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In Utero Nicotine Exposure Promotes M2 Activation in Neonatal Mice Alveolar Macrophages

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Abstract

Background—Maternal smoking *in utero* has been associated with adverse health outcomes including lower respiratory tract infections in infants and children, but the mechanisms underlying these associations continue to be investigated. We hypothesized that nicotine plays a significant role in mediating the effects of maternal tobacco smoke on neonatal alveolar macrophage (AM) function, the resident immune cell in the neonatal lung.

Methods—Primary AMs were isolated at postnatal day 7 from a murine model of *in utero* nicotine exposure. The murine AM cell line MH-S was used for additional *in vitro* studies.

Results—*In utero* nicotine increased IL-13 and transforming growth factor beta one (TGFβ1) in the neonatal lung. Nicotine-exposed AMs demonstrated increased TGFβ1 and increased markers of alternative activation with diminished phagocytic function. However, AMs from mice deficient in the α7 nicotinic acetylcholine receptor (α7 nAChR) had less TGFβ1, reduced alternative activation and improved phagocytic functioning despite similar *in utero* nicotine exposure.

Conclusion—*In utero* nicotine exposure, mediated in part via the α7 nAChR, may increase the risk of lower respiratory tract infections in neonates by changing the resting state of AM towards alternative activation. These findings have important implications for immune responses in the nicotine-exposed neonatal lung.

INTRODUCTION

Despite the well-known health risks of smoking, the use of cigarettes and tobacco continues to contribute significantly to healthcare problems in our society. Twenty-two percent of women of reproductive age smoke cigarettes exposing nearly 20% of pregnancies to this toxin (1–3). It is clear that *in utero* and postnatal exposure to smoking increases the risk of

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serious lower respiratory tract infections in infants and children (4). Specifically for the newborn, smoking during pregnancy increases the risks of prematurity, alters immune defenses, and increases risk factors for neonatal sepsis (5, 6). Despite advances in neonatal intensive care, infection increases mortality and morbidity, particularly for the premature newborn (7, 8).

Nicotine, the fat soluble and addictive component of tobacco, readily crosses the placenta, is present in the amniotic fluid, and adversely affects lung development (9). Levels of nicotine and its metabolites in the fetus closely mirror those observed in maternal plasma(10). *In utero* nicotine exposure alters multiple developing organs including the lung and affects branching morphogenesis (11–13). The effects of nicotine on the developing lung are likely mediated through theα7 nicotinic acetylcholine receptor (nAChR), one of a family of cation channels predominantly found in the nervous system (12, 14). However, the direct effects of nicotine or the role of the α7nAChR on inflammatory-mediated functioning of the resident immune cell of the lung, the alveolar macrophage (AM), are not fully known.

Several pathways govern the activation states of the normally resting AM (15). Classical activation of AM (TH1 response or M1 macrophage) is predominantly characterized by a robust cellular response to eliminate microbes with efficient phagocytosis, respiratory burst, and the generation of pro inflammatory cytokines. In contrast, alternative activation of AM (TH2 response or M2 macrophage) is currently defined by signature gene expression profiles and response to pathogenic stimuli that have been observed in mostly murine models. While the definition of alternative activation has been evolving, alternatively activated macrophages are presently characterized by a dampening of the immune response with decreased phagocytosis and respiratory burst. While the classical pathway involves microbial stimuli and efficient clearance of microbes, the alternative pathway can skew the AM response away from microbial clearance to leave the lung more susceptible to infections if the M2 phenotype persists. Another described feature of M2 activation is increased expression and activity of arginase 1 which shunts arginine away from the production of the antimicrobial nitric oxide and promotes remodeling and repair through enhanced collagen deposition (15).

In this study, we *hypothesized* that exposure to nicotine *in utero* places the neonate at risk for respiratory infections by influencing AM function through alternative activation (M2 activation). Using the mouse macrophage cell line MH-S and a mouse model of *in utero* nicotine exposure, the goals of this study were to: 1) determine whether chronic nicotine exposure polarized the AM towards an M2 phenotype and 2) whether these changes were mediated via the α7 nAChR.

RESULTS

Nicotine exposure increased α7 nAChR on the AM

By western blot techniques, chronic nicotine exposure to the MHS cell line significantly increased the protein expression of α7 nAChR 1.3-fold compared to control cells (Figure 1A). Similarly, primary P7 AM exposed to *in utero* nicotine demonstrated significant increase in α7 nAChR immunofluorescence as compared to control (Figure 1B).

