***Chlamydia muridarum* infection differentially alters smooth muscle function in mouse uterine horn and cervix**

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Abstract

Chlamydia trachomatis infection is a primary cause of reproductive tract diseases including infertility. Previous studies showed that this infection alters physiological activities in mouse oviducts. Whether this occurs in the uterus and cervix has never been investigated. This study characterized the physiological activities of the uterine horn and the cervix in a *Chlamydia muridarum* (*Cmu*) infected mouse model at three infection time-points 7-, 14- and 21 days post infection (dpi). *Cmu* infection significantly decreased contractile force of spontaneous contraction in the cervix (7- and 14dpi; $P<0.001$ and $P<0.05$, respectively) but this effect was not observed in the uterine horn. The responses of the uterine horn and cervix to oxytocin were significantly altered by *Cmu* infection at 7dpi ($P<0.0001$), but such responses were attenuated at 14- and 21dpi. *Cmu* infection increased contractile force to prostaglandin (PGF2α) by 53-83% in the uterine horn. This corresponded with the increased messenger ribonucleic acid (mRNA) expression of *Ptgfr* that encodes for its receptor. However, *Cmu* infection did not affect contractions of the uterine horn and cervix to PGE_2 and histamine. The mRNA expression of *Otr* and *Ptger4* were inversely correlated with the mRNA expression of *Il1b*, *Il6* in the uterine horn of *Cmu*-inoculated mice ($P<0.01$ – $P<0.001$), suggesting that the changes in the *Otr* and *Ptger4* mRNA expression might be linked to the changes in inflammatory cytokines. Lastly, this study also showed a novel physiological finding of the differential response to PGE_2 in mouse uterine horn and cervix.

Introduction

Chlamydia trachomatis infection is the most frequently reported sexually transmitted infection in western countries (19, 27, 42). The disease causes clinically important morbidities and causes significant financial burden to individuals and healthcare services (76). This infection is easily curable with antibiotics, but the majority of patients are often asymptomatic, thus, leaving the infection untreated (37). Subsequently, ascension of the bacteria into the upper reproductive tract occurs and cause serious complications that affect reproductive tract function. Pelvic inflammatory disease, infertility, ectopic pregnancy and chronic pelvic pain (13, 70) are often the result of chronic and recurrent *Chlamydia* infections (39, 62) . There is also a strong link between *Chlamydia trachomatis* reproductive tract infection on pre-term birth and miscarriage (2, 3, 58, 61). A better understanding of the processes that lead to the development of *Chlamydia*-associated pathologies, in particular its effects on reproductive tract function, is required.

In pre-clinical models, *Chlamydia* infection disrupts the pace-making activity of the mouse upper reproductive tract (i.e. oviducts), thus, affecting spontaneous contraction of these tissues (25, 26). This disrupted motility is a potential contributor to tubal infertility. Whether this extends to the lower region of the female reproductive tract is unknown. Like oviducts, the uterus and cervix contract spontaneously in the non-pregnant state in human and mouse (14, 15, 24, 36). Importantly, spontaneous contraction in the human female reproductive tract is believed to play an essential role in maintaining fertility. Although the exact functions of these contractions have not been fully elucidated, what is already known is that they help oocyte propulsion and menstrual shedding (43), sperm transportation (67) and perhaps the transportation or shedding of pathogens.

Apart from spontaneous contraction, smooth muscles along the female reproductive tract are also responsive to endocrine, paracrine and neuronally released signalling molecules.

For example, the hormone oxytocin, which is the prominent stimulant in the female reproductive tract, increases uterine (23, 34, 35, 64), cervical and vaginal motility (36). During parturition, increased expression of the oxytocin receptor along the female reproductive tract is essential for heightened uterine contraction during labour (65). Prostaglandin signalling is also recognized in modulating uterine motility (20). Of all the subtypes of prostaglandins, only PGF2α and PGE_2 are known to modulate motility in the female reproductive tract (56, 68). These prostaglandins are also associated with pain in dysmenorrhea (38). In addition, mast cell mediators such as histamine, also modulate smooth muscle contractions along the female reproductive tract (69). Importantly, these mediators are essential in regulating smooth muscle contractions during parturition (49).

In other viscera, notably the bladder and bowel, inflammation or infection are known to alter smooth muscle functions. This occurs in inflammatory bowel disease (53, 79), ulcerative colitis (48), interstitial cystitis (21), urinary tract infection (74) and cystic fibrosis (33). Inflammatory mediators can exert their effects by directly acting on smooth muscle cells. They can also stimulate the release of mediators from mast cells that have profound effects on smooth muscle function (63). In addition, these inflammatory mediators may also modify the sensitivity of smooth muscle cells to endogenous mediators by altering the expression of certain receptors (45). Evidence from preterm and term deliveries suggest that parturition is regulated by a series of inflammatory processes because inflammatory neutrophils and macrophages are observed in the uterus, decidua and cervix in both clinical and pre-clinical models during labour (32, 50). These immune cells, along with mast cells and chemokines, coordinate the timely contraction of the uterus, cervical ripening and dilation and rupture of the foetal membrane during parturition (1, 28, 32, 50). *Chlamydia* infection induces immunological alterations in the female reproductive tract (6, 8, 9, 29) but whether these affect physiological functions remain unknown.

86 It is now increasingly acknowledged that infection changes microbiota composition
87 (11, 22) in the infected region and may alter the physiological function of an organ (4, 30).
88 Evidence demonstrates that alterations in gut microbiota may lead to smooth muscle motility
89 dysfunction (30, 66). The alteration in gut microbiota is also suggested to be a factor causing
90 slow transit constipation (30, 55). The host and microflora interactions are essential in
91 maintaining organ health and any alteration in the microbiota composition may disrupt this
92 homeostasis. The changes in bacterial substances or end products of bacterial fermentation
93 may change the immune response and neuroendocrine factors which in turn affect the
94 physiological functions of the organ (4, 30). *Chlamydia* infection does not only alter the
95 immunological response (8, 29, 44), it is also suggested to change the microbiota composition
96 in the cervicovaginal region (4, 11). The collective evidence of alterations caused by
97 *Chlamydia* infection suggest there could be a change in the physiological function of the
98 uterus and cervix. Therefore, we compared smooth muscle contraction of mouse uterine horn
99 and cervix in *Chlamydia* infection at three time-points. We also assessed the relationship
100 between mRNA expression for receptors involved in smooth muscle contraction with
101 inflammatory cytokines following infection.
102

Materials and Methods

Ethics Statement

All procedures were conducted in accordance with the Australian Code for the Care and Use of Animals for Scientific Purposes 8th Edition (2013) as endorsed by the National Health and Medical Research Council (NHMRC), the Australian Research Council, the Commonwealth Scientific Industrial Research Organisation and Universities Australia. All protocols were approved by The University of Newcastle Animal Care and Ethics Committee (A-2011-109).

Mice

Naïve specific pathogen-free female wild-type C57BL/6 mice of reproductive age (3 – 7 months old; weighed 20-35g) were obtained from the Australian BioResources (Moss Vale, NSW, Australia) and housed 4 mice per cage in individually ventilated cages. Each cage was equipped with autoclaved corn cob bedding and a shelter, nesting paper, paper coils and a wooden tongue depressor for environmental enrichment. Mice were maintained on a 12-hour light-dark cycle in a room with controlled temperature (22 ± 2 °C) and humidity (30-70%). They were fed *ad libitum* with autoclaved standard rat and mouse cubes (Specialty Feeds, WA, Australia) and water throughout the experimental period. Mice acclimatised for at least 5 days prior to experimentation. Before the mice were assigned into groups, they were monitored for general signs of health and well-being and therefore represented the uniform population. All treated mice were monitored daily for clinical signs of disease as part of the approved protocol. Intervention by veterinary treatment or euthanasia was indicated by the development of signs of severe disease. There were no animal deaths or interventions required as a result of our protocol. Their weight, appearance and behaviour were within normal parameters prior to being assigned to groups. All mice were euthanised by pentobarbital (Virbac, Australia) overdose at the end of the treatment period.

128

129 **Mouse treatments**

130 To replicate *Chlamydia* reproductive tract infection in human, we infected mice
131 intravaginally with *Chlamydia muridarum* (*Cmu*) (52). This is a natural mouse pathogen that
132 induces upper reproductive tract inflammation and pathology, characterized by the
133 development of hydrosalpinx, similar to human *C. trachomatis* reproductive tract infection (6,
134 9, 29, 52). At day 1, animals were subcutaneously injected with medroxyprogesterone acetate
135 (PROVERA®, Pfizer, Australia; 2.5 mg in 200 μL saline) under isoflurane anaesthesia to
136 ensure all mice were in the diestrus stage of their estrous cycle (6, 9, 29). At day 8, mice were
137 allocated into two groups: sham-inoculated or *Cmu*-inoculated. Mice in the *Cmu*-inoculated
138 group were infected by intravaginal inoculation of 5×10^4 inclusion forming units of *Cmu*
139 (ATCC VR-123) in 10 μL sterile sucrose phosphate glutamate (SPG) buffer; while the sham-
140 inoculated mice were intravaginally inoculated with 10 μL of SPG buffer alone. All mice
141 were under ketamine-xylazine anaesthesia (Troy Laboratories, Australia; 80 mg/kg:5 mg/kg
142 IP; Ilium Ketamil® and Ilium Xylazil-20®) during the procedure. SPG buffer was used as the
143 vehicle as it maintains viability of *Chlamydia* (6, 9, 29). Mice were monitored daily for 7-,14-
144 and 21 days post-infection (dpi). Mice were sacrificed at the end of respective time-point by
145 sodium pentobarbitone (Lethabarb, Virbac, Australia) overdose, and vaginal lavages were
146 collected for estrous cycle stage confirmation as previously described (29, 36). The left
147 uterine horn was dissected out, trimmed of visceral fat, snap-frozen in liquid nitrogen and
148 stored at -80°C for subsequent quantitative polymerase chain reaction (qPCR). The right
149 uterine horn and cervix were placed in chilled physiological saline solution (PSS, 120 mM
150 NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 2 mM $MgCl_2$, 25 mM $NaHCO_3$, 1 mM NaH_2PO_4 and 10
151 mM glucose; gas with 95% O_2 and 5% CO_2).

