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Electronic Cigarettes and Pulmonary Immunity

By

MORGAN POINDEXTER

DISSERTATION

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Abstract

Electronic cigarettes (E-cigarettes) were originally designed in 2003 as smoking cessation devices intended to deliver nicotine without the need for combustion. E-cigarettes, also known as electronic nicotine delivery systems (ENDS), have since gained widespread popularity across the globe among both current and former tobacco smokers, and those who have never smoked before. Concerningly, e-cigarette popularity has also spread to adolescents with recent estimates that over 2 million middle and high school students currently use e-cigarettes. The efficacy of e-cigarettes as smoking cessation aids is highly contested, and the FDA has not yet approved any e-cigarette device for this purpose. There exists an ever-expanding body of knowledge regarding the potential health hazards of continued e-cigarette use.

E-cigarettes, which exist in many forms, or ‘generations’, that vary in design and efficiency at aerosolizing nicotine, share three core elements: a power source (battery), heating element (coil or atomizer) and a reservoir containing e-liquid (composed of the humectants propylene glycol (PG) and vegetable glycerin (VG), nicotine and flavoring chemicals). Aerosols, generated by heating of the coil to aerosolize e-liquid, are known to contain numerous toxic chemicals that are also found in cigarette smoke. In particular, research has consistently shown e-cigarette aerosols contain carbonyls, metals, free radicals, and nicotine by-products. Popular e-cigarette devices, called third-generation devices, allow users to adjust device settings like coil temperature and power, and can be used with low resistance “sub-ohm” coils made from numerous different metals and in many different coil configurations. Popular fourth-generation devices are less customizable but use highly bio-active nicotine salts, which increase nicotine delivery to users. E-cigarette research is challenging and complex due mostly to the numerous model types of e-cigarette devices available on the market, which evolved from first- to now

fourth- generation, with over 2,800 different models from 466 identified brands, and to the selection of more than 16,000 distinct e-liquid flavors. In addition, each combination of e-liquid variables (humectants, flavors, nicotine concentration) and device operating conditions (wattage and temperature settings) is unique and produces a specific toxicity profile.

In humans, e-cigarette use has been shown to cause acute adverse respiratory, cardiovascular, digestive, and neurologic health effects, among others. Evidence has also emerged that e-cigarette users can experience immunological disturbances leaving them at increased risk for respiratory infections, such as COVID-19 or influenza. Studies *in vitro* and *in vivo* in rodents have implicated e-cigarettes in disruption of pulmonary immune homeostasis leading to aberrant cytokine and chemokine production and altered immune cell functions. However, the exact mechanisms of action and the long-term health consequences of these changes are still largely unknown. Notably, vulnerable populations like fetuses, infants, children, pregnant women, and older adults may be uniquely vulnerable to immune related health risks associated with e-cigarette use.

Herein, we review the effects of e-cigarettes use on both maternal and fetal health outcomes, as well as consequences of early-life e-cigarette exposures in infants and children. We then investigate the effects of increased coil temperature on the chemical composition of aerosol from a third-generation e-cigarette device, and on pulmonary inflammation in murine lungs. We find that increased coil temperature does not significantly increase carbonyl compound concentrations, but that exposures to aerosols generated at higher temperatures can more significantly dampen pulmonary cytokine production and macrophage recruitment to lungs. We then investigate progressive exposure to e-cigarette aerosols and compare this to traditional cigarette smoke exposure in mice. We find that progressive e-cigarette exposure induces a

unique, acute inflammatory response compared to cigarette exposure, characterized by transient macrophage infiltration to lungs. We also find that progressive e-cigarette exposure reduces cytokine gene expression indicating a potentially immunosuppressive effect. Lastly, we investigate the effects of e-cigarettes on the pulmonary immune system and host responses to influenza A viral infection in both young and aged mice. We find that viral titers are reduced, viral clearance is impaired, and mortality from infection is decreased in e-cigarette exposed mice. We find that e-cigarette exposure caused significant shifts throughout infection in both innate and adaptive immune cell populations and cytokine profiles which persisted after resolution of infection, underscoring the potential health risks associated with e-cigarette use. Of note, during early infection, Natural Killer (NK) cell populations were significantly increased in both mouse strains and both ages, indicating a consistent phenotype which to our knowledge has not been reported previously.

The studies contained herein further the current understanding of the impacts of e-cigarettes on pulmonary immune homeostasis and underscore the need for continued regulation of e-cigarette sales to minimize negative public health outcomes.

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Chapter 1:

Introduction – Early Life Exposure to E-Cigarette Aerosols

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Epidemiological studies have shown in the past few years that the use of electronic nicotine delivery systems (ENDS) has significantly increased among youth and young adults, including women of childbearing age. Worldwide, ENDS are used by more than 35 million individuals (Besaratnia and Tommasi, 2019), with the European Union and the United States being the largest markets (WHO, 2020). The prevalence of ENDS use varies based on countries and their regulations. For example, in Brazil, the prevalence of vaping is below 1% (Besaratnia and Tommasi, 2019), in New Zealand it is 3.8% (WHO, 2020), and in European populations it ranges from 0.2 to 27% (Kapan et al., 2020). Among youth, in 2019 the prevalence of vaping was 0.7% in Japan (WHO, 2020), 18.4% in Ukraine (WHO, 2020), 2.7% in England, 5.7% in Canada, and 6.7% in the United States (Hammond et al., 2020). The latest figures from the United States Centers for Disease Control and Prevention (CDC), estimate ENDS use to ~ 8 million American adults and 3.5 million middle and high school students. Also, a dramatic 89% increase in the number of American high school girls utilizing ENDS occurred from 2017 to 2020 (Tobacco Free Kids Organization, 2021).

ENDS use continues to rise due in large part to the unproven belief that they are healthier and safer than conventional cigarettes. With this belief prominent in society, pregnant women and women of reproductive age are among the most frequent users who switch from combustible cigarettes to ENDS in an effort to reduce some of the known hazards of tobacco use (Wang et al., 2016). As women become pregnant, many are unaware of the harmful effects ENDS and nicotine exposures can have on their own health and the health of the unborn child from gestation and beyond. Most importantly, many pregnant women are not given adequate advice by their medical provider regarding the risks of vaping, as many physicians themselves are not fully aware of the potential risks. Based on an American study, it was previously reported that 13.5% of obstetricians

judged ENDS to be free of harm, 29% believed ENDS may have some negative adverse effects but less severe compared to tobacco cigarettes, 13.5% believed both ENDS and tobacco cigarettes to be equally harmful, while 36.5% were not sufficiently informed to provide an opinion (England et al., 2014). Over 90% of women reported their healthcare provider asked if they smoked conventional cigarettes, but only one quarter recalled being asked if they used ENDS. This finding indicates a critical need to educate healthcare providers about the importance of updating screening practices and how to describe the absolute harm of ENDS exposure during pregnancy to their patients (Dobbs et al., 2020). Currently, there are no guidelines in the United States for use of ENDS during pregnancy. This highlights the urgent need to bridge the clinical and scientific knowledge gap related to ENDS use during pregnancy.

In recent years, ENDS have become the most popular aid for smoking cessation in the United Kingdom, and a significant proportion of pregnant women report using them during pregnancy (Opondo et al., 2021). Due to these patterns of use and remaining challenges relating to reducing smoking in pregnancy, a multi-agency group comprised of some of the main health professionals and research organizations, along with health charities and mother and baby organizations in England (the Smoking in Pregnancy Challenge Group) have developed practical guidance on vaping during pregnancy (Bowker et al., 2018). This led to a positive policy framework related to ENDS use during pregnancy for women who cannot quit smoking (East et al., 2018; McNeill et al., 2018). Even though there is limited research on vaping during pregnancy, the fact that many physicians are unaware of how to provide guidelines to inform pregnant women on the risks of ENDS use (Hunter et al., 2021), illustrates why it is imperative that studies continue to provide information on whether ENDS use during pregnancy is safer and represents a better risk-to-benefit ratio for the developing fetus than conventional cigarettes, as well as to determine

whether ENDS use during pregnancy is harmful on its own, without using cigarettes as a comparison point.

Prevalence of ENDS use during pregnancy

Maternal smoking is considered the primary preventable factor for several pregnancy-related morbidity and mortality outcomes (Sheung et al., 2006). Whether ENDS use during pregnancy alleviates, aggravates, or give rise to similar pregnancy outcomes as maternal smoking is currently unknown. In the United Kingdoms, the prevalence of using ENDS during pregnancy is estimated to be 2.8% (Opondo et al., 2021), while in the United States, more than 10% of pregnant women vape during their pregnancy (US Surgeon General Report, 2016). Based on data collected in 2015, the prevalence of ENDS use among pregnant women in the states of Oklahoma and Texas was 10.4% before pregnancy and 7.0% around the time of pregnancy, and 1.4% during the last 3 months of pregnancy (Kim et al., 2020). Another study conducted in the United States showed that ENDS use during pregnancy, including exclusive ENDS users and dual users of ENDS devices and cigarettes, was approximately 13% (Wagner et al., 2017). Overall, research published between 2007 and 2017 are showing that up to 15% of pregnant women in the United States use ENDS (Schmidt et al., 2020). Alarmingly, of the pregnant women who use ENDS during their pregnancy, 75% are dual users of both conventional cigarettes and ENDS devices (Liu et al., 2019; Clemens et al., 2019). In fact, the prevalence of ENDS use is 29% among pregnant tobacco users in the United States (Kurti et al., 2017). In addition, it was found that at least three-quarters of pregnant smokers switched from cigarettes to ENDS because of their perceived reduced harm and their use as aid cessation devices for combustion tobacco products (Schmidt et al., 2020; Kim and Oancea, 2020). Also, reports show that 50% of the women who use ENDS continue to vape

after being pregnant (Spindle et al., 2016). This is partly due to many individuals perceiving ENDS as a safe alternative to smoking regular cigarettes during pregnancy (Kim and Oancea, 2020).

Overall, these prevalence findings on vaping during pregnancy are alarming, and since little is known about the health effects associated with inhaling ENDS aerosols, there is a clear urgent need for more clinical, epidemiological, and experimental research that investigate the health effects of vaping on pregnant mothers as well as on their developing fetuses.

Maternal health effects associated with ENDS use during pregnancy

Increasing evidence are showing adverse health effects of vaping in healthy adults (McDonough et al., 2021). This includes documented respiratory and cardiovascular effects in humans (Tarran et al., 2021). Thus far, clinical evidence shows that in dual users of both conventional cigarettes and ENDS, ENDS use a) leads to declines in lung function, in terms of lung impedance and airway flow resistance, and b) significantly increases the risk of having a myocardial infarction (Vardavas et al., 2012; Alzahrani et al., 2018). In exclusive ENDS users, pulmonary responses to ENDS aerosol containing nicotine include increase airway flow resistance, suggesting obstruction of the conducting airways (Antoniewicz et al., 2019). However, to our knowledge, no human study has investigated the specific effects of vaping on the overall health of pregnant women, excluding pregnancy outcomes (stillbirth, small for gestational age, preterm birth, etc.), which will be reviewed below.

Thus, human studies and epidemiological evidence are limited. Recommendations are based on decades of research on previous comparable studies, including studies on nicotine, maternal smoking, and secondhand smoke exposures during pregnancy, which have been extensively reviewed elsewhere (Zdravkovic et al., 2005; Metzger et al., 2013; Holbrook, 2016;

Abraham et al., 2017; Pereira et al., 2017; McGrath-Morrow et al., 2020). It was showed that excessive use of nicotine during pregnancy by mothers can reduce oxygen and nutrient delivery to the fetus (Holbrook, 2016; McGrath-Morrow et al., 2020; Suter and Aagaard, 2020). Thus, it is anticipated that nicotine delivery by ENDS can also reduce maternal vascular adaptations and decrease uterine artery blood flow. It is thought that the use of ENDS during pregnancy places excessive workload on the maternal cardiovascular system in an effort to counterbalance vascular adaptations that occur in the uterine artery which is the primary vessel that delivers oxygen and nutrients to the fetoplacental unit (Orzabal et al., 2019). Given the proven effects of ENDS aerosols on the cardiopulmonary system of healthy adults (Vardavas et al., 2012; Alzahrani et al., 2018; Antoniewicz et al., 2019), in addition to the documented effects of nicotine at the blood-placental interface in pregnant women (Zdravkovic et al., 2005; Suter and Aagaard, 2020), it can be predicted that ENDS use during pregnancy can pose significant risks to the pulmonary and cardiovascular health of expecting mothers. Thus, more clinical data and epidemiological evidence are needed in this particular area of research.

Fetal health effects associated with ENDS use during pregnancy

ENDS use during pregnancy has been shown to negatively affect pregnancy outcomes (Kim and Oancea, 2020; Regan et al., 2021; Regan and Pereira, 2021). Nicotine is a known teratogen, which, as a lipophilic molecule, readily crosses the placenta, accumulates in placental tissue, amniotic fluid, and fetal serum (Wickström, 2007; Whittington et al., 2018). Thus, nicotine concentrates in the fetus, where it binds to nicotinic acetylcholine receptors (nAChR) in the fetal nervous system, lungs, and other tissues (Wickström, 2007; Whittington et al., 2018). Research has established that nicotine exposure during pregnancy can cause developmental abnormalities in

the brain and lungs (Ernst et al., 2001; McEvoy and Spindel, 2017). Moreover, it is well-established that nicotine exposure can lead to preterm birth (National Academies of Sciences, 2018).

There are very few human studies that examined the effects of ENDS exposure on pregnancy outcomes. To date, we have identified 11 studies which investigated the pregnancy outcomes of women who were vaping during pregnancy in comparison to either non-users of tobacco products, dual users of ENDS plus cigarettes, and cigarette smokers (Cardenas et al., 2019; Clemens et al., 2019; Cardenas et al., 2020; Kim and Oancea, 2020; McDonnell et al., 2020; Wang et al., 2020a; Ashford et al., 2021; Hawkins et al., 2021; Opondo et al., 2021; Regan et al., 2021; Regan and Pereira, 2021). All but 2 of the 11 studies were conducted on American populations; the other 2 (McDonnell et al., 2020; Opondo et al., 2021) were conducted on European populations.

Using data from the 2016 – 2018 Pregnancy Risk Assessment Monitoring System (PRAMS) study with a sample size of 79,176 American women who delivered live birth, it was demonstrated that ENDS use before pregnancy was not associated with any adverse birth outcomes (Regan et al., 2021). Significant effects, however, were observed for ENDS use during gestation. There was a significant adjusted prevalence ratio (PR) of 1.33 for low birth weight (LBW) infants born from women who used ENDS and other tobacco products during their pregnancy compared to infants born from women who were non-users (Regan et al., 2021). In this same study, analysis of exclusive ENDS users revealed that there was a significant adjusted PR of 1.88 and 1.69 for low birth weight and preterm birth, respectively, for infants born from women who exclusively used ENDS during their pregnancy compared to infants born from women who were non-users (Regan et al., 2021). Another research group also analyzed data from the PRAMS study and evaluated pregnancy outcomes in more than 55,000 pregnant women (Kim and Oancea, 2020).

They found significant odd ratios (OR) of 1.76, 1.53, and 1.86 for small-for-gestational age (SGA), low birth weight and preterm birth infants, respectively, who were born from exclusive ENDS users when compared to non-user pregnant women (Kim and Oancea, 2020). They also noticed that exclusive ENDS users had similar adverse pregnancy outcome risks as cigarettes only users (Kim and Oancea, 2020). Other analyses based on extracted data from the PRAMS study on over 16,000 women who gave birth demonstrated a significant adjusted PR of 1.52 for low birth weight infants born from women using ENDS exclusively during their pregnancy when compared to infants born from women who were former smokers (Regan and Pereira, 2021). Also, significant adjusted PRs of 2.11 and 2.60 were observed for low birth weight and SGA infants born from women who were dual users during pregnancy compared to infants born from women who were former smokers (Regan and Pereira, 2021). Overall, data collected in the PRAMS study and analyzed by three independent research groups demonstrate that exclusive ENDS use during pregnancy can cause adverse pregnancy outcomes, including pre-term birth, low birth weight, and infants born SGA. These effects are in the same range as those observed with maternal smoking.

Further, in a United States-wide study conducted on more than 30,000 singleton live births, it was shown that women who were ENDS-only users during late pregnancy (3rd semester) had a significantly increased adjusted OR of 2.4-fold of having infants born SGA and the adjusted OR for women who were dual-users was 2.3, when compared to infants born from women who were non-users (Wang et al., 2020a). In this same study, no significant difference was observed for preterm birth between the various groups, and thus highlights that infant born of women who are exclusive ENDS users or dual users during late pregnancy may have experienced fetal growth restriction, as evidenced by the SGA status (Wang et al., 2020a).

Other studies have found significant effects in dual users but not in ENDS-only users. A cross-sectional study on American women (sample size > 57,000 participants from the 2016-2017 PRAMS study) showed that the dual-use of ENDS plus cigarettes significantly reduced the birth weight of newborns and significantly increased the OR by 1.93-fold of infants being born SGA when compared to infants born from women who did not use any tobacco products during their pregnancy (Hawkins et al., 2021). When results were analyzed for women using ENDS exclusively, no significantly different effects were noticed in comparison with non-users (Hawkins et al., 2021). Another study conducted in the United States (> 64,000 births from the 2016-2017 Arkansas Phase 8 PRAMS study) revealed significant adjusted risk ratios (RR) of 1.7- and 1.8-fold for SGA infants born from women who used cigarettes, as well as from women who were dual users of ENDS plus cigarettes during pregnancy, respectively, when compared to infants born from women who were non-users (Cardenas et al., 2020).

Also, a prospective cohort study (sample size 76 participants), which used nicotine hair levels to confirm nicotine exposure in pregnant women, found a significant RR of 8.3 and 7.3 for SGA infants born from women who are dual-users and smokers, respectively, when compared to infants born from pregnant women who did not use any tobacco products (Clemens et al., 2019). Moreover, in a cohort study of ~ 250 pregnant American women, the RR of having an infant born SGA was significantly increased to 5.1- and 3.8-fold for women who were exclusive ENDS users and multi-type tobacco users (including dual-users), respectively, when compared to infants born from women who were non-users (Cardenas et al., 2019). Overall, these four studies highlight that dual-use of ENDS with conventional cigarettes during pregnancy increases the risks for fetal growth restriction when compared to non-users and importantly, that risk is more substantial for

dual-users than for conventional smokers alone. This suggests a potentially additive risk associated with ENDS use during pregnancy.

Tobacco products use behavior and pattern may also be a factor that affects the health of the fetus. In a longitudinal cohort study (sample size 278 pregnant women from Kentucky), no effects on birth outcomes were observed between groups of pregnant women defined as ‘switchers’, i.e., women who reported tobacco products use during the 1st visit (including cigarettes, ENDS and dual-usage) and who switched to the use of another tobacco products during subsequent visits, and women defined as ‘no-switchers’, i.e., women who maintained the use of the same tobacco product (including ENDS) throughout their pregnancy (Ashford et al., 2021). A significantly higher birth weight of newborns, however, was observed between pregnant women defined as ‘quitters’, i.e., women who used a tobacco product, including ENDS, at the 1st visit and who quit afterwards, and ‘no-switchers’ (Ashford et al., 2021). This shows that switching tobacco products use behaviors can impact pregnancy outcomes.

We found only 2 epidemiological studies from Europe which investigated pregnancy outcomes in ENDS users and smokers (McDonnell et al., 2020; Opondo et al., 2021). A study based on data from 218 pregnant women in Ireland found that while the birth weight of infants born from exclusive ENDS users was similar to that of non-smokers, the birth weight of infants born from dual users was similar to that of infants born from smokers (McDonnell et al., 2020). These birth weights were significantly lower than those from infants born from pregnant women who did not use any tobacco product (McDonnell et al., 2020). Also, a cross-sectional study from England (sample size > 4,400 participants) found no significant association for birth weight and preterm birth between pregnant women who used ENDS and women who did not use any tobacco product during pregnancy (Opondo et al., 2021). Of important note, the levels of nicotine available

in ENDS products are different between the United States and European countries. In the United States ENDS products are available in concentrations ranging from 3 to 36 mg/mL for freebase nicotine and from 30 to 59 mg/mL for nicotine salts, while in Europe and the United Kingdoms, the nicotine concentration in ENDS products, regardless of its form (freebase or salt), do not exceed 20 mg/mL (McDonnell et al., 2020). Thus, exposure levels of pregnant women to nicotine from Europe and the UK can reasonably be assumed to be much lower than levels encountered by pregnant women in the United States. This may partly explain why epidemiological findings for pregnancy outcomes for women who vape during pregnancy are vastly different for the two continents. In addition, the sample size might also be an important differential factor, as study based in the United States benefited from larger sample size (e.g., PRAMS study: 16,000 – 79,000 individuals).

Taken together, these studies indicate that dual usage of ENDS plus cigarettes during pregnancy negatively affect pregnancy outcomes similarly or even worse than the effects associated with exclusive maternal smoking. Furthermore, exclusive ENDS use during pregnancy negatively impacts fetal growth as evidenced by the consistent findings of increased risk of infants born with LBW or SGA and can increase risks of adverse pregnancy outcomes such as preterm birth.

