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Modulation of Monocyte Chemotactic Protein-1 Expression during LPS-induced Preterm Delivery in the Pregnant Mouse

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Abstract

Objective—Preterm delivery is often associated with increased cytokine and chemokine production. These studies sought to characterize the expression of the chemokine, monocyte chemotactic protein-1 (MCP-1), in mice during lipopolysaccharide (LPS)-induced preterm delivery.

Methods—Uterine and other tissues were harvested from CD-1 mice on gestational day 15 after intrauterine LPS injection. Quantitative real-time reverse-transcriptase polymerase chain reactions (qRT-PCR) determined MCP-1 and toll-like receptor 4 (TLR4) mRNA expression during the 24 hours after LPS. MCP-1 protein expression was determined using a cytokine/chemokine protein array, by ELISA and by immunohistochemistry.

Results—Intrauterine LPS injection caused preterm delivery in CD-1 mice between 12 and 24 hours. Expression of MCP-1 mRNA significantly increased at 2 and 6 hours, while TLR4 expression did not significantly change over 24 hours. MCP-1 protein levels peaked by 2 to 6 hours in maternal serum, liver, lung, kidney and uterus. Immunohistochemistry confirmed MCP-1 in myometrium and endometrium.

Discussion—These studies have provided evidence suggesting that MCP-1 potentially plays an important role during the proinflammatory immune response leading to preterm labor in the mouse.

Keywords

CD-1 mouse; Preterm delivery; qRT-PCR; Chemokines

Introduction

Preterm entry into the world places a neonate at an immediate disadvantage with respect to its peers born at term. This infant will begin its life at a substantially higher risk of severe brain injury, chronic lung disease, and necrotizing enterocolitis, among other challenges, and this is only the beginning of a lifetime of increased medical, social, and special educational needs [1]. Approximately 12% of pregnancies in the U.S. result in preterm birth, a condition that makes the largest contribution to infant mortality during the first month of life [2].

Efforts to understand the multifactorial pathophysiologic mechanisms underlying preterm birth have identified activation of the proinflammatory pathways and components of the coagulation cascade as important contributors [3–9].

Numerous animal models of preterm birth have utilized activation of the inflammatory cascade to elucidate the mechanisms initiating preterm labor. Selected models include intra-amniotic group B *Streptococcus* (GBS) in rhesus monkeys [10], endoscopic inoculation of live *Escherichia coli* through the cervix in rabbits [11], and renal abscess caused by *E. coli* injection in CD-1 mice [12]. Lipopolysaccharide (LPS), derived from the gram-negative bacterial cell wall, causes a pronounced inflammatory response in animal and human tissues both *in vivo* and *in vitro*. Multiple reports have demonstrated that the LPS-induced proinflammatory response induces premature delivery in mice [4, 5, 12–16]. A 2003 report by Elovitz et al. [5] demonstrated the ability of intrauterine LPS injection to cause preterm delivery within 24–48 hours in pregnant CD1 mice.

Preterm delivery during human pregnancies is often associated with a robust proinflammatory response including an increase in cytokines. The LPS-induced inflammatory response in preterm delivery has also been associated with increased synthesis and activity of cytokines including interleukin-1 α (IL1 α), IL6, IL8, and tumor necrosis factor α (TNF α). This effect is mediated through stimulation of the toll-like receptor-4 (TLR4) and in part through activation of the transcription factor nuclear factor kappa B (NF κ B) [8, 13]. Activation of the innate immune response by LPS also results in the production of chemokines including monocyte chemoattractant protein-1 (MCP-1, also known as CCL2), cytokine-induced neutrophil chemoattractant (KC, also known as keratinocyte chemokine), the regulated upon activation, normal T cell expressed and secreted protein (RANTES), and LPS-induced CXC chemokine (LIX).

