Nicotine induces maternal and fetal inflammatory responses which predispose intrauterine infection risk in a rat model.

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ABSTRACT

Both smoking and infection adversely impact pregnancy. Previously, our group identified in a rodent model that 6 mg/kg/d nicotine increased the risk of fetal infection at gestation day (GD) 18. Here, we investigate lower nicotine doses. Methods. Pregnant Sprague-Dawley rats received nicotine infusion at 0, 1, or 3 mg/kg/d (no, low-, and mid-dose nicotine, respectively) from GD 6, with intravenous inoculation with Mycoplasma pulmonis (MP) at 107 CFU (N=20) or sterile broth (sham) (N=11) on GD 14. Uterus and fetuses were retrieved on GD 18 for MP culture and histopathologic evaluation of maternal and fetal inflammatory responses (MIR and FIR). Results. At 1 mg/kg/d nicotine, MP colonization rates were decreased, from 100% (9 of 9) to 40% (2 of 5) of MP-inoculated dams, (P=0.03), and 59% (66 of 111) to 39% (24 of 62) of fetuses (P=0.01), versus no nicotine. Low-dose nicotine resulted in increased MIR and FIR in the sham-inoculated group; in the MP-inoculated group, this resulted in reduced relative risk (RR) for placental colonization (RR, 95% CI with high MIR = 0.14, 0.02 to 0.65; FIR = 0.38, 0.12 to 0.93). In contrast, 3 mg/kg/d nicotine treatment did not alter colonization rates; furthermore, FIR was completely suppressed, even in the face of placental or amniotic fluid colonization. Conclusion. The 1 mg/kg/d nicotine dose decreased risk of intrauterine infection, with increased MIR and FIR. The 3 mg/kg/d nicotine dose inhibited FIR, and increased risk for intrauterine infection. Nicotine alterations of the intrauterine environment were markedly dose-dependent.
IMPLICATIONS

Nicotine exposure alters intrauterine infection and inflammation in a dose-dependent manner, potentially impacting fetal development and programming. Previous work in a rodent model showed that high-dose nicotine (6 mg/kg/d) exposure exacerbated intrauterine infection during pregnancy. The current study found that low-dose nicotine (1 mg/kg/d) exposure reduced colonization of placenta and amniotic fluid; this decrease was associated with increased intrauterine inflammation. Exposure to mid-dose nicotine (3 mg/kg/d) suppressed fetal inflammation. Elucidation of underlying mechanisms of these phenomena will inform public health and clinical care decisions, particularly in the context of risk assessment of nicotine replacement therapy during pregnancy for smoking cessation.
INTRODUCTION

The intrauterine environment during pregnancy influences fetal development and programming,¹ and altering perinatal and adult postnatal health outcomes, including mental health and chronic illness.² Further, intrauterine infection and inflammation may trigger preterm birth,³-⁸ which in turn results in neonatal morbidity and mortality as well as long-term postnatal effects.⁹-¹⁴ Cigarette smoke components, including nicotine, can exacerbate or otherwise modulate such impacts.¹⁵-¹⁷

In the past, concerns around cigarette smoking related to fetal exposure to carcinogens, toxins, and products of combustion (particulates, carbon monoxide, etc.). The recent rise in use of electronic nicotine delivery systems (ENDS; e.g. vape pens and E-cigarettes) by young adults,¹⁸ as well as the use of nicotine replacement therapies (e.g. nicotine patches or nicotine gum) for smoking cessation during pregnancy necessitates continued investigation of maternal exposure to nicotine and subsequent in utero and postnatal impacts. The proinflammatory response is modulated in part by nicotinic acetylcholine receptors (nAChR);¹⁹-²¹ therefore nicotine exposure during pregnancy potentially alters maternal and fetal responses to microbial invasion as well as the physiologic functions of inflammation in the reproductive tract.

Previously, our group identified that exposure to high-dose nicotine (6 mg/kg/d) during gestation in rodents had a dramatic impact on inflammatory cytokines; histologic lesions; and bacterial colonization in the endometrium, placenta, and fetal tissues; following inoculation with Mycoplasma pulmonis²² (MP), a pathogen of the reproductive tract. This outcome suggests that nicotine may increase the risk for fetal infection. The current study used the same rodent model of nicotine exposure and infection during pregnancy to evaluate impacts of 2 lower doses of nicotine on intrauterine infection and the inflammatory response to infection. Lower nicotine doses (1 or 3 mg/kg/d) were used for a closer approximation of nicotine exposure in pregnant women using nicotine replacement therapies²³-²⁵ or electronic/conventional cigarettes²⁶, respectively. We hypothesized that lower doses of
nicotine would have a similar increase of histologic inflammatory indicators as well as fetal
infection risk, but proportionately lower than the previous high-dose nicotine study.

