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EFFECTS OF *IN UTERO* NICOTINE EXPOSURE ON IMMUNE CELL DISPOSITION AFTER *P. AERUGINOSA* LUNG INFECTION

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THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in the College of Pharmacy at the University of Kentucky

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2017

ABSTRACT OF THESIS

EFFECTS OF *IN UTERO* NICOTINE EXPOSURE ON IMMUNE CELL DISPOSITION AFTER *P. AERUGINOSA* LUNG INFECTION

Current smoking cessation guidelines recommend nicotine replacement therapy (NRT) to assist pregnant smokers to quit, but this is without strong evidence for effectiveness and safety. Nicotine, the main addictive component of tobacco, is known to exert physiological effects by binding to its receptor, the nicotinic acetylcholine receptor (nAChR). Recent studies have identified the presence of nAChRs in non-neuronal cells, and in macrophages, functional alteration upon stimulation with nicotine has been documented.

To understand the impact of *in utero* nicotine exposure on various immune cell disposition and function, we designed preliminary studies using an *in vivo* model of *P. aeruginosa* infection. In this model, pregnant mice were exposed to nicotine and after weaning, offspring were infected intra-tracheally and humanely killed 5 days later.

Nicotine-exposed mice had a greater weight reduction post-infection. This was accompanied by a decreased number of neutrophil, resident macrophages, and B lymphocytes in the lungs, while the number of B lymphocytes in the lymph nodes were greater than that of the control group. In the lung lavage fluids, IL-6, MCP-1, and TNFα concentrations were elevated in nicotine-exposed mice. In an *in vitro* system using bone marrow-derived macrophages, a significantly reduced production of IFNγ was observed in nicotine-exposed mice when cells were stimulated with LPS.

To characterize and compare gene expression in macrophages isolated from neonates developmentally exposed to nicotine, we designed a clinical study to recruit pregnant mothers who 1) did not smoke during pregnancy, 2) smoked throughout pregnancy, or 3) used NRT during pregnancy. We found that successful RNA isolation can be achieved from neonatal tracheal aspirate samples and cell number and reagent volumes were important determinants of acceptable RNA quality and quantity.

Together, these preliminary findings demonstrate a possible alteration in immune response as a result of *in utero* nicotine exposure and sets a groundwork for future studies in identifying mechanisms underlying the impact of developmental nicotine exposure.

KEYWORDS: Pregnancy, cigarette smoke, nicotine, non-neuronal cholinergic system, macrophage, *Pseudomonas aeruginosa*

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A. Smoking and Pregnancy

a. Overview

Smoking is a significant contributor to several pathologies that lead to major public health problems, such as cancer, cardiovascular disease, pulmonary disease, and perinatal morbidity. It is the leading preventable cause of disease and death in the U.S. across all age groups and genders (1, 2). Various methodologies, including nicotine replacement therapy (NRT), have been studied and developed to aid smoking cessation and minimize nicotine withdrawal.

Current practice guidelines recommend the use of NRT during pregnancy if smoking cessation is not achieved with non-pharmacologic intervention alone (3). There is general consensus that NRTs are safer than cigarette smoking during pregnancy due to reduced exposure to the numerous toxins contained in cigarette smoke. Adverse effects observed during pregnancy from smoking are also observed with nicotine administration alone, signifying that NRT use in pregnancy should be extensively examined. Their use is without sufficient data to support safety and effectiveness in pregnant smokers, and importantly safety to the newborn has not been adequately evaluated.

Nicotine, the main addictive component of tobacco, is known to exert physiological effects by binding to its receptor, the nicotinic acetylcholine receptor (nAChR). Recently, studies have identified the presence of nAChRs on non-neuronal cells and have explored their role in normal cellular activities. The term non-neuronal cholinergic system (NNCS) distinguishes these alternate functions from traditional neuronal effects (4). The wide distribution of nAChR outside of the nervous system expands the potential impact of nicotine, much of which is yet to be fully understood. This contributes to another level of uncertainty regarding the safety and efficacy of NRT. Moreover, exposure to nicotine during the critical period of fetal development could induce abnormalities with regard to multiple aspects of cellular functions. This warrants the pursuit of definitive answers to proper use of NRT during pregnancy.

The Barker Hypothesis, which was proposed based on the observed association between birthweight and adult mortality outcomes, provides insight to the developmental

plasticity of the fetus and the importance of intrauterine conditions in shaping the proper tone of fetal health, structurally and functionally, which subsequently affects susceptibility to various diseases in adulthood (5). This concept highlights the critical role of maternal cigarette use and its impact on observed perinatal outcomes.

In this section, the epidemiology of smoking will be discussed to understand the prevalence of smoking, particularly in women during pregnancy, along with a review of smoking cessation options to understand their place in use and to describe concerns associated with current recommendations.

b. Epidemiology of tobacco use and smoking cessation guideline for general population

Epidemiology of tobacco use

It is estimated that 37 million of the U.S. adults aged 18 years and older actively smoke, and this behavior leads to the death of 480,000 people annually due to smoking related illnesses. When translated into overall healthcare cost, including direct medical cost and productivity loss, this equates to more than $300 billion per year (2). Association between cigarette smoking and negative health consequences is well established and highlighted by many *in vitro*, *in vivo*, and epidemiologic studies. The majority of patients suffering from chronic diseases, including pulmonary disease, cardiovascular disease, and cancer, have a strong link to a history of active and/or passive smoking (Reviewed in 6). This data is not surprising since the inverse relationship between cigarette smoking and positive health outcomes has been well-defined, and organizations encourage smoking cessation as the major preventative measure for improving life expectancy (6, 7). However, complete cessation is difficult to achieve due to various biological and physiological factors. For example, nicotine is almost 100% bioavailable from an average cigarette and easily passes the blood-brain barrier within 10-20 seconds due to its lipophilicity. Individuals with a long history of smoking are accustomed to repetitive behavior of cigarette smoking and continuous stimulation of the dependence-development pathway. Physiological effects mediated by cigarette smoke are favorable towards inducing a reward response, which ultimately lures the users to continue seeking this behavior. (8-10).

A concerning aspect of cigarette smoking is that ex-smokers and passive smokers also have increased risk for similar diseases that may be irreversible. Ex-

smokers account for 50% of patients with lung disease diagnoses, and the rate of diagnosis still exceeds that of never-smokers and persists even after 20 years of abstinence (11). Progression of atherosclerosis plaque formation was associated more with pack-years of smoking rather than current smoking status, suggesting cumulative and permanent changes induced by smoking (12). A range of health problems observed in passive smokers, such as lung and cardiovascular disease, are found in a similar incidence to that of smokers, and the risk of certain diseases, such as female breast cancer, allergic rhinitis, allergic dermatitis, and food allergy, may even be equally elevated in active and passive smokers (13, 14). Passive smoking also impacts the unborn fetus, as demonstrated by the detrimental effects observed in newborns exposed to cigarette smoke *in utero*. Prematurity, low birth weight, and sudden infant death syndrome are a few examples consistently reported to be linked to maternal smoking (15). The topic of *in utero* exposure to tobacco will be discussed more in depth in the following sections.

Smoking cessation guidelines

Various methodologies have been studied to reduce smoking prevalence, promote smoking cessation, improve life expectancy, and reduce smoking-related diseases (16). The United States Public Health Service (USPHS) established a clinical practice guideline to help health professionals identify tobacco dependence and assist patients with treatment strategies (3). The guideline emphasizes the importance of behavioral counseling to understand patient's readiness to quit, but also addresses the use of pharmacotherapeutic intervention to assist with cessation. It strongly recommends consistent intervention by clinicians and the use of a combination of behavioral counseling and pharmacotherapy, which is more effective than either alone. However, any intervention is more effective than no intervention and will significantly lower healthcare cost, as treatment strategies for tobacco dependence are cost-effective compared to other chronic diseases (3).

Currently, there are nicotine-based and non-nicotine based products approved by the FDA as smoking cessation aids (8, 17). The most commonly used category is nicotine containing products, known collectively as nicotine replacement therapy (NRT). NRT is one of the first-line pharmacologic agents and is regarded as safe and effective in the general population as it increases the quit rate by 50-70% (18-20). There are multiple dosage forms available over the counter (gum, lozenge, and patch) and by

prescription (inhaler and nasal spray), which provides additional benefit of a tailored approach based on patients' need and willingness to adhere to therapy. NRT is designed to slowly taper nicotine exposure and facilitate the transition from smoking to cessation without experiencing withdrawal and craving. Depending on the history and intensity of cigarette use, one can choose short-acting oral formulations or long-acting transdermal formulation of NRT, or a combination of both, to mimic smoking behavior (8). Although NRT use is associated with a variety of adverse events, those directly associated to these products are limited to local reactions, such as skin irritation with transdermal patch and mouth/throat soreness with oral administration. Systemic adverse events, such as insomnia and sleep disturbances, may be related to smoking cessation itself and not NRT use (21) (Table 1.1). The most serious adverse events reported were cardiovascular symptoms, such as heart palpitations and chest pain, but a systematic review and meta-analysis did not observe differences in the clinical incidence of myocardial infarction or death in the NRT users. Also, the guideline concluded that NRT is safe for patients with cardiovascular diseases (6, 21).

Other non-nicotine pharmacologic therapies include bupropion sustained release (SR) and varenicline (3). Bupropion is an atypical antidepressant that inhibits reuptake of dopamine and norepinephrine, ameliorating symptoms of reward, craving, and withdrawal (23, 24). Smokers frequently suffer from or have a history of major depression and they are less likely to achieve successful abstinence (25). Therefore, bupropion may be an appealing option for smokers with major depression or who may be at risk for developing depression upon quitting. It is also considered safe and tolerable in selected "difficult-to-treat" populations, such as patients with COPD, cardiovascular disease, or patients concerned for post-cessation weight gain. In one study comparing the efficacy of bupropion SR for 12 weeks in 411 heavy smokers (≥ 15 cigarettes per day) with mild or moderate COPD, significantly increased cessation rates were observed in those receiving bupropion SR compared to placebo. A significantly higher number of patients in this group remained abstinent at the 6 month follow-up (26). Another study validating the efficacy and safety of bupropion SR in 629 patients with cardiovascular disease observed significantly increased cessation rates in the treatment group after 12 months compared to placebo group. No clinically significant changes were noted in blood pressure all throughout the study period (27). Combination of NRT and bupropion led to a significant reduction in weight gain at 7 week post-cessation in a study comparing bupropion SR, a nicotine patch, combination of bupropion SR and a

nicotine patch, and placebo in 893 patients (28). Adverse events associated with bupropion use for depression are well reported, and similar events are expected when used for smoking cessation, including dry mouth and insomnia. Special caution should be utilized, however, in patients with risk factors for seizure, such as alcohol abuse and concomitant use of antipsychotics and antidepressants known to lower seizure threshold. Smokers with active seizure disorder or a history of seizures should not be challenged with bupropion therapy (24).

Varenicline is a partial agonist of the α4β2 subtype of nAChR with a higher affinity for the receptor than nicotine. With such properties, varenicline inhibits binding of nicotine to the receptor while its binding only partially stimulates the receptor with reduced effects. As a result, varenicline attentuates symptoms of withdrawal and reduces craving (29). A pooled analysis evaluating the efficacy of varenicline versus bupropion SR and placebo concluded greater continuous abstinence rates with varenicline use (44.0% vs. 29.7% vs. 17.7% for varenicline, bupropion SR and placebo, respectively) during weeks 9 to 12 of treatment, suggesting that varenicline may be more effective than bupropion (30). Post-marketing surveillance data raised concerns for possible neuropsychiatric adverse events, such as changes in behavior, depressed mood, and suicidal ideation, leading to a boxed warning mandated by the FDA in 2009. Subsequent analyses and large clinical trials found no difference in neuropsychiatric events between varenicline and placebo groups, and the boxed warning was removed by the FDA in December, 2016 (31). However, it is recommended that potential neuropsychiatric adverse events are communicated with the patients and their families/caregivers in detail, and to seek immediate assistance from a healthcare provider if any of the described events occur while on therapy (29, 32, 33). Therefore, although both NRT and non-NRT treatments are efficacious, the availability of NRT products without a prescription and mild systemic adverse events reported provide substantial advantages over the non-NRT products.

In summary, a large pool of evidence exists for smoking cessation aids, both pharmacologic and non-pharmacologic, and various strategies can be adopted based on patient-specific requirements and concerns as well as underlying medical conditions. It is clear that any mode of cigarette smoke exposure, direct or indirect, results in increased risks for acute and chronic diseases, and continuous encouragement and motivation are the keys to successful and complete abstinence. However, the use of these therapies during pregnancy requires additional consideration, as the complexity of the potential

impact on fetal development must be taken into account. The next section will begin to address the use of NRT in this vulnerable population.

Medication	Available Dose	Duration	Side Effects
Nicotine gum	2mg, 4mg	12 weeks	Jaw ache, hiccups, dyspepsia
Nicotine lozenge	2mg, 4mg	12 weeks	Nausea, hiccups, heartburn
Nicotine transdermal patch	7mg, 14mg, 21mg over 24 hrs 10mg, 15mg, 25mg over 16 hrs	10-12 weeks	Skin irritation, insomnia
Nicotine nasal spray	10mg/ml	3 months	Nasal irritation
Nicotine inhaler	10mg	6-12 weeks	Mouth and throat irritation, cough
Bupropion SR	150mg	7-12 weeks	Insomnia, dry mouth
Varenicline	0.5mg, 1mg	12-24 weeks	Nausea, abnormal dreams

Table 1.1. First-line pharmacotherapy for smoking cessation.[22]

c. Smoking during pregnancy

It is well demonstrated that smoking during pregnancy is associated with negative perinatal consequences, including miscarriage, prematurity, low birth weight, increased fetal respiratory symptoms, and sudden infant death syndrome (SIDS) (34-36). Although smoking is the leading preventable cause of perinatal morbidity and mortality, the National Vital Statistics Reports found that greater than 10% of U.S. pregnant women smoke during pregnancy (7). This percentage is likely to be underestimated by approximately 25% due to biases in self-reporting (37). Kentucky has more than double the national rate of pregnant women who smoke (Figure 1.2), with some regions reporting rates approaching 50%. Clearly, this is a significant public health concern that particularly affects women and neonates in Kentucky.

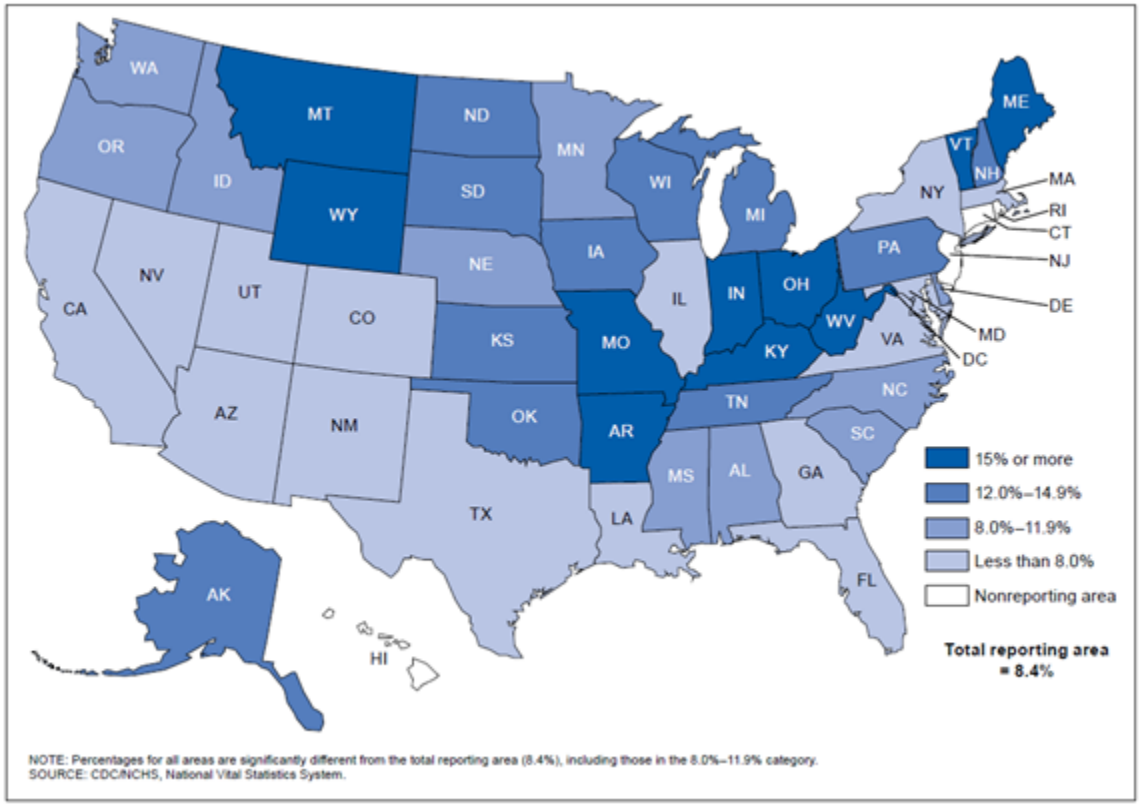


Figure 1.2. Prevalence of maternal smoking at any time during pregnancy: 46 states and District of Columbia, 2014.[7] Used with permission.

Multiple studies suggest that 20-30% of female smokers attempt to quit while pregnant, but success is often temporary. A large clinical study evaluating the efficacy and safety of NRT in pregnant smokers identified that the smoking rate declined from 21.3% to 9.4% at the time of delivery in the NRT group compared to a decline from 11.7% to 7.6% in the placebo group (38). In a small self-report study of 134 women, 25% of participants reported a relapse by 1 month postpartum, while another large study with 1550 women surveyed via telephone interview reported that the majority of participants relapsed by one year postpartum (39, 40). Factors affecting smoking relapse are similar to those that cause active smoking during pregnancy, with a stronger association with lack of motivation and exposure to cigarette smoke (41). This signifies the importance of support from family members and the society in achieving a successful smoking cessation. Pregnant women are likely to achieve short-term abstinence if their primary concern is the health of the fetus while those who are concerned about the health of both fetus and self are likely to achieve long-term abstinence (41). Pregnant smokers should be educated that postpartum abstinence is also important in protecting the mother and the newborn since nicotine accumulates in breastmilk, which can continue to affect neonatal development (42).

The intrauterine condition is critical for the proper growth and development of fetal organs, and the fetus is vulnerable to any subtle changes in this environment. In the early 1990s, a series of epidemiological studies reviewing birth and death records revealed the association between poor intrauterine nutrition and adult cardiac/metabolic diseases, suggesting the impact of “fetal programming” on adult health outcomes. This has become known as the Barker Hypothesis and has stimulated active research that expands beyond nutritional status during gestation to include the link between various *in utero* environmental exposures and negative health consequences, such as pregnancy smoking and fetal health (43, 44). Many studies and meta-analyses report intrauterine growth retardation in both genders, as measured by birth weights, compared to newborns of non-smoking mothers, accounting for 20-30% of low birthweight cases, in these infants. Other measures of growth retardation are suggested by data showing an impact on birth length as well as head circumference (45, 46). Dose-response relationships between maternal smoking and the risk of stillbirth, neonatal death, and perinatal death are also observed (47, 48). Other serious adverse outcomes include sudden infant death syndrome (SIDS), fetal congenital heart defects, decreased pulmonary function, obesity, neurobehavioral alterations such as decreased cognitive

function and attention deficit hyperactivity disorder (ADHD), and many more. These characteristics can be presented during neonatal/infant periods as well as during childhood (45, 49).

Consequences of developmental cigarette smoke exposure appear to be the greatest during the third trimester, and the rate of health complications in infants born to mothers who smoked only late in pregnancy is comparable to newborns whose mothers smoked throughout pregnancy (50, 51). For example, a study evaluating benefit of reducing the number of cigarettes smoked as opposed to a complete abstinence found that the association between the level of cigarette smoke exposure and birthweight was strongest during the third trimester. Interestingly, the association was only true when the number of cigarettes used daily was less than eight cigarettes (52). Additionally, early smoking cessation was associated with preterm birth rates comparable to nonsmokers (28.9% vs. 29.3%), while smoking during the third trimester or throughout pregnancy resulted in a significantly increased rate of preterm birth (43.9%) (53). This demonstrates that smoking cessation should be encouraged as soon as possible, prior to reaching the third trimester, and the use of less than eight cigarettes per day, and not any arbitrary reduction in cigarette use, may result in observable changes in the birth outcome. Although results vary based on an individual's tobacco dependence and lifestyle, interventions to support continued abstinence throughout pregnancy will certainly reduce perinatal morbidity and mortality.

Gene expression and cellular changes induced by developmental cigarette smoke exposure can cause damage that is amplified after birth. For example, a study of 2295 non-smoking patients who were exposed to parental cigarette smoke developmentally showed a decline in pulmonary function test in adulthood, measured by forced expiratory volume in 1 second (FEV_1). This decline was associated with maternal smoking, but not paternal smoking, and there was 3 to 5 years' loss of function if mothers smoked more than 25 cigarettes per day. This suggests that damage induced prenatally can become permanent (54). In a study that investigated the association between epigenetic modifications and *in utero* smoking exposure in cord blood of 1062 newborns identified differential DNA methylation patterns in several genes. These genes include CYP1A1, AHRR, and GF11, which are known to participate in detoxification and clearance of toxic tobacco components (55). Influence of maternal cigarette smoking on epigenetic changes of newborns is a growing area of study, and although limited in number, studies are starting to identify associations between altered gene methylation

from placenta, umbilical cord blood, and maternal blood with later offspring health outcomes or predispositions for adult health risks, such as mental and behavioral disorders (43, 45, 49).

The U.S. Preventive Services Task Force published clinical guideline for behavioral and pharmacotherapy interventions for smoking cessation in adults in 2015 and recommends all currently marketed pharmacotherapeutic agents to pregnant women for smoking cessation. However, the guideline states that there is limited evidence for a clear benefit of all NRTs as well as bupropion SR and varenicline (56). The FDA has assigned nicotine and nicotine containing products to Pregnancy Category D, which describes that the potential benefits may warrant product use despite potential risks found from investigational or marketing studies in humans (57). There are thousands of active compounds known to be fetal toxins in cigarette smoke, such as carbon monoxide, hydrogen cyanide, and nicotine. It is difficult to identify a causative agent responsible for the adverse effects of maternal smoking on fetal health (15). However, the main component of cigarette smoke, nicotine, easily crosses the placental barrier and can be detected in the fetal circulation at levels exceeding maternal concentrations by 15%, while amniotic fluid concentrations of nicotine are 88% higher than maternal plasma (58). Nicotine also accumulates in breast milk, which can be problematic due to subsequent ingestion by the infant (58). Therefore it is important to consider the potential effects of nicotine itself upon the development of those exposed prior to birth.

Teratogenic effects of nicotine on cognitive and neurobehavioral functions of newborns are very well studied in animal models at various doses, and alterations in motor, sensory, and cognitive functions are suggested to continue into childhood in humans (Reviewed in 59). In one study, rats exposed to nicotine prenatally displayed a significant delay in reflex to orientation and gravity, as measured by righting reflex and geotaxis tasks, and a decreased exploratory activity compared to saline exposed rats. Cognitive learning and memory functions were assessed by avoidance of stimulus, and it was observed that a greater percentage of rats exposed to nicotine *in utero* were poor learners. This was observed in rats both at 60 days and 6 months after birth, suggesting chronic adverse effects of gestational nicotine exposure (59).

Studies have not definitively stated whether NRT use achieves successful abstinence rates and is without adverse effects to mothers or newborns. For example, one clinical study comparing the efficacy and safety of NRT patches (15mg per 16

hours) to placebo during pregnancy concluded no significant benefit in increasing the rate of abstinence until delivery (38). While this study did not find differences in the rate of adverse pregnancy or birth outcomes, interpretation of findings are limited by a low adherence rate (7.2%), which is consistent with findings from other trial (60). One plausible explanation for ineffectiveness is the enhanced clearance of nicotine and its metabolite cotinine during pregnancy (60% and 140%, respectively) as well as a shorter half-life of cotinine (8.8 vs. 16.6 h) (61). However, dose adjustment of NRT patches to saliva cotinine levels still resulted in a comparable rate of relapse to placebo (62).

