/-) mice on E16 to investigate the role played by the CCL2/CCR2 system in the response to LPS.

Basally, lower numbers of monocytes and macrophages and higher numbers of neutrophils were found in the myometrium, placenta and blood of CCR2-/- vs. wt mice. After i.u. LPS, parturition occurred at 14 hours in both groups of mice. At 7 hours post injection, 70% of wt pups were dead vs. 10% of CCR2-/- pups, but at delivery 100% of wt and 90% of CCR2-/- pups were dead. Myometrial and placental monocytes and macrophages were generally lower in CCR2-/- mice, but this was less consistent in the circulation, lung and liver. At 7 hours post LPS, myometrial ERK activation was greater and JNK and p65 lower and the mRNA levels of chemokines were higher and of inflammatory cytokines lower in CCR2-/- vs. wt mice. Pup brain and placental inflammation was similar. Using the IP LPS model, we found that all measures of arterial pressure increased in CCR2-/- but declined in wt mice.

These data suggest that the CCL2/CCR2 system plays a critical role in the cardiovascular response to LPS and contributes to pup death but does not influence the onset of inflammation-induced PTL.

Introduction

Every year, an estimated 15 million babies are born preterm worldwide with rates ranging from 5% in several European countries to 18% in some African countries [1]. Globally, preterm birth complications are the leading cause of childhood mortality, responsible for over 1 million deaths in 2013 [2]. Preterm labor (PTL) is the most important cause of preterm delivery. Infection and inflammation are implicated as the main causative factors of PTL. The inflammatory process in human parturition is associated with activation of NFkB resulting in an increase in myometrial cytokine and chemokine levels [3, 4]. Inflammation is the first response to tissue injury and is driven by a chemokine-dependent infiltration of immune cells into the area of injury. The chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemotactic protein-1 (MCP-1), is a CC chemokine that mainly recruits monocytes, memory T cells and dendritic cells to the sites of inflammation [5, 6]. CCL2 has also been implicated in the pathogenesis of other diseases that involve monocyte infiltration. Its effects are mediated through the cell surface chemokine receptors CCR2 and CCR4, but of the two, CCR2, is the prime mediator of the effects of CCL2 and as such has been suggested to be an important therapeutic target [7].

In human term labor, the mRNA and protein levels of CCL2 are increased in the choriodecidua, myometrium and cervix [8, 9]; while increased amniotic fluid CCL2 levels are present in samples from women in both preterm and term labor [10, 11]. Similarly, in pregnant mice, serum and amniotic fluid CCL2 levels also increase with gestation [12]. While in rat studies, progesterone suppressed and RU486, an anti-progestin, increased myometrial CCL2 mRNA levels [13]. These data suggest that CCL2 may have a role in the onset of labor in different species, but although RS504393, an efficient CCR2 antagonist, reduced myometrial monocyte numbers in response to RU486, the onset of labor was not delayed [14], questioning the role of the CCL2/CCR2 system in the onset of labor. Indeed, Nelson's group found that CCR2 knockout mice had a normal pregnancy duration with similar litter sizes and pup birthweight [15]. Consistent with CCR2 knockout, on E18 myometrial F4/80 transcripts did not

increase, confirming defective monocyte trafficking, but Gr-1 transcript levels were normal suggesting granulocyte migration was normal [15].

The CCR2 knockout mouse model has been used mainly in studies of respiratory system, where the CCR2 defect markedly impaired monocyte recruitment to the site of inflammation. In CCR2-/- mice, after a *mycobacterium bovin* challenge, IFN-γ producing cells in draining lymph node were 70% lower [16]. Knockout of CCR2 also impaired dendritic cell trafficking, activation and maturation, again after *mycobacterium bovine* antigen challenge with reduced macrophage infiltration into the injected site. In the CLP induced mouse model of sepsis, TLRs regulated the CCR2 responsiveness in neutrophils via NF-kB and the absence of CCR2 or its inhibition improved survival [17], indicating direct or indirect effects on the CCL2/CCR2 axis on neutrophil as well as monocyte trafficking and function.

In this study, the response of the pregnant CCR2-/- mouse on E16 to LPS (10µg serotype 0111:B4) administered via intrauterine (i.u.) and intraperitoneal (i.p.) injection, which mimicked direct local myometrial inflammation and acute systemic inflammation respectively, is investigated. Specifically testing the hypothesis that CCL2/CCR2 system is critical to the onset of PTL, pup death and the systemic inflammatory and cardiovascular reaction to LPS.

Materials and methods

Animals

Animal studies were performed under UK Home Office License 70/6906 (University College London) or 70/7372 (Imperial College London) and in accordance with the UK Animals (Scientific Procedures) Act of 1986. Virgin female and stud male CD1 outbred mice were purchased from Charles River (Margate, UK) at 6-8 weeks of age. Female CCR2 knockout (CCR2-/-) on a C57BL/6 background were purchased from the Jackson Laboratory (Bar Harbor Maine, USA). All mice were maintained in open cages at 21 ± 1°C, with *ad libitum* access to food (RM1 diet, Lillico Biotechnology, UK) and water, and a 12:12 light/dark cycle regimen. All mice were acclimatized for a minimum of 72 hours before undergoing any regulated procedure or mating.

Outbreeding of transgenic strains

The CCR2 homozygous knockout mice were originally crossed to C57BL/6 mice. The strain was backcrossed to a CD1 background as CD1 mice are a particularly fecund strain and typically have much larger litters than C57BL/6. In addition, the greater robustness and reproduction of the outbred strain was considered to be sufficiently advantageous to offset the heterogeneity of the background and allow comparison with our inflammation-induced preterm labour model in CD1 mice. CCR2-/- female mice (classed as the F0 generation) were mated with CD1 outbred males. Heterozygous pups (F1 generation) from different litters were mated together, producing a heterogeneous F2 generation (25% CCR2-/-, 50% heterozygous and 25% wild type litters). Genotyping was performed to determine between CCR2-/-, heterozygous and wild type animals. The resulting CCR2-/- F2 generation (males and females) were interbred to expand the colony for experimentation.

Timed-mating

Timed mating was employed to enable accurate timing of injections (i.u. or i.p.). Female mice (wt; CCR2-/-) were mated with a stud wt male overnight. The presence of a copulatory plug in the vagina of the female indicated successful mating and was classed as E0 (day 0) of gestation.

Preterm labor mouse model

Mouse model of localized intrauterine inflammation and preterm delivery and tissue collection

Intrauterine administration of LPS to rodents has been described by Elovitz [18]. An adapted version of this model has subsequently been established in our group and further optimized. A laparotomy followed by intrauterine LPS (0111:B4, Sigma) injection was performed on day 16 (E16) of gestation as previously described [19]. The number of live fetuses per horn was determined before injection. Uterus of both wild type and CCR2 knockout mice were collected from naïve virgin non-pregnant mice and from time-mated mice (mated overnight with the following day termed E0). For pregnant mice, samples were collected during the morning of E6, E16, E18 and during labor (defined after the spontaneous delivery of at least one pup) and snap frozen for molecular analysis. For LPS mouse model, tissue collections were performed at 3h, 7h and the onset of labor after intrauterine injection of PBS or LPS. Dams were culled via exsanguination by cardiac puncture, followed by immediate cervical dislocation. For plasma collection, whole blood was centrifuged immediately at 1000x g for 10 minutes and snap frozen on dry ice. Maternal lungs, livers, myometrium, placenta and pup brain were collected. Tissue was processed as previously described [19].

Cell preparation and Flow Cytometry

Myometrial samples were weighed immediately after collection. Approximately 0.2g of tissue was processed for FACS using an adapted method previously published by our group [20]. Tissue samples were finely minced with scissors and added to a gentle MACS M tube (Miltenyi Biotec Ltd, Cologne,

Germany) containing 1ml of IC fixation buffer (eBioscience, San Diego, USA) and homogenized for 1 minute. 5ml of FACS Wash Buffer (FWB) (1x PBS, FCS 2%, Sodium Azide 0.1% and 2mM EDTA) was then added to the tube to stop fixation and the homogenate was then passed through a 40µm nylon cell strainer (BD Falcon, Bedford, USA), and the cell suspension was then centrifuged at 400xg for 5 minutes. Cells were then washed in PBS-FCS 2% and resuspended in permeabilising wash buffer (PWB) (1x PBS, 0.1% Sodium Azide, 0.5% BSA and 0.2% Saponin) and then incubated with antibodies at 4°C in the dark for 30 min. After staining, cells were washed in either FWB (unfixed samples) or PWB (fixed samples).

The fluorophore-conjugated rat anti-mouse MAbs antibodies used included: Ly6C (HK1.4), Gr1 (RB6-8C5), NK1.1 (PK136), F4/80 (CI:A3-1), CD45 (30- F11) (BioLegend), COX-2 (M-19) (Santa Cruz Biotechnology), CD86 (B7-2), CD11b (M1/70), MHCII (AMS-32.1), and F4/80 (BM8) (eBioscience). Levels of CD86 and COX-2 were calculated after subtraction of an isotype-matched control antibody to calculate MFI levels (IgG2a K Isotype Control (eBR2a, eBioscience)). FACS analysis was performed as previously described [19].