METHODS

Animals

All 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, with PHS Policy Assurance with OLAW and
USDA registration. Specific pathogen-free (SPF) adult male and primiparous female
Sprague Dawley rats (Crl:SD; Charles River Laboratories International, Inc., Wilmington,
MA) were used. 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 same-sex pairs
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. Upon sexual maturity, males were separated and
housed singly. 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

After breeding, pregnant dams were randomly assigned to 1 of 6 treatment groups:
saline infusion with 0 mg/kg/d nicotine and a sham inoculation of sterile broth injection
(Control); 1 mg/kg/d nicotine infusion and sterile broth injection (Low NIC only); 3 mg/kg/d
nicotine infusion and sterile broth injection (Mid NIC only); saline infusion and $10^7$ colony
forming units (CFU) *M pulmonis* inoculation (MP only); 1 mg/kg/d nicotine infusion and $10^7$
CFU *M. pulmonis* inoculation (Low-NIC+MP); 3 mg/kg/d nicotine infusion and $10^7$ CFU *M. pulmonis* inoculation (Mid-NIC+MP).

**Nicotine dose selection**

The selected doses were estimated to represent levels of both nicotine and the more stable nicotine metabolite, cotinine, of pregnant women smoking less than a pack per day or wearing a nicotine patch (1 mg/kg/d)\textsuperscript{23-25} or pregnant women smoking more than a pack a day (3 mg/kg/d).\textsuperscript{26} While data for nicotine and cotinine levels in pregnant women using ENDS are not widely available, we estimated that typical ENDS use by pregnant women would result in comparable levels to those of pregnant cigarette smokers. See the methods in the supplementary materials for additional discussion on nicotine dose selection.

**Infusion pump implantation**

At GD 6, pregnant dams were weighed, and anesthesia was induced in a chamber with 3\%–4\% isoflurane in 100\% oxygen; a surgical plane of anesthesia was maintained with 1\%–3\%, isoflurane in 100\% oxygen delivered by facemask. Lidocaine diluted to 0.5\% in sterile saline was injected subcutaneously near the surgical site. The surgical site was clipped and antiseptically prepared with povidone-iodine scrub and 70\% ethanol. A 28-day osmotic minipump (ALZET Osmotic Pump Model 2ML4, DURECT Corp., Cupertino, CA) was inserted subcutaneously via a small incision between the scapulae. The incision was closed in 2 layers using absorbable subcutaneous intradermal sutures and nylon skin sutures. The minipump was pre-filled with either sterile saline or nicotine tartrate (Sigma-Aldrich, St. Louis, MO) dissolved in sterile saline at a concentration sufficient to deliver 1 or 3 mg/kg/d based on the projected weight at midpoint of gestation. Insulated heating pads (SnuggleSafe, Lenric C21 Ltd. Littlehampton, West Sussex, UK) provided supplemental heat during anesthesia and recovery, and warmed subcutaneous fluids were administered. Carprofen 5 mg/kg/d and buprenorphine 0.03 – 0.05 mg/kg/8h SC were given subcutaneously for peri- and postoperative analgesia.
**Mycoplasma pulmonis preparation and intravenous injection**

To ensure identical inocula for all experiments, *M pulmonis* X-1048 was grown to late logarithmic phase in Frey’s medium, aliquoted, and frozen at -80 °C. The stock culture was thawed, and diluted in vehicle to achieve a concentration of $10^7$ CFU per 0.1 mL for inoculation of each rat. Dose concentration was confirmed by both optical density and culture.

At GD 14, dams were re-anesthetized as described above, and placed in dorsal recumbency to allow access to the right subclavian vein. The site was cleaned with 70% ethanol and an IV injection of $10^7$ CFU *M pulmonis* or sterile broth (vehicle) was delivered slowly over 10 to 15 s. This infection dose was selected based on previous experiments. 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 (1.5 mL Beuthanasia-D Special, Merck Animal Health, Summit, NJ). Tissues and swabs were collected using aseptic technique. Maternal spleen was minced in Frey’s broth culture medium; vaginal and endometrial mucosae were swabbed with calcium alginate swabs which were then inoculated into broth culture medium for serial dilution and culture. The uterus was opened, and fetal units were isolated and collected. Each fetus and associated membranes, amniotic fluid, cord, placenta, and attached uterus (including decidua, mesometrial triangle, and the uterine wall and endometrium associated with that unit) were treated as individuals (referred to herein as units, fetal units, or maternal-fetal units) for the purposes of sample collection and data analysis. For all units, amniotic fluid was swabbed for inoculation of broth medium, and a portion of the placental peripheral margin was minced in broth medium. Samples from MP only and NIC + MP groups were serially diluted 10-fold in Frey’s broth to $10^{10}$. Inoculated broths were incubated at 37 °C and monitored daily for color change. Half of the units of each litter were randomly selected for
fixation in 10% buffered formalin for histological analysis. Fixed tissues were transferred to 70% alcohol after 48 h of fixation.