152

153 **Measurement of cross-sectional area of oviducts**

154 The height and width of both oviducts were measured using a calliper and the cross-sectional
155 area was calculated. Higher cross-sectional area indicates swelling of the oviducts.
156 Development of hydrosalpinx and swelling of the oviducts indicate fluid accumulation which
157 is caused by scarring and blockage of the oviducts and is one of the key pathological features
158 of *Chlamydia* infections. Hydrosalpinx is the result of immune cell infiltration and
159 inflammation (5, 18, 77). We have used measurement of hydrosalpinx to identify the
160 development of *Chlamydia*-associated pathology in our model.

161

162 **Preparation of myometrial and cervical strips**

163 The detailed anatomy of our isolated preparations have been previously outlined relative to
164 smooth muscle actin ((36); see also (78)). Right uterine horns were trimmed of connective
165 tissue and opened along the mesometrial border. A uterine strip of 1 cm in length measured
166 from the end of oviduct was prepared. Uterine strips were tied at both ends with thread that
167 was subsequently attached to a hook at the base of the 4 mL tissue bath and a tension
168 transducer (Grass FT03) respectively. For cervical preparations, tissue immediately caudal to
169 the bicollis uterus was transected adjacent to the vaginal canals. The cervical incision was
170 made at the anterior fornix and extended longitudinally towards the uterine horns. The tubular
171 cervix was then uncoiled into a rectangular strip. Due to the firmer nature of the cervix tissue
172 piercings were made near the cut edge with a 30G needle. String was then looped through the
173 puncture and tissue was suspended in the tissue bath so the direction of force would be in
174 similar direction to a dilating cervix. Tissues were then equilibrated at tension of 10 mN
175 (cervix) and 5 mN (uteri) for 30 min in PSS at 37°C before being challenged with 60 mM
176 KCl (potassium chloride). Spontaneous contractions were recorded as previously described
177 (36). At the end of the experiment, tissues were blotted dry and wet weight measured.

Cervices were kept in RNA*later*® (Thermo Fisher Scientific, Scoresby, VIC, Australia), stored at 4℃ overnight, and finally stored at -80℃ until RNA extraction was performed for subsequent qPCR reaction.

Contractile activity

Contractile force was assessed by measuring peak amplitude relative to 60 mM KCl corrected for wet weight and expressed as %KCl while frequency of contractions were expressed as number of contractions per 5 minutes as outlined in (36). 60 mM KCl acted as the internal control for contractile force as it was not affected by *Cmu* infection in both cervix and uterine horns at the three time-points (**Fig S1 -** DOI: **https://doi.org/10.6084/m9.figshare.10317068.v1**). Each tissue was equilibrated for 30 min before stimulation with 60 mM KCl (~2 min). Following KCl stimulation preparations were left for a further 30 minutes to determine baseline contractility before application of drugs. Oxytocin, PGF2α and PGE_2 (Cayman Chemical, Ann Arbor, USA) were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO did not exceed 0.001%. Histamine (Sigma Aldrich, Missouri, USA) was dissolved in milli-Q water.

Cumulative oxytocin dose responses were obtained by adding increasing concentrations into the organ bath every 5 min. In separate experiments, PGF2α (1 μM and 3 μM), PGE_2 (1 μM) and histamine (1 mM) were tested. The concentration for each mediator was based on previous studies (17, 54, 59). Each mediator was applied for 5 min before wash-off. Tissues were washed 3-5 times before the next drug was added. At the end of the experiment, tissues were contracted with 60 mM KCl again to ensure tissue viability. The contractile force was extracted using automated quantification in LabChart 8 Reader (ADInstruments, Australia) and averaged over 5 min.

The change in contractile force of the spontaneous contractions evoked by PGF2α, PGE2 and histamine (Δ %KCl) was compared between the sham- and *Cmu*-inoculated mice.

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RNA isolation, reverse-transcription PCR (RT-PCR) and qPCR

Frozen tissues were thawed and squeezed dry of RNA*later*®. Tissues were then homogenized in 500 µL of TRIzol® (Invitrogen, Mount Waverly, VIC, Australia) using a Tissue-Tearor stick homogenizer (BioSpec Products, Bartesville, OK) on ice. Total RNA was extracted according to the manufacturer's instructions (TRIzol®, Invitrogen, Mount Waverly, VIC, Australia) (6, 9, 29) and was treated with DNase (Sigma-Aldrich). The end-product was reverse-transcribed to cDNA using M-MLV Reverse Transcriptase (Life Technologies, Thermo Fisher Scientific) and random hexamer primers (Bioline, Alexandria, NSW, Australia) in a T100™ Thermal Cycler (BioRad). qPCR was performed on a CFX384 Touch Real-Time PCR Detection System (Bio-Rad, Gladesville, NSW, Aus) using SYBR reagents (KAP Biosystems, MA, USA) and custom designed primers specific for *Cmu* ribosomal 16S rRNA; the inflammatory markers, *Stat1*, *Stat6*, *Ifng*, *Cxcl1*, *Cxcl2*, *Il10*, *Mmp9*, *Il1b*, *Il6*, *Cxcl15*, *Tnfa*; oxytocin receptor, *Otr*; and prostaglandin receptors, *Ptgfr*, *Ptger1*, *Ptger2*, *Ptger3* and *Ptger4*. mRNA expression was calculated using $2^{-\Delta\Delta Ct}$ relative to the reference gene hypoxanthine-guanine phosphoribosyl-transferase (*Hprt*) and expressed as relative expression. Primers used in this study are reported in Table 1.

Statistical Analysis

Tests for normality were performed on all data using Statistical Package for the Social Science v24 software (SPSS Inc., Chicago, IL, USA). GraphPad Prism Software v8 (San Diego, CA) was subsequently used for statistical analyses. All data are presented as means ± standard error of the mean (S.E.M.), with *n* representing the numbers of individual tissues

from different mice. Statistical significance was set at $P<0.05$. Statistical significance for comparisons between two groups was determined using either unpaired *t*-test for parametric data or Mann-Whitney U test for non-parametric data. Dose-response curves were fitted using Nonlinear Regression – log(agonist) vs. response (three parameters) model from GraphPad Prism v8, as previously described (47). Comparison of Fits under the Nonlinear Regression model was used for the comparison of best-fit curves, where the bottom plateau, EC_{50}, and top plateau of best-fit curves was compared between sham- and *Cmu*-inoculated data. For comparison of changes in mRNA expression of inflammatory markers versus receptors, a Spearman's correlation analysis of multi-variables was performed using GraphPad Prism v8 (San Diego, CA). Correlation coefficients r (rho) range from -1 to +1. Values lesser than 0 indicate an inverse relationship and values larger than 0 show a positive relationship. Significance level of $P<0.05$ indicates the two variables are significantly correlated.

Results:

Pathology of *Cmu* infection

We first confirmed productive *Cmu* infection in the upper reproductive tract by quantifying *Cmu* ribosomal 16S rRNA in the uterine horn. The presence of 16S rRNA indicates active infection (6). There was no 16S rRNA expression in sham-inoculated uterine horns while 16S rRNA was detected in the uterine horn of all *Cmu*-inoculated mice at 7-, 14- and 21dpi (**Fig 1A**). The highest bacterial load (peak infection) of 16S rRNA was observed at 7dpi. Higher cross-sectional area was also observed in both the oviducts of *Cmu*-inoculated mice compared to the sham (**Fig 1B** and **C**), confirming the successful development of pathology in our model. As noted in previous studies (6, 8, 9, 29), *Cmu* infection significantly increased inflammatory cytokines in the uterine horn (**Fig 2**) and cervix (**Fig 3**) at all three time-points.

Cmu reduced spontaneous contraction in the cervix but not in the uterine horn

Relative to 60 mM KCl contractions, spontaneous contractions were unaffected in the uterine horn of *Cmu*-inoculated mice at three time-points (**Fig 4A – E**). In contrast, spontaneous contraction in the cervix was reduced by 45.4% and 49.7% at 7- and 14dpi, respectively (**Fig 4F - I**). However, this phenomenon was restored to sham levels at 21dpi (**Fig 4I**). Contractile frequency of the cervix was not affected by *Cmu* infection at any time-point post infection (**Fig 4J**).

Effects of *Cmu* infection on mediator-induced contractions

Oxytocin

Cmu infection altered uterine and cervical contractions to oxytocin at the early infection time-point

In parallel with the study by Gravina and co-workers (36), oxytocin modified uterine contractile force and frequency in mice (**Fig 5A, D** and **G**). To investigate the effects of *Cmu* infection on the uterine contractions to oxytocin, best-fit dose-response curves were compared between the sham- and *Cmu*-inoculated mice. A significant decrease in both the contractile force and frequency to oxytocin in *Cmu*-inoculated mice was observed at 7dpi (**Fig 5B**). Despite the decrease in uterine response to oxytocin, there was no significant change in the EC_{50} of oxytocin between the two groups (EC_{50} - 32.3±1.4 nM in sham-inoculated; 42.4±1.9 nM in *Cmu*-inoculated; P=0.65).

To further investigate the reason for the decreased uterine contractions to oxytocin at 7dpi, we compared the mRNA expression of the oxytocin receptor – *Otr* in the uterine horn between the two groups. The *Otr* expression was significantly decreased in the *Cmu*-inoculated uterine horns at 7dpi (**Fig 5C**), while *Otr* expression remained unchanged at 14- (**Fig 5F**) and 21dpi (**Fig 5I**).

Cmu infection increased contractile force of the cervix to oxytocin at the early infection time-point

In contrast to the uterine horn, a significant increase in contractile force to oxytocin was observed in the cervix of *Cmu*-inoculated mice at 7dpi (**Fig 6A** and **B**) but contractile frequency was not affected by *Cmu* infection. The EC_{50} in the two groups was also unaffected (EC_{50} – 2.0±0.3 μM in sham-inoculated; 1.3±0.2 μM in *Cmu*-inoculated; P=0.75). The response of cervix to oxytocin in *Cmu*-inoculated mice was not significantly different to sham levels at 14 and 21dpi (**Fig 6E** and **H**).

In a separate cohort of mice, we found that 14dpi *Cmu* infection decreased mRNA expression of *Otr* in the cervix (refer to **Fig 6F**). However, there was no change in *Otr* expression in the cervix of *Cmu*-inoculated mice at 7- and 21dpi (**Fig 6C** and **I**).