In utero nicotine skewed cytokines in the neonatal lung and the AM towards TH2 via the α7 nAChR

At baseline, neonatal lungs exposed to nicotine demonstrated significantly increased IL-13 and decreased IL1β at the mRNA level at P7 (Figure 2A–B), suggesting that the cytokine milieu of the nicotine-exposed lung was shifted towards TH2. This shift towards TH2 was supported by significantly increased active TGFβ1 in the ELF of P7 pup exposed to *in utero* nicotine (Figure 3). The α7 nAChR modulated these nicotine-induced changes in active TGFβ1, since nicotine did not induce active TGFβ1 in the ELF of the α7 nAChR$^{-/-}$ neonatal mice.

The neonatal AM contributed to these nicotine-induced changes in IL-13 and TGFβ1. Nicotine *in vitro* significantly increased IL-13 and TGFβ1 in MHS cells at the mRNA level (Figure 4A and B). Blockage of the α7 nAChR with the addition of αBGT significantly blunted nicotine effects on both IL-13 and TGFβ1 mRNA. Furthermore, primary P7 AM demonstrated a significant increase in TGFβ1 via immunohistochemistry (Figure 4C), which was significantly attenuated in the α7K/O despite *in utero* nicotine exposure.

Nicotine exposure induced alternative activation in the AM via the α7 nAChR

In vitro exposure to nicotine induced markers of alternative activation in the MHS cell as demonstrated by significant increases in arginase-1 (Arg1), YM1 and FN at the mRNA level (Figure 5A–C). Correspondingly, *in utero* nicotine also demonstrated significant increases in these markers of alternative activation (Figure 5D–F). Blockade of the α7 nAChR with αBGT blunted *in vitro* nicotine-induced increases in Arg1, YM1 and FN on the MH-S cell line. Similarly, nicotine-induced increases in these markers were blocked in the P7 α7 nAChR deficient AM.

Nicotine-exposed AM promote FN expression in a paracrine fashion

In addition to increased FN induced by nicotine exposure, AM treated with nicotine *in vitro* can also promote FN transcription in adjacent cells. MH-S cells treated with nicotine *in vitro* and cultured on permeable membrane supports separating them from NIH 3T3 fibroblasts permanently transfected with a FN-luciferase reporter were able to induce fibronectin transcription in fibroblasts, as evidenced by increased luciferase production by the transfected NIH 3T3 cells (Figure 6). These effects were dependent onα7 nAChR signaling, since concurrent incubation with αBGT abrogated the nicotine effect. Increased fibronectin transcription was absent in fibroblasts cultured without MH-S cells in the membrane supports, demonstrating the nicotine exposed MH-S cells were capable of promoting fibronectin transcription in fibroblasts. Nicotine exposed MH-S cells also affected type I collagen expression in fibroblasts.

In utero nicotine impaired AM phagocytic function via the α7 nAChR

Lastly, we evaluated the effects of *in utero* nicotine exposure on the phagocytic function of the primary neonatal AM. *In utero* nicotine exposure significantly impaired the ability of the neonatal AM to phagocytose inactivated *staph aureus* by approximately 50% (Figure 7). Despite nicotine exposure, AM lacking the α7 nAChR maintained phagocytosis at control levels, suggesting that nicotine-induced deranged phagocytic function was modulated via the α7 nAChR.

DISCUSSION

Despite the known risk of cigarette smoke exposure on childhood health, maternal use of cigarettes during pregnancy remains an issue with approximately 10–12% of pregnant women admitting to tobacco use (16). Use of cigarettes during pregnancy exposes the developing neonatal lung to nicotine. There remains a gap of knowledge regarding the specific effects of nicotine on the characteristics and functioning of the developing AM, the resident immune cell in the lung. The current study demonstrated that *in utero* nicotine exposure induced a TH2 milieu in the neonatal lung at baseline and skewed the resting state of the neonatal AM towards M2 activation, impairing phagocytic function. The α7 nAChR played an important role in modulating nicotine's effect as demonstrated by the effects of αBGT *in vitro* and the attenuation of nicotine's effect *in vivo* in the α7 nAChR deficient neonatal mouse.