Health effects in prenatally and secondhand ENDS aerosol-exposed infants/children

Despite numerous animal studies on the implications of early life exposures to ENDS aerosols (reviewed below), currently, very few studies in humans evaluated the health outcomes in infants prenatally exposed to ENDS. Indeed, extensive information on the health impacts

associated with the use of ENDS products in early human development is largely undocumented, due to the relative novelty of ENDS products. To date, we found only one study in infants (n = 83) from the United Kingdom (Froggatt et al., 2020). In this study, despite similar pregnancy outcomes between groups (birth weight, gestation, and head circumference), it was shown that one-month old infants prenatally exposed to ENDS aerosols had an increased number of abnormal primitive reflexes and reduction of self-regulation abilities when compared to non-exposed infants (Froggatt et al., 2020). The levels in ENDS-exposed infants were comparable to those of infants exposed *in utero* to cigarette smoke (Froggatt et al., 2020). This indicates that prenatal ENDS exposure can result in neurobehavioral outcomes in infants, particularly a decline in motor maturity, similar to effects observed in offspring born from smoking mothers. These data thus suggest that prenatal ENDS exposure can cause adverse effects on fetal brain development.

Exposures of the pediatric population to tobacco-related secondhand and third-hand smoke is also of great concern (Protano et al., 2017). Exposures to secondhand cigarette smoke in children have been associated with increased risk of respiratory infections and lung diseases, including asthma (Protano et al., 2017). In contrast to combustion tobacco products, such as conventional cigarettes, ENDS do not produce combustion by-products, and close to 100% of the secondhand aerosol comes from the exhaled breath of the user (Collaco & McGrath-Morrow, 2018). However, secondhand ENDS aerosol is not harmless as ambient nicotine and particulate matter 2.5 (PM_{2.5}) levels are increased following vaping sessions (Schripp et al., 2013; Ballbe et al., 2014; Czogala et al., 2014; Ruprecht et al., 2014; Schober et al., 2014). In experimental settings, 1-hour after a natural vaping session, nicotine levels on average reached 3.32 µg/m³ and PM_{2.5} concentrations of 151.7 µg/m³ (Czogala et al., 2014). In homes of ENDS users, it was reported that the mean levels of nicotine were 0.13 µg/m³, which correlated with salivary cotinine levels in non-tobacco

product users of 0.19 ng/mL (Ballbe et al., 2014). These salivary cotinine levels were similar to those of non-tobacco product users exposed to secondhand cigarette smoke (Ballbe et al., 2014). This demonstrates that secondhand ENDS aerosol contains a sufficient level of nicotine to result in measurable levels of cotinine in bystanders, in addition to emitting PM (Flouris et al., 2013; Ballbe et al., 2014). Thus, infants, children and adolescents can be exposed to nicotine and PM_{2.5} emitted from secondhand ENDS aerosols in their household and indoor environments; however, little is currently known about the health effects associated with exposures to secondhand ENDS aerosols in the pediatric population.

When evaluating the health risks associated with secondhand ENDS exposure in pediatric populations, it is important to note that there are both anatomical and physiological differences between infant and adult lungs which can impact aerosol exposure risks. In particular, particle deposition increases as the size of the lungs decreases (Poorbahrami et al., 2021). Infants and children have small airways, which results in increased airflow velocity and thus, higher total and regional particle deposition, compared to lungs from an adult (Poorbahrami et al., 2021). In a study, experimental data were obtained by generating ENDS aerosols and were used to estimate secondhand ENDS aerosol exposures between 3-month-old infants and adults of 21 years of age using a multiple-path particle dosimetry (MPPD) model (Protano et al., 2017). Particle doses (particles/kg of body weight) from exposures to secondhand ENDS aerosols generated using a 1st generation ENDS device were approximately twice as high in 3-month-old infant respiratory systems than in adults. Due to the use of a 1st generation ENDS device, which generates significantly less aerosol than 3rd generation devices, predictably, particle concentrations from ENDS aerosols in the study were approximately 4 times lower than from conventional cigarettes. In recent years, 3rd generation ENDS devices are increasingly popular and therefore this study

likely represents a low estimation of possible secondhand ENDS aerosol particle doses. Of additional concern, the study also noted that infants and children were the age groups that inhaled the largest amounts of submicronic particles per kg of body weight, including particle sizes that can reach the deep lungs (Protano et al., 2017). Thus, demonstrating theoretically that environmental secondhand ENDS aerosols can lead to pulmonary particle deposition in infant and children.

Thirdhand ENDS aerosols exposure may also be of significant concern for infants and children. Recent evidence in mice suggests thirdhand ENDS aerosol exposure may, even in the absence of nicotine, impact lung function and serum inflammatory cytokine levels (Chen, et al., 2020). Indeed, a study has demonstrated that measurable levels of nicotine are present on hard surfaces, e.g., window, walls, floor, and metals, following exposure to ENDS aerosols (Goniewicz and Lee, 2014). Glass windows and floor showed the highest levels of nicotine, with concentrations reaching 205 $\mu\text{g}/\text{m}^2$ for the floor (Goniewicz and Lee, 2014). Another study testing thirdhand nicotine deposition on both glass and fabric, found that fabrics had approximately 3 times the concentration of nicotine following ENDS aerosol exposure (94.9 $\mu\text{g}/\text{m}^2$) (Marcham et al., 2019). Infants and children spend significant time indoors, and in addition to dermal contact with nicotine contaminated surfaces, ingestion exposures could also occur through hand-to-mouth interactions (McGrath-Morrow et al., 2015).

Taken together, since exposure to nicotine and the other chemicals found in ENDS secondhand and third-hand aerosols can occur through multiple routes for prenatally exposed fetuses to toddlers and adolescents, e.g., via lactation, inhalation, percutaneous, and ingestion, it is of utmost importance to investigate the currently unknown health effects of *in utero* and post-natal exposures to ENDS aerosols in those vulnerable populations where the brains and the lungs are

still undergoing development. This also highlights that there are significant implications regarding regulation and policy guidelines for the safe use of ENDS during pregnancy, as well as for indoor environments where infants and children spend the majority of their time.

Experimental animal studies

As noted from the section above, human studies on the health effects associated with early life exposures to ENDS aerosols are extremely limited. Epidemiological studies will take many more years to provide scientific evidence related to the safety or toxicity of ENDS use during pregnancy for fetal development. In these cases, the scientific communities and healthcare professionals rely on experimental animal studies to anticipate which adverse health effects to monitor more closely. To date, animal data clearly demonstrate that the use of ENDS during pregnancy is not harmless to the developing fetus, and in addition that early life exposures to ENDS aerosols can affect the offspring development (Chen et al., 2018a; Nguyen et al., 2018; Larcombe, 2019; Li et al., 2019; Wang et al., 2020b; Burrage et al., 2021). Adverse health effects were documented in the respiratory and cardiovascular systems, in addition to altered neurocognitive and metabolic behaviors (Chen et al., 2018b; Nguyen et al., 2018; Larcombe, 2019; Wang et al., 2020; Burrage et al., 2021; Hasan et al., 2021) (**Figure 1**). Hence, animal models are critical to exploring and understanding the potential outcomes and toxicity mechanism of ENDS use during pregnancy (Orzabal et al., 2019).

In the sub-sections below, we only describe animal studies where rodents were exposed to ENDS aerosols generated by ENDS devices, either *via* whole-body (the majority) or nose-only paradigm, to evaluate the implications of early life exposures to ENDS aerosols. Studies using other means of exposure, e.g., *via* drinking water or non-heated e-liquid, were excluded, as these

were considered physiologically irrelevant and non-representative exposure routes. Also, ‘preconception exposures’ are defined as exposures to ENDS aerosols starting before mating and throughout gestation, ‘prenatal exposures’ refer to exposures to ENDS aerosols occurring during gestation, ‘early life exposures’ represent exposures to ENDS aerosols during the first stages of life, including lactation, while the term ‘*in utero* exposures’ is used to expressed exposures to ENDS aerosols which occurred globally during fetal development (preconception and/or prenatal).

It is also important to bear in mind that prenatal exposures of fetuses to environmental contaminants are different from classical exposure routes (e.g., inhalation, ingestion, percutaneous), as *in utero* exposures do not involve any direct contact of the fetus with the contaminant, but rather the exposure occurs through the placenta and *via* the systemic circulation of the mother, also known as the blood-placenta barrier (Elliot et al., 2003). This passive *in utero* exposure leads to fetal organ uptake of nicotine and other harmful chemicals (e.g., carbonyls) found in ENDS aerosols (Elliot et al., 2003).

Neonatal outcomes (birth weight, body length, and body weight gain) in offspring exposed prenatally and in early life to ENDS aerosols

Fetal growth restriction is a typical phenomenon observed when exposures to toxicants (e.g., alcohol, pesticides, cigarette smoke) occur during pregnancy (Ng et al., 2006, Carter et al., 2013, Guo et al., 2014). This deficit in growth represents a well-known factor and a clinical indicator associated with increased risk for severe complications in neonatal morbidities, including lung and metabolic diseases (Su et al., 2000; Sin et al., 2004; Walter et al., 2009; Orzabal et al., 2019). A very limited number of experimental studies have directly investigated the effects of prenatal and early life exposures to ENDS aerosols on fetal outcomes, including intrauterine

growth restriction (IUGR), birth weight and body length of offspring. These studies are summarized below, and additional experimental detail are provided in **Table 1**.

A study conducted in rats directly investigated the effects of prenatal and early life exposures to ENDS aerosols on maternal uterine artery blood flow and fetal growth (Orzabal et al., 2019). Sprague-Dawley rats were exposed to non-flavored ENDS aerosols containing 50 to 100 mg/mL of nicotine produced by a third generation ENDS device. They found that fetal body weight and crown-to-rump length, measured at gestational day (GD) 20, was significantly reduced in fetuses prenatally exposed to ENDS aerosols when compared to air exposed controls (Orzabal et al., 2019). At post-natal day (PND) 10, offspring body weight and length were also significantly lower than those of offspring exposed to saline (Orzabal et al., 2019). These results in the fetuses and offspring correlated with hemodynamics data showing decreased maternal uterine artery blood flow and reduced fetal umbilical artery blood flow in the ENDS aerosols exposed group compared to the saline control group (Orzabal et al., 2019). Nicotine is a vasoconstrictive agent that can increase the vascular resistance of both the uterine and umbilical arteries, thereby decreasing the oxygen and nutrients levels delivered to the fetus (Orzabal et al., 2019). This suggests that the decreased maternal uterine and fetal umbilical artery blood flows may be the cause of ENDS aerosol exposure induced IUGR (Orzabal et al., 2019). These data overall suggest that both prenatal and early life exposures to ENDS aerosols may cause IUGR, which is associated with increased risk for health complications in offspring, including the development of chronic diseases later in life.

In vivo studies examining the effects of prenatal ENDS aerosol exposures on other organ systems (e.g., lungs or brains) either at birth or later in life often recorded birth weight and body length of offspring, even though these health outcomes were not the main focus of the study. Thus,

these studies, briefly described below, help provide additional data about how vaping during pregnancy affects fetal and neonatal physical growth.

Studies in BALB/c mice exposed *in utero* to cinnamon-flavored ENDS aerosols containing 36 mg/mL of nicotine recorded birth weight and body length (Noël et al., 2020; Cahill et al., 2021). Preconception and prenatal exposures to these ENDS aerosols produced by a third generation ENDS device led to significantly decreased body weight and length at birth compared to air exposed offspring (Noël et al., 2020). Moreover, this reduced body weight was maintained until PND28 in prenatally ENDS-exposed offspring (Noël et al., 2020). These effects on the physical growth of offspring correlated with lower maternal serum levels of placental growth factor (PIGF) and increased concentrations of 17- β -estradiol, both of which play important roles in gestation and fetal development (Noël et al., 2020). Unbalanced endocrine milieu can be caused by nicotine (Adeyemi et al., 2018), and in the context of pregnancy, can affect placental blood flow, possibly resulting in IUGR phenotypes. These effects of prenatal ENDS exposure, significantly reducing mice birth weight, were reproduced in Cahill et al., (2021), with decreased body weight being sustained until PND5. However, in this study, no differential effect on body length were observed between treated and control groups. Taken together, these studies show that *in utero* exposures to ENDS aerosol containing high levels of nicotine (36 mg/mL) can impair the physical growth of newborn mice.

ENDS aerosols containing lower concentrations of nicotine (e.g., 24 and 18 mg/mL) were also shown to affect the physical growth of newborn rodents. In Smith et al., (2015), prenatal and early life exposures of C57BL/6 mice to non-flavored ENDS aerosols containing either 0 or 24 mg/mL of nicotine produced by a second generation ENDS device, led to significant differences in body weight from PND8 to PND13 in offspring exposed to both ENDS aerosols (with and

without nicotine) compared to air exposed controls. In Sifat et al., (2020), CD-1 mice were exposed prenatally and during early life to ENDS aerosols containing 24 mg/mL of nicotine produced by a Blu ENDS device, and at PND8, ENDS exposed offspring exhibited significantly decreased body weight compared to controls. In Walayat et al., (2021), Sprague-Dawley rats were exposed prenatally to Blu Plus ENDS aerosols containing 24 mg/mL of nicotine, and both male and female offspring body weight at PND9 were significantly reduced by the *in utero* ENDS aerosols exposure when compared to controls. Further, in McGrath-Morrow et al., (2015), C57BL/6 mice exposed to non-flavored ENDS aerosols containing either 0 or 18 mg/mL of nicotine for the first 10 days of life, exhibited significantly decreased body weight compared to air exposed controls. Thus, these studies highlight that adverse fetal health outcomes can be observed at nicotine levels (18-24 mg/mL) that are preferred by adult ENDS users (Morean et al., 2016; Zare et al., 2018). In addition, the results described above from Smith et al., (2015) and McGrath-Morrow et al., (2015), where nicotine-free ENDS aerosols exposed offspring also exhibited decreased body weight, suggest that nicotine is not the only chemical in the ENDS aerosol contributing to the reduce physical growth of the offspring.

Further, some experimental studies have showed mixed effects of both nicotine-free and nicotine containing ENDS aerosols on the physical growth of offspring. In Chen et al., (2018a), preconception plus early life exposures of BALB/c mice to tobacco-flavored ENDS aerosols containing 18 mg/mL of nicotine significantly reduced the body weight of the offspring, while the ENDS aerosol without nicotine increased the body weight, when compared to sham controls at PND20. These physical growth effects of preconception plus early life ENDS exposure were reproduced in Li et al., (2019), where at PND20, offspring exposed to nicotine containing ENDS aerosol (18 mg/mL) had a reduced body weight, while offspring exposed to nicotine-free ENDS

aerosols had increased body weight when compared to sham controls. The significant differences observed at PND20 in these two studies may reveal that *in utero* exposures to ENDS aerosols, independent of nicotine content, disrupt metabolic activities in early life.

In addition to ENDS aerosols exposures affecting the physical growth of neonatal mice and rats in early life, experimental studies also showed that *in utero* ENDS aerosols exposures can alter the offspring body weight gain in later stages of life. In Church et al., (2020), female CD-1 mice prenatally exposed to non-flavored ENDS aerosols containing 16 mg/mL of nicotine, weighed significantly more than the air exposed counterparts at 12 weeks of age (Church et al., 2020). This is in contrast with results reported by Wetendorf et al., (2019), where preconception exposures of female C57BL/6 mice to non-flavored ENDS aerosols containing 24 mg/mL of nicotine produced by a third generation ENDS device, showed significantly reduced body weight when compared to air exposed counterparts at 8.5 month of age. These opposite weight gain effects in female adult mice observed in the two studies may imply that *in utero* exposures to ENDS aerosols also affect metabolic function later in life. Nonetheless, more research is needed in this particular area to determine precise metabolic effects.

Other studies in mice and rats did not observe any adverse effect on the physical growth of *in utero* ENDS aerosols exposed offspring. In Lauterstein et al., 2016, prenatal and early life exposures to tobacco-flavored ENDS aerosols containing either 0 or 13-16 mg/mL of nicotine produced by a second generation ENDS device, did not produce any significant differences in birth weight or weight gain in 1-month-old C57BL/6 mice offspring. While in Burrage et al., (2021), Sprague-Dawley rats exposed prenatally and in early life to French vanilla-flavored ENDS aerosols containing either 0 or 18 mg/mL of nicotine produced by a third generation ENDS device, did not exhibit any significant difference between the body weight of the offspring from all groups

at birth and at weaning. These studies show that not all animal models revealed adverse physical growth effects of offspring exposed *in utero* to ENDS aerosols. Besides the e-liquid ingredients used and their concentrations, the ENDS aerosol exposure parameters (e.g., generation of ENDS device used, ENDS device settings: power and voltage, vaping topography, and exposure duration), can all significantly contribute to the unique toxicity profile of the ENDS aerosol that is produced in a particular study, and this may add to the complexity of directly comparing experimental *in utero* ENDS aerosols animal studies.

Overall, physical growth outcomes (birth weight, body length, and body weight gain) in mice or rat offspring exposed *in utero* and in early life to ENDS aerosols show a combination of results, where ENDS aerosols containing high nicotine concentrations (> 24 mg/mL) seem to impair physical growth in early life (McGrath-Morrow et al., 2015; Smith et al., 2015; Chen et al., 2018a; Li et al., 2019; Orzabal et al., 2019; Sifat et al., 2020; Noël et al., 2020; Cahill et al., 2021; Walayat et al., 2021), while lower levels of nicotine containing ENDS aerosols (16-24 mg/mL) altered female offspring weight gain later in life, at 3 and 8.5 months of age (Wetendorf et al., 2019; Church et al., 2020). In addition, exposures to nicotine-free ENDS aerosols seem to either restrict or promote weigh gain in older neonatal offspring (Smith et al., 2015; McGrath-Morrow et al., 2015; Chen et al., 2018a; Li et al., 2019). However, in other studies, both nicotine-free and nicotine containing ENDS aerosols did not affect these outcomes (Lauterstein et al., 2016; Burrage et al., 2021). Thus, similarly with most human studies conducted in the United States, where *in utero* exposures to ENDS aerosols increased the risk of infants born SGA (see section 3), suggesting IUGR, the majority of the studies conducted in rodents point to *in utero* ENDS aerosols exposures impairing fetal/neonatal growth and altering weigh gain later in life. The implications of this negative health effect, observed in both epidemiological and experimental studies, are not

trivial, as reduced fetal/neonatal physical growth is associated with increased morbidity risk, both early and later in life.

Pulmonary health effects in offspring exposed prenatally and in early life to ENDS aerosols

The sequence of lung developmental events is well-conserved across humans and mice (Chao et al., 2015). Lung development occurs through a spacio-temporal evolution of four discrete histological phases (Chao et al., 2015). The lungs start to develop during 1) the pseudoglandular stage; in humans this occurs during weeks 4 to 17 of gestation, while in mice this period is covered from embryonic days 9.5 to 16.5. During this phase, among others, branching morphogenesis is initiated (Chao et al., 2015). This is followed by 2) the canalicular stage, characterized by subdivision of the respiratory bronchioles, which occurs in humans from gestational weeks 17 to 26 and in mice from embryonic days 16.5 to 17.5 (Chao et al., 2015). Subsequently, 3) the sacular stage begins in humans from gestational weeks 26 to 36 and in mice from embryonic days 17.5 to PND5. The growth of alveolar sacs and the production of pulmonary surfactant are hallmark events of the sacular stage (Chao et al., 2015). Lastly, 4) the alveolar stage expands from the gestational to the post-natal periods in humans, from gestational week 36 to approximately 8 years of age, while this stage occurs entirely post-natally in mouse, from PND5 to PND30. The onset of alveologenesis and lung maturation processes occur during this phase (Chao et al., 2015). Overall, mouse models of lung development and organogenesis are recognized as suitable means to investigate potential fetal and neonatal human respiratory health effects.

To date, there are only six experimental studies, which were conducted in mice, that investigated the effects of *in utero* and early life exposures to ENDS aerosols on offspring

respiratory health (McGrath-Morrow et al., 2015; Chen et al., 2018a; Noël et al., 2020; Wang et al., 2020b; Cahill et al., 2021; Orzabal et al., 2022). These studies included ENDS aerosols which were either nicotine-free or containing nicotine levels ranging from 16 to 100 mg/mL, as well as with and without flavoring chemicals (tobacco and cinnamon) (**Table 2**). Thus, experimental conditions between the various studies were different and since nicotine content and flavoring chemicals could trigger unique toxicological responses based on the chemical profile of the ENDS aerosol generated, the adverse effects from the studies described below may not be generalizable to ENDS aerosols produced by ENDS devices of all four generations. Also, while the mice offspring from these studies were all exposed either *in utero* or during early life, the time of biological outcome assessment were different, ranging from birth to 13 weeks of ages, representing distinct stages of lung organogenesis, including the saccular stage (PND0 to PND5), early, medial, and late alveolar stages (PND5 to PND28), as well as adolescence (6 weeks) and adulthood (13 weeks) (Warburton et al., 2010). Hence, below, the studies are grouped by the lung development stage at which the biological endpoints were evaluated.