Prostaglandins - PGF2α and PGE2 have differential effects on the cervix and uterine horn

PGE2 elicited a differential response in the cervix and uterine horn

Notably, we observed opposing responses elicited by PGE_2 in both the uterine horn and cervix. PGE_2 decreased contractile force in the uterine horn (**Fig 7A**) while it increased contractile force in the cervix (**Fig 7B**). To further assess if such differential responses to PGE_2 was due to differences in receptor expression, mRNA expression for PGE_2 receptors was compared between the uterine horn and cervix. The data were pooled from only sham-inoculated mice of 7-, 14- and 21dpi, and the comparison was made between the cervix and uterine horn. Other than *Ptger1*, the cervix generally showed lower mRNA expression of receptors – *Ptgfr*, *Ptger2*, *Ptger3* and *Ptger4* (**Fig 7C**; n=24-29; Mann-Whitney U test; P<0.0001) compared to the uterine horn. Interestingly, *Ptger2* expression was very low in the cervix compared to the uterine horn (relative expression of *Ptger2* in the cervix – 0.002±0.0004; uterine horn – 0.05±0.008).

Cmu infection increased uterine contraction to PGF2α

To investigate the effects of *Cmu* infection on the response to prostaglandins, PGF2α and PGE_2, we compared uterine contractions between sham-inoculated and *Cmu*-inoculated mice. *Cmu* infection did not alter uterine contraction to PGF2α at 7dpi (**Fig 8A** and **S2**) but it significantly increased uterine contractile force at 14- (**Fig 8C**) and 21dpi (**Fig 8E**). We then compared the mRNA expression of PGF2α receptors – *Ptgfr*, *Ptger3* and *Ptger4* between the two groups. At 7dpi, the mRNA expression of *Ptgfr*, *Ptger3* and *Ptger4* were significantly lower in the uterine horn of *Cmu*-inoculated mice compared to sham (**Fig 8B**). At 14dpi, a significant higher expression of *Ptgfr* was observed in the uterine horn of *Cmu*-inoculated

316 mice. Also, at 14dpi, *Ptger4* mRNA expression in the uterine horn was significantly
317 decreased by *Cmu* infection (**Fig 8D**). *Cmu* infection also significantly increased *Ptgfr*
318 mRNA expression in mouse uterine horn at 21dpi but did not alter the mRNA expression of
319 *Ptger3* and *Ptger4* (**Fig 8F**).
320
321 ***Cmu* infection did not alter cervical response to PGF2α**
322 PGF2α induced contraction in the cervix of sham- and *Cmu*-inoculated mice (**Fig S3** -
323 DOI: **https://doi.org/10.6084/m9.figshare.10317068.v1**) at 7-, 14- and 21dpi. However,
324 there was no significant difference in the cervical contraction to PGF2α between the sham-
325 and *Cmu*-inoculated mice (**Table 2**).
326
327 **PGE2**
328 ***Cmu* infection did not alter cervical and uterine contraction to PGE2**
329 The effects of *Cmu* infection on the cervical and uterine contractions to PGE_2 were
330 also investigated. *Cmu* infection did not alter physiological response of both the organs to
331 PGE_2 at all infection time-points (**Tables 2** and **3**).
332
333 **Histamine**
334 ***Cmu* infection did not alter cervical and uterine contractions to histamine**
335 Histamine is one of the mediators released during mast-cell degranulation and it is
336 also a potent modulator of smooth muscle contraction along the female reproductive tract (16,
337 59). Thus, the effects of *Cmu* infection on the cervical and uterine contractions to histamine
338 were also investigated. The cervical and uterine contractions to histamine were not different
339 between the sham- and *Cmu*-inoculated mice at 7-, 14- and 21dpi time-points (**Tables 2** and
340 **3**).

341

342 **Correlation of cytokines with receptor mRNA expression in the uterine horn**

343 Because *Cmu* infection significantly altered uterine contractions to mediators, we next
344 explored the relationship between cytokines and related receptors in the uterine horn,
345 specifically the relationship between *Il1b*, *Il6*, *Cxcl15* (analogue of human *Il8* (46)) and *Tnfa*
346 mRNA relative expression levels versus *Otr*, *Ptgfr* and *Ptger4* mRNA relative expression
347 levels. These cytokines are known to modulate female reproductive tract contraction (1) and
348 their mRNA was also significantly increased in the uterine horn of *Cmu*-inoculated mice (**Fig**
349 **2** and **Fig 3**). We combined the data of *Cmu*-inoculated uterine horn from the three time-
350 points for Spearman's correlation analysis. In our model, *Otr* was inversely correlated with
351 *Il1b* and *Il6* mRNA in the uterine horn (**Fig 9**; n=23; Spearman's correlation; $^{**}P<0.01$).
352 Similarly, *Ptger4* was also inversely correlated with *Il1b* and *Il6* mRNA in the uterine horn
353 (**Fig 9**; n=23; Spearman's correlation; $^{**}P<0.01$, $^{***}P<0.001$). There was no significant
354 correlation between *Ptgfr* mRNA and the selected cytokines (**Fig 9**).

355

Discussion and Conclusion

It has long been recognized that *Chlamydia* reproductive tract infections in women elicit a range of immunological responses in infected areas (40). Despite the alarming increase in *Chlamydia* reproductive tract infections, we do not know how these immunological changes affect the physiological functions of the female reproductive tract. Previous studies in mice showed that *Chlamydia* infection causes loss of pace-making ability in the oviduct, and this eventually impedes the transportation of the oocyte to the uterus (25, 26). However, no studies have asked whether sexually transmitted infection-associated pathology extends to the uterus or the lower reproductive tract. Here, we focused on the uterine horn and cervix, as these organs are the main routes for microbe ascension that lead to subsequent complications (67).

Cmu infection did not affect spontaneous contractions in the uterine horn, indicating that smooth muscle pace-making property was not affected. In contrast, *Cmu* infection reduced the force of spontaneous contractions in the cervix during early infection time-points (7- and 14dpi) when the *Cmu* load was high, but not at 21 days when *Cmu* load was reduced. This suggests that there might be an association between the *Cmu* load and smooth muscle motility. Although the mechanisms underlying this correlation are unclear, findings in the gastrointestinal tract demonstrated the association of changes in microbiota with disrupted smooth muscle function. Whether a similar mechanism occurs in the reproductive tract remains to be determined (4, 11, 22, 57). Also, we cannot discount the possibility that force changes are a secondary consequence of oedema within the connective tissue that the muscle fibres connect with (51, 71).

The responses of the uterine horn and cervix to oxytocin - the prominent stimulant for female reproductive tract motility, were also studied. Interestingly, oxytocin evoked an

increase in both baseline tone and contractile frequency in the uterine horn but only evoked an increase in contractile frequency in cervix.

We next compared the uterine contractions to oxytocin between the sham- and *Cmu*-inoculated mice and showed that *Cmu* infection significantly reduced contraction of the uterine horn to oxytocin at early infection (7dpi). This was concomitant with a significant decrease in the *Otr* mRNA expression. However, at 14dpi and 21dpi, *Cmu* infection did not affect oxytocin evoked uterine contraction or the mRNA expression of *Otr*. This suggests that the response of uterine horn to oxytocin may only be affected in acute *Cmu* infection. The cervical contractions to oxytocin between the sham- and *Cmu*-inoculated mice were also compared. At 7dpi, the EC_{50} of oxytocin in the cervix was not different between *Cmu*- and sham-inoculated mice, and there was no difference in the *Otr* mRNA expression. While the maximum contraction of oxytocin relative to spontaneous contraction at 7dpi was increased in *Cmu*-inoculated cervix, this was more due to the reduced contractile force in spontaneous contraction than the increased total force to oxytocin. At 14dpi, *Cmu* infection did not affect the amplitude of the cervical contractions to oxytocin even though *Otr* mRNA expression was significantly decreased. Presumably post-transcriptional receptor expression is maintained (72) or downstream pathways are amplified that compensate for any decrease in receptor expression (12, 41).

Cmu infection increased contractile force to PGF2α and the mRNA expression of *Ptgfr,* which encodes FP receptor, in the uterine horn at 14- and 21dpi. At 14dpi, there was also a marked decrease in the mRNA expression of *Ptger4* encoding the EP4 receptor in the uterine horn. PGF2α induces contraction via FP receptor while the binding of PGF2α to EP4 receptor mediates smooth muscle relaxation (10). Therefore, at 14dpi, both the potential increase in FP receptors and decrease in EP4 receptors would result in increased contractile force to PGF2α. On the other hand, at 7dpi, the uterine contractions to PGF2α was not

affected by *Cmu* infection despite the change in receptor mRNA expression. This further underscores the limitations of extrapolating changes in mRNA expression with physiological function (12, 72).

While *Cmu* infection did not change the responses of the uterine horn or cervix to PGE_2, one striking observation is that PGE_2 exerted opposing effects on these tissues in mice in diestrus phase of the estrous cycle. PGE_2 abolished spontaneous contractions in the uterine horn but induced contractions in the cervix. We also discovered differential mRNA expression for genes encoding EP receptors between cervix and uterine horn. Specifically, *Ptger2* mRNA was expressed at extremely low levels in the cervix compared to the uterine horn of C57BL/6 mice. Given the EP2 receptor is linked to smooth muscle relaxation, the differential response to PGE_2 in the uterine horn and cervix is perhaps not surprising. This is the first study to demonstrate a differential physiological response to PGE_2 in mouse cervix and uterine horn. Further investigation into physiological function by using receptor blockers will strengthen this finding.

Our results suggest that inflammation subsequent to *Chlamydia* infection drives changes in receptor expression or downstream signalling pathways. We found that *Il6* and *Il1b* mRNA expression was inversely correlated with the mRNA expression of *Otr* and *Ptger4* in the uterine horn. Several studies have implicated *Il6* with changes in *Otr* receptors. There are several *Il* response elements, including nuclear factor (NF)-*Il6*, which flanks both rat *Otr* and human *OTR* promoter regions (60, 80). Our findings were in line with a study conducted by Schmid and co-workers on human myometrial cells, where *Il1b* and *Il6* negatively regulated *Otr* gene expression (60). However, Fang and co-workers showed that there was no correlation between *Il6* and *Otr* in non-pregnant rat uterine tissue (28).

While our data shed light on altered physiology in the non-pregnant reproductive tract, extrapolations to pregnancy must be made with caution. We have previously shown that

cervix contractility is changed in late pregnancy compared with the non-pregnant state (36). Interestingly miscarriage is one of the complications associated with *Chlamydia* infections (2, 7, 31, 75). How this might relate to altered smooth muscle function requires future investigation (73, 75).

We have shown for the first time that *Chlamydia* infection can change smooth muscle motility in the female reproductive tract. Furthermore, *Cmu* infection differentially altered smooth muscle function and receptor mRNA expression of prostaglandin receptors (*Ptgfr, Ptger2* and *Ptger4*) in mouse uterine horn compared with cervix. Our results suggest that *Chlamydia*-induced changes to reproductive tract physiology need to be extended below the oviduct to the entire female reproductive tract.

