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Lipopolysaccharide-induced murine embryonic resorption involves changes in endocannabinoid profiling and alters progesterone secretion and inflammatory response by a CB1-mediated fashion

Manuel L. Wolfson [a,*], Fernando Correa [a], Emma Leishman [b], Claudia Vercelli [c], Cora Cymeryng [d], Julieta Blanco [a], Heather B. Bradshaw [b], Ana María Franchi [a]

[a] Laboratory of Physiopathology of Pregnancy and Labor, Center for Pharmacological and Botanical Studies, National Research Council, School of Medicine, University of Buenos Aires, Argentina
[b] Department of Psychological and Brain Sciences, Indiana University, Bloomington, IN, USA
[c] Biomedicine Research Institute of Buenos Aires, Partner Institute of the Max Planck Society (MPSP), National Research Council, Ciudad Autónoma de Buenos Aires (CABA), Buenos Aires, Argentina
[d] Laboratory of Molecular Endocrinology, Center for Pharmacological and Botanical Studies, National Research Council, School of Medicine, University of Buenos Aires, Argentina

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ABSTRACT

Genital tract infections are a common complication of human pregnancy that can result in miscarriage. We have previously shown that a lipopolysaccharide (LPS) induces embryonic resorption in a murine model of inflammatory miscarriage. This is accompanied by a dramatic decrease in systemic progesterone levels associated with a robust pro-inflammatory response that results in embryo resorption. Here, we tested the hypothesis that the endogenous cannabinoid system (eCS), through cannabinoid receptor 1 (CB1), plays a role in regulating progesterone levels and, therefore, the pro-inflammatory response. We show that LPS treatment in pregnant mice causes significant changes in the eCS ligands, which are reversed by progesterone treatment. We further show the CB1-*KO* mice maintain higher plasma progesterone levels after LPS treatment, which is associated with a feebler uterine inflammatory response and a significant drop in embryo resorption. These data suggest that manipulation of CB1 receptors and/or ligands is a potential therapeutic avenue to decrease infection-induced miscarriage.

1. Introduction

Genital tract infections caused by Gram-negative bacteria not only induce miscarriage but are also one of the most common complications in human pregnancy (Cram et al., 2002). The systemic presence of the main cell wall component lipopolysaccharide (LPS) in pregnant mice induces infiltration of the decidua with granulocytes and large granular lymphocytes (LGL) (Ogando et al., 2003), increased uterine and decidual production of nitric oxide (NO) and prostaglandins (Aisemberg et al., 2007; Ogando et al., 2003), and decreased plasma levels of progesterone (Aisemberg et al., 2013). These changes were associated with embryonic resorption (EmR) followed by fetal expulsion (Aisemberg et al., 2012, 2013; Ogando et al., 2003). We have previously shown that the endocannabinoid system (eCS) was involved in the effects of LPS on NO and prostaglandin production, and subsequent tissue damage during early embryonic loss (Vercelli et al., 2009a, 2009b, 2012).

Endogenous lipids of the eCS are composed of a growing family of *N*-acylamides and 2-acylglycerol esters, many of which are identified as ligands for specific GPCRs (*e.g.* CB1, CB2, GPR55, GPR18, GPR119) and TRPV receptors that participate in numerous physiological and pathological processes during pregnancy (Cella et al., 2008; Fonseca et al., 2010; Gebeh et al., 2013; Maccarrone et al., 2004; Pertwee et al., 2010; Raboune et al., 2014; Schuel et al., 2002; Sun et al., 2010; Taylor et al., 2011; Vercelli et al., 2009a, 2009b).

Abbreviations: 2-AG, 2-arachidonoylglycerol; 2-LG, 2-linoleoyl-*sn*-glycerol; 2-OG, 2-oleoyl-*sn*-glycerol; AEA, anandamide; AGly, *N*-arachidonoylglycine; CB1, cannabinoid receptor type 1; CB1-*KO*, CB1 receptor *knock-out* mice; CXCL-10, C-X-C motif chemokine 10; DEA, *N*-docosahexaenoylethanolamine; DGly, *N*-docosahexaenoylglycine; eCS, endocannabinoid system; EmR, embryonic resorption; IL, interleukin; i.p, intraperitoneal; LEA, *N*-linoleoylethanolamine; LGly, *N*-linoleoylglycine; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; NAEs, *N*-acylethanolamines; NAGly, *N*-acylglycines; NO, nitric oxyde; NOS, nitric oxyde synthase; OEA, *N*-oleoylethanolamide; OGly, *N*-oleoylglycine; PEA, *N*-palmitoylethanolamine; PCR, polymerase chain reaction; PGly, *N*-plamitoylglycine; RANTES, regulated on activation, normal T cell expressed and secreted; s.c, subcutaneous; SEA, *N*-stearoylethanolamine; SGly, *N*-stearoylglycine; TNFα, tumor necrosis factor alpha; WT, wild-type mice.

* Corresponding author. Centro de Estudios Farmacológicos y Botánicos (CEFyBO), Facultad de Medicina, Universidad de Buenos Aires, Paraguay 2155, Piso 16. C1121ABG, Ciudad Autónoma de Buenos Aires, Argentina. Tel.: +54 11 4508 3680 ext 111; fax: +54 11 4508 3680 ext 102.
E-mail address: manuwolfson@gmail.com (M.L. Wolfson).

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N-arachidonoylethanolamine (anandamide, AEA) was the first endocannabinoid to be isolated and characterized (Devane et al., 1992). *N*-acylethanolamines (NAEs) are the family of AEA endogenous analogs and have a variety of functions at different receptors including but not limited to TRPV1 receptors (Raboune et al., 2014). Similarly, another important endocannabinoid is 2-arachidonoylglycerol (2-AG) and its family of endogenous analogs, 2-acyl-*sn*-glycerols (Sugiura et al., 1999). Cumulative data support the concept that the eCS plays important roles in pregnancy.