Recently published human studies have suggested a unique role for MCP-1 during preterm labor and delivery. In 2003, Jacobsson et al. [17] reported finding elevated MCP-1 in the cervix of women in premature labor associated with intra-amniotic infection. A report in 2005 by Esplin et al. [18] described significantly elevated MCP-1 levels in the amniotic fluid of women in preterm labor, especially those associated with evidence of intra-amniotic infection. Interestingly, these investigators also noted elevated MCP-1 levels in the amniotic fluid of women delivering preterm, even without evidence of infection, compared to those women who delivered at term. In another 2005 report, Esplin et al. [19] described a 10-fold increase in MCP-1 protein and mRNA in myometrium of women in active labor compared to quiescent myometrium, and a greater myometrial increase in women in preterm labor, even without evidence of intrauterine infection. Further supporting an important role for MCP-1 during preterm labor, Törnblom et al. [6] reported elevated MCP-1 levels in cervical tissue in women undergoing preterm labor without clinical evidence of infection; notably, these women did have signs of cervical inflammation including increased levels of IL6 and IL8.

Considering what appears to be a potentially important role for MCP-1 during preterm delivery, the current studies sought to characterize the expression and modulation of MCP-1 in the pregnant uterus and other organs during intrauterine LPS-induced preterm delivery (PTD) in the mouse.

Methods

These studies utilized eighty-five CD-1 timed-pregnant mice purchased from Charles River Laboratories (Wilmington, MA). The animals shipped on day 9 after mating and acclimated in the University of Vermont's animal care facility for several days before undergoing

surgery on day 15 of gestation. All experiments accorded with National Institutes of Health guidelines for laboratory animals and had approval from the Institutional Animal Care and Utilization Committee (IACUC) at the University of Vermont. After induction of isoflurane anesthesia, a laparotomy was performed using sterile technique and the right uterine horn exposed. Lipopolysaccharide (LPS) dosed at 250 μ g or an equal volume of sterile saline (i.e. 100 μ L) was injected intrauterine between gestational sacs 2 and 3 on the right uterine horn. At the completion of surgery, mice received one dose of buprenorphine (50 μ g/kg) analgesic subcutaneously and were allowed to recover. Subsequently, the mice were euthanized at 2, 6, 12, 18, and 24 hours after LPS injection using isoflurane anesthesia and a lethal sodium pentobarbital injection (200 mg/kg intraperitoneal). The time zero (0-hour) control mice were euthanized without undergoing surgery or intrauterine injection.

The uterus, liver, lung, kidney, serum, and other tissues were harvested from the pregnant mice. Samples for protein assays were rinsed in normal saline, then immediately frozen in liquid nitrogen and stored at -80° C. Uterine tissue from the right uterine horn was typically designated for protein analysis, while the left uterine horn provided tissue for RNA analysis. Samples for RNA assays were rinsed in normal saline, placed in RNALater (Ambion, Inc., Austin, TX) and stored at 4° C for approximately 1 week, then removed from RNALater and stored at -80° C. For immunohistochemical studies, samples from the middle of the pregnant uterine horn were obtained at each of the time-points (i.e. time 0, 2, 6, 12, and 18 hour), then embedded in TissueTek O.C.T. compound (Sakura Finetek U.S.A., Torrance, CA) and immediately frozen in liquid nitrogen.

For the protein array and enzyme-linked immunosorbent assays (ELISA) studies, tissue was homogenized in ice cold 1X Cell Lysis buffer containing protease inhibitors. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockville, IL). For cytokine/chemokine protein array studies, uterine protein homogenates from four animals per time-point were equally pooled (125 μ g each) for a total concentration of 500 μ g protein. Expression of 40 mouse inflammation-related proteins was determined using the RayBio Mouse Inflammation Antibody Array kit (RayBiotech, Norcross, GA) (see Tables 1A and 1B for description of array). Membranes containing target inflammatory protein antibodies were incubated with blocking buffer, and then pooled protein samples were incubated with the membranes for 1–2 hours. The membranes were washed and then incubated in the biotinylated antibody solution at room temperature for 1–2 hours. The washing step was repeated, and diluted HRP-conjugated streptavidin was added to each membrane. After a 2-hour incubation, samples were washed a third time and developed to allow chemiluminescence visualization and densitometry using the Bio-Rad ChemiDoc XRS detection system (Bio-Rad Inc., Hercules, CA).

