N,N-Dimethylformamide Prevents LPS-Induced Preterm Birth in a Murine Model by Suppressing the Inflammatory Response

Zeng-Hui Wei
St John's University
Oluwabukola Salami
St. John's University
Jagadish Koya
Pharmaceutical Product Development Inc
Swapna Munnangi
Nassau University Medical Center
Ryan Pekson
Albert Einstein College of Medicine
Charles R. Ashby
St John's University
Sandra Reznik (rezniks@stjohns.edu)
St John's University https://orcid.org/0000-0002-6272-7198

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Abstract

Preterm birth accounts for the majority of perinatal mortality worldwide and there remains no FDA-approved drug to prevent it. Recently, we discovered that the common drug excipient, N,N-dimethylacetamide (DMA), prevents inflammation–induced preterm birth in mice by inhibiting NF-κB. Since we reported this finding it has come to light that a group of widely used, structurally related aprotic solvents, including DMA, N-methyl-2-pyrrolidone (NMP) and dimethylformamide (DMF), have anti-inflammatory efficacy. We show here that DMF suppresses LPS-induced TNFα secretion from RAW 264.7 cells and IL-6 and IL-8 secretion from HTR-8 cells at concentrations that do not significantly affect cell viability. *In vivo*, DMF decreases LPS-induced inflammatory cell infiltration and expression of TNFα and IL-6 in the placental labyrinth, all to near baseline levels. Finally, DMF decreases the rate of preterm birth in LPS-induced pregnant mice (P<.0001) and the rate at which pups are spontaneously aborted (P<.0001). In summary, DMF, a widely used solvent structurally related to DMA and NMP, prevents LPS-induced preterm birth in a murine model without overt toxic or teratogenic effects. Re-purposing the DMA/DMF/NMP family of small molecules as anti-inflammatory drugs is a promising new approach to preventing inflammation–induced preterm birth and potentially other inflammatory disorders as well.

Introduction

Preterm birth (PTB) is the leading cause of neonatal morbidity and mortality worldwide.[1,2] Fifteen million babies are born preterm each year and among industrialized nations, the United States has the highest rate of PTB.[2] Children born prematurely have an increased risk of developing neurological, respiratory and metabolic abnormalities,[3] including metabolic syndrome.[4,5] Although spontaneous PTB results from multiple causes, including oxidative stress[6], dysbiosis[7,8] and secretion of exosomes[9,10], inflammation, whether in the absence or presence of infection, accounts for most cases of spontaneous PTB.[11] Unfortunately, there is only one United States Food and Drug Administration approved drug to prevent PTB, hydroxyprogesterone caproate,[12, 13] which produces variable results and is not indicated for women in active labor[14]. Furthermore, this drug is ineffective in the following types of patients: 1) women with multiple gestation pregnancies,[14] 2) obese women[15, 16] and 3) African American women.[17, 18] Groups 2 and 3 are particularly concerning, because one in five pregnant women will be overweight by 2025[19] and African Americans have the highest rate of PTB.[20]

We and others have recently discovered that the common drug excipient, N,N-dimethylacetamide (DMA), and related compounds, have anti-inflammatory efficacy.[21–24] We were the first to report that DMA inhibits nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) nuclear translocation and protects nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα) from degradation[21]. Although it has been reported that DMA attenuates osteoporosis[23] and enhances bone healing impeded by inflammation[24] in *in vivo* models, we were the first to demonstrate that DMA has tocolytic activity. Interestingly, while our previous results indicated that monomethylacetamide (the primary metabolite of DMA) has no anti-inflammatory efficacy, [22] N,N-dimethylformamide (DMF), which

substitutes a hydrogen atom for one of the N-methyl substituents, is a novel cytokine suppressive anti-inflammatory compound, similar to DMA. In this work, we conducted experiments to determine if DMF has tocolytic as well as anti-inflammatory efficacy.

Methods

Cell viability assays

RAW 264.7 cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained in Dulbecco's Modified Eagle Medium supplemented with 10% FBS and 1% penicillin-streptomycin as described.[22] HTR-8/SVneo cells (HTR-8 cells) were kindly donated by Dr. Charles Graham (Queens's University, Ontario, Canada) and grown in Roswell Park Memorial Institute (RPMI) medium (Cellgro, Corning, NY) supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were maintained at 37^{o}C and 5% CO_2 and allowed to grow to 80-90% confluency before being sub-cultured or used in experiments. Cells were seeded at 20,000 cells per well in 96-well-plates and pre-incubated with N,N-dimethylformamide (DMF, Sigma-Aldrich, St. Louis, MO) (0.1 to 40 mM) for 2 h and then incubated with 1 ug/ml of LPS (*Escherichia coli* 026:B6, Sigma, St. Louis, MO) for 24 h. At the end of the 24 h incubation, 3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT, Alfa Aesar, Ward Hill, MA) was added to each well at a final concentration of 0.5 mg/ml. After incubation for 2 h at 37^{o}C and 5% CO_2, the media was aspirated and dimethyl sulfoxide (DMSO) (100 µl/well) (BDH, Randor, PA) was added to dissolve formed purple formazan crystals. The plates were shaken on an orbital microplate shaker for 10 min to ensure complete solubilization of the crystals and the absorbance of the resulting purple solution was measured at 570 nm using an Opsys MR microplate reader (Dynex Technologies, Chantilly, VA). Three independent experiments were performed in duplicate for each cell line.