Both cannabinoid receptors 1 and 2 (CB1, CB2) are expressed in human endometrium (Taylor et al., 2010a) with CB1 playing an important role in oviductal embryo transport (Wang et al., 2004) and uterine embryo receptivity (Paria et al., 2001). Low levels of AEA are favorable for implantation and trophoblast outgrowth whereas increased AEA concentrations are embryotoxic and lead to arrested embryo development and pregnancy failure (Paria and Dey, 2000). On the other hand, low fatty acid amide hydrolase (FAAH) activity in peripheral lymphocytes has been shown to correlate with miscarriages in humans (Maccarrone et al., 2000). Furthermore, high plasma levels of AEA have been associated with early pregnancy loss in humans (Habayeb et al., 2008). Maccarrone et al. (2002b, 2003) showed that a reduced peripheral FAAH activity and high plasma levels of AEA were correlated with low progesterone levels, even though Taylor et al. (2011) failed to find such correlation. FAAH activity is not limited to AEA; however, and has been shown to metabolize all of the NAEs (Cravatt and Lichtman, 2002). Much less is known about the role of 2-AG in the regulation of pregnancy; however, it has also been shown to be regulated in the uterus as a function of the ovarian cycle (Bradshaw and Allard, 2011). Therefore, the interplay between progesterone and the eCS is complex and not fully understood (Gebeh et al., 2013; Habayeb et al., 2008; Maccarrone et al., 2000; Paria and Dey, 2000; Taylor et al., 2011).

In addition to its role in establishing and maintaining pregnancy, progesterone is considered an immunosteroid (Correale et al., 1998) with a critical function in modulating the immune response during normal gestation (Szekeres-Bartho et al., 1996). It also has been shown that progesterone has anti-inflammatory effects by protecting the embryo from LPS-induced pregnancy loss (Aisemberg et al., 2013). It has been proposed that progesterone upregulates FAAH expression in human lymphocytes through the transcription factor Ikaros (Maccarrone et al., 2003) as well as CB1 mRNA expression in the endometrial stromal cells during the secretory phase (Resuehr et al., 2012). We have recently shown that LPS induced a downregulation of murine peripheral blood mononuclear cells' (PBMC) FAAH activity and that this effect was reversed by progesterone treatment (Wolfson et al., 2013).

Given that (a) CB1 is highly expressed in reproductive tissues (Taylor et al., 2010a; Vercelli et al., 2012), (b) LPS-induced high levels of endocannabinoids are associated with miscarriage (Habayeb et al., 2008; Maccarrone et al., 2000), and (c) progesterone exerts protective effects against pregnancy loss (Aisemberg et al., 2013), our aim for this work was to investigate whether progesterone exerts protective effects from the deleterious actions of LPS in early pregnancy and the role of the eCS.

2. Materials and methods

2.1. Reagents

LPS from *Escherichia coli* 05:B55 and progesterone were purchased from Sigma Chemical Co. (St. Louis, MI, USA). Trizol reagent, RNAse-free DNAse I, Moloney Murine Leukemia virus reverse transcriptase (M-MLVRT) and random primers were purchased from Invitrogen (Life Technologies, Argentina). GoTaq DNA Polymerase was purchased from Promega (Biodynamics, Argentina). All other chemicals were analytical grade.

2.2. Animals and treatments

Eight to 12-week-old virgin female Balb/c or CD1 (*wild-type* or CB1-*knock-out*) mice were paired with 8- to 12-week-old Balb/c or CD1 (*wild-type* or CB1-*knock-out*) males respectively. The day of appearance of a coital plug was taken as day 0 of pregnancy. CD1 CB1-*knock-out* mice were generated as previously described (Ledent et al., 1999). Animals were housed in cages under controlled conditions of light (12 h light, 12 h dark) and temperature (21–25 °C) and received murine chow and water *ad libitum*.

Next, 7-day pregnant Balb/c WT mice were divided into four groups: (1) control group received an i.p. and s.c. injection of vehicle, (2) LPS-treated group received an i.p. injection of LPS (1 μg/g of body weight in saline solution), (3) LPS plus progesterone-treated group received a s.c. injection of progesterone (2 mg/animal in corn oil) and an i.p. injection of LPS, and (4) progesterone-treated group received a s.c. injection of progesterone. Blood from the orbital sinus was extracted under CO_2 anesthesia 12 h after LPS or vehicle administration, followed by animal euthanization by cervical dislocation. The blood was collected in EDTA-coated tubes and centrifuged at 655 *g* for 10 min at 4 °C and the plasma fraction was stored at −70 °C until further use for lipid analysis as will be described later.

In the case of the CD1 mice, 7-day pregnant *wild-type* (WT) or CB1-*knock-out* (CB1-*KO*) mice were caged in two groups each: (1) control WT and control CB1-*KO* received an i.p. injection of vehicle, and (2) LPS-treated WT and LPS-treated CB1-*KO* received an i.p. injection of LPS (1 μg/g or 0.5 μg/g of body weight). Mice were euthanized 6, 12 or 24 h after LPS or vehicle administration. Blood from the orbital sinus was extracted under CO_2 anesthesia, followed by animal euthanization by cervical dislocation. The blood was allowed to clot and was centrifuged at 655 *g* for 10 min and the serum fraction was stored at −70 °C until used for progesterone level determination.

2.3. Ethics statement

The experimental procedures reported here were approved by the Animal Care Committee of the Center for Pharmacological and Botanical Studies of the National Research Council (CEFYBO–CONICET) and by the Institutional Committee for the Care and Use of Laboratory Animals from the School of Medicine (University of Buenos Aires), and were carried out in accordance with the Guide for Care and Use of Laboratory Animals (NIH). All blood extractions were performed under CO_2 anesthesia and all efforts were made to minimize suffering.

2.4. Determination of embryonic resorption rate

With the aim of assessing the rate of embryonic resorption, CD1 WT and CB1-*KO* mice were treated on day 7 with LPS (0.5 or 1 μg/g of weight) and euthanized by cervical dislocation 24 h later. The uteri were excised and examined macroscopically to count the number of healthy and reabsorbed embryos. The reabsorbed embryos were identified by their small size, extensive hemorrhage and necrosis. An embryo that fits these criteria was classified as resorbed. Resorption rates were calculated as: [number of resorbed embryos/(total number of embryos)] × 100.

2.5. Radioimmunoassay

Progesterone was measured in serum extracted from LPS treated CD1 WT and CB1-*KO* mice and control mice sacrificed 12 h after treatment. Blood from the orbital sinus was extracted under CO_2 anesthesia. Blood was allowed to clot and was centrifuged at 655 *g* for 10 min and stored at −70 °C until used. Progesterone was

measured by radioimmunoassay as previously described (Aisemberg et al., 2013). Values are expressed as ng/ml of serum progesterone.