The nAChRs are found in a number of cell types and non-neuronal organs, including the lung. Within the lung, nAChRs are present in epithelial cells, fibroblasts, smooth muscles cells and AM(12, 14). We have previously demonstrated that nicotine increased branching morphogenesis of the developing lung in the pseudoglandular stage through α7 nAChR-mediated signals (12). Others have shown that nicotine-inducedα7 nAChR expression in fetal lung, particularly around the airways (14). α7 nAChRs have been found in peripheral blood monocytes and macrophages and they are capable of influencing immune responses (17). An anti-inflammatory role for the α7 nAChR has been noted since a specific α7 nAChR agonist decreased TNF-α release in the lung, while α7 nAChR agonists, including nicotine, also inhibited NF-κB activity and LPS-induced release of TNF-α in the lung(18). However, literature also demonstrates that α7 nAChR has been associated with pro-inflammatory effects. Prostaglandins and cyclo-oxygenase 2 expression were enhanced by activation of α7 nAChR, whereas nicotine induced NF-κB and inducible nitric oxide synthase (iNOS) in peritoneal macrophages in a model of atherosclerosis (19, 20). Expression of nAChRs was upregulated by TNF-α, supporting a potential link between inflammation and nAChRs (21). For the developing AM, the current study suggested that nAChR activation via *in utero* nicotine shifted the baseline cytokine milieu towards TH2, increased TGFβ1 and skewed the resting AM state towards M2.

Currently available knowledge suggests the skewed responses of alternatively activated macrophages may leave the lung more susceptible to infections and remodeling. As a professional phagocyte within the lung, the AM patrols the lung, defending it against foreign particles and infection by initiating immune responses, participating in phagocytosis and particle clearance, and orchestrating subsequent inflammatory processes (22). Studies have demonstrated that complete absence of macrophages led to dramatically increased mortality after bacterial infection (23). Alternatively activated macrophages have also been shown to promote fibroblast proliferation as well as production of collagen and fibronectin production, extracellular matrix proteins important in remodeling (24). The current study shows that nicotine-induced alternative activation of the AM leads to increased FN within the alveolar macrophage and induction of FN transcription in fibroblasts in the setting of increased TGFβ1, a well known stimulus for fibronectin expression, potentially to promote airway remodeling (25). These effects on airway remodeling in the neonatal lung require additional investigation.

The nicotine-exposed neonatal lung and AM were hallmarked by increased TGFβ1, a well described anti-inflammatory mediator in monocytic cells and AM(26, 27). Furthermore, $TGF\beta_1$ contributes to the development of chronic lung disease (bronchopulmonary dysplasia) in the premature lung (28, 29). In a neonatal hyperoxia model, neutralization of $TGF\beta_1$ improved alveologenesis and microvascular development (30). Taken together, our data suggests that not only are the AM functionally impaired with *in utero* nicotine exposure, but the lung parenchyma and vasculature are at greater risk for injury in the setting of increased $TGF\beta_{1.}$

Many studies focus on the effects of cigarette smoke exposure on the lung, but the complex nature of cigarette smoke can potentially impede full mechanistic understanding of pathophysiology. In other examinations of adult human AMs, adult human AMs exposed to cigarette smoke similarly exhibit signs of M2 activation (31, 32). Taken together with previously published literature, our findings support the possible role of nicotine as a significant player in the pathophysiology behind the adverse effects of prenatal tobacco smoke exposure on the fetus since the effects from nicotine exposure alone mimic those seen with tobacco smoke exposure. Although cessation of cigarette smoking is advocated to all pregnant women, our results suggest that nicotine replacement therapy as a strategy for smoking cessation therapy may not be desirable in this population and alternative smoking cessation therapies may need to be considered.

MATERIALS AND METHODS

All animal and experimental protocols were approved by the Emory University Institutional Animal Care and Use Committee and the Office of Biosafety.

Model of *in utero* nicotine exposure and timed breeding

Female C57BL6/J mice and $Chrna7^{-/-}$ mice (α7 $nAChR^{-/-}$, α7K/O in C57BL6/J background, Jackson Laboratories, Bar Harbor, ME) were administered ± nicotine (100 μg/ml) in the drinking water ad libitum for 6–8 weeks prior to timed breeding. Pregnant female mice continued to drink nicotine treated water throughout the pregnancy. In this model, pregnant female mice are able to establish a steady state plasma level of nicotine similar to that seen in heavy smokers (12, 33). Post-natal day 0 (P0) was determined by the day of birth. Pups were evaluated at post-natal days 7 (P7). 100% of the pups in the nicotine group remained exposed to nicotine through maternal breast milk until P7.