**Histology scoring of intrauterine sites**

Tissue processing and histologic evaluation is described in detail in the supplementary material (Methods, Tables S1-S2 and Figures S1-S7). Briefly, the presence of neutrophil infiltration and of markers of chronic inflammation at certain microanatomical sites were evaluated, and a score was determined for each site for the severity of maternal and fetal inflammatory responses (MIR and FIR, respectively). The scoring methodology is further described in the Supplement. Stage 0 MIR (no inflammation) was defined as the absence of threshold-level lesions at any site. Stage 1 MIR was defined as the presence of lesions in the endometrium (an average score > 0 was the threshold for stage 1). Stage 2 MIR was defined by an average score of > 0.5 among the mesometrial triangle, the decidua, and the labyrinth. Stage 3 MIR was defined as the presence of purulent exudate associated with the decidua-yolk sac junction and/or the luminal aspect of the parietal and visceral membranes of the ruptured yolk sac; a score > 0.5 was the threshold for stage 3. Stage 0 FIR (no inflammation) was defined as the absence of threshold-level lesions at any site. Stage 1 FIR was defined as a chorionic vessel score > 0.75. Stage 2 FIR was defined by an average score > 0 among the umbilical vein and arteries. Stage 3 FIR was defined by a Wharton’s jelly score of 1. The data points analyzed below were the maximum MIR or FIR stage determined for each fetal unit.

**Data analysis**

Litter size data were analyzed with 2-way analysis of variance (ANOVA) with nicotine dose and infection as factors. Fetal weight differences among groups were determined by Kruskal-Wallis nonparametric and Dunn’s multiple comparison tests. Differences in the percentage of microbial colonization in maternal and fetal sites were determined by $\chi^2$ and Fisher exact tests, and relative risk (RR) and 95% confidence interval (CI) calculations using
the Koopman asymptotic score method. Differences in the microbial load recovered from maternal and fetal sites were determined by Kruskal-Wallis and Dunn tests. MIR and FIR among both noninfected and infected groups were determined by Kruskal-Wallis and Dunn tests. The relative proportion of high-stage and low-stage MIR and FIR among i) fetuses culture-negative for both placental and amniotic fluid sites (PL-neg/AF-neg); ii) fetuses culture-positive in placenta and culture-negative in amniotic fluid (PL-pos/AF-neg); and iii) fetuses culture-positive in both sites (PL-pos/AF-pos), within each nicotine dose was determined by the χ² test. Principle component and factor analyses of histologic site scores are described in detail in the supplemental materials. JMP version 15.0.0 (SAS Institute, Inc., Cary, NC) and GraphPad Prism version 8.3.0 for Windows (GraphPad Software, San Diego, CA) were used for statistical analyses and graph production.

RESULTS

Litter size was not impacted by nicotine dose or infection (2-way ANOVA, P > .36, Table 1). Nicotine alone did not impact fetal body weights in the Low NIC only group (Table 1, P = .28); however, a decrease in fetal body weights in the Mid NIC only group approached, but did not attain, statistical significance (Table 1, P = .08). Infection, alone (MP only, P = .004) or in concert with mid-dose nicotine (Mid-NIC+MP, P < .001), significantly decreased fetal body weight compared to the Control group (Table 1, Kruskal-Wallis and Dunn’s multiple comparison). Fetal body weight was not decreased in the Low-NIC+MP group (Table 1; P = .70). However, the Low-NIC+MP group was somewhat anomalous, due in part to a single litter of very large fetuses (see supplement Figure S8, Dam 6G litter), resulting in a wider range of fetal weights (Table 1) in the Low-NIC+MP group. This litter, in which all fetal body weights exceeded 2 grams, was from a dam with higher microbial load in spleen and uterus compared to other dams in the group (P = .056, approaching significance by Kruskal-Wallis test). The fetuses did not have any distinguishing differences from their counterparts in other litters from the same group with respect to placental and amniotic fluid cultures or maternal and fetal inflammatory responses. Therefore, the litter was included in
all analyses. However, if this single litter was excluded from the fetal body weight data, the Low-NIC+MP group, similar to the other MP-treated groups, had significantly lower fetal body weights ($P = .006$; in grams, median = 1.55; 1st and 3rd quartiles = 1.38 and 1.75) than Controls.