First, two reports included the assessment of respiratory effects associated with preconception and prenatal exposures to ENDS aerosols at birth (PND0) or PND1 in exposed mice offspring (Chen et al., 2018a; Noël et al., 2020). While both studies were conducted on the BALB/c mouse strain, Chen et al., (2018a) used a third generation ENDS device and tobacco-flavored e-liquid containing either 0 or 18 mg/mL of nicotine, whereas Noël et al., (2020) used a third generation ENDS device with a cinnamon-flavored e-liquid containing 36 mg/mL of nicotine. In Noël et al., (2020), preconception exposures to ENDS aerosols significantly increased the tissue fraction of the lungs at PND0, during the saccular stage, when compared to air-exposed controls. This was accompanied by the dysregulation of 75 genes associated with *Wnt* signaling, which is

crucial in lung organogenesis (De Langhe and Reynolds, 2008), in addition to the downregulation of several genes, including *Stat6*, *Gata3*, *Il-1b*, and *Tgfb β -1*. Thus, indicating pulmonary effects of *in utero* exposures to ENDS aerosols at the structural and molecular levels. In this same study, prenatal exposures to ENDS aerosols up-regulated the expression of 13 genes associated with *Wnt* pathway in addition to upregulating *Stat5a*, *Il-5*, *Il-13*, and *Hmox1*. In Chen et al., (2018a), preconception exposures to tobacco-flavored ENDS aerosols with and without nicotine significantly increased global DNA methylation in the lungs at PND1 when compared to sham controls. In addition, at this time point, ENDS aerosol exposure without nicotine upregulated the gene expression of *Il-5*, *Il-13* and *Tnf- α* , when compared to sham controls (Chen et al., 2018a). In addition to *Wnt* signaling, these two studies show that transcription of inflammatory markers is targeted by *in utero* ENDS exposures at birth. During lung development, inflammation markers play key roles in lung maturation processes, including alveogenesis, surfactant production, and lung function (Jobe and Ikegami, 2001; Warburton et al., 2010). Thus, these studies clearly demonstrate that *in utero* exposures to ENDS aerosols, with and without nicotine, induce molecular dysregulation of inflammation markers and affect DNA methylation patterns in the mouse developing lung at PND0 and PND1 while in the saccular stage. This is of particular importance as epigenetic changes, such as differential DNA methylation patterns, have been directly linked to adverse health outcomes involving childhood lung function, asthma, and respiratory development and morbidity (den Dekker et al., 2019).

Next, three reports assessed the effects of *in utero* and early life exposures to ENDS aerosols on the developing mouse lung while in the early and medial stages of alveogenesis (McGrath-Morrow et al., 2015; Cahill et al., 2021; Orzabal et al., 2022). In the study conducted by Cahill et al., (2021) a third generation ENDS device was used to expose BALB/c mice to

cinnamon-flavored e-liquid containing 36 mg/mL of nicotine. At PND5, the onset of alveologenesis in mice, *in utero* exposures to ENDS aerosols significantly reduced the lung fibrillar collagen content and dysregulated the expression of 121 genes associated with *Wnt* signaling or epigenetic chromatin modifications in female offspring, while 40 genes were dysregulated in male offspring. At PND11, a median time point in lung alveologenesis, increased Newtonian resistance was observed in ENDS-exposed offspring in addition to dysregulated expression of 27 genes in female offspring and 20 genes in male offspring. The formation of alveoli occurs through septation during this critical stage of lung development and alterations in elastin or collagen content as well as dysregulation of genes associated *Wnt* signaling can impair lung structure, maturation of alveolar cells, as well as lung function (Wongtrakool et al., 2012). Thus, this study provides experimental evidence that *in utero* exposures to ENDS aerosols impair lung structure and function, as well as induce sex-specific molecular changes in lungs in PND5 and PND11 mice offspring.

A recent study also showed that *in utero* exposures to nicotine containing ENDS aerosols in neonate mice led to alterations in lung structure, evidenced by development of an emphysematous phenotype, and to a decline in lung function, demonstrated by increased respiratory resistance and decreased compliance (Orzabal et al., 2022). In McGrath-Morrow et al., (2015) a second generation ENDS device was used to expose C57BL/6 mice to non-flavored e-liquid containing either 0 or 18 mg/mL of nicotine, they exposed mice to ENDS aerosols for the first 10 days of life and found that mice exposed to ENDS aerosol containing 18 mg/mL of nicotine had increased lung mean linear intercept (MLI) values, indicating alterations in lung structure, and reduced *Ki67* lung staining, a marker of cell proliferation, which is supportive of inhibition of alveolar growth. Thus, at PND10, early life exposures to ENDS aerosols containing nicotine

impaired alveolar growth. Overall, these three studies show that ENDS exposures, whether *in utero* or early life can impair and/or delay neonatal mouse lung maturation and alveologensis processes, which can translate into structural and functional adverse effects in early life.

In addition, the reports from Chen et al., (2018a) and Noël et al., (2020) described above, included the evaluation of pulmonary responses following *in utero* exposures to ENDS aerosols in later stages of alveologensis, e.g., at PND20 and PND28. In Chen et al., (2018a), preconception exposures to tobacco-flavored ENDS aerosols, both with and without 18 mg/mL of nicotine, significantly upregulated the lung gene expression of *Pdgf- α* , a marker of alveoli development, at PND20 in mice offspring. At PND28, mice offspring prenatally exposed to cinnamon-flavored ENDS aerosols contained 36 mg/mL of nicotine had increased lung MLI values, indicative of enlarged airspaces, accompanied by dysregulation of 3 *Wnt* signaling-related genes, including up-regulation of *FosII*, involved in cellular proliferation and differentiation (Noël et al., 2020). Taken together these two studies established that *in utero* exposures to ENDS aerosols in mice can cause sustained pulmonary adverse effects at the structural and molecular levels that persist until the late phases of the alveologensis period, PND20 to PND28.

Another study investigated the pulmonary effects of prenatal exposure to non-flavored ENDS aerosols containing either 0 or 16 mg/mL of nicotine in adolescent CD-1 mice (Wang et al., 2020b). They found that *prenatal* exposures dysregulated protein expression of developmental markers in the lungs of 6-week-old mice in a sex-dependent manner. For instance, in female offspring exposed to nicotine-free ENDS aerosols, lung protein expression of HDAC1, LEF-1, Fibronectin, and COL1A1 were significant increased compared to the air exposed offspring. In male offspring, PPAR- γ protein expression was increased, while Fibronectin and E-Cadherin were decreased, in the mice exposed to the ENDS aerosol containing nicotine compared to the air

exposed mice (Wang et al., 2020b). Further, the expression of proteins associated with extracellular matrix was also dysregulated by the *prenatal* exposure. Protein expression of PAI-1 was upregulated and MMP9 was downregulated in both female and male mice exposed to ENDS aerosols containing nicotine compared to air exposed mice, while p53, TIMP1 and MMP2 were elevated only in female mice exposed to ENDS aerosols with and without nicotine when compared to the air exposed mice (Wang et al., 2020b). Immunohistochemistry also revealed elevated lung protein expression of TGF- β and pSMAD2, in addition to increase Ashcroft fibrosis score, assessing lung collagen deposition, in male mice exposed to the nicotine-free ENDS aerosols (Wang et al., 2020b). Lastly, gene expression of lipogenic and myogenic markers were also altered by the *prenatal* ENDS aerosol exposures, with *Acta2* and *Fn1* being upregulated in female mice exposed to only nicotine-free ENDS aerosol, while *Adrp* was downregulated by both ENDS aerosols. In males, *Cnn1* and *Acta2* gene expression were upregulated in mice exposed to ENDS aerosols containing nicotine compared to air controls (Wang et al., 2020b). Overall, this study clearly showed that lasting, up to 6 weeks, and maybe permanent effects of *prenatal* exposures to ENDS aerosols, with or without nicotine, can occur in the lungs of exposed offspring. The patterns of expression of the various proteins analyzed were sex-specific and associated with extracellular matrix remodeling, fibrosis, and lipogenesis. These data thus suggest that *prenatal* exposures to ENDS aerosols may increase the susceptibility to development of lung diseases later in life.

Lastly, the study by Chen et al., (2018a) (described above), also investigated altered expression of inflammatory proteins in the lungs of 13-week-old mice offspring subjected to preconception exposures to ENDS aerosols. They found that the protein expression of IL-1 β was downregulated by both tobacco-flavored ENDS aerosols exposures (with and without 18 mg/mL of nicotine) when compared to the sham group. Protein levels for TNF- α , JNK, and p38 were also

elevated in the mice exposed to the nicotine containing ENDS aerosols compared to the sham group, while protein expression of ERK1/2, and p38 were up-regulated in the mice exposed to the nicotine-free ENDS aerosols when compared to the sham controls (Chen et al., 2018a). Here also, this *in utero* exposure seems to affect markers of inflammatory responses. Thus, these data suggest that the lungs of *in utero* ENDS aerosol exposed offspring may be more at risk of developing pulmonary impairment associated with dysregulation of inflammatory pathways later in life. Globally, these results indicate that *in utero* ENDS aerosol exposures, independent of nicotine content, can induce persistent molecular alterations in the lungs of exposed offspring, which can be sustained until adulthood (13 weeks of age).

In summary, these six experimental studies in mice on the pulmonary health effects of offspring exposed prenatally and in early life to ENDS aerosols clearly demonstrate that vaping during pregnancy is unsafe to the developing fetal lungs at critical stages of organogenesis, including the saccular and alveolar (early, medial, and late) stages (McGrath-Morrow et al., 2015; Chen et al., 2018a; Noël et al., 2020; Cahill et al., 2021). Further, *in utero* ENDS aerosol exposures may predispose offspring to the development of lung diseases associated with fibrosis (Wang et al., 2020b) and inflammation, e.g., asthma, in childhood or adulthood (Chen et al., 2018a). In addition, the persistent molecular lung effects, from birth to 13 weeks of age (adulthood in mice) (McGrath-Morrow et al., 2015; Chen et al., 2018a; Noël et al., 2020; Wang et al., 2020b; Cahill et al., 2021), induced by *in utero* exposures to ENDS aerosols, independent of nicotine content, may increase the risk of pulmonary adverse outcomes following exposures to a secondary environmental insult (e.g., allergen, air pollution) later in life. Thus, based on experimental evidence, the use of ENDS devices is not a ‘safe’ alternative to conventional combustion cigarettes in the context of pregnancy, as ENDS aerosols are not harmless to the developing lungs.

Given the myriad, and at times conflicting, data reported in the above sections, it is important to underscore that health outcomes associated with *in utero* exposures to ENDS aerosols may or may not be triggered depending on many factors, including but not limited to: 1) the window of *in utero* exposures, 2) the species or strain, including the genetic background, of the animals exposed, 3) the sex of the offspring, as well as 4) the ENDS aerosol exposure paradigm used (type of ENDS device, flavor and nicotine concentration in the e-liquid), vaping topography (puffs/minute, coil temperature, flow rate), and exposure duration (e.g., 1, 2 or 3 hours per day). The field of e-cigarette research is therefore inherently limited due to the significant variability in exposure parameters and a lack of a universally standardized exposure paradigm. Despite these limitations, overall, there exists significant experimental evidence indicating that *in utero* and early life exposures to ENDS aerosols can affect multi-organ systems both at birth and later in life in rodents. Indeed, *in utero* and early-life exposures to ENDS aerosol can alter the development of the central nervous system (Nguyen et al., 2018; Zelikoff et al., 2018), impair cognitive behaviors (Smith et al., 2015; Church et al., 2020), cause vascular dysfunction (Orzabal et al., 2019; Burrage et al., 2021), lead to decreased body weight (Wetendorf et al., 2019; Noël et al., 2020), and dysregulate genes critically associated with development of the lungs in offspring (Chen et al., 2018a; Wang et al., 2020b; Cahill et al., 2021). Even still, more research is needed to more precisely determine the developmental windows of sensitivity associated with increased susceptibility for post-natal health complications, including the manifestation of chronic diseases in childhood or adulthood.

Prenatal ENDS aerosol exposures and the developmental origin of health and disease (DOHaD) paradigm

The Barker hypothesis or the developmental origin of health and disease (DOHaD) stipulates that risk factors for the intrauterine milieu can affect the development of the fetus during specific windows of sensitivity, which could increase the risk of developing chronic diseases throughout the lifespan (Barker, 1990; Wadhwa et al., 2009; Harding and Maritz, 2012; Carpinello et al., 2018). Increasing epidemiological and experimental evidence have associated maternal exposures to cigarette smoke and secondhand smoke during pregnancy with the development of pulmonary diseases, including asthma and chronic pulmonary obstructive disease (COPD) (Zacharasiewicz, 2016; Eisner et al., 2010; Lovasi et al., 2010). The exact mechanisms by which *in utero* exposures to environmental insults lead to the development of chronic diseases later in life are still unclear. However, it was showed that prenatal exposures to environmental pollutants, including tobacco smoke, dysregulate the epigenome and increase the risk for childhood or adult diseases (Perera and Herbstman, 2011; Li et al., 2017; Rauschert et al., 2019). Epigenetic dysregulation through differential DNA methylation patterns caused by maternal smoking has been shown to persist well into adulthood, indicating these alterations are not transient (Richmond et al., 2018). Indeed, recent findings demonstrated that *in utero* exposures to cigarette smoke and secondhand smoke alter the methylation status of genes, effects that can last for almost two decades after the exposure, thus pointing towards the involvement of epigenetic mechanisms (Joubert et al., 2016; Christensen et al., 2017; Meyer et al., 2017). Thus, substantial evidence now indicates that *in utero* and early life exposures to toxicants can cause epigenetic changes, resulting in dysregulated gene expression, either through silencing or increased expression, which may increase the development of specific diseases across the lifespan (Lauterstein et al., 2016). These

in utero exposures may impact fetal programming and have long-term consequential effects throughout life.

To date, no epidemiological or experimental study have investigated or established any association between *in utero* ENDS aerosols exposures and exacerbated lung diseases, e.g., asthma or COPD, later in life. However, experimental studies in mice have clearly demonstrated the effects of *in utero* ENDS aerosols exposures on both the offspring's lungs and brain methylation status (Chen et al., 2018a; Nguyen and al. 2018) as well as on the dysregulation of epigenetics-related transcriptomes (Lauterstein et al., 2016; Nguyen et al., 2018; Wang et al., 2020b; Cahill et al., 2021). For the effects on the lungs, BALB/c mice exposed *in utero* to tobacco-flavored ENDS aerosols (18 mg/ml of nicotine) exhibited a hyper DNA methylation status at PND1 (Chen et al., 2018a), while Cahill et al., (2021) reported that, in BALB/c mice exposed *in utero* to cinnamon-flavored ENDS aerosols containing 36 mg/ml of nicotine at PND5, 1 and 60 epigenetics related genes were dysregulated in male and female offspring, respectively. These dysregulations were mostly resolved by PND11 where 4 and 17 genes associated with epigenetics were dysregulated in male and female offspring, respectively (Cahill et al., 2021). Although no one has yet studied the effects of *in utero* ENDS aerosols exposures on increased susceptibility to develop lung diseases following a second environmental insult, the experimental data collected thus far, showing methylation changes and epigenetic transcriptomic effects in the *in utero* exposed offspring's lungs (Chen et al., 2018a; Wang et al., 2020b; Cahill et al., 2021), suggest, based on the DOHaD paradigm and its origin in epigenetic alterations, that there is a strong possibility that *in utero* ENDS aerosols exposures, as a baseline effect, may increase the offspring vulnerability to develop detrimental pulmonary effects in the postnatal lifespan. More research is needed to either reject or confirm this hypothesis.

Regarding the effects on the brain, Lauterstein et al., (2016) showed significant dysregulation of gene expression (dysregulated genes range from 152 to 2,630) in the frontal cortex of 1-month-old C57BL/6 male and female mice exposed *in utero* plus during lactation to tobacco-flavored ENDS aerosols with or without nicotine (13-16 mg/mL). In addition, in Nguyen et al., (2018), BALB/c mice exposed *in utero* to tobacco-flavored ENDS aerosols without nicotine showed hyper DNA methylation status in brain tissue at PND1 and PND20. Further, the gene expression of epigenetic chromatin modification enzymes, including DNA methyltransferases and histone acetyltransferases, in the brain were significantly dysregulated by both ENDS aerosols (with and without 18 mg/mL of nicotine) either at PND1, PND20 or at 13 weeks of age. Thus, showing that *in utero* ENDS aerosols exposures, independent of nicotine content, can affect the brain methylome in the exposed offspring.

Furthermore, two studies to date have investigated the ability of *in utero* ENDS aerosol exposures to enhance neurobehavioral outcomes following a secondary insult, such as hypoxic-ischemic brain injury (Sifat et al., 2020; Walayat et al., 2021). In Sifat et al., (2020), CD-1 mice were exposed prenatally and during early life to ENDS aerosols containing 24 mg/mL of nicotine produced by a Blu ENDS device, and offspring susceptibility for hypoxic-ischemic brain injury was assessed. They found that following induced neonatal hypoxic-ischemic brain injury at PND8-9, mice exposed *in utero* to ENDS aerosols exhibited enhanced brain injury and increased edema after 24 hours compared to control offspring. Also, in those exposed mice, they observed decline in neurological outcomes (impaired memory, learning, and motor coordination) at PND40-45.

Using a similar model, Sprague-Dawley rats were exposed prenatally to Blu Plus ENDS aerosols containing 24 mg/mL of nicotine, and offspring were evaluated at PND9 for brain damage following induced hypoxic-ischemic brain injury (Walayat et al., 2021). Following hypoxic-

ischemic brain injury, there were significantly larger infarctions in the brains of male rats exposed to ENDS aerosol *in utero*, compared to controls; this trend was generally observed in female rats but did not reach statistical significance. In both males and female brain tissue, levels of ROS and nicotinamide adenine dinucleotide phosphate hydrogen oxidase (NOX2) were significantly increased in the *in utero* ENDS exposed group compared to controls. Moreover, *in utero* ENDS aerosols exposures resulted in hypermethylation of rat brain and downregulation of autophagy-related proteins (Walayat et al., 2021). Thus, these data suggest that a brain phenotype which is sensitive to induced hypoxic-ischemia may be caused by ENDS aerosols. This is plausibly induced through changes in fetal programming as evidenced by alterations in DNA methylation status and dysregulation of autophagy related pathways. Since the main mechanisms related to epigenetic alterations associated with the initial cause of DOHaD, include DNA methylation and histone modification; as seen in this study, DNA methylation may be an essential mechanism, and the missing link, associated with the exacerbated hypoxic-ischemic induced brain injury observed in *in utero* ENDS exposed offspring (Walayat et al., 2021). Taken together, *in utero* ENDS aerosols exposures are a prenatal insult which may have lasting effects on both cognitive and motor functions into the postnatal life.

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Table 1.1: Summary of experimental findings for neonatal outcomes (birth weight, body length, and body weight gain) in offspring exposed *in utero* to ENDS aerosols.

Publication	Species	Device Generation	PG/VG	Nicotine	Flavoring	Gestational Exposure	Exposure duration: Hours/day and days/week	Aerosol Concentration/ TPM	Serum Nicotine or Cotinine	Study findings
McGrath-Morrow et al., 2015	C57BL/6J mice	2nd	Not reported	0 or 18 mg/mL (0% or 1.8%)	No	PND1-10	Intermittent - 2 times per day. Total of 0.66 hours/day. 7 days/week.	Not reported	Plasma cotinine (pups PND10): 62.3 +/- 3.3 ng/mL; Urine cotinine (pups PND10): 892.5 +/- 234 ng/mL	Compared to controls, <i>in utero</i> ENDS aerosol exposures with AND without nicotine resulted in: - Pup weight significantly decreased at PND10.
Smith et al., 2015	C57BL/6J mice	2nd	100:0	0 or 24 mg/mL (0% or 2.4%)	No	GD15-19 and PND 2-16	20 mins/ day. 7 days/week.	Not reported	Plasma cotinine (pups PND16): 23.7 +/- 4.2 ng/mL	Compared to controls, <i>in utero</i> ENDS aerosol exposures with AND without nicotine resulted in: - Pup weight significantly decreased PND2-16 (no differences in weight at 14 weeks of age).
Lauterstein et al., 2016	C57BL/6J mice	1st	Not reported	0 or 13-16 mg/mL (0% or 1.3-1.6%)	Tobacco	GD0-21 and PND4~ PND21 (weaning)	3 hrs/day. 5 days/week.	Nicotine chamber: 25.6 mg/m ³ Nicotine free chamber: 30.7 mg/m ³	Urine cotinine (dams 2-3 hours post exposure on GD16-19): 664 - 1,972 ng/mL	Compared to controls, <i>in utero</i> ENDS aerosol exposures with AND without nicotine resulted in: - No significant differences in pup weight from PND1-25.
Chen et al., 2018a	BALB/c mice	3rd	50:50	0 or 18 mg/mL (0% or 1.8%)	Tobacco	Dams only: 6 weeks prior to mating, GD0-21, and PND1-20	Intermittent - 2 times per day. Total of 0.5 hours/day. Days/week: not reported.	Not reported	Plasma cotinine (pups PND20): 9.12 +/- 1.17 ng/mL	Compared to controls, <i>in utero</i> ENDS aerosol exposure with nicotine resulted in: - Pup weight significantly decreased PND20 (but not at PND1 or 13 week of age); - Offspring liver weight significantly increased at PND20 (but not at PND1 or 13 weeks of age); - Offspring retroperitoneal fat mass significantly increased at PND20 and 13 weeks of age. Compared to controls, <i>in utero</i> ENDS exposure without nicotine resulted in: - Pup weight significantly increased at PND20 (but not at PND1 or 13 weeks of age); - Offspring liver weight was significantly decreased at 13 weeks of age (but not at PND1 or PND20); - Offspring retroperitoneal fat mass significantly increased at PND20 and 13 weeks of age; - Offspring epididymal fat mass significantly increased at PND20 (but not at 13 weeks of age).