Mouse model of systematic inflammation and telemetry

Telemetry device implantation, recording, acquisition and data analysis

Telemetry probes (PA-C10, Data Sciences International) were surgically implanted as previously described [21]. On day 8 after surgery, continuous 24 hour recording (data collection) began using the Dataquest ART Acquisition System (Data Sciences International, v4.1) for at least 48 hours to collect data in non-pregnant animals (non-pregnant baseline). These data allowed animals to act as their own controls. Radiotelemeters were magnetically activated and deactivated. Whilst recording, animals were kept in their home cages with access to food and water ad libitum; however these cages were placed in a separate room for recording. This environment was chosen to cause fewer disturbances

from other facility users and staff. Collected parameters included mean arterial pressure (MAP), systolic arterial pressure (SAP), diastolic arterial pressure (DAP), heart rate (HR), and activity. Haemodynamic data were collected for 10 seconds every 30 seconds. Labor time and litter sizes were also recorded. Accurate labor time was enabled by the use of infrared IP cameras in conjunction with the AVerDiGi (NV3000 Series) HYBRID Surveillance Platform (RF Concepts, Dundonald, UK).

Intraperitoneal (i.p.) injection of LPS

After telemetry implantation surgery, the intrauterine administration of LPS, which requires another laparotomy, was not possible under the current Procedure Project License (PPL). Therefore we aimed to establish a systemic endotoxaemic model using the intraperitoneal (i.p.) route.

Dams received a single i.p. injection of 10µg LPS (serotype O111:B4; Sigma-Aldrich, Poole, UK) per dam (in 100µL PBS) which resulted in no maternal mortality and gave the most consistent outcomes in latency to labor and pup survival or vehicle control (PBS) were administered systemically (i.p.). Pregnant wt and CCR2-/- mice were anaesthetized under isoflurane for i.p. injection to minimize potential harm to fetuses and were carried out between 8am and 10am.

Model reproducibly and effect sizes

For i.u and i.p LPS models, animals were randomly selected for LPS or vehicle control administration. Both groups injected alongside each other during every surgical session to avoid bias. Surgeries were performed at the same time during each experimental session (between 1000 and 1200 h) to ensure that the gestational stage was as similar as possible between mice, thus reducing variation in the model. Intermediate collection time points were determined once the latency to labor post-LPS administration was calculated. Due to pregnancy rates and to again avoid collection bias, multiple time point collections were performed on the same day, while laboring samples were collected over various days. We mated enough animals to ensure that we had a minimum of six animals at each time point. The sample size was chosen based on previous *in vivo* work [22] and the requirements for

statistical normality testing. When the mating proved to be more successful than expected, we used the excess animals to increase numbers at the different time points. A single pregnant dam represents a single experimental unit throughout this study.

Genomic DNA extraction and genotyping PCR

The ear clips from tested mice were taken and put in 75µl of solution 1 (NaOH 25mM, EDTA Na2 0.2mM), then vortexed. The sample was heated to 95^{o}C for 10min and cooled down to 4^{o}C. Solution 2 (Tris-HCl, pH8.0 40mM) was added and, the whole solution was vortexed and spun down. 4µl of final solution was used and mixed with PCR reaction mix which containing 2 µl PCR Buffer (×10), 2 µl dNTP's (2mM), 0.6 µl Mgcl2 (50mM), 1 µl of primer F (10µM), 1 µl of primer R (10µM), 4 µl of DNA, 9.3 µl dH2O and 0.1 µl Taq (5U/ µl). Genotyping PCR thermocycler condition for CCR2-/- was 95^{o}C for 5min, 95^{o}C for 30sec, 65^{o}C for 1min, 72^{o}C for 2min and, after 35 cycles heated 75^{o}C for 10min and 4^{o}C for 10min.

Quantitative RT-PCR

Total RNA was extracted and purified from cell or tissue samples using RNeasy mini kit (Qiagen Ltd, UK). After quantification, 1.0 µg was reverse transcribed with oligo dT random primers using MuLV reverse transcriptase (Applied Biosystems Ltd, UK). 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) that continually measures fluorescence caused by the binding of the dye to double-stranded DNA. In order to correct the variations, data were expressed relative to the amount of constitutively expressed housekeeping gene.

Western blot analysis

Denatured protein samples (40-60ug) were loaded in 10-15 well gels (Invitrogen, UK) and run on 80-130 volts. After that, an electrophoretic transfer onto a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA) was performed. Membranes were

blocked in washing buffer (5M NaCL, Tris-HCL and Tween) containing 5% milk for 1 h at room temperature, washed in washing buffer and hybridized with the primary antibody over night at 4^{o}C. Membranes were washed again and then incubated with secondary antibody at a dilution of 1:2000 for 2 h at room temperature. SuperSignal West Pico Chemiluminescent Substrate (Pierce, USA) was used for detection. Protein band size was determined using Novex Sharp Pre-stained Protein Standard (Invitrogen, UK).

ELISA

ELISA assay procedure was followed according to manufacturer's instructions (R&D Systems, Abingdon, UK). Plasma samples were diluted 1:2 in Assay Diluent RD1-14 and mixed well. The calculation of concentrations was performed using standard concentrations for comparison fit with a 4-parameter logistic curve.

Statistical analysis

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

Results

Myometrial chemokine mRNA levels before and during pregnancy.

In myometrial samples obtained before and during pregnancy, we measured the mRNA levels of chemokines that we had found previously to be elevated in myometrial samples taken at the time of human labor [3](supplementary figure 1A-E). We found that the myometrial mRNA levels of CCL2, CCL5 and CXCL1 were initially low in wt mice on E6, but increased to a peak on E18, in contrast, CXCL5 mRNA levels remained static throughout gestation and those of CCL20 declined. In CCR2-/- mice, myometrial chemokine mRNA levels were higher at baseline, through pregnancy and with the onset of parturition than in wt mice (p=0.05-0.01). With advancing gestation and the increase in wt myometrial chemokine mRNA levels, the differences were less consistent (supplementary figure 1A-E).

Inflammatory cell distribution on E16

We determined myometrial myeloid cell numbers in different compartments (expressed per g of tissue or per ml of blood) on E16 (Fig.1) in CCR2-/- and wt mice. In the myometrium, placenta and blood, the pattern was similar, with higher neutrophils and lower Ly-6C^{high} and Ly-6C^{low} monocytes in the CCR2-/- mice ($p<0.05$-0.001; Fig.1A-C); in the myometrium and placenta, macrophage counts were also lower in the CCR2-/- mice ($p<0.05$ and $p<0.001$ respectively; Fig.1A&B). In the lung and liver, neutrophil counts were similar, but Ly-6C^{high} and Ly-6C^{low} monocytes were again lower in the CCR2-/- mice ($p<0.05$-0.01; Fig.1D&E). In the myometrium, the expression of cell activation markers, COX-2 and CD86 was greater on CCR2-/- macrophages in CCR2-/- vs. wt mice (p=0.01 and 0.001 respectively; supplementary figure 2A&B).

Pregnancy outcomes after LPS

To investigate the significance of the modified myometrial response in CCR2-/-, we used the localized septic insult model of sepsis i.u. injection of 10µg serotype 0111:B4. LPS labor started after 14 hours

in both groups (Fig.2A). However, at 7 hours post LPS, in wt mice, 70% of pups were dead vs. 10% in the CCR2-/- mice ($p<0.001$), but with parturition, 100% of wt and 90% of CCR2-/- pups were dead (Fig.2B).

Inflammatory cell responses to LPS on E16

Next, we compared numbers of inflammatory cells in different compartments in both wt and CCR2-/- mice after i.u. LPS. Intriguingly, in the circulation, inflammatory cells numbers were comparable in the two groups at both 7 hours post LPS and in labor (Fig.3C). In contrast, in the myometrium Ly-6C^{low} monocytes were consistently lower at both time points (both $p<0.05$), neutrophil numbers were higher and macrophage numbers were lower at 7h (both $p<0.05$), both were similar in labor, and Ly-6C^{high} monocytes were comparable at both time points in CCR2-/- vs. wt mice (Fig.3A). In the placenta, Ly-6C^{low} monocytes at 7h and Ly-6C^{high} monocytes at labor were lower ($p<0.001$ and $p<0.05$ respectively), macrophages were lower at both time points (both $p<0.01$) and neutrophils similar at both time points (Fig.3B) in CCR2-/- vs. wt mice. In the lung, like the circulation, cell numbers were similar with the exception of Ly-6C^{low} monocytes, which were lower at 7 hours ($p<0.001$; Fig.3D); similarly in the liver, except that the Ly-6C^{high} monocytes were lower in labor in CCR2-/- vs. wt mice ($p<0.05$; Fig.3E).

Myometrial cytokine mRNA and kinase responses to LPS on E16

We compared the effect of LPS on myometrial cytokine mRNA levels in CCR2-/- compared to wt mice. At 7h post i.u. LPS, the myometrial mRNA levels of CCL2 ($p<0.01$) and CCL5 ($p<0.05$) were higher and of IL-1β ($p<0.01$) and TNFα ($p<0.05$) were lower (Fig. 4A). In labor, the myometrial mRNA levels of CCL5 ($p<0.01$) were higher and of CCL2 ($p<0.05$) and IL-1β ($p<0.01$) were lower in CCR2-/- mice (Fig. 4B).

These changes in cytokine mRNA levels were associated with greater phosphorylation of ERK1/2 ($p<0.05$-0.01) in CCR2-/- myometrial samples at 7h and labor and reduced phosphorylation of p65 at 7h ($p<0.05$) and of JNK ($p<0.01$) in laboring samples; COX-2 levels were lower at 7h only ($p<0.05$;

Fig.4C&D). In basal, non-pregnant and E16 myometrium from wt and CCR2-/- mice, ERK1/2 activity was greater in the CCR2-/- mice (data not shown).