**Low-dose nicotine (1 mg/kg/d) reduced the colonization rate, colonization risk, and microbial load of M. pulmonis in intrauterine sites**

Success of infection in MP groups was confirmed by culture of *M. pulmonis* in at least one maternal site. Table 2 presents the *M. pulmonis* colonization rate (percentage of culture-positive fetal units for each site) among MP infection treatment groups. The percentage of fetal units with culture-positive intrauterine sites including endometrium, placenta, and amniotic fluid all were lower in Low-NIC+MP dams compared to MP only dams ($P < .03$). There were no significant differences in the colonization rate of spleen, vagina, or intrauterine sites in the Mid-NIC+MP treatment group as compared to the MP only group. The relative risk (RR) analyses are reported with 95% confidence intervals (CI) in Table S3. The Low-NIC+MP group had significantly reduced endometrial, placental, and amniotic fluid colonization risks compared to both MP only (RR = 0.00, 95% CI = 0 to 0.73; RR = 0.74, 95% CI = 0.58 to 0.93; and RR = 0.69, 95% CI = 0.56 to 0.86; for respective sites) and Mid-NIC+MP (RR = 0.25, 95% CI = 0.08 to 0.83; RR = 0.62, 95% CI = 0.42 to 0.89; and RR = 0.41, 95% CI = 0.21 to 0.72; for respective sites).

Table 2 also provides the mean and standard deviation (SD) of microbial load (log color-changing units, CCU) of sites among culture-positive fetal units. There was a marked effect of nicotine dose on the microbial load of culture-positive fetal units. Specifically, low-dose nicotine significantly decreased the mean microbial load in the placenta ($P < .01$); microbial load in the amniotic fluid was not impacted by this dose ($P \geq .08$). In contrast, microbial load in the amniotic fluid was significantly increased in the Mid-NIC+MP group ($P = .03$).
Impact of nicotine dose and infection on maternal and fetal inflammation

The maternal and fetal inflammatory response stages (MIR and FIR, respectively) were defined as none (stage 0), mild (stage 1), moderate (stage 2), and severe (stage 3). Stages are shown for Control, Low NIC only, Mid NIC only (Figure 2A and C); and for MP only, Low-NIC+MP, and Mid-NIC+MP (Figure 2B and D) treatment groups. The median MIR stage was not significantly different among nicotine doses in the sham-inoculated ($P = .074$) nor *M pulmonis*-infected groups ($P = .33$, Figure 2A and B). However, it is notable that the distribution of MIR scores in the Low NIC only group are clustered at stage 2 MIR (Figure 2A), without earlier stages represented. Unlike the MIR response, the median FIR stage (Figure 2C and D) was significantly impacted by both nicotine dose and infection. Among sham-inoculated groups (Figure 2C), a subset of fetuses in the Low NIC only group showed moderate to severe FIR, with median stage significantly higher than either the Control ($P < .001$) or Mid NIC only ($P = .006$) groups, in which no fetal units had a fetal inflammatory response. When animals were infected with *M pulmonis* (Figure 2D), no fetal units in the Mid-NIC+MP group developed moderate to severe FIR, which was significantly different from the FIR response in both the MP only ($P < .003$) and Low-NIC+MP ($P < .001$) groups.

MIR and FIR data were divided by high (moderate to severe/stage 2-3), and low (none to early/stage 0-1) fetal units. The relative risk (RR) analyses with 95% confidence intervals (CI) for high MIR and FIR are provided in Table S4. Among the sham-inoculated groups, risks for high MIR and FIR were increased in the Low NIC only group relative to Control (MIR RR = 1.81, 95% CI = 1.24 to 2.40; FIR RR ≥ 2.00, 95% CI = 2.00 to infinity) and Mid NIC only (MIR RR ≥ 1.50, 95% CI = 1.50 to infinity; FIR RR = 2.14, 95% CI = 1.33 to 4.03). Among the *M pulmonis*-infected groups, the risk for high FIR was decreased in the Mid-NIC+MP group fetal relative to both MP only (RR = 0.59, 95% CI = 0.45 to 0.73) and Low-NIC+MP (RR = 0.32, 95% CI = 0.25 to 0.75).
Impact of nicotine dose and maternal and fetal inflammation on *M. pulmonis* colonization of placenta and amniotic fluid

Figure 3 compares maternal and fetal inflammatory response severity among treatment groups and colonization subgroups as defined by presence of *M. pulmonis* in placenta and amniotic fluid. In the MP only as well as in the Mid-NIC+MP groups, moderate to severe MIR was observed in >50% of all fetal units from the infected animals, regardless of placental infection status. However, in the Low-NIC+MP group, placentas with moderate to severe MIR were more likely (*P* < .01) to have culture-negative placentas. A strikingly different pattern among treatment groups was observed for FIR. In the Mid-NIC+MP group, no fetal units showed any evidence of FIR regardless of colonization of the placental and amniotic fluid. In both the MP only and Low-NIC+MP groups, a lower FIR (*P* < .01) was found in fetal units where both the placenta and amniotic fluid were colonized by *M. pulmonis*. The treatment groups also differed with respect to overall placental colonization (Table 2), with *M. pulmonis* isolated from the placenta of 85% of the Mid-NIC+MP group, 64% of the MP only group, and only 45% of the Low-NIC+MP group.