2.6. Mass spectrometric analysis of lipids in fractions

For lipid analysis, 0.5 ml of plasma was obtained from each Balb/c pregnant mouse, flash frozen and stored at −70 °C until used for extraction preparation, plasma was allowed to thaw on ice in a covered container, then 2 ml of HPLC-grade methanol was added. [^{2}H8]-AEA (200 pmol) was added to each sample and diluted with HPLC grade water to make a 75% aqueous solution. Lipids were extracted as previously described (Bradshaw et al., 2006; Rimmerman et al., 2011). Briefly, 500 mg C18 Bond Elut solid phase extraction columns (Varian) were conditioned with 5 ml HPLC-grade methanol, followed by 3.0 ml HPLC water. The 75% aqueous solutions containing the fractions were loaded onto separate columns, which were then washed with 2.5 ml water. Four sequential elutions (1.5 ml each of 40, 75, 85 and 100% methanol) were collected for mass spectrometric analysis. As described previously (Bradshaw et al., 2006), sample analysis of lipids was carried out as follows. An aliquot of each of the eluates was loaded using a Shimadzu SCL10Avp (Wilmington, DE, USA) autosampler onto a reversed phase Zorbax 2.1 × 50 mm C18 column maintained at 40 °C. HPLC gradient formation at a flow rate of 200 ml/min was achieved by a system comprising a Shimadzu controller and two Shimadzu LC10ADvp pumps. Lipid levels in the samples were analyzed in multiple reaction monitoring (MRM) mode on a triple quadrupole mass spectrometer, using either the API 3000 (Applied Biosystems/MDS SCIEX, Foster city, CA, USA), with electrospray ionization. Methods for lipid analysis were created and optimized by flow injection of lipid standards. All calculations for quantitation experiments were based on calibration curves using synthetic standards as previously described (Bradshaw et al., 2006).

2.7. Histology

CD1 pregnant mice were divided into four groups (control-WT, LPS-WT, control-CB1-*KO* and LPS-CB1-*KO*) and treated as described before. For each animal, two implantation sites from pregnant mice on day 7 of gestation were removed and fixed in formol 4% in phosphate-buffered saline. The tissue sections were dehydrated and embedded in paraffin. Sections of 5 μm were made with a microtome and mounted on 2% silane-coated slides. The sections were stained with hematoxylin–eosin, and observed by light microscopy (Nikon Eclipse 200, NY, USA) to evaluate tissue morphology and cell types. Hematoxylin–eosin-stained sections for the four groups were scored for the density of leukocytes by counting ten fields (100× objective) pooled from the mesometrial decidua of each implantation site. Ten animals were used per treatment, and two sites of implantation of each animal were randomly selected. Hematoxylin–eosin staining of sites of LPS-treated and control animals at 6 h after injection was performed.

2.8. Determination of NOS activity

Briefly, the uterus and decidua from each implantation site from both CD1 WT and CB1-*KO* mice were separated and decidual tissue was immediately frozen at −70 °C until NOS activity determination. A modification of the method of Bredt and Snyder (1989) was used. This method measures the conversion of [^{14}C]arginine to [^{14}C]citrulline, as citrulline remains in the sample, whereas the equimolar amounts of NO produced are rapidly destroyed. Briefly, decidual tissue was homogenized and incubated at 37 °C in a buffer containing 20 mmol/l HEPES, 10 μM [^{14}C]arginine (0.3 μCi), 25 mM valine, 1 mM DTT, 0.45 mM $CaCl_2$ and 1 mM NADPH. Valine, which inhibits the conversion of L-arginine to L-citrulline by arginases, was included in the reaction mixture to increase assay specificity. After 15 min of incubation, samples are centrifuged for 10 min at 3000 *g* and applied to a 1 ml DOWEX AG50WX8 column (Na^+ form) and [^{14}C]citrulline was eluted in 3 ml water. The radioactivity was measured by liquid scintillation counting. Enzyme activity is reported as pmol [^{14}C]citrulline per 15 min per 100 mg wet weight.

2.9. Semi-quantitative RT-PCR analysis

Total RNA from uterine explants from CD1 WT and CB1-*KO* mice was isolated using Trizol reagent according to the manufacturer's recommendations (Invitrogen, California, USA). Following extraction, RNA was quantified and further treated with RNAse-free DNAse I to digest contaminating genomic DNA. Subsequently, RNA was quantified and cDNA was synthesized from total RNA (3 μg) using M-MLVRT, random primers and ribonuclease inhibitor. PCR was performed with specific primers designed using the Primer3 Software package and checked for self-complementarity with OligoCalc Software package. CXCL-10 forward primer: 5′-TGCCGTCATTTTCTGCCTCA-3′; CXCL-10 reverse primer: 5′-AGGCTCGCAGGGATGATTTC-3′. MCP-1 forward primer: 5′-ATGC AGTTAACGCCCCACTC-3′; MCP-1 reverse primer: 5′-GCACAG ACCTCTCTCTTGAGC-3′. RANTES forward primer: 5′-CACTCCC TGCTGCTTTGC-3′; RANTES reverse: 5′-CACTTGGCGGTTCCTTCG-3′. IL-4 forward: 5′-AGGACTGGGACTAGAGGCAG-3′; IL-4 reverse: 5′-CTACCACCTGACCACCAACC-3′. IL-10 forward primer: 5′-TGCTAT GCTGCCTGCTCTTA-3′: IL-10 reverse primer:_ 5′-TCATTTCCGAT AAGGCTTGG-3′. IL-6 forward primer: 5′-TTGGGACTGATGCTGGTGAC-3′; IL-6 reverse primer: 5′-TCTCTCTGAAGGACTCTGGCT-3′. TNFα forward primer: 5′-TCAGCCTCTTCTCATTCCTGC-3′; TNFα reverse primer: 5′-TTGGTGGTTTGCTACGACGTG-3′. IL-1β forward primer: 5′-GCAACTGTTCCTGAACTC-3′; IL-1β reverse primer: 5′-CTCGG AGCCTGTAGTGCA-3′. β-actin forward primer: 5′-TGTTACCAA CTGGGACGACA-3′, β-actin reverse primer: 5′-TCTCAGCTGT GGTGGTGAAG-3′. PCR cycle parameters were as follows: an initial denaturing step at 94 °C for 5 min followed by 30 cycles of 94 °C for 40 s, 60 °C (RANTES, CXCL-10, MCP-1, IL-4, IL-10, IL-6), 57 °C (β-actin) or 55 °C (TNFα, IL-1β) for 30 s and 72 °C for 60 s followed by 72 °C for 5 min. A 2% agarose gel was loaded with 18 μl of the PCR reaction and separated DNA bands were visualized on a transilluminator after ethidium bromide staining. Images were taken using a digital camera Olympus C-5060 and analyzed using the Image J Software package (open source). Relative amount of target PCR product was calculated as a ratio of each target PCR product *versus* β-actin PCR product. Next, relative fold change was expressed as the ratio of LPS-treated cases *versus* control cases.