In vitro Nicotine exposure

The MH-S murine AM cell line (ATCC, Manassas, VA) was cultured in RPMI media containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were exposed to ± nicotine (50 μg/ml) for 2–48 hrs. In some experiments, the cells were incubated with α-bungarotoxin (αBGT, 5 nM), an inhibitor of α7 nAChR (34).

Recovery of primary AM and epithelial lining fluid (ELF)

AM were isolated from P7 neonatal pups as previously described (27).

Measurement of ELF cytokine levels

Pooled pup ELF was evaluated for IL-13 and active TGF-β1 via commercial ELISAs per the manufacturers' instructions (IL-13- R&D Systems, Minneapolis, MN, Active TGFβ1- Promega, WI). Values were normalized to protein as determined by the BCA protein assay (Bio-Rad, Hercules, CA). Data are presented as the mean (IL-13 or TGF-β1 in pg/μg protein) ± SEM (27).

Measurement of α7 nAChR

Levels of AM α7 nAChR were measured by Western blot analysis as previously described (35, 36). Primary P7 mouse AM obtained from pooled bronchoalveolar lavages were evaluated via immunofluorescence for α7 nAChR and markers of M2 activation using methods previously described (27).

RNA extraction, semi-quantitative bioluminescent, and quantitative real time RT-PCR

The determination of mRNA levels was done by a semi-quantitative bioluminescence-based RT-PCR assay and quantitative real time RT-PCR as previously described (12). The primers used were synthesized based on GenBank published sequences: IL-13: 5′ GGAGCTGAGCAACATCACACA 3′ and 5′ GGTCCTGTAGATGGCATTGCA 3′; TGF-β1: 5′ CCCACTCCCGTGGCTTC 3′ and 5′ TAGTAGTCCGCTTCGGGCT 3′; IL1-β: 5′ GAGCACCTTCTTTTCC 3′ and 5′ GGAAAAAGAAGGTGGTC 3′; Ym1/2: 5′ TTATCCTGAGTGACCCTTCTAAG 3′ and 5′ TCATTACCCAGATAGGCATAGG 3′; arginase I: 5′ TGGACCTGGCCTTTGTTGA 3′ and 5′ GGTTGTCAGGGGAGTGTT 3′;fibronectin: 5′ CTGTGACAACTGCCGTAG 3′ and 5′ACCAAGGTCAATCCACAC 3′.

Fibronectin luciferase assay

MH-S cells were cultured on a permeable membrane Transwell® support with NIH 3T3 fibroblasts permanently transfected with the fibronectin promoter attached to a luciferase reporter (FN-Luc) in the bottom well. After overnight serum starvation, MH-S cells on the permeable membrane were then treated with 5 nM αBGT for 1 hour prior to addition of 50 μg/ml of nicotine. After 24 hours, the MH-S cells and membrane supports were removed and fibronectin transcription in the transfected 3T3 fibroblasts was measured by luciferase activity. Relative luciferase units were recorded and are presented as mean ± SD (37).

Measurement of phagocytosis

Phagocytosis was measured in freshly isolated P7 AM as previously described (27, 38).

Statistical Analyses

Sigma Stat for Windows (Systat Software, San Jose, CA) was used for all statistical analyses. ANOVA or ANOVA on Ranks was used as appropriate. Student Newman Keul's or Dunn's test was used for multiple comparisons, respectively. A $p \leq 0.05$ was considered significant. Each *n* represents a separate mouse litter or a separate experimental condition.

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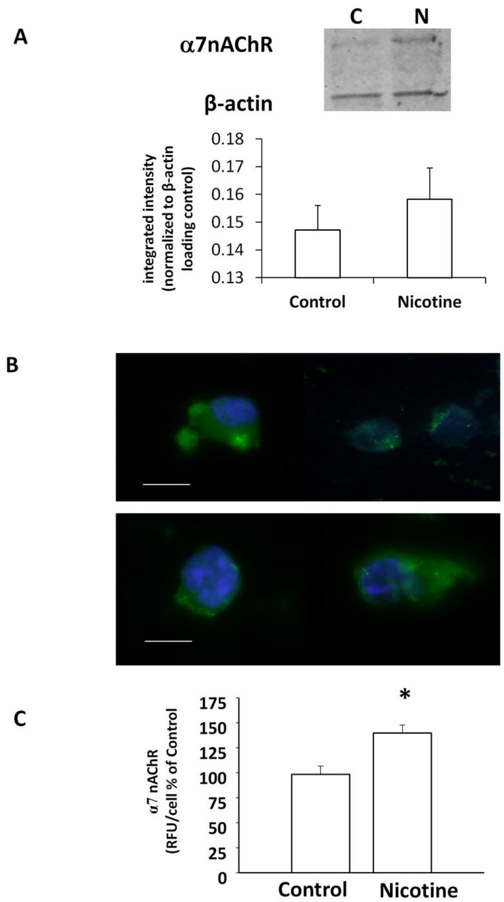