Relative risk (RR) analyses and Chi-square *P* values are provided in Table S5. The risk of placental infection was decreased in Low-NIC+MP fetal units with high MIR (RR = 0.14, 95% CI = 0.02 to 0.69). The risk for placental infection with high FIR was decreased in both MP only (RR = 0.56, 95% CI = 0.29 to 0.95) and Low-NIC+MP (RR = 0.38, 95% CI = 0.12 to 0.93) groups. Although not statistically significant, there appeared to be a trend towards increased risk of placental colonization with high MIR in the Mid-NIC+MP group (RR = 3.11, 95% CI = 0.90 to 10.43). The risk of amniotic fluid infection among placenta-positive fetal units with high FIR was decreased in the MP only group (RR = 0.51, 95% CI = 0.24 to 0.86).
DISCUSSION

Here we report a previously unrecognized effect of low-dose (1 mg/kg/d) nicotine: protection against intrauterine colonization by *M* pulmonis during pregnancy at sites including endometrium, placenta, and amniotic fluid. Further, maternal and fetal inflammatory responses (MIR and FIR, respectively) were altered by low-dose nicotine. In our previous study, we used a higher dose of nicotine (6 mg/kg/d), which resulted in increased infection accompanied by severe inflammation at gestation day (GD) 18. This inflammation, characterized by a copious mucopurulent exudate, was not observed with the lower doses of nicotine used in the present study. In the previous study, the exacerbated maternal inflammatory response likely contributed to necrosis and placental barrier tissue damage, facilitating microbial invasion into the fetal compartment. In the current study, we expected to see incremental increases in infection in a dose responsive manner. While there was no difference in colonization rates between the MP only and Mid-NIC+MP groups, there was a dramatic and unexpected decrease in both maternal and fetal colonization in the Low-NIC+MP group (see Table 2). Notably, all fetal units in the low-dose nicotine group had increased MIR as well as decreased uterine colonization, suggesting that these animals may have been better prepared in general to prevent colonization from an infectious agent. Increased fetal inflammation was not observed in any Control nor mid-dose nicotine fetal units, but an increased FIR was present in a subset of fetal units from the low dose nicotine group. Thus, dams and fetal units receiving low dose nicotine appeared to have higher MIR and FIR, even in the absence of an infectious stimulus, than did other treatment groups.

In the face of *M* pulmonis infection, the overall maternal response pattern was similar with all groups, regardless of nicotine dose. However, the FIR response was quite different. Both the MP only and Low-NIC+MP groups had a subset of fetal units with elevated FIR. In marked contrast, none of the fetal units in the Mid-NIC+MP group had a fetal response. The ability of *M* pulmonis to breach the placental barrier and colonize the amniotic fluid was different across treatment groups (see Table 2), and virtually all fetal units where this had
occurred were characterized by no to mild FIR. Strikingly, the Mid-NIC+MP group did not show any FIR, even in the presence of colonization of 65% of the placentas and a seemingly appropriate MIR response. This suggests that in the Mid-NIC+MP group, the activation of the MIR response was either not adequate to direct the activation of FIR or the fetal tissues were not responsive.

Our findings are consistent with recent studies of transcriptomic changes in innate immune cell types in women with maternal and/or fetal inflammatory responses. Maternal and fetal innate immune cells in the fetal membranes and amniotic fluid appear to drive pro-inflammatory responses in both placental and fetal tissues, and these responses are further layered with complexity if infection is present in the fetal compartment. Within that framework, we suggest that the response to nicotine is not a simple dose continuum as we had originally believed. In the absence of nicotine, no fetal inflammation is present, yet maternal cells can respond effectively to an infectious challenge and direct an appropriate fetal response; the collective response can control or limit the ability of the pathogen to colonize the fetal compartment. At the low dose of nicotine, both the maternal and a subset of individual fetal responses are elevated before the infectious insult, thus permitting a more rapid response and more effective clearance of the pathogen. At the mid dose of nicotine, however, the maternal response does not appear to drive the fetal response, and the fetal response is not activated even in the face of colonization of the fetal compartment. Because the pattern of maternal inflammatory response coupled with the colonization rate of the placentas is similar between MP only and Mid-NIC+MP dams, we suggest that the failure to activate a fetal response may be due to an effect of nicotine at the level of the fetal compartment, though future work will be needed to explore this hypothesis. At the high dose of nicotine, the maternal response is so profound that the tissue damage to the placental barrier facilitates the ability of the pathogen to breach the barrier and gain access to the amniotic fluid and fetus.
There are some caveats with our current study. Unfortunately, a standard histologic scoring system has not been established for rodent maternal and fetal inflammatory responses. Because of the extensive tissue damage in the high-dose nicotine study, our approach in the current study differed slightly with respect to scales and cutoffs used to define severity. Our histologic scoring system also differs from the well-established human criteria in order to accommodate the anatomic differences in rodents, including the prominent mesometrial triangle and the apposition of the endometrium to the yolk sac membranes. In our experience, the assessment of intrauterine inflammation at the multiple distinct sites included in our analyses was necessary to fully characterize the host responses.