Enzyme linked immunosorbent assay

RAW 264.7 and HTR-8 cells were grown in the absence or presence of DMF (0.1 to 30 mM) for 2 h and then incubated with LPS (1 µg/ml) for another 24 h. The levels of tumor necrosis-alpha (TNFα), interleukin (IL)-6, IL-8 and monocyte chemoattractant protein (MCP)-1 in the supernatants were determined using Ready-SET-Go! sandwich ELISA kits (eBioscience, San Diego, CA) as per the manufacturer's protocol. The ELISA for each experimental sample was performed in duplicate. Three independent experiments were performed in duplicate for each cytokine.

In vivo **model**

Eight-week-old C57Bl/6 mice were purchased from Taconic Biosciences (Rensselaer, NY) and housed at 23 ± 1°C and humidity of 50 ± 10% in the vivarium on a 12:12 h light/dark cycle. All experimental protocols were approved by the St. John's University Institutional Animal Care and Use Committee (IACUC) and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

A total of 21 timed pregnant dams at E15.5 were randomly divided into four groups: 1) the LPS group (n=8), which were injected intraperitoneally (i.p.) with 100 µL of phosphate buffered saline (PBS) at t = -15 min and 10 h and with 50 mg/kg LPS (serotype 026:B6) at t = 0; 2) the DMF rescued group (n = 7), which were injected i.p. with 2.6 g/kg DMF at t = -15 min and 10 h and with 50 mg/kg LPS at t = 0; 3) the sham group (n = 4), which were injected i.p. with 100 µL of PBS at t = -15 min and 10 h and with 500 µL of PBS at t = 0; and 4) the DMF control group (n = 2), which were injected with 2.6 g/kg DMF at t = -15 min and 10 h and with 500 µL of PBS at t = 0. (See Supplementary Table 1 for a summary of the mouse groups). In accordance with our IACUC's regulations, the mice were sacrificed within 2 h of delivery and the mice that had not gone into labor were euthanized at t = 24 h. All mice were necropsied to confirm pregnancy and the retained pups were counted and examined.

Histological analysis

One placenta was randomly selected from each dam, fixed in formalin, embedded in paraffin, sectioned at 4 µm, and stained with hematoxylin and eosin (H & E) as previously described.[21, 25, 26] Sections were examined under a Nikon Eclipse 80i light microscope (Nikon Inc., Melville, NY) by three blinded observers (OOS, SM and SER), one of whom is a practicing placental pathologist (SER). Sections were graded for degree of inflammatory cell infiltration on a four-point scale (Grades 0-3). Sections were initially scanned at a magnification of 100x to identify the three most active areas of inflammation. Each of those areas was then observed at 400x and graded based on the number of inflammatory cells per field as follows: Grade 0 = 0-5 cells, Grade 1 = 6-50 cells, Grade 2 = 51-100 cells and Grade 3 = more than 100 cells.

Immunohistochemistry

The semi-quantitative analysis and immunolocalization of TNFα and IL-6 in formalin-fixed paraffin embedded mouse placental tissue sections was carried out using DAKO-Labeled Streptavidin-Biotin 2 System and horseradish peroxidase (LSAB2-system HRP, Dako, Carpinteria, CA), as previously described.[25, 27] Tissues were rehydrated and were immersed in citrate buffer (8.2 mmol/L sodium citrate and 1.8 mmol/L citric acid, pH 6.0, containing 0.01% Triton X-100), heated to 95^{o}C - 98^{o}C for 10 min and then cooled to room temperature to achieve antigen retrieval. Tissues were incubated in 3% hydrogen peroxide for 10 min to saturate native peroxidases and washed in PBS (Sigma-Aldrich) for 3 min. The sections were incubated in 1% bovine serum albumin in a humidified chamber for 90 min to reduce non-specific binding, and then incubated overnight at 4^{o}C with antibodies directed against TNFα or IL-6 (Sigma-Aldrich) diluted 1:100 in 1% BSA. The tissue sections were then washed in PBS for 10 min and incubated with secondary antibody biotinylated link for 1 h at room temperature in the humidified chamber, washed in Tris buffered saline-Tween and incubated with Streptavidin-horse radish peroxidase for 10 min with subsequent washing in PBS for 3 min. The slides were developed using 3,3'-diaminobenzidine. The sections were observed under a Nikon Eclipse 80i light microscope at 400X magnification and images were captured with SPOT Advanced Software and analyzed with Image-Pro Premier software (Media Cybernetics, Rockville MD).

