

Research Article

# Impact of gestational nicotine exposure on intrauterine and fetal infection in a rodent model<sup>†</sup>

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## Abstract

We investigated the interaction between prenatal nicotine exposure and intrauterine infection using established rat models. Beginning at gestation day (GD) 6, dams were continuously infused with either saline or 6 mg/kg/day nicotine (Nic). At GD 14, dams received either sterile broth or 10<sup>5</sup> colony-forming units *Mycoplasma pulmonis* (MP), resulting in four treatment groups: control (4 dams, 33 fetal units); MP only (5 dams, 55 fetal units); Nic only (5 dams, 61 fetal units), and Nic + MP (7 dams, 82 fetal units). At GD 18, nicotine exposure significantly increased ( $P \leq 0.02$ ) the percentage of amniotic fluids and fetuses infected by MP but did not impact colonization rates of maternal sites. Nicotine exposure significantly reduced the numbers of MP in the placenta required for high microbial loads ( $\geq 10^4$  color-changing units) in the amniotic fluid ( $P < 0.01$ ). Fetal inflammatory response lesions were most extensive in the Nic only and Nic + MP groups ( $P < 0.0001$ ). Control and MP only placentas were interleukin (IL)10-dominant, consistent with an M2/Th2 environment. Placentas exposed to nicotine shifted to a neutral environment, with equivalent levels of interferon gamma (IFNG) and IL10. Both IL6 and tumor necrosis factor (TNF) levels in amniotic fluid were highly elevated when both nicotine and infection were present. Our study suggests that prenatal exposure to nicotine increases the risk for intrauterine infection, lowers the infectious dose required to breach the placental barrier and infect the amniotic fluid and fetus, and alters the pathology and inflammatory profile associated with maternal and fetal sites.

## Summary Sentence

A modifiable risk factor (nicotine) can directly impact what has been considered an immutable risk factor (infection) resulting in increased risk of pathogen transmission across the placental barrier with subsequent impacts on placental pathology and maternal/fetal immune environment.

**Key words:** nicotine, infection, rodent model, inflammation, placental pathology, pregnancy.

## Introduction

Maternal smoking during pregnancy is a well-established cause of perinatal complications [1–5] including preterm birth. Cigarettes contain a complex assortment of chemicals, but nicotine is one of the main pathogenic components and has the potential to impact placental function [6–9]. Accordingly, smoking cessation strategies are routinely recommended for women during pregnancy [10, 11]. Nicotine replacement therapies (NRTs), such as transdermal patches or gum [12, 13], have been recommended, but safe and effective doses of nicotine exposure via these devices during pregnancy have not been definitively established [14–16]. Electronic nicotine delivery systems (ENDS), including e-cigarettes, are also being promoted for smoking cessation [17, 18]. Unfortunately, ENDS can be perceived as a safe alternative to smoking by the general public [19–24] and are gaining popularity among young reproductive age and pregnant women [20, 21, 24, 25]. Furthermore, the level of nicotine delivered by ENDS increases significantly with user experience and duration of puffing period [26–30], and ENDS which contain higher nicotine concentrations actually result in significantly higher plasma levels of nicotine than conventional cigarettes [31]. Thus, nicotine exposure during pregnancy remains a significant health issue.

Another significant but separate risk factor for perinatal complications and preterm birth is microbial infection of the reproductive tract, both at the time of conception and during pregnancy [32–35]. Infection generally is considered to be an immutable risk factor for perinatal complications while smoking is considered a mutable risk factor (<http://www.nap.edu/catalog/11622.html>) [36]. Most epidemiological studies considered smoking and infection as potential confounders, and therefore excluded either smoking or infection in data analyses. Recent studies [2, 4, 37] considering both risk factors showed that women who smoke are at significantly higher risk for pathologic intrauterine infection, indicating an interaction between these risk factors.

Based on these new emerging data, this study was undertaken to evaluate the prenatal interaction between nicotine exposure and infection at the maternal:fetal interface. We hypothesized that nicotine exposure during pregnancy would lower the microbial threshold required to establish intrauterine and fetal infection. To test our hypothesis, we combined a maternal nicotine infusion model that achieves high levels of plasma nicotine and cotinine without causing fetal loss or changes in birth weight [38–41] with a defined model for intrauterine and fetal infection [42–46]. Here we report that prenatal nicotine exposure does reduce the threshold of placental microbial load required for infection of the amniotic fluid and alters both the pathology and inflammatory profile associated with maternal and fetal sites.

## Materials and methods

### Ethics statement

All experimental procedures involving animals were performed in accordance with the University of Florida Institutional Animal Care and Use Committee-approved protocols. The University of Florida is an AAALAC-accredited institution.

### Mycoplasma preparation and culture

To ensure identical inocula for all experiments, *Mycoplasma pulmonis* strain X-1048 was grown to late logarithmic phase in

SP4 medium, aliquoted, and frozen at  $-80^{\circ}\text{C}$ . For each experiment, the stock culture was thawed and an inoculum dose of  $10^5$  colony-forming units (CFU) per  $0.25\ \mu\text{L}$  was used for inoculation of a rat. Dose concentration was confirmed by both optical density and culture.

### Animals, husbandry, and breeding

Specific pathogen-free (SPF) adult male and female Sprague Dawley rats (CrI:SD; Charles River Laboratories International, Inc., Wilmington, MA) were used.

All animal handling took place within a laminar airflow hood. Animal rooms were maintained on a 12:12 h light:dark cycle. Rats were initially housed in an SPF barrier facility in individually ventilated cages (Microisolator, Lab Products, Inc., Maywood, NJ) and provided ad libitum access to food (LabDiet 5053, Purina Mills International, St Louis, MO) and water. Prior to receiving inoculations, rats were relocated to an ABSL-2 containment facility where they were housed singly in static filter-top cages (Microisolator, Lab Products, Inc., Maywood, NJ). Control animals were housed on separate shelves above the infected groups and were always handled first and always within a biosafety cabinet.

For breeding, females were paired with males overnight and were checked daily for evidence of copulation as determined by either visual observation of a vaginal plug or sperm on vaginal cytology. The morning of plug or sperm detection was considered the beginning of gestation day (GD) 0. Bred females were housed in pairs until surgical implantation of the osmotic pump at GD 6, after which they were housed singly.

### Experimental design and treatment groups

All pregnant dams were randomly assigned to one of four treatment groups: normal saline infusion and sterile broth inoculation (control); nicotine infusion and sterile broth inoculation (Nic only); normal saline infusion and *M. pulmonis* inoculation (MP only); and nicotine infusion and *M. pulmonis* inoculation (Nic + MP).

### Infusion pump implantation

At GD 6, all pregnant dams received a subcutaneous injection of carprofen (5 mg/kg; Rimadyl, Zoetis, Florham Park, NJ) prior to surgery and were anesthetized with 3%–4% isoflurane (Iso-thesia, Abbott Laboratories, Abbott Park, IL) and maintained with 1%–3% isoflurane in 100% oxygen delivered by facemask. A 28-day osmotic minipump (ALZET Osmotic Pump Model 2ML4, DURECT Corp., Cupertino, CA) was inserted subcutaneously via a small incision between the scapulae and the incision was closed with wound clips. The minipump was filled with either sterile saline (control and MP only rats; Baxter Veterinary Supplies, Round Lake, IL) or nicotine tartrate (Sigma-Aldrich, St. Louis, MO) dissolved in sterile saline at a concentration sufficient to deliver 6 mg/kg/day (Nic only and Nic + MP rats). Supplemental heat was provided by insulated heating pads (SnuggleSafe, Lenric C21 Ltd. Littlehampton, West Sussex, UK) during anesthesia and recovery.

### Intravenous inoculation

Based on our previous studies [42–44, 46], GD 14 is the most efficient time point for inducing fetal infection, and intravenous (IV) administration allows for delivery of a known dose at a precise time during gestation. Consequently, at GD 14, dams were re-anesthetized

(isoflurane) as described above, and placed in dorsal recumbency to allow access to the right subclavian vein. After the injection site was cleaned with a sterile 70% ethanol swab, pregnant dams received an IV inoculation via the subclavian vein with either  $10^5$  CFU *M. pulmonis* (MP only and Nic + MP groups) or 0.25 ml of sterile SP4 broth (control and Nic only rats), delivered slowly over 10–15 s. Pressure was applied to the injection site for 30–60 s following withdrawal of the needle.