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Disclosure

No conflicts of interest, financial or otherwise, are declared by the authors.

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456 **Author Contributions**

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459 Funding acquisition: JCH, PMH

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Figures and Legends

Fig 1: Intravaginal inoculation of *Cmu* successfully promoted bacterial ascension into the upper reproductive tract. (A) 16S rRNA expression was only detected in the uterine horn of *Cmu*-inoculated mice at three different time-points (***P*<0.01, ****P*<0.001; Mann-Whitney U test). (B-C) Higher cross-sectional area (mm^2) was observed in both the oviducts of *Cmu*-inoculated mice at three time-points (**P*<0.05, ***P*<0.01, ****P*<0.001; unpaired *t*-test). Data are presented at mean ± S.E.M. (*n* = 8-14).

Fig 2: mRNA expression of cytokines in the uterine horn of sham- and *Cmu*-inoculated mice at 7-, 14- and 21dpi. Data are presented at mean ± S.E.M. (*n* = 7-14; unpaired *t*-test or Mann-Whitney U test; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

Fig 3: mRNA expression of cytokines in the cervix of sham- and *Cmu*-inoculated mice at 7-, 14- and 21dpi. Data are presented at mean ± S.E.M. (*n* = 7-14; unpaired *t*-test or Mann-Whitney U test; **P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001).

Fig 4: The contractile force and frequency of spontaneous contractions in the uterine horn and cervix of sham-inoculated and *Cmu*-inoculated mice at 7-, 14- and 21 days post-infection (dpi). (A-C; F-H) Representative traces of spontaneous contractions of mouse uterine horns and cervices. (D-E) *Chlamydia muridarum* (*Cmu*) did not affect the spontaneous contraction of the uterine horn at 7-, 14- and 21dpi. (I) *Cmu* infection significantly reduced the contractile force of the spontaneous contractions of cervix at only 7- and 14dpi (* *P*<0.05, ****P*<0.001; unpaired *t*-test) but not at 21dpi. (J) *Cmu* also did not alter

the contractile frequency of the spontaneous contractions in the cervix at the three time-points. Data are presented as mean ± S.E.M. (n = 8-14).

Fig 5: *Cmu* infection significantly reduced contractile force and frequency of the uterine horn to cumulative doses of oxytocin at7dpi. (A, D, G) Representative traces of the 7-, 14- and 21dpi uterine horns in response to increasing concentrations of oxytocin. (B, E, H) Dose-response curves of the uterine horn responses to cumulative doses of oxytocin (n=6-10; Nonlinear Regression – Comparison of Fits; ****P<0.0001). (C, F, I) mRNA expression of *Otr* receptor in the uterine horns (n=6-12; unpaired t-test or Mann-Whitney U test; **P<0.01). Data are presented as mean ± S.E.M.

Fig 6: *Cmu* infection significantly reduced the contractile force of the cervix at 7dpi. (A, D, G) Representative traces of the 7-, 14- and 21dpi cervices in response to increasing concentrations of oxytocin. (B, E, H) Dose-response curve of the cervix response to cumulative doses of oxytocin (n=6-10; Nonlinear Regression – Comparison of Fits; ****P<0.0001). (C, F, I) mRNA expression of *Otr* receptor in the cervices (n=6-10; unpaired t-test; ****P<0.0001). Data are presented as mean ± S.E.M.

Fig 7: PGE_2 induced relaxation in the uterine horn but induced contraction in the cervix. (A and B) Representative traces of the uterine horn (A) and cervix (B) in response to 1μM PGE_2. (C) The comparison of combined mRNA expression of prostaglandin receptors in the cervices and uterine horns of sham-inoculated mice in three time-points (n=24-29; Mann-Whitney U test; ****P<0.0001). Data are presented as mean ± S.E.M.

774 **Fig 8: *Cmu* infection significantly increased the contractile force of the uterine horn to**
775 **1μM and 3μM PGF2α at 14- and 21dpi and altered mRNA expression of prostaglandin**
776 **receptors.** (A, C, E) The change in peak contractile force of the spontaneous contractions of
777 the uterine horn evoked by 1μM and 3μM PGF2α was compared between the sham- and
778 *Cmu*-inoculated mice (*n*=8-10; unpaired *t*-test; **P*<0.05, ***P*<0.01). (B, D, F) mRNA
779 expression of prostaglandin receptors (*Ptgfr, Ptger3* and *Ptger4*) in the uterine horn (*n*=6-12;
780 unpaired *t* test or Mann-Whitney U test; **P*<0.01, ***P*<0.01, *****P*<0.0001). Data are
781 presented as mean ± S.E.M.
782
783 **Fig 9: Correlation coefficients of inflammatory cytokine gene expression with *Otr*, *Ptgfr***
784 **and *Ptger4* gene expression in the uterine horn of *Cmu*-inoculated mice.** Numbers in the
785 figure refer to the correlation coefficients r value. The data was combined from three time-
786 points for correlation analysis. The mRNA expression of *Otr* and *Ptger4* genes was inversely
787 correlated with the mRNA expression of *Il1b* and *Il6* genes in the uterine horn of *Cmu*-
788 inoculated mice (*n*=23; Spearman's correlation; ***P*<0.01, ****P*<0.001).
789
790 **Fig S1 *Cmu* infection did not alter the responses of the cervix and uterine horn to 60mM**
791 **KCl at three time-points.** Data are presented as mean ± S.E.M. (*n* = 8-14).
792
793 **Fig S2: Representative traces of uterine contractions to PGF2α at 7-, 14- and 21dpi.**
794
795 **Fig S3: Representative traces of cervical contractions to PGF2α at 7-, 14- and 21dpi.**
796

Chlamydia muridarum infection differentially alters smooth muscle function in mouse uterine horn and cervix

Tables:

Gene	Forward primer	Reverse primer
Hprt	5'-AGGCCAGACTTTGTTGGATTTGAA-3'	5'-CAACTTGCGCTCATCTTAGGCTTT-3'
16S rRNA	5'-GCGGCAGAAATGTCGTTTT-3'	5'-CGCTCGTTGCGGGACTTA-3'
Stat1	5'-CCCGAATTTGACAGTATGATGA-3'	5'-GAAGGAACAGTAGCAGGAAGGA-3'
Stat6	5'-GCACCTTGGAGAGCATCTATCAGAG-3'	5'-TTGAGTTCTTCCTGCTTCCGATG-3'
Ifng	5'-TCTTGAAAGACAATCAGGCCATCA-3'	5'-GAATCAGCAGCGACTCCTTTTCC-3'
Cxcl1	5'-GCTGGGATTCACCTCAAGAA-3'	5'-CTTGGGGACACCTTTTAGCA-3'
Cxcl2	5'-TGCTGCTGGCCACCAACCAC-3'	5'-AGTGTGACGCCCCCAGGACC-3'
Il10	5'-AGGCGCTGTCATCGATTTCT-3'	5'-ATGGCCTTGTAGACACCTTGG-3'
Mmp9	5'-CGAACTTCGACACTGACAAGAAGT-3'	5'-GCACGCTGGAATGATCTAAGC-3'
Il1b	5'-TGGGATCCTCTCCAGCCAAGC-3'	5'-AGCCCTTCATCTTTTGGGGTCCG-3'
Il6	5'-AGAAAACAATCTGAAACTTCCAGAGAT-3'	5'-GAAGACCAGAGGAAATTTTCAATAGG-3'
Cxcl15	5'-AAGGAAGTGATAGCAGTCCCAAA-3'	5'-GCCAACAGTAGCCTTCACCC-3'
Tnfa	5'-TCTGTCTACTGAACTTCGGGGTGA-3'	5'-TTGTCTTTGAGATCCATGCCGTT-3'
Otr	5'-CCTGGAGAGACGAGCATTAGC-3'	5'-TCATGCCGAGGATGGTTGAG-3'
Ptgfr	5'-ATAATGTGCGTCTCCTGCGT-3'	5'-GCGGAGAGCAAAAAGTGTCG-3'
Ptger1	5'-ACGCACACGATGTGGAAATG-3'	5'-CAGGGAGTTAGAGTTCCAGC-3'
Ptger2	5'-GAGAGGACTTCGATGGCAGAG-3'	5'-AGGTCCCACTTTTCCTTTAGGG-3'
Ptger3	5'-ATGTGTGTGCTGTCCGTCTG-3'	5'-TGTCTTGCATTGCTCAACCG-3'
Ptger4	5'-CTCATCTGCTCCATTCCGCT-3'	5'-GGTTCACAGAAGCAATCCTG-3'

Table 1: Forward and reverse primers of selected genes

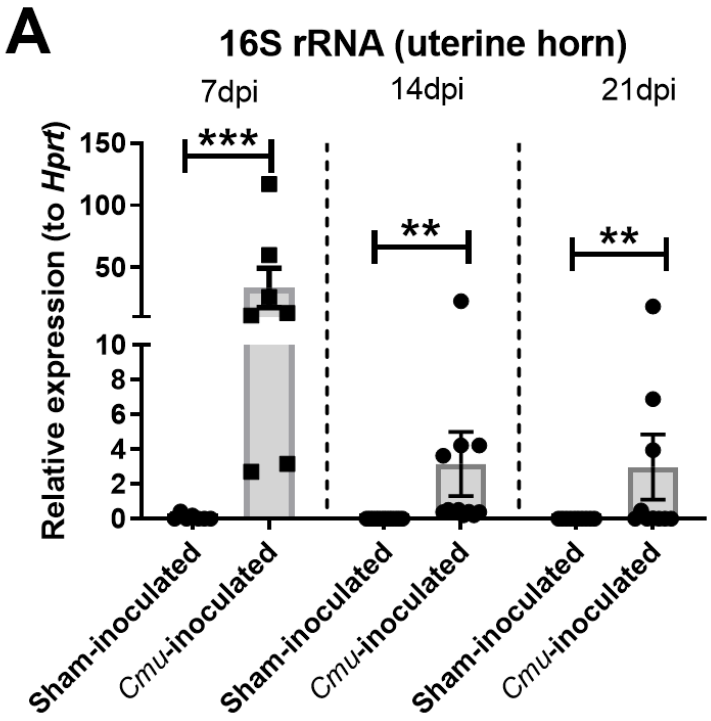
	Relative increase or decrease in cervical peak force in the presence of agonists					
	7 dpi		**14 dpi**		**21 dpi**	
	Sham	***Cmu***	**Sham**	***Cmu***	**Sham**	***Cmu***
n	8	9	8	8	10	10
1 µM PGF2α	25.68±6.56	32.76±3.58	38.24±6.95	31.37±6.46	28.53±9.26	38.97±13.06
3 µM PGF2α	37.00±7.15	27.62±4.75	34.53±6.64	27.36±6.82	23.21±5.85	31.57±8.19
1 µM PGE_2	-6.28±3.03	-5.09±4.38	15.50±5.93	25.70±7.27	9.25±7.61	11.65±5.86
1mM histamine	9.90±4.86	2.60±3.97	1.83±4.18	9.91±5.63	-1.66±2.87	7.43±5.57

Table 2: Comparison of cervical contractile force in sham-inoculated and *Cmu*-inoculated mice to prostaglandins and histamine. The change in contractile force evoked by agonists as a percentage of maximal KCl evoked force. There was no significant difference in the cervical contractions between the two groups at these doses. Data are presented as mean ± S.E.M.