2.10. Statistical analyses

Treatments were assigned completely random to experimental units. Data were analyzed by means of one or two way ANOVA procedures and means were compared by Tukey or LSD Fischer *post hoc* tests. Differences between means were considered significant when p value was 0.05 or less. Different letters indicate significant differences between means. Normality and homoscedasticity were tested by Shapiro–Wilk (modified) and Levene tests, respectively. Statistical analysis was performed using the Infostat Software package (Córdoba, Argentina).

3. Results

3.1. Progesterone reverses the LPS-induced increase in NAEs as well as the LPS-induced decrease in N-acylglycines (NAGlys) and 2-acyl-sn-glycerols plasma levels

LPS administration to 7-day pregnant Balb/c WT mice induced an increase in the plasma levels of all NAEs and a decrease in all

Table 1
LPS administration to 7-day pregnant Balb/c mice induces an increase in the plasma levels of several NEAs, such as AEA, OEA, PEA, LEA, SEA, and DEA. The administration of progesterone, 2 hours previous to LPS, was able to reverse the LPS-induced increase on OEA and SEA plasma concentration while not having the same of effect on PEA, DEA and LEA. Progesterone treatment was unable to restore AEA plasma levels. Progesterone supplementation alone had differential effects: DEA and LEA, OEA, PEA, and SEA levels were unaffected meanwhile AEA levels were increased. Regarding *N*-acylglycines: LPS induced a decrease in the plasma levels of all measured NAGlys. Progesterone treatment was able to restore the plasma levels of OGly, SGly, LGly and DGly, not having the same magnitude of effect on PGly, and lacking any effects on AGly. Progesterone alone did not have any effect in AGly, PGly, SGly plasma levels while OGly, LGly and DGly plasma levels were slightly decreased. In the case of 2-acyl-*sn*-glycerols, LPS administration to 7-day pregnant Balb/c mice induced a marked decrease in 2-AG, 2-OG and 2-LG plasma levels. Progesterone treatment was able to restore the plasma levels of all three 2-acyl-*sn*-glycerols with progesterone alone having no effects on their basal levels (n = 5–10).

Mouse plasma	LPS Δ rel. to control	LPS plus progesterone Δ rel. to control	Progesterone Δ rel. to control	LPS plus progesterone Δ rel. to LPS	LPS Δ rel. to progesterone	LPS plus progesterone Δ rel. to progesterone
***N*-acyl ethanolamine**						
PEA	↑↑↑↑			↓↓	↑↑↑↑	↑↑
SEA	↑↑↑			↓↓	↑↑↑	↑↑
OEA	↑↑↑	↑↑		↓↓	↑↑↑↑	↑↑↑
LEA	↑↑↑			↓↓↓	↑↑↑↑	
AEA	↑↑	↑↑	↑			
DEA	↑↑			↓	↑↑↑	↑↑
***N*-acyl glycine**						
PGly	↓	↓			↓	↓
SGly	↓↓↓	↓		↑	↓↓	↓
Ogly	↓↓↓	↓	↓	↑↑	↓↓	
LGly	↓↓		↓	↑↑	↓↓	
AGly	↓↓↓	↓↓			↓↓	↓
DGly	↓↓↓	↓	↓↓	↑↑		↑
2-Acyl-*sn*-glycerol						
2-AG	↓↓↓↓			↑↑↑↑	↓↓↓	
2-LG	↓↓↓↓			↑↑↑↑	↓↓↓↓	
2-OG	↓↓↓↓↓			↑↑↑↑↑	↓↓↓↓↓	

Keys

	Significant increase $p < 0.05$
	Trending increase $p < 0.1$
	Trending decrease
	Significant decrease
↑↑↑↑↑	10 or more times higher
↑↑↑↑	3–9.99 times higher
↑↑↑	2–2.99 times higher
↑↑	1.50–1.99 times higher
↑	1–1.49 times higher
↓	1–1.49 times lower
↓↓	1.50–1.99 times lower
↓↓↓	2–2.99 times lower
↓↓↓↓	3–9.99 times lower
↓↓↓↓↓	10 or more times lower

N-acylglycines (NAGlys) and 2-acyl-*sn*-acylglycerols tested here (Table 1; Supplementary Table S1). Given that we previously demonstrated that progesterone exerts protective effects against LPS-induced pregnancy loss (Aisemberg et al., 2013) and that this hormone was able to restore FAAH activity levels in PBMCs from LPS-treated mice (Wolfson et al., 2013), we tested the hypothesis that treatment with progesterone would normalize the plasma concentration of these NAEs in our LPS-treated pregnant mice. As shown in Table 1, progesterone was able to counteract LPS-induced increase in *N*-oleoylethanolamine (OEA), *N*-stearoylethanolamine (SEA) plasma concentration, it did not have the same magnitude of effect on *N*-plamitoylethanolaine (PEA), docosahexaenoylethanolamine (DEA) and *N*-linoleoylethanolamine (LEA) and it did not have any effects on AEA plasma levels. Interestingly, progesterone supplementation alone had differential effects on plasma NAEs, wherein DEA and LEA, OEA, PEA, and SEA levels were unaffected and AEA levels were increased (Table 1). Conversely, we found that LPS induced a decrease in the plasma levels of several NAGlys, of which at least *N*-arachidonyl glycine (AGly) is a known metabolite of AEA (Bradshaw et al., 2009). Progesterone treatment was able to restore the plasma levels of *N*-oleoylglycine (OGly), *N*-stearoylglycine (SGly), *N*-linoleoylglycine (LGly) and *N*-docosahexaenoylglycine (DGly), it did not have the same magnitude of effect on *N*-plamitoylglycine (PGly), and no effect on AGly. Interestingly, progesterone alone did not have any effect in AGly, PGly, or SGly plasma levels while OGly, LGly and DGly plasma levels were slightly decreased (Table 1). Similar to NAGlys, when we measured the plasma levels of several 2-acyl glycerols in response to LPS and/or progesterone, we found that LPS administration to 7-day pregnant Balb/c WT mice induced a marked decrease in 2-AG, 2-oleoyl-*sn*-glycerol (2-OG) and 2-linoleoyl-*sn*-glycerol (2-LG) plasma levels. In this case, progesterone was able to restore the plasma levels of all three 2-acyl glycerols with progesterone alone having no effects on their basal levels (Table 1). Taken together these effects add further evidence to the interrelationships between the endocrine and endocannabinoid signaling systems.