Statistical analysis

The MTT assay and ELISA data were analyzed with one-way analysis of variance (ANOVA) and Dunnett's test for post-hoc analysis. The significance of the effect of DMF on the number of dams delivering prematurely and on the number of pups being prematurely delivered was determined using the Chi square test and Fisher's exact test, respectively. The effect of DMF on dams delivering preterm, pups being delivered preterm and pup survival over the 24-h experimental period was analyzed using log rank test (Mantel Cox). Differences in inflammatory cell infiltration in placentas harvested from the different mouse groups were analyzed using the Mann-Whitney U test, along with Tukey's multiple comparison *post hoc* analysis, using GraphPad Prism. The optical density of the immunohistochemical staining was determined by DAB analysis Image-Pro Premier software and these data were exported to GraphPad Prism for statistical analysis using ANOVA and Tukey's post test.

Results

DMF inhibits cytokine secretion from RAW 264.7 and HTR-8 cells

We first determined if DMF inhibited the secretion of an acute phase inflammatory cytokine in LPS stimulated RAW 264.7 cells. No loss of cell viability was detected in RAW 264.7 cells incubated with concentrations of DMF from 0.1 to 30 mM (Supplementary Figure 1, A); therefore, concentrations of DMF up to 30 mM were used in this assay. As previously reported, LPS (1 µg/ml) increased the production of TNFα in RAW 264.7 cells approximately 18-fold (P<.0001, Figure 1, A). This effect was partially prevented by DMF in a concentration-dependent manner, with decreases in TNFα secretion of 19% (P<.01) and 30% (P<.001) in cells incubated with 20 and 30 mM DMF, respectively (Figure 1, A). Interestingly, DMF was at least as effective in reducing TNFα secretion as NF-kB inhibitor BAY 11-7082, which was used as a positive control.

As placental trophoblast cells play an important role in inflammatory signaling at the maternal fetal interface,[28] we next assessed DMF's effect on cytokine secretion in LPS-stimulated placental trophoblast HTR-8 cells. No loss of cell viability was detected in HTR-8 cells incubated with concentrations of DMF from 0.1 to 10 mM (Supplementary Figure 1, B); therefore, concentrations of DMF up to 10 mM were used in this assay. LPS, at 1 µg/ml, increased IL-6 secretion in HTR-8 cells more than 4-fold (P<0.001, Figure 1, B). DMF (0.1 to 10 mM) attenuated the LPS induced increase in IL-6 in a concentration-dependent manner, with 10 mM DMF reducing IL-6 secretion by more than 40% (P<.05, Figure 1, B). The same concentration of LPS increased IL-8 secretion more than 8-fold in HTR-8 cells (Figure 1, C). DMF attenuated this LPS-induced increase in IL-8 in a concentration-dependent manner as well, with 10 mM DMF reducing IL-8 secretion by 30% (P<.01, Figure 1, C). Finally, LPS increased MCP-1 secretion in HTR-8 cells approximately 5-fold and DMF produced a concentration-dependent downward trend in MCP-1 secretion (Figure 1, D). NF-κB inhibitor BAY 11-7082 was used as a positive control in all experiments (Figure 1, A-D).

DMF attenuates LPS induced inflammatory cell infiltration of the placental labyrinth

Given DMF's effect on LPS-induced cytokine secretion *in vitro*, we hypothesized that it would decrease LPS-driven inflammatory cell infiltration *in vivo* in mouse placenta. Microscopic examination indicated a marked inflammatory cell infiltrate in the placental labyrinth in the LPS group compared to the sham group (P<.0001, Figure 2, B-D). This response, however, was significantly attenuated in placentas from the DMF rescued group (P<.0001, Figure 2, A, B and D).

DMF attenuates LPS-induced increases in TNFα and IL-6 in the placental labyrinth

Immunohistochemical staining for TNFα in the placental labyrinth was increased almost four-fold in the LPS group as compared to the sham group (P<.0001, Figure 3, B-D). In contrast, immunohistochemical staining for TNFα was significantly reduced in the DMF rescued group compared to the LPS group (P<.0001, Figure 3, A. B and D) and, in fact, was similar to basal levels seen in the sham group (Figure 3, A, C and D). Similarly, immunohistochemical staining for IL-6 was increased approximately 20-fold in the LPS group compared to the sham group (P<.0001, Figure 4, B-D), whereas IL-6 was reduced by approximately 15-fold in the DMF rescued group compared to the LPS group (P<.0001, Figure 4, A, B and D).