Figure 1. Nicotine induced α7 nAChR expression in alveolar macrophages

Cultured murine alveolar macrophages (MH-S cells) were serum starved overnight and treated with 50 μg/ml of nicotine for 24 hours. By Western blot (n=4), nicotine (**N**) treatment increases expression of α 7 nAChR in MH-S cells after 24 hours when compared to untreated control (**C**)(1A). Female C57 BL/6J mice were administered 100 μg/ml of nicotine in the drinking water for 6–8 weeks prior to timed breeding. AM were harvested from the offspring at P7. In AM harvested from pups exposed to nicotine (bottom color panel, 1B), expression of α7 nAChR in relative fluorescent units per cell (RFU/cell) was significantly increased compared to untreated control (top color panel, 1B) when analyzed with confocal microscopy (1C). * $p<0.05$ vs. Control, 4 separate litters in each condition were analyzed; image is representative image at 100X magnification. Scale bar represents 5 μm.

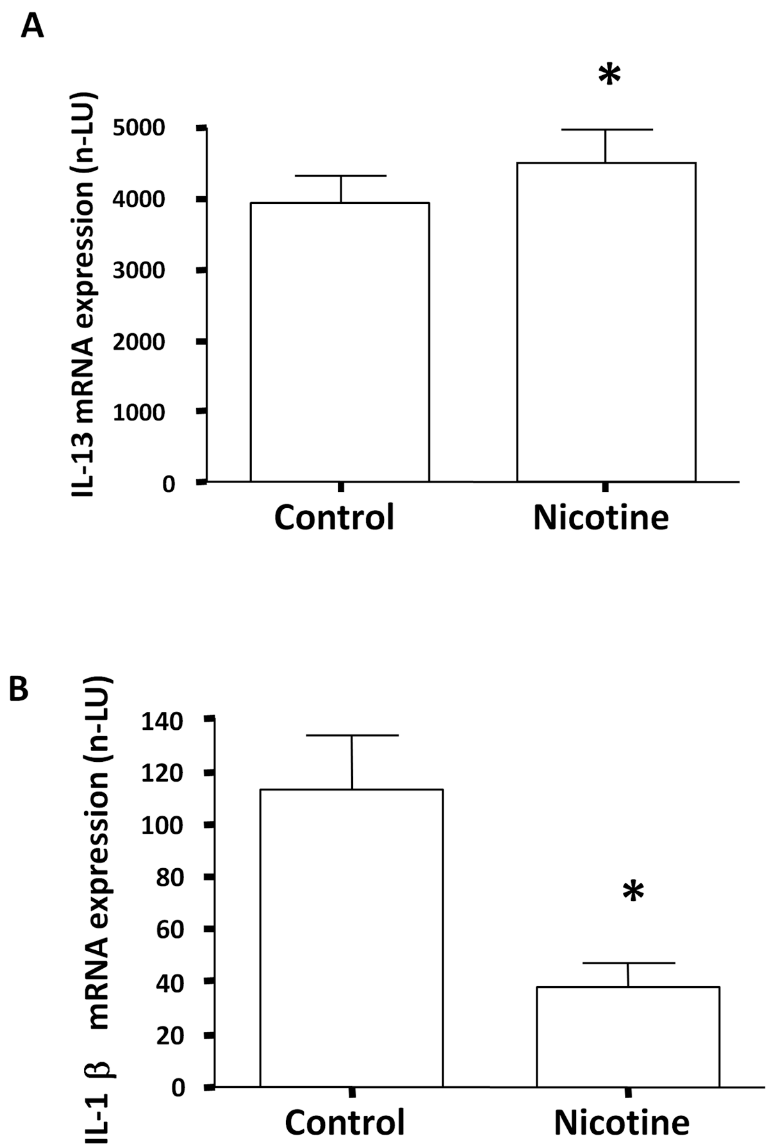

Figure 2. Neonatal lungs exposed to nicotine *in utero* exhibited a shift towards a Th2 profile

Female C57 BL/6J mice were administered 100 μg/ml of nicotine in the drinking water for 6–8 weeks prior to timed breeding. Whole lung homogenates were collected from the offspring at P7 for mRNA analysis. Using semi-quantitative bioluminescent RT-PCR, P7 lungs had increased mRNA expression of IL-13 (2A) and decreased mRNA expression of IL-1β (2B). * $p<0.05$ vs. Control. n=4. n-LU denotes luciferase units normalized to endogenous loading control.