3.2. *CB1*-KO mice are resistant to LPS-induced early embryonic resorption

Since evidence suggests that an increase in AEA levels is associated with several adverse effects leading to pregnancy failures (Paria and Dey, 2000) and since CB1 is prominently expressed in reproductive tissues (Taylor et al., 2010a), we decided to investigate the role of this cannabinoid receptor in our LPS-induced embryonic resorption model. Our first approach was to compare the effect of two doses of LPS (1 and 0.5 μg/g of weight) on *wild-type* CD1 (WT) and CB1-*knock-out* CD1 (CB1-*KO*) pregnant mice. As shown in Fig. 1, both doses of LPS induced a high percentage of embryonic resorption (EmR) in WT mice whereas in CB1-*KO* mice, it showed a lesser effect (Fig. 1A, $^{*}p < 0.05$). Since we achieved a high percentage of EmR in WT mice with the lower dose of LPS (0.5 μg/g of weight) and that

A

Treatment	WT	CB1-*KO*
Control	0.0 ± 0 0 %	0.0 ± 0.0 %
LPS (0.5 µg/g weight)	69.4 ± 22.0 % (*)	3.4 ± 1.4 %
LPS (1 µg/g weight)	82.0 ± 11.9 % (*)	28.5 ± 3.0 %

B

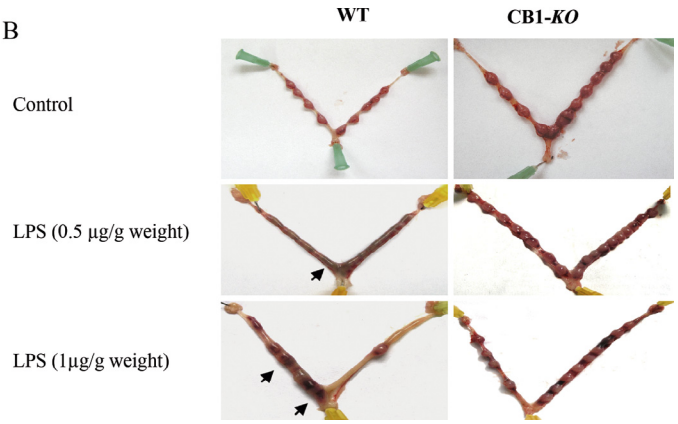


Fig. 1. LPS induces a high percentage of embryonic resorption (EmR) in WT mice when compared to CB1-*KO* mice. The embryonic resorption rate in LPS-treated or control mice was assessed by examining the uteri of pregnant mice 24 h after the LPS or saline administration. The uteri were excised and examined macroscopically to count the number of healthy and reabsorbed embryos. The reabsorbed embryos were identified by their small size, extensive hemorrhage and necrosis, as shown in panel B (arrows). Statistics: $^{*}p < 0.05$ (n = 5).

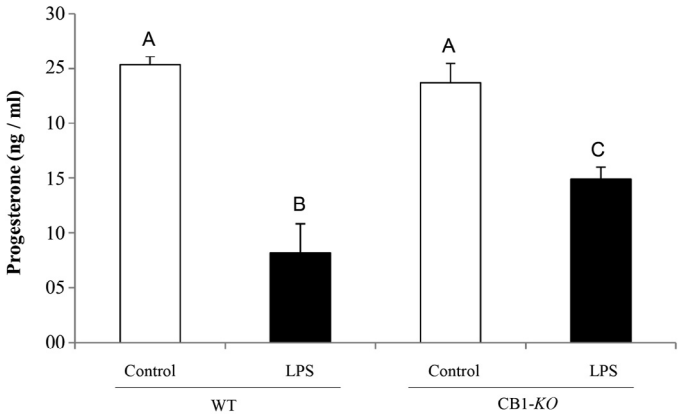


Fig. 2. CB1-*KO* mice show a diminished LPS-induced drop in serum progesterone (P) concentration when compared to WT mice. Twelve hours post-administration of LPS (0.5 µg/g body weight), blood was collected and serum P level was assessed by radioimmunoassay. Statistics: A ≠ B ≠ C, $p < 0.05$ (n = 8).

this dose was unable to induce any EmR in the CB1-*KO* mice, we performed all our next experiments using 0.5 µg LPS/g of weight.

3.3. *CB1*-KO mice show a diminished LPS-induced drop in progesterone levels when compared to WT mice

We have previously shown that LPS (1 µg/g body weight) induced a drop in serum progesterone levels in 7-day pregnant Balb/c mice at 6, 12 and 24 h post-injection (Aisemberg et al., 2013). Therefore, we decided to analyze whether LPS had a similar effect on serum progesterone concentrations in 7-day pregnant CD1 WT and CB1-*KO* mice. LPS (0.5 µg/g body weight) was administered intraperitonally and 12 h later, blood was collected from these mice and serum progesterone level was assessed. Both WT and CB1-*KO* control mice had equivalent baseline concentrations of serum progesterone (Fig. 2). Interestingly, though both groups had a significant drop in circulating progesterone when exposed to LPS, the magnitude of the decrease in CB1-*KO* mice was significantly less (38%) when compared to WT mice (70%) ($p < 0.05$).

3.4. *Increased leukocyte infiltration in the decidua of LPS-treated CD1 WT mice in comparison to LPS-treated CD1 CB1*-KO *mice*

Our LPS-induced EmR model is associated with an increase in the number of leukocytes infiltrating the decidua (Ogando et al., 2003). When we analyzed the infiltrating leukocytes in our LPS-treated CD1 WT and CB1-*KO* mice, we found an elevated number of white blood cells infiltrating the decidua from LPS-treated WT mice when compared to control WT mice. Interestingly, this effect was absent in LPS-treated CB1-*KO* mice, in which we observed very few leukocytes infiltrating the decidua when compared to LPS-treated WT mice (Fig. 3).