Despite several concerns and uncertainty regarding efficacy and safety of NRT, many international guidelines generally advocate its use during pregnancy for those who may benefit from therapy, such as individuals who failed to achieve successful cessation with behavioral therapy alone. The US Public Health Service Clinical Practice Guideline, the Society of Obstetricians and Gynecologists of Canada, and the US Preventive Services Task Force together address this topic and make a statement that while NRT reduces the number of cigarettes smoked in general population, studies have not demonstrated the same results during pregnancy (3, 56, 63). In this regard, it is suggested that physicians clearly communicate pros and cons of NRT use to their patients and make clinical judgement when recommending such therapy.

Increased risk of respiratory anomalies has been suggested from a large study investigating the association between pregnancy NRT use with major congenital anomalies (MCA) in offspring (35). This study included nearly 200,000 children born in the UK over a 10 year period based on the availability of mother-child primary care records for diagnoses of MCA and for the prescription of NRT. By designating the NRT group as those who had a prescription during the first trimester or 1 month prior to conception, the study investigated the effects of *in utero* nicotine exposure during the early stages of fetal development. The absolute risk of MCAs was comparable between the NRT group and the smoking group, both of which were higher than the control group. No statistically significant changes were observed in the risk of all MCA combined in the NRT group compared to the smokers or the control group, but the risk of respiratory anomalies was significantly higher than the control group as well as the smoking group (OR: 4.65, 99% CI 1.76-12.25; $p<0.001$ and OR: 3.49, 99% CI 1.05-11.62; $p<0.007$, respectively). This study highlights that the use of NRT in pregnancy is not without serious health consequences, some of which may be comparable to the effects caused by smoking (35).

Moreover, studies assessing maternal smokeless tobacco use demonstrate a negative association with perinatal outcomes, including preterm birth, stillbirth, and neonatal apnea, that are comparable to those observed with cigarette smoking (64-66). These studies were conducted using birth records of approximately 600,000 Swedish citizens, and the pregnant women were divided into smokeless tobacco users, light smokers (1-9 cigarettes/day), heavy smokers (≥ 10 cigarettes/day), and non-smokers based on the information collected before the 15th week of gestation. Compared to non-smokers, risk of preterm birth and stillbirth was increased in both smokeless tobacco users and smokers, although heavy smokers were associated with a greater increase than smokeless tobacco users or light smokers (64, 65). Results of neonatal apnea demonstrated approximately a twofold increase in the smokeless tobacco users that remained unchanged when adjusted for gestational age, fetal growth, and gender, while cigarette smokers were associated with a 50% increase only before the adjustment but not after (66). This is surprising as this data suggests that smokeless tobacco has a greater negative impact on neonatal apnea. The authors proposed differences in PK parameters of nicotine from smokeless tobacco, which is similar to those of NRT but not cigarette smoking, as a possible explanation.

It is impossible to identify a single agent responsible for the negative perinatal outcomes as various factors, including the timing and the level of tobacco exposure, influence the outcomes collectively. Furthermore, physiological effects mediated by smoking may differ from one organ system to another. Nonetheless, these studies strongly suggest that nicotine is a critical toxin in cigarette smoke whose effects should not be underestimated nor be considered a safer alternative to smoking during pregnancy.

B. Smoking/nicotine and Immunity

a. Overview

Cigarette smoke contains thousands of compounds with immunotoxic properties, including nicotine and polycyclic aromatic hydrocarbons (15). In addition to commonly known perinatal adverse health effects, cigarette smoke affects both the innate and adaptive immune responses in a number of ways, including an impact on macrophage function. Decreased levels of pro-inflammatory cytokines and impaired bactericidal properties have been observed in pulmonary macrophages exposed to cigarette smoke (67). Maternal smoking both before and after birth is a major threat to respiratory health of newborns, and the timing and the level of exposure appear to have a significant impact on the proper development of immune function and its capacity, as the development of the immune system continues after birth (68).

Despite advances in the development of anti-infectives and other public health measures, infectious diseases remain a major contributor of widespread morbidity and mortality in the United States as well as worldwide. Rates of hospitalization from infectious diseases fluctuate due to outbreaks of antibiotic-resistant organisms (69). In a 2009 report, the leading cause of infectious disease hospitalization was attributed to respiratory tract infection, and the burden was highest among young children (aged < 5 years) (69). Similar observations were reported from retrospective case-control analyses evaluating infectious diseases hospitalization and mortality outcomes in infants developmentally exposed to maternal cigarette smoke (70). There was a dose-dependent association between maternal smoking and morbidity outcome due to infectious disease, and infection was a significant contributor of infant mortality, particularly within the first two days of hospitalization (32.4%). Among several types of infections, respiratory infection had the strongest association with infant hospitalization. Additionally, this observation was independent of birthweight and gestational-age, suggesting other potential mechanisms that adversely affect immune function in infants exposed to cigarette smoke *in utero* (70).

Cigarette smoke is reported to have both pro-inflammatory and anti-inflammatory effects that are mediated by different toxic components. The overall impact of cigarette smoke on the function of immune cells and health of an individual depends on the chronicity of the tobacco use as well as the sum of the effects caused by the toxins, and

the extrapolation of this data should be cautiously considered when used in making therapeutic recommendations, especially to a pregnant smoker (71).

In this section, the effects of cigarette smoke, and those specifically attributed to nicotine, on the respiratory immune cell properties and functions, particularly the alveolar macrophages, will be reviewed. Additionally, data describing the impact of cigarette smoke on immunity will be compared to what is known of the impact of nicotine alone, because the distinction between the two is important when considering the degree to which NRT should be recommended during pregnancy. Finally, the impact of developmental nicotine exposure on fetal immunity will be discussed, as this will set the groundwork for establishing safety of NRT during pregnancy and highlight whether NRT can be considered less harmful than smoking in pregnancy.

b. Respiratory Immune Defense

The risk of respiratory infection is greatly increased with both active and passive cigarette smoke exposure and alterations of host defense are responsible for increased susceptibility to infections in smokers. Normal flora of the respiratory tract are disrupted, allowing colonization of pathogenic bacteria, such as *H. influenza, S. pneumoniae*, and *M. catarrhalis* (72). *Pseudomonas aeruginosa*, which is an opportunistic gram-negative nosocomial pathogen, rarely causes lung infection in healthy population, but the risk is increased in patients with underlying medical conditions, such as chronic obstructive pulmonary disease (COPD) and malignancy (73, 74). Two possible mechanisms describe how cigarette smoke contributes to the altered respiratory immune defense and therefore increased risk of infection by pathogenic or opportunistic bacteria: 1) physiological and structural changes in the host and 2) disruption of normal immune function. These changes occur simultaneously and all modulations mediated by cigarette smoke collectively alter the overall balance of the immune system (72).

Lungs physiologically can be divided into two compartments: the conducting airways, comprised of airway epithelial cells and secretory cells that act as the first line of defense, and the lung parenchyma comprised of alveoli where gas exchange occurs (75). The respiratory tract is constantly exposed to the external environment and homeostasis is maintained by distinct groups of immune cells that mediate inflammatory and anti-inflammatory responses to antigens in a coordinated manner (Figure 2.1). In the conducting airways, the epithelial cells form physical barriers to exclude incoming

antigens and particles that have gained access. These particles are removed from the lungs by the ciliated pseudostratified columnar epithelial cells. Additionally, a highly developed network of immune cells, including dendritic cells and macrophages, populate the airways and the alveolar spaces to properly respond to a wide range of pathogens and to initiate inflammation (76). The lung parenchyma is populated mainly by the alveolar macrophages (AM) that make up 90% of the total immune cells, with the remainder composed of dendritic cells, T cells, and B cells. Upon exposure to pathogenic environmental antigens, airway epithelial cells detect conserved structural motifs of microorganisms and allergens via surface receptors, such as toll-like receptors (TLR), to signal both the innate and adaptive immune responses. They also secrete a large array of antimicrobial substances and effector molecules to kill infectious agents, recruit other immune cells, and support their function and survival during infection. For example, nitric oxide (NO) released from epithelial cells is a free radical with potent antimicrobial effects and tissue damaging effects, augmenting the inflammatory response at the site of infection. Chemokines and cytokines, such as neutrophil attractant IL-8 and granulocyte/macrophage colony-stimulating factors (GM-CSF) aid the survival and recruitment of inflammatory cells (75, 77).

Dendritic cells (DCs) are known as professional antigen presenting cells that develop from bone marrow-derived precursor cells and migrate to various tissues, including the airways. They have the ability to recognize a variety of stimuli through the expression of pathogen-associated molecular pattern (PAMP) receptors (78). During active infection, DCs take up antigens and migrate to the draining lymph nodes, where they interact with naïve T lymphocytes that recognize these molecules and undergo proliferation and differentiation into subtypes of T cells, including helper, cytotoxic, and regulatory T cells. Subsequently, T cells migrate back to the inflamed tissue via the lymphatics and circulation and remove specific pathogens (78). Similarly, antibody producing B lymphocytes are activated by antigens presented by DCs, which cause B cells to migrate to the T lymphocyte zone in the lymph nodes, allowing for T and B lymphocyte interactions required for optimal response (79).

Macrophage interaction with DCs is also important in maintaining homeostasis at steady-state and inducing robust inflammatory responses during active infection. Macrophages initially limit antigen acquisition by DCs by phagocytosing various antigens inhaled from the air. This mechanism prevents unnecessary stimulation of the immune response and lung damage from inflammation. However, macrophages become

saturated with actively replicating microbial antigens upon infection, which causes spillover of antigens into DCs and induces a robust immune response (80). Smoking, and specifically nicotine, exposure alters the ability of macrophages to perform these functions.

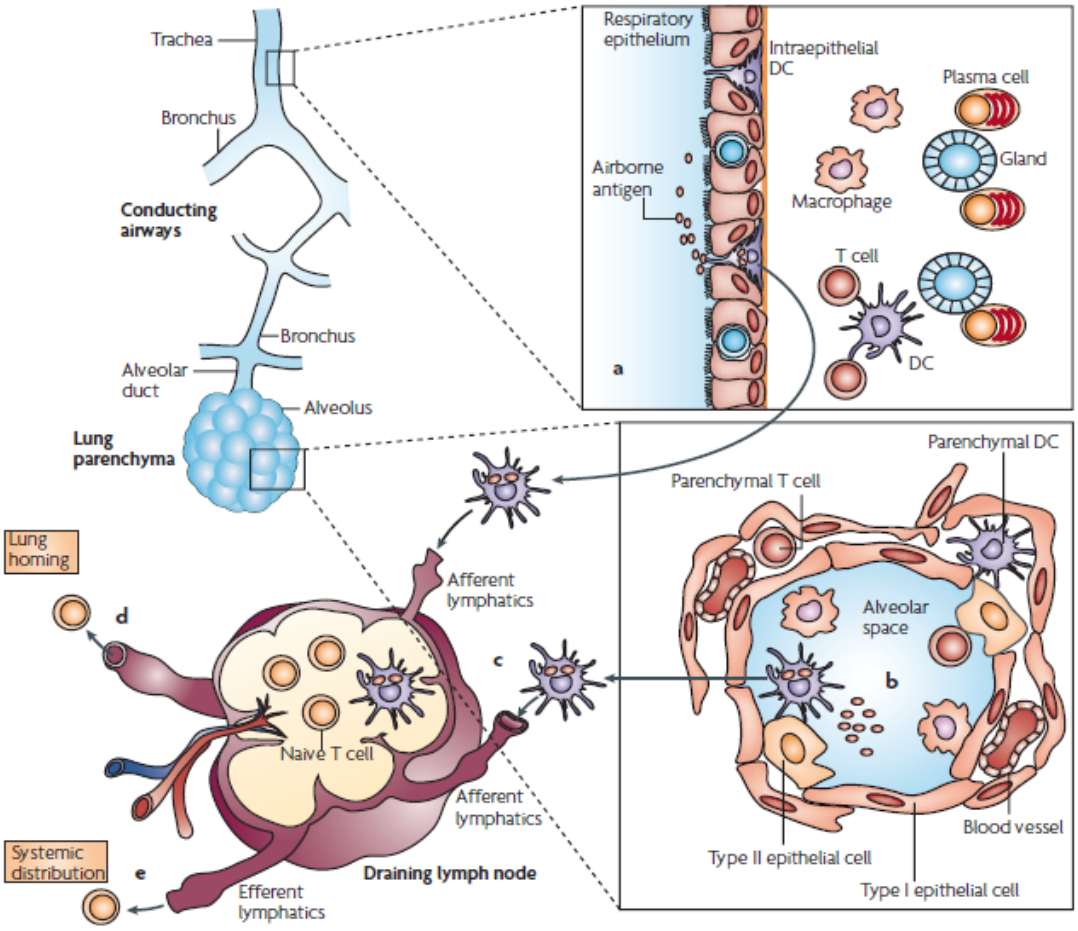


Figure 2.1 Antigen acquisition and transport in the lungs.[75] Used with permission.

Smokers are known to have increased risk of respiratory infection and other pulmonary diseases, in part due to epithelial cell modifications induced by toxic substances in tobacco that alter the integrity of epithelial defense mechanisms. Exposure of human bronchial epithelial cells to 2.5% and 5% cigarette smoke extract (CSE) for 28 days was shown to significantly increase the number of secretory Clara cells and goblet cells while the number of ciliated cells was significantly reduced (81). Similarly, mice exposed to cigarette smoke inhalation exhibited hypertrophy of the Clara cell as well as hyperplasia of the basal and squamous cells when exposed to the combination of cigarette smoke and the carcinogen N-nitroso-N-methylurethane (NMUT) via nebulization. These mice were kept in a chamber for 192 days (6 hours/day, 5 times/week), thus demonstrating chronic effects of cigarette smoke exposure (82). Additionally, alterations in epithelial and alveolar permeability are also associated with cigarette smoke exposure, and this was linked to the damage and reduction in the number of gap junctions (83). In this study, human bronchial epithelial cells (16HBE14o-) were exposed to 10% cigarette smoke extract (CSE) for 72 hours, and proteins associated with tight junctions, such as occludin (OCLN) and Zonula occludens (ZO-1, ZO-2, ZO-3), were visualized via immunofluorescence staining. Compared to untreated cells, those exposed to 10% CSE demonstrated discontinuous, fragmented tight junctions, suggesting the negative effects of CSE on cell integrity (83). While it is certain that these toxic cellular effects influence infection directly, these changes in the airway epithelium likely also impact the function of immune cells in the airways.

The ability of immune cells to respond to and clear bacteria are compromised upon cigarette smoke exposure (84, 85). Cigarette smoke leads to an influx of innate immune cells, including neutrophils, macrophages, and dendritic cells, but their ability to phagocytose microbes, generate respiratory burst, and present antigens are impaired. Downregulation of surface receptors involved in pathogen recognition has also been reported, signifying a defect in the initial sensing of pathogens (86, 87). These aspects will be discussed in Section d of this chapter. Dendritic cells, critical in bridging the innate and adaptive immune responses, have reduced expression of the co-stimulatory molecules CD80 and CD86 as well as antigen presenting MHC Class II molecules. Additionally T-cell activation, proliferation, and production of cytokines are suppressed by cigarette smoke exposure (72, 86). While multiple cell types are impacted, this thesis will focus mainly on macrophage function and characteristics in response to nicotine exposure, which will be discussed in the next section.

c. Macrophages

Macrophages are a subset of innate immune cells derived from circulating peripheral blood mononuclear cells (PBMC) that differentiate into tissue-resident cell phenotypes, such as microglia in the brain, Langerhans cells in the skin, Kupffer cells in the liver, and alveolar macrophages in the lungs. Alveolar macrophages reside at the interphase between the external environment and lung tissue and function in part to remove innocuous substances as well as cell debris generated during normal cellular processes in a healthy host. All macrophages have similar function as the primary phagocytes of the innate immune system. Upon contact with a pathogen, alveolar macrophages secrete various pro-inflammatory mediators, such as IL-1, IL-8, and TNFα, to recruit neutrophils to the site of infection and generate subsequent inflammatory responses for rapid clearance of the bacteria. After the initial surge of inflammatory response and resolution of infectious challenge, macrophages remove apoptotic neutrophils and other debris and secrete anti-inflammatory cytokines, such as TGFβ and IL-10, to prevent lung injury from prolonged inflammation and to maintain tissue homeostasis (88).

In order to function accordingly to a dynamically changing microenvironment, macrophages take on different phenotypes defined by gene expression patterns that induce various functions. Two such subsets are classically activated macrophages (M1) and alternatively activated macrophages (M2). This classification is derived from the Th1/Th2 paradigm of the CD4+ T helper cells, which also take on two different phenotypes. Differentiation into Th1 and Th2 cells depend on the cytokine milieu of the environment, with IL-12 and IL-4 inducing Th1 and Th2 responses, respectively. Subsequently, Th1 cells produce its signature cytokine IFNγ that mediates a strong pro-inflammatory response. IFNγ generates antimicrobial effector molecules, such as NO, and pro-inflammatory cytokines and induces M1 macrophage polarization. M1 macrophages also produce IL-12, which further promotes Th1 polarization, thus perpetuating the pro-inflammatory cycle. Conversely, Th2 polarized cells produce IL-4, IL-5, and IL-13 that induce M2 polarization and also amplify proliferation and differentiation. Late in response to bacterial pathogens, M2 macrophages mediate anti-inflammatory response by producing low levels of IL-12, shifting the balance away from Th1 cells, and generating mediators involved in tissue remodeling, such as TGFβ1 (89, 90).

One main distinction between M1 and M2 macrophages is highlighted by a shift of L-arginine metabolism into urea and ornithine by upregulating arginase expression in M2 macrophages, which results in cell repair through enhanced collagen synthesis and cell growth. M1 macrophages, on the other hand, produce inducible nitric oxide synthase (iNOS) that competes with arginase for the same substrate, L-arginine, but produces citrulline and NO, which have potent antimicrobial activities (Figure 2.2) (90, 91). M1 macrophages also secrete high levels of the pro-inflammatory cytokines IL-6 and TNFα as well as reactive oxygen species (ROS). In addition to arginase production, M2 macrophages are characterized by the expression of surface molecules Ym1, Fizz1, CD36, and CD206 and by production of high levels of IL-10 and low levels of IL-12 (90). In the absence of iNOS, M2 macrophages display more effective phagocytic activity than bactericidal activity. These two opposing functions of M1 and M2 macrophages maintain balance between homeostasis and inflammation, and dysregulation of this balance may explain why certain populations, such as smokers, are more prone to immunologic diseases.

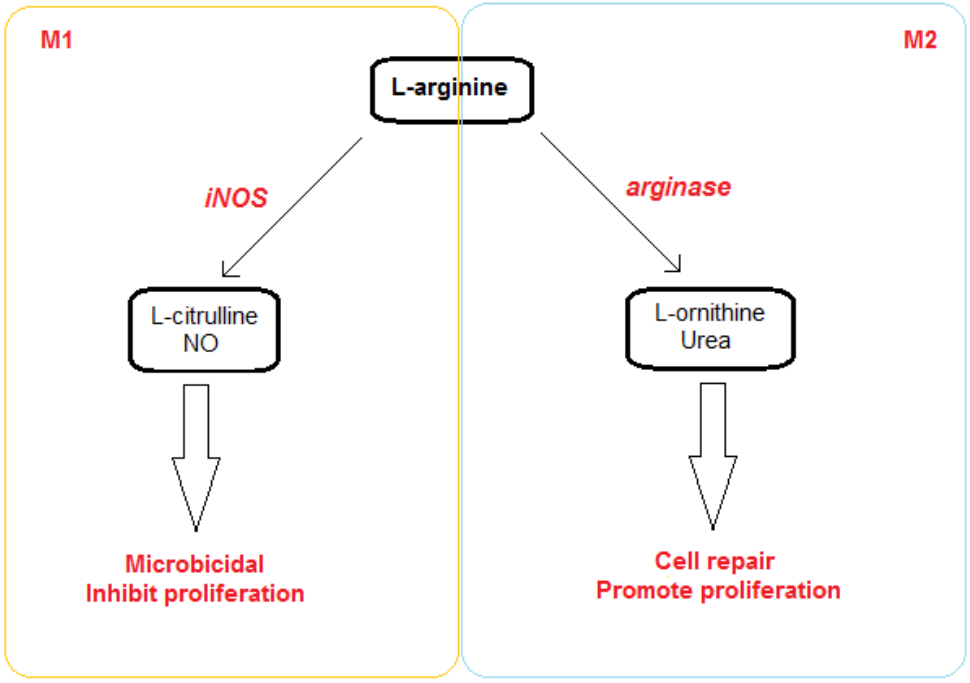


Figure 2.2 Catabolism of L-arginine in M1 and M2 macrophages.[90] Reprinted with permission.

d. Effects of cigarette smoke and nicotine exposure on macrophage functional characteristics

Effects of cigarette smoke

Numerous *in vitro* and *in vivo* studies suggest functional and phenotypic alterations of macrophages upon exposure to cigarette smoke. Mice exposed to cigarette smoke (CS) for 6-8 weeks showed a worsened clinical status after *P. aeruginosa* infection as measured by weight loss (74). Several observations from the mice in this study provide possible explanations for the effects of CS on outcomes. First, CS-exposed mice were associated with increased bacterial burden in the lungs and an increased inflammatory response, suggested by higher numbers of neutrophils and mononuclear cells in the lungs. These mice also had increased levels of pro-inflammatory cytokines, including TNFα, IL-1β and IL-6, and monocyte chemo-attractant protein-1 (MCP-1) and macrophage inflammatory protein-2 (MIP-2) chemokines in the lungs post-infection. Investigators then studied the impact of CS exposure on alveolar macrophage responsiveness to an infection as a possible explanation for these observations. Interestingly, alveolar macrophages isolated from CS-exposed mice produced significantly lower levels of TNFα and IL-6 than sham-exposed mice, as opposed to the observed increase of these cytokines in the lungs. This may be a result of a feedback response to a dampened production of inflammatory mediators by alveolar macrophages (74). Similarly, intratracheal lung infection with *Streptococcus pneumoniae* in mice exposed to CS for 5 weeks resulted in a worsened clinical outcome, measured by clinical appearance score and core body temperature. Post-infection bacterial burden was significantly higher in the lungs of mice exposed to CS, and this group was associated with higher levels of IL-1β, IL-6, IL-10, MIP-2, and TNFα in their lungs 48 h post-infection. Furthermore, phagocytic capacity of macrophages isolated from CS-exposed mice was decreased by 40%, suggesting reduced bacterial clearance and prolonged infection that led to increased inflammatory cytokine production (92).

The impacts of CS on human alveolar macrophages appear to parallel those observed in animal studies. Alveolar macrophages isolated from lung lavage fluids of smokers and non-smokers exhibited a similar phagocytic ability against *Listeria monocytogenes* infection but the mean phagocytic index was decreased in smokers. Moreover, they were inefficient at killing ingested bacteria compared to those of non-smokers, as measured by bactericidal activity (93).

Other important aspects of macrophage function involve recognition of and interaction with pathogenic molecules through surface receptors. CD14 and TLR4 are macrophage surface receptors that recognize and bind to bacterial endotoxin (LPS). Activation of these receptors induces downstream signaling pathways that initiate the production of pro-inflammatory cytokines, such as TNFα, IL-1β, IL-6, and IL-8 (94). Pre-treatment of alveolar macrophages isolated from murine lung lavage fluids with CS resulted in a significant reduction of receptors expressed compared to non-CS treated macrophages (95). This observation may explain the decreased production of pro-inflammatory cytokines, as a result of reduced recognition, by CS-exposed macrophages, as discussed earlier. Another important surface receptor, CD11b, is highly expressed by monocytes/macrophages and has an important role in phagocytosis of apoptotic cell debris. The percentage of macrophages expressing CD11b was reduced when mice were exposed to CS for 10 days (95). This can lead to a critical functional deficit in regulation and resolution of inflammation. In fact, alveolar macrophages isolated from smokers displayed insufficient phagocytosis of apoptotic cells when compared to those of non-smokers (87). In this study, alveolar macrophages were purified from the lung lavage fluid of patients with COPD who were smokers or ex-smokers, healthy smokers without COPD, and non-smokers. Compared to non-smokers, healthy smokers and both COPD groups were associated with macrophages that had decreased ability to phagocytose apoptotic epithelial cells (87). Similarly, phagocytosis of apoptotic neutrophils was depressed when murine alveolar macrophages were pre-treated with CS (96). Apoptosis is programmed cell death that is tightly regulated to maintain an intact cell membrane during the process and to prevent release of toxic substances to neighboring cells. Inefficient ability to phagocytose apoptotic cell debris by macrophages upon CS exposure is concerning, as this can lead to tissue damage by inappropriately releasing noxious cytoplasmic contents into the environment (86, 88).