Total RNA was extracted from maternal mouse uterus, liver, lung, and kidney tissue using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). Subsequently, genomic DNA was removed from samples using TURBO DNA-free (Ambion, Austin, TX). The RNA concentrations were determined using a NanoDrop spectrophotometer (NanoDrop, Inc., Wilmington, DE). Intact total RNA was confirmed by analysis of the 18S and 28S band patterns after formaldehyde-agarose gel electrophoresis.

For qualitative analysis of MCP-1 mRNA expression using reverse-transcriptase polymerase chain reaction (RT-PCR), cDNA was synthesized from 1 μ g of RNA template using the iScript cDNA Synthesis Kit (Bio-Rad) with random primers. Subsequently, the PCR was performed using the iTaq DNA polymerase kit (Bio-Rad) and mouse-specific sense and antisense primers for MCP-1 and the constitutively expressed gene beta-2-microglobulin (B2m) (see Table 2 for primer sequences). The MCP-1 primers were designed over exon-exon splice sites to eliminate the possibility of amplifying genomic DNA. Tris borate EDTA

(TBE) gels were made with 1–1.2% agarose and stained with GelRed (Biotium, Inc., Hayward, CA). Densitometric analysis of gels was performed using the Bio-Rad ChemiDoc XRS chemiluminescence detection system. Toll-like receptor 4 (TLR4) mRNA expression was also determined in uterine tissue using mouse TLR4 sense and antisense primers.

Real-time quantitative RT-PCR (qRT-PCR) was performed on an ABI Prism 7000 Sequence Detection System using the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). For each sample, the cDNA was generated as described previously and used to amplify MCP-1, TLR4, and three constitutively expressed genes: B2m, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide (Ywhaz), and hypoxanthine guanine phosphoribosyl transferase (Hprt) (see Table 2 for primer sequences). Reactions were performed in triplicate for each sample. Following the qRT-PCR runs, standard curves generated for each primer set were used to calculate the relative quantities of all samples. The geometric mean of the quantities of the three constitutively expressed genes was used to normalize the target gene quantities. Negative (water) controls were run for each primer set in the qRT-PCR reaction to ensure the reagents were not contaminated and that no secondary primer structures were amplified. Specific sense and antisense primers used in RT-PCR and qRT-PCR runs were designed to span at least one exon-exon junction. PCR amplicons for all genes were sequence-verified by the University of Vermont's DNA Analysis Core using an ABI 3130x1 Genetic Analyzer (16 Capillary) (Applied Biosystems).

The MCP-1 protein concentrations in individual tissue and serum samples were determined by ELISA using the mouse MCP-1 Assay kit (Invitrogen Corp.). The tissue samples were homogenized in 1X Cell Lysis buffer, and the total protein concentration was determined using BCA assays as previously described. Samples were added to MCP-1-antibody-coated microtiter wells and incubated for 2 hours. After washes, biotinylated MCP-1 antibody solution was incubated in the wells for 45 minutes. Streptavidin-HRP solution was then incubated in the wells for 45 minutes, followed by additional washes and the addition of Stabilized Chromogen reagent. After 20 minutes, Stop Solution was added, and absorbance of each well was read at 450 nm using a Synergy HT Multi-well Plate Reader (BioTek Instruments, Winooski, VT). The range for the ELISA was 9–2500 pg/ml of MCP-1.

For the immunohistochemical studies, 25 μ m sections of uterine tissue were fixed in 4% paraformaldehyde on glass slides, then washed twice in phosphate buffered saline (PBS), and the endogenous peroxidases were quenched by incubating slides in a solution of 0.3% hydrogen peroxide in methanol. The slides were washed, blocked with rabbit serum and incubated in goat anti-MCP-1 polyclonal primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Subsequently, the slides were washed in PBS, then incubated in anti-goat biotinylated IgG polyclonal antibody (Vector Laboratories, Burlingame, CA). The slides were then developed using the Vectastain Elite ABC kit (Vector Laboratories) and counterstained with hematoxylin.

Statistical analyses were performed using the one-way analysis of variance (ANOVA) or the nonparametric Kruskal-Wallis ANOVA on ranks, followed by multiple comparisons tests including the Student-Newman-Keuls test, Dunn's test (for the ELISA studies), and Dunnett test (for the qRT-PCR and kidney ELISA studies). Statistical significance occurred when $p < 0.05$.