### Necropsy

Pregnant dams were euthanized at GD 18 by intraperitoneal injection of euthanasia solution (Beuthanasia-D Special, Merck Animal Health, Summit, NJ). A full gross necropsy was performed, including examination of surgical and injection sites. Tissues were collected using aseptic technique to prevent cross-contamination of tissue sites. Broths were incubated in ambient air at 37°C, and checked daily for growth to determine color-changing units (CCU). Maternal spleen, vagina, and endometrial mucosa were swabbed with calcium alginate swabs (Fisherbrand, Thermo Fisher Scientific, Inc., Waltham, MA) for culture. The uterus was opened and each fetus and associated membranes, amniotic fluid, cord, placenta, and attached uterus/mesometrium were treated as individual units for the purposes of sample collection and data analysis. Fetal units were processed identically regardless of treatment group. Each fetal unit was arbitrarily assigned a number. For all units, amniotic fluid was collected aseptically for culture and a portion of the placenta was minced in SP4 medium and serially diluted for culture. Samples from MP only and Nic + MP groups were serially diluted 10-fold in SP4 broth to  $10^{-10}$ ; samples from control and Nic only groups were diluted to  $10^{-3}$ . For odd-numbered fetal units, the fetus was minced for culture, and the placenta with attached uterine wall and fetal membranes were fixed in 10% buffered formalin for histological analysis. Even-numbered fetal units were fixed in 10% buffered formalin with fetus and attached tissues intact. All fixed tissues were transferred to 70% alcohol after 48 h of fixation.

### Histopathology and lesion scoring of placental tissues

Tissues were trimmed and transected so that a cross-sectional view of the placental disk with associated decidua and attached visceral yolk sac, amnion, and umbilical cord was present. Tissues were processed routinely and stained with hematoxylin and eosin. Some fetal units had missing structures that were lost during collection, handling, or processing. Tissue examined included the following areas: choriodecidua (decidual layer between metrial triangle and junctional zone, and decidua/junctional zone at margins of placental disk); subchorion (maternal blood sinuses within the labyrinth zone); chorionic plate (epithelium and stroma); visceral yolk sac (endodermal villi, vitelline vessels, and mesothelium); amnion; chorionic vessels within the plate (lumen and endothelium); lumen and endothelium of umbilical vein and umbilical arteries; and Wharton's jelly, including perivascular stroma. Lesion scores were assigned based on presence of neutrophils, with infiltration of intact neutrophils indicating acute inflammation. Fragmented neutrophils as well as necrotic or degraded cells and connective tissue indicated chronic inflammation. A score of 0–3 was assigned for both acute and chronic inflammation, with 0 indicating little to no inflammation, 1 = mild inflammation, 2 = moderate inflammation, and 3 = severe inflammation. The overall scores for each site were added together for a maximum score of 6. In a few cases where tissue damage was so advanced that neutrophils could not be assessed, a score of 7 was

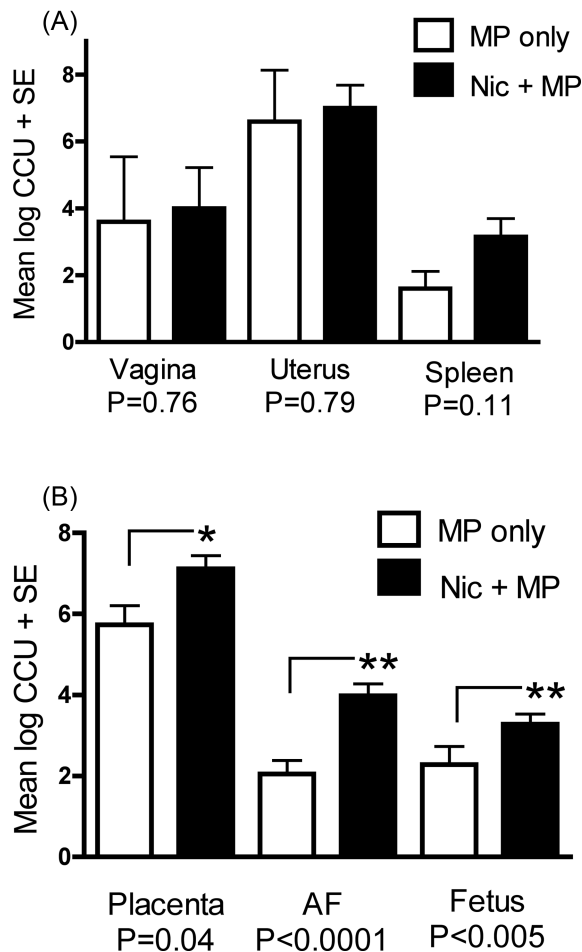
assigned to indicate maximum lesion severity. The maternal inflammatory response (MIR) was obtained by adding the lesion scores for the subchorion and choriodecidua; the fetal inflammatory response (FIR) was obtained by adding the lesion scores for chorionic vessels, umbilical arteries, umbilical vein, and Wharton's jelly. The inflammatory response in the chorionic plate, visceral yolk sac, and amnion tissues was considered as a mixture of both maternal and fetal responses; therefore, these lesion scores were added together to give a score for an inflammatory response potentially involving both maternal and fetal components (MIXED).

### Cytokine analysis

Placental tissues were weighed and placed in a Biomasher II homogenizer (Kimble Chase). One milliliter of lysis buffer (T-PER protein extraction reagent containing HALT protease inhibitor cocktail with EDTA according to the manufacturer's instructions; Pierce Biotechnology, Inc.) per 100 mg of tissue was added and the placental tissue was mechanically homogenized. Tissue homogenates were centrifuged at  $10\,000 \times g$  at 4°C and supernatants were transferred to clean tubes and frozen at –20°C prior to analysis. Placental protein extracts, amniotic fluids, and serum from dams were diluted 1:5 in the Reagent Diluent (R&D Systems) and analyzed for the presence of interferon gamma (IFNG), tumor necrosis factor (TNF), interleukin (IL6), and IL10 using rat DuoSet ELISA kits (R&D Systems). Cytokine values were expressed as pg/mg placental tissue or pg/ml amniotic fluid or serum. Standard curves were constructed using recombinant cytokines provided in the kit, and these curves were included for each assay. For placental homogenates, the recombinant proteins were diluted in Reagent Diluent:T-PER (4:1 vol/vol).

### Data analysis

Culture data were compared between the two infection groups (MP only and Nic + MP) using the Mann-Whitney U test. Categorical data for culture status (positive vs. negative) were compared by Chi Square with Yates correction factor. The relationships of microbial load in placenta, amniotic fluid, and fetus were compared by Spearman  $r$  and regression models were fit to the data. Lesion scores were analyzed by Kruskal-Wallis one-way nonparametric ANOVA followed by the Dunn multiple comparison test. Distribution of severe lesions among treatment groups was assessed by the Fischer exact test. Cytokine data were transformed using  $\log(Y+1)$ , where  $Y$  was an individual data point and analyzed by Kruskal-Wallis one-way nonparametric ANOVA followed by the Dunn multiple comparison test. MP only and Nic + MP groups were subdivided based on infection status (culture-positive or culture-negative) of placenta and amniotic fluid. Transformed cytokine data for these subgroups were analyzed by the unpaired  $t$  test or Kruskal-Wallis one-way nonparametric ANOVA. When indicated, the Dunn test was used for relevant multiple comparisons. As an indicator of the overall M2/Th2 profile in individual placenta and amniotic fluid samples, the IL10 level was subtracted from the IFNG level. A negative value was indicative of a more M2/Th2, IL10-dominant environment; positive values were indicative of a more M1/Th1, IFNG-dominant environment, and values near zero indicated a neutral environment. The IFNG minus IL10 values were analyzed using the Kruskal-Wallis test and Dunn multiple comparison tests between groups. For all analyses, a probability of  $P \leq 0.05$  was considered significant.



**Figure 1.** Prenatal exposure to nicotine increased microbial load in fetal but not maternal tissues. (A) Microbial load in maternal tissues was not significantly impacted by nicotine exposure. (B) Microbial load in the fetal unit [placenta, amniotic fluid (AF), and fetus] was significantly increased with maternal exposure to nicotine. Data are expressed as the mean log CCU + SE.  $P$  value < 0.05 was considered significant. *Mycoplasma pulmonis* was never isolated from any control or Nic only rats.