Macrophage phenotypic changes triggered by cigarette smoke are also noteworthy as this leads to restricted functional capacity during a host's response to infection. Alveolar macrophages recovered from bronchoalveolar lavage (BAL) of healthy smokers revealed gene profiles and protein production characteristics that were associated with a macrophage shift toward an M2 phenotype, both at steady-state and after LPS stimulation, when compared to that of healthy non-smokers. This was characterized by decreased expression and secretion of TNFα, IL-1β, IL-6, and IL-8 and

elevated expression of genes related to IL-4 and a minimal to modest elevation of IL-10 expression and secretion (97, 98).

In summary, findings from animal and human studies suggest that smoke exposure causes a defect in alveolar macrophages to recognize and respond to pathogens, which result in exaggerated and prolonged inflammation as well as worse clinical outcomes. Macrophages display distinct features upon exposure to CS that are different from those not exposed to CS.

Effects of nicotine

There are thousands of chemical compounds in cigarette smoke capable of mediating various biochemical effects in exposed tissues. It is difficult to isolate a single agent responsible for the observed immunosuppressive effects on macrophages, and little is known about the effects of cigarette components separately. However, many studies suggest that nicotine may be driving the biochemical changes. Furthermore, because smoking cessation products contain nicotine as the main component to help with withdrawal symptoms and craving, its impact on immune function should be closely evaluated, as recommendation of such product use during pregnancy introduces two major issues: 1) effects of nicotine directly on immune cells and 2) effects of *in utero* nicotine exposure on development of the immune network and response.

Several studies report nicotine-induced changes in macrophage characteristics both at steady state and after stimulation that display a decrease in pro-inflammatory protein production. In one study, murine alveolar macrophage cell lines were infected with *Legionella pneumophila* and treated with various concentrations of nicotine to determine its immunosuppressive properties as well as the mechanism. Results were similar to the changes observed after the exposure to cigarette smoke, highlighted by enhanced bacterial growth, downregulated secretion of IL-6, IL-12, and TNFα and minimal effect on IL-10 concentrations. Additionally, the same modulations were observed when dimethylphenylpiperazinium (DMPP), a nonselective nicotinic receptor agonist, was administered (99), suggesting that different types of nAChR may be involved in mediating these changes. Another study observed a nicotine-induced shift from M1 to M2 phenotypes, characterized by expression of surface markers, cytokine profile upon LPS stimulation, and T cell proliferation (100). In this study, human peripheral blood monocytes (PBMCs) were differentiated into M1 or M2 macrophages with granulocyte-macrophage colony-stimulating factor (GM-CSF) or macrophage

colony-stimulating factor (M-CSF), respectively, and also in the presence of nicotine to generate nicotine-exposed M1 (Ni-M1) and nicotine-exposed M2 (Ni-M2) cells. Although Ni-M1 retained M1 characteristics, increased expression of CD14 and CD163 and decreased expression of CD206 and CD11b suggest possible skewing of Ni-M1 into an M2-like phenotype. Upon stimulation with LPS, cytokines produced by Ni-M1 were different from M1 polarized macrophages, suggested by a significantly lower IL-12 production and high MCP-1 production. Macrophage/T-cell co-culture experiments further suggested that Ni-M1 macrophages resemble M2 macrophages rather than M1 macrophages, as demonstrated by a reduced production of IFNγ and elevated IL-10 production (100).

Overall, data suggest strong similarities between the changes driven by cigarette smoke and nicotine alone in various host defense mechanisms. This signifies that harmful effects can be caused by nicotine administration. Nicotine replacement products could modulate immune responses and increase the risk of infection, especially in vulnerable populations, such as pregnant women and their developing fetuses and newborns.

e. Impact of *in utero* nicotine exposure

NRT use during pregnancy should be cautiously recommended due to the ability of nicotine to cross placental barrier easily, given its lipophilicity. Cigarette contains immunomodulatory molecules and infants exposed *in utero* have a greater risk for several developmental impairments, such as intrauterine growth retardation, premature birth, compromised lung function, and altered immune defense. It is likely that these impairments result from an accumulation of physiological changes mediated *in utero* (48, 101). However, little is known about the contribution of nicotine alone to observed impairments and the extent of its contribution during development, thus raising the question as to whether nicotine can be considered less threatening than smoking during pregnancy.

Epidemiologic and animal data raise concern for the role of developmental nicotine exposure in inducing changes that result in negative health outcomes. Increased risk of childhood allergic airway disease, such as asthma, is one example that has a strong association with exposure to cigarette smoke during fetal development (102). Animal study data supplements the general notion that nicotine may be

responsible for inducing parameters associated with increased risk of asthma (103). When pups were exposed to nicotine during gestational periods, they had increased total airway resistance, decreased total airway compliance, and increased tracheal constriction after birth. In another study, baseline cytokine profiles of neonatal lung from *in utero* nicotine exposed mice displayed increased IL-13 and decreased IL-1β mRNA expression as well as increased TGFβ1 protein in the epithelial lining fluid at postnatal day 7 (104). This demonstrates a shift towards an anti-inflammatory set point. Furthermore, *in utero* nicotine exposure resulted in elevated mRNA expressions of M2 specific markers at baseline, including arginase-1, Ym-1, and fibronectin. A profound reduction in phagocytic ability of neonatal alveolar macrophages was also observed after *in utero* nicotine exposure (104). Importantly, most of the observations mirror the changes demonstrated with post-natal nicotine exposure.

T lymphocytes have also been shown to be affected by developmental nicotine exposure. Lymphoid cell precursors are generated in the bone marrow and migrate to the thymus, where they undergo stages of positive and negative selections and differentiate into subsets of T cells. Therefore, it is crucial that proper development of the fetal thymus is maintained during the gestational period to establish fully functioning and well-balanced T cell lineages. Th1 and Th2 cells, as discussed earlier, are two subsets of Th cells whose balance is important in regulating the immune response and maintaining homeostasis. However, daily injection of nicotine in pregnant mice generated offspring with a shift towards a Th2 bias (105). In this study, immunoglobulin (IgG2a and IgG1) levels were used as a marker of Th1 and Th2 responses, respectively. Upon immunization with an antigen, serum IgG2a levels were reduced while IgG1 levels were increased significantly in the pups. Additionally, IL-4 concentrations were higher in the serum of pups developmentally exposed to nicotine. Abnormal development of thymus tissue through exposure to nicotine was suggested by increased apoptosis of T cells and lower thymus weights in fetal thymus harvested at gestational day 18. Moreover, changes in T cell phenotypes, including cells that were double negative for CD4 and CD8 expression and single positive CD4 or CD8 expression, were observed in this group. Improper generation of T cell subsets as well as reduced output of T cells as a result of increased apoptosis can lead to dysfunctional effector T cells in the periphery (105).

Prenatal nicotine also affects lung development and function postnatally. Animal studies demonstrated a reduction in pulmonary function parameters due to altered

airway structure as well as alveolarization of the lungs induced by prenatal nicotine exposure (106). This provides a possible explanation for the increased susceptibility to respiratory infections in neonates and children exposed to nicotine developmentally.

C. Non-neuronal cholinergic system and immunity

a. Overview

Acetylcholine (ACh) is a molecule known to scientists for more than 150 years. It has been extensively studied and understood for its function as a neurotransmitter. Both unicellular and multicellular organisms appear to have the capability to synthesize ACh and possess components of the cholinergic system to various extents. This demonstrates that ACh is a phylogenetically ancient molecule whose function in non-neuronal cells precedes that in neuronal cells (107). Recent interest in the role of ACh apart from the nervous system has led to a better understanding of the wide distribution of its synthesis and expression of its receptors in non-neuronal cells as well as its critical function in human health and disease beyond the brain.

There are two types of ACh receptors, nicotinic and muscarinic receptors, and both types are expressed in non-neuronal cells (4). As the name implies, nicotinic ACh receptors (nAChR) can be stimulated by endogenous ACh as well as exogenous nicotine, whereas muscarinic ACh receptors (mAChR) are stimulated by endogenous ACh as well as muscarine, a molecule derived from a poisonous mushroom. Although all nAChRs share this feature, affinity and specificity of ligands to the receptor may vary based on the composition of receptor subunits, which can ultimately lead to various degrees of physiological effects (108). This is of particular interest and concern for pregnant women and developing fetuses prone to direct and indirect exposure to environmental toxins capable of stimulating nAChRs, such as nicotine. As mentioned earlier, children born to smoking mothers are at a higher risk of respiratory infection, and cigarette smoke and nicotine are known to have immunomodulatory effects. Prevalence of ACh and its components in non-neuronal cells early in the embryonic stage suggests a possible role in normal development of fetal lymphoid organs and establishing the underlying immune tone (109). Hyperstimulation of nAChR through exposure to cigarette smoke and nicotine-containing products can lead to undesirable cellular and organ development set at birth (110).

In this section the non-neuronal cholinergic system will be discussed with regard to its expression, function, and role in human health with particular emphasis on macrophages and the potential developmental effects of *in utero* nicotine exposure.

b. Non-neuronal cholinergic system and human diseases

The term “cholinergic system” refers to the neurotransmitter ACh and the components of its synthesis, transport, receptor binding, and degradation. ACh was the first neurotransmitter identified in the 1920s and since then, its role in the neurons of the central and peripheral nervous systems has been extensively studied. Choline acetyltransferase (ChAT) is an enzyme responsible for catalyzing ACh from coenzyme A (CoA) and choline in the cytoplasm of cholinergic neurons. Once synthesized, ACh is stored in the synaptic vesicle and released upon depolarization of the neuron into the synapse by exocytosis. Ligand bound ACh receptors (AChR) cause changes in intracellular calcium concentration via ion-gated channel opening or by regulating downstream signaling effector molecules. Unbound ACh is quickly hydrolyzed by acetylcholinesterases (AChE) and the degraded components are recycled (4). There are two different classes of ACh receptors, ionotropic nicotinic and metabotropic muscarinic receptors, as their stimulation is induced by nicotine and muscarine, respectively (111). Both types can be activated by endogenous ACh, and they are widely distributed through the neuronal and non-neuronal cells. nAChR will be the main focus and therefore will be discussed more in depth.

The nAChR is a member of the ligand-gated ion channel receptor family that produces structures from combinations of five polypeptide subunits forming a functional core (other members include $GABA_A$ receptors, $5HT_3$ receptors, and glycine receptors) (112). Types of nAChR subunits include muscle subunits (α1, β1 γ, δ, ε) and neuronal subunits (α2-7, α9-10, and β2-4), which can be further classified into α-bungarotoxin sensitive α7-9 homopentamers and α-bungarotoxin insensitive heteropentamers (113). Once endogenous ACh or exogenous nicotine binds to a receptor, it is stabilized in the open conformation and causes depolarization of the membrane via cation influx. Various combinations of these subunits contribute to the diversity of nAChR properties and functions that are tissue specific. For example, receptors that contain α4 and β2 subunits, which are mainly found in the brain, have the highest affinity for nicotine. Depending on the stoichiometry of the subunits, receptors may be more or less sensitive for upregulation when a ligand is bound (113, 114). α7 nAChRs have the highest permeability to calcium, which can lead to calcium-dependent downstream signaling and

result in physiological responses, such as proliferation, angiogenesis, and apoptosis (115).

Despite the vast majority of study dedicated to understanding the neuronal cholinergic system, the complex and intricate network connecting nAChR to cellular functions beyond the nervous system have only recently garnished attention. ACh has been found to play a role in various non-neuronal cells, including epithelial, endothelial, mesothelial, and immune cells. (108). In fact, ACh is ubiquitously expressed in prokaryotic and non-neuronal eukaryotic cells, such as protozoa, fungi, and plants, and components of the cholinergic system are also detected in these cells, demonstrating the role of cholinergic communication in non-neuronal systems (111). Evolutionarily, this indicates that the non-neuronal cholinergic system existed before the neuronal cholinergic system. Therefore, the term “non-neuronal cholinergic system” has been introduced to distinguish the newly discovered role of ACh and its components acting as a local signaling “cytotransmitter” molecule, whereas the conventional “neuronal cholinergic system” describes the role of ACh as a neurotransmitter, mediating rapid communication between neurons and effector cells (116). The importance of the non-neuronal cholinergic system in human health is highlighted in many studies and nAChRs have become a potential target for pharmacotherapy in a variety of disease states.

The role of the non-neuronal cholinergic system in pathogenesis is supported by *in vivo* and *in vitro* studies of cancer, immune-related diseases, respiratory diseases, and many more. Patients suffering from cystic fibrosis (CF) and COPD have impaired mucociliary clearance, mucus hyperviscosity, increased risk for lung infection and pulmonary function decline. It was observed that nAChRs are expressed at the apical membrane of the ciliated lung epithelium and its co-localization with the cystic fibrosis conductance regulator (CFTR) may be responsible for its regulatory role in CFTR function. Absence of α7 nAChR led to lower mucus transport as well as higher electrolyte concentrations in the mucus, similar to what is observed in CF patients. As mentioned earlier, α7 nAChR has the highest permeability to calcium, which is an important mediator in activating signaling cascade that ultimately activates CFTR-mediated ion transportation. Coupling of α7 nAChR and CFTR function was further validated by α7 nAChR agonist-induced calcium influx and activation of effector molecules involved in CFTR activation as well as delocalization of CFTR in α7 nAChR-deficient mice. (117). In a human breast cancer cell line, elevated α9 nAChR mRNA and protein expression levels were observed after 6 h of nicotine treatment (118).

Proliferative properties induced by nicotine treatment were also demonstrated in other cancer cell lines, including those from the lung, pancreas, stomach, and colon, mediated via other nAChR subunits. One important component of cancer cells is the ability to migrate and metastasize to a new region. Chronic nicotine treatment to lung and breast cancer cell lines resulted in an altered regulation of adhesion molecules, including E-cadherin and β-catenin, and mesenchymal proteins, including fibronectin and vimentin. As a result, nicotine-exposed cells demonstrated the ability to undergo an epithelial-mesenchymal transition and gain a migratory phenotype (119, 120).

In non-neuronal cells, ACh acts in both an autocrine and a paracrine manner. For example, ACh secreted from airway epithelial cells targets neighboring epithelial cells, monocytes and resident macrophages that express AChRs (Figure 2.3). Stimulation of nAChR expressed on circulating monocytes or macrophages leads to inflammatory or anti-inflammatory effects (121). Reciprocally, nAChR expression levels can be regulated by effector molecules of the immune cells, demonstrated by upregulation of α4β2 nAChR levels by TNFα released from macrophages. ACh released from airway epithelial cells regulates their ciliary activity and proliferation, and this is supported by the fact that small cell lung cancer cell growth is promoted by ACh released as an autocrine growth factor (121).

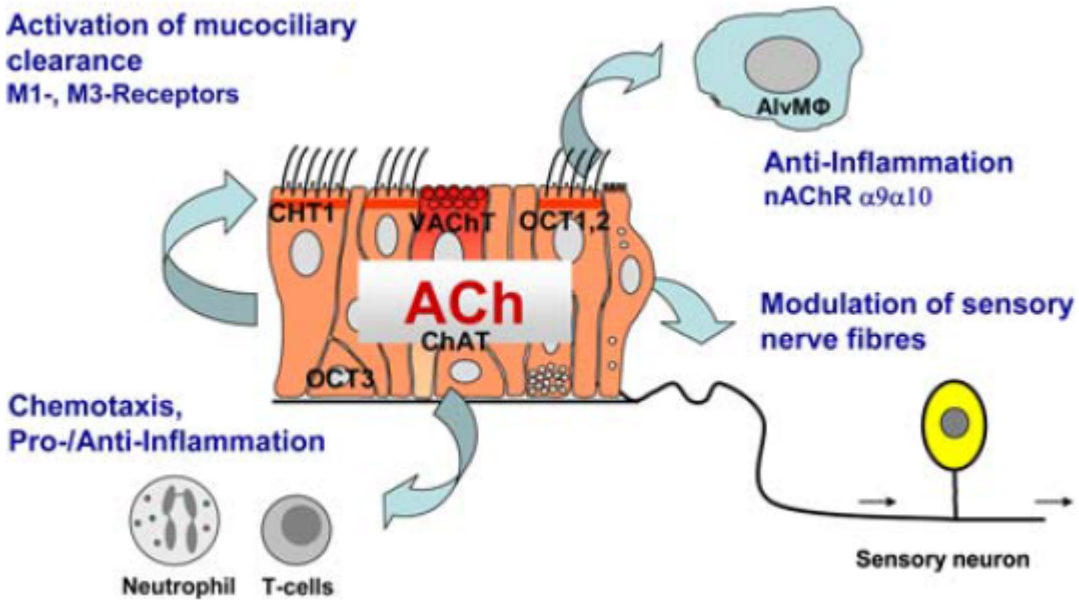


Figure 2.3 Auto/paracrine role of epithelial ACh.[121] Used with permission.

c. Non-neuronal cholinergic system in immune cells and nicotine

Although ACh was first reported in the 1930s to be present in mammalian blood, it was not until the 1980s that a sensitive and specific radioimmunoassay (RIA) for detecting ACh was available to confirm its presence in mammalian plasma. The origin of ACh in the bloodstream was found to be immune cells, particularly mononuclear leukocytes (MNL). Nicotine administration in rabbits led to an increase in plasma ACh concentration with a reduction in blood ACh levels in the presence of an AChE inhibitor. Further analysis of blood components detected ACh contained in the MNL fraction only and not in polymorphonuclear leukocytes (PMN) or the red blood cells. Subsequently, the expression of choline acetyltransferase (ChAT) mRNA from a human leukemic T cell line and mammalian blood and the expression of both mAChR and nAChR on most immune cells led to the conclusion that ACh is synthesized by the immune cells independent of that released from neurons (Reviewed in 122, 123).

The presence of cholinergic components in immune cells suggests that ACh must have a role in regulating the immune response. In fact, stimulation of mAChRs or nAChRs results in downstream signaling process and cellular response, such as differentiation of CD8+ T cells, changes in the pattern of cytokine release, and proliferation (124-127). Depending on the levels of expression and types of mAChR and/or nAChR present on individual cell types, the overall biological changes induced by stimulation of these receptors may vary. For example, M_1 mAChRs appear to participate in the differentiation of CD8+ T cells into cytolytic T cells while deletion of M_3 or M_5 or combination of M_2 and M_4 mAChRs has no effect (124). Also, M_1/M_5 mAChR-deficient mice showed reduced level of the pro-inflammatory cytokines IL-6 and IFNγ, whereas α7 nAChR-deficient mice were associated with increased level of the pro-inflammatory cytokine TNFα (125, 127).

Although details concerning each type of AChR and the composition of its subunits and their expression on immune cells are still being actively investigated, the role of the cholinergic system in immune related diseases is apparent and has been demonstrated in several disease models, including sepsis, colitis, and arthritis (128). Particular attention was given to the role of the anti-inflammatory effects of α7-nAChR stimulation on macrophages as a part of the ‘cholinergic anti-inflammatory pathway.’

ACh treated human macrophages that were exposed to LPS displayed inhibition of TNFα release through a post-transcriptional mechanism (129). Comparable inhibition was observed with nicotine administration. Using the α7 nAChR antagonist, α-bungarotoxin, the study concluded that α-bungarotoxin-sensitive nAChRs are responsible for mediating the observed effects (129). Additionally, release of other pro-inflammatory cytokines, including IL-1β, IL-6, and IL-18, were also inhibited by ACh treatment while no changes were observed in anti-inflammatory cytokine IL-10 release. Subsequent studies identified that the α7 subunit is necessary for cholinergic-induced anti-inflammatory responses, and this was further validated *in vivo* using α7 subunit-deficient mice. This study showed that cells release significantly higher amount of TNFα after administration of endotoxin compared to wild-type mice (127). Later, it was identified that T cells are responsible for producing ACh and regulating macrophage cytokine release patterns as well (130).

It was discussed earlier that cigarette smoke and nicotine can suppress inflammatory response partially by shifting macrophages towards an M2 phenotype. Different compositions of nAChR subunits are capable of mediating this change with the most studies performed to investigate the α7 subunit. Nicotine-induced suppression of pro-inflammatory cytokines as well as antimicrobial activities to *Legionella pneumophila* were observed in alveolar macrophages that express only α4β2 nAChR subunits, and these effects were reversed with *d*-tubocurarine, a non-selective nAChR antagonist (99). In a sepsis-induced acute lung injury (ALI) model with *Escherichia coli*, reduction in MIP-2 production in bronchoalveolar lavage fluid and neutrophil migration into the lungs were observed upon administration of α7 nAChR agonists, including nicotine. Pre-treatment with nicotine led to a reduction in LPS-induced MIP-2 and TNFα cytokine production by alveolar macrophages, which was reversed with α7 nAChR specific antagonist methyllycaconitine (MLA). Similar results were seen with wild-type neutrophils and α7 nAChR-deficient neutrophils treated with nicotine, suggesting the expression of α7 nAChR on neutrophils and the functional regulation by α7 nAChR. Overall outcomes in mice indicate that nicotine treatment aids in survival after intratracheal *E. coli* infection and α7 nAChR plays an important role (131).

There is insufficient data regarding the role of non-neuronal cholinergic system in immune cell development. However, it was observed that the non-neuronal cholinergic system is developed and expressed early in life, during fetal hematopoiesis. This is particularly important because, unlike in adults, hematopoietic stem cells (HSC) are

generated initially in the yolk sac and then migrate to the fetal liver. Homing of HSC from fetal liver to the bone marrow occurs towards the end of the gestational period. The intrauterine developmental period is, therefore, crucial in the process of lymphopoiesis and any disturbance in this process can lead to imbalanced immune cell populations that can be amplified as a defect in the overall regulation of the immune system (132).

In fact, nicotine treatment was shown to change environments regulating hematopoietic stem cells in the fetal bone marrow. The presence of ChAT, AChE, and nAChRs was confirmed by gene expression profiling from embryonic stem cells, and nicotine treatment led to impaired colonization of hematopoietic stem cells in the fetal bone marrow. Furthermore, the cytokine production profile from bone marrow cells was changed upon nicotine treatment such that hematopoiesis-supportive cytokines, such as G-CSF and GM-CSF, were suppressed and Th2 T lymphocyte produced cytokine IL-4 was upregulated (109). Another study demonstrated that alveolar macrophages isolated from mice exposed to nicotine developmentally had suppressed inflammatory response mediated by α7 nAChR (104). Expression of α7 nAChR was increased in mice exposed to nicotine *in utero* and its role in regulating macrophage function was demonstrated. One such example is the increased expression of TGFβ1 by alveolar macrophages harvested from the pups on post-natal day 7. However, this increase was attenuated in α7 nAChR knock-out mice that were exposed to nicotine developmentally, suggesting that α7 nAChR and other nAChR may be involved in mediating these changes. Additionally, cells expressing markers of alternative activation, including arginase-1, Ym1, and fibronectin, were increased in pups exposed to nicotine prenatally. This observation was reversed in α7 nAChR knock-out mice. Reduction in phagocytic activity against *Staphylococcus aureus* was displayed only in the macrophages expressing α7 nAChR, which is another noteworthy impact of *in utero* nicotine exposure and the role of non-neuronal cholinergic system in mediating these effects. These findings raise concern regarding the effects of *in utero* nicotine exposure on programming of macrophage function and its tone set at birth, which can predispose neonates to various immune-related diseases (104).