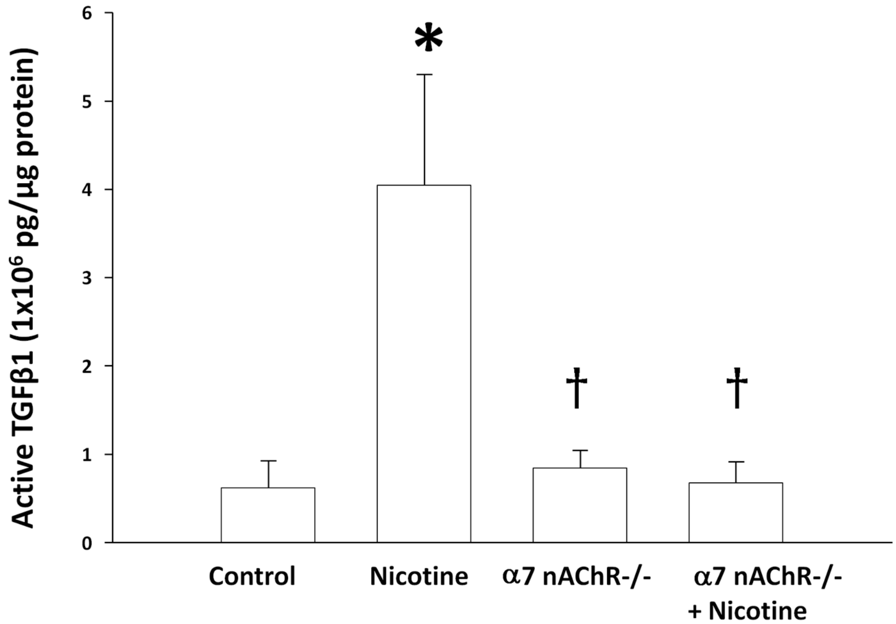

Figure 3. α7 nAChR signaling mediated increased active TGFβ1 found in the alveolar ELF of P7 pups exposed to nicotine *in utero*

Alveolar ELF was collected from P7 litters and pooled for measurement of active TGFβ1 by ELISA. *In utero* nicotine exposure induced increased active TGFβ1 expression in P7 pups. *In utero* nicotine exposure did not induce active TGFβ1 expression in α7 nAChR$^{-/-}$ pups. * $p<0.05$ vs. Control, †$p<0.05$ vs. Nicotine. Minimum of 4 separate litters in each experimental condition..

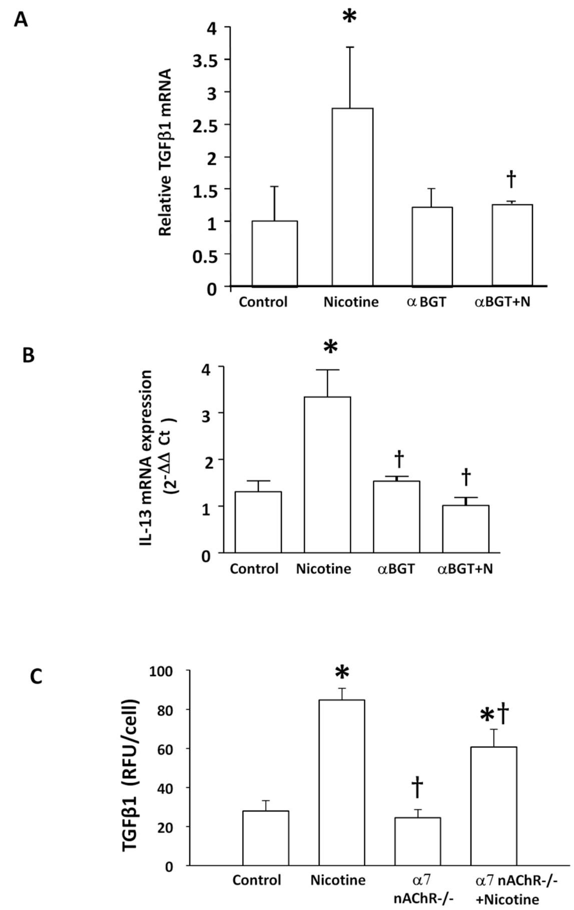

Figure 4. Nicotine-induced increases in mRNA expression of TGFβ1 and IL-13 was dependent on α7 nAChR mediated signals