## Results

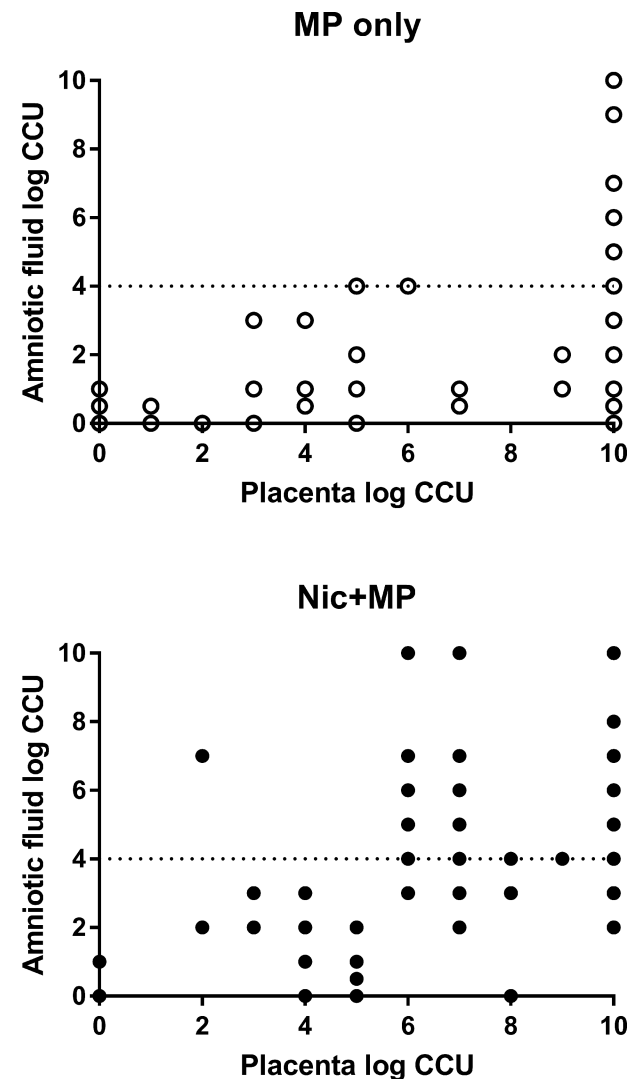
### Nicotine exposure increases risk of infection in the fetal compartment (Figure 1, Table 1)

The mean, standard deviation, and minimum-maximum for litter sizes for the treatment groups were control,  $11.5 \pm 3.3$ , 5–16; MP only,  $12.6 \pm 0.9$ , 12–14; Nic only,  $11.0 \pm 3.1$ , 9–16; and Nic + MP,  $11.9 \pm 2.8$ , 9–16. Three control dams (fetal units = 33), four Nic only dams (fetal units = 36), five MP only dams (fetal units = 63), and seven Nic + MP dams (fetal units = 83) were used for data analysis. Exposure to nicotine (Nic + MP) did not alter the percent colonization of placenta or any of the maternal tissues (vagina, endometrial surface, spleen) when compared to MP only treatment; however, nicotine exposure did significantly increase the percentage of infected fetuses and amniotic fluids (Table 1). Although the percentage of placentas colonized by *M. pulmonis* was not influenced by nicotine exposure, the numbers of *M. pulmonis* (microbial load) recovered (Figure 1) from culture-positive placentas in the Nic + MP group was higher compared to the MP only group ( $P = 0.04$ ). The microbial load in the amniotic fluids ( $P = 0.0001$ ) and fetuses

**Table 1.** Nicotine did not impact the percentage of maternal sites colonized by *M. pulmonis*.

Site	MP only	Nic + MP	$P$ value
Uterus	5/5 (100%)	7/7 (100%)	NA
Placenta	53/61 (87%)	74/79 (94%)	0.28
Amniotic fluid	43/57 (75%)	73/80 (91%)	<b>0.02</b>
Fetus	22/28 (79%)	36/36 (100%)	<b>0.01</b>
Spleen	4/5 (80%)	7/7 (100%)	0.86
Vagina	3/5 (60%)	7/7 (100%)	0.29

Statistically significant differences (Chi Square with Yates correction factor) are shown in bold. All fetal units had placental and amniotic fluid cultures performed; however, fetal cultures were available only for a subset of the total fetal units. *Mycoplasma pulmonis* was never isolated from any control or Nic only rats.



**Figure 2.** Correlation of microbial load in the placenta with microbial load in the amniotic fluid. Rats without nicotine exposure (MP only) attained levels of infection > log 4 CCU (dotted line) only when the placental load was ≥ log 10 CCU. Rats with nicotine exposure (Nic + MP) attained levels of infection > log 4 CCU (dotted line) when the placental load was ≥ log 6 CCU. Results are expressed as the log CCU recovered. Each data point represents an individual fetal unit. *Mycoplasma pulmonis* was never isolated from any control or Nic only rats.

**Table 2.** Relationship of fetal culture status to presence of *M. pulmonis* in the placenta and amniotic fluid.

Fetus positive <sup>a</sup>	MP only (n = 22)	Nic + MP (n = 33)
PL (+) AF (+) <sup>b</sup>	19/22 (86.5%)	32/33 (97%)
PL (+) AF (-)	2/22 (9%)	1/33 (3%)
PL (-) AF (+)	0/22 (0%)	0/33 (0%)
PL (-) AF (-)	1/22 (4.5%)	0/33 (0%)
Fetus negative	MP only (n = 5)	Nic + MP (n = 0)
PL (+) AF (+)	3/5 (60%)	0
PL (+) AF (-)	1/5 (20%)	0
PL (-) AF (+)	0/5 (0%)	0
PL (-) AF (-)	1/5 (20%)	0

<sup>a</sup>Fetal colonization was more likely ( $P < 0.05$ ) to occur with nicotine exposure (MP only, 81.5% (22/27) culture-positive fetuses; Nic + MP, 100% (33/33) culture-positive fetuses).

<sup>b</sup>Fetal colonization was most likely ( $P < 0.01$ ) to occur when both the placenta and amniotic fluid were culture positive, regardless of treatment group.

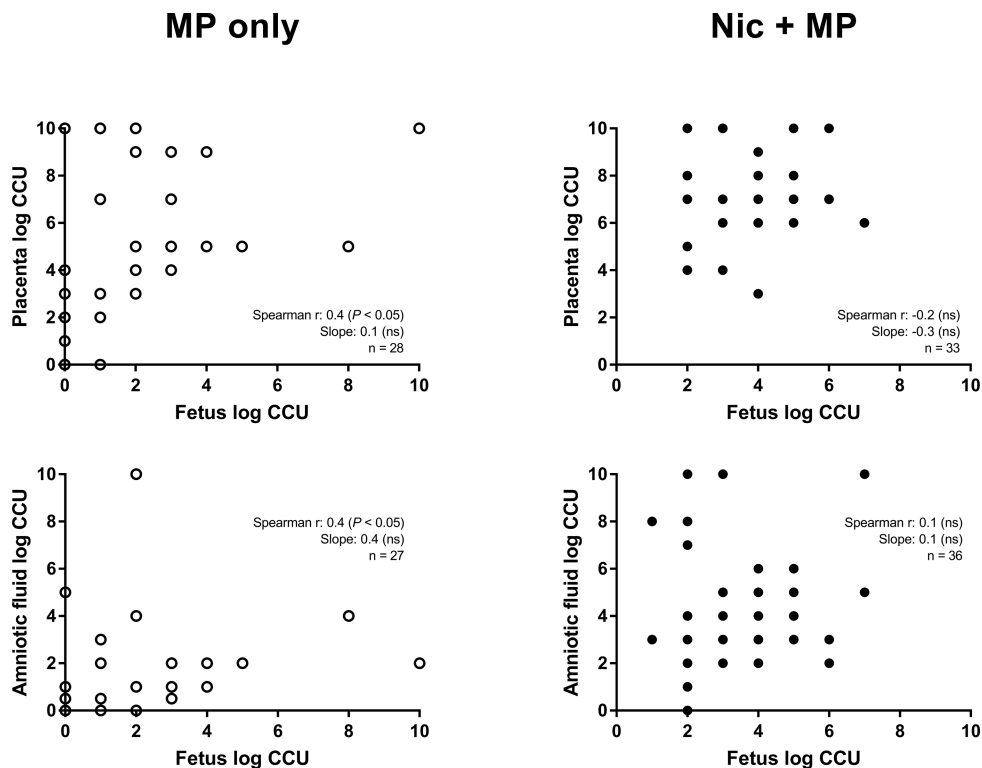
( $P = 0.005$ ) also was higher in Nic + MP rats compared to the MP only group. *Mycoplasma pulmonis* was never isolated from any Control or Nic only rats.