Results

Consistent with a previous report [5], the intrauterine LPS injection caused preterm delivery in the majority of the treated mice by 12 hours after LPS injection (see Figure 1); in contrast,

none of the saline-injected control mice had delivered by 24 hours. During our studies, no mice had delivered at 2 hours and delivery was rare before 6 hours, with only one of 13 females delivering (7.7%). By 12 hours 71.4% of the mice had delivered (n=14); by 18 and 24 hours the delivery rate had reached 91.7% (n=12) and 75% (n=12), respectively. All pups delivered were dead, as expected for the gestational age of 15–16 days.

Densitometry was used for semi-quantitative measurements of the RayBio Inflammation Protein Array data (data not shown); these studies confirmed early intrauterine expression of several proinflammatory cytokines including IL1 α , IL6, IL12 (p40p70), and granulocyte colony stimulating factor (GCSF) in response to LPS (Figure 2). Other inflammatory modulators such as tissue inhibitor of metalloproteinases-1 (TIMP1) and the soluble tumor necrosis factor receptor type II (sTNFRII) also appeared, albeit later (i.e. 18–24 hours). Interestingly, with these studies, we were unable to demonstrate an increase in interferon gamma (IFN γ) or TNF α . The RayBio array studies also demonstrated robust increases in the expression of several chemokines including KC and MCP-1 at 2 hours, RANTES at 6 hours, and B-lymphocyte chemoattractant (BLC) at 24 hours in response to LPS injection when compared with the time 0 and the saline injected control mice, as observed in Figure 2B.

The qualitative RT-PCR studies confirmed expression of MCP-1 mRNA in uterus and maternal lung, kidney, and liver in control and LPS-injected mice as observed in Figure 3. Densitometric analysis suggested upregulation of MCP-1 in all of these tissues by 2 hours (data not shown). These qualitative RT-PCR studies also confirmed expression of TLR4 mRNA in mouse uterine tissue; however, the level of expression did not appear to increase after LPS injection (data not shown). The real-time quantitative RT-PCR studies demonstrated early upregulation of MCP-1 mRNA in maternal uterus, liver, kidney, and lung tissues, followed by a tissue-specific pattern of decline over time (Figure 4). Uterine tissue showed an 80-fold increase in MCP-1 mRNA at 2 hours and 51-fold at 6 hours (both statistically significant), followed by 10-fold increases at 12 and 18 hours (Figure 4A). MCP-1 mRNA also increased significantly in the liver by 168-fold at 2 hours and by 34-fold at 6 hours (Figure 4B). In the kidney, MCP-1 mRNA expression increased to 244-fold at 2 hours and 42-fold at 6 hours (Figure 4C); whereas in lung, MCP-1 mRNA increased to 73-fold at 2 hours, then plateaued at about half this value for the remainder of the time-points (Figure 4D). Interestingly, the real-time quantitative RT-PCR studies showed no significant change in TLR4 mRNA expression in uterine tissue after LPS injection as observed in Figure 5. Although the TLR4 mRNA levels had decreased to 50% of baseline levels at the 18 hour time-point and even further by the 24 hour time-point, these changes were not statistically significant.

The ELISA studies were consistent with the quantitative RT-PCR studies, showing significant increases in MCP-1 protein after intrauterine LPS. In the serum, MCP-1 protein peaked at 2 hours and 6 hours, with 88-fold and 49-fold respective increases over the 0 hour time-point, and then declined dramatically during the 12 to 24 hour time-points as observed in Figure 6. In the uterine tissue, MCP-1 protein demonstrated a significant 8-fold increase at 2 hours when compared with 0 hours, which subsequently decreased to a plateau of about 50% of this value at 12 to 18 hours (Figure 7A). In the maternal liver, MCP-1 protein peaked at 2 hours with a significant 1.6-fold rise over the 0 hour time-point followed by a decline to near baseline levels (Figure 7B). Maternal lung levels of MCP-1 protein were significantly elevated at 2 hours and 6 hours, showing 8.8-fold and 11.7-fold increases, respectively (Figure 7C); whereas in the kidney MCP-1 protein levels did not peak until 6 hours after LPS injection, then rapidly returned to near baseline levels (Figure 7D).