Pup brain and placental and cytokine mRNA response to LPS on E16

To understand whether differences in pup inflammation were responsible for the more acute demise of the wt pups, we studied the mRNA levels of cytokines in pup brain and placenta obtained at 3h and 7h post LPS and during labor (Fig5A-F).

In the pup brains, at 3 hours, the mRNA levels of CXCL1 were higher ($p<0.01$) and IL-1β lower ($p<0.05$) in CCR2-/- pup brains (Fig.5A); at 7h the mRNA levels of CCL2, CCL5 and IL-1β were higher in CCR2-/- pup brains ($p<0.05$-0.001; Fig.5B); and in labor, the mRNA levels of CCL2, CCL20, CXCL1 and IL-1β were higher and of IL-6 and were lower in CCR2-/- pup brains ($p<0.05$-0.001; Fig.5C).

In the placenta at 3 hours, cytokine mRNA levels were similar in both groups, at 7h they tended to be lower in CCR2-/- mice but were only significantly lower in the case of CXCL1 ($p<0.01$; Fig.5E). In laboring samples, placental mRNA levels of CCL2, CCL5, CCL20 and CXCL1 were lower in CCR2-/- mice (Fig.5F; $p<0.05$-0.01). These changes in gene expression were associated with reduced placental phosphorylation of ERK1, JNK and p65, COX-2 levels at 7h in CCR2-/- vs. wt mice (all $p<0.05$; Supplementary figure 3A). In laboring samples of placenta, the findings were similar with reduced phosphorylation of ERK1/2 and p65 and lower COX-2 levels in CCR2-/- vs. wt mice ($p<0.05$-0.001; Supplementary figure 3B).

Circulating IL-6 levels in response to i.u. LPS on E16

Plasma IL-6 level in both wt and CCR2-/- mice were measured before and during pregnancy, no differences were observed (Supplementary figure 4). In mice injected with i.u. LPS (Supplementary figure 4A) and in sham mice injected with i.u. PBS (Supplementary figure 4B), the levels of IL-6 were

295 increased at 3 hours, they remained elevated in both wt and CCR2-/- mice injected with LPS at 7 hours
296 and in labor, but declined in those injected with PBS (Supplementary figure 4A&B). There were no
297 differences between the 2 groups.
298
299 *Haemodynamic response to LPS*
300 We used the i.p. LPS PTL model to assess the effect of a direct systemic septic challenge on the
301 haemodynamic response in pregnant wt and CCR2-/- mice. We found that, in contrast to the fall in
302 blood pressure in wt mice, there was a transient increase in all blood pressure parameters in response
303 to LPS in CCR2-/- mice, with a peak elevation in MAP of Δ28.24 ± 8.5 mmHg at 5 hours post-LPS
304 administration ($p<0.05$-0.001; Fig.6A-C). Blood pressure parameters were then maintained at baseline
305 levels, which were higher than in wt mice (Fig.6A-C). The nadir in MAP of Δ-31.1 ± 7.06 mmHg in wt
306 mice was seen at 12 hours post-LPS, after which, this was maintained and did not resolve (Fig.6A-C).
307 The HR fell acutely in both wt and CCR2-/- mice, reaching a nadir at 1-3 hours post LPS. The decline in
308 HR and recovery to baseline was less marked and quicker respectively in CCR2-/- mice than in wt mice
309 ($p<0.05$; Fig.6D). The onset of labor and pup survival post IP LPS were similar for both groups.
310

Discussion

In this study, we have confirmed previous data showing a normal pregnancy outcome in CCR2-/- mice [15]. We demonstrated marked differences in basal myometrial chemokine mRNA levels and inflammatory cell numbers with increased neutrophils and reduced monocyte/macrophage numbers. Similar changes were observed in the placenta, blood, lung and liver. Despite these basal differences, there was no delay in the onset of LPS-induced preterm birth, despite a reduced myometrial inflammatory response, and although pup death was delayed, it was ultimately similar in both wt and CCR2-/- mice. The marked hypotensive response to LPS we previously reported was apparent in wt mice [21] but prevented in CCR2-/- mice. These data suggest that the CCL2/CCR2 system mediates some of the inflammatory response to LPS, but that although pup demise is delayed, ultimately neither is the onset of labor delayed nor pup survival improved by inhibition of CCL2/CCR2 system. In contrast, the hypotensive response to LPS was blocked, suggesting a potential approach to the management of septic shock.

Myometrial chemokine mRNA levels were higher in non-pregnant CCR2-/- mice as has been reported in other tissues previously for CCL2, CCL10, CCL23 (MIP-3) and CXCL2 (MIP-2α) in CCR2-/- mice [23-25]. With pregnancy and advancing gestation, chemokine levels remained static in CCR2-/- mice and rose in wt animals to similar levels by E18 in most cases. On E16, myometrial inflammatory cells showed marked differences with lower numbers of monocytes and an increase in neutrophils, the latter presumably driven by the increase in chemokines. The lower levels of monocytes are consistent with previous observations [25-28] and neutrophil and lymphocyte infiltration has been reported to be intact in CCR2-/- mice [23, 29], with a relatively increased neutrophil tissue infiltration in some circumstances [25, 28] including in an influenza mouse model [30, 31]. The greater neutrophil numbers were suggested to be the result of increased neutrophil CXCR2 expression or perhaps the increased levels of eotaxin [30, 31], although the exact effect of eotaxin on neutrophils is debated [32]. The macrophage population did show greater expression of activation markers, suggesting that although numbers were reduced they may be more functionally active. The differences in cell numbers

and activity was associated with higher ERK1/2 and lower JNK phosphorylation and increased in COX-2 protein levels. How changes in CCR2-/- status might result in increased ERK1/2 and reduced JNK phosphorylation is difficult to explain and the importance of the differential activation of MAPK subtypes unclear since there seems to have been lower inflammatory cytokine mRNA levels in the myometrium of CCR2-/- compared to wt mice.

Despite the difference in basal myometrial inflammatory cell numbers, the onset of LPS-induced PTL was not delayed in CCR2-/- vs. wt mice, with the onset of labor occurring some 14 hours later in both groups similar to previous reports [33-35]. This is intriguing as previously, the administration of a broad-spectrum chemokine antagonist was able to inhibit LPS-induced PTL [36]. It is possible that although the monocyte and macrophage counts were lower in CCR2-/- mice after LPS, the greater numbers of neutrophils may have been able to compensate. Equally, inflammatory cytokine mRNA levels were lower and, although the greater phosphorylation of ERK1/2 persisted post LPS, both p65 phosphorylation and COX-2 protein levels were lower. The greater activation of ERK may have contributed to the increase in neutrophil numbers as ERK is necessary for both neutrophil extracellular trap formation [37] and adhesion of neutrophils [38]. In terms of the lower COX-2, CCL2 has been shown to enhance COX-2 expression in endothelial cells [39] and the loss of the CCL2 effect may have contributed to the lower levels of myometrial COX-2. These data suggest that the myometrial inflammatory response was reduced in CCR2-/- mice, however the time of onset of parturition was similar in both wt and CCR2-/- mice. These data imply that LPS does not act via cells of the monocyte-macrophage lineage to induce labor, as suggested by the finding that LPS acts via monocytes to enhance myometrial contractility [40]. Our observations are supported by the fact that we found that reducing the RU486-induced monocyte infiltration of the myometrium did not delay the onset of labor [14].

Interestingly, pup survival was improved at 7 hours post LPS in CCR2-/- mice. This proved to be only a temporary phenomenon as by the onset of labor virtually all pups were dead in both wt and CCR2-/-

mice. Inflammatory cytokine mRNA levels were only reduced for IL-1ß in CCR2-/- pup brains at 3 hours, suggesting that pup inflammation was not significantly lower in CCR2-/- pups. Placental LY6C^{low} monocytes and macrophages numbers were reduced vs. wt mice at 7 hours but cytokine mRNA levels were similar, again suggesting that inflammation was not reduced in the placenta either. Previous studies on the impact of LPS in rat pregnancy suggest that fetal demise was a result of reduced placental perfusion [41], indeed this was our impression when we studied the effect of LPS in CD1 mice [19]. Others have reported that LPS can induce preeclampsia-like symptoms in rats, but Yamashita et al. showed that 80 mg/kg intraperitoneal (i.p.) LPS caused a greater than 90% mortality in WT (C57bl/6) male mice and triggered a severe and progressive drop in mean arterial pressure (MAP) of about 60 mm Hg, a 40% reduction from pretreatment values (mean 97 mm Hg) [42]. In our study, mice received intraperitoneal LPS (10 µg) on E16 induced a greater fall in blood pressure in pregnant mice. This was consistent with our previous study [21] and was reproduced here in the wt mice. Remarkably, we found that this was reversed in the CCR2-/- mice. Indeed, rather than a hypotensive response, we recorded a transient increase in all blood pressure parameters. Previously, we suggested that the LPS-induced hypotensive response may be mediated through the local activation of inflammatory cells and their release of nitric oxide or another vasoactive compound. This suggestion was based on the observation of a greater lung margination by inflammatory cells in pregnant compared to non-pregnant mice [21]. Here, in the CCR2-/- mice, other than a reduced lung margination of Ly6C^{low} monocytes at 7h and lower Ly6C^{high} monocytes numbers in laboring liver samples, we observed a surprisingly similar inflammatory cell response to LPS in terms of circulating, lung and liver inflammatory cell changes and the response of circulating IL-6 was similar, again suggesting that the systemic inflammatory response was equivalent in both groups implying that there must be another explanation for the difference in cardiovascular response. It is possible that there was a differential activation of the inflammatory cells or that the CCL2/CCR2 system has direct effects on vascular function. Chemokines are thought to regulate blood pressure via the control of inflammation in the vessels and in the heart. Inflammatory cell infiltration and oxidative stress in

388 vessel walls contributes to an increase in blood pressure [42]. However, the lack of functional CCR2
389 receptors has been associated with hypertension in humans [42, 43], also CCL2 modulates pulmonary
390 vascular endothelial function [44], suggesting that the CCL2/CCR2-/- system does have an effect on
391 vascular function. Other studies investigated the impact of CCR2 inhibition in the response to sepsis
392 and found improved survival in the caecal ligation and puncture model [17]; the benefits were
393 suggested to be through reduced neutrophil organ infiltration [17], but the cardiovascular response
394 was not assessed in these studies. The improvement in sepsis outcomes mediated by IL-10 is thought
395 to be mediated at least in part through the repression of neutrophil CCR2 expression [45]. Once the
396 mechanism of action of CCR2 antagonism in sepsis is understood, this approach may prove to be
397 helpful in the management of sepsis particularly as an alternative to vasopressors in the maintenance
398 of blood pressure. In the LPS-PTL model, pup demise may still be because of reduced utero-placental
399 perfusion, which may have contributed to the increase in systemic blood pressure, but this possibility
400 awaits further study.