Publication	Species	Device Generation	PG/VG	Nicotine	Flavoring	Gestational Exposure	Exposure duration: Hours/day and days/week	Aerosol Concentration/ TPM	Serum Nicotine or Cotinine	Study findings
Li et al., 2019	BALB/c mice	3rd	50:50	0 or 18 mg/mL (0% or 1.8%)	Tobacco	Dams only: 6 weeks prior to mating, GD0-21, and PND1-20	Intermittent - 2 times per day. Total of 0.5 hours/day. Days/week: not reported.	Not reported	Not reported	<p>Compared to controls, <i>in utero</i> ENDS aerosol exposure with nicotine resulted in:</p> <ul style="list-style-type: none"> -Pup weight significantly decreased PND20 (but not at PND1 or 13 week of age); - No changes in offspring kidney weight at PND1, PND20 or at 13 weeks of age. <p>Compared to controls, <i>in utero</i> ENDS exposure without nicotine resulted in:</p> <ul style="list-style-type: none"> - Pup weight significantly increased at PND20 (but not at PND1 or 13 weeks of age); - No changes in offspring kidney weight at PND1, PND20 or at 13 weeks of age.
Orzabal et al., 2019	Sprague-Dawley rats	3rd	80:20	GD5-8: 50 mg/mL (5%) GD11-21: 100 mg/mL (10%)	No	For 'maternal vaping': GD5-19 For 'maternal and pup vaping': GD5-21 and PND 4-10	For 'maternal vaping': 3 hrs/day For 'maternal and pup vaping': 2 hrs/day. 5 days/week	Not reported	Serum nicotine (dams GD11): ranged from 7.30-27.69 ng/mL (peak at 6 hours after starting exposure)	<p>Compared to controls, <i>in utero</i> ENDS aerosol exposures with nicotine resulted in:</p> <ul style="list-style-type: none"> - Fetal weight significantly decreased at GD20; - Crown to rump length significantly decreased at GD20 and PND10; - Significantly decreased maternal uterine and fetal umbilical blood flow.
Wetendorf et al., 2019	C57BL/6J mice	3rd	55:45	24 mg/mL (2.4%)	No	Fertility trial: 4 months beginning on the first day of mating. Implantation Study: 4 weeks prior to mating and GD0-21	3 hrs/day. 5 days/week.	Not reported	Not reported	<p>Compared to controls, <i>in utero</i> ENDS aerosol exposures with nicotine resulted in:</p> <ul style="list-style-type: none"> - Female offspring weight significantly decreased at 8 months of age; - No changes in male offspring weight at 8 months of age.
Church et al., 2020	CD-1 mice	1st	50:50	0 or 16 mg/mL (0% or 1.6%)	N/A	GD0.5-17.5	3 hrs/day. 7 days/week.	Nicotine chamber: 130.25 +/- 8.59 mg/m ³ Nicotine free chamber: 131.85 +/- 7.93 mg/m ³	Urine cotinine (dams averaged values of GD5.5, GD10.5 and GD15.5): 0.026 +/- 0.00726 ng/mL	<p>Compared to controls, <i>in utero</i> ENDS aerosol exposures with AND without nicotine resulted in:</p> <ul style="list-style-type: none"> - No significant differences in pup weight from PND21 or 12 weeks of age (when combining male and female offspring); - Of note, nicotine exposed female offspring had significantly increased body weight at PND21 but not at 12 weeks of age.
Noel et al., 2020	BALB/c mice	3rd	50:50	36 mg/mL (3.6%)	Cinnamon fireball	For 'preconception group': M&F 12 days prior to mating and F GD1-19 For 'prenatal group': GD6-19	2 hrs/day. 7 days/week.	0.23 mg/puff +/- 0.05 SEM	Serum cotinine (male breeder mice): 150.4 +/- 22 ng/mL	<p>Compared to controls, <i>in utero</i> ENDS aerosol exposures with nicotine resulted in:</p> <ul style="list-style-type: none"> - Birth weight and length significantly decreased (prenatally exposed mice had maintained decreased body weight until PND28); - Maternal serum PIGF and 117-b-estradiol significantly decreased.

Publication	Species	Device Generation	PG/VG	Nicotine	Flavoring	Gestational Exposure	Exposure duration: Hours/day and days/week	Aerosol Concentration/ TPM	Serum Nicotine or Cotinine	Study findings
Sifat et al., 2020	CD-1 mice	1st	Not reported	24 mg/mL (2.4%)	No	Dams only: GD5-21 and PND 1-7	Intermittent - 6 times per day. Total of 1.6 hrs/day. 7 days/week.	Not reported	Plasma and brain nicotine and cotinine taken at GD18 from dams and fetuses	Compared to controls, <i>in utero</i> ENDS aerosol exposures with nicotine resulted in: - Offspring weight significantly decreased at PND8 (no differences in weight at PND45).
Burrage et al., 2021	Sprague-Dawley rats	3rd	75:25	0 or 18 mg/mL (0% or 1.8%)	French Vanilla	Dams only: GD2-21 and PND1-21	1 hr/day. 7 days/week.	Nicotine chamber: 134 +/- 51 mg/m ³ Nicotine free chamber: 117 +/- 49 mg/m ³ (+/- =SD)	Not reported	Compared to controls, <i>in utero</i> ENDS aerosol exposures with AND without nicotine resulted in: - No significant differences in offspring weight at 1, 3 or 7 months of age.
Cahill et al., 2021	BALB/c mice	3rd	50:50	36 mg/mL (3.6%)	Cinnamon fireball	GD1-21	2 hrs/day. 7 days/week.	0.165 mg/puff +/- 0.11 SD	Not reported	Compared to controls, <i>in utero</i> ENDS aerosol exposures with nicotine resulted in: - Birth weight and weight until PND5, significantly decreased; - No changes in birth crown to rump length or average litter sizes.
Walayat et al., 2021	Sprague-Dawley rats	1st	Not reported	24 mg/mL (2.4%)	No	GD4-20	Intermittent - 12 times a day. Total 0.3 hours/day. 7 days/week.	Not reported	Not reported	Compared to controls, <i>in utero</i> ENDS aerosol exposures with nicotine resulted in: - Offspring weight and brain weight significantly decreased at PND9; - Offspring brain-to-body weight ratio significantly increased.

Table 1.2: Summary of experimental findings for pulmonary health effects in offspring exposed *in utero* to ENDS aerosols.

Publication	Species	Device Generation	PG/VG	Nicotine	Flavoring	Gestational Exposure	Exposure duration: Hours/day and days/week	Aerosol Concentration/TPM	Serum Nicotine or Cotinine	Study findings
McGrath-Morrow et al., 2015	C57BL/6J mice	2nd	Not reported	0 or 18 mg/mL (0% or 1.8%)	No	PND1-10	PND1-2: 20 mins/day PND3-9 Intermittent 2 times per day. Total 0.66 hours/day. 7 days/week.	Not reported	Plasma cotinine (pups PND10): 62.3 +/- 3.3 ng/mL Urine cotinine (pups PND10): 892.5 +/- 234 ng/mL	Compared to controls, <i>in utero</i> ENDS aerosol exposures with nicotine resulted in: - Significantly higher mean linear intercept values in offspring lung tissue at PND10; - Significantly decreased Ki67 expression in offspring lung tissue at PND10, indicating possible cell proliferation impairment.
Chen et al., 2018a	BALB/c mice	3rd	50:50	0 or 18 mg/mL (0% or 1.8%)	Tobacco	Dams only: 6 weeks prior to mating, GD0-21, and PND1-20	Intermittent - 2 times per day. Total 0.5 hours/day. Days/week: not reported.	Not reported	Plasma cotinine (pups PND20): 9.12 +/- 1.17 ng/mL	Compared to controls, <i>in utero</i> ENDS aerosol exposure with nicotine resulted in: - Significantly increased TNF- α protein expression in offspring lungs at 13 weeks of age. Compared to controls, <i>in utero</i> ENDS exposure without nicotine resulted in: - Significantly increased protein expression of IL-5, IL-13, and TNF- α in offspring lungs at PND1. Both resulted in: - Significantly upregulated alveoli developmental marker PDGFR in offspring lung at PND20 (but not PND1); - Significantly decreased IL-1b protein expression in offspring lungs at 13 weeks of age; - Significantly increased p38 protein expression in offspring lungs at 13 weeks of age; - Significantly increased global cytosine methylation in offspring's lungs at PND1.
Noel et al., 2020	BALB/c mice	3rd	50:50	36 mg/mL (3.6%)	Cinnamon fireball	For 'preconception group': males and females 12 days prior to mating and females GD1-19 For 'prenatal group': GD6-19	2 hrs/day. 7 days/week.	0.23 mg/puff +/- 0.05 SEM	Serum cotinine (male breeder mice): 150.4 +/- 22 ng/mL	Compared to controls, preconception ENDS aerosol exposures with nicotine resulted in: - Significantly decreased fetal lung gene expression of Tgf β 1, IL-5, IL-13, IL-1 β , Stat6, Gata3, Stat5 α and Notch2 at PND0; - Significantly increased fetal lung tissue fraction compared with airspace at PND0 - Increased club cell percentage in fetal lungs at PND0; - Significant downregulation of 75 lung genes involved in <i>Wnt</i> signaling, essential to lung organogenesis at PND0 (in particular, genes associated with decreased growth and proliferation of lung cells). Compared to controls, prenatal ENDS aerosol exposures with nicotine resulted in: - Significantly decreased fetal lung gene expression of IL-1b, IL-6, and Foxp3; - Significantly increased fetal lung gene expression of IL-5, IL13, Stat5 α and Hmox1; - Significantly higher mean linear intercept values in fetal lung tissue at PND28, indicating lung structural damage; - Significant upregulation of <i>Wnt</i> signaling genes such as Mmp7, Wnt10 α , and Frzb at PND0 which return to baseline at PND28;

Publication	Species	Device Generation	PG/VG	Nicotine	Flavoring	Gestational Exposure	Exposure duration: Hours/day and days/week	Aerosol Concentration/TPM	Serum Nicotine or Cotinine	Study findings
Wang et al., 2020b	CD-1 mice	Nor reported	50:50	0 or 16 mg/mL (0% or 1.6%)	No	GD0.5-21	3 hrs/day. 5 days/week.	Not reported	Urine cotinine (dams GD5.5, GD10.5 and GD15.5): data not reported	<p>Compared to controls, <i>in utero</i> ENDS aerosol exposure with nicotine resulted in:</p> <ul style="list-style-type: none"> - Significant upregulation of protein expression of PPAR-γ in male offspring lungs at 6 weeks of age; - Significant upregulation of protein expression of PAI-1 in male and female offspring lungs at 6 weeks of age; - Significant downregulation of protein expression of extracellular matrix protein markers (fibronectin and E-Cadherin) in male offspring lungs at 6 weeks of age; <p>- Significant upregulation of Phospho-p53, Timp1 and MMP-2 in female offspring lungs at 6 weeks of age;</p> <p>- Significant downregulation of MMP9 in male and female offspring lungs at 6 weeks of age.</p> <p>Compared to controls, <i>in utero</i> ENDS aerosol exposure without nicotine resulted in:</p> <ul style="list-style-type: none"> - Significant upregulation of protein expression of lipogenic and myogenic markers (LEF-1 and HDAC-1) and extracellular matrix protein markers (fibronectin, COL1A1, MMP-2 and Timp1) in female offspring lungs at 6 weeks of age; - Significant upregulation of protein expression of E-Cadherin, PAI-1 and pSmad2 (in alveolar area) in male offspring lungs at 6 weeks of age.
Cahill et al., 2021	BALB/c mice	3rd	50:50	36 mg/mL (3.6%)	Cinnamon fireball	GD1-21	2 hrs/day. 7 days/week.	0.165 mg/puff +/- 0.11 SD	Not reported	<p>Compared to controls, <i>in utero</i> ENDS aerosol exposure with nicotine resulted in:</p> <ul style="list-style-type: none"> - Significantly decreased total lung fibrillar collagen content at PND5; - Significantly downregulated gene expression of IL-4, IL-10, IL-13 and Hmox1 in male offspring lungs at PND5; - Significantly downregulated gene expression of IL-4 and IL-6 and significantly upregulated gene expression of Gata3 in female offspring lungs at PND5; - Significantly increased Newtonian resistance of offspring lungs at PND11 indicating narrowing of the conducting airways; - Dysregulation of epigenetic and <i>Wnt</i> signaling associated genes at PND5 and PND11; significant sex differences were present (males = 40 and 20 respectively, females = 121 and 27 respectively).

Publication	Species	Device Generation	PG/VG	Nicotine	Flavoring	Gestational Exposure	Exposure duration: Hours/day and days/week	Aerosol Concentration/TPM	Serum Nicotine or Cotinine	Study findings
Orzabal et al., 2022	Sprague-Dawley rats	3rd	80:20	GD5-8: 50 mg/mL (5%) GD11-21: 100 mg/mL (10%)	No	GD5-20 excluding GDs 9,10,16 and 17	3 hrs/day. 5 days/week.	Not reported	Serum nicotine previously reported to be 27.7 ng/mL using this vaping system	<p>Compared to controls, <i>in utero</i> ENDS aerosol exposure with nicotine resulted in:</p> <ul style="list-style-type: none"> - Significant downregulation of 984 genes in fetal lungs at GD21 and significant up-regulation of 2,322 genes in fetal lungs at GD21 representing 159 disrupted cellular pathways; - Significantly decreased offspring fixed lung weight (but not lung weight to body weight ratio) at PND4; - Significantly higher mean linear intercept values and significantly decreased radial alveolar counts in fetal lung tissue at PND4 indicating fewer and larger distal air spaces; - Though not statistically significant ($0.1 < p > 0.05$), trends exist showing decreased static lung compliance (Cst) and increased respiratory system resistance (Rrs). <p>Compared to controls, <i>in utero</i> ENDS aerosol exposure without nicotine resulted in:</p> <ul style="list-style-type: none"> - Significant downregulation of 7 genes in fetal lungs at GD21 and significant up-regulation of 41 genes in fetal lungs at GD21 representing 207 disrupted cellular pathways.

ENDS use during pregnancy

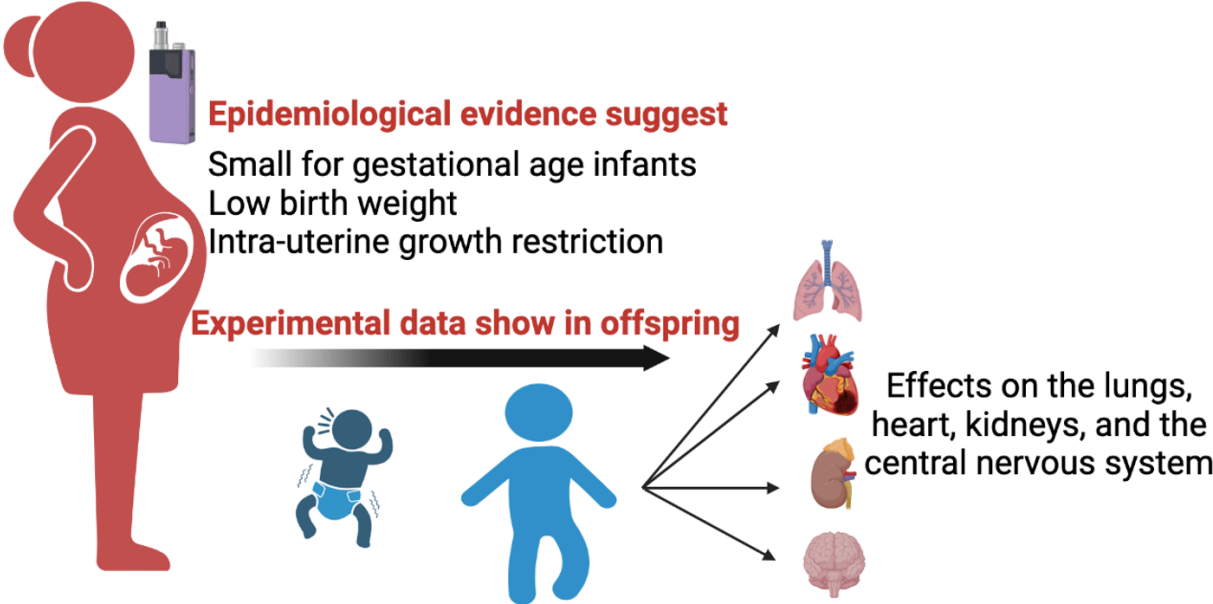


Figure 1.1: Summary of the health effects associated with ENDS use during pregnancy based on current epidemiological and experimental evidence.

CHAPTER 2:

Increasing Coil Temperature of a Third-Generation E-Cigarette Device Alters
Aerosol Chemical Composition Which Modulates Murine Lung Immune Cell
Composition and Cytokine Milieu

Abstract:

In recent years there has been a rise in popularity and availability of third-generation e-cigarettes that allow users to precisely control both power and temperature settings during vaping. The health implications of vaping at higher temperatures are largely unknown, though chemical compositions studies suggest that vaping at higher temperatures induces higher aerosol generation, thus increasing concentrations of potentially harmful carbonyl compounds in the aerosol. Direct sampling from the third-generation e-cigarette device used in the present study confirmed those findings. To isolate pulmonary effects of coil temperature increases alone during vaping, we exposed mice to e-cigarette aerosols generated at either low (375°F) or high temperature (475°F) while maintaining a constant aerosol concentration. C57BL/6 mice were placed in whole body e-cigarette exposure chambers for 3 hours per day for 3 days. Serum, bronchoalveolar lavage fluid (BALF), and whole lung tissue were collected immediately following the last exposure. Under both low and high temperature conditions, respectively, chamber aerosol concentrations and aerosolized nicotine concentrations were consistent, resulting in equally consistent nicotine and serum cotinine concentrations in the serum. Despite equivalent aerosol concentrations in whole-body exposure chambers, formaldehyde and acetaldehyde output was significantly lower from high compared to low coil temperature exposures. Total bronchoalveolar cells were significantly decreased in mice exposed to high coil temperature aerosols – primarily driven by reduced macrophage influx. Compared to controls, gene expression of IFN β , IL-1 β , TNF α , and IL-10 in mouse lung tissue samples were also significantly decreased following e-cigarette exposures at both conditions, compared to filtered air exposure, with higher temperature exposure exacerbating downregulation of IFN β and IL-1 β . Collectively, these data suggest that higher temperature vaping can have stronger adverse effects

on the pulmonary immune system. Understanding the implications of high temperature vaping could be critical for e-cigarette users, who may unknowingly increase their exposure risks by using their devices at high heat settings.

Introduction:

Electronic cigarettes (e-cigarettes) continue to be popular electronic nicotine delivery systems (ENDS) used by both adult and youth populations. According to the latest statistics from the Center for Disease Control, in the United States in 2023, 4.8 million middle and high school students (1 in 6) have tried e-cigarettes, while 2.13 million (1 in 13) are current e-cigarette users (Birdsey et al., 2023). For comparison, 430,000 (1 in 60) students currently smoke traditional cigarettes. Therefore, e-cigarettes are approximately 5x more likely than cigarettes to be the tobacco product of choice for youths. Additionally, in the US in 2021, 11.7 million adults (1 in 22) were current e-cigarette users, with younger adults (age 18-24) being more likely than older adults (age 45+) to use e-cigarettes (1 in 9 vs 1 in 50) (E. A. Kramarow & Elgaddal, 2021). Despite e-cigarettes being marketed as smoking cessation tools, public health experts have voiced concerns that growing e-cigarette use is contributing to rising nicotine addictions/dependencies (Boakye et al., 2022; Obisesan et al., 2020). A 2021 study found that of all adults currently using e-cigarettes, 20% had never smoked cigarettes (Erhabor et al., 2023). Among younger respondents aged 18-24, that number grew to a staggering 60.5%, underscoring the role of e-cigarettes in promoting nicotine use in otherwise tobacco-free adults.