DMF rescues mice from LPS-induced preterm birth

Based on the *in vitro* and *in vivo* histologic data described above, and on the structural similarity between DMF and DMA, we hypothesized that DMF would rescue LPS challenged mice from developing preterm delivery. All of the mice in the LPS group (n = 8) delivered within 24 h with a mean delivery time of t = 11 h and 35 min (Table 1, Figure 5). Only 1 out of the 7 mice in the DMF rescued group, however, delivered within the 24 h experimental period (at t = 10 h), reflecting DMF's significant effect on PTB risk in our *in vivo* model (100% vs. 14.3%, P<.05, Table 1). As expected, none of the mice in the sham and DMF control groups delivered preterm. DMF had an even more significant effect on pups at risk of spontaneous abortion. While 38 of 60 (63.3%) pups were delivered within 24 h in the LPS group, all of which were non-viable, only 6 out of 53 (11.3%) pups were spontaneously aborted in the DMF rescued group (P<.0001, Table 1). The effect of DMF on LPS challenged mice developing preterm labor and spontaneous abortions over time is shown in Figure 5. Based on analysis by log-rank test (Mantel Cox), DMF has a significant effect on the rate of dams developing PTB (P<.0001, Figure 5, A), the rate of pups being spontaneously aborted (P<.0001, Figure 5, B) and, as illustrated by the Kaplan-Meier survival curve, the rate of pups being rescued (P<.0001, Figure 5, C). A total of 150 pups were involved in this study, 63 of which were exposed to DMF. There were no overt teratogenic effects of DMF and no macroscopic differences observed between pups exposed or unexposed to DMF.

Table 1

DMF prevents preterm birth and rescues pups from preterm delivery. The asterisk indicates P<.05 and the quadruple asterisks indicate P<.0001.

GROUPS	LPS	DMF Rescue	DMF Control	Sham
Number of dams injected	8	7	2	4
Number of dams that delivered within 24 h	8 (100%)	1* (14.3%)	0 (0%)	0 (0%)
Total number of pups	60	53	14	23
Number of pups delivered within 24 h	38 (63.3%)	6**** (11.3%)	0 (0%)	0 (0%)

Comment

Principal Findings: In this report, using both *in vitro* and *in vivo* approaches, we show that DMF significantly decreases LPS-induced cytokine secretion from both macrophages and placental trophoblasts, cytokine expression in the placental labyrinth and immune cell infiltration at the maternal-fetal interface. Finally, we show here, for the first time, that DMF prevents inflammation-induced preterm birth and rescues pups from spontaneous abortion without producing overt toxic effects in the mother or overt teratogenic effects in the fetuses. DMF's anti-inflammatory and tocolytic efficacies are, in fact, similar to those of DMA.[21, 22]

Results (in the context of what is known): Recent studies have shown that the aprotic solvents, DMA and NMP, which are structurally related to DMF, have anti-inflammatory efficacy.[21–24] We were the first to show that DMA inhibits NF-κB *in vitro* and prevents inflammation driven PTB in an *in vivo*.[21] In the work presented here, we show that the closely related aprotic solvent, DMF, has anti-inflammatory efficacy by decreasing the levels of the cytokines TNFα, IL-6 and IL-8 and, specifically, prevents inflammation-induced PTB *in vivo*.

Clinical Implications: As there are currently no FDA approved drugs to arrest preterm labor, the discovery of a new anti-inflammatory compound that prevents inflammation-driven birth is of significant clinical importance. Apart from its important effect on PTB risk, DMF's ability to decrease the levels of inflammatory cytokines has an additional significant benefit for the fetus, namely the prevention of fetal inflammatory response syndrome, which has been associated with elevated levels of IL-6.[29] The elevated IL-6 levels in inflammatory response syndrome can result in several types of injury to the developing fetal brain, including cytokine-induced periventricular leukomalacia,[30] hippocampal inflammation[31] and dysregulation of hippocampal glutamatergic homeostasis.[31] The fact that no overt toxic effects were observed in the dams and no overt teratogenic effects were observed in the pups suggests that DMF is a

promising candidate for a novel tocolytic drug. Further toxicity studies followed by clinical trials are warranted to fully assess the efficacy and safety of this molecule.

Research Implications: We and others have described various mechanisms of action of DMA and NMP, which are structurally related to DMF, including inhibition of NF-κB,[21–23] inhibition of the mitogen-activated protein kinase pathway[24] and inhibition of gene transcription by acting as bromodomain ligands.[23, 24] The precise mechanism of action of DMF is yet to be elucidated.