401 This study shows that CCR2-/- does not prevent LPS-induced preterm delivery but reverses its
402 hypotensive effect. Basally, and in response to LPS, monocyte and macrophage numbers are reduced
403 and neutrophil numbers increased in most, but not all compartments, suggesting that monocytes do
404 not play an essential role in the LPS-induced onset of PTL, but are important in the cardiovascular
405 response to LPS during pregnancy. As such CCR2 antagonism may be useful adjunct to the
406 management of sepsis in pregnancy.

407

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Legends

Figure 1 Inflammatory cell numbers on E16 in wild type and CCR2-/- mice.

Leukocyte cell count and densities in the myometrium (A), placenta (B), circulation (C), lung (D) and liver (E) from untreated CCR2-/- and wild type mice on E16. Data were expressed as median, interquartile range and range and were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when the data were not. * indicates a difference of $p<0.05$, ** of $p<0.01$, *** of $p<0.001$). n=5-6.

Figure 2 Effect of iu LPS on interval to parturition and pup survival on E16 in wild type and CCR2-/- mice.

Wild type and CCR2-/- mice on E16 were administered intrauterine LPS (10µg serotype 0111:B4) or PBS. **A:** Interval to delivery (hours). **B:** The percentages of live pups. Data were expressed as median, interquartile range and range and were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when the data were not. ** indicates a significant difference of $p<0.01$, n=6.

Figure 3 Cell density of leukocytes in the myometrium, circulation, placenta, lung and liver of wild type and CCR2 KO mice following intrauterine injection of LPS

Leukocyte cell count and densities in the myometrium (A), placenta (B), circulation (C), lung (D) and liver (E) from CCR2-/- and wild type mice on E16 after intrauterine LPS (10µg serotype 0111:B4). Data were expressed as median, interquartile range and range and were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when the data were not. Statistical significance was indicated by * indicating a difference of $p<0.05$, ** of $p<0.01$, *** of $p<0.001$, n=5-6.

Figure 4 Effect of LPS on myometrial cytokine mRNA, COX-2 protein, phosphorylated ERK1/2, c-Jun and p65 levels.

Myometrium was obtained at 7 hours and in labor after the administration of LPS (10µg serotype

0111:B4) from wild type and CCR2-/- mice. **A-B:** Myometrial cytokine mRNA levels were measured using rtPCR. Data were expressed as median, interquartile range and range. **C-D:** Myometrial COX-2 and phosphorylated ERK1/2, c-Jun and p65 levels measured by western analysis and densitometry. Data were expressed as mean±SEM. The data were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when the data were not.* indicates a significant difference of $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$.

Figure 5 The effect of LPS on wild type and CCR2-/- pup brain and placental cytokine mRNA levels

Pup brain **(A-C)** and placenta **(D-F)** were obtained from the left horn at 3 hours, 7 hours and during labor after intrauterine LPS (10µg serotype 0111:B4) injection. Cytokine mRNA levels were measure by rtPCR. Data were expressed as median, interquartile range and range and were analysed using an unpaired t-test when normally distributed and a Mann Whitney test when the data were not. * indicates a significant difference of $p<0.05$, ** indicates $p<0.01$.

Figure 6 The haemodynamic response to LPS-induced inflammation in CCR2-/- mice.

The haemodynamic response in CCR2-/- and wild type mice was recorded for 24-hours following LPS administration (10µg serotype 0111:B4; intraperitoneal) on E16. (A) ΔMAP (B) ΔSAP (C) ΔDAP (D) HR. *$p<0.5$, **$p<0.01$, ***$p<0.001$, trends were compared using 2-way ANOVA and individual time points were compared using 2-way ANOVA and Bonferroni post-hoc test. Data are expressed as a mean change from pre-LPS baseline (pre) compared to PBS controls ± SEM (wt, n=7; CCR2-/-, n=4).

Supplementary figures

Figure 1 Wild type and CCR2KO mouse myometrial chemokine expression.

Myometrial samples were obtained from non-pregnant and pregnant wild type and CCR2KO mice and chemokine, CCL2 (A), CCL5 (B), CCL20 (C), CXCL1 (D) and CXCL5 (E) mRNA levels measured by rt-PCR.

606 Data are expressed as median, interquartile range and range and were compared using an unpaired
607 t-test when normally distributed and a Mann Whitney test when the data were not. * indicates a
608 difference of $p<0.05$, ** of $p<0.01$, *** of $p<0.001$, n=6 at each time point.

609

610 **Figure 2 Wild type and CCR2KO mouse myometrial macrophage activation markers.**
611 The activation status of myometrial macrophages as assessed by COX-2 and CD86 expression in the
612 myometrium from untreated wild type mice and CCR2 KO mice on E16. Data are expressed as mean
613 ±SEM and were compared using an unpaired t-test ** indicates a difference of $p<0.01$ and *** of
614 $p<0.001$, n=6.

615

616 **Figure 3 The effect of LPS on wild type and CCR2-/- mice placenta**
617 Placenta were obtained from the left uterine horn at 3 hours and in labor after the administration of
618 LPS (10µg serotype 0111:B4) from wild type and CCR2-/- mice. COX-2 and phosphorylated ERK1/2, c-
619 Jun and p65 levels measured by western analysis and densitometry. Data were expressed as
620 mean±SEM and were analysed using an unpaired t-test when normally distributed and a Mann
621 Whitney test when the data were not. * indicates a significant difference of $p<0.05$, ** indicates
622 $p<0.01$, *** indicates $p<0.001$.