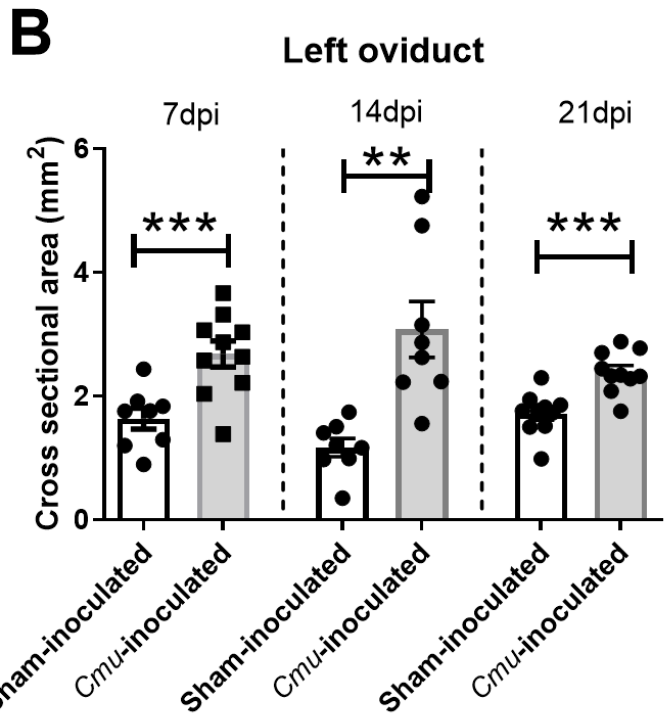
	Relative increase or decrease in uterine peak force in the presence of agonists					
	7 dpi		**14 dpi**		**21 dpi**	
	Sham	***Cmu***	**Sham**	***Cmu***	**Sham**	***Cmu***
n	8	9	8	8	10	10
1 µM PGE_2	-15.34±3.12	-19.50±4.04	-17.63±6.09	-15.50±5.24	-24.40±4.50	-31.31±5.70
1 mM histamine	0.57±2.60	4.87±4.03	9.01±3.48	15.91±3.36	12.69±3.30	13.36±4.49

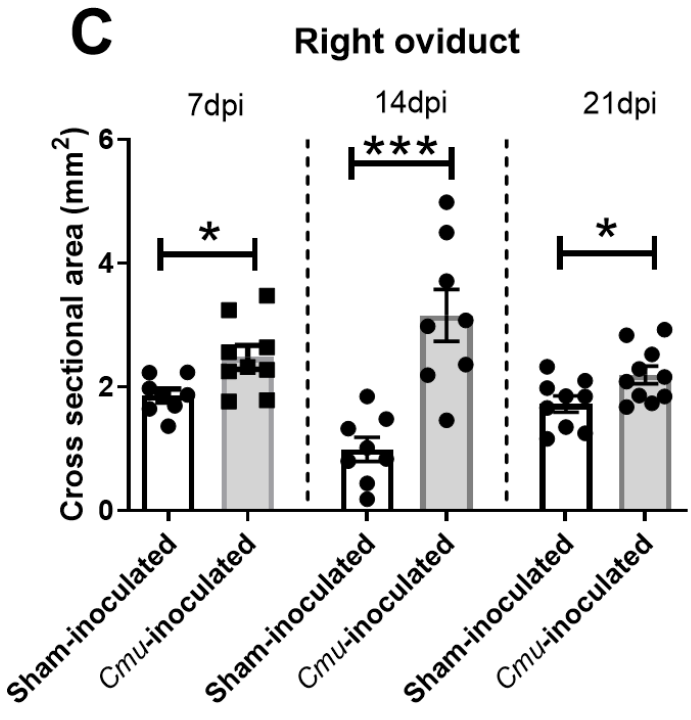
Table 3: Comparison of uterine contractile force in sham-inoculated and *Cmu*-inoculated to prostaglandins and histamine. The change in contractile force evoked by agonists as a percentage of maximal KCl evoked force. There was no significant difference in the uterine contractions at these doses. Data are presented as mean ± S.E.M.