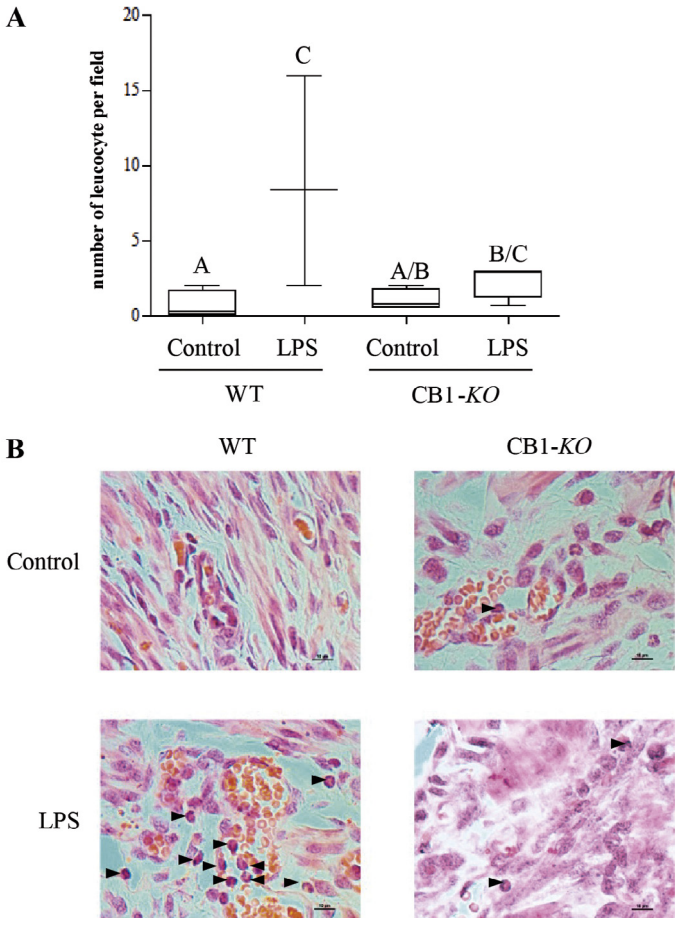
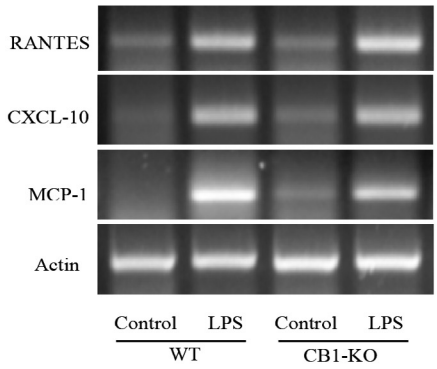


Fig. 3. Histological analyses of hematoxylin–eosin stained deciduas show that LPS administration induced an increased infiltration of leukocytes in LPS-treated WT mice when compared to LPS-treated CB1-*KO* mice. Ten random fields per decidua were chosen and the number of infiltrating leukocytes was assessed. Data were transformed by calculating normal scores for statistical analysis. Statistics: A ≠ B ≠ C, $p < 0.05$ (n = 3–4).

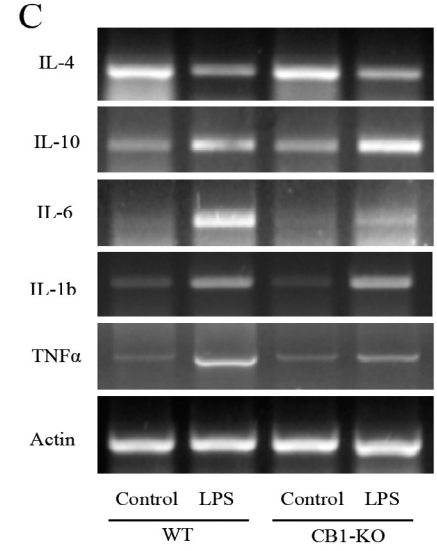
A



B

Chemokines	Relative fold increment: LPS WT (vs Control WT)	Relative fold increment: LPS CB1-*KO* (vs Control CB1-*KO*)
RANTES	2.81 ± 0.28	2.06 ± 0.19
CXCL-10	2.89 ± 0.45	2.12 ± 0.20
MCP-1	2.47 ± 0.38	2.41 ± 0.14

C



D

Cytokines	Relative fold increment: LPS WT (vs Control WT)	Relative fold increment: LPS CB1-*KO* (vs Control CB1-*KO*)
IL-4	0.62 ± 0.07	0.68 ± 0.03
IL-10	2.10 ± 0.23	2.78 ± 0.40
IL-6	11.00 ± 0.93	4.44 ± 0.61 **(*)**
IL-1β	2.56 ± 0.62	2.29 ± 0.46
TNFα	1.90 ± 0.14	1.54 ± 0.14 **(*)**

Fig. 4. Semi-quantitative RT-PCR analyses of the mRNA expression of chemokines in the deciduas of control and LPS-treated mice show no difference between WT-mice and CB1-*KO* mice. A representative experiment of six independent experiments is shown (n = 6). LPS-treated CB1-*KO* mice show an altered pattern of mRNA expression of pro-inflammatory cytokines in the decidua when compared to LPS-treated WT mice. LPS-treated CB1-*KO* mice showed lower levels of decidual IL-6 and TNFα mRNA expression when compared to LPS-treated WT mice. No differences were observed in the decidual mRNA expression of IL-4, IL-10 and IL-1β. Statistics: *$p < 0.05$ (n = 4–6).

3.5. CB1-KO mice show changes in the decidual pro-inflammatory/anti-inflammatory milieu when compared to WT mice

The lower leukocyte infiltration observed in the decidua of CB1-*KO* mice could be due to an altered expression of chemokines in these mice when compared to WT mice. Therefore, our next set of experiments aimed at analyzing the chemokine mRNA patter of expression in WT and CB1-*KO* mice decidual tissue. Interestingly, PCR analysis of chemokine mRNA expression showed no differences in RANTES, CXCL-10 or MCP1 levels between CB1-*KO* and WT mice (Fig. 4A and 4B).

CB1-*KO* mice show a diminished LPS-induced drop in serum progesterone levels which is associated with a lower percentage of EmR when compared to WT mice. Progesterone has also been shown to exert anti-inflammatory effects (Hardy et al., 2006). Therefore, we hypothesized that CB1-*KO* mice express lower levels of pro-inflammatory cytokines and/or higher levels of anti-inflammatory cytokines in decidual tissues after exposure to LPS. PCR analysis of LPS-induced changes in mRNA expression of two anti-inflammatory cytokines (IL-4, IL-10) showed no difference between genotypes. Next, we studied the mRNA expression profile of the pro-inflammatory cytokine. For both IL-6 and TNFα, LPS induced a smaller increase in mRNA in CB1-*KO* compared to WT mice. In the case of IL-6, the difference was more striking (4.44-fold increase in CB1-KO *versus* 11-fold increase in WT) (Fig. 4C and 4D).