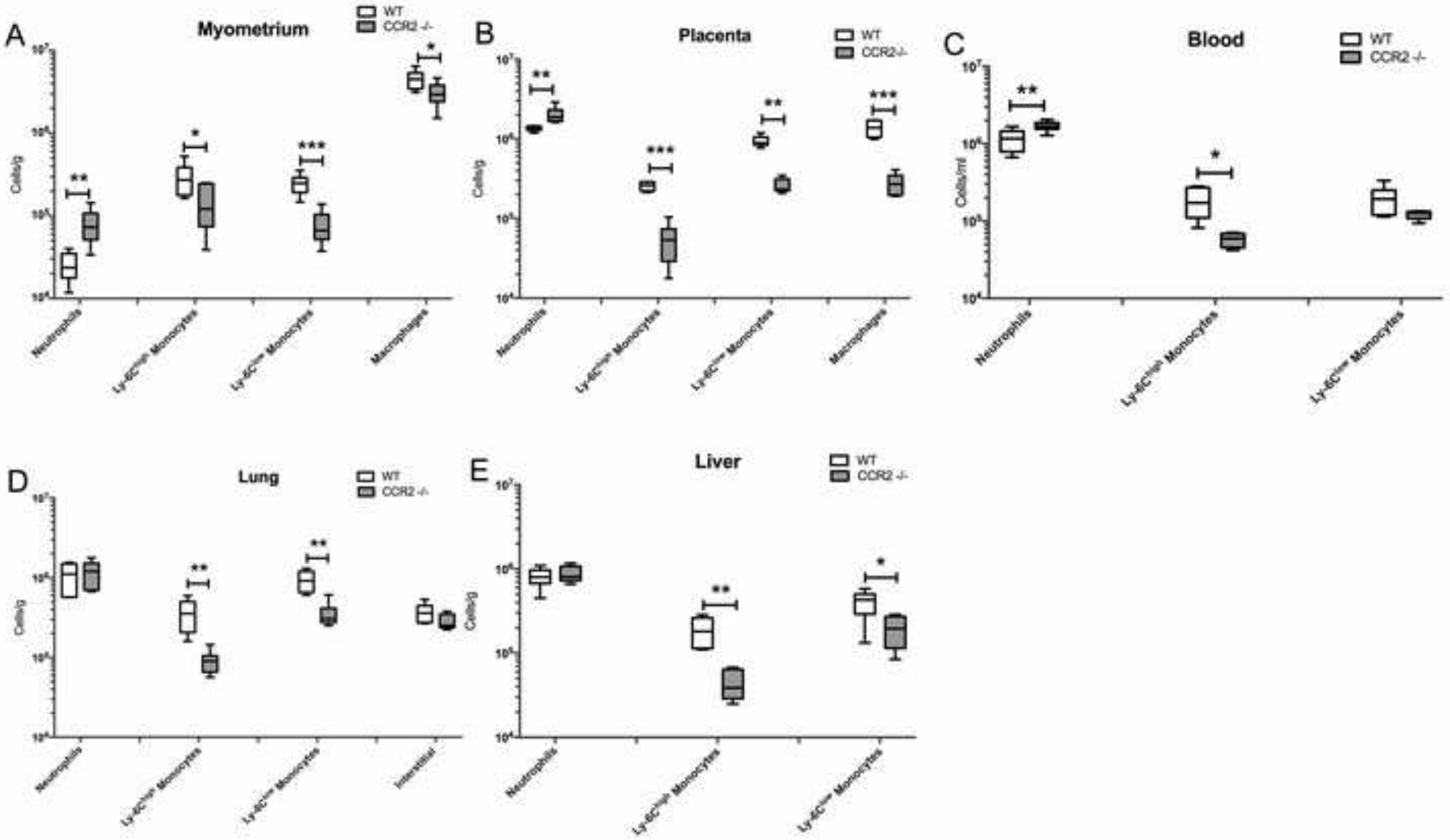
623

624 **Figure 4 The effect of LPS/PBS on wild type and CCR2-/- plasma IL-6 level**
625 Plasma samples were obtained from wild type and CCR2-/- mice before conception, at E16 and during
626 labor. Further samples were obtained from pregnant wild type and CCR2-/- mice after intrauterine
627 PBS or LPS (10µg serotype 0111:B4). Plasma IL-6 levels were measured by ELISA, expressed as median,
628 interquartile range and range and analysed using an ANOVA and Bonferroni post-hoc test. ** indicates

629 a significant difference of $p<0.01$.

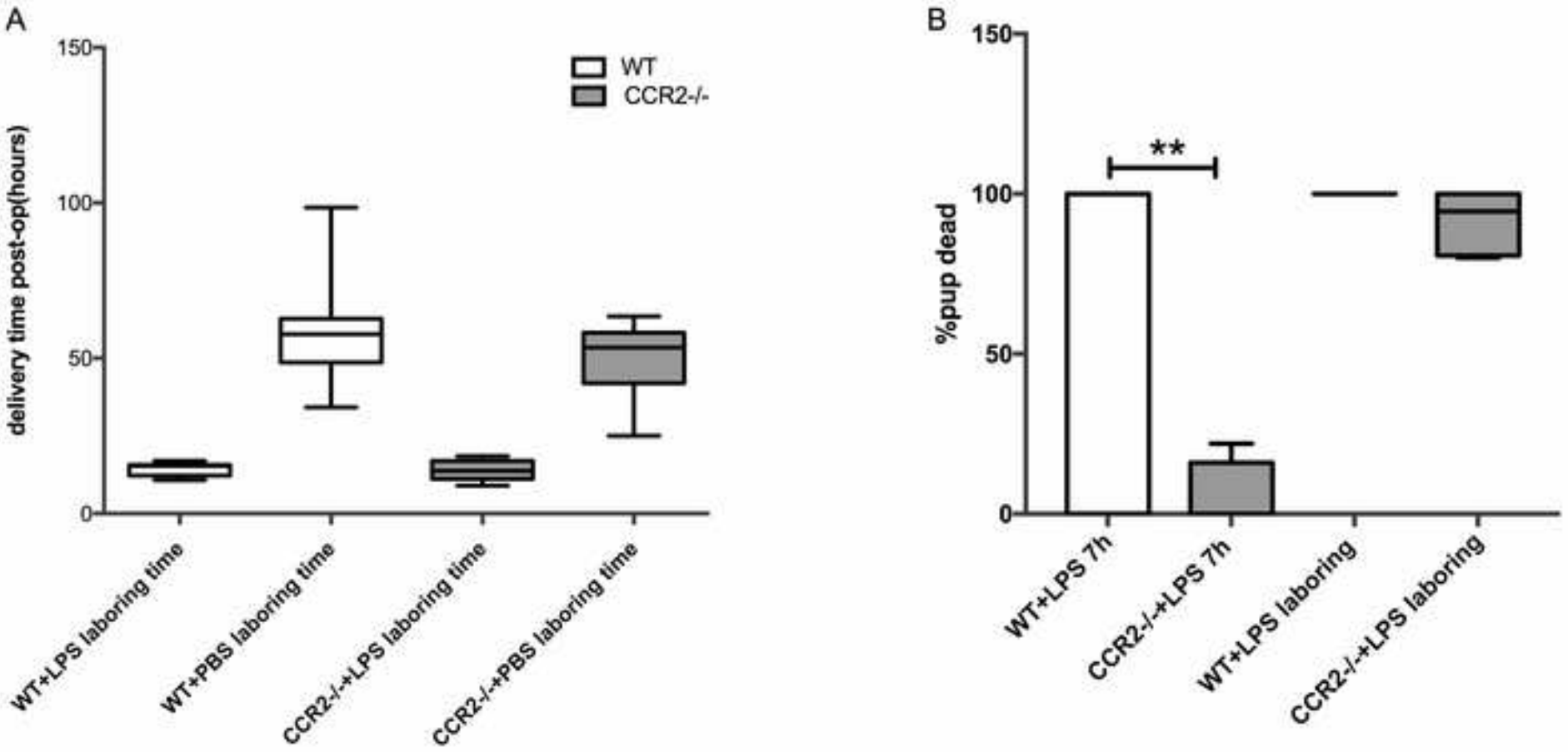
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Figure 1



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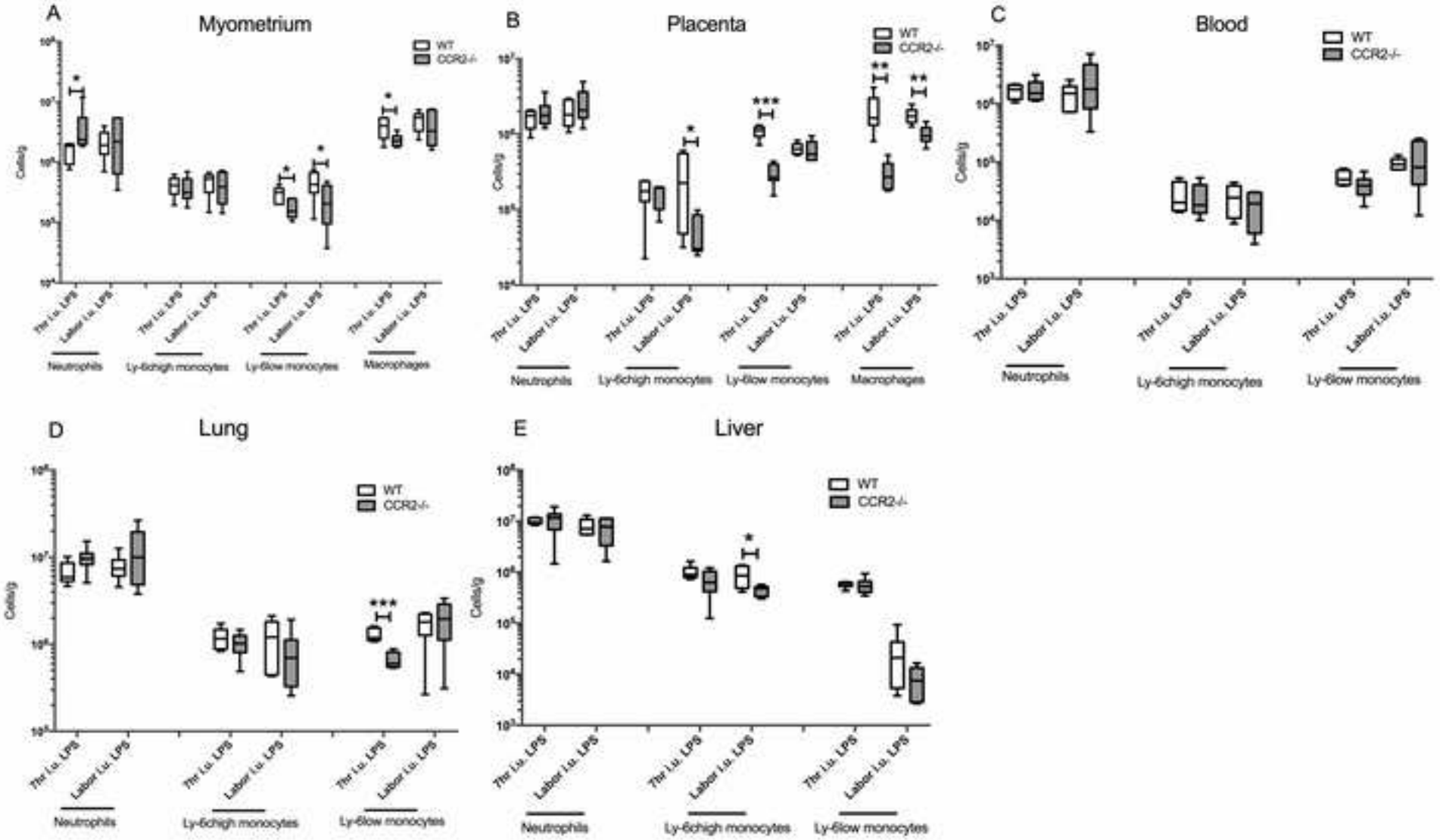
Figure 2