D. Project Overview

The use of NRT during pregnancy is still without strong efficacy and safety evidence. It should not be neglected or underestimated that nicotine itself poses harmful effects to human health and potentially to proper development of fetal immunity. In order to state that NRT is less harmful than smoking during pregnancy and recommend its use to pregnant smokers, it is crucial to understand how *in utero* nicotine exposure affects offspring and the extent of its effects. In this study, we administered nicotine to pregnant mice via their drinking water to generate offspring that were exposed to nicotine *in utero*. The immune response was then observed following *P. aeruginosa* lung infection. Additionally, bone marrow cells were obtained from offspring mice and differentiated into macrophages, which were then stimulated with LPS and various cytokines to observe cellular responses *ex vivo*. Finally, a clinical study has been developed to examine the impact of NRT on macrophage gene expression in neonates. Tracheal aspirate samples from neonates will be collected and gene expression signatures will be compared to correlate findings to different modes of *in utero* nicotine exposure. This step will aid in identifying impacted genes and targeting future studies in this area. The central hypothesis of this project is that *in utero* nicotine exposure will alter immune cell disposition and function in response to *P. aeruginosa* lung infection. This will be investigated through *in vivo* infection as well as *ex vivo* macrophage stimulation with LPS.

Chapter 2. Methods

A. Animal Work

a. Mice

C57BL/6 mice with a GFP tag on α4 nicotinic receptors were provided from the Stitzel lab (University of Colorado at Boulder) and bred in-house for the experiments. Mice were fed with either saccharin dissolved in water or nicotine dissolved in water (200 μg/L) ad libitum before and throughout pregnancy. This is a well-established model for chronically administering nicotine to experimental animals and achieves plasma cotinine levels within the range reported in human smokers (133). All animal studies and procedures were approved by the University of Kentucky Institutional Animal Care and Use Committee.

b. Intratracheal Infection

After weaning, 4-6 week old offspring were infected intratracheally with the clinically derived mucoid strain *P. aeruginosa* M57-15. Bacteria were thawed from the stock vial and grown in Trypticase soy broth (TSB) to late log phase at 37°C, which were then incorporated into agarose beads by adjusting temperature of mineral oil and TSB and swiftly mixing them. Once the beads had formed, they were washed multiple times with phosphate buffered saline (PBS) to retrieve beads of various sizes ranging in 10-100 μm. Visualization of beads was performed using an inverted microscope to confirm the sizes and numbers of beads (134). The number of CFU was determined by homogenizing the beads and growing the bacteria on Trypticase soy agar (TSA) plates in multiple dilutions. The *P. aeruginosa*-laden agarose beads were diluted to the target CFU for each infection. Beads were kept at 4°C and used within 1 week of preparation to assure their quality and integrity. On the day of infection, mice were lightly anesthetized with isoflurane aerosolized and 100uL of beads were instilled intratracheally using a curved 24-gauage needle. The same volume was plated on Pseudomonas isolation agar

(PSA) plates to ensure that mice were infected with desired inoculum. Daily weights were recorded as a measure of morbidity.

c. Tissue Harvest

On post-infection day 5, mice were humanely euthanized by injecting 0.1mL of SOMNASOL Euthanasia-III Solution (Henry Schein Animal Health, Dublin, OH) intraperitoneally. Bronchoalveolar lavage fluid was collected by instilling 5 mL of buffered solution containing 0.3 μM EDTA in 1 mL aliquots. This is representative of immune cells located in the airway compartment and will be referred to as lung lavage (LL). The first 1 mL was collected and centrifuged to separate out cells and measure cytokine concentration in the supernatant. Pelleted cells from this collection was combined with the remainder 4 mL of lavage fluid samples. Lungs were then collected in RPMI medium (Mediatech, Manassas, VA), minced with scissors, and incubated with 1mg/mL collagenase A and 50 U/mL DNase for 1 h at 37°C. Digested lung tissue was then pushed through a 70 μm mesh screen to create a single cell suspension and washed with red blood cell lysis buffer (Quality Biological, Inc., Gaithersburg, MD) and PBS. This is representative of immune cells located in the lung interstitium and will be addressed as lung digest (LD). An aliquot of LD from each mouse sample was plated on PSA plates in multiple dilutions to determine bacterial burden. Lastly, tracheobronchial lymph nodes (LN) were collected and pushed through 70 μm mesh screens to create a single cell suspension. Cells were then washed with red blood cell lysis buffer and PBS.

d. Flow Cytometry

Cells from LD, LL, and LN were incubated with panels of fluorescently labeled antibodies (CD11b, CD11c, Ly6G, CD4, CD8, CD19, and CD44) to determine their characteristics by surface marker expression. In excess of 50,000 events per sample were acquired by the Attune Acoustic Focusing Cytometer (Life Technologies, Carlsbad, CA), and data were analyzed by FlowJo software (Tree Star, Ashland, OR). Acquired cells were gated for leukocytes and the percentage of each subset was multiplied by the total number of cells.

e. Cytometric Bead Array

Cytokine concentration from the first lung lavage wash as well as from the supernatants collected from the human tracheal aspirate samples were measured using cytometric bead array (BD Biosciences). The following cytokines were measured: IL-6, IL-10, MCP-1, IFN_{γ}, IL-12p70, and TNF-α. Briefly, detection beads are conjugated with antibodies specific for each cytokine in the sample. Sample was mixed with the beads and the mixture was added to phycoerythrin-conjugated detection antibody for 2 hours. Fluorescence intensity for each analyte was then assessed via flow cytometry, with each distinguished by gating on specific bead size, and compared to a standard curve of known concentrations.

f. Isolation and *ex vivo* stimulation of bone marrow-derived macrophages (BMDM)

Primary macrophage cells derived from bone marrow were isolated and cultured *ex vivo* to determine their responses upon stimulation and polarization. C57BL/6 mice with GFP tag on nicotinic receptors described previously were used. Briefly, 4-6 week old offspring from mice fed with either nicotine or saccharin dissolved in water during gestation were humanely euthanized. Femurs and tibias from both legs were dislocated by cutting off the patellar tendon and the foot, and the bones were isolated by removing tissues and muscles. Bones were then flushed with 5 mL RPMI media by inserting 25-gauge 5/8 inch needle into the bone cavity. This was repeated until all bone marrow cells were removed and the bone appeared clear. Collected cells were centrifuged at 1200 rpm for 5 minutes and red blood cells were lysed using the lysis buffer. Bone marrow cells were then washed and resuspended in complete RPMI (supplemented with 10% fetal bovine serum, $2x10^{-5}$M 2-mercaptoethanol, 1% penicillin/streptomycin, 1% glutamine) as well as L929 supernatant, which contains macrophage colony-stimulating factors (M-CSF) to allow differentiation of hematopoietic bone marrow stem cells into macrophages (135). Cells were initially cultured in petri dishes with media changed every 2 days. On day 7, cells were replated in 24-well plates at $2x10^5$ cells per well in

complete RPMI media (without L929 supernatant) and treated with IFNγ (20 ng/mL), IL4/13 (10 ng/mL), and LPS (50 ng/mL) for determination of arginase activity or cytokine levels. Cells were incubated at 37°C and 5% CO_2 for 24 hours.

g. Arginase Activity

Arginase activity was assessed by measuring urea concentration produced in the arginase reaction. Arginase converts arginine into urea and ornithine, and the activity is measured by the intensity of urea-chromogen complex, which produces a color change. Prior to the assay, cell lysates were prepared using 0.4% Triton X-100 in 10 mM Tris-HCl buffer with protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL). Arginine buffer was preheated to 37°C for 10 minutes and then combined with Mn solution to form a substrate buffer. 10 µL of the substrate buffer was added to 40 µL of each sample on a 96 well plate and incubated at 37°C for 2 hours. The arginase reaction was terminated by adding 200 µL of urea reagent and incubating the plate for 1 hour at room temperature. Optical density (OD) of each sample was read using a 430 nm filter, and the readings were normalized using OD of blank sample and water. One unit of arginase is responsible for conversion of 1 µmole of L-arginine to ornithine and urea per minute, and the values were additionally normalized to total protein concentration, using the bicinchroninic acid protein assay (BCA) (Pierce Biotechnology, Rockford, IL).

B. Human Study

a. Study Design

Pregnant women at the University of Kentucky Medical Center who gave birth to pre-term (>24 weeks) and term infants were screened for inclusion. Initially, a collaborating neonatologist identified potential participants based on general health of the mothers and the newborns. After obtaining a consent from the mothers, a detailed smoking history and medical history were obtained through a series of questionnaires (Appendix I) and used to include or exclude patients from the study. Use of ≥ 5 cigarettes daily during pregnancy was required to be enrolled in the smoking group. Women who used nicotine replacement products during pregnancy were stratified into the replacement group. The control group was defined by no direct exposure to nicotine during pregnancy. Informed consent and parental permission forms to include the neonates in the study were provided at recruitment and signed informed consent was required prior to participant enrollment (Appendix II). Patients were excluded if there were known major fetal abnormalities, chemical/alcohol dependence, contraindication to NRT, use of any forms of tobacco other than cigarettes or e-cigarettes, and any active infection for either the mother or newborn at the time of delivery, before sample collection. Premature infants requiring mechanical ventilation for reasons other than assisting “physiologic normalcy” were excluded from the study. Chronic use of medications known to pose immunomodulation, such as steroids, or have potential for causing immunomodulation also met exclusion criteria.

b. Sample Collection and RNA isolation

Baseline data (date of birth, hospital number, ethnicity, medical history, daily number of cigarettes smoked during pregnancy, time from last cigarette smoked/nicotine replacement product use, partner’s smoking status, gestational age, signed consent form, a list of acute/chronic medications, medications administered during current

hospitalization, and indication of participant's contact details) were collected through a series of questionnaires as well as from the subject's medical record (Appendix III).

Within the first week of birth, 0.5 to 1 mL of tracheal aspiration samples were collected for a total of 2-3 samples per baby (up to 5 samples). Samples were centrifuged at 4°C for 7 min at 300g to pellet the cells, and supernatants were saved and frozen at -80°C for cytokine measurement. Cells were then washed 3 times with RPMI and 1-2x10^6 cells were aliquoted and kept frozen in TRizol at -80°C for RNA isolation. The remainder of cells were seeded at 2x10^5 cells per well in 24-well plates and incubated at 37°C and 5% CO_2 for 2 hr to isolate alveolar macrophages. After removing non-adherent cells, macrophages were stimulated with 10 ng/mL of LPS from *Escherichia coli* O55:B5 from EMD Millipore and 20 ng/mL of human interferon-γ (eBioscience) (IFNγ) for 6 hours. Supernatants were saved for post-stimulation cytokine measurement. Cells were placed in TRizol and frozen at -80°C for RNA isolation. RNA isolation was performed using the Qiagen RNeasy Kit (Qiagen Sciences, Germantown, MD) and quantified using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE).

Once patients are recruited for the study, 3 groups (cigarette smoke vs. NRT vs. no exposure) will be compared for 1) baseline RNA expression immediately upon collection of the specimen and 2) after stimulation with LPS and IFNγ. Upon isolation of RNA, microarray will be performed, using the TaqMan® Gene Expression Array Plates (Applied Biosystems, Foster City, CA). TaqMan® arrays are flexible, affordable, and convenient for gene expression analysis screening for specific biological pathways, processes, diseases, or can be customized. These arrays each consist of 48 genes of interest, such as TNFa, IL-1, IL-10, and CD86. A list of genes that will be assessed is provided in Appendix I. RNA samples (0.1-10ug) will be converted into cDNA, and using 10-100 ng of cDNA per plate, Applied Biosystems real-time quantitative PCR instrument will allow amplification of target genes. Gene expression can be measured by the quantitation of cDNA relative to a calibrator sample, which serves as a physiological reference. All quantitations will be also normalized to an endogenous control to account for variability in the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction.

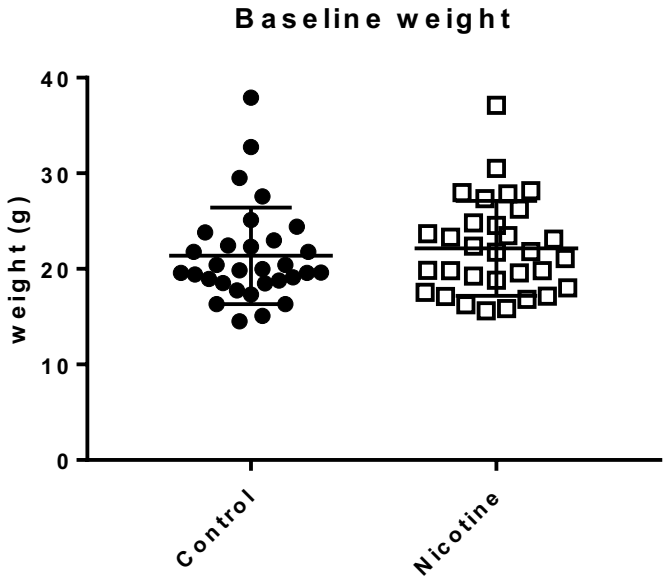
c. Statistics

Data are reported as mean ± SD and compared using GraphPad Prism (GraphPad Software, La Jolla, CA, USA). Data were compared via two-way ANOVA, followed by Bonferroni's post-test individual comparisons, or t-test where appropriate. Differences were deemed statistically significant at a p value < 0.05.

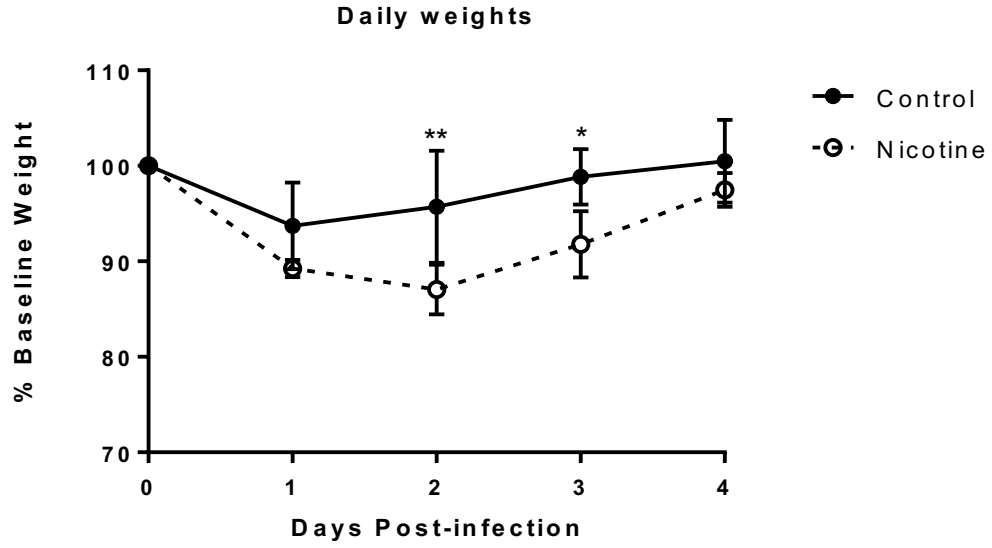
Chapter 3. Results

***In utero* nicotine exposure negatively affects morbidity outcome**

Mice exposed to either saccharin (Control) or nicotine *in utero* were infected with *P. aeruginosa* to assess the impact of developmental exposure on complete immune response. Daily weights were measured and mice that had weight reduction greater than 20% prior to the harvest were humanely killed. Pre-infection weights were comparable between the two groups with the average weight of 21.37 g and 22.15 g for control and nicotine group, respectively (Figure 3.1a). Infected mice lost weight initially but recovered by the end of study period. While the pattern of weight loss was similar between two groups, mice exposed to *in utero* nicotine displayed a greater weight reduction, particularly on Days 2 and 3 (Figure 3.1b). This experiment was repeated three times and, although no statistically significant differences were observed between the groups in pooled data, similar weight loss pattern was observed with more pronounced reduction in the nicotine group (Figure 3.1c).



a)



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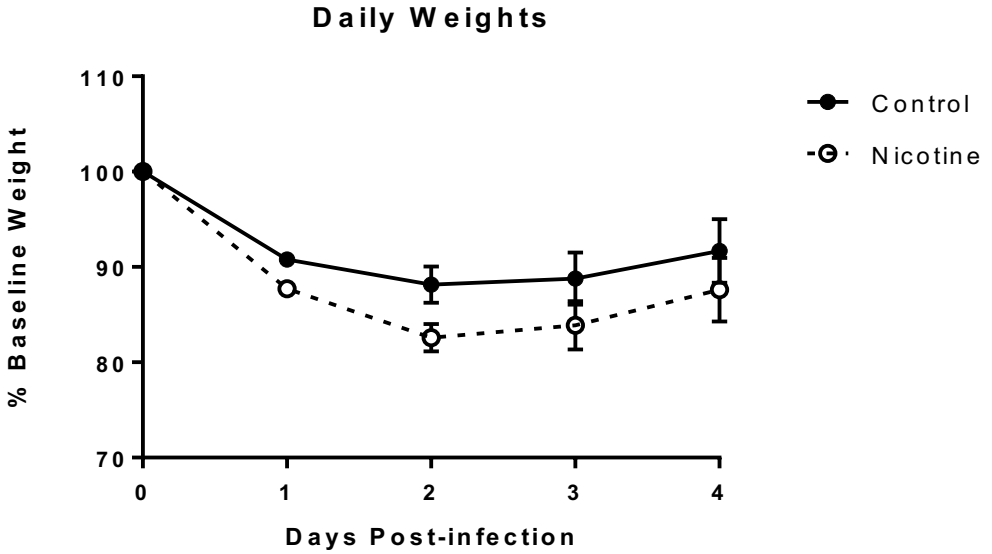


c)

Figure 3.1 Baseline weight and post-infection weight reduction as a measure of morbidity. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy. 4-6 weeks post-birth, mice were infected with agarose beads containing bacteria. a) Baseline weight pre-infection. b) Post-infection weight changes from a single experiment. c) Post-infection weight reduction from pooled data. Data represents the mean ± SD of b) 7 or c) 16 mice per group. Significance is indicated for p values < 0.05 (*) and < 0.01 (**). Data was analyzed by two-way ANOVA.

Pre- and post-infection cellular characteristics

A group of mice born to those fed with saccharin- or nicotine-dissolved water was sacrificed without an infection to assess baseline characteristics (pre-infection, Day 0) while another group underwent intratracheal infection with *P. aeruginosa* and were sacrificed to compare cellular characteristics on Day 5 post-infection. Cells were enumerated by Trypan blue staining using an automated cell counter and the percentage of immune cell subsets in the lung digest, lung lavage, and lymph nodes were determined by the surface receptor expression. The absolute number of each subset was obtained by multiplying the percentage by the total cell numbers.

First, we compared the total cell numbers in the airway compartments and the lymph node between the groups pre- and post-infection (Figure 3.2). There was no difference in the number of cells between the groups in any of the three compartments pre-infection, suggesting that *in utero* nicotine exposure is not associated with changes in cell numbers at baseline (Figure 3.2a, c, e). However, post-infection cell numbers increased significantly from the baseline only in the lung digest of the control group ($p=0.0411$), and this number was significantly higher than that of the nicotine group (Figure 3.2a, b). In the lung lavage samples and the lymph nodes, comparable cell numbers were observed between the groups as well as pre- and post-infection (Figure 3.2c-f).

We then analyzed cells for markers of neutrophils (Ly6G+), resident alveolar macrophages (CD11b-CD11c+), infiltrating monocytes (CD11b+Ly6G-), and lymphocytes in different compartments to characterize subsets of total cells. Through cytometric analysis, total cells were gated for granulocyte/monocyte population to separate out lymphocytes and apoptotic cells, based on forward/side scatter characteristics. Respective surface marker designations were used to obtain percent values for neutrophils, resident alveolar macrophages, and infiltrating monocytes within the gates. CD4, CD8, and CD19 cell populations were enumerated by gating for lymphocyte population from the total cells. A representative flow cytometry plot is shown in Figure 3.3, which depicts the gating scheme for neutrophils, resident macrophages, and infiltrating monocytes in the lung digest from control (Top) and nicotine (Bottom) groups.

Resident alveolar macrophages are the first-line defense against pathogens in the airways, and they are characterized by a high expression of CD11c and low expression of CD11b. When they fail to sufficiently control and maintain homeostasis

against pathogens, circulating monocytes and neutrophils are recruited to the site of infection, following cytokine/chemokine gradients, to supplement the initial burst of inflammatory response (136). Infiltrating monocytes express a high level of CD11b, and after they migrate to the lungs, their phenotype gradually begins to resemble that of the resident macrophages (137). Neutrophils, another infiltrating cell type during an infection, have a short lifespan but their survival is prolonged during an infection (136). Release of neutrophils from the bone marrow pool further supplements their accumulation in the lungs. This influx is critical for clearing bacteria and inducing adaptive immune response (136). We defined neutrophil subset as a positive expression of Ly6G on the cellular surface.

Both groups exhibited a significant increase in the number of neutrophils from baseline in the post-infection lung digest (control: $p<0.0001$, nicotine: $p=0.0184$) (Figure 3.4a, b). However, post-infection neutrophil numbers were significantly higher in the control group compared to nicotine group ($p=0.0266$, denoted) (Figure 3.4b). Similarly, while baseline resident macrophage numbers were comparable, post-infection numbers were significantly higher in the lung digest of the control group ($p=0.0170$, denoted) (Figure 3.4c, d). Post-infection infiltration of monocytes was observed in the lung digest of both groups, but unlike neutrophils, this influx was comparable between the treatment groups (Figure 3.4e, f). Interestingly, these differences were not observed in the lung lavage fluids (Figure 3.5a-f). Pre- and post-infection cell numbers remained similar for neutrophils, resident macrophages, and infiltrating monocytes in both control and nicotine groups, although there was a trend towards increased post-infection influx of neutrophils in the nicotine group ($p=0.056$) (Figure 3.5a, b). No significant differences in the number of cells were observed between the groups.

Next, we analyzed cells of the adaptive immunity, CD4+ and CD8+ T lymphocyte and B lymphocyte characteristics in each compartment. B lymphocytes were characterized by the expression of CD19. T and B lymphocytes have a delayed response compared to innate immune cells and become effective 5-7 days post-infection (138). This is because dendritic cells are needed to first transport antigens into draining lymph nodes and activate naïve T cells that are specific to a particular antigen. Subsequently, antigen-specific T cells are selected, differentiated, proliferated, and travel back to the inflamed tissue to remove pathogens or remain in the lymph nodes to activate B cells. Therefore, we compared subsets of lymphocytes in the lung tissue and lymph nodes on post-infection day 5.

Nicotine groups displayed a significant increase in the number of CD4+ and CD8+ T cells in the lung digest post-infection ($p=0.0004$ and $p=0.0273$ for CD4+ and CD8+ T lymphocytes, respectively) compared to baseline (Figure 3.6a-d). However, both pre- and post-infection CD4+ and CD8 T cell numbers were similar to those of control group. Interestingly, the control group had comparable pre- and post-infection CD4+ and CD8+ T cell numbers. On the contrary, the nicotine group had a significantly lower number of B lymphocytes post-infection ($p=0.0482$) from baseline, and this number was significantly lower than that of the control group ($p=0.0109$, denoted) (Figure 3.6e, f). Neither group nor time after infection had an impact on CD4+ T lymphocyte counts in the lymph nodes (Figure 3.7a, b). However, a significant increase in the CD8+ T lymphocytes was noted in the lymph node of control group, compared to baseline ($p=0.0235$) (Figure 3.7c, d). Unlike in the lung digest, post-infection B lymphocytes of the nicotine group increased significantly compared to the baseline ($p=0.0247$) as well as compared to control in the lymph nodes ($p=0.0386$, denoted) (Figure 3.7e, f).