MH-S cells were serum starved overnight and treated with 50 µg/ml of nicotine (**N**) after 1 hour pre-treatment with 5 nM of α-bungarotoxin (αBGT), a snake venom toxin that is an inhibitor of α7 nAChR. Cells were harvested after 24 hours for mRNA analysis using quantitative real-time PCR. Nicotine induced mRNA expression of TGFβ1 (4A) and IL-13 (4B) and pre-treatment with αBGT blocked induction of TGFβ1 (4A) and IL-13 (4B) by nicotine. Primary AM were harvested from the offspring at P7. Immunofluorescent staining for TGFβ1 was significantly increased after *in utero* nicotine exposure in primary AM while TGFβ1 was not increased in α7 nAChR$^{-/-}$ pups despite nicotine exposure (4C). * $p<0.05$ vs. Control, †$p<0.05$ vs. Nicotine. 3–4 separate litters in each experimental condition.

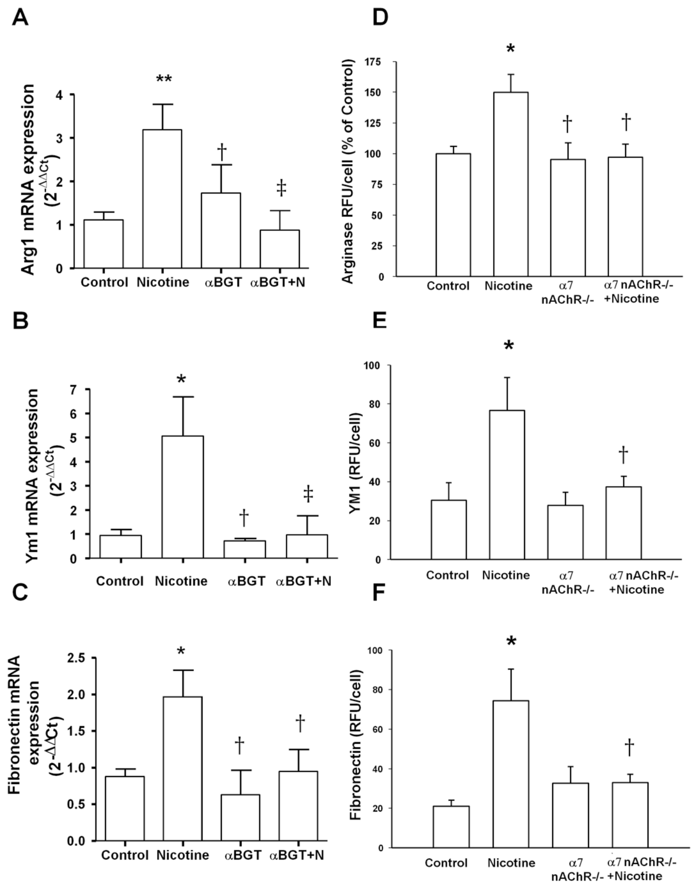

Figure 5. Nicotine promoted M2 activation of AM *in vitro* and *in vivo*

Primary AM were harvested at P7. In addition, MH-S cells were serum starved overnight and treated with 50 μg/ml of nicotine after 1 hour pre-treatment with 5 nM of αBGT. Cells were harvested after 24 hours for mRNA analysis using quantitative real-time PCR. Nicotine induced mRNA expression of the following markers of M2 activation: Arg1, Ym1, and fibronectin (5A–C, respectively). Similarly, increased expression of Arg1, Ym1 and fibronectin were seen by immunofluorescent staining in relative fluorescent units per cell (RFU/cell) using confocal microscopy in primary AM at P7 (5D–F, respectively). Pre-treatment with αBGT blocked in the nicotine induced changes in mRNA expression. *In utero* nicotine exposure did not induce changes in mRNA expression of Arg1, Ym1, and fibronectin in primary AM from α7 $nAChR^{-/-}$ pups. *p<0.05 vs. control, **p<0.01 vs. control, †p<0.05 vs. Nicotine, ‡p<0.01 vs. Nicotine. Figure is a composite of 3–5 separate litters in each experimental condition.