3.6. LPS treatment induces an increase in NOS activity in the decidua of WT mice but not in the decidua of CB1-KO mice

Nitric oxide (NO) is a potent pro-inflammatory mediator, which has a central role in the pathophysiology of embryonic and fetal loss (Aisemberg et al., 2007; Ogando et al., 2003). Here we examined the relationship of NO production via decidual NO synthase (NOS) activity with LPS treatment and CB1 deletion. We found that basal NOS activity in the decidua of CB1-*KO* mice was higher than in the decidua of control WT mice. However, treatment with LPS for 6 h to 7-day pregnant mice failed to increase NOS activity in the decidua of CB1-*KO* mice in contrast with the higher activity levels found in the decidua of WT mice (Fig. 5).

4. Discussion

Several studies have shown an increase in endocannabinoid synthesis during sepsis (Wang et al., 2001) and septic shock (Maccarrone et al., 2002a). Therefore, we hypothesized that endocannabinoids and related lipids would likewise increase in our model of LPS-induced early embryonic resorption. Indeed, we observed, in agreement with previous reports, an increase in the plasma level of all NAEs measured here including the endocannabinoid AEA, 12 h post-LPS administration. Cumulative evidence shows a link between alterations of the eCS and miscarriage. Maccarrone et al. (2000)

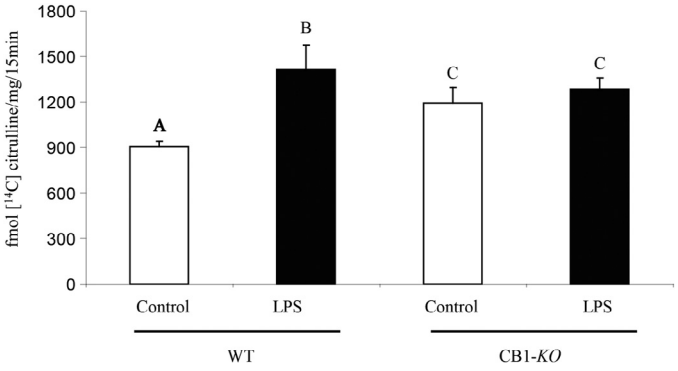


Fig. 5. Increased NOS activity in the decidua of LPS-treated WT mice but not in LPS-treated CB1-*KO* mice. Basal NOS activity in the decidua of CB1-*KO* mice was higher than in the decidua of control WT mice. NOS activity was measured in the decidua of control and LPS-treated WT and CB1-*KO* mice by assessing the conversion of [^{14}C]arginine to [^{14}C]citrulline, accordingly to the modified method of Bredt and Snyder (1989). Statistics: $A \neq B \neq C$, $p < 0.05$ ($n = 5–9$).

reported a correlation between FAAH activity in peripheral lymphocytes and miscarriage in humans. Similarly, Habayeb et al. (2008) found that high plasma levels of AEA were associated with early pregnancy loss in humans, suggesting that plasma AEA levels need to be tightly regulated for a successful pregnancy. These observations are in agreement with the findings in animal models in which low levels of AEA are favorable for implantation and trophoblast outgrowth, whereas increased AEA concentrations are embryotoxic and lead to arrested embryo development and pregnancy failure (Paria and Dey, 2000).

Interestingly, it has been reported that there are relatively higher concentrations of 2-AG in rat reproductive tissues, that are likewise regulated by the steroids (Bradshaw and Allard, 2011) and that this endocannabinoid has a similar role to AEA during uterine remodeling (Fonseca et al., 2010) and embryo implantation (Wang et al., 2007). Indeed, the distribution of the *sn*-1-diacylglycerol lipase (DAGL), the main enzyme of 2-AG synthesis, and of monoacylglycerol lipase (MAGL), the main enzyme for degradation of 2-AG, are distributed during the implantation process in order to minimize the exposure of the embryos to excessive levels of 2-AG (Fonseca et al., 2010). Nonetheless, the role of 2-AG during the different stages of pregnancy is not fully understood.

When we co-administered progesterone to LPS-treated mice and measured NAE plasma levels, we found that progesterone reversed the LPS-induced increase in OEA and SEA, while having a lesser effect on PEA, DEA and LEA, and having no effect on AEA. Conversely, progesterone reversed the LPS-induced decrease in the plasma levels of several NAGlys, such as OGly, SGly, LGly and DGly, with a lesser effect on PGly and no effects on AGly. Metabolically, AEA and AGly have been shown to be linked where AEA is a precursor for AGly through (1) a FAAH-dependent pathway and (2) a FAAH-independent alcohol dehydrogenase pathway involving the oxidation of ethanolamine to glycine (Bredt and Snyder, 1989). Whether or not additional NAEs and NAGlys are linked in this way is not known. Similarly to NAGlys, progesterone treatment reversed the LPS-induced decrease of 2-AG, 2-OG and 2-LG plasma levels. Therefore, progesterone seems to differentially regulate the plasma levels of these endocannabinoids. Since NAEs, NAGlys and 2-acylglycerols are potential substrates of FAAH, a possible explanation for the effects of progesterone on the plasma levels of these endocannabinoid could be due, at least partially, to the modulation of FAAH activity, which could essentially alter the pool of fatty acid substrates in such a way as to shift this entire pool of signaling lipids. In this sense, we have previously reported that progesterone reversed the LPS-induced downregulation of murine peripheral blood mononuclear cells' (PBMC) FAAH activity (Wolfson et al., 2013). Therefore, it could be hypothesized that progesterone, by upregulating PBMC's FAAH activity, differentially modulates the plasma levels of several endocannabinoids, driving decreases in NAEs with a compensatory increase in NAGlys and 2-acylglycerols. Additionally, it could also be hypothesized that progesterone interferes with MAGL activity resulting in an increase in 2-acylglycerols with a compensatory decrease in other lipid metabolites. Ultimately, more experiments are needed in order to understand the complex interplay between progesterone and the eCS.

The endocannabinoids AEA and 2-AG are ligands for the cannabinoid receptors CB1 which is highly expressed in the human endometrium (Taylor et al., 2010a) and it has been shown to play an important role in oviductal embryo transport (Wang et al., 2004) and uterine embryo receptivity (Paria et al., 2001). Since local uterine AEA levels are important not only for a normal pregnancy but also to participate in LPS-induced tissue damage (Vercelli et al., 2009a, 2009b, 2012), we hypothesized that CB1-*KO* mice would be resistant to LPS-induced embryo loss. Indeed, LPS administration (0.5 or 1 μg/g of weight) to CB1-*KO* mice resulted in fewer EmR when compared to WT mice, with the embryo loss rate of the lower dose being no different from vehicle. To the best of our knowledge, we are the first group to report that CB1-*KO* mice are resistant to LPS-induced EmR.