Overall, this data shows that the baseline cell numbers were not affected by *in utero* nicotine exposure in any of the three compartments analyzed. However, some key differences in the immune response were observed upon Pseudomonal lung infection. Although robust neutrophil influx was observed post-infection in both groups, this was less pronounced in the nicotine group. Additionally, a significant reduction in the number of B cells in the lung interstitium but a significant elevation in the lymph nodes in the nicotine group suggests a potential defect in migration and/or proliferation of B lymphocytes, due to *in utero* nicotine exposure.

Characteristics of cell composition and disposition

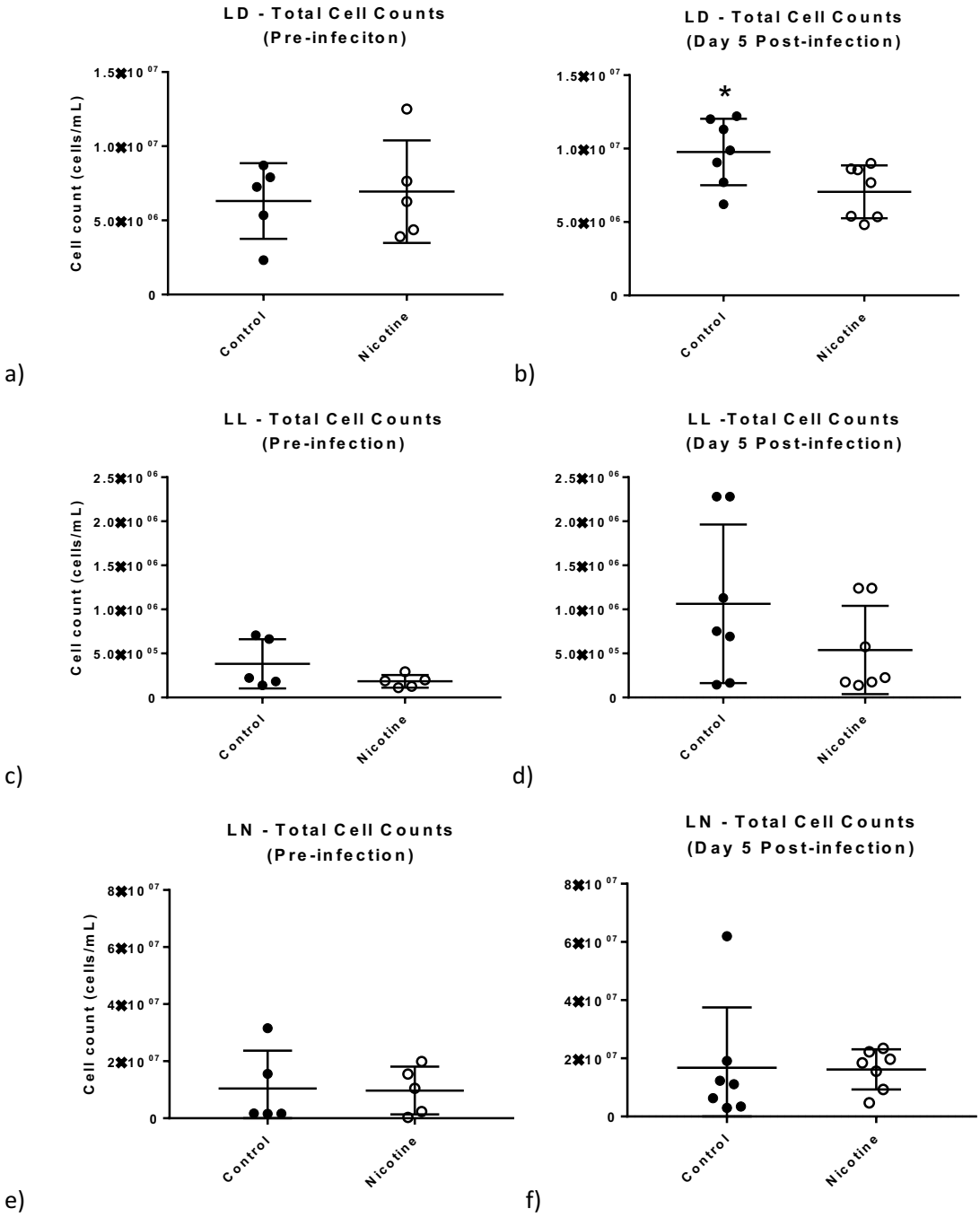


Figure 3.2 Pre- and post-infection cell counts in the lung digest, lung lavage, and lymph nodes. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy and humanely killed at 4-6 weeks of age before and after intratracheal infection with *P. aeruginosa*. Immune cells were quantified by flow cytometry in (a-b) lung digest, (c-d) lung lavage fluid, and (e-f) lymph node. Data represents the mean ± SD, and statistical significance is indicated for p value < 0.05 (*) between the groups. (*) denotes significance between groups. Data was analyzed by two-way ANOVA for comparison of the groups and t-test for comparison of pre- and post-infection

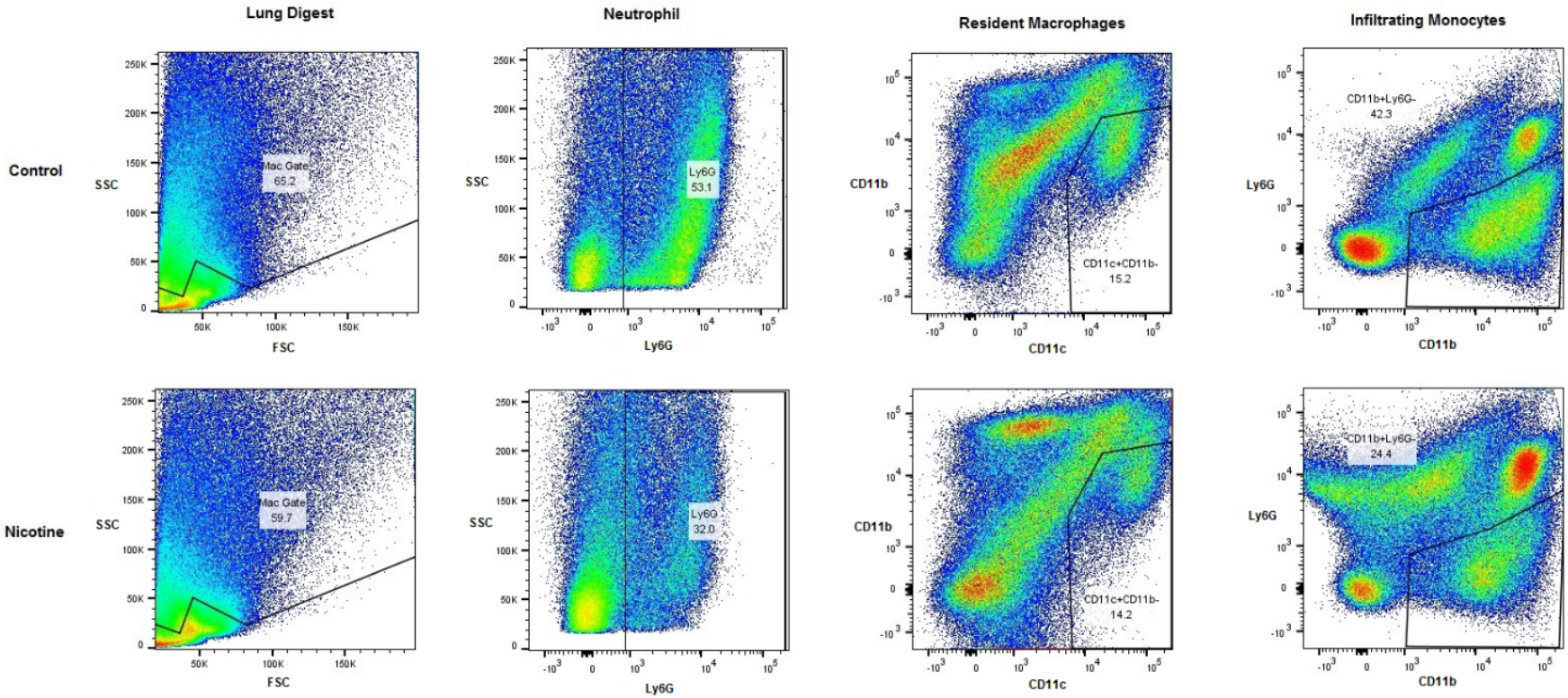


Figure 3.3 Lung parenchyma cell analysis by flow cytometry. On post-infection day 5, lungs were harvested from mice exposed to saccharin (Top) or nicotine (Bottom) developmentally. Single cell suspension was created by incubating lung tissue with collagenase and DNase. Cells were stained with fluorescently labeled antibodies to determine surface marker expression. Initially, cells were gated to isolate granulocyte/monocyte population. Subsequently, neutrophil (Ly6G+), resident macrophage (CD11b-CD11c+), and infiltrating monocyte (CD11b+Ly6G-) populations were analyzed by their respective surface marker expressions. SSC, side scatter; FSC, forward scatter.

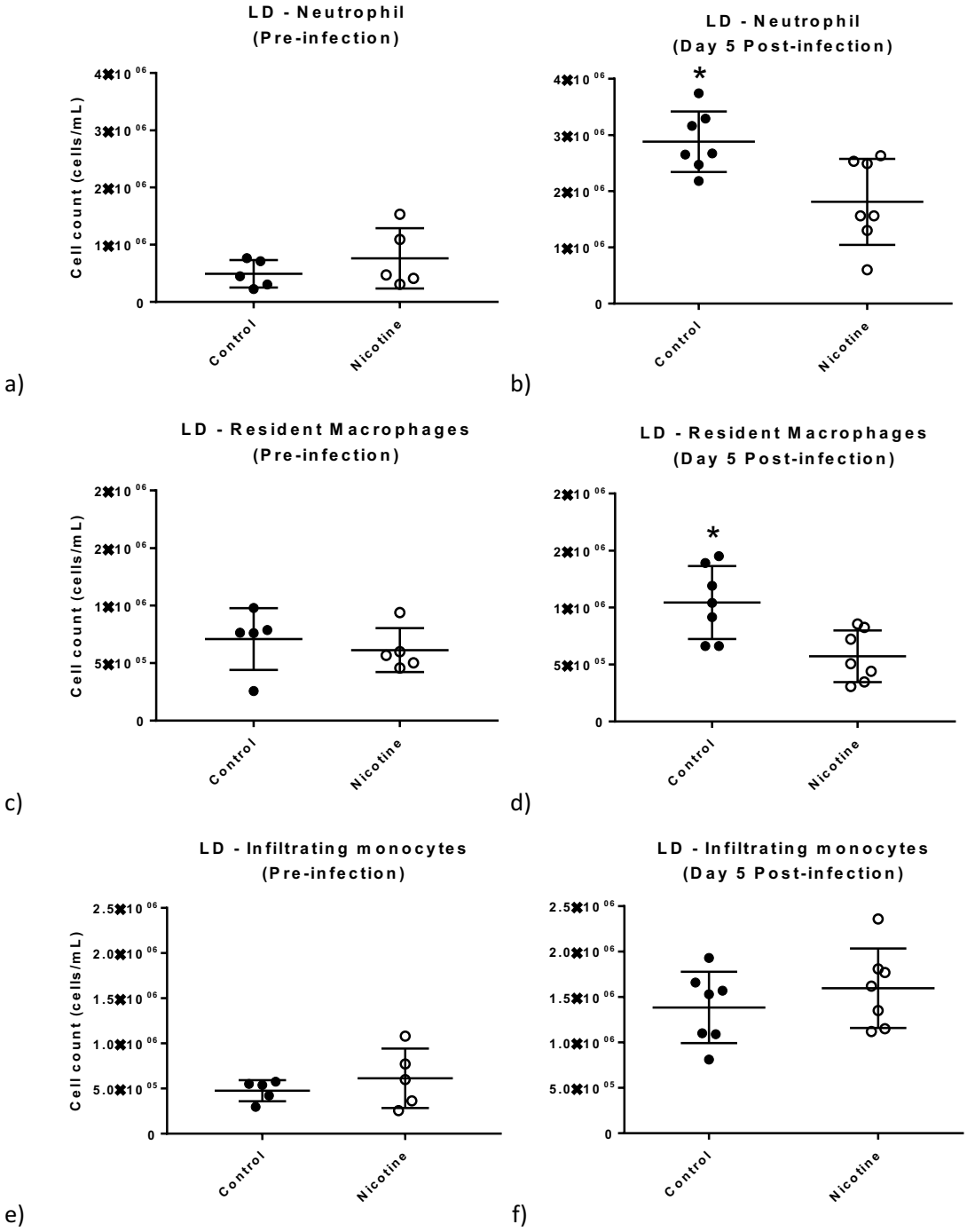


Figure 3.4 Pre- and post-infection neutrophil, resident macrophages, and infiltrating monocytes in lung digest. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy and humanely killed before and after intratracheal infection with *P. aeruginosa*. Immune cells were quantified by flow cytometry in the lung digest for markers of (a-b) neutrophils (Ly6G+), (c-d) resident macrophages (CD11b-CD11c+), and (e-f) infiltrating monocytes (CD11b+Ly6G-). Data represents the mean ± SD, and statistical significance is indicated for p value < 0.05 (*). (*) denotes significance between groups. Data was analyzed by two-way ANOVA for comparison of the groups and t-test for comparison of pre- and post-infection.

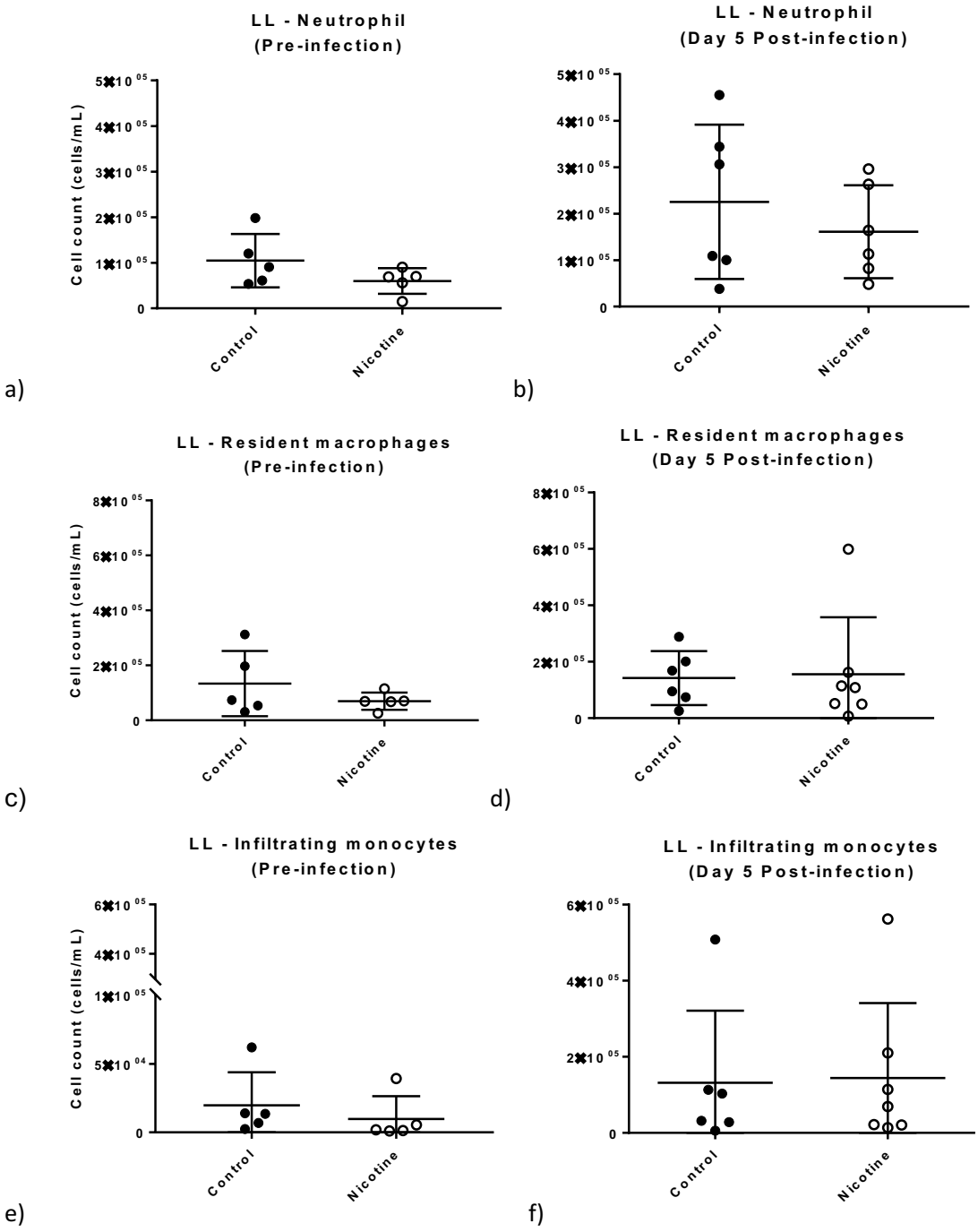


Figure 3.5 Pre- and post-infection neutrophil, resident macrophages, and infiltrating monocytes in lung lavage. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy and humanely killed before and after intratracheal infection with *P. aeruginosa*. Immune cells were quantified by flow cytometry in the lung lavage samples for markers of (a-b) neutrophils (Ly6G+), (c-d) resident macrophages (CD11b-CD11c+), and (e-f) infiltrating monocytes (CD11b+Ly6G-). Data represents the mean ± SD. Data was analyzed by two-way ANOVA for comparison of the groups and t-test for comparison of pre- and post-infection. Differences were not statistically significant.

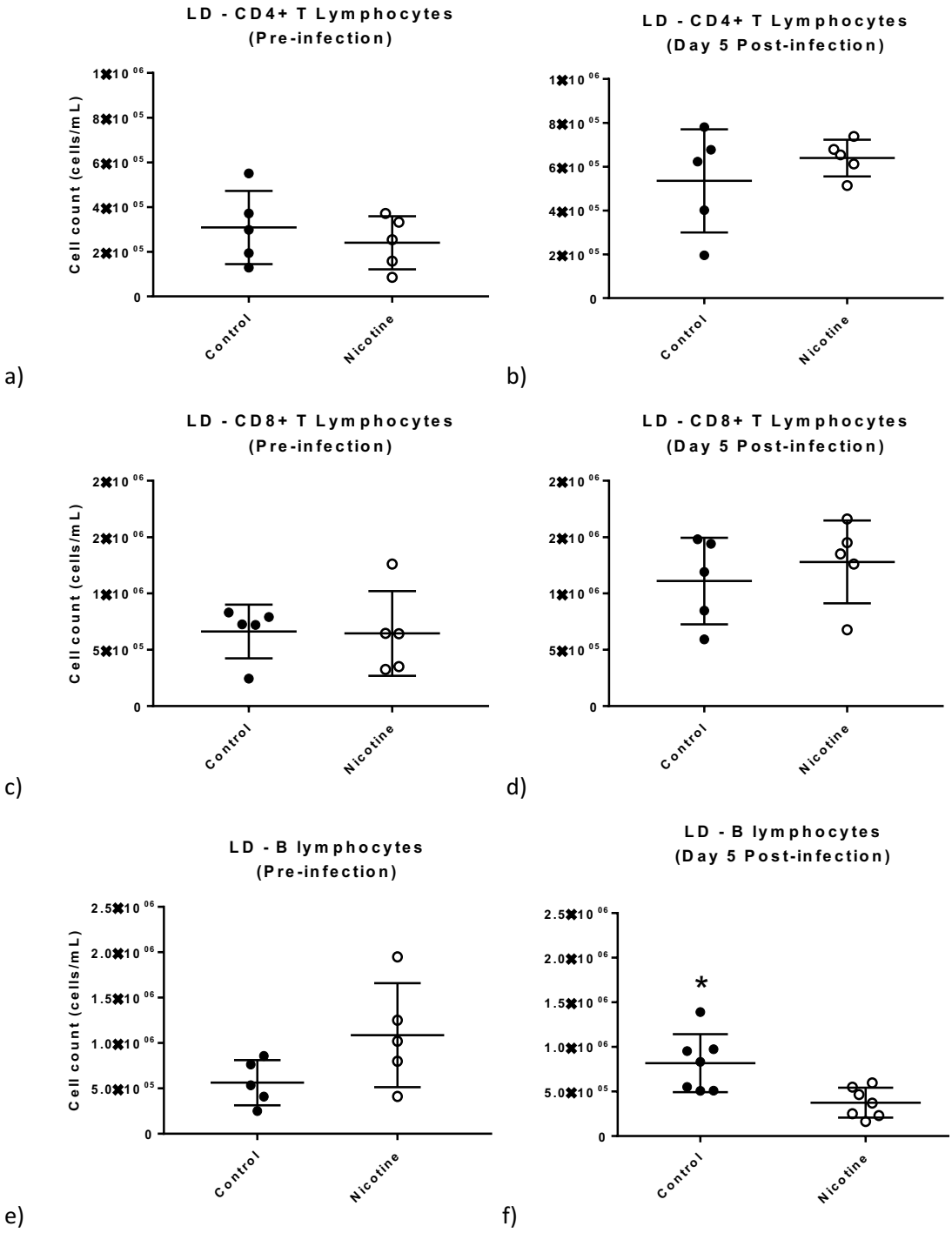


Figure 3.6 Pre- and post-infection T lymphocytes and B lymphocytes in lung digest. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy and humanely killed before and after intratracheal infection with *P. aeruginosa*. Immune cells were quantified by flow cytometry in the lung digest for markers of (a-b) CD4+ T lymphocytes, (c-d) CD8+ T lymphocytes, and (e-f) B lymphocytes (CD19+). Data represents the mean ± SD, and statistical significance is indicated for p value < 0.05 (*). (*) denotes significance between groups. Data was analyzed by two-way ANOVA for comparison of the groups and t-test for comparison of pre- and post-infection.