E-cigarettes, which share three common components: a power source (battery), heating element (coil or atomizer) and a reservoir containing e-liquid (composed of the humectants propylene glycol (PG) and vegetable glycerin (VG), nicotine and flavoring chemicals), have undergone many design evolutions, which can broadly be delineated into so-called “generations”. First-generation “cig-alike” devices and second generation “vape pen” devices were popular from the market’s inception in 2007 until around 2009-2011. These generations, which were succeeded by third-generation “mod” or “tank” devices and fourth-generation “pod”

and “disposable pod” devices, have been more thoroughly reviewed elsewhere (Williams & Talbot, 2019). Third-generation e-cigarettes are defined by their large re-fillable e-liquid reservoirs and adjustable device settings, which allow users to modify the power, voltage, and temperature the device operates at. These mods are compatible with numerous coil configurations (single, double, mesh, etc.), coil metals (kanthal, Nichrome, stainless steel etc.) and e-liquid formulations (PG/VG ratios, nicotine concentrations, etc.), making them the most customizable category of devices. Third-generation devices were the first to utilize “sub-ohm” vaping, characterized by coils that have low resistance (less than 1Ω) and subsequently require high power (20-200W) to operate, resulting in relatively high coil temperatures (300-500°F or 150-250°C). These mods are typically used by experienced e-cigarette users to achieve specific sensory vaping experiences, such as increased clouds upon exhalation. Fourth generation “pod” devices are defined by their use of highly bioactive nicotine salts and convenient designs, featuring fully disposable devices or disposable pods with rechargeable batteries. Popular fourth-generation devices include Elf Bars (EBCREATE), Esco Bars (Pastel Cartel), and JUULpods (JUUL). Together, third- and fourth-generation e-cigarette devices represent most of the current e-cigarette market. Pod devices are more popular with younger demographics, while mod devices are used more frequently by adult e-cigarette users. Among US middle and high school student current e-cigarette users who identified their e-cigarette by device type, 6.4% used third-generation mods while 93.7% used fourth-generation pods (Birdsey et al., 2023). In a 2020 survey of US adults, 30% of e-cigarette users exclusively used mods, 43% exclusively used pods and 27% were dual users (Tillery et al., 2023) indicating third-generation devices have maintained a large following, despite the rise in fourth-generation brand popularity.

Traditional cigarettes combust tobacco and myriad chemical additives to aerosolize nicotine, in the process creating thousands of toxic chemical compounds in the resultant smoke (Rodgman & Perfetti, 2012). E-cigarettes, on the other hand, deliver electrical energy to a heating coil in contact with nicotine containing e-liquid to aerosolize the e-liquid, which is inhaled by the user. E-liquid is known to have a simpler chemical composition than traditional cigarette contents, and the thermal degradation process undertaken during vaping is known to create fewer chemical byproducts than combustion does. Yet, cigarette smoke and e-cigarette aerosols contain some of the same harmful or potentially harmful constituents (HPHCs) detrimental to human health (Cunningham et al., 2020; Margham et al., 2021; Wagner et al., 2018). Indeed, *in vitro* and *in vivo* animal studies consistently report inflammation and/or immune dysregulation as a result of e-cigarette exposure (Ganapathy et al., 2017; Higham et al., 2018; Husari, Shihadeh, Talih, Hashem, el Sabban, et al., 2016; Scott et al., 2018; Vasanthi Bathrinarayanan et al., 2018; Ween et al., 2017a). The contribution of specific chemicals to these effects is not clearly understood. Moreover, how immune homeostasis is impacted by changes in e-cigarette device settings such as power or coil temperature is largely unknown at present.

HPHCs include broad categories such as carbonyls (Farsalinos & Gillman, 2018; Flora et al., 2017; Lee et al., 2017; Lorkiewicz et al., 2022; Ogunwale et al., 2017; Samburova et al., 2018) and free radicals (Goel et al., 2015) generated through thermal degradation and oxidation reactions (Jensen et al., 2017), metals likely leached into aerosol from the coil and e-cigarette housing (Arnold, 2018; Ko & Kim, 2022; Olmedo et al., 2018), and tobacco-specific nitrosamines generated from nicotine or tobacco alkaloids (Farsalinos et al., 2015; Jin et al., 2021). Carbonyls, such as formaldehyde, acrolein, and acetaldehyde, are consistently found in e-cigarette aerosols from many different types of e-cigarette devices, including third-generation

devices. They are of particular concern due to their known carcinogenic or toxic effects on humans (Bein & Leikauf, 2011; Conklin, 2016; IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, 2006). Aerosolized carbonyl outputs are known to vary significantly due to variations in many aspects of e-cigarette use, such as device settings (coil metal material or resistance, coil configuration, power, temperature etc.), puffing profile (puff length, flow rate, etc.) or e-liquid formulation (nicotine concentration, PG/VG ratio, flavoring chemicals, etc.). Studies evaluating carbonyl output exhibit considerable experimental variation making comparisons difficult and determinations of exact causality for specific variables challenging, if not impossible (Soulet & Sussman, 2022). Further complicating the process is the inherently linked nature between device settings and coil properties through Ohm's law and other thermodynamic principles which relate voltage, resistance, power, energy, and temperature (Saleh et al., 2020).

Even still, aerosolized carbonyl outputs from third-generation devices are higher than those from lower powered, higher resistance e-cigarette devices that operate at lower coil temperatures (Cirillo, Urena, et al., 2019; Farsalinos & Gillman, 2018; Geiss et al., 2016; Li et al., 2021; Noël et al., 2020; Sleiman et al., 2016; Son et al., 2020; Talih et al., 2023; Zelinkova & Wenzl, 2020). However, these carbonyl data are primarily reported on a per puff basis. Because low resistance coils produce more total aerosol mass per puff than higher resistance coils (Ko & Kim, 2022), and because these carbonyl output data are not normalized to aerosol mass, it is challenging to determine if carbonyl output increases proportionally as power and temperature increase.

At present, no federal regulations exist in the United States establishing a maximum allowable power or temperature setting for e-cigarette devices. Therefore, high power and/or

high coil temperature could expose e-cigarette users to increased doses of carbonyls. Isolating the effect of increasing temperature on aerosol and carbonyl output in third-generation devices is therefore of importance. In this study, we investigated the effect of increased coil temperature in a third-generation e-cigarette device on aerosol and carbonyl outputs and on pulmonary immune responses in mice.

Materials and Methods:

Exposure Conditions. E-cigarette aerosols were generated and introduced into a whole-body exposure chamber (TE-2epv E-Cig Machine by Teague Enterprises Inc., Woodland, CA) using a third-generation modular vaping device with an Evolv DNA 75 Color computer chip (Evolv LLC., Hudson, Ohio) and a rechargeable battery with a variable output voltage (0.2–9 V) and power (0–75 W) (**Supp. Fig. 1**). The device was equipped with a commercially available FreeMax Mesh Pro Sub-Ohm Tank and M Pro stainless steel (SS316L) single mesh 0.12-ohm coils (FreeMax Technology Inc., Shenzhen, China). Pure propylene glycol, vegetable glycerin and nicotine (>99% purity, Sigma-Aldrich Inc., St. Louis, MO) were combined to make a 50/50 PG/VG (% w/v) e-liquid containing 6 mg/mL nicotine. Coils were inspected prior to use in the study to verify their integrity (resting resistance of between 0.118 and 0.122 ohms). During exposure, coils were inspected daily for signs of burning from overheating and were replaced every 5 days (following approximately 1,800 puffs). The e-cigarette device was robotically operated by a custom linear actuator (TE-2e, Teague Enterprises Inc., Woodland, CA). Puff rate was adjusted throughout each exposure to maintain a chamber aerosol concentration of approximately 1,800 mg/m³. Rates varied from 0.5 to 2 puffs/min. A 3 second puff duration was used during all exposures. Based on the manufacturer-suggested operating voltage of the 0.12-ohm coil and on common self-reported operating temperatures by users, 375°F and 475°F were chosen to represent the low and high coil temperature experimental conditions, respectively. Temperature was calculated indirectly by the Escribe software and e-cigarette device with material-specific temperature coefficient of resistance (TCR) equations using direct real-time coil resistance measurements. The device was connected to a laptop and Evolv Escribe software (Evolv LLC., Hudson, Ohio) was used to set the power (60-75W) to achieve the desired coil

temperature as measured by a flexible Kapton-insulated K type thermocouple (Oakton instrument Inc., Vernon Hills, IL) in contact with the center of the coil surface. Coil temperature from both Escribe software and the thermocouple were recorded for, a minimum of 30 consecutive puffs during each exposure for validation of Escribe software temperature accuracy. During the period of exposure, as puffs were generated, the flow rate through the chamber and through the device was 5 L/min resulting in an air exchange rate of 6.8 exchanges/hour. Mice (male and female C57BL/6; 6-8 weeks old; n=12 per group) were placed in polycarbonate cages with wire lids in the exposure chamber for 3 hours/day, for 3 consecutive days.

Exposure Characterization. Total particulate matter (TPM) concentrations (mg/m^3) were measured every hour during exposure using a DryTest Meter to measure precise air volumes. Filters were used to collect chamber air samples to gravimetrically determine particulate mass concentrations during sample collection. Nicotine samples were collected every hour during exposure on XAD-4 sorbent tubes (226-30-11-04-GWS, SKC West, Fullerton, CA) then extracted and analyzed via gas chromatography (Varian 3740). Nicotine concentrations (mg/m^3) were calculated via area ratio of nicotine/quinoline in each sample.

Carbonyl Characterization. Carbonyls are derivatized in situ into hydrazones with 2,4-dinitrophenylhydrazine (2,4-DNPH) cartridges (350 mg DNPH, Supelco Inc., Bellefonte, PA) and extracted with 2 mL of acetonitrile (LC-MS grade, Fisher Scientific Inc., Hampton, NH) prior to analysis. Carbonyl-DNPH extracts were analyzed for molecular composition using an Agilent 1100 HPLC with an Agilent Poroshell EC-C18 column ($2.1 \times 100 \text{ mm}$, $2.7 \mu\text{m}$, 120 \AA) coupled to a linear-trap-quadrupole Orbitrap (LTQ-Orbitrap) mass spectrometer (Thermo Corp.,

Waltham, MA) at a mass resolving power of $\sim 60\,000$ $m/\Delta m$ at m/z 400. All analyses were performed in triplicate. Concentrations of formaldehyde, acetaldehyde, acetone, acrolein, and propionaldehyde in e-cigarette aerosols were quantified by analytical standards (**Supp. Fig. 2**), and those of other carbonyls were quantified using theoretical calculations of relative sensitivity in the ESI negative mode ionization (**Supp. Fig. 3**). The $\pm 1\sigma$ uncertainty of the analysis is 10–20% when using analytical standards and 30–50% when using the theoretical model. The HPLC-HRMS data for carbonyls derivatized as hydrazones were corrected to remove the mass contribution of DNPH.

Animal Protocol. This study was conducted in compliance with regulations set by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC) under NIH guidelines. C57BL/6 mice (6-8 weeks old) were purchased from Envigo. Animals were housed 3 per cage in a 12-h light/ 12-h dark cycle with Purina 5001 regular laboratory rodent diet (Newco Distributors, Rancho Cucamonga, California) and water provided ad libitum. Immediately following exposure on day 3, mice were euthanized via 0.2 mL intraperitoneal injection of Beuthanasia-D pentobarbital solution (65 mg/kg body weight; Nembutal Cardinal Health, Sacramento, CA). Cardiac puncture was performed, and blood was collected in a 1.5 mL microcentrifuge tube, maintained for 20 minutes at room temperature to allow for coagulation, and then centrifuged for 15 minutes at 3,000 rpm. Serum was collected (100uL aliquots) and stored at $-80\text{ }^{\circ}\text{C}$. Serum samples were sent to University of California San Francisco (San Francisco, CA) for GC-MS analysis of nicotine and cotinine concentrations in serum (ng/mL).

Bronchoalveolar Lavage. Following cardiac puncture, the trachea was cannulated with a 22-gauge blunt end needle sutured in place. Whole lungs were lavaged twice with 0.8 mL of sterile phosphate-buffered saline (PBS, Sigma Aldrich, St.Louis, MO). Each aliquot was instilled and recovered 3 times before collecting the bronchoalveolar lavage fluid (BALF). The two BALF samples were combined and centrifuged for 15 minutes at 2,000 rpm at 4°C. BALF supernatant was collected and stored at -80 °C for total protein determination by Lowry assay, while the BAL cell pellet was resuspended in 1.5 mL of PBS. Cell counts and cell viability were determined via Trypan Blue using a hemocytometer. Cytospin slides were prepared using 100uL of BAL cell suspension stained with Diff-Quik for cell differentials. Following collection of BALF, the right main stem bronchus was sutured closed and right lung lobes (cranial, middle, caudal and accessory) were collected and flash frozen in liquid nitrogen and stored at -80 °C.

qRT-PCR. Caudal lobes were homogenized, and RNA was isolated using Quick-RNA Miniprep RNA (Zymo Research, Irvine, CA) according to manufacturer's protocol. RNA was converted to complementary deoxyribonucleic acid (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. Gene-specific forward and reverse primer (0.2 μm), cDNA (2 μl/reaction), and SYBR Green nucleic acid stain (10 μl/reaction; Applied Biosystems) were used for quantitative polymerase chain reaction (qPCR) (*Table 2.1*). Using the $\Delta\Delta$ -Ct method normalized to β -actin, gene expression of inflammatory markers: interleukin-1 β (IL-1 β), interleukin-10 (IL-10), interleukin-6 (IL-6), interferon alpha (IFN α), interferon beta (IFN β), interferon gamma (IFN γ), tumor necrosis factor- α (TNF- α), and oxidative stress marker hemeoxygenase-1 (HO-1) were analyzed and standardized to the expression of the housekeeping gene, β -actin.

Statistical Analysis. Statistical analysis was performed using Graph Pad Prism 9. ROUT(Q=1%) was used to remove outliers. Analysis between two groups was done using two-tailed Student's t-test with Welch's correction. Analysis between three or more groups was done using one-way ANOVA with multiple comparisons. Unless otherwise specified, data is presented as the mean +/- SEM.

Results:

Verification of temperature control accuracy

Since accurate temperature measurement was critical for our investigation, a thermocouple was placed in contact with the metal coil surface centrally inside the e-cigarette device. Temperature data from the thermocouple and indirect temperature calculations from the e-cigarette device software were compared. During operation of e-cigarette devices at both 375°F and 475°F coil temperatures, the per puff recorded Escribe software and thermocouple temperatures were stable and not significantly different from each other (**Fig. 1a**). This resulted in similar average software and thermocouple temperatures for both experimental conditions over the entire exposure ($377.1 \pm 1.2^\circ\text{F}$ vs $378.8 \pm 1.6^\circ\text{F}$; $484.8 \pm 0.6^\circ\text{F}$ vs $492.6 \pm 0.4^\circ\text{F}$), which confirms that the Escribe software accurately predicts coil temperatures (**Fig. 1b**). Dibaji et al. reported similar Escribe accuracy when using 0.15 ohm nickel coils (Dibaji et al., 2018).

Validation of consistent aerosol and nicotine delivery to mice

As increasing coil operation temperatures are known to increase aerosol concentrations, e-cigarette puff rate (puffs/minute) was adjusted in the chamber to ensure both low and high temperature exposure conditions were maintained at similar aerosol concentrations. This was done to ensure that potential chemical composition changes in the aerosol could be attributed directly to the increasing coil temperatures, not simply from an increase in total aerosol concentration. Mice were placed in whole-body exposure chambers for 3 hours per day for 3 days and exposed to e-cigarette aerosol generated from coils operated at either low or high temperatures. Daily total particulate matter (TPM) of both low and high temperature exposure chambers ranged from 1,700 to 2,000 mg/m³ resulting in an average TPM of 1,800 mg/m³ in

both chambers (**Fig. 2a**). Similarly, daily aerosolized nicotine ranged from 5.1 to 7.1 mg/m³ resulting in an average of 6.0 mg/m³ in both exposure chambers (**Fig. 2b**). Likewise, murine nicotine metabolism, as evidenced by serum nicotine and cotinine concentrations, was consistent in mice exposed to aerosols generated from low or high temperature coils (**Fig. 3a, b**). These data together validate that in this study, aerosol and nicotine delivery in the chambers were constant for both low and high coil operating temperatures.

Coil temperature and carbonyl compounds in e-cigarette aerosols

In the exposure chambers operated at both low and high coil temperatures, concentrations of aerosolized carbonyls were determined, which are byproducts of thermal degradation of PG and VG in e-liquid known to be hazardous to human health. Both acetaldehyde and formaldehyde were found in significantly lower quantities when coils were operated at 475°F compared to 375°F, while acetone and propionaldehyde were similar between the two coil operating temperatures (**Fig. 4**).

Using the same e-cigarette device, puffs were generated at coil temperatures ranging from 225°F to 525°F and sampled directly from the device, not from whole-body exposure chambers as before. Total aerosol was quantified from five independent coils. As expected, particle mass per puff increased as coil temperature increased, such that coil temperatures of 475°F generated approximately 3-fold larger aerosol concentrations per puff than 375°F (**Fig. 5a**). This is consistent with the experimental observation that on average, to maintain a concentration of 1,800 mg/m³ in the exposure chambers, the 375°F coil device was puffed once every 30 seconds while the 475°F coil device was puffed once every 90 seconds. Per puff carbonyl outputs at increasing coil temperatures were also directly sampled and quantified.

Concentrations of all nine carbonyls, including formaldehyde, acetaldehyde, acetone and propionaldehyde, increased consistently as coil temperature increased (**Fig. 5b**).

Together, these data demonstrate that operation of an e-cigarette device at a higher coil temperature produces significantly higher volumes of total aerosol and aerosolized carbonyls, but that when aerosol concentration is kept constant, the relative proportion of carbonyls is significantly decreased in the case of formaldehyde, and acetaldehyde, or unchanged in the case of acetone and propionaldehyde. These data are important to understanding the relative risks to users of e-cigarettes which allow for temperature control, or which operate at high coil temperatures.

Coil temperature, pulmonary immune cell influx and pro-inflammatory cytokine expression

Compared to filtered air (FA) controls, exposure of mice to aerosols generated from coils at 375°F significantly increased total airway cellularity, primarily driven by macrophage influx into BAL (**Fig. 6a, b**). In contrast, exposure to aerosols generated at 475°F did not significantly alter immune cell influx as compared to FA controls (**Fig. 6**). Mice exposed to aerosol generated from coils at 475°F had significantly fewer total BAL cells and macrophages compared to mice exposed to aerosols generated at 375°F (**Fig. 6a, b**). No significant differences were observed in neutrophil or lymphocyte influx as a result of e-cigarette aerosol exposure (**Fig. 6c, d**). Therefore, increasing coil temperature did not appear to exacerbate murine lung inflammation. In fact, aerosols generated using lower temperature coils induced significantly more macrophage influx to airways than higher temperature coils (**Fig. 6b**).

Compared to FA exposed control lung tissue, both 375°F and 475°F exposures significantly reduced IFN β , TNF α , and IL-10 expression, while IL-1 β expression was only

significantly reduced by 475°F exposures (**Fig. 7**). No changes were observed in IL-6 or IFN γ expression compared to controls. Of note, IL-1 β and IFN β expression were significantly reduced in mice exposed to aerosols generated at 475°F compared to 375°F.

Taken together, this data demonstrates that e-cigarette exposures can dampen pro-inflammatory cytokine expression in the lungs, and that increased coil temperatures can further promote this reduction in gene expression.

Discussion:

The goal of this study was to investigate the impact of increasing coil temperature of a third-generation e-cigarette device on the formation of aerosolized carbonyls and respiratory immune responses in mice, while maintaining a constant aerosol concentration. The rationale for this approach is based on the manner in which e-cigarettes are vaped by the user to titrate puff volume, regardless of coil temperature to achieve the desired level of nicotine. Understanding how customizable user controls, like high temperature or power settings, can impact health risks and toxicant exposures is important for individual e-cigarette user risk management and, importantly, for the creation of public health guidelines and product regulations/ oversight of e-cigarette devices to minimize exposure risks. In the present study, we demonstrate that formaldehyde and acetaldehyde concentrations were proportionally lower in aerosols generated at 475°F than those generated at 375°F when total aerosol concentrations were kept constant. Additionally, exposure of mice to e-cigarette aerosols generated using high coil temperatures resulted in reduced macrophage trafficking to lung airways, and enhanced reductions in expression of the pro-inflammatory cytokines IL-1 β and IFN β .

To evaluate the impact of coil temperature on carbonyl output, as a proportion of total aerosol mass, we experimentally maintained a constant aerosol concentration in the low (375°F) and high (475°F) temperature exposure chambers. To do this, the puff rate (puffs/minute) during exposures was adjusted. To maintain an average of 1,800 mg/m³ aerosol concentration, the e-cigarette at 375°F puffed at a rate of 1 puff every 30 seconds, which was 3 times faster than the 1 puff every 90 seconds rate needed for the 475°F device. This indicates the 475°F device was generating roughly 3 times as much aerosol per puff than the 375°F device. When sampling directly from our e-cigarette device, instead of through the sampling port of the whole-body

exposure chamber, we found a similar magnitude (approximately 5 mg/puff vs 15 mg/puff) of aerosol increase from 375°F to 475°F (**Fig. 5a**). These data are consistent with previous reports that increased power or temperature causes increased total aerosol mass generation (Dibaji et al., 2022; Ko & Kim, 2022; Tran et al., 2023).