The present study also differed from other animal studies of nicotine and infection or inflammation. In pregnant rat models, 1 mg/kg/d nicotine administered acutely within 48 hours prior to, or 72 hours after, lipopolysaccharide (LPS) toxin exposure reduced proinflammatory cytokines in placental tissue and plasma; decreased leukocyte infiltration in placental tissue, and restored normal litter size and fetal weight. Our model deviates from the acute nicotine LPS model described above in two major aspects. First, we used chronic and continuous administration of nicotine beginning 8 days prior to insult. Second, our use of a live microbe may have resulted in different immune modulatory effects than that of the toll-like receptor (TLR)-4-specific binding in the LPS model. For example, Ureaplasma spp. surface lipoproteins and whole bacterium are detected by binding of TLR-2/6 and 9, respectively, additionally, recognition of various Mycoplasma spp. relies on TLR-2 in combination with either TLRs 1 or 6. This creates an additional layer of complexity, as chronic nicotine exposure may differentially alter receptor expression or functionality.

Our model takes advantage of the similar reproductive effects of the human reproductive pathogen U parvum and the rat reproductive pathogen M pulmonis. Because the rat is the natural host to M pulmonis, we believe the tropism, colonization, and disease
Pathogenesis is more organic to the human host-pathogen relationship which we are modeling. While experimental infections using human pathogens are valuable, a rodent-adapted human pathogen may not capture the full host-pathogen relationship. Routes of infection, including both intravaginal and intravenous inoculation, have been defined for this model previously. Since the microbial load in maternal and fetal tissues was a critical outcome to permit assessment of treatment effects, it was imperative to ensure that each dam in each treatment group received the same infectious dose at the same gestational time. In order to establish consistent placental/fetal infections by the vaginal route, dams have to be infected 10 days prior to breeding. Therefore, using intravaginal inoculation to model an ascending route of infection would not allow us to know the exact timing of exposure to *M. pulmonis* or infectious dose to which the placenta and fetal unit are exposed. Similarly, using direct instillation of *M. pulmonis* into the amniotic fluid would bypass the placental barrier and potentially abrogate our ability to assess the impacts on maternal sites. Based on our previous studies, intravenous administration of *M. pulmonis* at GD 14 ensures that all dams and fetal units have an equal chance of becoming infected. The integrity of the cervical barrier is a key factor in limiting ascending infections in mouse models, and a caveat to our approach is that it does not allow us to assess the impact of nicotine on the cervix. However, once the cervical barrier is breached, the uterus must be colonized and the placental barrier also must be breached. Our model was designed to permit us to assess the impact of nicotine on colonization of both the uterus and the placental, but not the cervical, barrier.

In summary, we demonstrated an unexpected protective effect of low-dose nicotine on intrauterine infection. At higher nicotine doses, the ability to generate a strong fetal inflammatory response was impacted, which may in turn impact pregnancy outcome, fetal and neonatal development and programming, and adult postnatal outcomes. While we believe studies of animal models and their natural pathogens are essential to discovery, we also recognize animal models of human pathogen infection, in vitro study of human tissues
and primary cells, clinical case studies, and epidemiologic analyses are necessary to put our findings in context. The low-dose nicotine (1 mg/kg/d dose) used in the present study was chosen to model clinically relevant exposure levels comparable to nicotine replacement therapies. While the safety and efficacy of nicotine replacement therapy used in smoking cessation for pregnant women is still an issue of debate, a recent review suggests evidence is accumulating which would argue for a more favorable cost-benefit ratio. At this time, the current recommendations from the American College of Obstetricians and Gynecologists include conditional use of nicotine replacement therapy to aid in smoking cessation in pregnant women. The results of our study suggest that the implications of intrauterine infections and inflammation should be considered when studying nicotine replacement therapy use during pregnancy. Continued research on the mechanisms by which nicotine alters the response to infection as well as the in utero and postnatal impacts of nicotine exposure is vital for navigating the growing epidemic of e-cigarette use among youth and for making informed decisions regarding nicotine replacement therapy use for smoking cessation during pregnancy.
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DECLARATIONS OF INTEREST

The authors declare no conflict of interest.