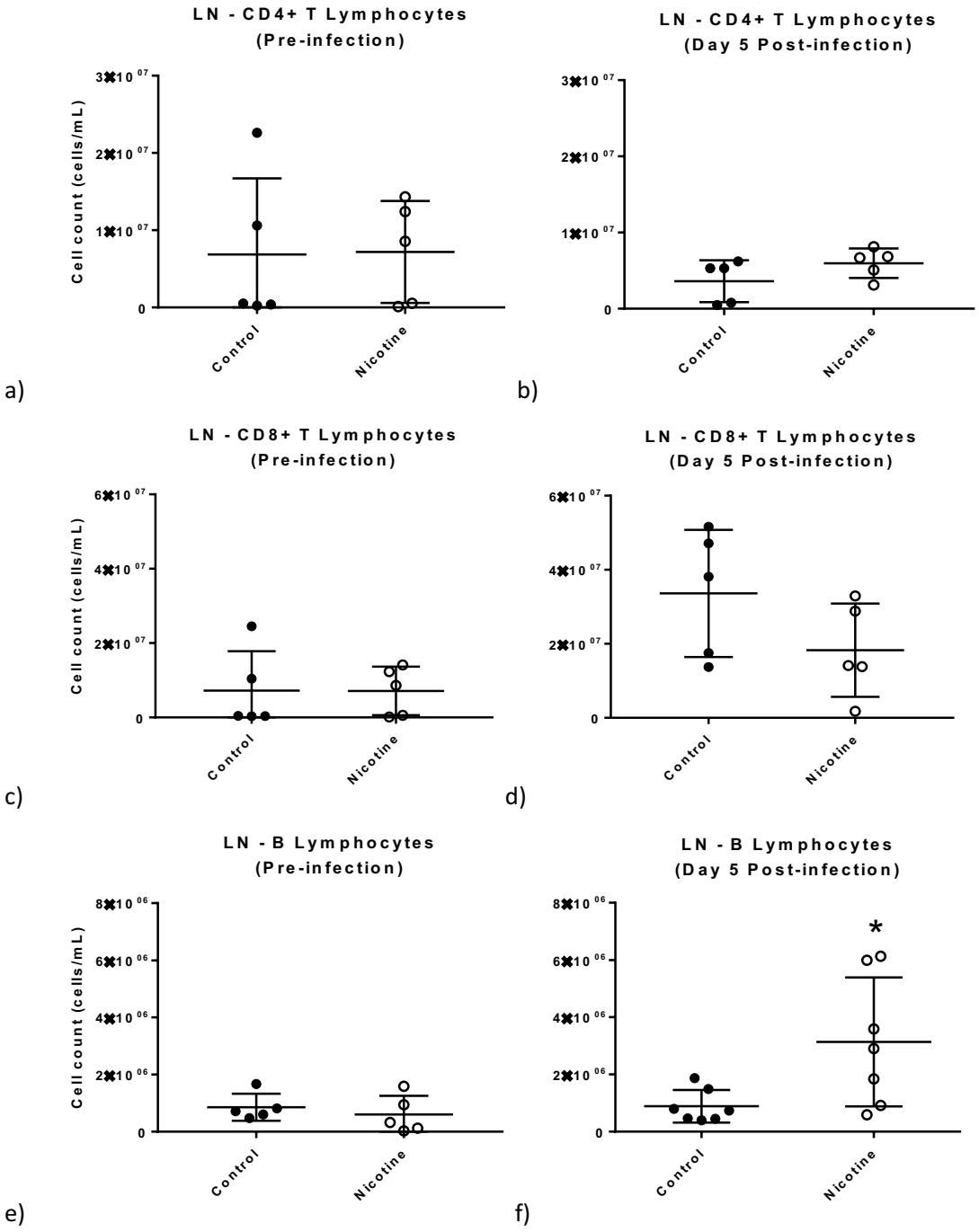
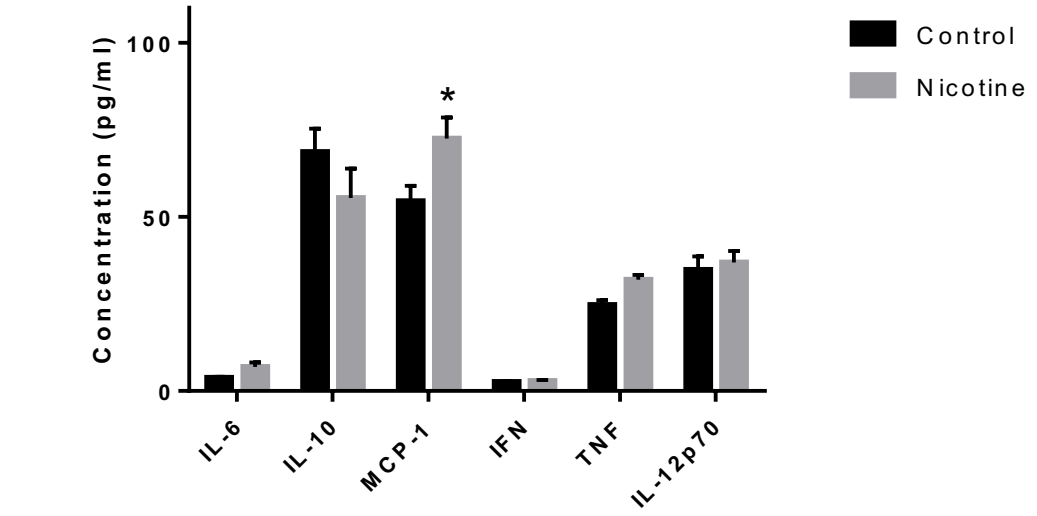


Figure 3.7 Pre- and post-infection T lymphocytes and B lymphocytes in lymph node. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy and humanely killed before and after intratracheal infection with *P. aeruginosa*. Immune cells were quantified by flow cytometry in the lymph nodes for markers of (a-b) CD4+ T lymphocytes, (c-d) CD8+ T lymphocytes, and (e-f) B lymphocytes (CD19+). Data represents the mean ± SD, and statistical significance is indicated for p value < 0.05 (*). (*) denotes significance between groups. Data was analyzed by two-way ANOVA for comparison of the groups and t-test for comparison of pre- and post-infection.

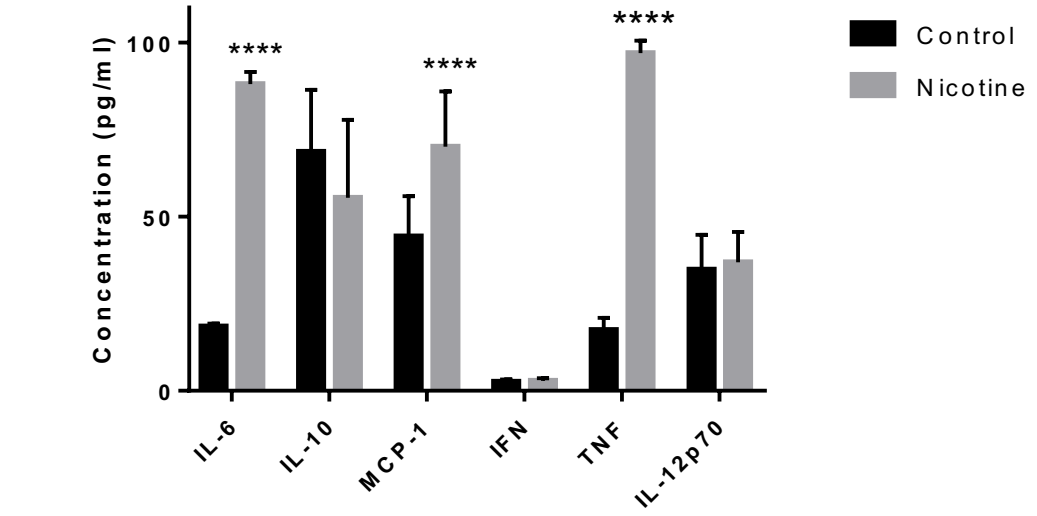
Cytokine production in the lung lavage fluid of *P. aeruginosa* infected mice

Various pro- and anti-inflammatory cytokines are produced in response to an infection, and the balance of these mediators is crucial in amplifying or suppressing the immune response at a proper timing. IL-6, IFNγ, TNFα, and IL-12 are secreted from M1 polarized macrophages in response to an infection and IL-12 also promotes differentiation of T cells into the Th1 subset, potentiating the pro-inflammatory response. On the contrary, IL-10 is secreted by Th2 cells to regulate exaggerated inflammatory responses and to reduce tissue damage. Therefore, we assessed levels of IL-6, IL-10, MCP-1, IFNγ, TNFα, and IL-12p70 in the lung lavage fluids of mice 5 days post-infection to compare cytokine milieu in response to an infection.

The most prominent difference was observed in MCP-1 level, which is a chemoattractant protein for monocytes. MCP-1 is produced in high levels during infection by both immune and non-immune cells. As the name implies, it promotes recruitment of monocytes to the inflamed tissue and also attract neutrophils during severe infection (139). We observed higher concentrations of MCP-1 in the lung lavage fluid of the nicotine group in a single experiment and it remained elevated when results are pooled from 3 separate cohorts. The pro-inflammatory cytokines IL-6 and TNFα were additionally found to be increased from pooled data while IL-10, IFNγ, and IL-12 levels were not different between groups (Figure 3.8a, b).



a)

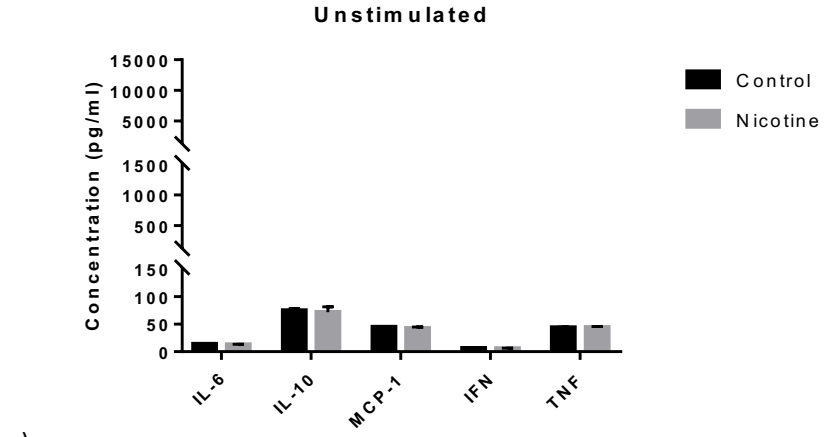


b)

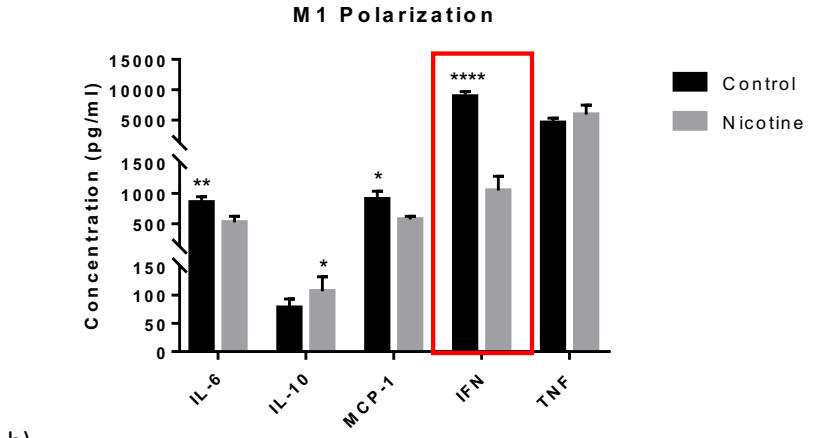
Figure 3.8 Cytokine concentrations in the lung lavage fluid of mice after *P. aeruginosa* infection. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy and humanely killed before and after intratracheal infection with *P. aeruginosa*. On post-infection Day 5, lung lavage samples were collected and cytokine levels were measured by CBA. Figure a) describes one cohort and figure b) is pooled data from 3 separate experiments. Data represents the mean ± SD, and statistical significance is indicated for p value < 0.05 (*) and < 0.0001 (****). Data was analyzed by multiple t-test.

Cytokine production from macrophage cell culture

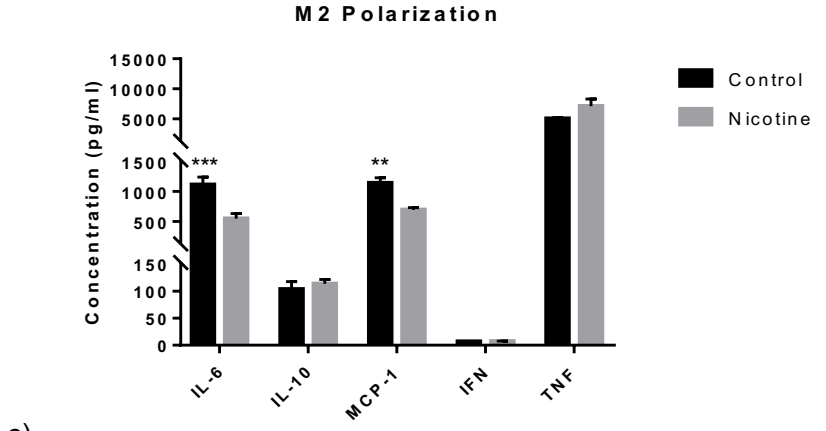
In order to assess macrophage cytokine profile at baseline and upon M1 or M2 polarized states, we harvested murine bone marrow cells and stimulated them *ex vivo*. Bone marrow cells were obtained from mice exposed to saccharin (control) or nicotine developmentally and differentiated into macrophages for 7 days *ex vivo*, as described in Methods. When fully differentiated, bone marrow-derived macrophages were stimulated with LPS and IFNγ or IL-4/13 to polarize them into M1 or M2 macrophages, respectively. Cytokine concentrations in the supernatants were measured to assess cellular response to stimulation and polarization. Cytokine levels were comparable between the two groups without any stimulation (media only). However, several differences were noted between control and nicotine groups upon stimulation and polarization. First, production of all cytokines were elevated upon polarization to M1, except for IL-10, which is an anti-inflammatory cytokine produced mainly by M2 macrophages (Figure 3.9a, b). This was true for both control and nicotine groups, and the nicotine group was associated with higher production of IL-10 compared to both media treatment and the M1 polarized control group. IL-10 levels were elevated upon M2 polarization for both groups and the levels were comparable between the two groups (Figure 3.9a, c). The most prominent finding was the production of IFNγ, which was significantly increased upon M1 polarization, but not upon M2 polarization, in both groups (Figure 3.9a-c). One caveat is that IFNγ was added to the culture in M1 polarized cells but not M2 polarized cells. However, significantly elevated production of IFNγ in the control group compared to the nicotine group is noteworthy (Figure 3.9b). Similarly, increased TNFα levels were observed upon M1 polarization for both groups (Figure 3.9b), but interestingly this was comparable to M2 polarized macrophages (Figure 3.9c). The nicotine group was associated with significant reduction in production of IL-6 and MCP-1 compared to control group for both M1 and M2 polarizations (Figure 3.9b, c). This is reverse of what was observed from lung lavage fluids of mice infected with *P. aeruginosa* (Figure 3.8b).



a)



b)

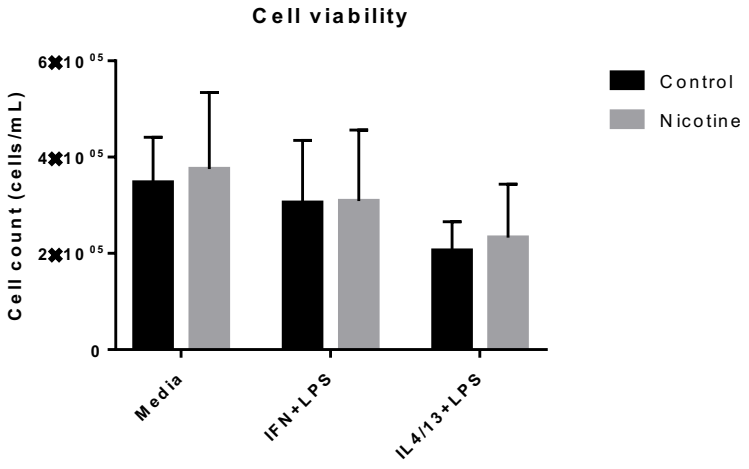


c)

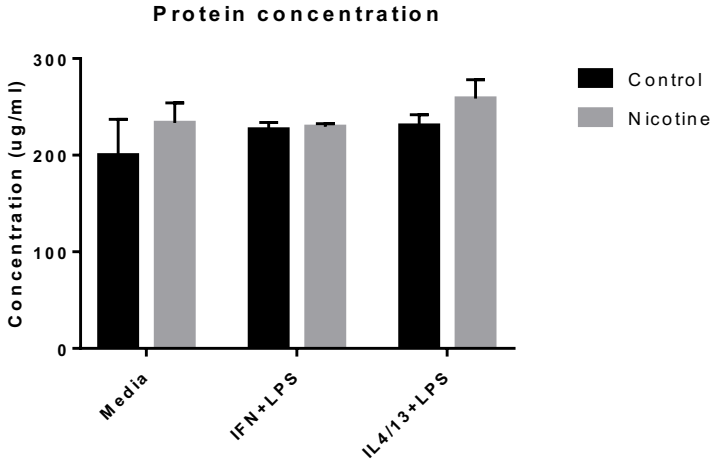
Figure 3.9 Cytokine production in the supernatants of *ex vivo* BMDM. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy. Bone marrow-derived macrophages (BMDM) were stimulated with LPS and treated with IFNγ or IL-4/13 for M1 or M2 polarization, respectively. After overnight incubation, supernatants were collected to determine cytokine concentrations by CBA. Figures describe a) baseline cytokine levels, b) cytokine levels when polarized to M1, and c) cytokine levels when polarized to M2. Data represents the mean ± SD, and statistical significance is indicated for p value < 0.05 (*), < 0.01 (**), < 0.001 (***), and < 0.0001 (****). Data was analyzed by two-way ANOVA.

Arginase activity

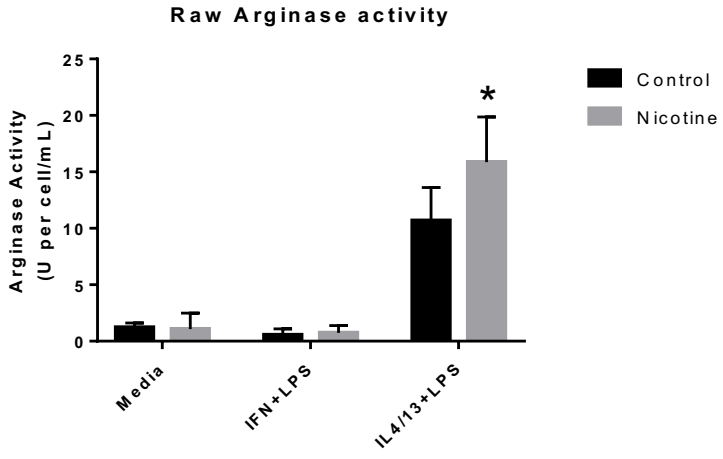
A previous study reported that *in utero* nicotine exposure shifts the resting state AM into an alternative phenotype (104). We utilized BMDM from control and nicotine mice to measure arginase activity as a marker of alternative activation when stimulated with LPS *ex vivo*. Cells were treated with media only, IFNγ+LPS to induce M1 polarization, or IL-4/13+LPS to induce M2 polarization. Cell lysates were obtained to assess arginase activity. First, we assessed cell viability with and without stimulation to determine whether developmental nicotine exposure affects this parameter. There was no difference in cell viability between control and nicotine groups when polarized into M1 or M2 (Figure 3.10a). Additionally, protein concentrations were comparable between the groups as well as across the cytokine treatments (Figure 3.10b). We found significantly increased arginase activity in cells polarized to the M2 phenotype in both control and nicotine groups, and this increase was more pronounced in the nicotine group ($p=0.0588$, denoted) (Figure 3.10c). Low levels of arginase activity were detected in cells treated with media only as well as M1 polarized cells, and this was similar between control and nicotine groups (Figure 3.10c). When normalized to protein concentration, increased arginase activity in M2 polarized cells of nicotine group was less pronounced, resulting in a similar arginase activity between M2 polarized cells of control and nicotine groups (Figure 3.10d). Similarly, the two groups were comparable when arginase activity was normalized to cell counts (Figure 3.10e).


Cell viability
Cell count (cells/mL)
6✖10 05
4✖10 05
2✖10 05
0
Media
IFN+LPS
IL4/13+LPS
Control
Nicotine

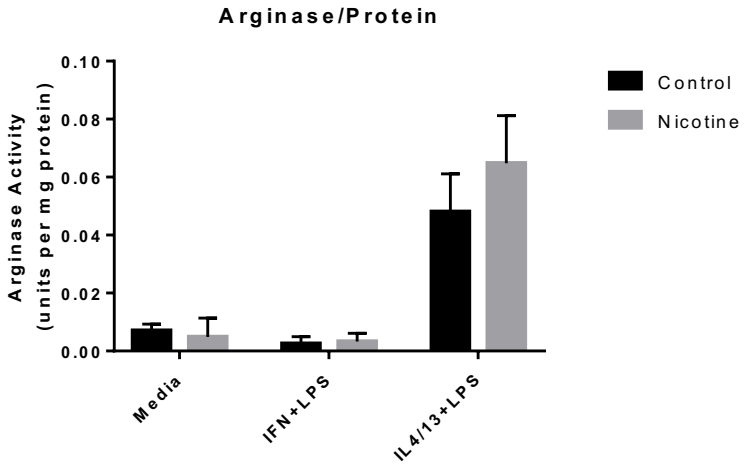
a)


Protein concentration
Concentration (ug/ml)
300
200
100
0
Media
IFN+LPS
IL4/13+LPS
Control
Nicotine

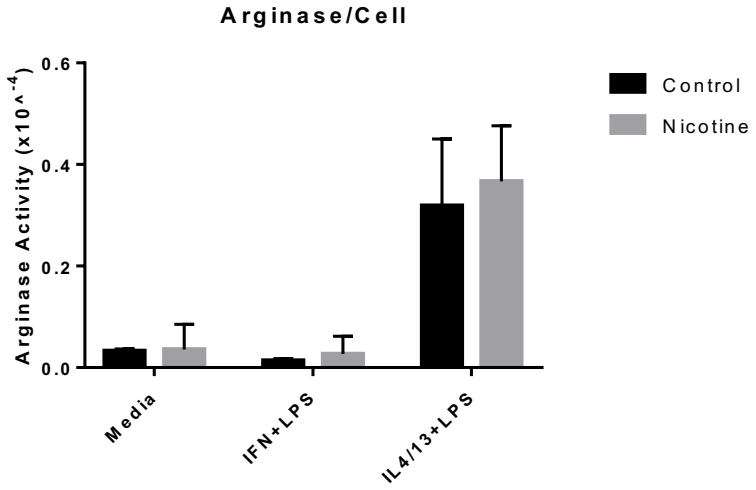
b)


Raw Arginase activity
Arginase Activity (U per cell/mL)
25
20
15
10
5
0
*
Media
IFN+LPS
IL4/13+LPS
Control
Nicotine

c)



d)



e)

Figure 3.10 Arginase activity at baseline and upon M1 and M2 polarization. *In utero* nicotine exposed mice were generated from pregnant mice fed with saccharin- or nicotine-dissolved water throughout pregnancy. Bone marrow-derived macrophages (BMDM) were stimulated with LPS and treated with IFNγ or IL-4/13 for M1 or M2 polarization, respectively. After overnight incubation, cell lysates were collected to measure arginase activity. Figures describe a) cell viability as measured using Trypan blue staining, b) protein concentration, c) raw arginase activity, d) arginase activity normalized to protein concentration, and e) arginase activity normalized to cell numbers. Data represents the mean ± SD, and statistical significance is indicated for p value < 0.05 (*). Data was analyzed by multiple t-test.

Human Tracheal Aspirate Sample Processing

In our murine model, animals were allowed to age for 4-6 weeks prior to performing various experiments described above. This is equivalent to approximately young adult age in humans, at which nicotine effects mediated during the critical periods of fetal development are likely to be less pronounced due to post-birth factors (140). To better understand the impact of *in utero* nicotine exposure when it is most pronounced and to investigate its impact in humans, we designed a clinical study, recruiting neonates from the University of Kentucky Medical Center born to mothers who 1) did not smoke during pregnancy, 2) smoked throughout pregnancy, or 3) used NRT during pregnancy.

The study was designed to characterize and compare alveolar macrophage gene expression obtained from neonates who are placed on a ventilator in the NICU. As a standard care measure, tracheal aspirate samples are suctioned to maintain patency of the ventilation tubes and discarded immediately. For this study, we attempted to collect these samples and transfer them to the laboratory to purify alveolar macrophages and isolate RNA 1) immediately upon purification and 2) after stimulating with LPS to determine macrophage response.

Prior to enrolling patients, we performed preliminary work to establish successful RNA isolation techniques with human samples. Approximately 1 mL volume was recovered from tracheal aspirate samples obtained from each patient in the NICU, and samples were processed according to the protocol described in the Methods section. Quality and quantity of isolated RNA were determined using the UV spectrophotometer, and the A_{260}/A_{280} ratio of 1.8-2.1 was used as a target of quality. First, the entire sample was purified, immediately upon receiving, to isolate RNA, using 350 µL or 1 mL of TRizol® reagent. When low volume of TRizol® was used, A_{260}/A_{280} ratio did not fall in the desired range, suggesting impurities and unreliable results. Higher volume of TRizol® led to successful isolation, and this was true only with $2x10^6$ cells/mL (Table 2a). Next, we cultured purified alveolar macrophages and stimulated them with LPS 10 ng/mL prior to isolating RNA. After 6 h incubation, cells were collected and RNA was isolated, following the same procedure and using 1 mL TRizol®. We observed that A_{260}/A_{280} ratio fell within the desired range from these samples (Table 2b). Overall, the results suggest that successful RNA isolation can be achieved by using the described methods and using neonatal tracheal aspirate samples, and cell number and reagent volumes are important determinants of acceptable RNA quality and quantity. This preliminary data sets the

groundwork for our proposed clinical study of investigating the alveolar macrophage gene expression profile in neonates exposed to *in utero* nicotine via various methods.

a)

Sample number	Cell counts	A_{260}/A_{280} ratio
1	$1.5x10^6$ cells/mL	1.16
2	$1x10^6$ cells/mL	1.32
3	$2x10^6$ cells/mL	1.82
4	$1x10^4$ cells/mL	1.49

b)

Treatment	A_{260}/A_{280} ratio
Media	1.8
Media	1.47
LPS 10 ng/mL	1.71
LPS 10 ng/mL	1.86

Table 2.1 RNA isolation results from clinical tracheal aspirate samples. Samples were collected from neonates placed on a ventilator and processed to purify alveolar macrophages. A) RNA isolated immediately after obtaining samples from patients. Samples 1 and 2 were saved in 350 µL TRizol and samples 3 and 4 were saved in 1 mL TRizol. B) RNA isolated from cells cultured in the presence or absence of LPS.