Despite this, we did not observe increased concentrations of any thermal carbonyls at a higher coil temperature, which are primarily generated through heat-induced dehydration reactions (Klager et al., 2017; Laino et al., 2011, 2012; Li et al., 2021; Sleiman et al., 2016). Interestingly, we observed decreased formaldehyde and acetaldehyde in the 475°F chamber, compared to the 375°F chamber, and no changes in acetone or propionaldehyde. Using third- and fourth-generation e-cigarettes, previous studies have reported increased carbonyl outputs, on a per puff basis, as a result of either increased coil temperature (T, in °F/C), power (P, in watts (W)) or voltage (V, in volts (V)), or decreased coil resistance (R, in ohms (Ω)) (Cirillo, Urena, et al., 2019; Geiss et al., 2016; Gillman et al., 2016a; Li et al., 2021; Noël et al., 2020; Son et al., 2020; Talih et al., 2023; Tran et al., 2023; Zelinkova & Wenzl, 2020). However, from most of these studies it is not immediately clear whether carbonyl concentrations increase proportionally, or simply as a factor of overall aerosol mass increases. Of these studies, several did not report sufficient total aerosol mass information to determine proportional carbonyl concentrations (Cirillo, Urena, et al., 2019; Geiss et al., 2016; Son et al., 2020; Talih et al., 2023), making direct comparisons to the present study impossible. Two studies (Noël et al., 2020; Zelinkova & Wenzl, 2020) provided enough information about total aerosol or total aerosolized nicotine concentrations to roughly analyze the proportional carbonyl outputs. Zelinkova et. al measured carbonyl outputs of a 0.15 Ω coil at various power levels and found that when power increased from 50W to 70W, formaldehyde concentrations increased proportionally to aerosol mass by

roughly 1.5 times. Noël et. al did not provide total aerosol information, but did include aerosolized nicotine, which could be used to normalize carbonyl concentrations. They found that for a 0.15 Ω coil operated at 4.8V compared to 2.8V, where increasing voltage corresponds to increasing power levels, produced more acetaldehyde, under both flavoring conditions, relative to nicotine concentrations. Interestingly they report both increased and decreased formaldehyde concentrations relative to nicotine, depending on the flavoring additives tested (Zelinkova & Wenzl, 2020).

Additionally, two studies normalized carbonyl concentrations by total aerosol mass (Gillman et al., 2016a; Tran et al., 2023). In line with our findings, Gillman et. al report that using a 0.72 Ω coil, increasing the power from 10W to 25W decreased formaldehyde and acetaldehyde concentrations relative to aerosol mass by 30% and 45%, respectively. Tran et. al showed that when comparing third- and fourth-generation devices, carbonyl yields increase as resistance increases and as power and temperature decrease, which is consistent with our results. That comparison has some limitations as this study, unlike ours, did not compare the same third-generation coil operated at various temperatures or power settings but rather compared carbonyl output from e-cigarette devices, which have significantly different coil resistances, power and coil temperatures to draw conclusions. Nevertheless, the authors suggest a possible reason for the seemingly contradictory finding that increased coil temperature leads to decreased thermal carbonyl formation. The explanation draws on Ohm's law, and other equations relating voltage (V), current (I), resistance (R), power (P), energy (E), and temperature (T). Tran et. al posit that because lower power and subsequently, lower temperature, e-cigarette devices are equipped with higher resistance coils (like those used in fourth-generation devices), these devices will create comparably larger voltage drops across the high resistance coils than high power devices (like

third-generation devices). These larger voltage drops, they suggest, may lead to greater energy dissipation in the form of thermal energy, or heat, which could cause greater localized heating of the e-liquid around the coil than that seen in third-generation devices. Though the difference in voltage drops between our low and high temperature experimental conditions are likely much smaller than the differences seen between third- and fourth-generation e-cigarettes, it is possible that, if proven correct, this theory could explain our experimental results. To our knowledge, only two studies have measured e-liquid temperature directly (Ko & Kim, 2022; Ranpara et al., 2023), though not in the context of varied power, coil temperature or coil resistance, which would be needed to confirm this theory. More research measuring both coil temperature and e-liquid temperature, with a focus on how power, energy and temperature interact within the system, is needed to further explore this claim.

In summary, we found both confirmatory and contradictory literature reports with respect to the effect of temperature, or power, on carbonyl outputs as a relative proportion of total aerosol mass. These studies varied in their methodological approaches, data collection and analysis, and e-cigarette device products used, which likely explains the observed discrepancies.

Standardization of both experimental conditions and data reporting could facilitate more useful comparisons. Still, more research is needed to elucidate the complex physical and chemical relationships behind coil temperature and carbonyl concentrations.

It should be noted that if increasing coil temperature does not increase thermal carbonyl generation proportionally, this does not definitively reduce all risks associated with high temperature vaping. Depending on e-cigarette use and nicotine titration behavior, which has been shown to be influenced by device power (Hiler et al., 2020), users could still plausibly increase their daily carbonyl exposures by vaping at higher temperatures consistently. Additionally, high

coil temperatures introduce other potential health risks unrelated to thermal carbonyls. Studies have demonstrated that toxic and carcinogenic metals, such as nickel and chromium, are significantly more abundant in e-cigarette aerosols from devices operated at higher temperatures and power settings (Ko & Kim, 2022; Rastian et al., 2022; Zhao et al., 2019). Increasing power and temperature has also been shown to effect e-cigarette aerosol particle size and therefore lung deposition, potentially creating smaller particles which can deposit more deeply into the lungs (Floyd et al., 2018; Lechasseur et al., 2019). Other phenomenon such as unintentional dry puffing and film boiling, which are associated with increased volatile organic compound (VOC) release, are also more likely to occur at higher e-cigarette coil temperatures (Talih et al., 2020). Taken together, it is clear that high temperature vaping is not without risks.

Immune dysregulation, both pro- and anti-inflammatory, as a result of e-cigarette exposure, is well documented and can result in changes to leukocyte populations, leukocyte functions, and inflammatory gene expression. However, few studies have investigated how increased temperature or power effects the biological and immunological outcomes from third- or fourth-generation e-cigarette aerosol exposure. In the present study, we report that increased coil temperature significantly altered both leukocyte trafficking and pro-inflammatory cytokine gene expression in the lungs of mice. Exposure for 3 days to aerosols generated at 375°F resulted in significantly greater numbers of macrophages in murine lungs compared to aerosols generated at 475°F, potentially indicating either a more significant acute inflammatory response to lower temperature aerosols, or an immunosuppressive effect of higher temperature aerosols. Additionally, analysis of inflammatory gene expression in lungs found that both aerosol exposure conditions induced suppression of IL-1 β , IFN β , TNF α , and IL-10 compared to unexposed FA controls. Compared to mice exposed to aerosols generated at 375°F, those

exposed to 475°F experienced significantly greater downregulation of both IL-1 β and IFN β , potentially indicating more potent immunosuppression as a result of high temperature exposures. To our knowledge, two studies have been published which evaluate lung inflammation after exposure to aerosols generated by the same coils at different power levels (Kleinman et al., 2020; Shi et al., 2022). These studies in rats seem to validate our findings that increasing temperature can increase immune dysregulation. Shi et. al reports that rat lung cytokine gene expression was induced above control levels after exposure to aerosols generated at 70W but not 45W (Shi et al., 2022), while Kleinman et. al described EVALI-like lung injury in rat lungs exposed to aerosols generated at 70W, but not 60W (Kleinman et al., 2020). It is important to note that both studies did not indicate the resistance of the coils used in the studies, making it more difficult to directly compare these studies to the present one. Four other studies investigated the impact of temperature or power on different coils, where sub-ohm coils (0.15 Ω or 0.25 Ω) are compared to high resistance coils (1.5 Ω) (Cirillo, Urena, et al., 2019; Cirillo, Vivarelli, et al., 2019; Noël et al., 2020) or fourth-generation (1.6 Ω) devices (Pinkston et al., 2023). Direct comparisons between these studies to the present study are very limited in their usefulness. Still, three of the four studies find that high power, and therefore higher temperatures, results in greater decreases in cell viability (Cirillo, Urena, et al., 2019; Noël et al., 2020), more drastic alterations in inflammatory gene expression (Cirillo, Vivarelli, et al., 2019; Pinkston et al., 2023), and greater increases in reactive oxygen species (ROS) release (Cirillo, Urena, et al., 2019) *in vitro*. These results are generally in accordance with our findings. Of note, Cirillo et. al reported a significant decrease in blood lymphocytes in rats exposed to aerosol generated by a higher-powered e-cigarette device. This combined with our findings of significantly reduced macrophage

populations in the airways, provides further evidence that higher-powered e-cigarettes may suppress leukocyte populations more than lower-powered e-cigarettes.

In contrast, the fourth study reports that lower-powered fourth-generation aerosol exposure results in greater macrophage cell death, dampening of cytokine gene expression (particularly IL-6), and oxidative stress levels *in vitro* compared to higher-powered third-generation devices (Pinkston et al., 2023). Though these results may be contradictory to ours and the three previously mentioned studies, it is difficult to conclude this with confidence since the focus of this study was on comparison between third- and fourth-generation e-cigarettes instead of comparing device settings of third-generation e-cigarettes. Taken together, it is clear that further research is required to properly determine if and how coil temperature alters biological outcomes.

In conclusion, the present study adds to the growing body of evidence demonstrating that adjustments to basic e-cigarette settings can significantly alter aerosolized carbonyl concentrations, which in murine models, can alter pulmonary immune responses. Therefore, we conclude that high temperature vaping is not without risk. Policy makers and public health professionals should be aware of the potential health risks of high power or high temperature vaping, and regulations should be considered that limit the allowed maximum coil temperatures of third-generation e-cigarette devices.

Table 2.1: qRT -PCR Primer Sequences

	Forward Primer	Reverse Primer
β -actin	CCT CTA TGC CAA CAC AGT GC	CCT GCT TGC TGA TCC ACA TC
IL-1 β	GCC CAT CCT CTG TGA CTC AT	AGG CCA CAG GTA TTT TGT CG
IL-10	CCA AGC CTT ATC GGA AAT GA	TTT TCA CAG GGG AGA AAT CG
IL-6	AGT TGC CTT CTT GGG ACT GA	TCC ACG ATT TCC CAG AGA AC
IFN γ	ACT GGC AAA AGG ATG GTG AC	TGA GCT CAT TGA ATG CTT GG
TNF α	AGC CCC CAG TCT GTA TCC TT	CTC CCT TTG CAG AAC TCA GG
IFN β	CCC TAT GGA GAT GAC GGA GA	CTG TCT GCT GGT GGA GTT CA

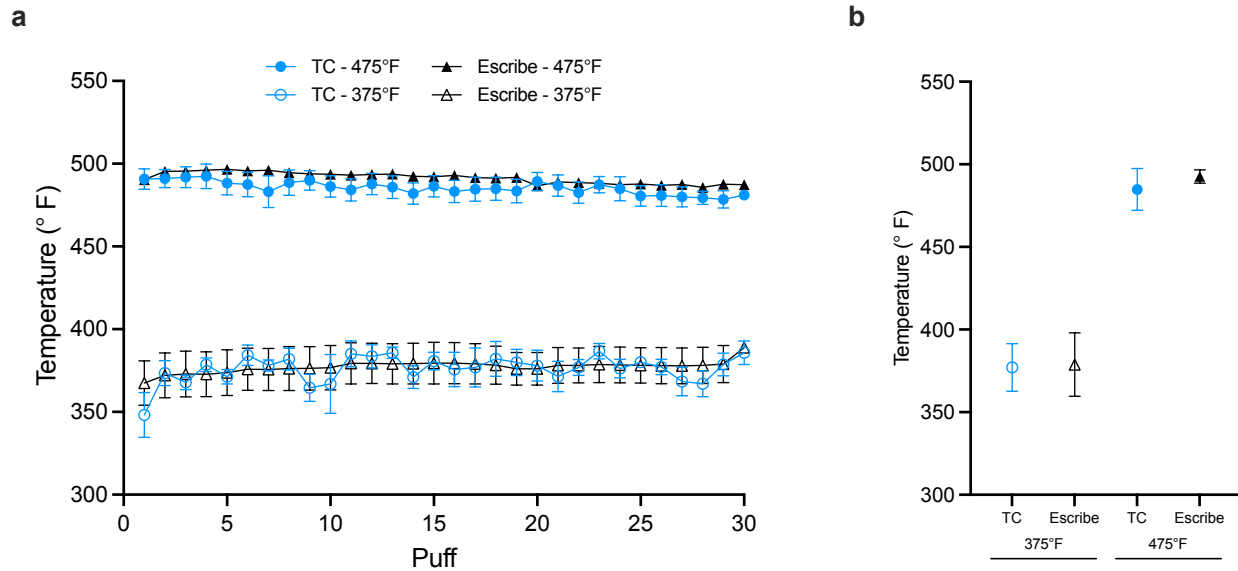


Figure 2.1: Escribe software accurately predicts average coil temperature. (a, b) A thermocouple (TC) was placed in contact with the metal surface of the e-cigarette coil in a vertically and horizontally central location. During each 3 second puff, the peak temperature of the thermocouple device was recorded. Simultaneously, the e-cigarette device was connected via USB to Escribe software and puff temperature data was automatically recorded. **(a)** Thirty consecutive puffs were recorded per day at the start of both low (375°F) or high (475°F) temperature exposures using both temperature sensing methods simultaneously. **(b)** Cumulative average temperatures during exposures are presented. Data are presented as mean \pm SEM. n=6 from 6 independent exposure days.

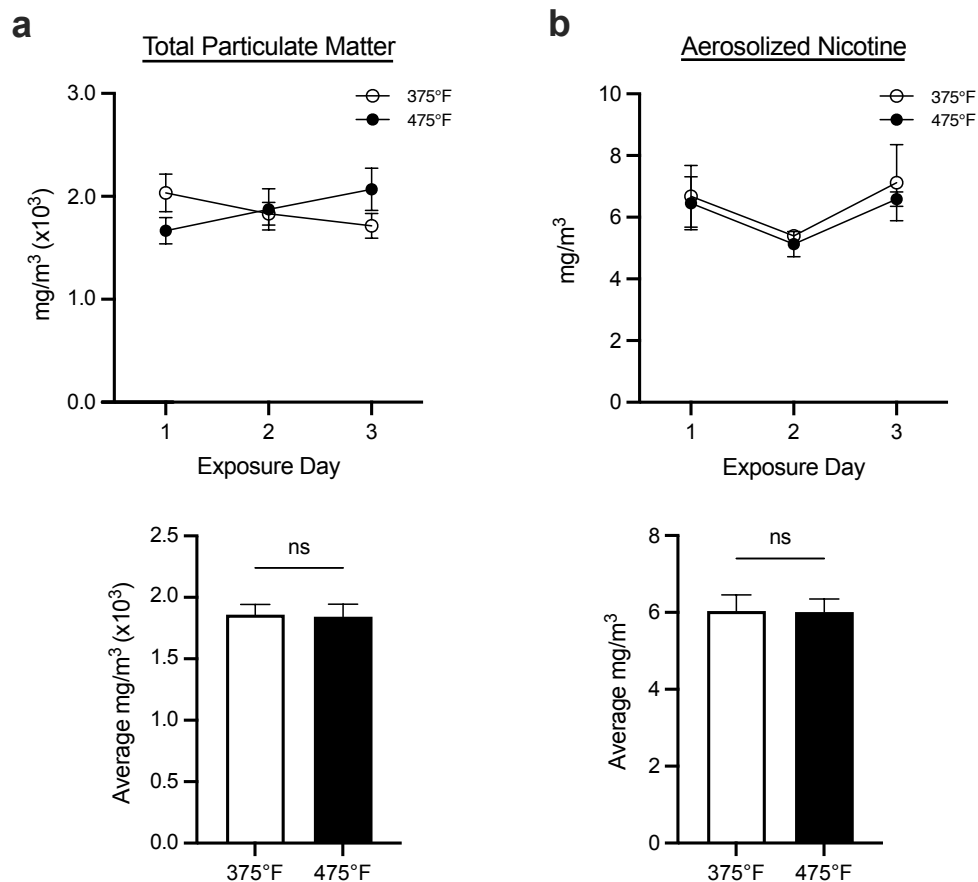


Figure 2.2: Average daily aerosol and nicotine concentrations during low and high temperature ECIG exposures. (a) Total particulate matter (TPM) (mg/m³) was measured gravimetrically once per hour during e-cigarette exposures using either a low (375°F) or high (475°F) temperature coil **(b)** Aerosolized nicotine samples (mg/m³) were collected once per day using XAD-4 cartridges, then analyzed via gas chromatography **(a, b)** Top: daily average; Bottom: total exposure averages. Data are presented as mean ± SEM. n=6 from 6 independent exposure days. Statistical significance determined by two-tailed Student's *t*-test with Welch's correction.

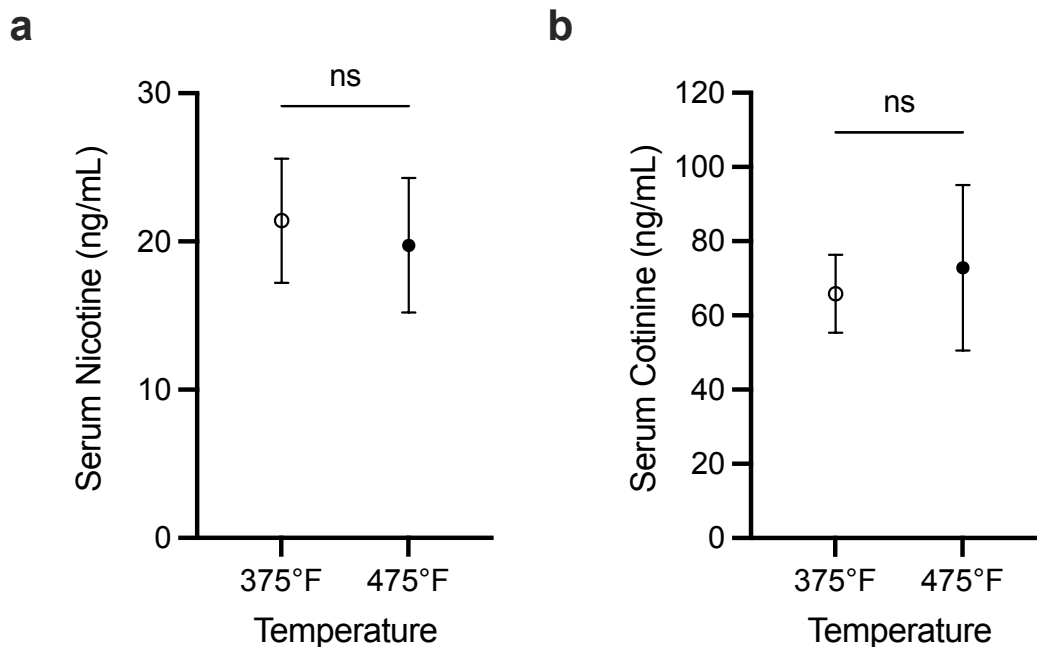


Figure 2.3: Serum nicotine and cotinine concentrations. (a, b) Mice were exposed to e-cigarette aerosol generated using either a low (375°F) or high (475°F) temperature coil for 3 hours/day for 3 days. Immediately after completion of the final exposure, serum was collected and analyzed via gas chromatography mass spectrometry for nicotine (a) and cotinine (b) concentrations. n=6 per group. Data are presented as mean \pm SEM. Statistical significance determined by two-tailed Student's *t*-test with Welch's correction.

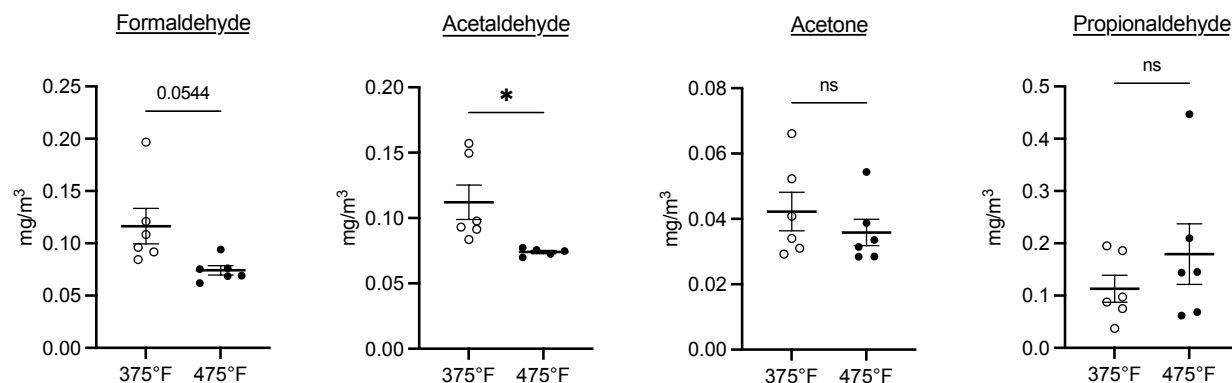


Figure 2.4: Aerosolized carbonyl concentrations in chambers during low and high coil temperatures e-cigarette exposures. Chamber aerosol samples were collected one to three times daily during exposure using 2, 4 dinitrophenylhydrazine (2, 4-DNPH) cartridge for quantification of carbonyls. Samples were extracted with acetonitrile and characterized then quantified by High Performance Liquid Chromatography – High Resolution Mass Spectrometry (HPLC-HRMS) using analytical standards. Data are presented as mean \pm SEM. $n=6$ from 6 independent exposure days. Statistical significance was determined by two-tailed Student's t -test with Welch's correction. $*p<0.05$.