Figure

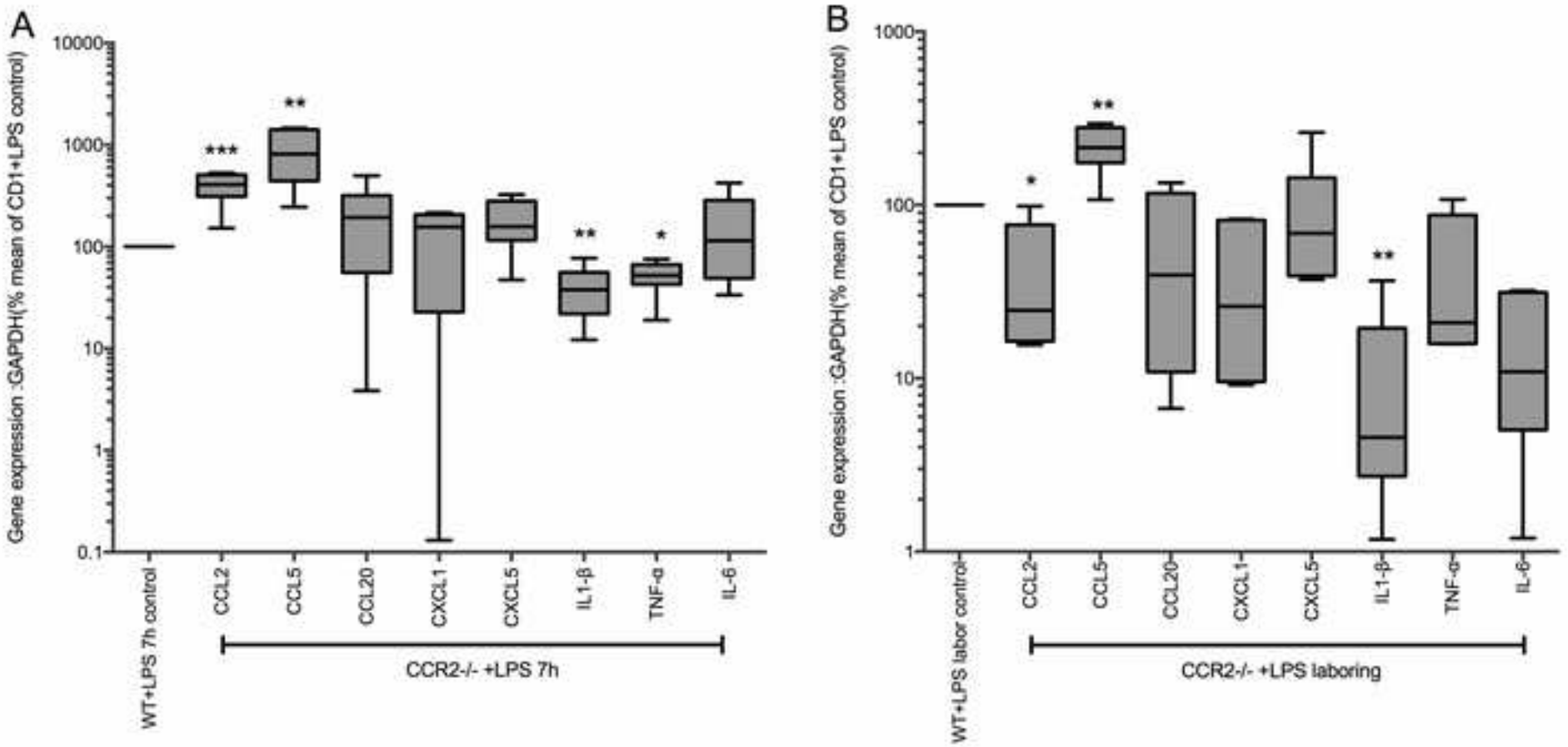
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Figure 3



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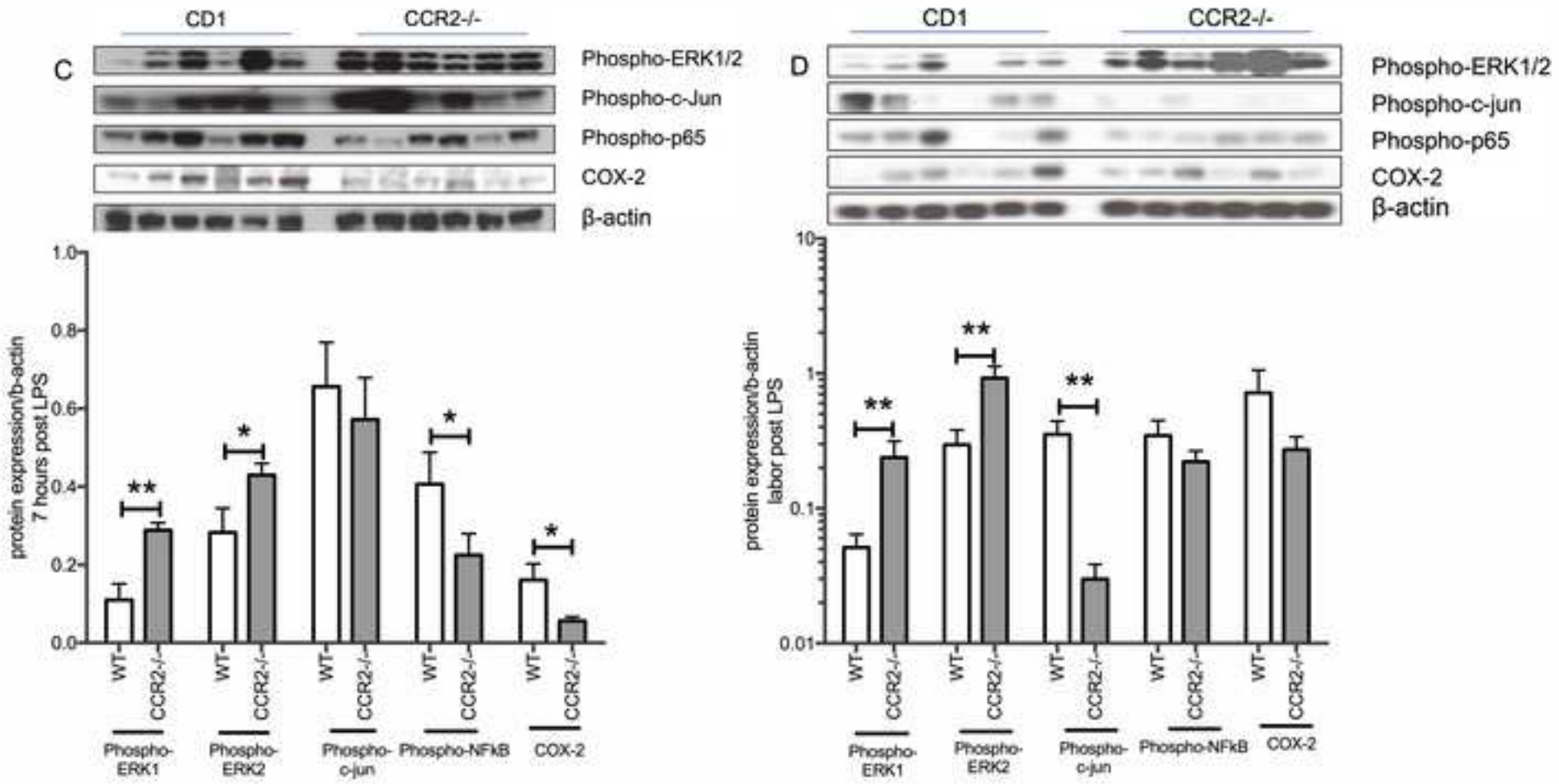
Figure 4 Myometrium



Figure

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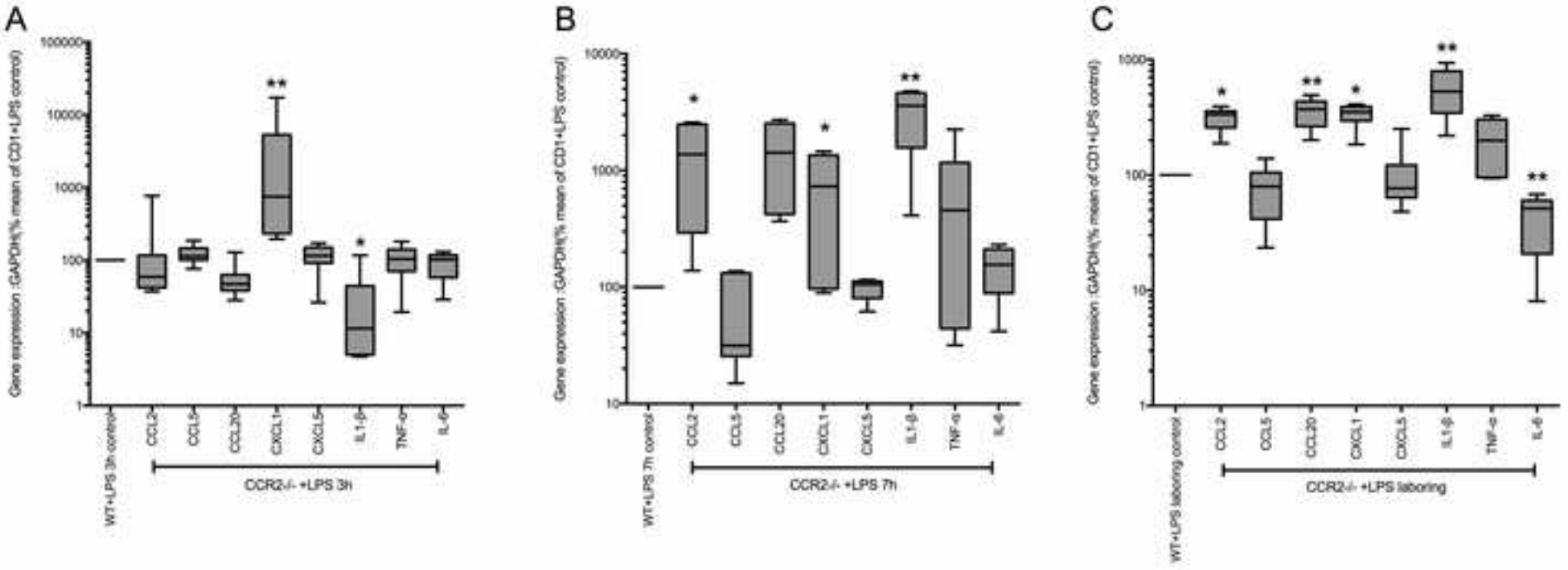
Figure 4 Myometrium