### Nicotine exposure decreases the placental threshold associated with increased microbial load in amniotic fluid (Figure 2)

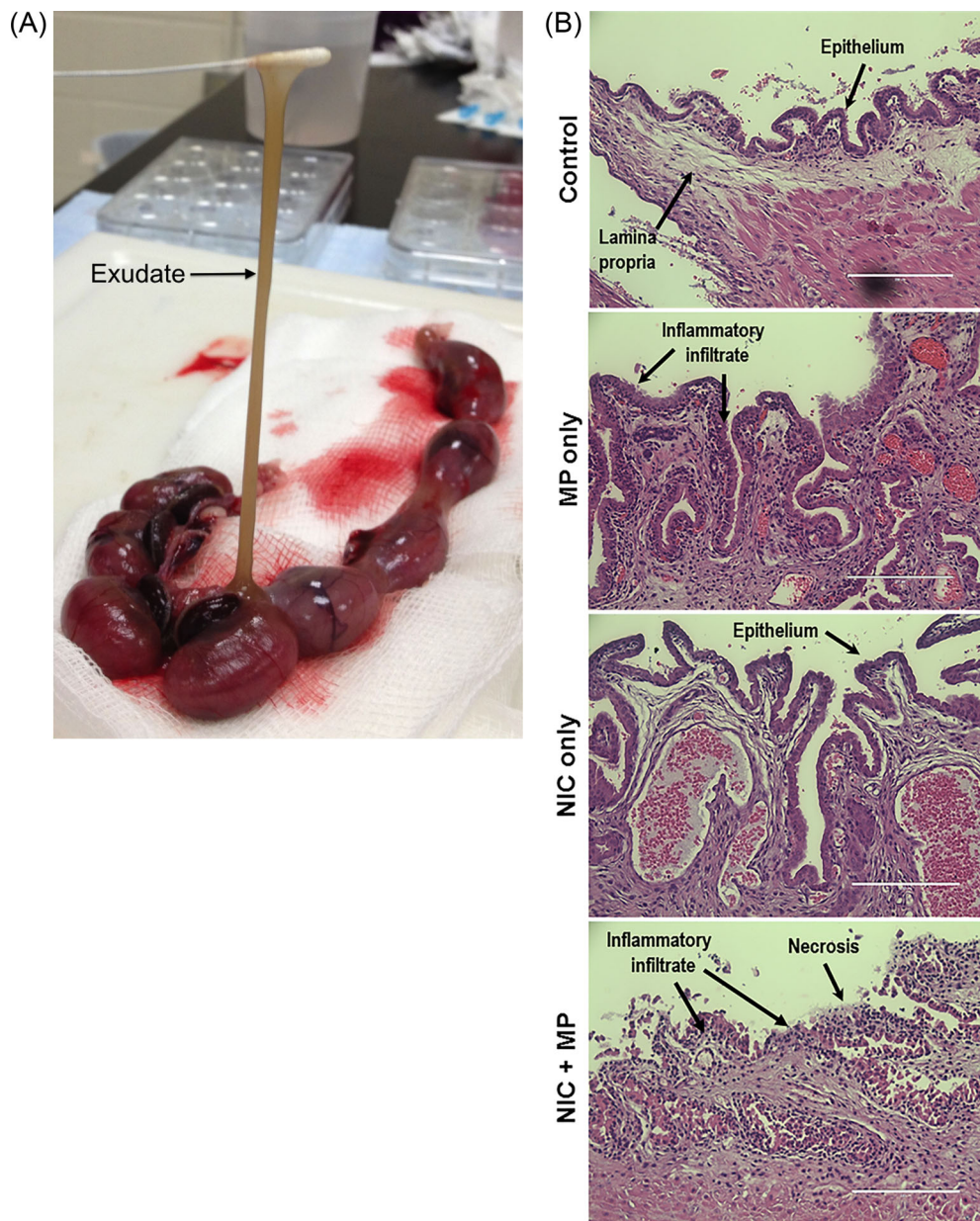
Previously, we demonstrated that the microbial load in the placenta was correlated with the microbial load in the amniotic fluid [46]. To determine if nicotine altered this relationship, the microbial load

of amniotic fluid as a function of microbial load in the placenta in both treatment groups was analyzed. In MP only fetal units, a high microbial load (defined as  $>10^4$  CCU) was seen in the amniotic fluid only when the microbial load in the placenta was  $\geq 10^9$  CCU (Figure 2). In contrast, in the Nic + MP group, the threshold of placental microbial load associated with high amniotic fluid loads was much lower. Consistently high numbers of *M. pulmonis* were recovered from amniotic fluids with placental microbial load  $\geq 10^6$  CCU, and in a few instances, at even lower placental loads. When nicotine exposure was present, the relationship between placental and amniotic fluid microbial load was altered ( $P = 0.001$ , best fit regression curves; MP only,  $y = 0.4x - 0.1$ ; Nic + MP,  $y = 0.5x + 0.8$ ).

Complete culture data (Table 2, Figure 3) were available for 27 (MP only) and 33 (Nic + MP) fetal units (placenta, amniotic fluid, and fetus). Fetal colonization was more likely to occur with nicotine exposure ( $P < 0.05$ ; MP only, 81.5% positive; Nic + MP, 100% positive). Furthermore, in both MP only and Nic + MP treatment groups, fetal colonization was most likely ( $P < 0.01$ ) to occur when both the placenta and amniotic fluid were culture positive (Table 2). A linear correlation between fetal microbial load and both the placental (Spearman  $r = 0.4$ ,  $P < 0.05$ ,  $n = 28$ ) and amniotic fluid (Spearman  $r = 0.4$ ,  $P < 0.05$ ,  $n = 27$ ) microbial loads was present in the MP only group but not in the Nic + MP group (for fetus vs. placenta microbial loads: Spearman  $r = -0.2$ ,  $P = 0.2$ ,  $n = 33$ ; for fetus vs. amniotic fluid: Spearman  $r = 0.1$ ,  $P = 0.7$ ,  $n = 36$ ). A two-way ANOVA for treatment group (MP only and Nic + MP) and intrauterine site (placenta, amniotic fluid, and fetus) showed that both treatment group and intrauterine site, as well as an interaction between the two, contributed to variation in



**Figure 3.** Correlation of microbial load in the fetus with microbial load in both the placenta and amniotic fluid. In the MP only group, there was a positive correlation between fetal microbial load and placental and amniotic fluid microbial loads (left panels). A correlation was not observed in the Nic + MP group (right panels). The correlation coefficient (Spearman  $r$ ) and slope of linear regression line with corresponding  $P$  values (if  $< 0.05$ ) are indicated.



**Figure 4.** Acute endometritis was observed in both MP only and Nic + MP groups. (A) Copious brown mucoid exudate (arrow) was observed in the uterine lumen of Nic + MP dams (3 of 7, 43%), but was never observed in MP only dams or controls. (B) Representative endometrial histology from control, MP only, Nic only, and Nic + MP dams. Scale bar is equivalent to 200  $\mu\text{m}$ ; hematoxylin and eosin stain. All microscopic images were obtained with an EVOS Auto FL imaging system (Life Technologies).

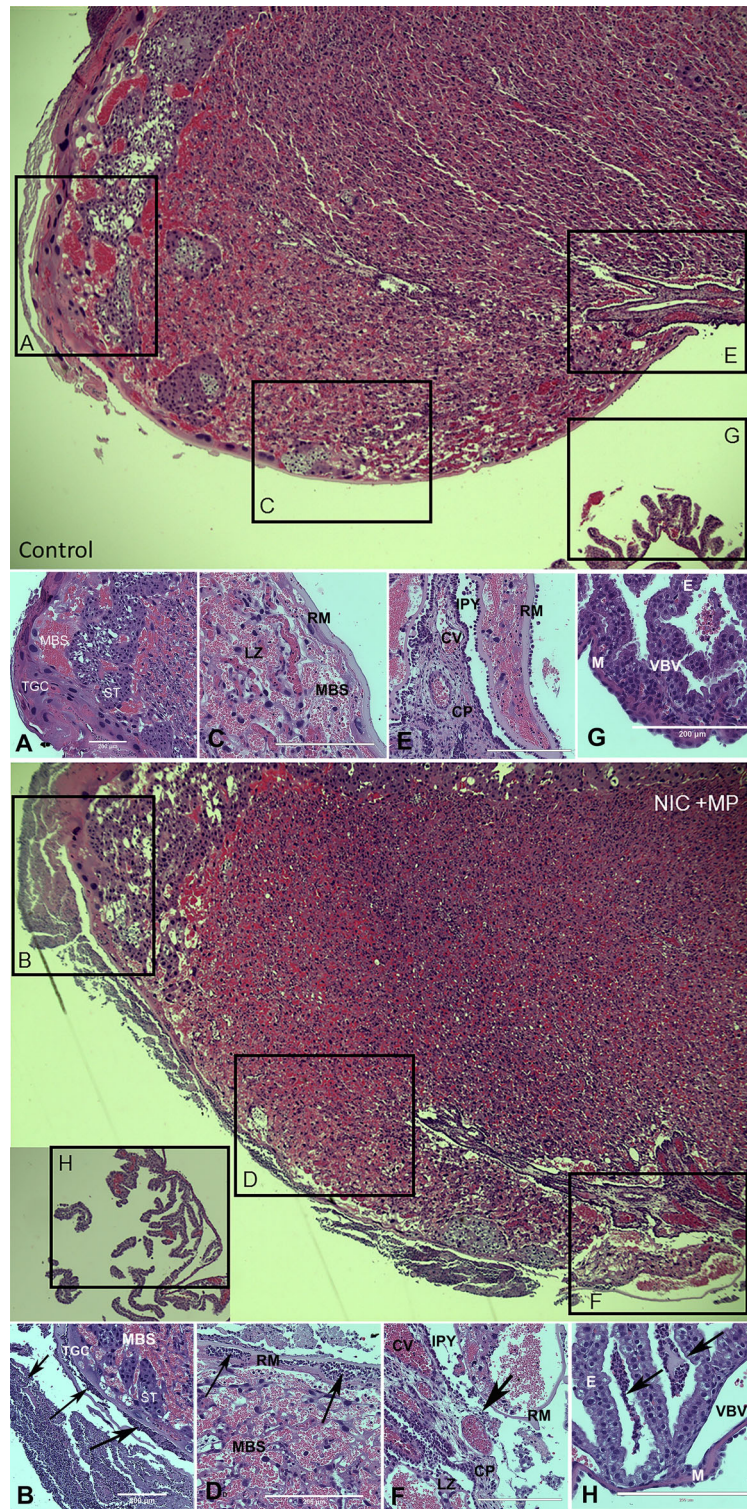
microbial load. Intrauterine site contributed to 78% of the variation ( $P < 0.0001$ ) while treatment group contributed to 17% of the variation ( $P < 0.0001$ ), and the interaction contributed to 1% of the variation ( $P < 0.0001$ ).