16S rRNA

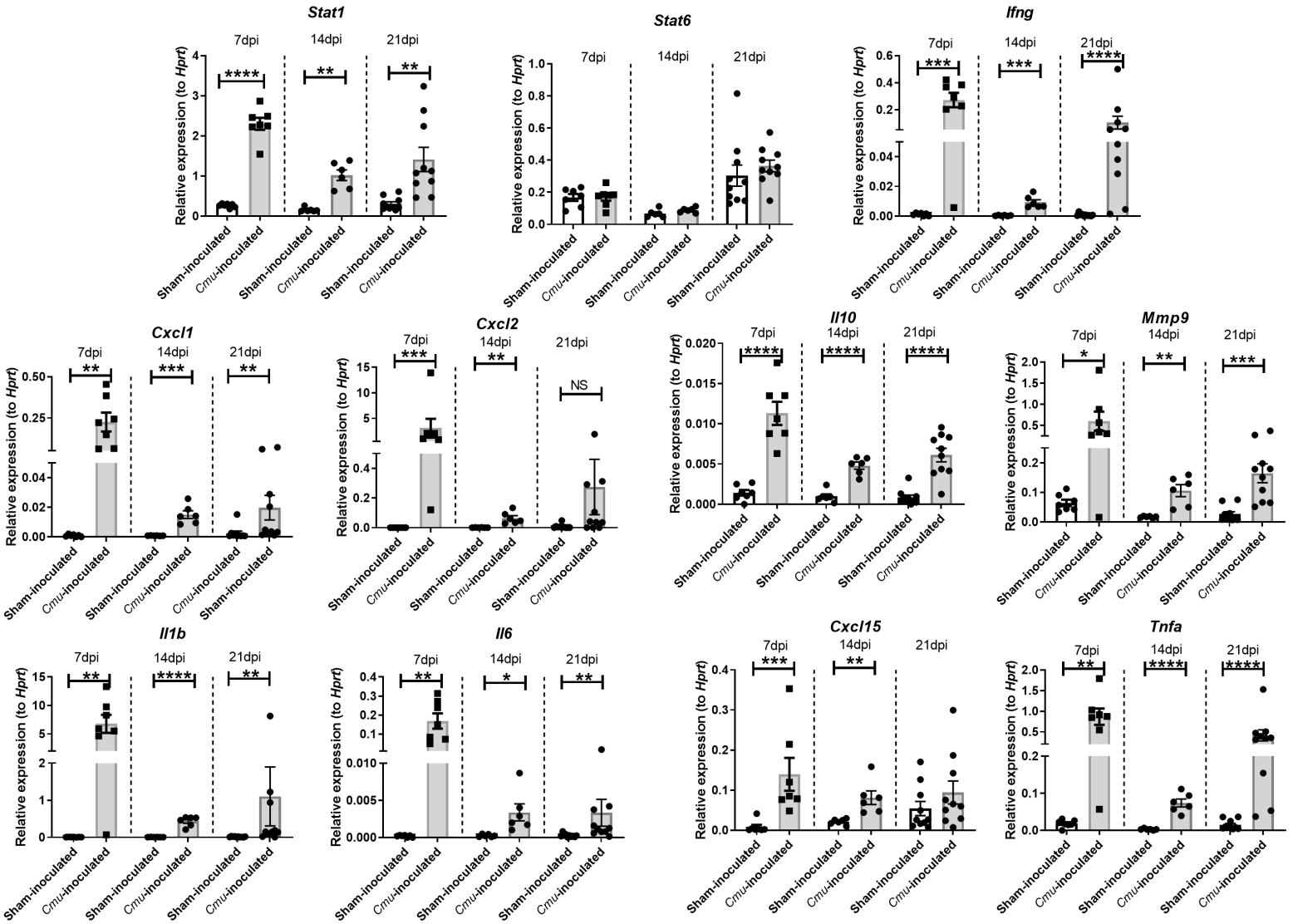


Cross-sectional area of oviducts

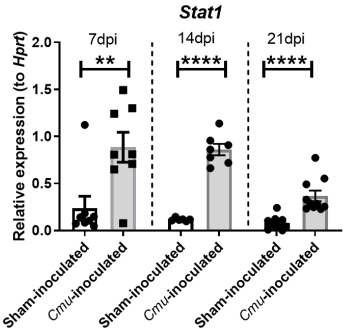


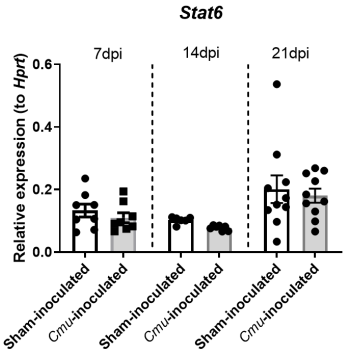


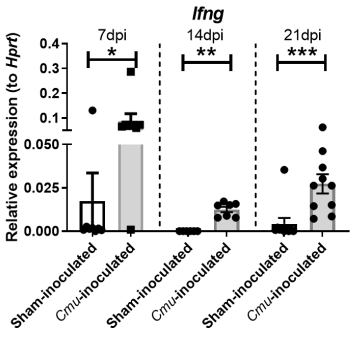
Uterine horn

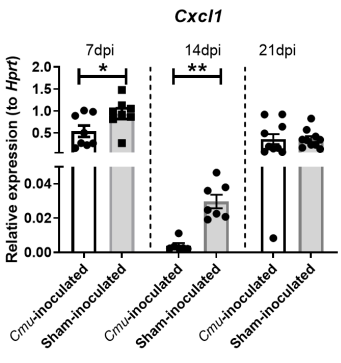


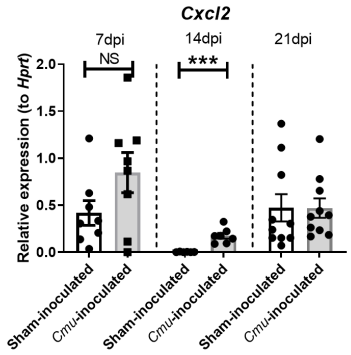
Cervix

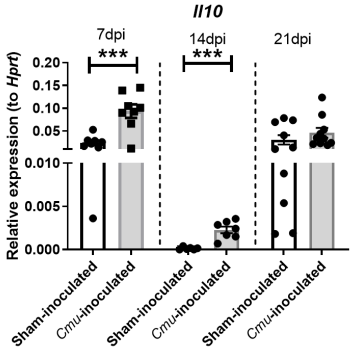

Stat1
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

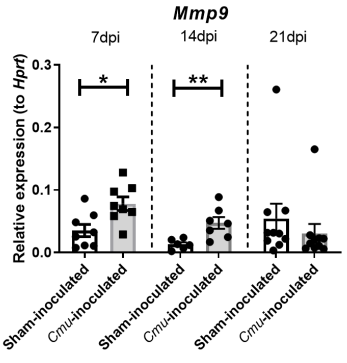

Stat6
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

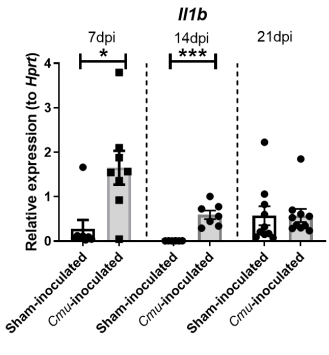

Ifng
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

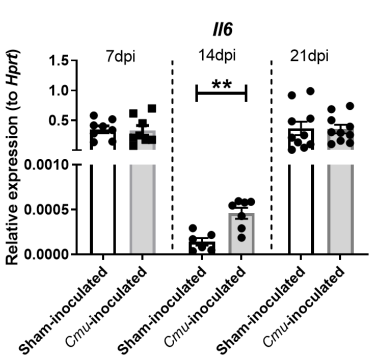

Cxcl1
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Cmu-inoculated
Sham-inoculated