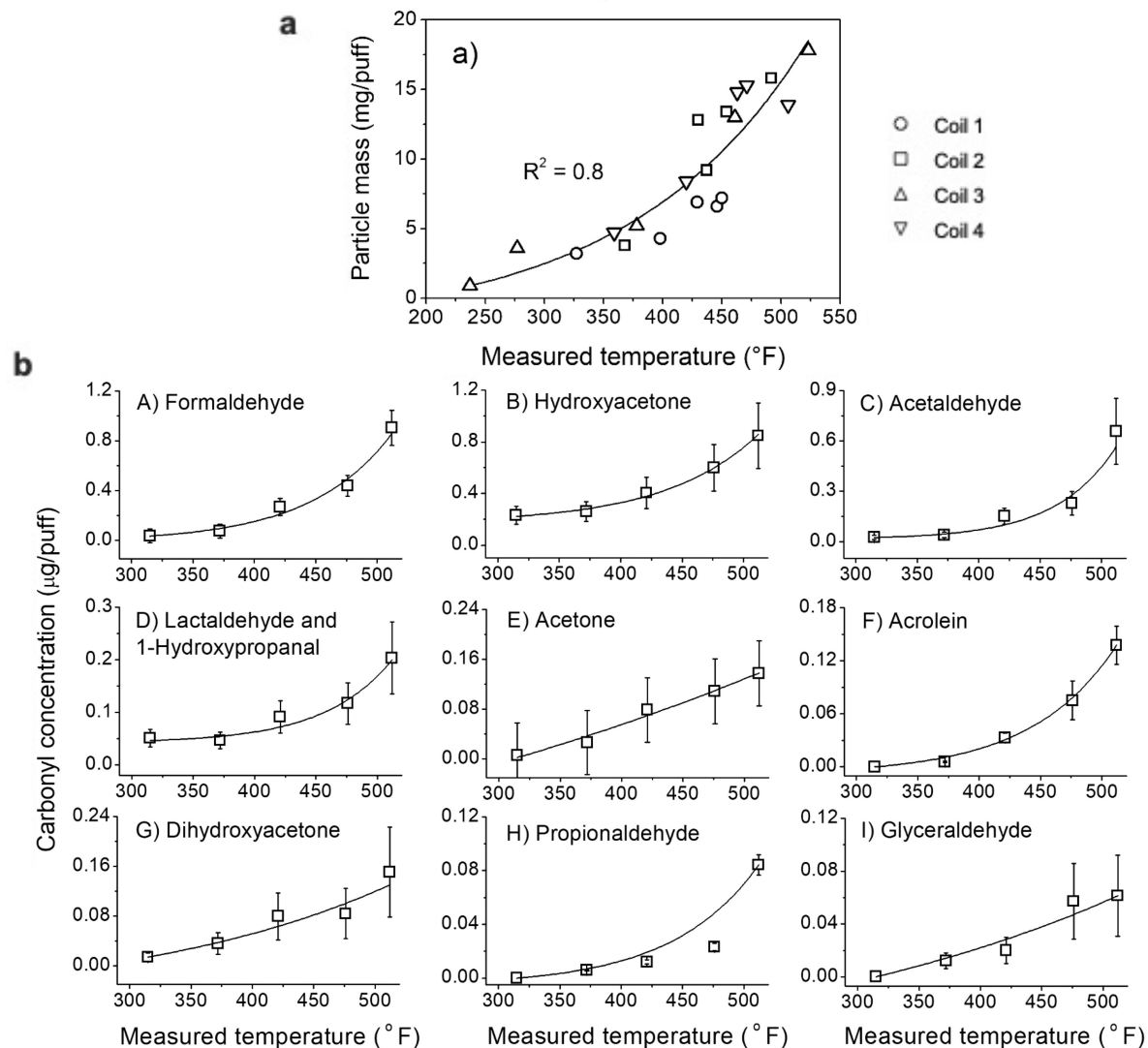


Figure 2.5: Carbonyl concentrations in e-cigarette aerosol increase exponentially as particle mass increases. (a, b) Thirty puffs were captured directly from a third-generation e-cigarette device operated at various coil temperatures (325°F - 500°F) (a) Particle mass was determined gravimetrically using hydrophilic polytetrafluorethylene (PFTE) membrane filters from four independent coils. (b) Carbonyl concentrations were determined using 2, 4 dinitrophenylhydrazine (2, 4-DNPH) cartridge. Samples were extracted with acetonitrile and characterized then quantified by High Performance Liquid Chromatography – High Resolution Mass Spectrometry (HPLC-HRMS) using analytical standards. Data are presented as mean \pm 95% CI. (Figure adapted from Li Y. et al, 2021)

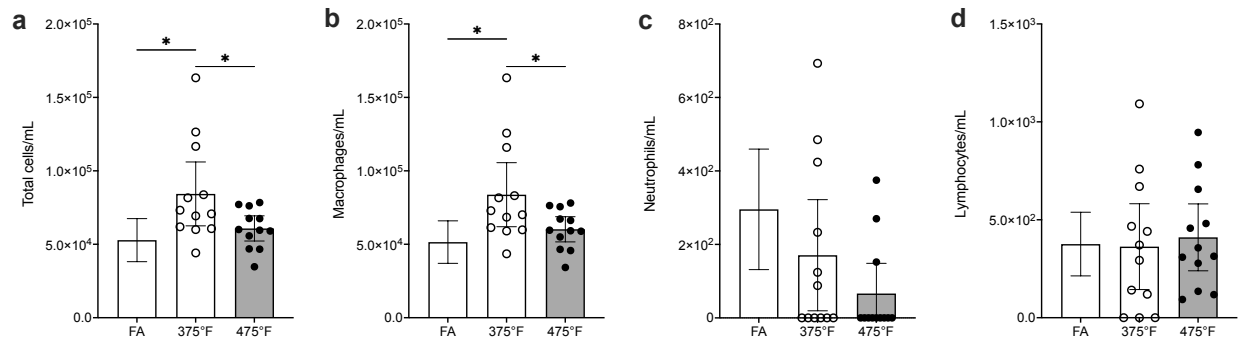


Figure 2.6: Increasing coil temperature does not exacerbate leukocyte influx to lungs. (a-d) Mice were exposed to e-cigarette aerosol generated using either a low (375°F) or high (475°F) temperature coil for 3 hours/day for 3 days. Immediately after completion of the final exposure, bronchoalveolar lavage fluid was collected. Data are presented as mean \pm SEM of total BAL cells (a), macrophages (b), neutrophils (c), and lymphocytes (d), as assessed by Cytospin. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. $n=12$ per group. * $p<0.05$.

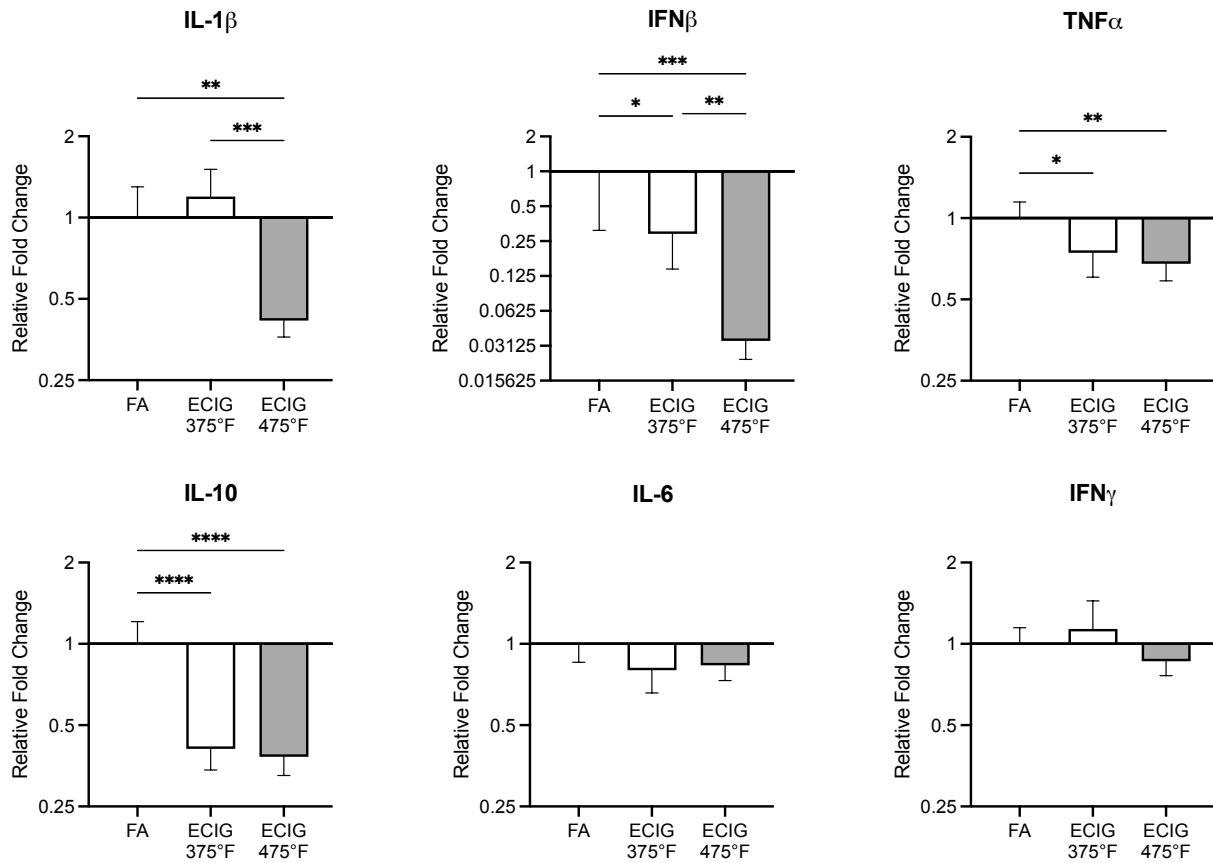


Figure 2.7: Inflammatory cytokine gene expression in lung tissue is further dampened following high coil temperature e-cigarette exposure. Mice were exposed to e-cigarette aerosol generated using either a low (375°F) or high (475°F) temperature coil for 3 hours/day for 3 days. Immediately after completion of the final exposure, lung tissue fluid was collected. Lungs were homogenized, RNA was extracted, amplified, and quantified via qRT-PCR. n=12 per group. Data are presented as mean \pm SEM. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

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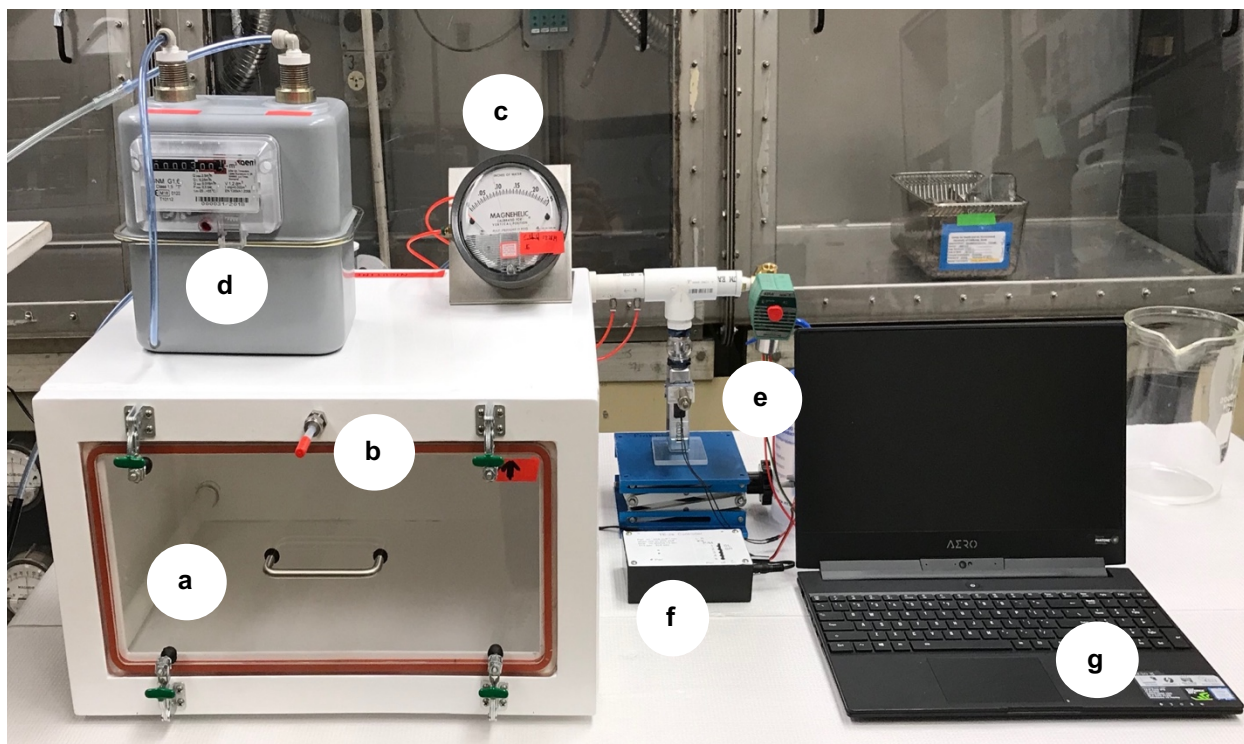
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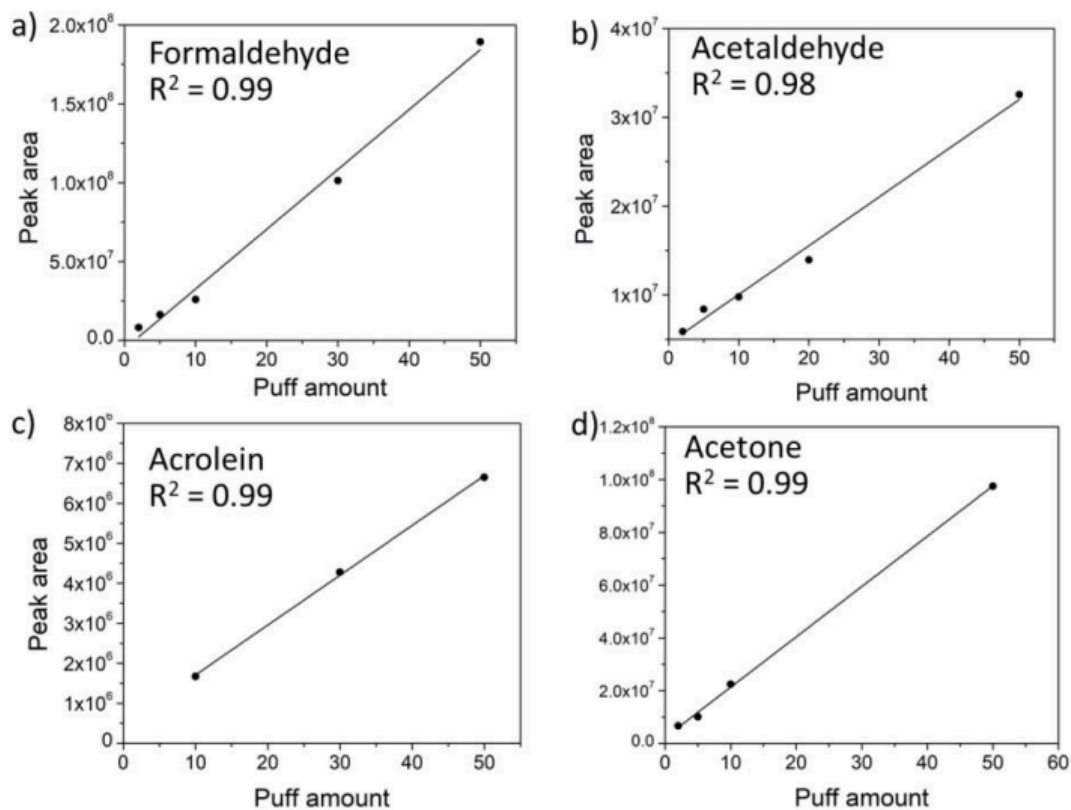
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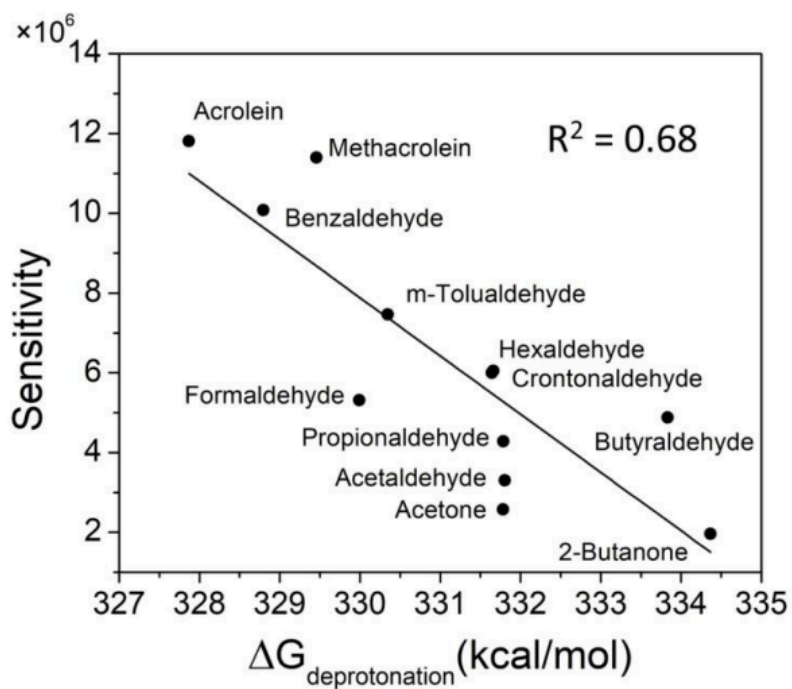
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Supplemental Figure 2.1: E-cigarette exposure chamber set-up. Whole-body exposure chamber (a) with sampling port (b), magnehelic (c) and dry test meter (d), third- generation e-cigarette device (e), puffing controller (f), and laptop (g) to monitor puff topography and characteristics.



Supplemental Figure 2.2: Linear dynamic range of the HPLC-HRMS analysis of DNPH hydrazones in the range of 2-50 puffs for representative carbonyl compounds: formaldehyde (a), acetaldehyde (b), acrolein (c) and acetone (d). (Figure reproduced with permission from Li Y et al., 2021)



Supplemental Figure 2.3: Correlation between the observed ESI sensitivities of standard carbonyl-DNPH hydrazones and calculated gas phase basicity ΔG_d . (Figure reproduced with permission from Li Y et al., 2021)

CHAPTER 3:

Comparative Effects of Progressive Exposure to E-Cigarette Aerosol and Cigarette

Smoke *in vitro* and *in vivo*

Abstract:

E-cigarettes are popular devices that deliver nicotine to users by heating e-liquid: a mixture of propylene glycol (PG), vegetable glycerin (VG), nicotine, and artificial flavors. Popularization of brands, such as JUUL, which use highly bioactive nicotine salts, has increased public health concerns that rising e-cigarette use may increase nicotine addictions. Since e-cigarette devices are relatively new, the long-term health effects of inhaling e-cigarette aerosols are largely unknown. A comprehensive understanding of the immunomodulatory effects of e-cigarette use has not yet emerged, as both pro- and anti-inflammatory effects have been attributed to e-cigarette exposure. Lack of standardization in exposure regimens, coupled with a lack of detailed reporting of exposure conditions further complicates the ability to compare reported biological and immunological outcomes. To this end, herein we provide a detailed report of ECIG exposure conditions, and the resulting biological outcomes in mice. We investigated how *in vivo* controlled progressive exposure of mice to high nicotine content e-cigarette aerosol (ECIG) from a third-generation device impacts immune cell influx and lung cytokine expression, in comparison to controlled progressive exposure to cigarette smoke (CS). Nicotine metabolism, evidenced by serum cotinine to nicotine ratios, was significantly different in mice following ECIG and CS exposure. Compared to CS exposure, acute ECIG exposure induced similar, or at times more significant, macrophage infiltration, which is not sustained over longer duration exposures. Progressive ECIG exposure dampens pro-inflammatory cytokine gene expression in murine lung tissue. Furthermore, *in vitro* exposure of human macrophages and alveolar epithelial cells to ECIG conditioned media decreased pro-inflammatory cytokine gene expression. Taken together, these data indicate the ability of ECIG to disrupt pulmonary immune homeostasis, in a similar fashion to CS, while also significantly suppressing immune modulators.

Introduction:

E-cigarettes, invented in 2003 by a Chinese pharmacist named Hon Lik, are nicotine delivery devices consisting of three primary components: a power source (usually a rechargeable battery), a heating element (an atomizer coil), and a reservoir containing e-liquid. The e-liquid is comprised of solvents - commonly propylene glycol (PG) and/or vegetable glycerin (VG), nicotine in either free-base or nicotine salt forms, and various flavoring chemicals. Heating of the atomizer coil aerosolizes the e-liquid, which is inhaled by the user. The devices were introduced to the United States in 2007 and have since gained massive popularity around the world among youths and adults. In the past 10 years, e-cigarette use has consistently increased with recent reports estimating approximately 7.6% of youths and 5.1% of adults in the U.S. are current e-cigarette users, while as many as 6.6 million youths have tried vaping (Boakye et al., 2022; Gentzke et al., 2022).