Figure

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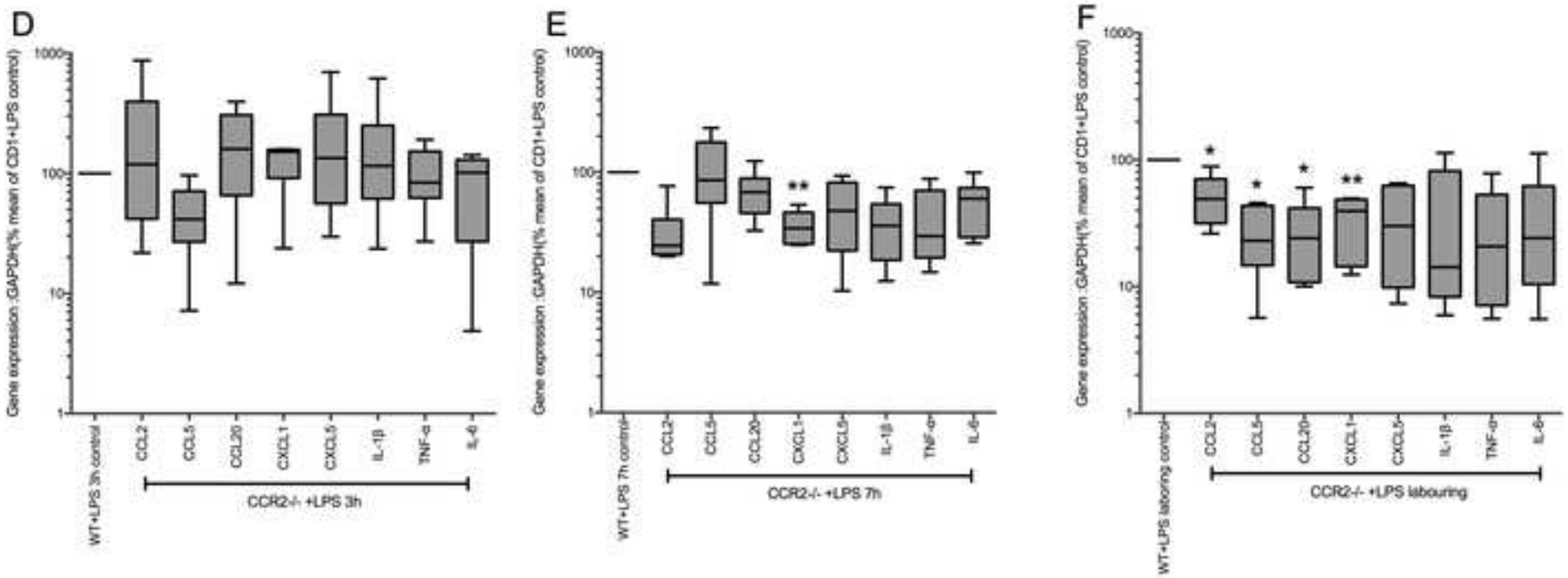
Figure 5 Pup brain



Figure

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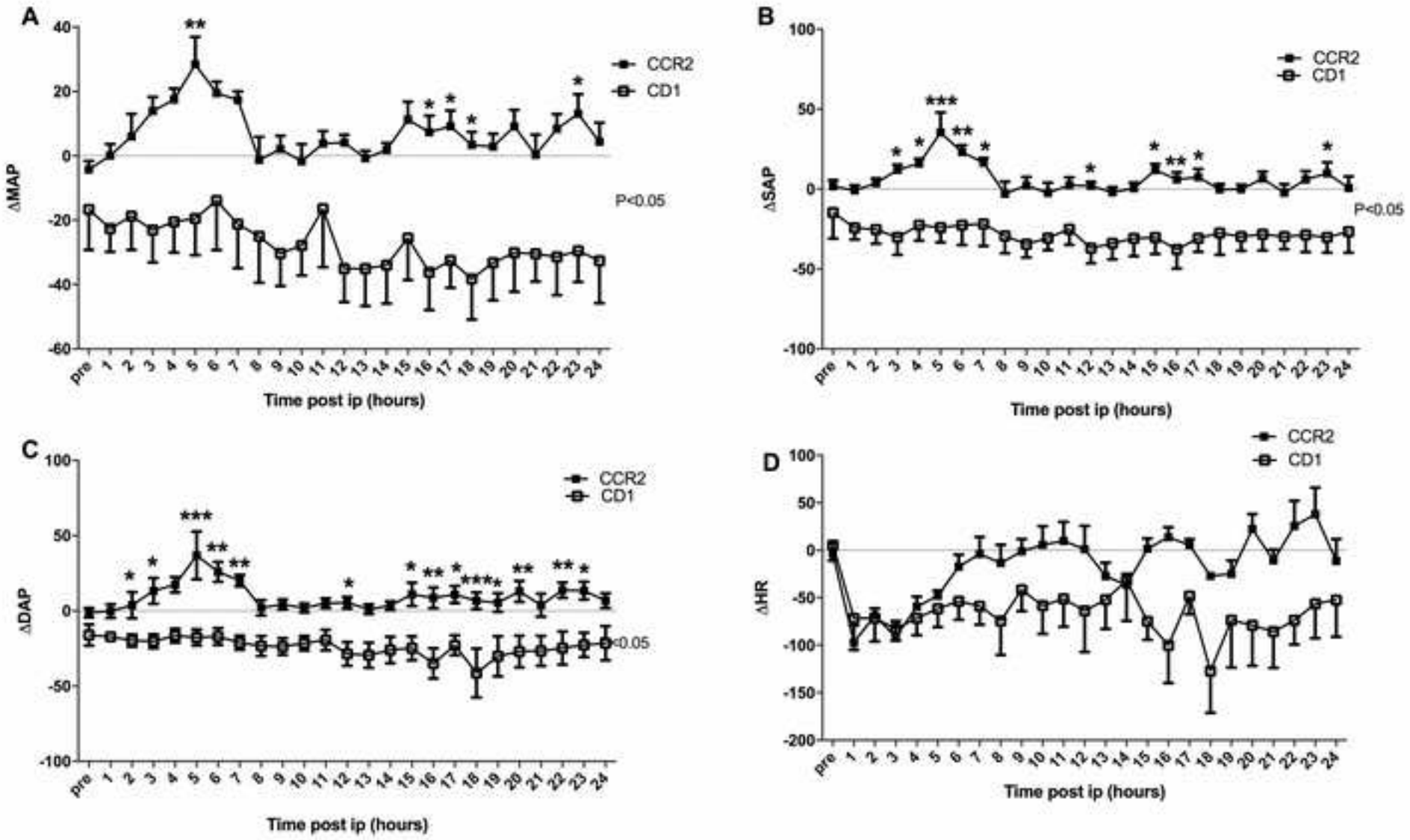
Figure 5 Placenta



Figure

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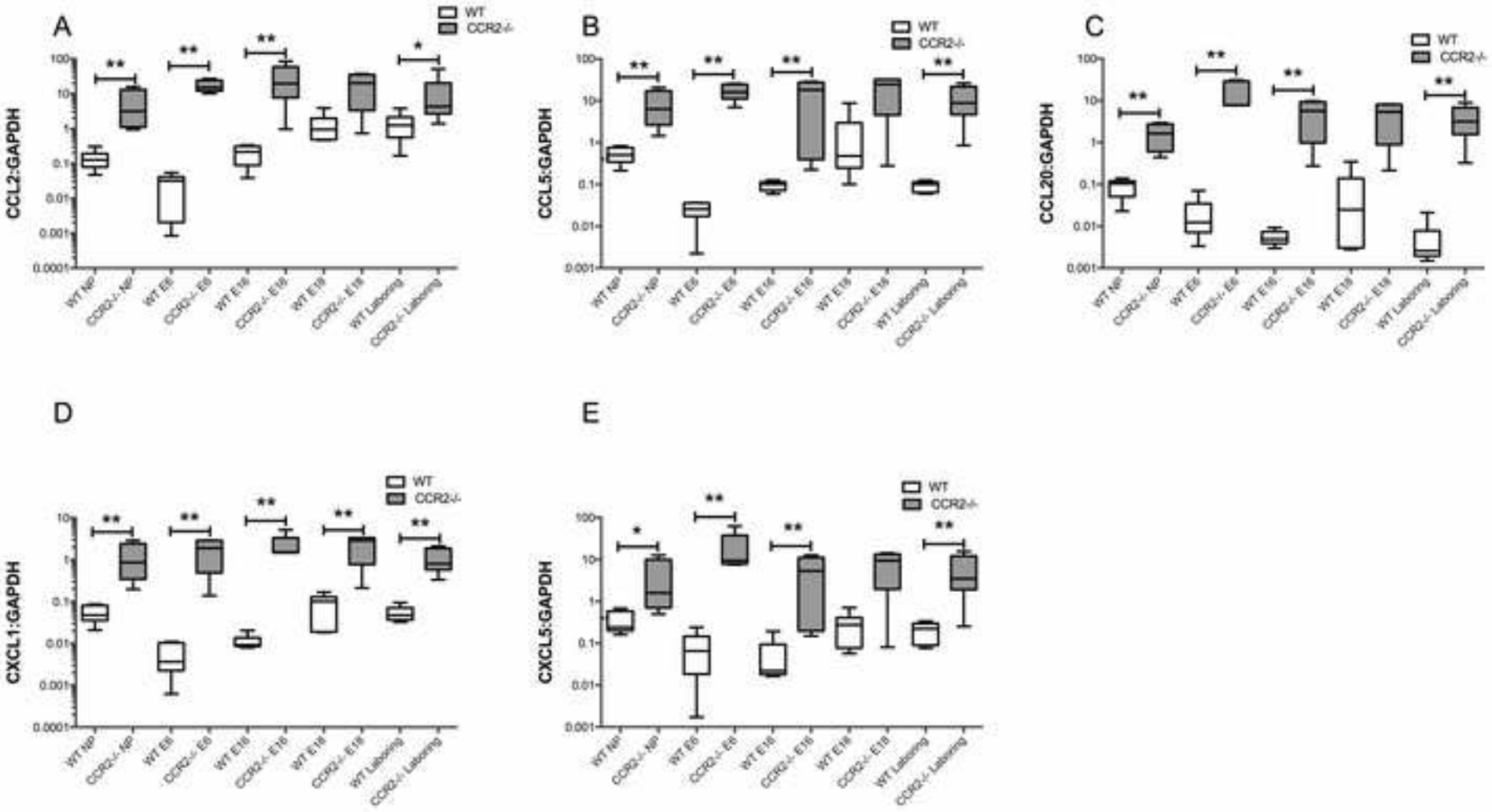
Figure 6