Strengths and Limitations: This study has several important strengths. First, it addresses the urgent need for novel pharmacologic approaches to PTB, a serious clinical problem with no effective treatment.[32–34] Second, it includes both *in vitro* and *in vivo* methods to test our hypothesis. Third, it adds to the growing body of evidence that the DMA/DMF family of aprotic solvents has anti-inflammatory properties of potential clinical importance. Fourth, it shows that DMF's desirable anti-inflammatory properties are present at concentrations below cytotoxic levels *in vitro* and below toxic or teratogenic levels *in vivo*. A weakness of this study is that, due to IACUC restrictions, the effect of DMF on LPS-challenged mice was only followed for 24 h.

Conclusions: Inflammation has been implicated in a wide range of human disorders, including cardiovascular disease,[35, 36] cancer,[37–40] neurodegenerative disorders[41–43] and of course PTB.[11] While pharmacotherapy exists for some of these medical challenges, many clinical needs related to inflammation remain unmet. In the United States, apart from inflammation resulting from infection, sterile inflammation is driven by dietary, environmental and even social factors. In fact, we have recently shown that consumption of a high fat diet during pregnancy activates the oxidative stress/inflammatory axis[8, 26] and leads to increased rates of PTB in rodent models. The DMA/DMF family of aprotic solvents may represent a novel, safe, accessible and affordable approach to treat inflammation driven preterm birth as well as other disorders resulting from inflammation for which existing drug therapy has not been fully efficacious.

Declarations

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AVAILABILITY OF DATA AND MATERIALS

The data that support the findings in this study are available from the corresponding author upon reasonable request.

DECLARATIONS

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

Z.-H. W. and O.O.S. performed all of the experiments and contributed equally to this manuscript. J.K. assisted in the performance of the *in vitro* experiments. S.M. assisted in the performance of the *in vivo* experiments. R.P. assisted with the statistical analyses. C.R.A. contributed to the original idea of the project and assisted with the writing of the manuscript. S.E.R. contributed to the original idea of the project, wrote the manuscript and provided funding.

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Figures

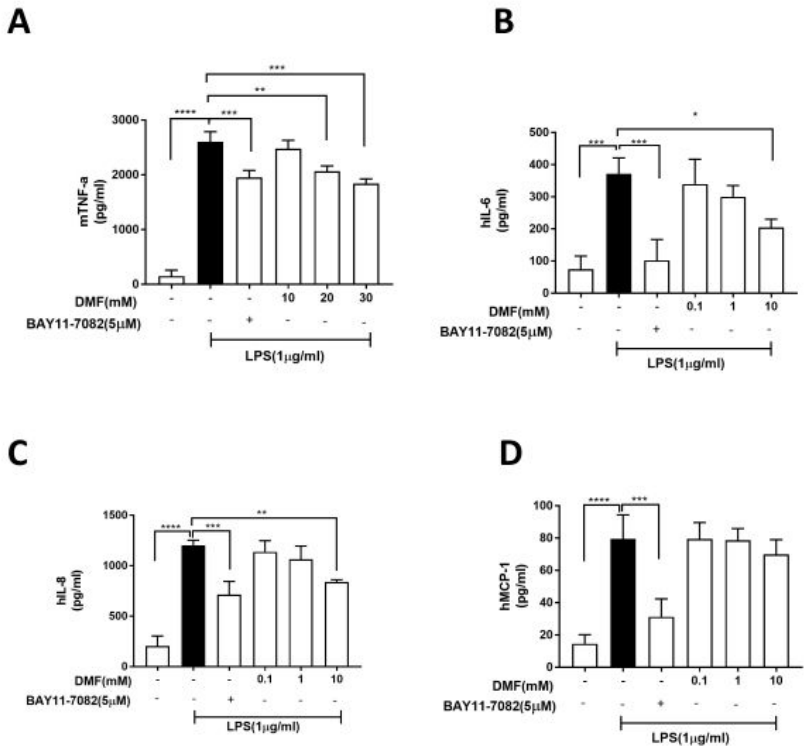

Figure 1

DMF suppresses cytokine secretion in vitro A, RAW 264.7 cells or B-D, HTR-8 cells were stimulated with 1 μg/mL of LPS for 24 h in the absence or presence of various concentrations of DMF, which was added to the media 2 h before the LPS. Concentrations of A, TNFα; B, IL-6; C, IL-8 and D, MCP-1 in the media were determined by ELISA. BAY 11-7082 (5 μM) was included as a positive control. The asterisk indicates P<.05; the double asterisks indicate P<.01; the triple asterisks indicate P<.001; the quadruple asterisks

indicate P<.0001. DMF, N,N-dimethylformamide; HTR-8, HTR-8/SVNeo; LPS, lipopolysaccharide; TNFα, tumor necrosis factor alpha; IL-6, interleukin-6; IL-8, interleukin-8; MCP-1, monocyte chemoattractant protein-1; ELISA, enzyme linked immunosorbent assay.