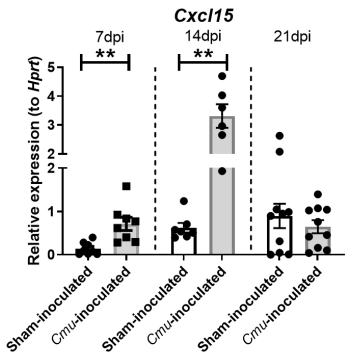

Cxcl2
7dpi
NS
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

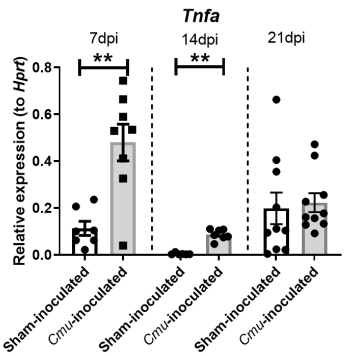

Il10
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

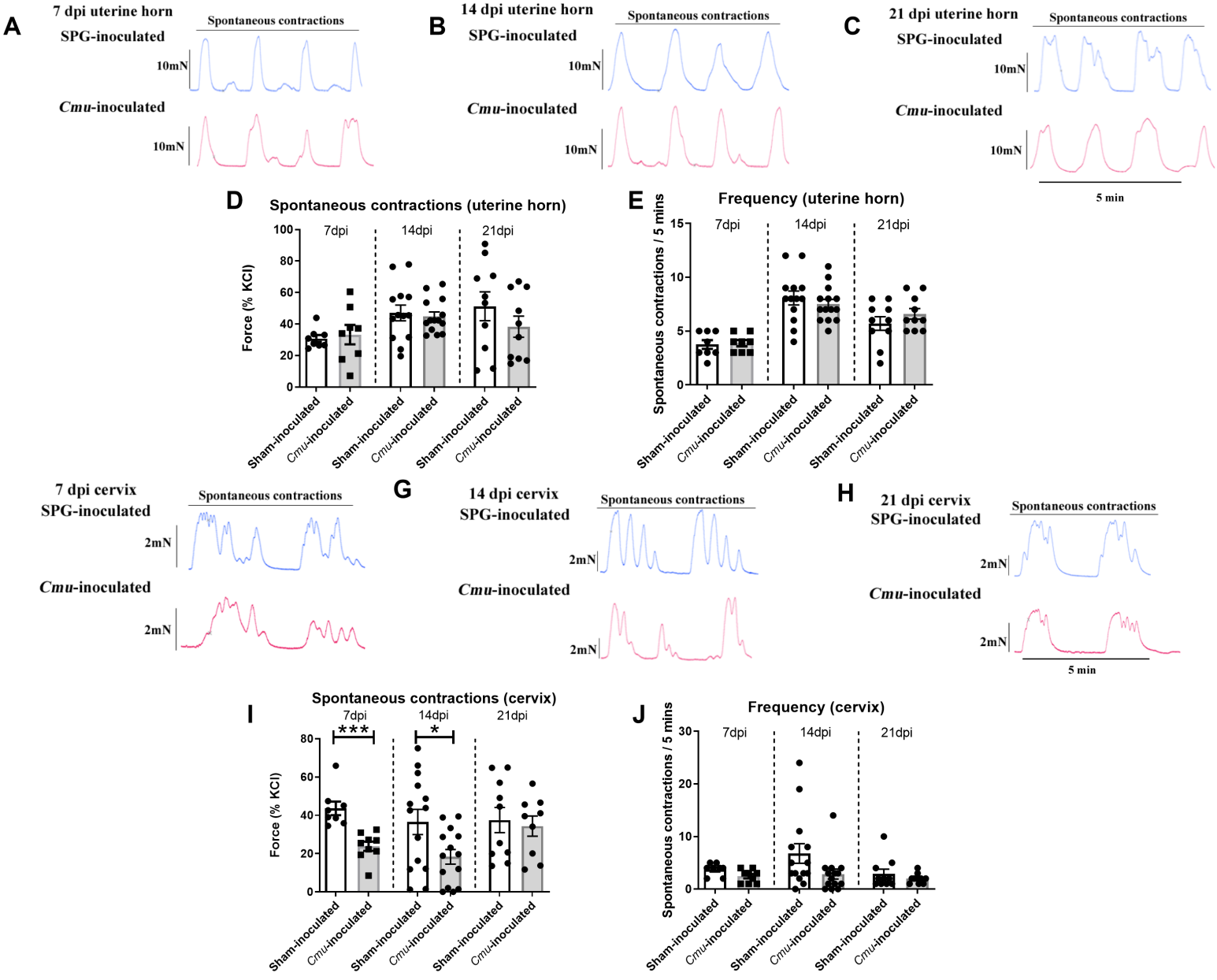

Mmp9
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

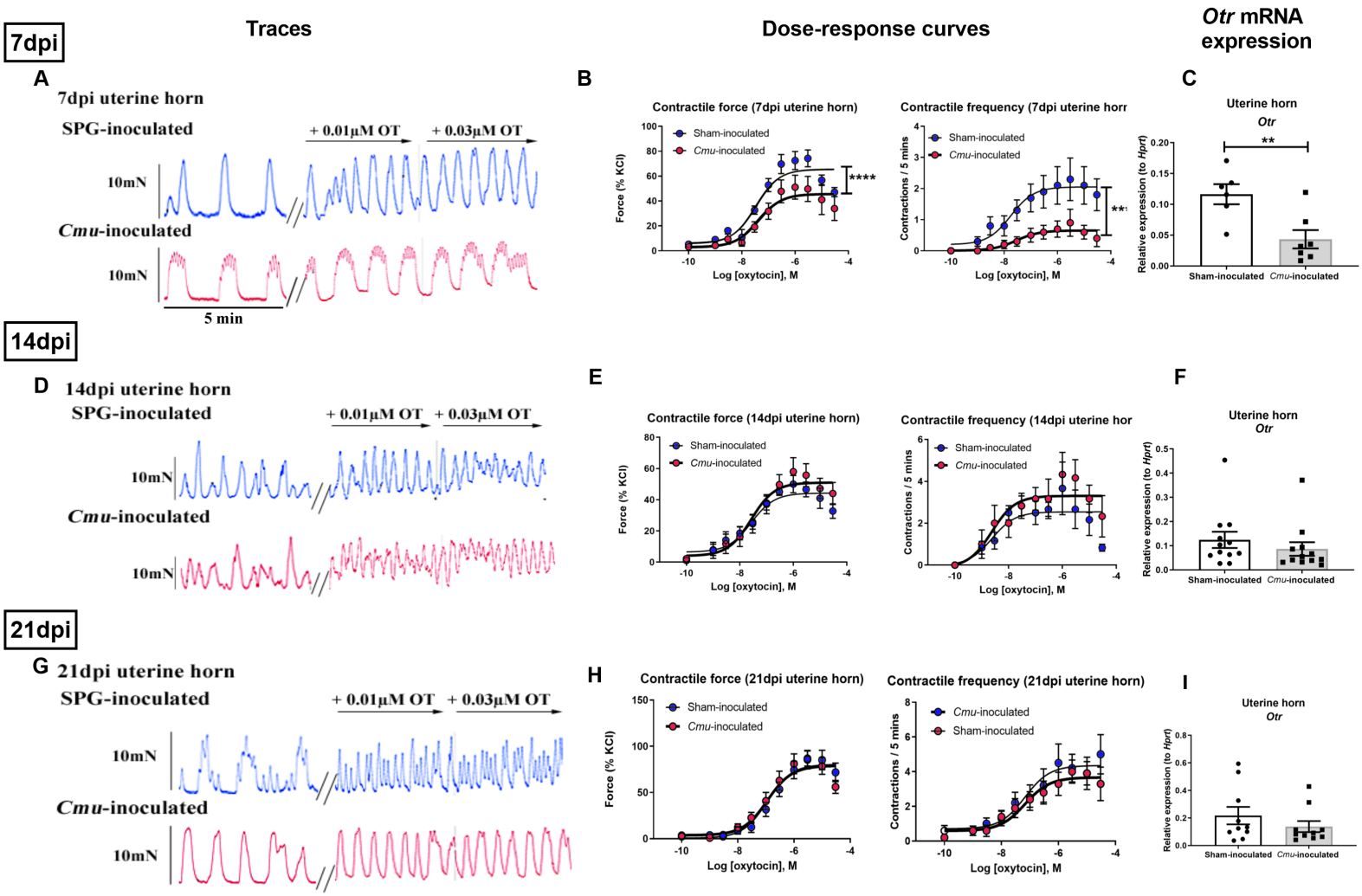

Il1b
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

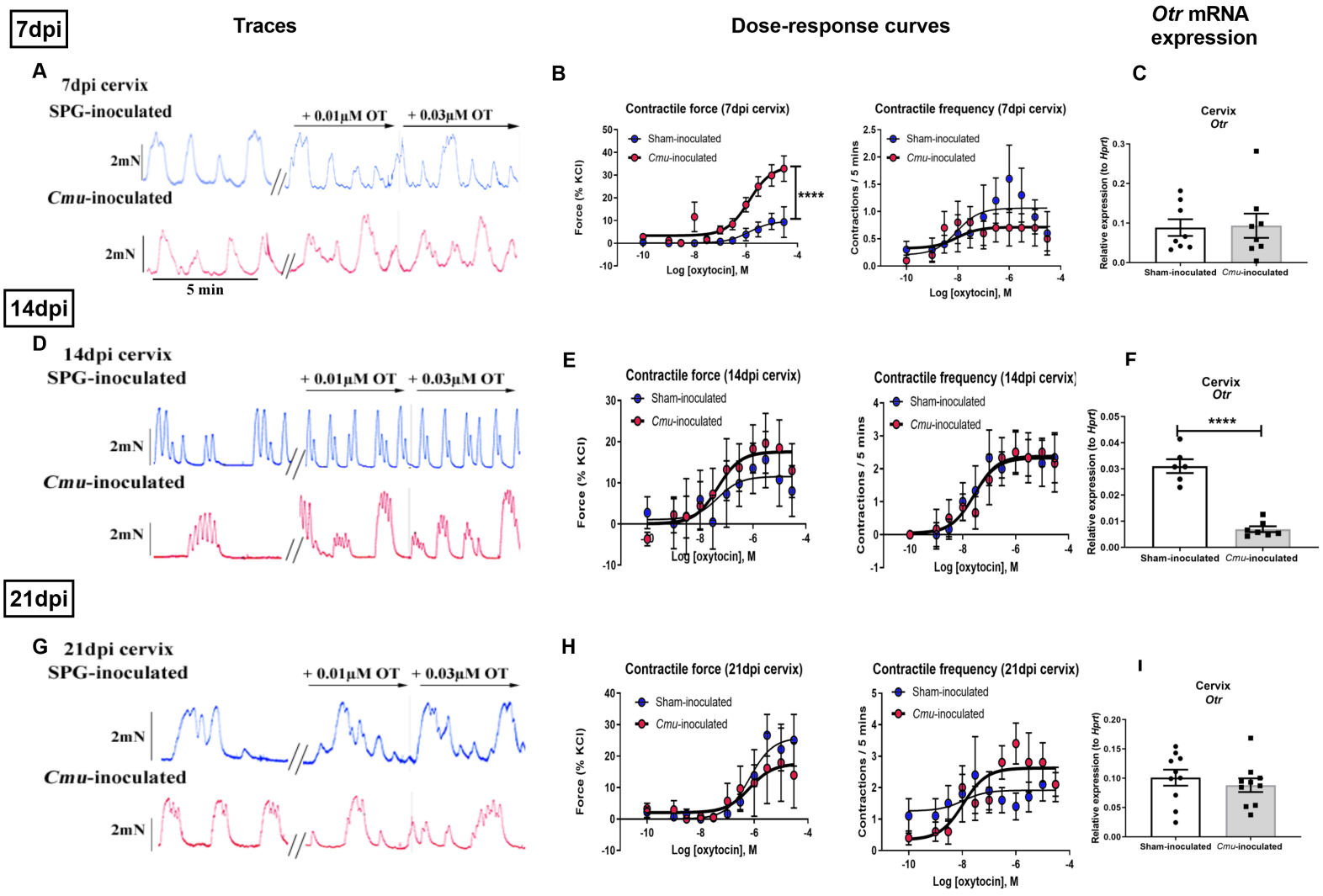

Il6
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

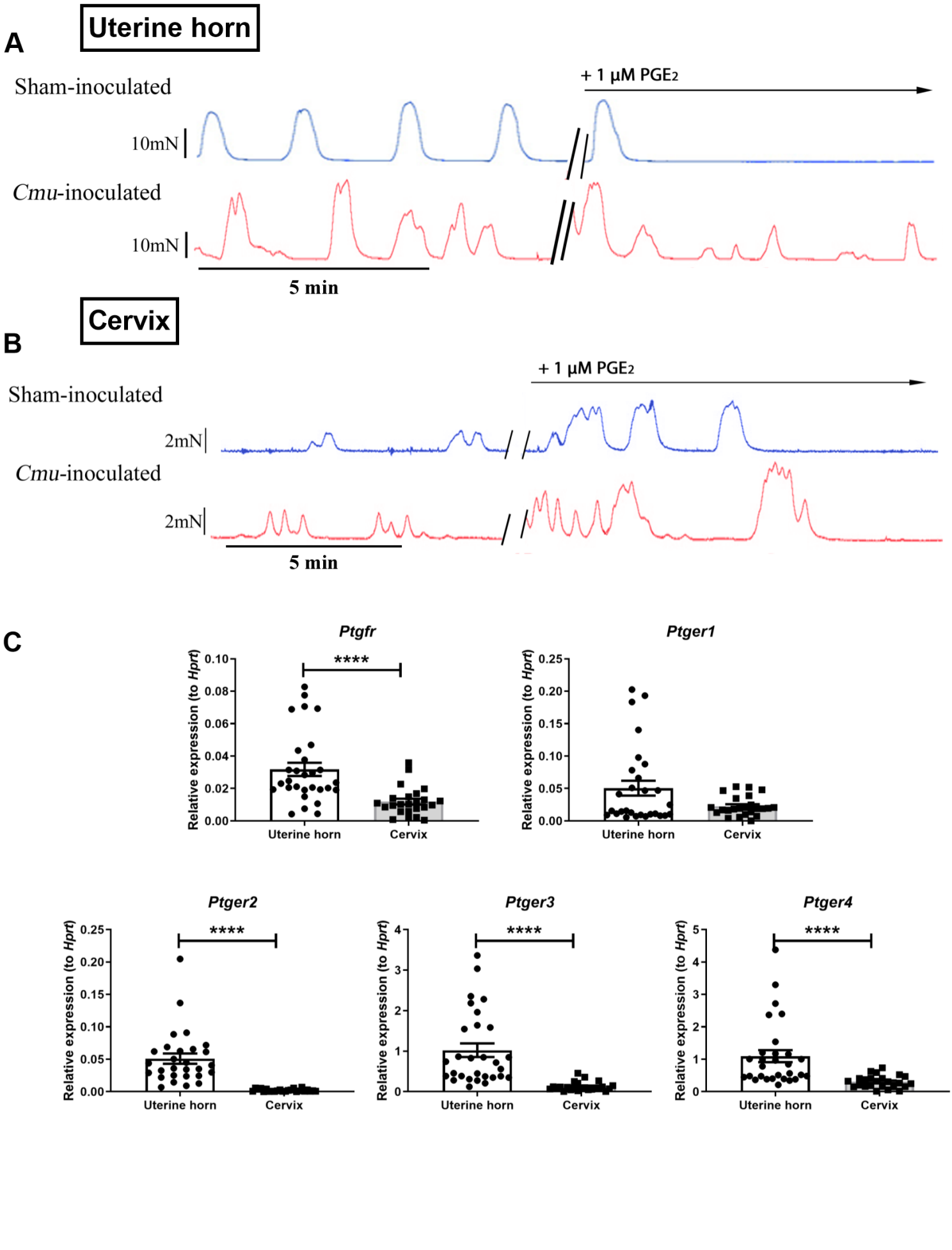

Cxcl15
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

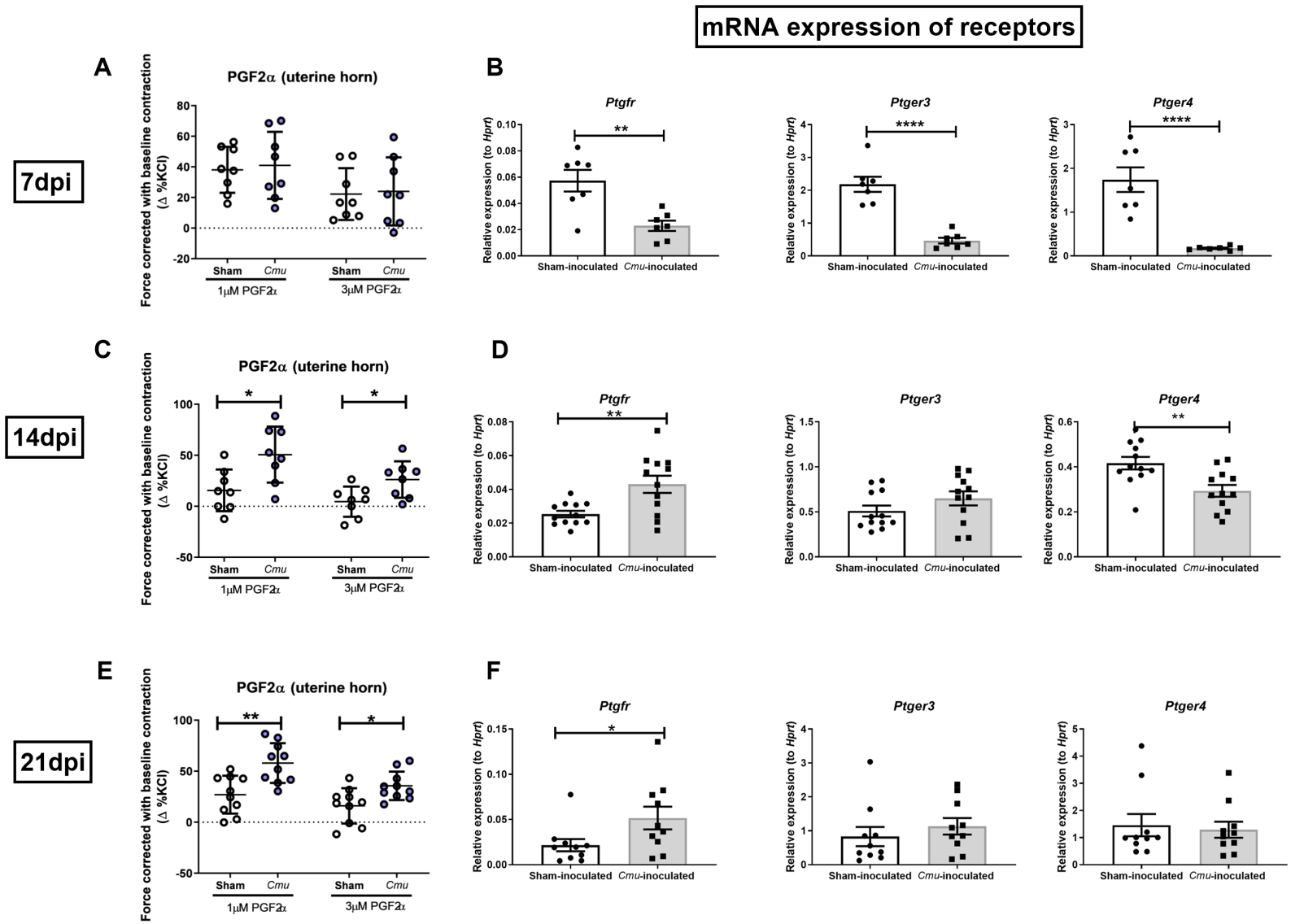

Tnfa
7dpi
14dpi
21dpi
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated

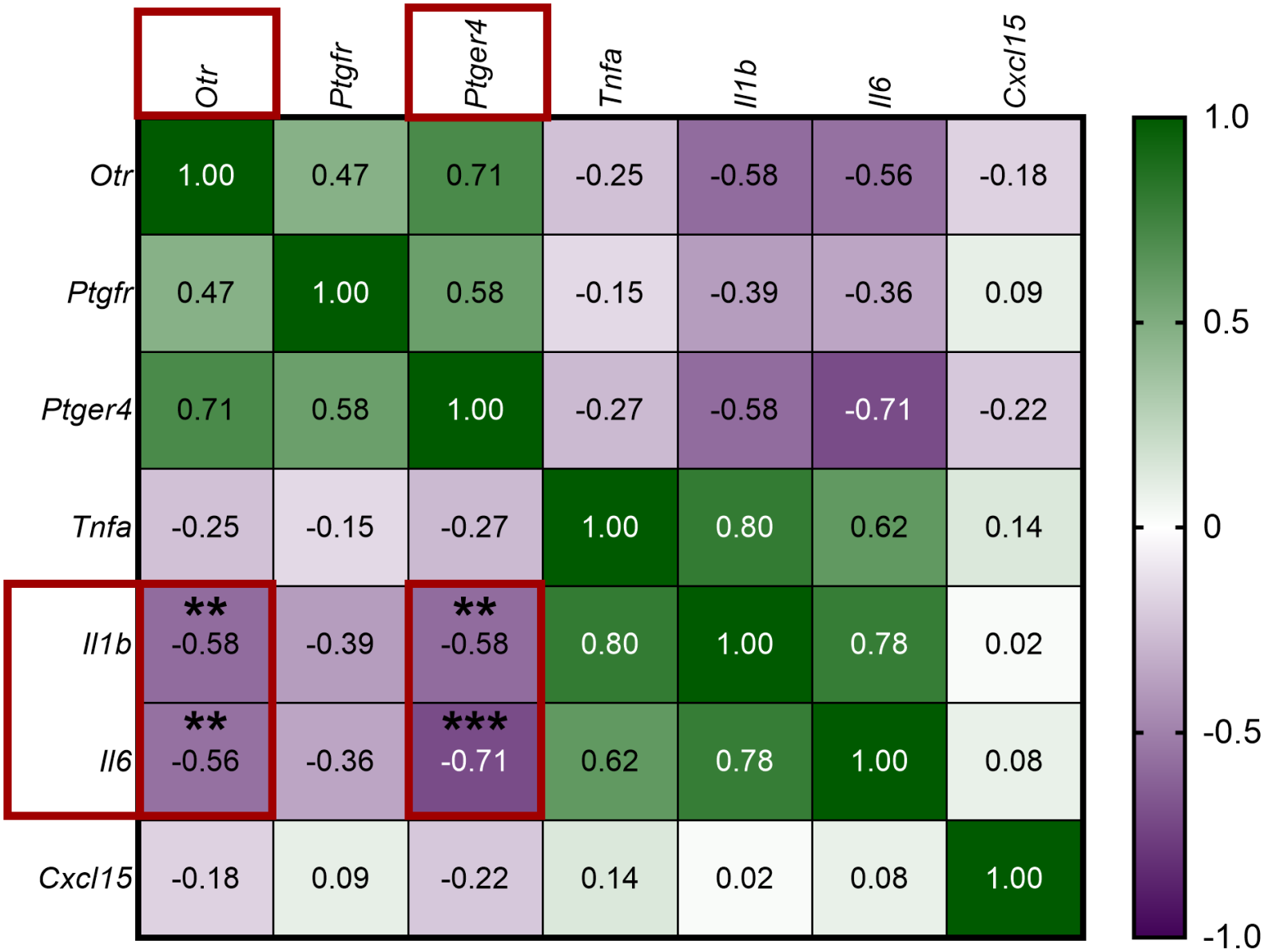

A
7 dpi uterine horn
SPG-inoculated
Spontaneous contractions
10mN
Cmu-inoculated
10mN
B
14 dpi uterine horn
SPG-inoculated
Spontaneous contractions
10mN
Cmu-inoculated
10mN
C
21 dpi uterine horn
SPG-inoculated
Spontaneous contractions
10mN
Cmu-inoculated
10mN
5 min
D
Spontaneous contractions (uterine horn)
Force (% KCl)
7dpi
14dpi
21dpi
Sham-inoculated
Cmu-inoculated
E
Frequency (uterine horn)
Spontaneous contractions / 5 mins
7dpi
14dpi
21dpi
Sham-inoculated
Cmu-inoculated
F
7 dpi cervix
SPG-inoculated
Spontaneous contractions
2mN
Cmu-inoculated
2mN
G
14 dpi cervix
SPG-inoculated
Spontaneous contractions
2mN
Cmu-inoculated
2mN
H
21 dpi cervix
SPG-inoculated
Spontaneous contractions
2mN
Cmu-inoculated
2mN
5 min
I
Spontaneous contractions (cervix)
Force (% KCl)
7dpi
14dpi
21dpi
***
*
Sham-inoculated
Cmu-inoculated
J
Frequency (cervix)
Spontaneous contractions / 5 mins
7dpi
14dpi
21dpi
Sham-inoculated
Cmu-inoculated


Traces
Dose-response curves
Otr mRNA expression
7dpi
A
7dpi uterine horn
SPG-inoculated
+ 0.01μM OT
+ 0.03μM OT
10mN
Cmu-inoculated
10mN
5 min
B
Contractile force (7dpi uterine horn)
Sham-inoculated
Cmu-inoculated
Force (% KCl)
****
Log [oxytocin], M
Contractile frequency (7dpi uterine horn
Contractions / 5 mins
**
C
Uterine horn
Otr
**
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated
14dpi