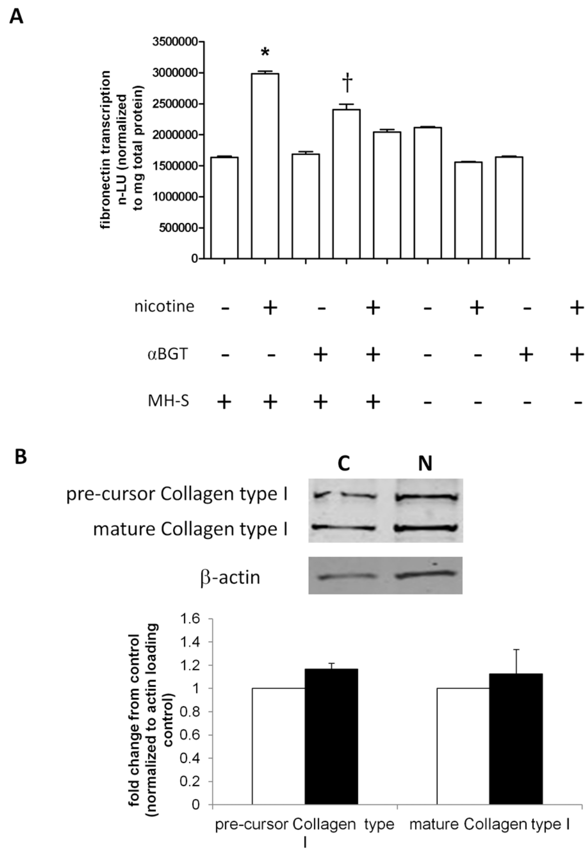

Figure 6. Nicotine promotes expression of extracellular matrix proteins associated with alternative activation

MH-S cells were serum starved overnight, pre-treated with 5 nM of BGT to inhibit α7 nAChR, then treated with 50 μg/ml of nicotine and co-cultured with fibroblasts permanently transfected with a fibronectin-luciferase reporter for 24 hours. MH-S cells treated with nicotine promoted transcription of fibronectin in co-cultured fibroblasts as measured by luciferase assay (6A). Pre-treatment with αBGT blocked in the nicotine induced increase in fibronectin transcription. Collagen type I expression was also analyzed in the MH-S cells co-cultured with fibroblasts in Figure 6A. Nicotine treated MH-S cells (**N, black bars**) also had increased collagen type I expression by Western blot analysis after 24 hours when compared to untreated MH-S (**C, white bars**) (6B). Image is a representative Western blot. * $p<0.05$ vs. Control, †$p<0.05$ vs. Nicotine. n=3.

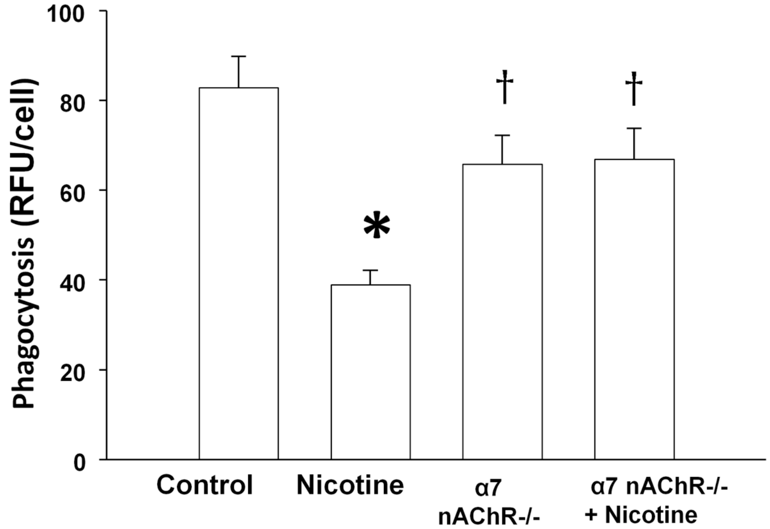

Figure 7. *In utero* nicotine exposure impaired the phagocytic response of AM and nicotine's effect was dependent on α7 nAChR mediated signals

Primary AM were harvested at P7 and were administered pH rhodo-labeled *S. aureus*, which fluoresces when incorporated into an acidic phagolysosome. AM from *in utero* nicotine exposed pups demonstrated diminished phagocytosis, but AM from α7 $nAChR^{-/-}$ pups maintained their phagocytic ability despite *in utero* nicotine exposure. * $p<0.05$ vs. Control, †$p<0.05$ vs. Nicotine. Minimum of 4 separate litters in each experimental condition.