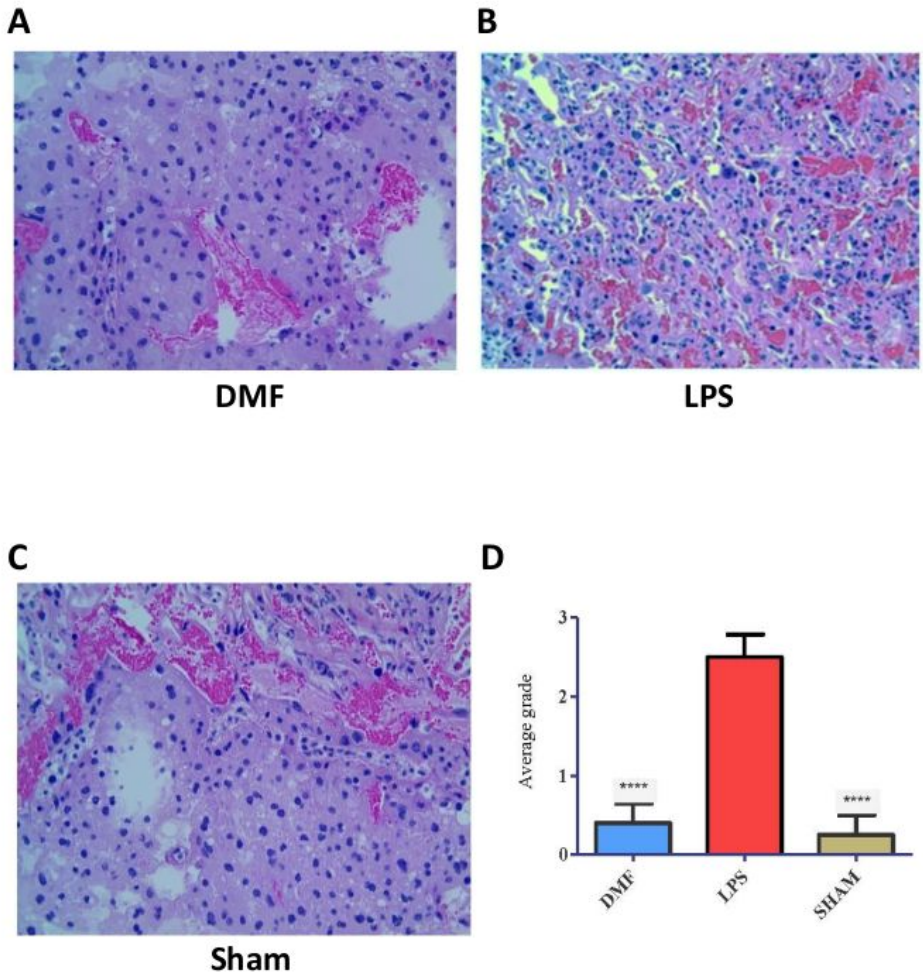

Figure 2

DMF suppresses inflammatory cell recruitment to the mouse placental labyrinth One placenta per litter was randomly selected for histology from each of the A, DMA rescued (n=7); B, LPS (n=8); and C, sham

(n=4) E16 dams. A-C, Representative H & E sections from each of the groups. D, Sections were graded for degree of inflammatory cell infiltration on a four-point scale (Grades 0-3). Original magnification 400X. The quadruple asterisks indicate P<.0001, as compared to the LPS challenged group. DMF, N,N-dimethylformamide; LPS, lipopolysaccharide.