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Litter size and fetal weight for each treatment group are shown. Litter size is the count of fetuses in the litter at necropsy on gestation day 18. Resorptions (early fetal losses) were not counted. Median fetal weight is the median fetal body weight in grams of fetuses pooled across litters in the same treatment group. A two-way ANOVA of nicotine dose and infection demonstrated that there were no significant contributions of these factors to the variation of litter size (P > .36). The Kruskal-Wallis nonparametric test found significant variation in pooled fetal body weights among treatment groups (P < .001). The Dunn’s test compared each treatment group to the Control group. Min = minimum of range; max = maximum of range. Q₁ = 1st quartile/25th percentile; Q₃ = 3rd quartile/75th percentile. Body weight data was not available for some fetuses.

| Treatment group | Median litter size (min-max) | N  | Median fetal weight (Q₁ – Q₃) | N  | P value |
|-----------------|-----------------------------|----|------------------------------|----|---------|
| Control         | 12 (9-14)                   | 5  | 1.69 (1.55-1.81)             | 35 | NA      |
| Low NIC only    | 14 (11-14)                  | 3  | 1.59 (1.50-1.67)             | 39 | .28     |
| Mid NIC only    | 11 (10-13)                  | 3  | 1.56 (1.47-1.67)             | 34 | .08     |
| MP only         | 12 (10-16)                  | 9  | 1.56 (1.48-1.64)             | 110| .004    |
| Low-NIC+MP      | 13 (11-13)                  | 5  | 1.69 (1.41-1.85)             | 62 | .70     |
| Mid-NIC+MP      | 12 (4-14)                   | 6  | 1.52 (1.44-1.59)             | 65 | < .001  |
Table 2. Culture results for maternal systemic and intrauterine sites

| Site              | MP only | Low-NIC+MP | Mid-NIC+MP | P value |
|-------------------|---------|------------|------------|---------|
| Spleen            | 7 of 9  | 5 of 5     | 6 of 6     | .49<sup>a</sup> |
| Vagina            | 6 of 9  | 2 of 5     | 4 of 6     | .57     |
| Endometrium       | 9 of 9  | 2 of 5     | 6 of 6     | .03<sup>a</sup> |
| Placenta          | 66 of 111 | 24 of 62  | 40 of 65   | .01     |
| Amniotic Fluid    | 38 of 109 | 8 of 62   | 25 of 63   | <.01    |

Microbial load mean ± SD log CCU

| Site              | Mean ± SD |
|-------------------|-----------|
| Spleen            | 1.6 ± 1.0 |
| Vagina            | 2.8 ± 1.8 |
| Endometrium       | 3.4 ± 2.4 |
| Placenta          | 3.8 ± 2.0 |
| Amniotic Fluid    | 1.7 ± 1.2 |

Colonization rate (% culture-positive) for maternal systemic sites (spleen, vagina) and intrauterine sites (endometrium, placenta, and amniotic fluids). Arrows and bold text indicate a significant difference as compared to at least one other group. P values for the χ² test are reported; where conditions for the χ² test were not met, a Fisher’s exact test was performed between each group (lowest P values are reported and indicated by superscripted letter a). For spleen colonization rate, MP only vs. Low-NIC+MP P = .50; MP only vs. Mid-NIC+MP P = .49; Low-NIC+MP vs. Mid-NIC+MP P > .999. For endometrium colonization rate, MP only vs. Low-NIC+MP P = .03; MP only vs. Mid-NIC+MP P > .999; Low-NIC+MP vs. Mid-NIC+MP P = .06.

Microbial load (mean ± standard deviation (SD) log color changing units (CCU)) for culture-positive fetal units for each site. P values in the table were derived from Kruskal-Wallis test. Where P < .05, the Dunn’s multiple comparison test was performed between all groups and P values are as follows. For placental microbial load, MP only vs. Low-NIC+MP P = .01; MP only vs. Mid-NIC+MP P > .999; Low-NIC+MP vs. Mid-NIC+MP P < .01. For amniotic fluid microbial load, MP only vs. Low-NIC+MP P > .999; MP only vs. Mid-NIC+MP P = .03; Low-NIC+MP vs. Mid-NIC+MP P = .08. Sample sizes are indicated by the numerator value in the percentage culture-positive portion of the table.
FIGURE LEGENDS