#### Prenatal nicotine and/or infection exposure induced site-specific pathology (Figures 4 and 5; Table 3)

Consistent with previous studies [42, 44–46], no exudate in the uterine lumen was observed in dams from control or MP only groups; we also observed no exudate in the Nic only group. In contrast, the combination of nicotine and infection resulted in a dramatic and unexpected inflammatory response in the uterus. At gross necropsy, three of seven Nic + MP dams had copious brown mucoid exu-

date present in the uterine lumen (Figure 4A). The exudates were positive for growth of *M. pulmonis* and negative for other aerobic bacteria. Representative histological sections of the endometrium between placental implantation sites of both uninfected and infected dams are shown in Figure 4B. No inflammatory lesions within the endometrium were observed in uninfected control or Nic only dams. In contrast, both MP only and Nic + MP dams had evidence of acute endometritis, but severe necrosis was seen only in Nic + MP dams.

Lesion severity scores for sites indicative of the MIR, FIR, or a mixture (MIXED) of both maternal and fetal components are presented in Table 3. The detailed inflammatory scores for MIR (subchorion and choriodecidua), FIR (chorionic vessels, umbilical arteries, umbilical vein, and Wharton jelly), and MIXED responses



**Figure 5.** Normal and representative severe histological lesions in the placenta. Low magnification images of control (top) and Nic + MP (bottom) placental disks with boxed areas indicating select sites subject to scoring: choriodecidua (boxes A and B), subchorion (boxes C and D), chorionic plate (boxes E and F), and visceral yolk sac (boxes G and H). Boxes correspond to the location of the structures shown in the high magnification image panels (control: A, C, E, G; Nic + MP: B, D, F, H). Arrows indicate neutrophils. Panel A shows normal choriodecidua. In Panel B, the decidua is obliterated by neutrophil infiltrates. Panel C shows normal subchorion. In Panel D, neutrophils infiltrate the maternal blood spaces at the periphery of the labyrinth zone. Panel E shows normal chorionic plate. Panel F shows epithelial effacement, neutrophil infiltration, and structural breakdown of the chorionic plate. Panel G shows normal visceral yolk sac. In Panel H, purulent exudate is seen among the endodermal villi of the visceral yolk sac. All images were obtained with an EVOS Auto FL imaging system (Life Technologies). Panels represent  $\times 10$  (A and B) or  $\times 20$  magnification. All scale bars are equivalent to  $200 \mu\text{m}$ . Structural abbreviations: decidua (D), maternal blood sinus (MBS), trophoblastic giant cell (TGC), spongiotrophoblast (ST), labyrinth zone (LZ), Reichert's membrane (RM), intraplacental yolk sac (IPY), chorionic plate (CP), chorionic vessel (CV), endoderm (E), vitelline blood vessel (VBV), and mesothelium (M).

**Table 3.** Lesion severity in sites indicative of maternal inflammatory response (MIR), fetal inflammatory response (FIR), or a potential mixture (MIXED) of both maternal and fetal components was impacted by treatment group.

Group	MIR <sup>a,b,c</sup>	Severe MIR <sup>c</sup>	FIR <sup>a,b,c</sup>	Severe FIR <sup>c</sup>	MIXED <sup>a,b,c</sup>	Severe MIXED <sup>c</sup>
Control	2.3 ± 1.2	0/12 (0%)	3.2 ± 1.6	0/12 (0%)	1.6 ± 1.4	0/12 (0%)
MP only	<b>4.5 ± 2.0</b>	5/18 (28%)	7.2 ± 2.6	0/12 (0%)	<b>5.5 ± 2.7</b>	3/17 (18%)
Nic Only	<b>4.8 ± 1.5</b>	<b>8/26 (31%)</b>	<b>13.0 ± 7.5</b>	<b>7/20 (35%)</b>	<b>7.3 ± 4.7</b>	<b>8/27 (30%)</b>
Nic + MP	<b>6.0 ± 2.1</b>	<b>17/28 (61%)</b>	<b>11.0 ± 6.3</b>	<b>8/25 (32%)</b>	<b>5.8 ± 4.0</b>	7/28 (25%)

Results are presented as mean lesion score ± SD, or as proportion of total having severe scores

<sup>a</sup>A score of 0–3 was assigned for both acute and chronic inflammation at a specific site; 0 = little to no inflammation, 1 = mild inflammation, 2 = moderate inflammation, and 3 = severe inflammation, with a maximum score of 6. If tissue damage was so advanced that neutrophils could not be assessed, a score of 7 was assigned to indicate maximum lesion severity.

<sup>b</sup>MIR lesion score = sum of subchorion and choriodecidua lesion scores; maximum possible score = 14; severe MIR = lesion score ≥ 6. FIR lesion score = sum of chorionic vessels, umbilical arteries, umbilical vein, and Wharton's jelly lesion scores; maximum possible score = 28; severe FIR = lesion score ≥ 12. MIXED lesion score = sum of chorionic plate, visceral yolk sac, and amnion tissues lesion scores; maximum possible score = 21; severe MIXED = lesion score ≥ 9.

<sup>c</sup>Statistically significant differences (Kruskal-Wallis) among the groups for overall lesion score are shown in bold. MIR lesion scores: All treatment groups > control,  $P < 0.05$ . FIR lesion score: both Nic only and Nic + MP > control ( $P < 0.0001$ ). MIXED: all treatment groups > control ( $P < 0.003$ ). Statistically significant differences (Fischer exact test) among the groups for severe lesions are shown in bold. Severe MIR: Nic + MP group > control ( $P < 0.0003$ ) and both MP only and Nic only ( $P < 0.05$ ) treatment groups; Nic only group > control group ( $P < 0.05$ ). Severe FIR: Both Nic only and Nic + MP > both control and MP only groups ( $P < 0.05$ ). Severe MIXED: Nic only ( $P < 0.05$ ) > control group.

(chorionic plate, visceral yolk sac, and amnion tissues) are shown in Table S1, supplementary material. For reference and orientation, low magnification placental images (Figure 5) showing the location of the choriodecidua (box A, B), subchorion (box C, D), chorionic plate (box E, F), and visceral yolk sac (box G, H) are provided. The corresponding high magnification image panels for the control placenta (A, C, E, G) are a reference for the normal architecture. Selected examples of severe lesions are shown in the high magnification image panels (B, D, F, H). The primary lesion observed was neutrophil infiltration. In severe lesions, the infiltrate was associated with severe tissue damage. For example, epithelial effacement, neutrophil infiltration, and structural breakdown of the chorionic plate are shown in Figure 5F. All treatment groups had increased MIR and MIXED lesion severity relative to controls (Table 3,  $P < 0.025$ ). However, the most severe MIR lesions were seen in the Nic + MP group relative to the control ( $P < 0.0003$ ), MP only ( $P < 0.05$ ), and Nic only ( $P < 0.05$ ) treatment groups, suggesting some synergy between the risk factors. Nicotine, alone or in concert with infection, promoted FIR ( $P < 0.0001$ ), with increased lesion severity ( $P < 0.05$ ) in all FIR sites (refer to Table S1, supplementary material).

#### Nicotine exposure alone or in combination with infection impacts cytokine levels in the placenta (Figure 6)

Placental IL6 levels did not vary among groups. Relative to the control group, Nic only exposure resulted in increased placental levels of IFNG ( $P < 0.05$ ) but did not alter levels of IL10 or TNF. Placental levels of IFNG and TNF were statistically higher ( $P < 0.01$ ) in the Nic + MP group as compared to both control and MP only groups. Furthermore, IL10 placental levels were higher in the Nic + MP group than in all other groups ( $P < 0.001$ ). In general, levels in the MP only group were comparable to control values.

#### Nicotine and infection, independently and in combination, impact cytokine levels in the amniotic fluid (Figure 7)

In contrast to what was observed in the placenta, IFNG levels in amniotic fluid were decreased ( $P < 0.0001$ ) in all treatment groups relative to the control group. IL10 was significantly decreased ( $P < 0.05$ ) in the MP only and Nic only groups relative to the con-

trol group. However, in the Nic + MP group, the IL10 levels did not decrease in amniotic fluid and were similar to control levels. Nicotine or infection did not independently impact TNF levels, but when combined (Nic + MP), TNF levels were significantly increased ( $P < 0.0001$ ) as compared to all other groups. Similarly, IL6 amniotic fluid levels in Nic + MP were increased ( $P < 0.0001$ ) as compared to all other groups.