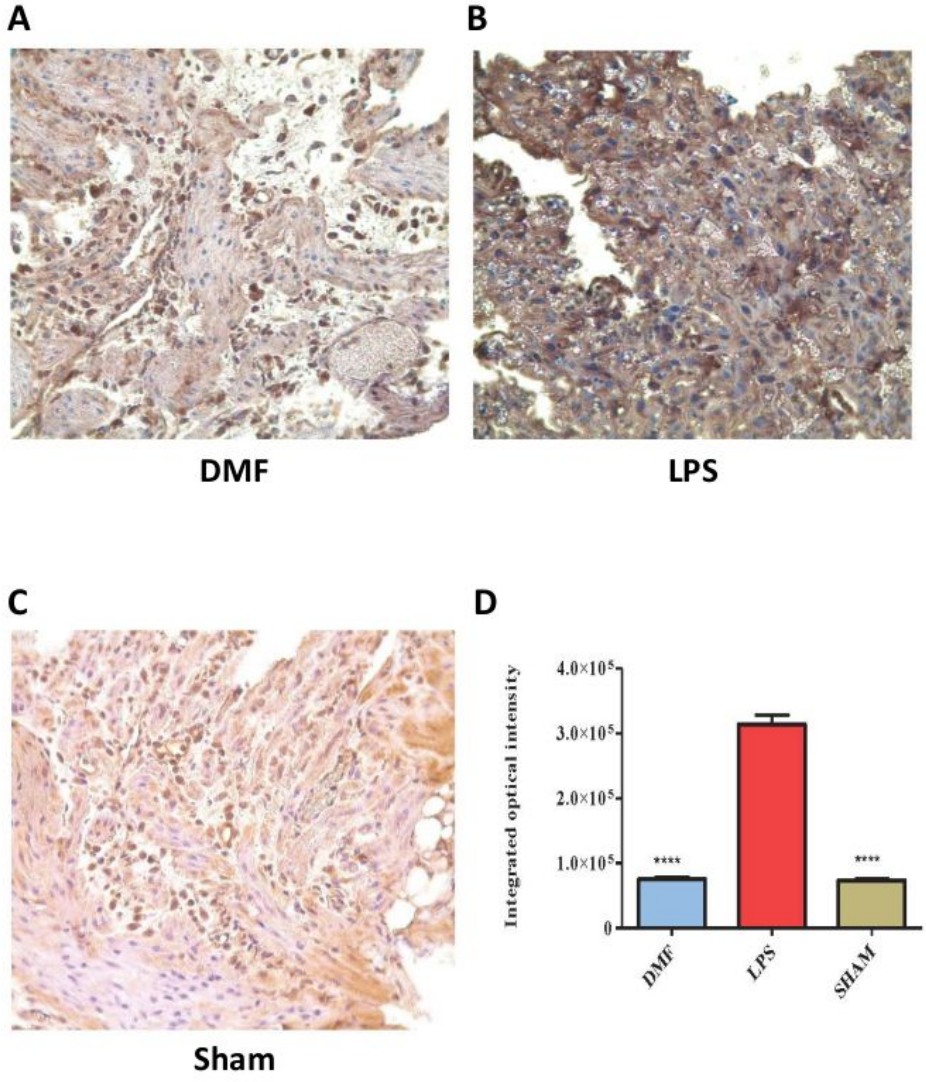

Figure 3

DMF attenuates TNFα expression in the mouse placental labyrinth One placenta per litter was randomly selected for immunohistochemistry from each of the A, DMA rescued (n=7); B, LPS (n=8); and C, sham (n=4) E16 dams. A-C, Representative sections reacted with primary TNFα antibodies from each of the groups. D, Staining intensity in the various groups, analyzed with Image-Pro Premier software. Original magnification 400X. The quadruple asterisks indicate P<.0001, as compared to the LPS group. DMF, N,N-dimethylformamide; TNFα, tumor necrosis alpha; LPS, lipopolysaccharide.