Figure 1: Definition of maternal and fetal inflammatory response (MIR and FIR) stages. The figure depicts the tissues of the maternal-fetal unit including the uterus, placenta, umbilical cord, and fetal membranes. Black boxes (MIR sites) and lines (FIR sites) indicate the various sites evaluated for stage of neutrophil infiltration (sites affected) and number of markers of chronicity for scoring purposes. Stage 1 MIR: endometrial epithelium surrounding the attachment site of the placenta (1). Stage 2 MIR: mesometrial triangle (2a) decidua (2b) and/or the labyrinth zone (2c). Stage 3 MIR: decidua-yolk sac junction and/or the luminal surface of the parietal and visceral yolk sac (PYS and VYS) membranes. Stage 1 FIR: chorionic vessels at the level of the chorionic plate. Stage 2 FIR: umbilical vein and/or arteries. Stage 3 FIR: Wharton’s jelly. H&E stain. Photomicrograph captured by Image Scope software viewing an Aperio-scanned slide. Scale bar is 4 mm.

Figure 2: Maternal and fetal inflammatory responses (MIR: panels A and B; and FIR: panels C and D, respectively) for sham inoculated (A, C) and infected (B, D) treatment groups, respectively. The medians and quartiles are denoted by thick and thin dashed lines respectively. Outliers were identified by the ROUT method (Q = 1%) and eliminated prior to analyses. The Kruskal-Wallis and Dunn’s tests were performed and significant differences and P values are noted. The number of maternal-fetal samples included in the MIR analysis were Control: n = 26; Low NIC only: n = 13 with 3 outliers removed; Mid NIC only: n = 11; MP only: n = 49; Low NIC + MP: n = 22; Mid NIC + MP: n = 31. The number of maternal-fetal samples included in the FIR analysis were Control: n = 18 with 8 outliers removed; Low NIC only: n = 13 with 3 outliers removed; Mid NIC only: n = 8 with 3 outliers removed; MP only: n = 49; Low NIC + MP: n = 22; Mid NIC + MP: n = 21 with 9 outliers removed.

Figure 3: High vs. low maternal and fetal inflammatory responses (MIR and FIR) observed with progressive *M. pulmonis* (MP) colonization of fetal sites. MIR and FIR data are represented as percent of fetal units with moderate to severe response (“high”, stage 2-3,
darker fill), or none to mild stage response ("low", stage 0-1, lighter fill). Colonization is presumed to progress from maternal to placental to amniotic fluid sites. Fetal units within each nicotine dose group were divided into subgroups based on MP culture result of placental (PL) and amniotic fluid (AF) sites, either MP culture-positive (-pos) or MP culture-negative (-neg). The N indicates the number of fetuses having a given colonization profile that also had a tissue sample available for MIR and FIR staging. The Chi-square analysis was not valid for differences among subgroups due to some having few numbers in each high vs low or colonization category. The Fisher exact test was used to compare the high versus low stage of inflammatory response between two subgroups within each nicotine dose. The asterisks and bars represent significant differences (α = 0.05; one * for \(P\)-values <.05; two ** for \(P\) < .01, and three *** for \(P\) < .001).
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Figure 1

Stage 1 MIR
Endometrium

Stage 2 MIR
a. Mesometrial triangle
b. Decidua
c. Subchorion (labyrinth)

Stage 3 MIR
a. Decidua-yolk sac junction
b. PYS, VYS

1 FIR: Stage Chorionic vessels
2 FIR: Umbilical vein/arteries
3 FIR: Wharton's jelly
Figure 3

- **MIR**
  - MP only
    - PL-neg/AF-neg (N = 17)
    - PL-pos/AF-neg (N = 11)
    - PL-pos/AF-pos (N = 19)
  - Low NIC + MP
    - PL-neg/AF-neg (N = 12)
    - PL-pos/AF-neg (N = 7)
    - PL-pos/AF-pos (N = 3)
  - Mid NIC + MP
    - PL-neg/AF-neg (N = 6)
    - PL-pos/AF-neg (N = 6)
    - PL-pos/AF-pos (N = 15)

- **FIR**
  - MP only
    - PL-neg/AF-neg (N = 17)
    - PL-pos/AF-neg (N = 11)
    - PL-pos/AF-pos (N = 19)
  - Low NIC + MP
    - PL-neg/AF-neg (N = 12)
    - PL-pos/AF-neg (N = 7)
    - PL-pos/AF-pos (N = 3)
  - Mid NIC + MP
    - PL-neg/AF-neg (N = 6)
    - PL-pos/AF-neg (N = 6)
    - PL-pos/AF-pos (N = 15)

- Bar graphs show the percentage of fetal units in different conditions.
- Moderate/severe (stage 2-3)
- None/mild (stage 0-1)