#### Presence of live microbes impacts cytokine levels at the site of infection (Figures 8 and 9)

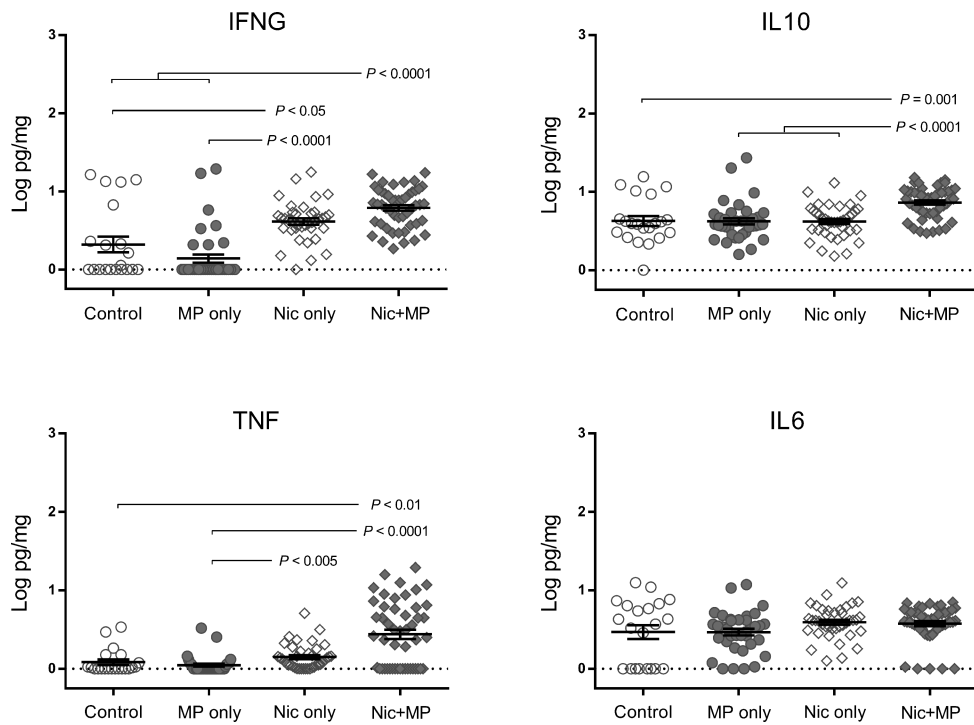
We wished to determine if the cultural status of the placenta and amniotic fluid impacted cytokine levels. Therefore, the MP only and Nic + MP groups were subdivided by culture-positive and culture-negative status at the level of the placenta (Figure 8) or amniotic fluid (Figure 9). None of the cytokine levels in the MP only group were impacted by culture status of the placenta ( $P > 0.05$ ). In contrast, in the Nic + MP group, IFNG and IL10 ( $P < 0.005$ ) and TNF ( $P < 0.05$ ) were all elevated when infection was present. Furthermore, the presence of nicotine in concert with MP infection resulted in higher placental levels of IFNG, IL10, TNF ( $P < 0.01$ ), and IL6 ( $P < 0.05$ ) when compared with MP only culture-positive placentas.

In the MP only group, levels of IFNG in amniotic fluid were higher when infection was absent ( $P < 0.05$ ); IL10, TNF, and IL6 levels were not altered by the presence of infection. Within the Nic + MP group, when *M. pulmonis* was isolated from amniotic fluid, TNF ( $P < 0.01$ ) levels were elevated but levels of IFNG, IL10, or IL6 were not impacted. When culture-positive amniotic fluids in the Nic + MP and MP only groups were compared, log increases in cytokine levels were observed for TNF ( $P < 0.0001$ ), IL10 ( $P < 0.005$ ), and IL6 ( $P < 0.0001$ ) in the Nic + MP group. For IL6 ( $P < 0.01$ ), the increases were also observed in the absence of microbial colonization of the amniotic fluid. These data suggest that nicotine and infection can act synergistically at the local site of infection to modulate cytokine levels.

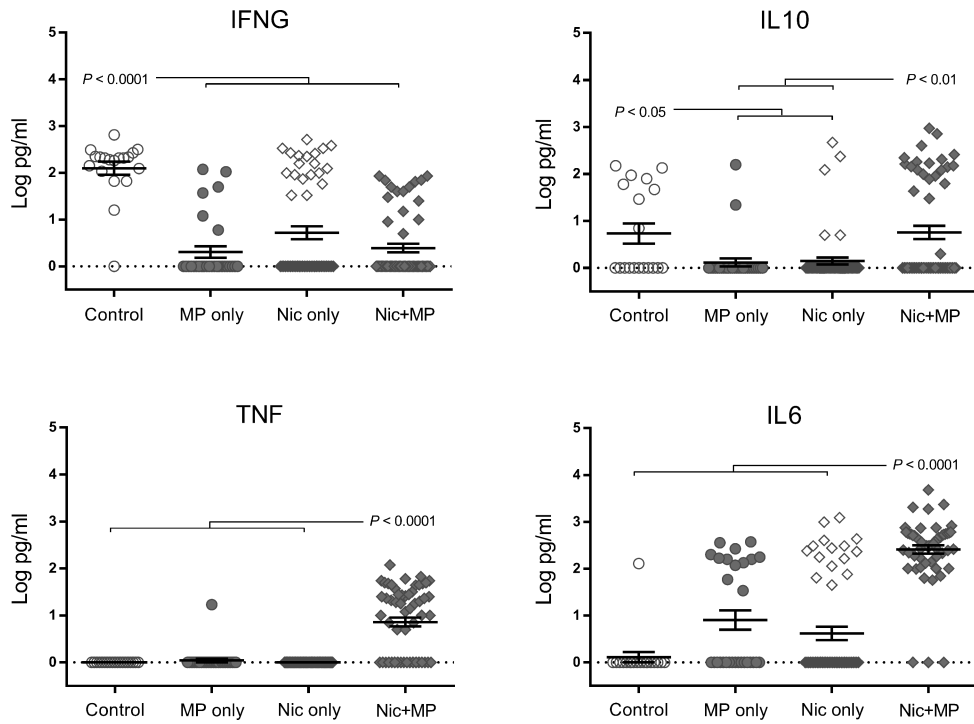
#### Nicotine alters the cytokine environment in both the placenta and the amniotic fluid (Figure 10)

Because the M2/Th2 phenotype is present in the normal pregnancy environment, we wished to determine if the combination of infection and nicotine altered key cytokines in the placenta and amniotic fluid.

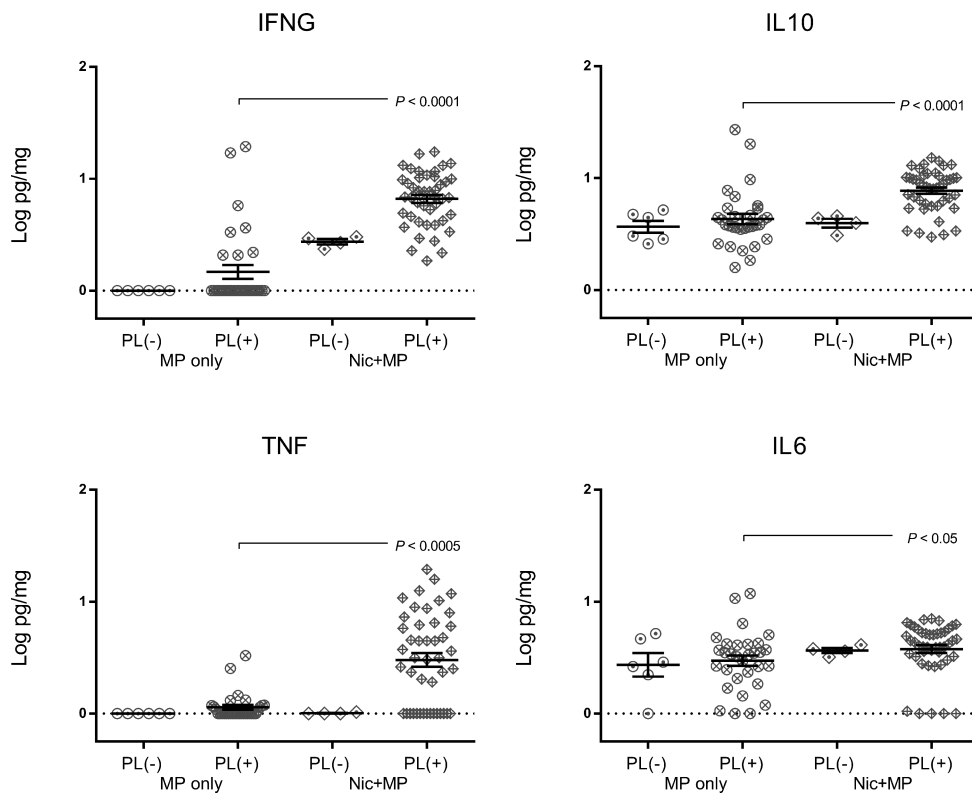




**Figure 6.** Combined nicotine and infection impacted cytokine levels in the placenta. Log-transformed cytokine levels for IFNG, IL10, TNF, and IL6 are shown. Graphs show individual data points as well as bars for mean and SEM. Cytokine data for control (n = 21), Nic only (n = 39), MP only (n = 38), and Nic + MP (n = 49) placentas were analyzed by Kruskal-Wallis tests. P values < 0.05 indicate significant differences between groups.