The immunohistochemistry studies confirmed MCP-1 protein in both the longitudinal and circular layers of the pregnant myometrium, as well as in the endometrium of the mouse uterus after LPS injection as observed in Figure 8.

Discussion

The studies described in this report have demonstrated significant elevations in MCP-1 production both within the pregnant uterus and systemically over the course of 24 hours during LPS-induced preterm delivery in the day 15 pregnant CD-1 mouse. During these studies, LPS caused PTD in the majority of mice by 12 hours, peaking at 91.7% of mice delivering by 18 hours; an effect consistent with a previous report using this model [5]. The current study has demonstrated that intrauterine LPS was associated with a robust increase in MCP-1 protein expression by 2 and 6 hours in the pregnant uterus as confirmed by cytokine/chemokine protein arrays and by ELISA studies. The quantitative real-time RT-PCR studies have confirmed significant increases in MCP-1 gene expression in the uterus in response to LPS. The immunohistochemical studies have confirmed that MCP-1 protein is expressed within both the endometrial and myometrial layers of the pregnant mouse uterus. The ELISA studies demonstrating significant increases in MCP-1 protein in maternal serum, liver, lung and kidney have confirmed that LPS produces a systemic proinflammatory response despite being injected directly into the uterus. Quantitative RT-PCR studies with these other mouse tissues have demonstrated that MCP-1 gene expression is actually occurring in the liver, lung and kidney as part of the systemic inflammatory response.

The innate immune response to LPS is mediated by the TLR4 membrane receptor, leading to increased production of the proinflammatory cytokines including IL1 α , IL1 β , IL6, TNF α , and IFN γ , in part through activation of the transcription factor NF κ B [7, 20]. These proteins enter a positive feedback loop with NF κ B that amplifies the inflammatory response by increasing cytokine production which stimulates further activation of NF κ B [14]. The cytokines, among other putative effects, recruit leukocytes to inflamed tissues, ultimately leading to the events resulting in parturition, such as increased prostaglandin synthesis, increased uterine contractile activity, and collagen degradation. PTD has been reported to be associated with increased production of proinflammatory cytokines including IL1 β , IL6, TNF α and others [3, 7–9]. In this study, we confirmed increased expression of several of these proinflammatory cytokines along with increased expression of proinflammatory chemokines in mice undergoing LPS-induced PTD. The limited sensitivity of the protein arrays appears to have prevented us from observing an expected increase in TNF α and IFN γ .

In human pregnancies, Adams et al [21] have reported that the TLR4 membrane receptor is expressed in non-inflamed human placental tissue consistently from 10 weeks until term. These investigators also demonstrated that LPS stimulated increased TLR4 expression in vitro in normal term placentas [21]. Elovitz [5] confirmed the requirement for TLR4 to produce LPS-induced preterm delivery in the CD-1 mouse. In this report, Elovitz [5] also demonstrated that TLR4 expression in mouse uterine tissue at 6 hours after LPS injection decreased in the lower uterine segment (the area around the lower two gestational sacs) and increased in the fundus (i.e. area around the upper gestational sacs). In contrast, the current studies have shown an insignificant decline over 24 hours in TLR4 mRNA expression in uterine tissue (combined upper, middle, and lower segments) after LPS injection.

Reznikov [14] used pregnant knockout mice to show that IL1 β upregulates NF- κ B, TNF α , and IFN γ . IL6, IL8, and IL1 β increase in mouse cervical tissue at term due to leukocyte migration to the cervix [7]. Hirsch [3] showed how bacterially-induced IL1 and IL6, along with TNF α , can induce PTD and what might be intermediate steps in the delivery cascade in the mouse, such as elevated prostaglandin synthesis. However, these cytokines are neither

necessary nor sufficient for labor [3, 4, 16], showing that the innate immune response is complex and redundant. Our documented association between increased IL1 α , IL6, IL12 (p40p70), GCSF, KC, MCP-1, LIX, BLC, and RANTES, and preterm delivery, supports this model of complexity. The cytokine responses confirm results documented previously in mouse models [4, 7, 14].