Chapter 4. Conclusion

Despite known perinatal health risks of smoking during pregnancy, many pregnant smokers fail to achieve complete cessation (7). Although NRTs are considered first-line treatment options during pregnancy, there is paucity of data to suggest NRT is safe and effective during pregnancy (3). Furthermore, many recent studies observed that the physiological effects of nicotine are not only limited to the nervous system but can modulate functions of non-neuronal cells, (4, 107). This is mediated through the nicotinic acetylcholine receptors (nAChR) present in non-neuronal cells, including the immune cells. Investigation of the role of nAChR is an active area of research, and so far it has been observed that stimulation of nAChR can dampen inflammatory responses from macrophages and stimulate proliferation of T cells. Most of what is known regarding the effect of nicotine on the immune system suggests that nicotine is an anti-inflammatory molecule, as suggested by suppressed secretion of inflammatory mediators (99, 100, 109). This is an important area of research particularly for smokers who use NRT under the notion that there is no harm to their health and regard NRT as a safer option. Moreover, this introduces concerns for pregnant smokers whose fetuses are exposed to nicotine through the placental barrier with the potential to modulate cells directly as well as indirectly through developmental alterations. Therefore, we sought to investigate the effect of *in utero* nicotine exposure on the immune response to *P. aeruginosa* lung infection as well as *ex vivo* LPS stimulation.

The infection model we have employed in this study is an excellent method in assessing chronic lung infection commonly seen in smokers. As discussed in this thesis, smokers have an increased susceptibility to infection, and many of them suffer from chronic inflammation of the lungs and repetitive respiratory infections as a result of damages induced by cigarette smoke (73). By administering *P. aeruginosa*-laden agarose beads intratracheally, we can assess the immune response against bacteria in orchestrating the balance between pro- and anti-inflammatory responses and monitor the overall clinical status, such as animal weight, over time. Any alterations of the immune cell functions, due to developmental nicotine exposure, that result in an inadequate control of the infection is manifested by poor clinical status, as this parameter is commonly used to determine the response to an infection (74, 92, 134). This model is extensively used in other investigations associated with chronic lung infection, such as in cystic fibrosis (134).

In this study, we hypothesized that *in utero* nicotine exposure would alter immune cell disposition and function in response to *P. aeruginosa* lung infection. We found that the nicotine group had a worsened clinical outcome, as measured by weight-loss over time post-infection. Additionally several differences were observed with regard to immune cell disposition and cytokine profile in the lung tissue, lung lavage fluid, and lymph nodes post-infection. However, these results are not conclusive but rather preliminary. The work accomplished to this point sets the groundwork for addressing our hypothesis but does not answer it. Importantly, findings from our study appear to contradict some of what is known in the current literature. For example, maternal smoking or nicotine exposure is generally known to cause low birthweight, which was not observed in our study. We had a total of 62 offspring mice (31 mice in each group) generated from mice fed with saccharin- or nicotine-dissolved water, and their baseline weights prior to infection were not different. This may be explained by the time elapsed between birth and the day of infection, which was approximately 4-6 weeks. It is possible that weight differences, if any, may have been minimized during postnatal development. Due to the cannibalistic behavior of the mothers, neonatal mice could not be manipulated from the cages prior to weaning. However, post-infection weight changes suggest that *in utero* nicotine exposure may impact the overall clinical outcome.

Our study did not find any baseline differences in the number of cell subpopulations. Cell numbers were comparable to those of control group for all subsets analyzed, and differences were observed only in post-infection numbers, including pronounced dampened neutrophil influx, increased CD4 and CD8 cell numbers in lung tissue, and increased B cells in the lymph nodes. This is similar to the results of other studies investigating the effects of prenatal nicotine exposure on neonatal and adult animal immune cell populations (141, 142). Despite similar baseline numbers, one study observed that CD8+ T-lymphocyte activity was significantly reduced when exposed to cigarette smoke developmentally, which suggests that there is an impact of prenatal cigarette smoke exposure on functional capacity of CD8+ T cells (141). We did not assess this parameter for CD8+ T cells, but the impact observed on the cytokine profile from *in vivo* and *ex vivo* studies may support the notion of altered function. In our study, *in utero* nicotine exposure was associated with significantly increased production of the inflammatory cytokines IL-6, MCP-1, and TNFα on day 5 post-infection in the lung lavage fluid. Interestingly, the opposite occurred during our *ex vivo* experiments. IL-6 and MCP-1 levels were higher in the control group, and additionally IFNγ concentrations

were significantly elevated in the control group. One possible explanation for this observation is the difference in time when the cytokine levels were measured. M2 polarization induced by developmental nicotine exposure suppresses inflammatory response 6 hours after stimulation with LPS, as observed with our *ex vivo* data. This initial response perhaps leads to delayed resolution and prolonged inflammation, which results in increased production of pro-inflammatory cytokines 5 days after *P. aeruginosa* infection, as demonstrated *in vivo*.

Fetal and neonatal T cell immunity is known to be unbalanced, with a bias towards Th2 cells, which increases neonatal susceptibility to infectious diseases compared to adults (143). This does not necessarily indicate that neonates are born with immunodeficiency, as they are able to generate effective immune responses, such as induction of Th1 cells upon stimulation with antigen. Th1 cell apoptosis was observed, however, when cells were re-challenged with the antigen while Th2 cells mediated a secondary response. Subsequent research to understand specific mechanisms underlying this phenomenon suggests that IL-4 produced by Th2 cells mediates Th1 cell apoptosis (143). Development of the immune system continues to occur postnatally, and the fetal and neonatal period is considered a critical timeframe that requires sequential stimulation to induce differentiation, proliferation, and degeneration (143). Unbalanced Th1/Th2 stimulation in fetuses and neonates is, therefore, in its transitory period and will ultimately adapt to the environment and establish functional capacities during postnatal developmental periods when stimulated appropriately.

Our study and previous studies report that *in utero* nicotine exposure is associated with a shift towards a Th2 response, and towards an M2 macrophage response which mediates regulation of inflammation (100, 104). We observed increased activity of arginase in M2 polarized cells in mice developmentally exposed to nicotine compared to the control group, although no differences were observed at baseline. Wongtrakool et al. reported an increased number of macrophages expressing arginase-1, as well as Ym1 and fibronectin, when exposed to nicotine *in utero* (104). Furthermore, cytokine profiles in the lung were associated with Th2 responses, as suggested by increased TGFβ1 and IL-13 mRNA expression. Currently available literature generally suggests biased Th2 cell responses in part due to Th1 cell apoptosis. It could be speculated that developmental stimulation of nAChR affects apoptosis/survival signal specific to Th cell phenotypes and potentiate a Th2 response, which ultimately interferes with establishing a well-balance Th1/Th2 system. One such example is demonstrated

through α7 nAChR, whose pro-survival role was demonstrated in M2 macrophages and not in M1 macrophages (144). In this study, activation of the STAT3 signaling pathway was observed upon α7 nAChR stimulation, which is known to be involved in the regulation of cell growth and survival. This observation, however, was specific to M2 polarized bone marrow-derived macrophages only (144). Therefore, it is plausible that stimulation of α7 nAChR, and perhaps other nAChRs, during development via maternal use of nicotine containing products can modify survival of immune cells and render the immune tone set at birth.

Epigenetic regulation of immune cell development is another possible mechanism through which *in utero* nicotine modulates immune cell disposition and function. Epigenetic modifications involve post-transcriptional acetylation and methylation of histone proteins that can result in different cell phenotypes without affecting DNA sequence (145). Studies report altered histone modification and methylation in the brain and lungs of mice exposed to nicotine developmentally, and these patterns were similar to those in individuals with behavioral alterations, including drug addiction (145). Intrauterine exposure to cigarette smoke was also shown to affect the DNA methylation pattern which is negatively associated with neuronal content in the fetal brain (146). Epigenetic control of immune cell differentiation is best characterized in T cells. For example, demethylation of the IFNγ gene promoter occurs in the Th1 cell lineage while demethylation of the IL-4 gene occurs in the Th2 cell lineage. Concomitantly in Th2 cells, DNA methylation of the IFNγ gene occurs, which ultimately silences the expression of Th1-associated genes when Th2-associated genes are expressed (147). Prenatal cigarette smoke exposure is known to be associated with increased methylation of peripheral blood genes (148). Patil et al. analyzed the association between prenatal cigarette smoke exposure and DNA methylation pattern and observed that there is an interaction between the pattern of methylation and maternal smoking as well as a gene variant for IL-13 that, together, affect lung function (149). Although limited at this time, the influence of maternal cigarette smoking on epigenetic changes in newborns is a growing area of study, and associations between altered epigenetic patterns and health outcomes can lead to a better understanding of predisposition for health risks.

The result of developmental nicotine exposure on the overall immune network of an individual is complex and multi-layered. First, the effects of nicotine directly on immune cells introduce a potential effect. Second, the effects of *in utero* nicotine

exposure on development of the immune cells and response is another major issue that is yet to be fully understood. One other aspect of *in utero* nicotine exposure influencing the immune network is the cholinergic regulation of the immune system. There is evidence that vagus nerve stimulation results in an inhibition of peripheral inflammatory cytokine production by splenocytes, and that this is mediated through α7 nAChR. One such example is the exaggerated inflammatory response, manifested by greater tissue damage, in the models of colitis, septic shock, and pancreatitis in α7 nAChR knock-out mice. This has become known as the 'cholinergic anti-inflammatory pathway' (Reviewed in 128). Although the study of prenatal cigarette smoke and nicotine exposure on brain development and neurobehavioral outcomes in the offspring have been active areas of research for many years, how this impacts the network of immune regulation is rather novel, especially when referring to the interconnected function between neuronal and non-neuronal cholinergic systems. This study targets the issue from one direction, from the perspective of the immune cells. Consistent with other studies, findings from our preliminary results suggest that *in utero* nicotine exposure leads to an unbalanced macrophage tone, with a shift in macrophage function towards an anti-inflammatory M2 phenotype. This could potentially affect the development of the reflex network of neuronal regulation of inflammation by changing the milieu of the cytokine profile in the local environment. Furthermore, hyperstimulation of the nAChR, out of sequence, may generate under- or over-expressed cholinergic anti-inflammatory pathways. Future studies addressing the impact of developmental nicotine exposure on the neuronal stimulation of the immune network, and whether this results in an aberrant immune reflex, will additionally address the overall impact in an individual's baseline immune tone and response.

There are several limitations in this study. First, our animal model had considerable variation in regard to the level of infection between and within the cohorts. We utilized intratracheal infection with *P. aeruginosa* embedded in agarose beads to ensure a chronic, stable infection. This model had been previously established and widely utilized by other investigators for the assessment of *P. aeruginosa* lung infection (134, 150, 151). Although we confirmed the amount of bacteria for inoculation prior to infection, it is difficult to know if all mice received the same amount of bacteria. This may explain the intra-experiment variability we observed with post-infection weight loss as well as other data. If mice died early prior to analysis, this resulted in unbalanced sample sizes, which may be skewing some of the data. Additionally, preparation of *P.*

aeruginosa embedded beads requires adequate bacterial growth and production of beads in various sizes. This is a difficult task and requires timely coordination of two separate procedures. Moreover, reproduction of same sized beads is unlikely between experiments, increasing inter-experiment variability. In order to reduce these variabilities, we modified our infection model by separately preparing bacterial culture and sterile agarose beads and mixing them prior to infection. This would allow us to keep a stock of beads that could be used for several experiments, reducing the inter-experiment variability. We ensured that bacteria were grown to late log phase and determined dilutions of stock beads and the amount of bacteria sufficient to induce a stable infection when mixed together. However, alternate method resulted in a similar level of intra- and inter-experiment variability and required higher number of bacteria to induce similar level of post-infection weight loss we had observed with our initial experiments.

The amount of maternal nicotine ingestion may affect the extent of developmental alterations in the fetus, and classification or sub-analysis of offspring by the amount of *in utero* exposure may provide a greater understanding of dose-dependent effects. We utilized C57BL/6 mice with a GFP tag on nicotinic receptors that were bred in-house. Mice were fed with either saccharin dissolved or nicotine dissolved water ad libitum before and throughout pregnancy to generate *in utero* nicotine exposed offspring. This is a well-established model for experimental administration of nicotine. Other methodologies include parenteral nicotine injection several times a day or installing an osmotic minipump (152). However, these alternative methods have several disadvantages, such as stress of daily injections over a long-time period as well as the expense for installing and replacing the pumps, depending on the timeframe needed for the study. In study by Rowell et al, mice weighing 18-22g were fed ad libitum with either plain water or nicotine solutions (20-100 µg/mL) for up to 4 weeks to address the validity of such a methodology as an investigational model for chronic nicotine administration. Although reduction in drinking behavior was observed when nicotine concentrations were greater than 20 µg/mL, this behavior was comparable to the control group even at nicotine concentrations of 100 µg/mL when saccharin was added to the solution. Gradual increase in nicotine amount also led to a normal fluid intake behavior. Other studies have utilized 200 µg/mL and have seen biochemical efficacy as well as neurodevelopmental alterations without significant effects on perinatal outcomes, such as maternal and offspring weights or fluid intake (153, 154). In our study, we were unable to assess nicotine, or practically cotinine, levels in mothers or the offspring after

delivery due to cannibalistic behaviors in nurturing mothers upon manipulation of animal cages or the offspring. In order to accurately measure and control the amount of nicotine administered, other modes of administration could be considered, such as daily injection or implantation of pumps. However, these methods are invasive and require several manipulations of pregnant mice, which would be a stressful event during the critical periods. For future studies, sacrifice of neonatal mice for determination of cotinine levels by ELISA can be considered to maintain the sample size for post-infection analyses.

We attempted to compare gene expression in neonates born to non-smoking mothers, to mothers either using NRT or who continued to smoke throughout pregnancy and correlate changes in gene expression patterns to the mode of nicotine exposure. Despite IRB approval and initiation of the clinical study, we were unable to recruit patients, resulting in obtaining samples for practice trials only. However, validation of the experimental methods has been successfully accomplished, and continued recruitment of study candidates is ongoing. Once a sufficient number of patients is recruited to statistically analyze the results, we can make a correlation between *in utero* nicotine exposure and changes in macrophage gene expression that can guide future study directions. For example, any abnormal expression can be targeted and explored for underlying mechanisms involved in its expression and the consequences from observed changes.

This study provides preliminary data to understand potential research areas and several preliminary findings noted from the study highlight that *in utero* nicotine exposure leads to changes in immune response against *P. aeruginosa* lung infection. Any robust observation in the future will positively impact current smoking cessation guidelines for pregnant women by providing safety data that is currently lacking. Moreover, the clinical study in neonates will enable correlation of macrophage gene expression changes mediated by different modes of nicotine exposure and provide a better understanding of individual's predisposition for infection.

Appendices

A. Appendix I: List of genes for gene expression analysis

Well	Gene	Fisher #
A1	CD86	Hs01567026_m1
A2	CD64/CD64	Hs00417598_m1
A3	ITGAM	Hs00167304_m1
A4	CD14	Hs02621496_s1
A5	CD68	Hs02836816_g1
A6	CD23/FCER2	Hs00233627_m1
A7	CD40	Hs01002915_g1
A8	STAT1	Hs01013996_m1
A9	SOCS3	Hs02330328_s1
A10	SLAMF1	Hs00234149_m1
A11	**RNA18S5**	**manufacturing control (housekeeping gene)**
A12	TNF	Hs00174128_m1
A13	IL6	Hs00174131_m1
A14	IL12B	Hs01011518_m1
A15	IL1B	Hs01555410_m1
A16	IL10	Hs00961622_m1
A17	TGFB1	Hs00998133_m1
A18	IL12A	Hs01073447_m1
A19	IL8/CXCL8	Hs00174103_m1
A20	IL23A	Hs00372324_m1
A21	IL4RA	Hs00965056_m1
A22	IL27RA	Hs00945029_m1
A23	HLA-DRA	Hs00219575_m1
A24	MARCO	Hs00198937_m1
B1	CCL17	Hs00171074_m1
B2	CCR2	Hs00704702_s1
B3	TREM2	Hs00219132_m1
B4	CCL22	Hs01574247_m1
B5	CCL18	Hs00268113_m1
B6	NOS2	Hs01075529_m1
B7	RETNLB	Hs00395669_m1
B8	ARG1	Hs00163660_m1
B9	PPARG	Hs01115513_m1
B10	IKBKB	Hs01559460_m1

B11	ARG2	Hs00982833_m1
B12	IDO1	Hs00984148_m1
B13	IRF1	Hs00971965_m1
B14	MRC1/CD206	Hs00267207_m1
B15	NLRP1	Hs00248187_m1
B16	CASP1	Hs00354836_m1
B17	MAP1LC3B	Hs00797944_s1
B18	AKT1	Hs00178289_m1
B19	PCNA	Hs00427214_g1
B20	ATG5	Hs00169468_m1
B21	MTOR	Hs00234508_m1
B22	GCN2/EIF2AK4	Hs01010957_m1
B23	GAPDH	Hs02786624_g1
B24	CCND1/cyclin D1	Hs00765553_m1

B. Appendix II: Survey Questionnaire and Data Collection Form

Impact of *in utero* nicotine exposure on neonatal *ex vivo* macrophage responses study

Survey Questionnaire

1. Are you a present/former smoker?
   - ☐ Yes
   - ☐ Yes, e-cigarette use
   - ☐ No
2. If answered **Yes** in #1, how often do you usually smoke cigarettes (**Before you became pregnant**)?
   - ☐ Every day
   - ☐ On most days
   - ☐ Less than most days
3. What was your smoking status during pregnancy? Check all that apply (*Please provide in detail on Page 2*)
   - ☐ Every day/on most days
   - ☐ Smoking cessation aids: Nicotine replacement therapy (NRT)
   - ☐ Did not smoke at all/Smoked occasionally
   - ☐ Never smoked
   - ☐ Other: Please specify_____
4. If you smoked during pregnancy, daily number of cigarettes smoked: _____ Please indicate "0" if you did not smoke during pregnancy (*Please fill out Page 2 for details*)
5. If you smoked/used nicotine replacement therapy (NRT) during pregnancy, which of the following best describes you?
   - ☐ Smoked cigarettes only
   - ☐ Tried using nicotine replacement therapy (NRT) but mostly smoked cigarettes
   - ☐ Smoked cigarettes and used nicotine replacement therapy (NRT) equally
   - ☐ Used nicotine replacement therapy (NRT) more often/consistently than smoking
   - ☐ Used nicotine replacement therapy (NRT) only
   - ☐ Not sure
   - ☐ N/A
6. Approximately how many cigarettes have you smoked in the last week?

   _____
7. If you used nicotine replacement therapy (NRT) during pregnancy at any point, what type of aid(s) did you use? Check all that apply.
   - ☐ Nicotine patch
   - ☐ Nicotine gum
   - ☐ Nicotine inhaler
   - ☐ Nicotine lozenge
   - ☐ Nicotine spray

8. When was the last time you smoked/used nicotine replacement therapy (NRT)? Please indicated "N/A" if you never smoked during pregnancy
    - ☐ 1-2 days ago
    - ☐ 3-4 days ago
    - ☐ 5-6 days ago
    - ☐ More than 1 week ago
    - ☐ N/A

Trimester/Month	Approximate # of Cigarettes/day	Nicotine Replacement Therapy (NRT)
1st Trimester (1)		
1st Trimester (2)		
1st Trimester (3)		
2nd Trimester (4)		
2nd Trimester (5)		
2nd Trimester (6)		
3rd Trimester (7)		

3rd Trimester (8)		
3rd Trimester (9)		

- *Please indicate the type of nicotine exposure in detail (smoking, e-cigarette, form of NRT) and describe as appropriate*

Nicotine Study Data Collection Form

Date: _____

Patient ID (Mother/Baby's MR#) : _____

Mother's Age/DOB: _____

Gestational Age at Birth: _____

Gender of infant: M F

Race: _____

Study ID: _____

Samples Collected:

Date Collected	Age (Days)	Time Collected	Time Processed	Notes
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

Mode of nicotine exposure (circle one): Smoking NRT BOTH/OTHER

If BOTH/OTHER, specify: _____

Comorbidities: _____

Procedures

Type of Procedure	Date
_____	_____
_____	_____
_____	_____

Any active infection (circle one): YES NO

Immune altering underlying condition (circle one): YES NO

Immune altering medication (circle one): YES NO

Medications

Drug	Start Date	Stop Date	Notes
_____	_____	_____	_____
_____	_____	_____	_____

_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____
_____	_____	_____	_____

NOTES: _____

C. Appendix III: Consent Forms

Please leave enough space at the top of your consent form to accommodate a box this size. DO NOT INCLUDE THIS BOX

Combined Consent and Authorization to Participate in a Research Study

PLEASE NOTE: When "YOU" is referenced in the consent, it include "YOU and YOUR BABY"

IMPACT OF IN UTERO NICOTINE EXPOSURE ON NEONATAL EX VIVO MACROPHAGE RESPONSES

WHY ARE YOU BEING INVITED TO TAKE PART IN THIS RESEARCH?

You are being invited to take part in this research study about effects of nicotine exposure during fetal development. You are being invited to take part in this study because you are having a baby being delivered prematurely and we are interested in the effects of nicotine on the formation of cells in the lungs.

WHO IS DOING THE STUDY?

The persons in charge of this study are Drs. Hubert Ballard and David Feola of the University of Kentucky, Department of Pediatrics – Division of Neonatology and Department of Pharmacy Practice and Science. There may be other people on the research team assisting at different times during the study.

WHAT IS THE PURPOSE OF THIS STUDY?

By doing this study, we hope to learn how nicotine exposure during pregnancy, either from smoking tobacco or using nicotine replacement products, will affect the development of your baby's ability to fight infection.

ARE THERE REASONS WHY YOU SHOULD NOT TAKE PART IN THIS STUDY?

Study personnel will make sure that you are eligible for this study. You should not participate if you know or have been informed that you and your baby have any conditions that can affect the immune system. Also if you take any medications chronically that can affect you and your baby's immune system, you may not be eligible to participate in the study.

WHERE IS THE STUDY GOING TO TAKE PLACE AND HOW LONG WILL IT LAST?

The research procedures will be conducted at UK Medical Center, and tracheal aspirate samples will be transferred to the laboratory of Dr. Feola for further analyses. There will be no additional clinic visits or hospital stay due to participation in the study. Cells will be obtained during the first 2 weeks after delivery.

WHAT WILL YOU BE ASKED TO DO?

If you wish to take part in the study, you will be provided with detailed information regarding the study before signing the consent and permission forms. You will also be given a series of questions to assess your smoking history. Your smoking history will be used to put you into one of three group, which are 1) smoking group, 2) nicotine replacement group, 3) control group, or non-smoking group. No blood sample will be collected for the sole purpose of this study. You will receive usual hospital care as necessary and there will be no additional testing or hospital visits after discharge. Upon delivery, your baby will also receive usual hospital care in the intensive care unit. In order to make sure breathing tubes are not clogged, nurses usually clear out the tube by suctioning out the secretions (which are mostly mucous and some cells) and discard them. For this study, we will collect these samples and keep for research purposes. No additional procedures will be done to you or your baby at any time.