**Figure 7.** Combined nicotine and infection impacted cytokine levels in the amniotic fluid. Log-transformed cytokine levels for IFNG, IL10, TNF, and IL6 are shown. Graphs show individual data points as well as bars for mean and SEM. Data for control (n = 12), Nic only (n = 57), MP only (n = 30), and Nic + MP (n = 58) amniotic fluids analyzed by Kruskal-Wallis tests. P values < 0.05 indicate significant differences between groups.



**Figure 8.** Cytokine levels in the placenta were impacted by nicotine and by presence of the microbe. Log-transformed cytokine levels for IFNG, IL10, TNF, and IL6 for MP only ( $n = 38$ ) and Nic + MP ( $n = 49$ ) placentas (PL) were further subdivided based on positive (+) and negative (-) culture status of the placenta and analyzed by the Kruskal-Wallis test.  $P$  values  $< 0.05$  indicate significant differences between groups. Graphs show individual data points as well as bars for mean and SEM.

The IFNG-to-IL10 ratio can be used to assess the general immune environment in the placenta. In some samples, the cytokine levels were below the detection limit of our assays and were reported as zero. Therefore, we were unable to use a ratio but instead determined the difference (IFNG minus IL10) for each individual sample. A negative value was indicative of a more M2/Th2, IL10-dominant environment; a positive value was indicative of a more M1/Th1, IFNG-dominant environment, and a value near zero indicated a neutral environment. In control placentas, IL10 levels dominated over IFNG, while in Nic only placentas, the levels were equivalent and significantly different from control ( $P = 0.005$ ). MP only resulted in an IL10-dominant environment similar to control. However, when both nicotine and infection were present, a shift to a neutral balance was observed ( $P < 0.0001$ ). Unlike in the placenta, the general immune environment in control amniotic fluids was IFNG dominant while all other treatment groups shifted to a neutral environment ( $P < 0.0005$ ). Interestingly, when both nicotine and infection were present (Nic + MP), the shift from the IFNG-dominant control environment was more pronounced, resulting in an IL10-dominant environment in the amniotic fluid ( $P < 0.0001$ ).

#### Circulating serum cytokines were not impacted

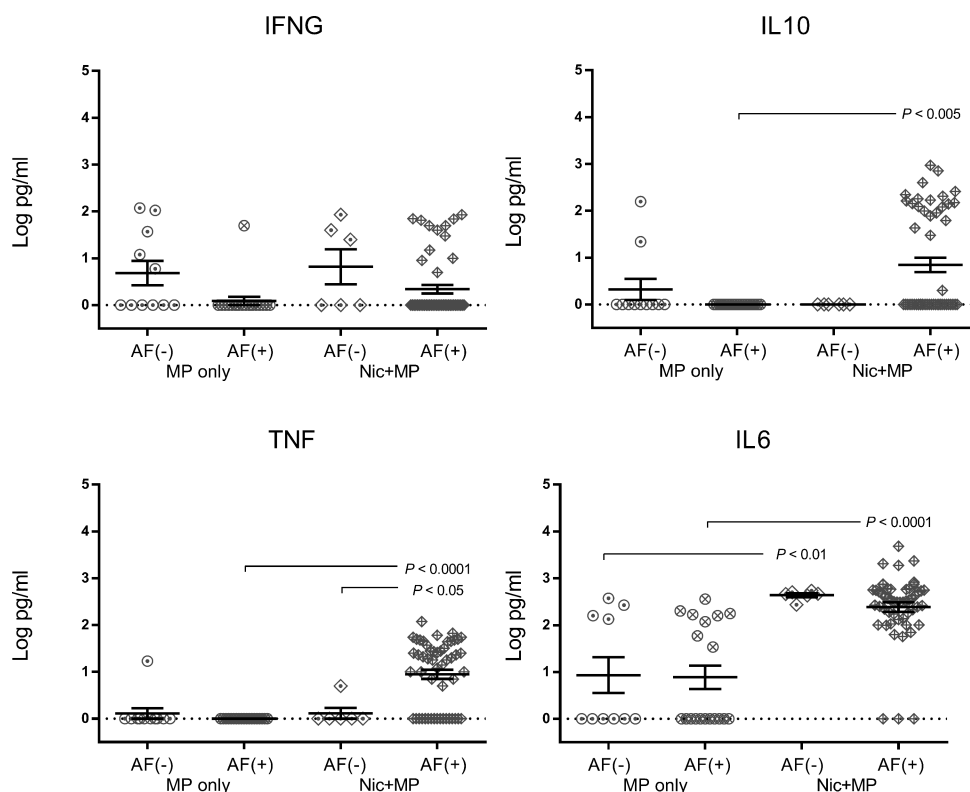
No significant differences (Kruskal-Wallis) among treatment groups were found in maternal serum cytokine levels.

#### Discussion

Prenatal nicotine exposure alone has been associated with a host of adverse fetal/neonatal outcomes [8, 38–40, 47–53]. The results of

this study provide new evidence demonstrating that nicotine exposure also increases the risk of fetal infection. Specifically, prenatal exposure to 6 mg/kg/day nicotine significantly increased the incidence of both amniotic fluid and fetal infection with *M. pulmonis*. The placental microbial load needed to trigger amniotic fluid infection was significantly decreased in Nic + MP dams, suggesting that prenatal nicotine exposure compromises the placental barrier protection against infection.

While causal relationships of adverse pregnancy outcomes can be inferred from retrospective and prospective epidemiological studies, animal models are essential to elucidate mechanisms of pathogenesis [54–57]. The rat is a good model for studies focused on the maternal:fetal interface because, similar to that in humans, this species has both hemiochorial placentation and deep trophoblast invasion of spiral arteries [58]. The primary limitation of the rat as a model is that rats resorb fetuses rather than deliver prematurely. One potential caveat is that we used a high dose of nicotine in our study. The 6 mg/kg/day we chose does not impact maternal body weight, number of pups per litter, pup weight, or placental weight, but does significantly alter neurotransmitter balance within the central nervous system and postnatal behavior [38, 39, 41]. We coupled this nicotine dose with an infectious dose of *M. pulmonis* that results in consistent colonization of the placenta but variable colonization of the fetal compartment [45, 46]. Because there are no studies that have examined the interaction between nicotine and intrauterine microbial infection, we reasoned that using the highest dose of nicotine not associated with adverse pregnancy outcomes coupled with a moderate infectious dose would provide the best opportunity to document an interaction between nicotine and infection. In fact, in



**Figure 9.** Cytokine levels in the amniotic fluid were altered by nicotine and by presence of the microbe. Log-transformed cytokine levels for IFNG, IL10, TNF, and IL6 for MP only (n = 30) and Nic + MP (n = 58) amniotic fluids (AF) were further subdivided based on positive (+) and negative (-) culture status of the amniotic fluid and analyzed by Kruskal-Wallis test. P values < 0.05 indicate significant differences between groups. Graphs show individual data points as well as bars for mean and SEM.