MCP-1, a 12–13 kilo-Dalton size CC chemokine, is chemotactic for monocytes, macrophages, lymphocytes, and basophils, and also induces histamine production [22]. MCP-1 appears to have intrinsic immune functions, as demonstrated by the observations that monocyte and macrophage recruitment to sites of inflammation was impaired, IFN γ expression decreased, and IL4 and IL5 production diminished in MCP-1 knockout mice [23]. However, the contributions of MCP-1 to the inflammatory response appear to be pleiotropic. For example, MCP-1-deficient mice have been shown to have comparable brain leukocyte recruitment as controls but less IL1 β and TNF α [24]. MCP-1 has also been shown to be required for survival during acute septic peritonitis [25], and it appears to play a role in skeletal muscle regeneration after injury via its receptor, CCR2 [26].

MCP-1 appears to play a role in both preterm and term human labor, both with and without evidence of infection. Women experiencing PTL before 34 weeks of gestation who delivered within 7 days with positive amniotic fluid cultures had significantly elevated cervical and amniotic fluid MCP-1 levels, as well as increased IL6 and IL8 [17]. MCP-1 was significantly higher with delivery prior to 34 weeks than at or after 34 weeks. Amniotic fluid MCP-1 correlates with preterm premature rupture of membranes (PPROM) in women with intra-amniotic infection and histological chorioamnionitis during PTD [18]. With term labor, MCP-1 increased 10-fold in myometrium and 2-fold in the membranes compared with quiescent tissue [19]. Human cervical tissue in women in preterm labor without clinical evidence of infection contained increased IL6, IL8, and MCP-1, with no change in RANTES [6]. The origin of the elevated MCP-1 levels is unclear in the human, though we present data suggesting an organism-wide systemic response.

Redundancy in molecular communication networks results in flexibility responding to challenges such as LPS, and has been demonstrated using mice deficient in IL6 and IL1 that still underwent PTD at the same rates as controls. However, absence of MCP-1 led to a markedly reduced inflammatory response in other tissues [23, 24], suggesting a unique function in the innate immune response. Human studies of MCP-1 in labor [6, 19] show upregulation in term and preterm labor especially in the presence of intrauterine infection, suggesting that MCP-1 may play an intrinsic role during the process of parturition which is amplified in the presence of infection.

In the current studies, MCP-1 protein levels peaked in serum at 2 and 6 hours, at or after the time when gene expression and protein synthesis, evidenced by mRNA levels, also peaked in all tissues examined. MCP-1 was expressed and increased significantly in response to LPS in the four tissues we examined. Lund et al. [27] reports increased MCP-1 in mouse brain tissue four hours after brain LPS injection. Based upon these consistent results from five mouse tissues, we postulate that LPS, even when given intrauterine, provokes an increase in MCP-1 as part of the systemic proinflammatory immune response. The elevated serum levels reflect the increased MCP-1 expression in all tissues.

Interestingly, our studies failed to demonstrate a positive feedback effect of LPS on TLR4 expression in the pregnant mouse uterus. During these studies, TLR4 mRNA expression actually appeared to decrease at 18 and 24 hours; however, these changes were not statistically significant. These results contrast those reported in a study using LPS and human amniotic epithelial cells [21]. Our results suggest that intrauterine upregulation of

TLR4 is not necessary to maintain the robust increase in uterine MCP-1 expression observed in response to LPS.

In summary, these studies have provided support for an important and possibly mechanistic role for MCP-1 during inflammation-induced preterm delivery in the mouse. These observations are consistent with the previously discussed human studies which demonstrated a robust increase of MCP-1 in the pregnant uterus of women undergoing preterm delivery, especially in the presence of intrauterine infection [17–19]. Interestingly, however, MCP-1 was also noted to be elevated, although not as high, in human pregnancies in preterm labor without evidence of infection and in term uncomplicated parturition [6]. Our mouse studies have provided a model which will make possible elucidation of the actual role and mechanisms of action for MCP-1 during preterm delivery in the future.

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Figure 1. Preterm delivery rates of Day 15 timed-pregnant CD-1 mice injected intrauterine with 250 μ g LPS or saline via survival surgery. Each time-point included n=10–14 females and n=87–173 pups. 0 hour mice did not undergo surgery.