Supplementary Figure

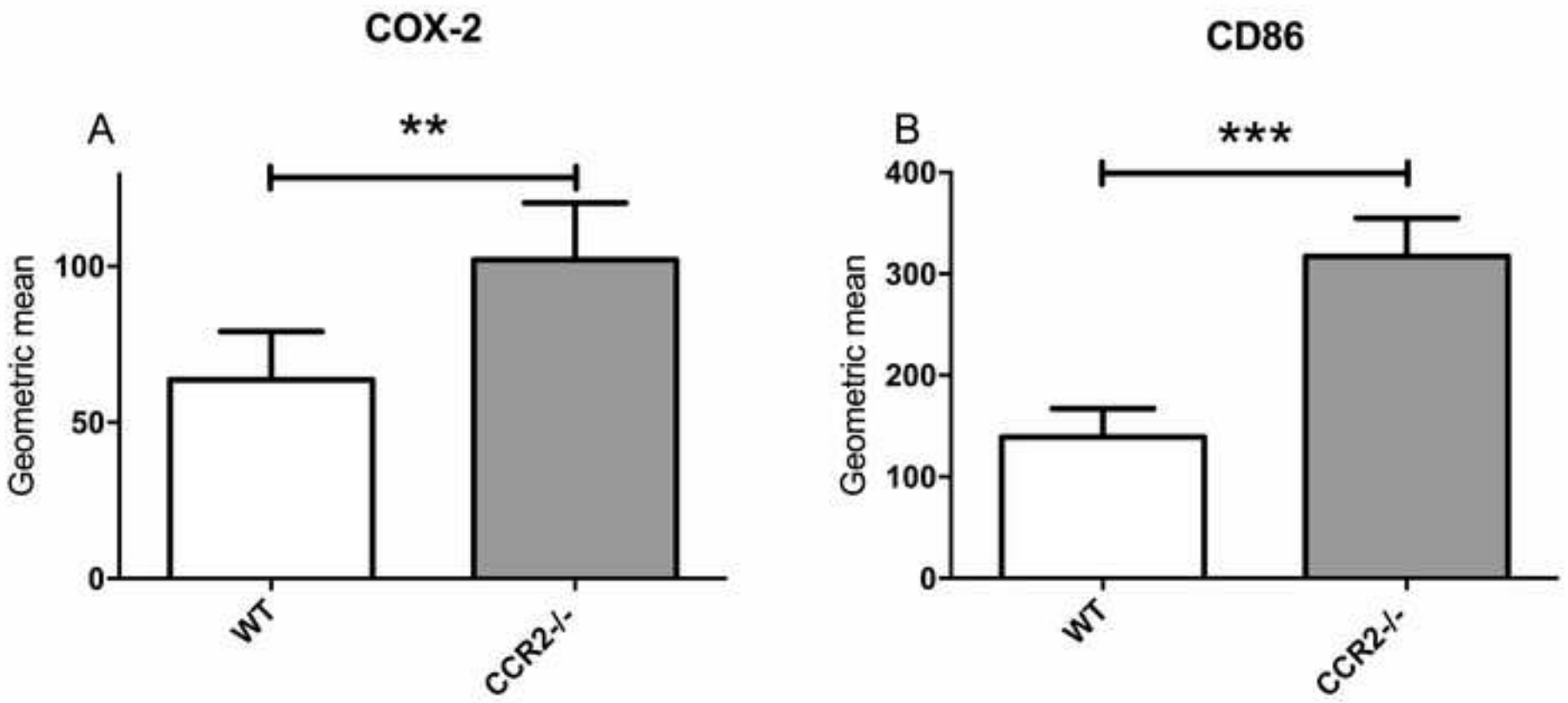
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Figure 1



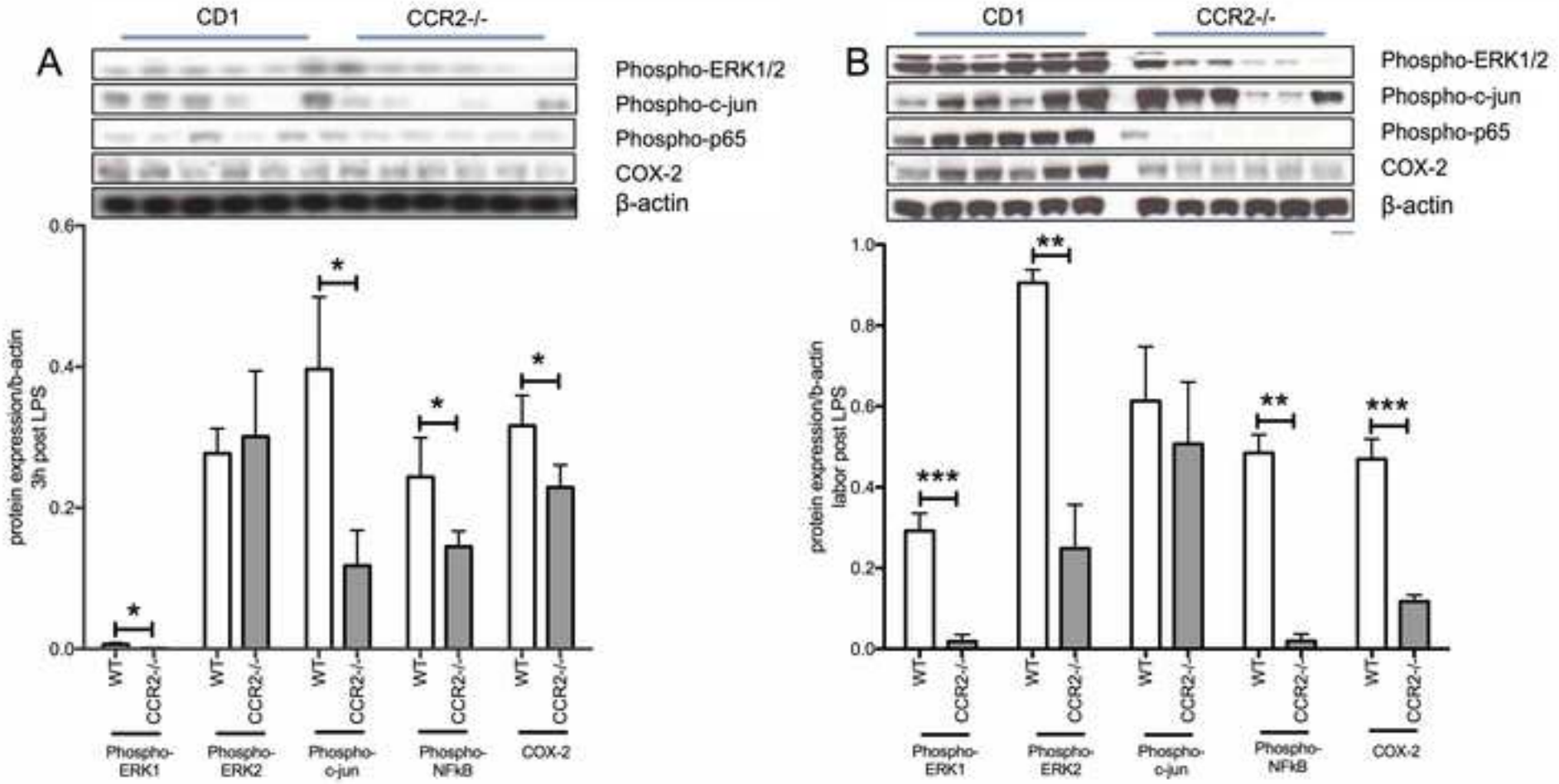
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Figure 2



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Figure 3



Supplementary Figure

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Figure 4