D
14dpi uterine horn
E
Contractile force (14dpi uterine horn)
Contractile frequency (14dpi uterine hor
F
21dpi
G
21dpi uterine horn
H
Contractile force (21dpi uterine horn)
Contractile frequency (21dpi uterine horn)
I


7dpi
Traces
Dose-response curves
Otr mRNA expression
A
7dpi cervix
SPG-inoculated
+ 0.01µM OT
+ 0.03µM OT
2mN
Cmu-inoculated
2mN
5 min
B
Contractile force (7dpi cervix)
Sham-inoculated
Cmu-inoculated
Force (% KCl)
****
Log [oxytocin], M
Contractile frequency (7dpi cervix)
Contractions / 5 mins
Log [oxytocin], M
C
Cervix
Otr
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated
14dpi
D
14dpi cervix
SPG-inoculated
+ 0.01µM OT
+ 0.03µM OT
2mN
Cmu-inoculated
2mN
E
Contractile force (14dpi cervix)
Sham-inoculated
Cmu-inoculated
Force (% KCl)
Log [oxytocin], M
Contractile frequency (14dpi cervix)
Contractions / 5 mins
Log [oxytocin], M
F
Cervix
Otr
****
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated
21dpi
G
21dpi cervix
SPG-inoculated
+ 0.01µM OT
+ 0.03µM OT
2mN
Cmu-inoculated
2mN
H
Contractile force (21dpi cervix)
Sham-inoculated
Cmu-inoculated
Force (% KCl)
Log [oxytocin], M
Contractile frequency (21dpi cervix)
Contractions / 5 mins
Log [oxytocin], M
I
Cervix
Otr
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated


A
Uterine horn
Sham-inoculated
+ 1 μM PGE2
10mN
Cmu-inoculated
10mN
5 min
B
Cervix
Sham-inoculated
+ 1 μM PGE2
2mN
Cmu-inoculated
2mN
5 min
C
Ptgfr
Relative expression (to Hprt)
****
Uterine horn
Cervix
Ptger1
Ptger2
Ptger3
Ptger4


mRNA expression of receptors
A
B
7dpi
PGF2α (uterine horn)
Force corrected with baseline contraction (Δ %KCl)
Sham
Cmu
1μM PGF2α
3μM PGF2α
Ptgfr
Ptger3
Ptger4
Relative expression (to Hprt)
Sham-inoculated
Cmu-inoculated
C
D
14dpi
E
F
21dpi


Otr
Ptgfr
Ptger4
Tnfa
Il1b
Il6
Cxcl15
Otr 1.00 0.47 0.71 -0.25 -0.58 -0.56 -0.18
Ptgfr 0.47 1.00 0.58 -0.15 -0.39 -0.36 0.09
Ptger4 0.71 0.58 1.00 -0.27 -0.58 -0.71 -0.22
Tnfa -0.25 -0.15 -0.27 1.00 0.80 0.62 0.14
Il1b ** -0.58 -0.39 ** -0.58 0.80 1.00 0.78 0.02
Il6 ** -0.56 -0.36 *** -0.71 0.62 0.78 1.00 0.08
Cxcl15 -0.18 0.09 -0.22 0.14 0.02 0.08 1.00
1.0
0.5
0
-0.5
-1.0