Figure 2.

A) RayBio Mouse Inflammation Protein Arrays showing inflammatory mediators expressed in uterine tissue in response to intrauterine injection of 250 μ g LPS. Each protein is represented in two spots for an internal control. B) RayBio Arrays performed with homogenates of uterine tissue from mice at time 0 (pre-injection), 2 hours, 6 hours, 18 hours and 24 hours after intrauterine LPS injection, and 24 hours after vehicle (sterile saline) injection. MCP-1 spots have been circled in each of the six arrays.

Figure 3.

Representative RT-PCR gels demonstrating MCP-1 mRNA expression in uterus, liver, lung, and kidney in day 15 timed-pregnant CD1 mice, at 2, 6, 12, 18, and 24 hours after LPS intrauterine injection. MCP-1 amplicon (132 bp long) was designed to span at least one exon-exon junction; sequencing has confirmed the amplicon identity.

Figure 4.

Quantitative RT-PCR results showing relative MCP-1 mRNA levels in uterus, liver, lung, and kidney tissues harvested from day 15 timed-pregnant mice at 2, 6, 12, 18, and 24 hours after LPS intrauterine injection compared with uninjected 0 hour control animals. Asterisks denote significance, $p < 0.05$, when compared with 0 hour levels (ANOVA on Ranks, Dunnett test). Each bar represents the mean $\pm$ SD for mRNA from $n=6$ animals at each time-point in uterus, and $n=4$ pregnant animals for each time-point in liver, lung, and kidney.

Figure 5.

A) Qualitative RT-PCR gel showing TLR4 mRNA expression in uterine tissue harvested from day 15 timed-pregnant mice at 2, 6, 12, 18, and 24 hours after LPS intrauterine injection compared with 0 hour uninjected control animals. Amplicon (334 bp long) was designed to span at least one exon-exon junction; sequencing has confirmed amplicon identity. B) Quantitative RT-PCR results showing relative TLR4 mRNA levels. Each bar represents the mean $\pm$ SD for mRNA from n=6 pregnant animals at each time-point. Amplicon for qRT-PCR was 133 bp long, designed to span at least one exon-exon junction; sequencing has confirmed amplicon identity.

Figure 6.

ELISA results showing MCP-1 protein levels in serum collected from day-15 timed-pregnant CD1 mice at 2, 6, 12, 18, and 24 hours after LPS intrauterine injection. 0-hour mice received no LPS; saline controls were harvested at 24 hours. Each bar represents the mean $\pm$ SD for protein from $n=4$ animals at each time-point. Asterisks denote significance, $p<0.05$, when compared with 0 hour levels (ANOVA).

Figure 7.

ELISA results showing MCP-1 protein levels in uterine, liver, kidney, and lung tissues harvested from day-15 timed-pregnant CD1 mice at 2, 6, 12, 18, and 24 hours after LPS intrauterine injection. 0-hour mice received no LPS; saline controls were harvested at 24 hours. Each bar represents the mean $\pm$ SD for protein from $n=4$ animals at each time-point. Asterisks denote significance, $p < 0.05$, when compared with 0 hour levels (ANOVA).

Figure 8. Immunohistochemical expression of MCP-1 in pregnant mouse uterine tissue 2 hours after intrauterine injection of LPS. Tissue is from the right-mid uterine horn. MCP-1 is clearly visible in both myometrial layers as well as endometrium at 40X (a) and 400X (b), as compared with unstained controls at 40X (c) and 400X (d).