A decrease in serum progesterone levels is associated with events leading to pregnancy loss (Babalioğlu et al., 1996; Daya, 1989; Fidel et al., 1998). We have previously shown that LPS administration to 7-day Balb/c WT pregnant mice induced a drop on progesterone plasma levels as early as 6 h post-LPS injection, with the biggest effect at 12 h post-LPS administration (Aisemberg et al., 2013). We suggested that LPS-induced embryonic loss was in part due to a deficiency in progesterone plasma levels in Balb/c WT mice (Aisemberg et al., 2013). Therefore, we sought to investigate whether LPS induced a similar drop in progesterone plasma levels in our CD1 WT and CD1 CB1-*KO* mice. Similarly to the previously reported LPS-induced drop in progesterone plasma levels in Balb/c WT mice, we observed a significant drop in this hormone plasma levels in our CD1 WT mice 12 h post-LPS injection. Interestingly, at the same time post-LPS administration, CB1-*KO* mice showed a statistically significant lower drop in progesterone plasma levels when compared to WT mice. This finding correlates with the reports in which it is observed that CB1 or CB2 receptor agonists decreased progesterone secretion in luteal tissue from cows (Weems et al., 2009) and ewes (Tsutahara et al., 2011). Therefore, the lower LPS-induced drop of progesterone plasma levels in CB1-*KO* could explain the lower EmR ratio observed in these mice, suggesting a role for CB1 receptor in the EmR of the endotoxin.

Leukocytes are present in the decidua in early pregnancy (Warning et al., 2011) with their relative numbers varying during different stages of pregnancy (Blois et al., 2011; Croy et al., 2012). The phenotype and function of decidua-resident leukocytes are tightly regulated (Krishnan et al., 2013). The administration of LPS to pregnant mice induces decidual infiltration with peripheral leukocytes which is associated with embryo toxicity and resorption (Ogando et al., 2003). We found that the decidua from LPS-treated CB1-*KO* mice showed fewer infiltrating leukocytes when compared to LPS-treated WT mice, which could result in a lower pro-inflammatory milieu in the decidua and therefore, a reduced embryotoxic effect. It has been shown that AEA inhibits the chemokine-induced T lymphocyte migration (Joseph et al., 2004). We, therefore, hypothesized that the disparate decidual infiltration of leukocytes in WT and CB1-*KO* mice in response to LPS could be due to a differential expression of chemokines induced by the endotoxin. However, when we analyzed the decidual pattern of expression of RANTES, MCP-1 and CXCL-10 induced by LPS treatment, we did not find any differences between WT and CB1-*KO* mice,

suggesting that the disparate level of leukocyte infiltration is not due to a differential expression of chemokines.

Recent evidence suggests that different stages of pregnancy are associated with either a more pro-inflammatory environment or a more anti-inflammatory one (Mor and Cardenas, 2010). In any case, a tightly regulated network of cytokines is essential for a successful pregnancy (Battista et al., 2012; Krishnan et al., 2013). LPS treatment of pregnant mice results in an imbalance of the cytokine environment, with an overproduction of pro-inflammatory cytokines that are detrimental for the embryo and results in miscarriage and embryo resorption (Aisemberg et al., 2007, 2012; Ogando et al., 2003). The response of CB1-*KO* mice to LPS induction of TNFα and IL-6 mRNA is blunted compared to that of WT mice. A lower production of pro-inflammatory cytokines likely results in a diminished inflammatory response and in a lower embryo resorption rate. We postulate that, even though there are no changes in the decidual levels of mRNA expression of IL-4, IL-10 or IL-1β, the lower production of IL-6 and TNFα changes the inflammatory milieu in the decidua of CB1-*KO* mice, protecting the embryos from inflammation-induced damage. Interestingly, progesterone plasma levels after LPS treatment remain higher in these animals, contributing to the modulation of the inflammatory response by affecting the cytokine production (Correale et al., 1998; Szekeres-Bartho et al., 1996). Therefore, the lower embryo resorption rate observed in LPS-treated CB1-*KO* mice could be due to the relatively permanent higher levels of progesterone which exerts immunomodulatory and protective effects in the embryos, allowing the pregnancy to continue normally.

Nitric oxide (NO) has been proposed to be an important regulator of the physiology of the reproductive system. NO exerts important functions during pregnancy and its effects are largely dependent on the levels of this gas. Thus, low concentrations of NO reduced uterine prostaglandin synthesis whereas high concentrations augmented it (Cella et al., 2010). Furthermore, reduced serum NO levels in pregnant women are associated with miscarriage (Paradisi et al., 2007). We have previously shown that decidual and uterine production of NO is increased by the treatment with LPS, causing fibrinolysis and infiltration of mesometrial decidua with iNOS positive macrophages (Ogando et al., 2003). Moreover, the inhibition of NO production rescues LPS-induce embryo resorption in mice (Athanassakis et al., 1999; Ogando et al., 2003). Here, we demonstrate that CB1-*KO* mice show no modifications in decidual NOS activity in response to LPS treatment whereas WT mice show an increase in decidual NOS activity when compared to control mice. Again, when compared to WT mice, CB1-*KO* mice seem to mount a feebler inflammatory response to LPS in the decidua which results in a fewer embryo loss. Progesterone was able to down-regulate NO levels in the *ex vivo* culture of uterine tissue, thus exerting anti-inflammatory effects (Aisemberg et al., 2013). In this sense, in our model, the relatively sustained levels of progesterone observed in our LPS-treated CB1-*KO* mice could be responsible for the lack of increased NOS activity in response to the endotoxin.

Collectively, our results suggest a participation of CB1 receptors in the embryotoxic effects of LPS. While the mechanism of action is still unknown, these data suggest that LPS induces a systemic change in eCB signaling ligand production that is regulated in large part by circulating progesterone levels, which in turn regulates the pro-inflammatory response associated with embryo resorption. In spite of the treatment with LPS, CB1-*KO* mice maintain relatively high levels of progesterone, which likely protects embryos by modulating the production of the pro-inflammatory cytokines IL-6 and TNFα (thus reducing the impact on the Th2/Th1 cytokine network) and preventing an increase in NO production, leukocyte infiltration and ultimately allowing embryo maintenance. Taken together, our results point to the CB1 receptor as an important mediator of the embryotoxic effects of LPS.

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Appendix: Supplementary material

Supplementary data to this article can be found online at doi:10.1016/j.mce.2015.04.032.

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