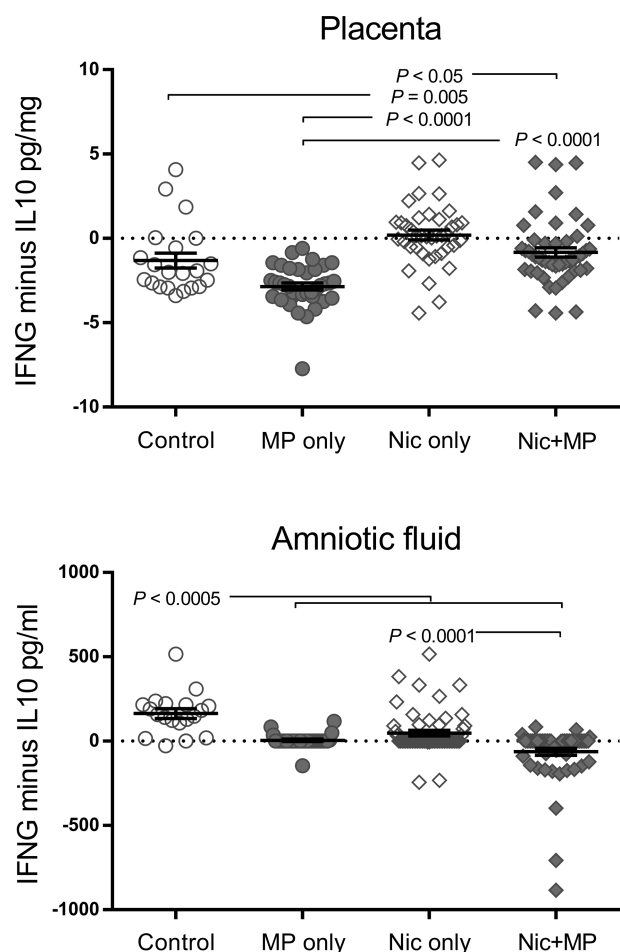
a pilot study in which both nicotine and infection were combined, at term pregnancy we saw an unexpected reduction in litter size and few surviving pups (see supplemental data). Ethical concerns for animal welfare precluded continued studies at term, but it is important to note that these severe outcomes were not observed in term dams with nicotine or infection exposure alone. Therefore, use of this model for term or postnatal studies will need to incorporate lower doses of nicotine.

One of the strengths of our model system is that we use viable *M. pulmonis* that can replicate, establish in the placenta, and disseminate throughout the fetal unit [42, 46, 59], similar to what is likely to occur during intrauterine infection in humans. *Mycoplasma pulmonis* genital disease is a naturally occurring reproductive disease in laboratory rats [60, 61]. The model is well characterized, and there is an established dose response and reproducible pattern of colonization of maternal and fetal tissues [42, 45, 46] as well as defined pathology [44, 46]. Experimentally infected dams develop varying complications as well as histological lesions similar to ureaplasma-associated reproductive disease of humans [62–65], including chorioamnionitis, fetal infection of the lung and central nervous system, low birth weight, and fetal and neonatal death [42, 44–46, 59, 66]. Routes of infection, including both intravaginal and IV inoculation, have been defined for this model previously [43–46]. This study was designed to ensure that all inoculated dams became infected. Intravenous administration provided the precise timing and dose delivery to all pregnant dams. The uterus was infected in all dams, and there was no difference between MP only and Nic + MP with respect to numbers of microbes recovered from the uterus.

Therefore, all fetal units had an equal chance of becoming infected. By design, the infectious dose chosen did not result in 100% of placentas becoming infected in the MP only group, permitting us to identify differences between the infected treatment groups. There was no difference in the percentage of placentas that were colonized between groups. What was dramatically different was the increased microbial load in the placenta in the Nic + MP group coupled with the lowered threshold required for *M. pulmonis* to breach the placental barrier in this group.

In this study, we used an established paradigm of prenatal nicotine exposure to model the impact of maternal smoking (either cigarettes or ENDS) on fetal infection or susceptibility to infection using viable microbes as opposed to purified endotoxin. Administration of a purified toxin like LPS is often used as a surrogate for maternal infection and can induce premature birth and/or evidence of resorption in rats and mice [55–57]. LPS intoxication stimulates innate immune responses, induces prematurity, and has provided valuable insights into the specific mechanism by which LPS alone or in concert with nicotine impacts pregnancy [67–70]. However, intoxication is not adequate to examine the mechanisms by which viable pathogens invade and breach the placental barrier. To our knowledge, very few animal studies have investigated the interaction between nicotine exposure and true maternal infection on fetal outcome.

For much of gestation, the placenta induces an M2/Th2 phenotype in decidual leukocytes [71–74]. The relative balance of IFNG to IL10 in placental tissue was used as an indicator of M2/Th2 profile. In our study, nicotine exposure altered the IFNG to IL10 balance.



**Figure 10.** The normal environments of the placenta (M2/Th2) and amniotic fluid (M1/Th1) are impacted by nicotine, alone or in concert with infection. As an indicator of the overall M2/Th2 profile in individual placenta and amniotic fluid samples, the IL10 level was subtracted from the IFNG level. A negative value was indicative of a more M2/Th2, IL10-dominant environment; positive values were indicative of a more M1/Th1, IFNG-dominant environment, and values near zero indicated a neutral environment. Cytokine data for control (n = 21), Nic only (n = 39), MP only (n = 38), and Nic + MP (n = 49) placentas, and for control (n = 12), Nic only (n = 57), MP only (n = 30) and Nic + MP (n = 58) amniotic fluids, were analyzed by the Kruskal-Wallis test.  $P$  values  $< 0.05$  indicate significant differences between groups. Graphs show individual data points as well as bars for mean and SEM.

As expected, placentas from controls were characterized by  $IL10 > IFNG$  levels. Placental IFNG levels were increased relative to IL10 with Nic only, shifting to a neutral balance. In the MP only group, the normal IL10-dominant environment persisted, independent of the presence of live microbes in the placenta. Thus, even in the face of overt infection, the placenta was able to maintain the appropriate M2/Th2 environment. However, in the presence of nicotine and placental colonization with a live microbe, the balance was again shifted to neutral. When considered in context with lesion severity, this suggests that at 6 mg/kg/day, nicotine might prime the placental tissues to a stronger cell-mediated, M1/Th1 inflammatory response when a pathogen is encountered.

A compelling contrast was observed in the amniotic fluid. In controls, the amniotic fluid, unlike the placenta, was IFNG dominant, suggesting an M1/Th1 environment. In Nic only and MP only animals, this environment shifted to a neutral balance. When both

nicotine and infection were present, the amniotic fluid environment was shifted from the M1/Th1 profile seen in the controls to become IL10 dominant. Interestingly, the Nic + MP group had dramatically increased levels of both TNF and IL6 relative to all other treatment groups. In the case of IL6, this increase was independent of the presence of *M. pulmonis* in the amniotic fluid. This interaction between nicotine and maternal infection to elevate IL6 levels without regard to amniotic fluid colonization is interesting. The magnitude of the increases in amniotic TNF and IL6 is striking. Levels of TNF and IL6 in amniotic fluid from the Nic + MP group were one to three logs higher than levels seen in control amniotic fluids, with TNF levels up to 117 pg/ml and IL6 levels up to 4791 pg/ml. These concentrations are comparable to those reported from human amniotic fluids with fetal inflammatory syndrome and intrauterine infection [75–77]. TNF does not cross the placental barrier; additionally, activated macrophages expressing TNF increase dramatically from GD 15 to GD 17 in the amniotic fluid of the mouse [78]. IL6 from maternal circulation does cross the placental barrier [79] but given the magnitude of the increase in IL6 in the amniotic fluid, it is unlikely that the maternal circulation or the placenta is the primary source of IL6 in the amniotic fluid. This draws into question whether it is maternal leukocytes present in the amniotic fluid or fetal cells that are the source of these cytokines. Given that IFNG levels are high in the placenta and minimal to absent in the amniotic fluid, it is possible that priming and activation occurs within the placenta prior to leukocyte entry into the amniotic fluid. Another intriguing possibility is that nonimmune fetal cells might be the source of the fetal inflammatory response [80, 81]. The cells involved in the fetal inflammatory response to infection in the rat have not been fully characterized. Thus, there is a need to determine the specific cell types producing the cytokines in response to both nicotine and infection.

In summary, our study demonstrated that nicotine exposure at 6 mg/kg/day enhances the ability of the pathogen to cross the placental barrier, colonize the amniotic fluid, and gain access to the fetus. Additional studies are needed to further elucidate the specific mechanism(s) by which nicotine facilitates microbial invasion of the fetal compartment, as well as to determine if the responses observed are dose dependent. Most importantly, these data demonstrate that a modifiable risk factor (nicotine exposure) can directly impact what has been considered an immutable risk factor (infection) for perinatal complications. This study highlights the need for continued education of reproductive age women on the potential dangers of ENDS use as well as the need for in-depth epidemiological studies on pregnancy outcomes and risks associated with ENDS use.

## Supplementary data

Supplementary data are available at [BIOLRE](https://doi.org/10.1093/biolre/btx011) online.

**Supplemental Table S1.** Median lesion severity scores and interquartile range for placental sites evaluated.

**Supplemental Figure S1.** Adverse pregnancy outcome in term dams. A. Dam euthanized at GD 25 after failure to deliver. Arrows indicate fetuses that were removed from uterus at necropsy. Fetuses were large and misshapen, with bruising and swelling of muzzle, abdomen, and/or hind limbs. Granulomatous inflammation was widespread in placental disks. B. Dam euthanized at GD 25 after normal delivery of 2 pups at GD 22. Two macerated fetuses were retained in the uterus and were coated with viscous material.

Placentas appeared necrotic. C. Upon excision of the uterus below the cervix, purulent exudate oozed from the vaginal canal.

*Conflict of interest:* The authors have declared that no conflict of interest exists.

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