Table 1

A. Pattern of Proteins in Mouse Inflammation Antibody Array												
	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	POS	POS	POS	Blank	Blc	CD30L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
B	NEG	NEG	NEG	NEG	Blank	Blc	CD30L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
C	GM-CSF	IFN γ	IL-1 α	IL-1 β	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12 p70
D	GM-CSF	IFN γ	IL-1 α	IL-1 β	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12 p40p70	IL-12 p70
E	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1a	MIP-1g
F	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1a	MIP-1g
G	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNF α	s TNF RI	s TNF RII	Blank	Blank	Blank
H	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNF α	s TNF RI	s TNF RII	Blank	POS	POS

B. Proteins in Mouse Inflammation Antibody Array		Full Name
Abbreviation		
POS		Positive control spots
NEG		Negative control spots
Blank		Empty spaces
Bic		B-Lymphocyte Chemoattractant
CD30L		CD30 Ligand
Eotaxin		Eosinophil Chemokine
Eotaxin 2		Eosinophil Chemokine-2
Fas Ligand		Ligand for Fas Receptor
Fractalkine		CX3CR1 Receptor Ligand
GCSF		Granulocyte Colony Stimulating Factor
GM-CSF		Granulocyte Macrophage Colony Stimulating Factor
IFN γ		Interferon γ
IL-1 α		Interleukin 1 α
IL-1 β		Interleukin 1 β
IL-2		Interleukin 2

B. Proteins in Mouse Inflammation Antibody Array	
Abbreviation	Full Name
IL-3	Interleukin 3
IL-4	Interleukin 4
IL-6	Interleukin 6
IL-9	Interleukin 9
IL-10	Interleukin 10
IL-12p40p70	Interleukin 12 p40/p70 heterodimer
IL-12p40	Interleukin 12 p40 subunit
IL-13	Interleukin 13
IL-17	Interleukin 17
I-TAC	Interferon-induced T-cell Chemoattractant
KC	Keratinocyte Chemokine
Leptin	Leptin protein
LIX	Lipopolysaccharide (LPS)-induced CXC Chemokine
Lymphotactin	Lymphocyte Chemokine
MCP-1	Monocyte Chemoattractant (Chemoattractant) Protein-1
MCSF	Mononuclear Phagocyte Colony Stimulating Protein
MIG	Mig Chemokine
MIP-1 α	Macrophage Inflammatory Protein-1 α
MIP-1 γ	Macrophage Inflammatory Protein-1 γ
RANTES	Regulated upon Activation, Normal T cell Expressed and Secreted
SDF1	Stromal cell-Derived Factor-1
TCA-3	T-Cell Activator 3
TECK	Thymus-Expressed Chemokine
TIMP-1	Tissue Inhibitor of Metalloproteinases-1
TIMP-2	Tissue Inhibitor of Metalloproteinases-2
TNF α	Tumor Necrosis Factor α
sTNF RI	Soluble TNF Receptor I
sTNF RII	Soluble TNF Receptor II

Table 2

Primers used in RT-PCR and qRT-PCR studies

Gene	Primer sequence	Amplicon length	NCBI Accession Number
<i>MCP-1 sense</i>	TGATCCCAATGAGTAGGCTGGAG	132 bp	NM_011333.2
<i>MCP-1 antisense</i>	ATGTCTGGACCCATTCCTTCTTG		
<i>TLR4 sense</i>	CTAGGACTCTGATCATGGCACTGT	334 bp	NM_021297.2
<i>TLR4 antisense</i>	CCTGGGGAAAACTCTGGATAG		
<i>TLR4 sense (for qRT-PCR)</i>	CCTGATGACATTCCTTCTTCAAC	133 bp	NM_021297.2
<i>TLR4 antisense (for qRT-PCR)</i>	TTGTTTCAATTTCACACCTGGATAAA		
<i>B2m sense</i>	ATGCTATCCAGAAAACCCCTCAAA	79 bp	NM_009735.2
<i>B2m antisense</i>	CAGTTCAGTATGTTTCGGCTTCCC		
<i>Hprt sense</i>	CAGTCCCAGCGTCGTGAT	137 bp	NM_013556.2
<i>Hprt antisense</i>	CAAGTCTTTCAGTCCTGTCCATAA		
<i>Ywhaz sense</i>	GCAACGATGTACTGTCTCTTTTGG	149 bp	NM_013556.2
<i>Ywhaz antisense</i>	GTCCACAATTCCTTTCTTGTCATC		

Sense and anti-sense primer sequences used in RT-PCR and quantitative real-time RT-PCR (qPCR). Primers were designed to span at least one exon-exon junction to avoid amplifying genomic DNA remaining after DNase treatment. Amplicons for all primers were sequence-verified.