We will take suctioned secretions to the laboratory and test to see their responses and any changes in their genes. This genetic testing will not be a part of your permanent medical record and will not involve testing your genetic makeup, only the level at which certain genes are turned on/off. You will not be given the results of these genetic tests.

We will also look at your medical records for information regarding your general health, smoking history, and recent labs you have had.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

You and your baby will not undergo any interventions that are not part of the usual care during hospitalization. Therefore, there will be minimum risk to participating in the study and this will be no more than what may be posed by the usual perinatal care at the hospital.

WILL YOU BENEFIT FROM TAKING PART IN THIS STUDY?

You will get no direct benefit for being in the study. Your willingness to take part, however, may, in the future, help doctors better understand about nicotine exposure during pregnancy and make proper recommendation to pregnant smokers for the health of themselves and their babies.

DO YOU HAVE TO TAKE PART IN THE STUDY?

If you decide to take part in the study, it should be because you really want to volunteer. You will not lose any benefits or rights you would normally have if you choose not to volunteer. You can stop at any time during the study and still keep the benefits and rights you had before volunteering. If you decide not to take part in this study, your decision will have no effect on the quality of medical care you receive.

IF YOU DON'T WANT TO TAKE PART IN THE STUDY, ARE THERE OTHER CHOICES?

If you do not want to be in the study, there are no other choices except not to take part in the study.

WHAT WILL IT COST YOU TO PARTICIPATE?

There will be no additional cost to participating in the study. Investigators will pay for laboratory measures performed outside of what is normally reported but they will not be paying for your hospitalization and other medical cares as these will be part of usual cares provided.

WHO WILL SEE THE INFORMATION THAT YOU GIVE?

We will make every effort to keep confidential all research records that identify you to the extent allowed by law.

Your information will be combined with information from other people taking part in the study. When we write about the study to share it with other researchers, we will write about the combined information we have gathered. You will not be personally identified in these written materials. We may publish the results of this study; however, we will keep your name and other identifying information private.

We will make every effort to prevent anyone who is not on the research team from knowing that you gave us information, or what that information is. Information will be secured in a locked cabinet in a private office that has limited access. Electronic records can only be accessed using passwords.

CAN YOUR TAKING PART IN THE STUDY END EARLY?

If you decide to take part in the study you still have the right to decide at any time that you no longer want to continue. You will not be treated differently if you decide to stop taking part in the study.

ARE YOU PARTICIPATING OR CAN YOU PARTICIPATE IN ANOTHER RESEARCH STUDY AT THE SAME TIME AS PARTICIPATING IN THIS ONE?

You may not take part in this study if you are currently involved in another research study that requires administration of certain medications chronically. It is important to let the investigator/your doctor know if you are in another research study. You should also discuss with the investigator before you agree to participate in another research study while you are enrolled in this study.

WHAT HAPPENS IF YOU GET HURT OR SICK DURING THE STUDY?

If you believe you are hurt or if you get sick because of something that is due to the study, you should call Dr. Feola at 859-323-8751 or Dr. Ballard at 859-323-5481 immediately.

It is important for you to understand that the University of Kentucky does not have funds set aside to pay for the cost of any care or treatment that might be necessary because you get hurt or sick while taking part in this study. Also, the University of Kentucky will not pay for any wages you may lose if you are harmed by this study.

The medical costs related to your care and treatment because of research related harm will be

paid by the investigators for medical expenses incurred by treating injuries that directly result from participating in the study, with some exceptions. The exceptions are instances such as your failure to follow the sponsor's directions or the investigator's failure to follow the sponsor's directions;

WILL YOU RECEIVE ANY REWARDS FOR TAKING PART IN THIS STUDY?

You will not receive any rewards or payment for taking part in the study.

WHAT IF YOU HAVE QUESTIONS, SUGGESTIONS, CONCERNS, OR COMPLAINTS?

Before you decide whether to accept this invitation to take part in the study, please ask any questions that might come to mind now. Later, if you have questions, suggestions, concerns, or complaints about the study, you can contact the investigator, Dr. Dave Feola at 859-323-8751. If you have any questions about your rights as a volunteer in this research, contact the staff in the Office of Research Integrity at the University of Kentucky between the business hours of 8am and 5pm EST, Mon-Fri at 859-257-9428 or toll free at 1-866-400-9428. We will give you a signed copy of this consent form to take with you.

WHAT IF NEW INFORMATION IS LEARNED DURING THE STUDY THAT MIGHT AFFECT YOUR DECISION TO PARTICIPATE?

If the researcher learns of new information in regards to this study, and it might change your willingness to stay in this study, the information will be provided to you. You may be asked to sign a new informed consent form if the information is provided to you after you have joined the study.

WHAT ELSE DO YOU NEED TO KNOW?

There is a possibility that the data/tissue/specimens/blood collected from you may be shared with other investigators in the future. If that is the case the data/tissue/specimen/blood will not contain information that can identify you unless you give your consent/authorization or the UK Institutional Review Board (IRB) approves the research. The IRB is a committee that reviews ethical issues, according to federal, state and local regulations on research with human subjects, to make sure the study complies with these before approval of a research study is issued.

AUTHORIZATION TO USE OR DISCLOSE YOUR IDENTIFIABLE HEALTH INFORMATION

The privacy law, HIPAA (Health Insurance Portability and Accountability Act), requires researchers to protect your health information. The following sections of the form describe how researchers may use your health information.

Your health information that may be accessed, used and/or released includes:

- Age, medical history, smoking history, family history, and your babies gestational age

The Researchers may use and share your health information with:

- The University of Kentucky's Institutional Review Board/Office of Research Integrity.
- Law enforcement agencies when required by law.
- University of Kentucky representatives.

The researchers agree to only share your health information with the people listed in this document.

Should your health information be released to anyone that is not regulated by the privacy law, your health information may be shared with others without your permission; however, the use of your health information would still be regulated by applicable federal and state laws.

You may not be allowed to participate in the research study. If you decide not to sign the form, it will not affect your:

- Current or future healthcare at the University of Kentucky
- **Current or future payments to the University of Kentucky**
- **Ability to enroll in any health plans (if applicable)**
- **Eligibility for benefits (if applicable)**

After signing the form, you can change your mind and NOT let the researcher(s) collect or release your health information (revoke the Authorization). If you revoke the authorization:

- You will send a written letter to: Dave Feola to inform him of your decision.
- Researchers may use and release your health information **already** collected for this research study.
- Your protected health information may still be used and released should you have a bad reaction (adverse event).

The use and sharing of your information has no time limit.

If you have not already received a copy of the Privacy Notice, you may request one. If you have any questions about your privacy rights, you should contact the University of Kentucky's Privacy Officer between the business hours of 8am and 5pm EST, Mon-Fri at: (859) 323-1184.

You are the subject or are authorized to act on behalf of the subject. You have read this information, and you will receive a copy of this form after it is signed.

_____ _____

Signature of research subject (*if applicable:*) Date
or *research subject's legal representative

_____ _____

Printed name of research subject (*if applicable:*) Relation to
or*research subject's legal representative research subject

*(*If, applicable)* Please explain Representative's relationship to subject and include a description of Representative's authority to act on behalf of subject:

_____

_____

_____ _____

Name of [authorized] person obtaining informed consent/HIPAA authorization Date

_____

_____

Signature of Principal Investigator or
Sub/Co-Investigator

D. Appendix IV: Impact of *In utero* Nicotine Exposure on Neonatal *Ex vivo* Macrophage Responses Study IRB Research Description

1. **Background:**

Smoking during pregnancy is associated with adverse perinatal outcomes, including miscarriage, prematurity, low birth weight, and neonatal or sudden infant death. Numbers of studies show an increase in respiratory symptoms and altered immune defense in infants and children exposed to maternal smoking during pregnancy (1-3). Although smoking is a preventable risk factor of pregnancy related morbidity and mortality, >10% of pregnant women in high-income countries smoke during pregnancy and the rates are increasing in low- and middle-income countries (3, 4). Medications, including nicotine replacement therapy (NRT), have been developed and approved by the FDA to assist smoking cessation but there is a paucity of data regarding the safety and effectiveness of therapy during pregnancy and its effects on fetal development. Yet there is a general consensus internationally that recommends the use of NRT during pregnancy, assuming that nicotine replacement will reduce the symptoms of craving and withdrawal while reducing the exposure of toxins from cigarette smoke (3, 4). A recent study on the use of NRT patches during pregnancy until delivery found that there is no difference in the rate of abstinence from smoking or the risk of adverse perinatal outcomes compared to placebo, although the rate of abstinence was higher at 1 month of therapy in the NRT group (4). There are various factors that could lead to the observed outcomes, and conclusions from the study should be derived with careful interpretation since compliance rate in both groups were very low (< 10%).

Nicotine, one of the main components of tobacco and a pharmacologically active compound in NRT, is an agonist for nicotinic acetylcholine receptor (nAChR). nAChR are mainly found in the central and peripheral nervous systems. The expression of these receptors are also found in non-neuronal cells, modulating various cellular functions such as proliferation, differentiation, and migration via paracrine/autocrine fashion (6, 7). In immune cells, functional alteration of macrophages upon stimulation with nicotine or modulation of nAChR are well documented. Previous studies show that nicotine exposure drives macrophages into an alternative M2 phenotype, suggested by the characteristics of surface markers and cytokine production profile (8, 9). Macrophages that were polarized into classical M1 phenotype and alternative M2 phenotype in the presence and absence of nicotine showed that nicotine exposed M1 polarized macrophages (Ni-M1) demonstrated surface marker expressions similar to those seen in M2 polarized macrophages. Upon stimulation with LPS, cytokines produced by

Ni-M1 were different from M1 polarized macrophages, suggested by a significantly lower IL-12 production. Although cytokine profile of Ni-M1 did not result in the same profile seen in M2 polarized macrophages, the investigators reported skewed macrophage differentiation towards M2 phenotype with nicotine exposure (9).

While it is meaningful to understand the alteration of immune cells upon nicotine exposure, it is significant to understand the impact of this alteration as a defense mechanism. In vitro model of alveolar macrophages (AM) infected with *Legionella pneumophila* shows changes in antimicrobial activity and cytokine production of AM upon treatment with nicotine (10). This effect was reversed by nAChR antagonist treatment. In this study, enhanced growth of *L. pneumophila* in nicotine treated macrophages was observed, which was associated with reduction in production of cytokines IL-6, IL-12, and TNF-α by AM. Nicotine did not contribute to direct antimicrobial activity, suggesting that nicotine probably decreases phagocytic activities of AM.

Recently, the effects of *in utero* nicotine exposure on neonatal mice AM were evaluated. Both in vitro and in vivo studies demonstrated markers of AM shifted into M2 phenotype, characterized by increases in arginase-1, YM1, and FN. These alterations contribute to the baseline profile of neonatal lung characterization into Th2 immune response, which is further supported by an increased IL-13 and TGFβ1 expression. This study also demonstrated impaired phagocytic activity by *in utero* nicotine exposed AM upon *Staphylococcus aureus* infection. Using α7 nAChR knockout model, the involvement of such receptor subunit in immunomodulatory effects of nicotine was observed, which is consistent with previous studies (8). This model demonstrates possible effects of NRT on the development of fetal immune system and the inflammatory "tone" of neonatal AM set at birth.

Our preliminary findings with mice exposed to nicotine developmentally suggested that these mice have greater morbidity and increased inflammatory cytokine production upon *Pseudomonas aeruginosa* lung infection compared to those not exposed to nicotine developmentally. Taken all together, the effect of nicotine from NRT on fetal immune development and thus neonatal immune response signifies the importance of investigating the immune function of pulmonary macrophages during the neonatal period, especially without strong evidence of the effectiveness and safety of *in utero* nicotine exposure via NRT use in pregnant mothers

2. **Objectives:** This study is designed to observe the effects of developmental nicotine exposure, either from tobacco smoking or from nicotine replacement therapy, on neonatal alveolar macrophage characteristics obtained from tracheal aspirate (TA) samples.

3. **Study Design:** This study will be a prospective, single-center, observational investigation. There will be no study medications administered by the investigators. Participants will be grouped into three different arms, Smoking

group vs. Replacement group vs. Control, based on their smoking status during pregnancy.

4. **Study Population:** Mothers aged 18-50 years old whose newborns are placed on a ventilator in the Neonatal Intensive Care Unit (NICU) will be contacted for potential enrollment of their babies in the study. There will be three different groups with 10 participants in each group: 1) smoking group vs. 2) nicotine replacement group vs. 3) control group. Use of 5 or more cigarettes daily during pregnancy will be required to be enrolled in the smoking group while use of nicotine replacement products (patch, gum, inhaler, nasal spray, lozenges) during pregnancy and continued abstinence will be required for their infants to be enrolled in the replacement group. Control group will be defined by no exposure to nicotine during pregnancy. Although best efforts will be made to include participants whose mothers have smoked consistently throughout pregnancy, it is reasonable that some pregnant smokers may only smoke periodically. As long as there was no record of using NRT at any point during pregnancy, these participants will be enrolled in the smoking group. Similarly, smokers using NRT will often-times relapse. Based on smoking history obtained, investigators will make a decision whether to include their infants in the replacement group and such data will be handled statistically. Participants will be excluded if there is known major fetal abnormalities, chemical/alcohol dependence, contraindication to NRT, use of any forms of tobacco other than cigarettes and e-cigarettes, and any active infection, both mother and the newborn, at the time of delivery, before sample collection. Premature infants requiring mechanical ventilation for reasons other than assisting “physiologic normalcy” will be excluded from the study as well. Mothers taking any chronic medications known to pose immunomodulation, such as steroid, or have potential for causing immunomodulation will also be excluded from the study. Since this study is designed to observe the effects of developmental nicotine exposure on alveolar macrophage characteristics of neonates, inclusion of newborns are crucial.

5. **Subject Recruitment Methods and Privacy**: Participants will be identified by hospital number and date of birth. Initial contact with potential participants will be made after the delivery and they will be inquired about their interest in participating in the study. Detailed information about the study and its objectives will be provided by the study investigator, and signed informed consents and parental permission form will be obtained before enrollment. A series of questions will be asked to accurately determine the number of cigarettes smokes, time of the last cigarette smoked or the time of initiation of nicotine replacement therapy and the last NRT product used to enroll participants to corresponding study group.

6. **Informed Consent Process**: Informed consent and parental permission form to include babies in the study will be provided at recruitment and signed

informed consent will be required prior to participant enrollment. This will be collected by the study investigators to make sure participants fully understand the purpose and procedures involved in the study and to answer any questions. Upon the receipt of the signed consent form, record of such action will immediately be documented electronically and maintained throughout study period.

7. **Research Procedures:** All participants will undergo initial screening process for inclusion/exclusion criteria on the day of recruitment. Signed informed consent and parental permission form by the potential participant will be required to be enrolled in the study. Baseline data (date of birth, hospital number, ethnicity, medical history, daily number of cigarettes smoked during pregnancy, time from last cigarette smoked/nicotine replacement product use, partner's smoking status, gestational age, signed consent form, a list of acute/chronic medications, medications administered during current hospitalization, and indication of participant's contact details) will be collected through a series of questionnaires as well as from the subject's medical record (see attached data collection tools). **For TA collection**: TA suctioning is performed as part of routine care in the neonatal intensive care unit and these specimens are discarded thereafter. For the purpose of this study, 0.5 to 1 mL of suctioned specimens will be collected within the first week of birth for 2-3 samples per baby (up to 5 samples) or less if extubated earlier. Specimens will be collected in pre-labeled tubes on ice and immediately transported to the laboratory of Dr. Feola.

   For TA samples: specimen will be centrifuged to pellet the cells, then cells will be washed 3 times with RPMI media and counted using a hemocytometer. Cells will be seeded at 1-2x10^5 cells per well in 24-well plates, removing non-adherent cells after 1-2 hours, leaving macrophages intact. Lipopolyssacharide (LPS) from *Escherichia coli O55:B5* from EMD Millipore will be used to stimulate inflammatory responses. mRNA from cells will be isolated and purified using the Qiagen RNeasy Kit (Qiagen Sciences, Germantown, MD). mRNA concentration and sample purity will be calculated by measuring ultraviolet absorbance at 230, 260, and 280 nm using a spectrophotometer. Samples will be frozen at -80℃ for microarray analysis.

   Microarrays will be performed on mRNA samples using the TaqMan® Gene Expression Array Plates (Applied Biosystems, Foster City, CA). TaqMan® arrays are flexible, affordable, and convenient for gene expression analysis screening for specific biological pathways, processes, diseases, or can be customized. These arrays each consist of 48 genes of interest, such as TNFa, IL-1, IL-10, and CD86. RNA samples (0.1-10ug) will be converted into cDNA, and using 10-100 ng of cDNA per plate, Applied Biosystems real-time quantitative PCR instrument will allow amplification of target genes. Gene expression can be measured by the quantitation of cDNA relative to a calibrator sample, which serves as a physiological reference. All quantitations will be also normalized to an endogenous control to account for variability in

the initial concentration and quality of the total RNA and in the conversion efficiency of the reverse transcription reaction.

Additionally, cytokine concentrations from cell culture supernatant will be quantified using BD™ Cytometric Bead Array (CBA Kits (BD Biosciences, San Jose, CA). Bead populations distinct fluorescence intensities are coated with capture antibodies specific for each cytokine to be measured. These beads will be incubated with fluorochrome-conjugated detection antibodies, and then incubated with 50uL of each sample for 3 hours at room temperature. Sandwich complexes are then formed, after which the beads are washed, and the fluorescence intensity is assayed by flow cytometry. These intensities are then compared to a standard curve generated for each cytokine to determine the concentration in each sample.

Gene and protein expression levels in TA will be statistically analyzed through principal component analyses. These expression levels will then be compared among groups and correlated to clinical outcomes (demographic information, smoking status, gestational age, etc.) acquired through retrospective review of the chart.

8. **Resources:** This will be a single center study, performed at the University of Kentucky Medical Center. In addition to the investigators listed, nurses who routinely work in the neonatal intensive care unit under the medical direction of neonatologist Dr. Hubert Ballard will assist in collection of TA sample. Upon sample collection, this will be transported to the laboratory of David Feola at the Department of Pharmacy Practice and Science at University of Kentucky for further analysis.

   Dr. Feola's research laboratory space at the College of Pharmacy will be utilized. The PI operates wet-lab functions in approximately 1000 ft^2 of space. The lab employs the use of Class II biosafety cabinets and is approved for biosafety level 2 works through the Institutional Biosafety Committee. This lab also contains all needed equipment to conduct this research, including refrigerators, freezers, incubators, centrifuges, and microscopes.

9. **Potential Risks:** Potential risks to the patient from this study are minimal and include unanticipated breaches of confidentiality, in which case the IRB will be notified immediately. No invasive procedure will be performed on patients enrolled in this study for the sole purpose of the study, and TA specimens, which will be suctioned out and discarded as a routine care, will be collected for the study. There will be no study drug administered to participants in this study. Strict adherence to confidentiality requirements will ensure the patients' data and demographic information is protected.

10. **Safety Precautions:** In order to minimize the risks of breaching confidentiality or invasion of privacy, paper documents that contain patient information will be stored in a locked cabinet in room 231 of BioPharm Complex and any electronic data will be made accessible with password. All standard perinatal care will be provided to participants, and their enrollment in

the study will not prevent them from receiving any additional interventions necessary for unanticipated problems during hospitalization. If mothers raise any concerns or questions about the procedures for themselves as well as the newborns, they are able to withdraw from the study at any point after enrollment.

11. **Benefit vs. Risk:** Samples collected from this study will be obtained as a part of routine standard measures and there is minimum risk to participants. No blood will be drawn for the sole purpose of this study. TA suctioning is performed as part of routine care in the neonatal intensive care unit, and these specimens will be stored and transferred to Dr. Feola's laboratory for analysis.

12. **Available Alternative Treatment(s):** There will be no study drug administered/provided by the investigators and the sources of samples obtained during the study will be part of standard care.

13. **Research Materials, Records, and Privacy:** Baseline data (please refer to #7) from mothers and newborns, and TA samples from newborns will be collected. They will be individually labeled with corresponding participant's hospital number followed by the type of sample and the date of collection. TA samples will be collected as described above and processed immediately. All data outline above, including chart information, smoking status, immune system genetic profile, cytokine concentration will be recorded electronically and will be made accessible with password and secured in a locked office (231 College of Pharmacy).

14. **Confidentiality:** Baseline data collected from the participants will be saved in a locked cabinet in room 231 of BioPharm Complex, accessible to only the research investigators. Any information obtained electronically will require password-protected access. Upon collection of specimen, they will be initially stored in a limited access environment and will be transported immediately to the laboratory of Dr. Feola for storage/analysis, at which de-identification will occur by re-assigning sample number to a unique identifier code consisting of a number to designate each participant followed by letters to designate sequence of samples collected from that individual. This will be recorded electronically and access will require password. Patient data and specimens will be stored for perpetual maintenance for the accuracy of results interpretation.

15. **Payment**: Not applicable. There are no incentives or payments for participation in this study.

16. **Costs to Subjects**: There will be no costs that are the participant's responsibility as a consequence of participating in the research. All costs

associated with the procedure beyond the usual standard of care procedures will be directed to the research investigators.

17. **Data and Safety Monitoring:** This study has a minimal risk to the participants as all the procedures involved in obtaining patient information and samples are part of usual standard of care measures and will be under the medical direction of Dr. Ballard. Therefore, no monitoring is required.

18. **Subject Complaints:** Participants will be able to raise any concerns or questions regarding the study and study procedures at any point, and study investigators will make best efforts to meet with them face-to-face to clarify and address their concerns. Additionally, they will be able to withdraw from the study at any point, and the use of collected data for analysis will be discussed.

19. **Research Involving Non-English Speaking Subjects or Subjects from a Foreign Culture:** Mothers who cannot speak and understand English will not be enrolled in the study. Very few Hispanic mothers smoke, therefore it will not significantly decrease potential pool of subjects

20. **HIV/AIDS Research:** Not applicable

21. **PI-Sponsored FDA-Regulated Research:** Not applicable

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PROFESSIONAL POSITIONS

Clinical Staff Pharmacist, Surgery — December, 2014 - Present
University of Kentucky Chandler Medical Center
Lexington, KY

Post-Graduate Year 1 Pharmacy Resident — July, 2013 – June, 2014
Hartford Hospital
Hartford, CT

PRESENTATIONS/ABSTRACTS

Kang N, Housman ST, and Nicolau DP. Predictive Surrogate Susceptibility of Oxacillin and Cefoxitin for Ceftriaxone and Commonly Utilized Agents against Methicillin-susceptible *Staphylococcus aureus*. Presented at International Union of Microbiological Societies, 2014. Montréal, Canada.

Kang N, Pauly JR, and Feola DJ. Effects of *in utero* nicotine exposure on immune responses. Presented at 44th Autumn Immunology Conference 2015, Chicago, IL; Drug Discovery and Development Symposium 2015. Lexington, KY; Infectious Disease Research Day, 2015. Lexington, KY; Infectious Diseases Pharmacotherapy Fellowship Forum, 2016. Aspen, CO.

PUBLICATIONS

Kang N, Housman ST, and Nicolau DP. Assessing the Surrogate Susceptibility of Oxacillin and Cefoxitin for Commonly Utilized Parenteral Agents against Methicillin-

susceptible Staphylococcus aureus: Focus on Ceftriaxone Discordance between Predictive Susceptibility and In Vivo Exposure. *Pathogens*. 2015; 4(3): 599-605.

Kang N, Forcello N. Targeted cancer therapy: Class related toxicities. *Conn Med*. 2015 Feb; 79(2): 109-113.

Kang N, Sobieraj, DM. Indirect treatment comparison of new oral anticoagulants for the treatment of acute venous thromboembolism. *Thrombosis Research.* 2014; 133(6):1145-1151.

Eckman AM, Tsakalozou E, **Kang NY**, Ponta A, and Bae Y. Drug release patterns and cytotoxicity of PEG-poly(aspartate) block copolymer micelles in cancer cells. *Pharmaceutical Research* 2012; 29(7): 1755-1767.