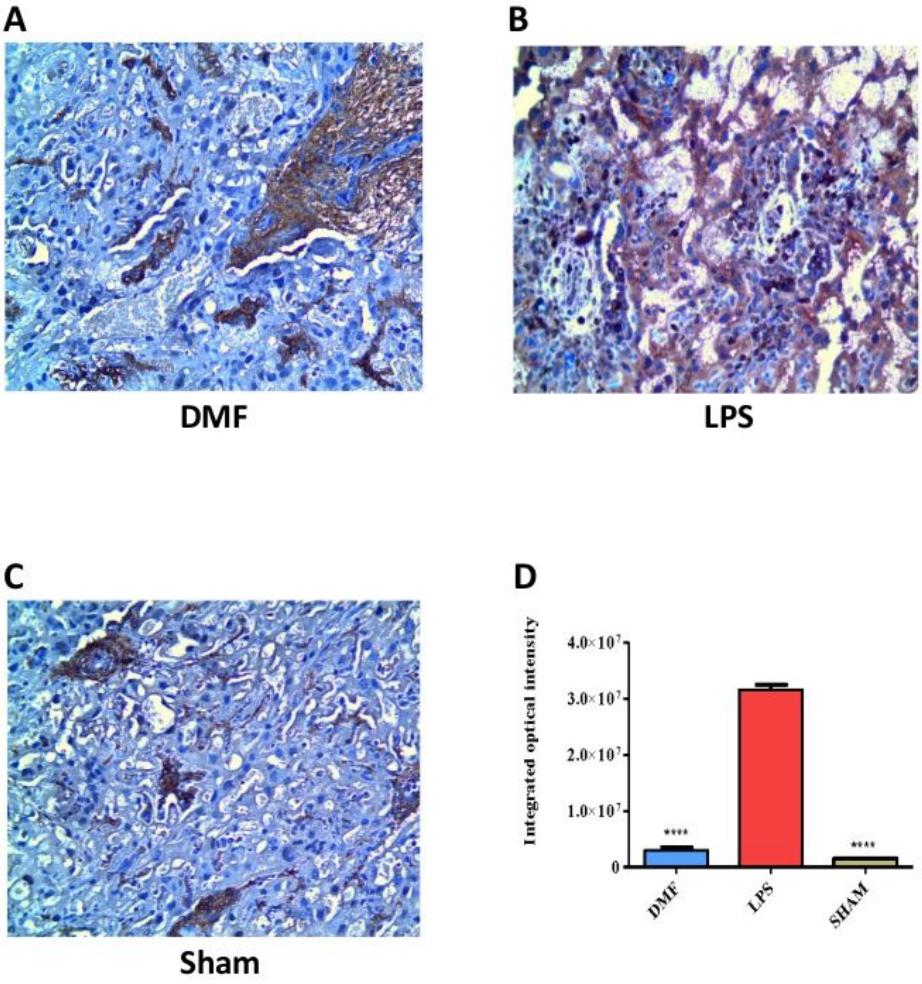

Figure 4

DMF attenuates IL-6 expression in the mouse placental labyrinth One placenta per litter was randomly selected for immunohistochemistry from each of the A, DMA rescued (n=7); B, LPS (n=8); and C, sham (n=4) E16 dams. A-C, Representative sections reacted with primary IL-6 antibodies from each of the groups. D, Staining intensity in the various groups, analyzed with Image-Pro Premier software. Original magnification 400X. The quadruple asterisks indicate P<.0001, as compared to the LPS group. DMF, N,N-dimethylformamide; IL-6, interleukin-6; LPS, lipopolysaccharide.

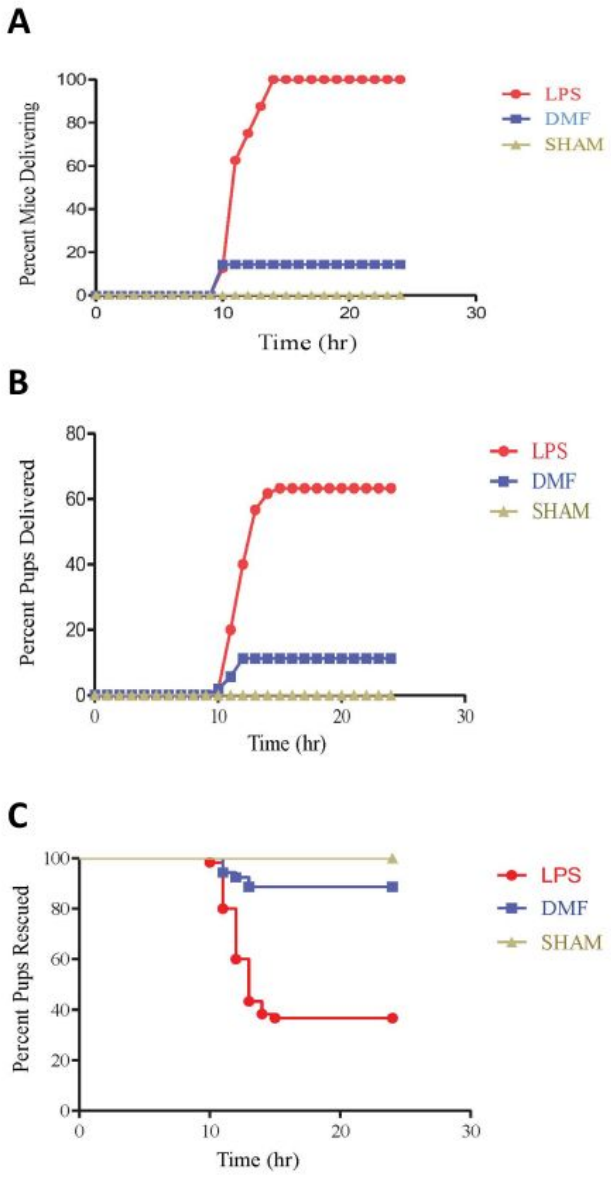

Figure 5

DMF rescues mice from inflammation driven preterm birth DMF reduces A, the rate of LPS challenged E15.5 mice delivering within 24 h (P<.0001) and B, the rate of pups being spontaneously aborted in LPS challenged E15.5 mice within 24 h (P<.0001). C, The Kaplan-Meier plots show DMF's ability to increase survival of pups in LPS-challenged mice over 24 h (P<.0001). DMF, N,N-dimethylformamide; LPS, lipopolysaccharide.