Though commonly advertised as smoking cessation devices, the efficacy of e-cigarettes for this purpose is contested (R. Chen et al., 2022; Hartmann-Boyce et al., 2021; Quigley et al., 2021). Coupled with targeted marketing to teens and young adults, e-cigarettes are considered by many to represent a serious risk to public health. Troublingly, the use of these devices among never-tobacco smokers is increasing, suggesting that the use of these devices could further increase, not decrease, the prevalence of nicotine addictions/dependencies (Boakye et al., 2022; Obisesan et al., 2020). Indeed, as these devices have rapidly evolved and with the addition of highly bioactive nicotine salts, e-cigarettes have become increasingly more efficient nicotine delivery devices. They are now able to match or surpass the amount of nicotine delivered per puff by cigarettes (Prochaska et al., 2022). With the rise in popularity of the brand JUUL, which uses nicotine benzoate salt, other compact and easily concealable disposable pod systems with

high nicotine e-liquids have flooded the market. These and the highly modifiable third-generation e-cigarette devices, which use ‘sub-ohm’ resistance coils and can be customized by consumers to maximize aerosol output and re-filled with high nicotine e-liquid, justify the need to study e-cigarette exposure conditions with high aerosol and nicotine concentrations. To date, most studies have been carried out studying low to moderate nicotine exposures, while high nicotine concentration exposures are relatively rare (Husari, Shihadeh, Talih, Hashem, El Sabban, et al., 2016; Q. Wang et al., 2020a).

The negative health effects of cigarette smoke (CS) are well characterized (Dai et al., 2022). They include cardiovascular diseases such as coronary heart disease and myocardial infarction, respiratory diseases such as chronic obstructive pulmonary disease (COPD) and pneumonia, and myriad cancers such as lung, stomach, liver, kidney, bladder, and oral cancers (Onor et al., 2017). In addition, users of cigarettes experience wide-spread deleterious health effects that increase their risk of such conditions as diabetes, cataracts, hip fractures, and respiratory infections (Sherman, 1991).

CS is a complex mixture of thousands of chemicals formed through combustion, many of which have been characterized and implicated in disease pathologies as both toxicants and carcinogens. Such chemicals include formaldehyde, acetaldehyde, acetone, acrolein, metals, nitrosamines, polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds (VOCs), and free radicals (Caruso et al., 2009; Talhout et al., 1990). Many of these compounds have consistently been detected in e-cigarette aerosols, albeit generally at lower concentrations (Geiss et al., 2016; Gillman et al., 2016b; Kosmider et al., 2014; Lorkiewicz et al., 2022; Ogunwale et al., 2017). Indeed, in the short period since e-cigarettes have been on the market, multiple adverse effects of e-cigarettes on human health have been reported by consumers (Hua et al.,

2020). Although the long-term health effects of e-cigarette use are largely unknown, numerous medical case studies have documented adverse health outcomes. The most common respiratory complications from e-cigarette use are pneumonia and bronchiolitis, with variable involvement of macrophages or neutrophils. More rare findings involve eosinophil or lymphocyte accumulations (Tzortzi et al., 2020). Additional *in vitro* and *in vivo* studies using human cells and murine models, respectively, report significant alterations in lung immune cell populations as well as cytokine/chemokine production in response to e-cigarette exposures, strongly suggesting their ability to dysregulate pulmonary immunity (Ganapathy et al., 2017; Higham et al., 2018; Husari, Shihadeh, Talih, Hashem, el Sabban, et al., 2016; Scott et al., 2018; Vasanthi Bathrinathan et al., 2018; Ween et al., 2017a). There is emerging evidence that these alterations may impact susceptibility to and recovery from viral and bacterial infections (Corriden et al., 2020; Madison et al., 2019; Masso-Silva, Moshensky, et al., 2021; Rebuli, Brocke, et al., 2021; Sussan et al., 2015; Wu et al., 2014). However, contradictions regarding the impact of e-cigarette aerosols on immunity are common in the published literature and, thus far, a universal consensus has not been reached. For example, exposure of C57BL/6 mice to e-cigarette (ECIG) aerosols for 3 days was reported to have no effect (Lerner et al., 2015), while others reported significant increases in macrophages in the bronchoalveolar lavage (BAL) (Glynos et al., 2018). Similarly, IL-6 protein concentrations in BAL from C57BL/6 mice following acute e-cigarette exposure were reported to be increased (Q. Wang et al., 2019) and decreased (Sussan et al., 2015).

These contradictory data might be explained in part by the fact that unlike conventional tobacco cigarette research, which utilizes standard exposure parameters and research-grade cigarettes, e-cigarette research suffers from a lack of standardized exposure methods. Variations

in device type, coil material, puffing regimen, flow rate, exposure duration, PG/VG ratio, nicotine concentration or type, and flavoring chemicals can significantly impact biological outcomes of e-cigarette exposure and make comparisons between studies difficult. Compounding this problem is the inconsistency in reporting of specific exposure parameters. Standardized reporting of device/e-liquid specifications (e.g., generation, coil material, PG/VG ratio, nicotine concentration), device settings (e.g., voltage, temperature), puffing regimens (e.g., puff duration, puffs/minute), exposure conditions (e.g., flow rate, aerosol and aerosolized nicotine concentration, duration/day), and resulting nicotine absorption (e.g. serum nicotine/cotinine concentration) would be invaluable for clarifying the actual biological effects. Outcomes data from e-cigarette exposure on pulmonary and immunological parameters lack consistency in reporting. Therefore, evaluating contradictory findings of immune cell influx and cytokine regulation resulting from e-cigarette exposure, is challenging. To allow more direct comparisons and to provide the appropriate context for reported findings, the present study seeks to couple detailed reporting of exposure conditions with biological outcomes.

The objective of this study is to investigate how *in vivo* controlled progressive exposure to high nicotine content e-cigarette aerosols from a third-generation device impacts immune cell influx and lung cytokine expression, in contrast to controlled progressive exposure to CS. To that end, female BALB/c mice were exposed to either CS, ECIG, or filtered air (FA) for 1, 3, 5, or 10 days. Furthermore, we investigated the impact of acute ECIG or CS conditioned media exposure on gene expression in human cell lines, U937 and A549.

Materials and Methods:

Exposure Conditions. E-cigarette aerosols were generated and introduced into a whole-body exposure chamber (TE-2epv E-Cig Machine by Teague Enterprises Inc., Woodland, CA) using a third-generation modular vaping device with an Evolv DNA 75 Color computer chip (Evolv LLC., Hudson, Ohio) and a rechargeable battery with a variable output voltage (0.2–9 V) and power (0–75 W). The device was equipped with a commercially available FreeMax Mesh Pro Sub-Ohm Tank and M Pro SS316L single mesh 0.12-ohm coils (FreeMax Technology Inc., Shenzhen, China). Coils were inspected prior to use to verify their reliability (resting resistance of between 0.118 and 0.122 ohms). During exposure, coils were inspected daily for signs of burning from overheating and replaced every 5 days (following approximately 1,800 puffs). The device was robotically operated by a custom linear actuator (TE-2e, Teague Enterprises Inc., Woodland, CA) to achieve a puff rate of 2 puffs/min and a 3 second puff duration during three hours of daily exposure. The device was connected to a laptop and Evolv Escribe software (Evolv LLC., Hudson, Ohio) was used to set the power at 45W and temperature at 450°F and to monitor the device for malfunctions for the duration of the exposures. Pure propylene glycol, vegetable glycerin and nicotine (>99% purity, Sigma-Aldrich Inc., St. Louis, MO) were combined to make a 50/50 PG/VG (% w/v) e-liquid containing 12 mg/mL nicotine. During the period of exposure, the flow rate through the chamber and e-cigarette device was 5 L/min resulting in a chamber air exchange rate of 6.8/hour. Mice (BALB/c females; 6-8 weeks old; n=6 per group per timepoint) were placed in polycarbonate cages with wire lids in the exposure chamber for 3 hours/ day, 5 days/ week for 1, 3, 5 or 10 days. ECIG conditioned media was created by pipetting 100 mL of DMEM or RPMI into a petri dish and placing that into the ECIG

chamber for 3 hours using either 12 mg/mL nicotine e-liquid (ECIG + Nic) or 0mg/mL nicotine e-liquid (ECIG – Nic).

Cigarette smoke was generated using 3R4F research cigarettes and an automatic metered puffer set to Federal Trade Commission conditions (1 puff per minute of 35-mL volume for a duration of 2 seconds), using a previously described whole-body cigarette exposure chamber system (Teague et al., 2008). Mice were exposed for 3 hours/day, 5 days/week for 1, 3, 5 or 10 days. CS conditioned media was created in a similar manner as ECIG media by placement of media in a petri dish in the CS chambers for 3 hours. Filtered Air (FA) controls were housed in separate filtered air exposure chambers for the duration of the experiment.

Exposure Characterization. From both ECIG and CS chambers, aerosol concentrations (mg/m^3) were measured every hour during exposure using a DryTest Meter to measure precise air volumes. Filters were used to collect chamber air samples to gravimetrically determine particulate mass concentrations during sample collection. Nicotine samples were collected every hour during exposure on XAD-4 sorbent tubes (226-30-11-04-GWS, SKC West, Fullerton, CA) followed by extraction and analysis by gas chromatography (Varian 3740). Nicotine concentrations (mg/m^3) were calculated using the area ratio of nicotine/quinoline in each sample.

Animal Protocol. All animal studies were conducted in compliance with regulations set by the University of California, Davis, Institutional Animal Care and Use Committee (IACUC) under NIH guidelines. Female BALB/c mice (6-8 weeks old) were purchased from Envigo. Animals were housed 3 per cage in a 12-h light/ 12-h dark cycle with Purina 5001 regular laboratory rodent diet (Newco Distributors, Rancho Cucamonga, California) and water provided ad libitum.

Six mice were assigned randomly to each of the 4 treatment and 4 control groups corresponding to the 4 timepoints studied (day 1, 3, 5, and 10). Group size was n=6/group.

On days 1, 3, 5, or 10 immediately following exposure, mice were euthanized via 0.2 mL intraperitoneal injection of Beuthanasia-D pentobarbital solution (65 mg/kg body weight; Nembutal Cardinal Health, Sacramento, CA). Cardiac puncture was performed, and blood was collected in a 1.5 mL microcentrifuge tube, maintained for 20 minutes at room temperature to allow for coagulation, and then centrifuged for 15 minutes at 3,000 rpm. Serum was collected (100uL aliquots) and stored at -80 °C. Serum samples were sent to University of California San Francisco (San Francisco, CA) for GC-MS analysis of nicotine and cotinine concentrations in serum (ng/mL).

Cell Culture. A549 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 100U/mL penicillin G, 100 µg/ml streptomycin, and 10% fetal bovine serum (FBS). U937 monocytic cells were cultured in complete RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin then differentiated into macrophage-like cells with 50 ng/mL PMA for 72 hours. After reaching 80-90% confluence, cells were seeded into 12 well plates and rested for one day, after which cells were treated with either untreated media, ECIG conditioned media (with or without nicotine), or CS conditioned media (diluted to 20%). After 24 hours, cells were lysed, and RNA was isolated for qPCR. ECIG conditioned media with or without nicotine was not diluted as it was found to induce similar levels of cell viability as 20% CS conditioned media.

Bronchoalveolar Lavage. Following cardiac puncture, the trachea was cannulated with a 22-gauge blunt end needle that was sutured in place. Whole lungs were lavaged twice with 0.8 mL of sterile phosphate-buffered saline (PBS, Sigma Aldrich, St.Louis, MO). Each aliquot was instilled and recovered 3 times before collecting the bronchoalveolar lavage fluid (BALF). The two BALF samples were combined and centrifuged for 15 minutes at 2,000 rpm at 4°C. BALF supernatant was collected and stored at -80 °C for total protein determination by Lowry assay, while the BAL cell pellet was resuspended in 1.5 mL of PBS. Cell counts and cell viability were determined via Trypan Blue using a hemocytometer. Cytospin slides were prepared using 100uL of BAL cell suspension stained with Diff-Quik for cell differentials. Following collection of BALF, the right main stem bronchus was sutured closed and right lung lobes (cranial, middle, caudal and accessory) were collected and flash frozen in liquid nitrogen and stored at -80 °C.

qRT-PCR. Caudal lobes were homogenized, and RNA was isolated using Quick-RNA Miniprep RNA (Zymo Research, Irvine, CA) according to manufacturer's protocol. RNA was converted to complementary deoxyribonucleic acid (cDNA) using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's protocol. Gene-specific forward and reverse primer (0.2 μm), cDNA (2 μl/reaction), and SYBR Green nucleic acid stain (10 μl/reaction; Applied Biosystems) were used for quantitative polymerase chain reaction (qPCR) (*Table 3.1*). Using the $\Delta\Delta$ -Ct method normalized to β -actin, gene expression of inflammatory markers interleukin (IL)-1 β , IL-10, IL-6, interferon (IFN) α , IFN β , IFN γ , tumor necrosis factor- α (TNF- α), and oxidative stress marker hemeoxygenase-1 (HO-1) were analyzed and standardized to the expression of the housekeeping gene, β -actin.

Statistical Analysis. Statistical analysis was performed using Graph Pad Prism 9. ROUT(Q=1%) was used to remove outliers. One-way ANOVA with multiple comparisons was performed to determine statistical significance. Unless otherwise specified, data are presented as the mean with SEM error bars.

Results:

E-cigarette and cigarette smoke progressive exposure conditions

Average daily total particulate matter (TPM) in the ECIG chamber ranged from $2,960 \pm 117 \text{ mg/m}^3$ on day 2 to $1,200 \pm 235 \text{ mg/m}^3$ on day 5, with an average of $2,120 \pm 186 \text{ mg/m}^3$ across the 10 days of exposure (**Fig. 1a**). Average daily nicotine in the ECIG chamber ranged from $21.1 \pm 2.4 \text{ mg/m}^3$ on day 3 to $11.3 \pm 1.6 \text{ mg/m}^3$ on day 5, with an average of $16.3 \pm 1.1 \text{ mg/m}^3$ across the 10 days of exposure (**Fig. 1a**). Variability in TPM and nicotine concentrations were not intentional. Instead, they were attributable to differential device performance and aerosol output under identical experimental settings.

As is standard for CS inhalation studies, TPM in the CS chamber was gradually increased over the course of exposure to acclimate the mice. Average daily TPM in the chamber ranged from $76 \pm 2 \text{ mg/m}^3$ on day 1 to $128 \pm 10 \text{ mg/m}^3$ on day 10, with an overall average of $93 \pm 5 \text{ mg/m}^3$ across 10 days of exposure (**Fig. 1b**). Average daily nicotine in the CS chamber ranged from $4.7 \pm 0.9 \text{ mg/m}^3$ on day 6 to $17.0 \pm 0.9 \text{ mg/m}^3$ on day 10, with an overall average of $9.4 \pm 1.1 \text{ mg/m}^3$ across 10 days of exposure (**Fig. 1b**). TPM concentrations of ECIG and CS exposure chambers were not in the same range (**Fig. 1c**) while nicotine concentrations from both exposure systems attained similar levels with progressive exposure (**Fig. 1d**).

All mice were placed in exposure chambers at the same time for at each timepoint under study: 1, 3, 5 or 10 days. For each timepoint, one group of six mice was removed and necropsied immediately following exposure. Due to the observed exposure variability, the cumulative average TPM and nicotine exposure over the duration of the experiment were calculated for each group (**Fig. 2**) to validate that similar exposure conditions were achieved between groups. There were no significant differences in cumulative average TPM or nicotine concentrations in mice

exposed for any length of time to CS (**Fig. 2b, e**). Therefore, any biological changes observed between progressive CS exposure groups were due to the duration of exposure and not to variations in exposure conditions between groups. However, mice exposed to e-cigarette aerosol for 1 day experienced a significantly higher mean TPM concentration than mice exposed for 10 days. Mice exposed for 3 days also had significantly higher TPM concentrations than mice exposed for both 5 and 10 days (**Fig. 2a**). While it is possible that differences in biological outcomes between these groups could have arisen from inhalation of a higher concentration of e-cigarette aerosols (mg/m^3), the cumulative average nicotine concentrations between progressive ECIG exposure groups were not significantly different (**Fig. 2d**), making this possibility less likely. Additionally, based on the inherent variability in e-cigarette device output despite the rigorous controlled experimental conditions used here, these variations in cumulative TPM likely represent the variability a consumer could experience using an e-cigarette device. Therefore, we do not believe this is a serious limitation of the study.

As it is well known that third-generation e-cigarette devices create significantly more aerosol than cigarettes do, all ECIG groups were exposed to significantly higher cumulative average TPM and nicotine concentrations than all CS groups (**Fig. 2c, f**).

ECIG exposure results in differential metabolism of nicotine than CS exposure

Nicotine is an important driver of immunological changes seen following CS or ECIG exposure. Given the observed differences in exposure conditions, we tested whether similar deliveries of nicotine to the bloodstream were achieved so that direct comparisons of ECIG and CS exposures could be made. Indeed in the ECIG group, mice had similar serum nicotine and cotinine levels at all timepoints studied. The single exception was in mice exposed to ECIG for 3

days, which had significantly higher serum nicotine concentrations than mice exposed to ECIG for 10 days (196 vs 73 ng/mL) (**Fig. 3a**). Serum cotinine between the two timepoints followed the same trend but the difference did not reach statistical significance ($P= 0.0525$) (**Fig. 3d**). Nicotine concentrations in the ECIG chamber peaked at day 3 (**Fig. 2d**), explaining this observation. Despite the lack of significant differences in cumulative mean chamber nicotine exposures between CS exposed mice groups (**Fig. 2e**), mice exposed for 10 days had significantly higher serum nicotine and cotinine concentrations (135 and 431 ng/mL, respectively) than mice exposed to CS for 1, 3 or 5 days (**Fig. 3b, e**).

Importantly, despite the significant differences in chamber nicotine concentrations between the ECIG and CS groups, all mice had similar serum nicotine (**Fig. 3c**) and cotinine concentrations (**Fig. 3f**) with the exception of mice exposed to ECIG for 3 days. Thus, allowing a direct comparison of the two exposure conditions.

Due to the observation that significantly higher chamber concentrations of nicotine (mg/m^3) (**Fig. 2f**) resulted in similar serum nicotine and cotinine values (**Fig. 3c, f**), we investigated the relationship between nicotine and cotinine in each mouse. Plotting nicotine vs. cotinine concentrations in each animal, revealed that the CS and ECIG experimental groups segregated with distinct slopes of linear regression (**Fig. 4a**). Exposure to ECIG resulted in a significantly higher cotinine to nicotine ratio relative to CS exposure (4.3:1 vs 2.4:1) (**Fig. 4b**). Together the data indicate that nicotine from CS and ECIG is metabolized differently in these mice.

Bronchoalveolar lavage and cell differentials following ECIG and CS exposure

ECIG exposure for 1 day significantly increased absolute numbers of total cells (**Fig. 5a**), macrophages (**Fig. 5b**), neutrophils (**Fig. 5c**), and eosinophils (**Fig. 5d**) in BAL compared to FA controls. One day of ECIG exposure had no effect on lymphocyte numbers (**Fig. 5e**). Progressive exposure to ECIG for 3, 5 and 10 days did not result in significant elevations of leukocytes indicating that cellular influx was not sustained over longer durations of aerosol exposure. Rather cellular influx represented an initial acute response to e-cigarette aerosols. Of note, macrophages frequencies were significantly higher than in the FA control mice after 3 days of ECIG exposure (98.2% vs 99.5%) (**Table 1**). No other significant changes in the proportion of leukocytes were observed at any timepoint.

As exposure duration increased, total BAL cells and macrophages decreased significantly ($P=0.0269$ and 0.0195 , respectively). Inversely, neutrophil, eosinophil and lymphocyte populations seemed to increase as ECIG exposure durations increased, though this trend did not reach statistical significance. Interestingly, neutrophils, eosinophils and lymphocytes were reduced the most following 3 days of ECIG exposure. Mice exposed to 3 days of ECIG had significantly fewer neutrophils and eosinophils than mice exposed for only 1 day (**Fig. 5c, d**). Eosinophils were also significantly lower in mice exposed to ECIG for 5 days compared to 1 day (**Fig. 5d**). After 10 days, neutrophils and eosinophils were no longer significantly decreased indicating that this innate immune cell suppression likely does not persist on a subacute or chronic exposure timescale. Taken together, the data suggest that high nicotine e-cigarette aerosols induce a significant, but transient, inflammatory response causing immune cell influx to the lungs. With longer duration exposures, e-cigarette aerosols may dysregulate immune cell populations, particularly innate cell proportions.

Unlike e-cigarette exposure, CS exposure for 1 day did not significantly alter BAL cellular profiles compared to FA controls (**Fig. 6a-e**). Only after 10 days did CS induce a significant increase in BAL cells and specifically macrophages in mice (**Fig. 6a, b**). Macrophages as a proportion of total cells were the only cell population to be significantly changed by CS exposure. Macrophage frequencies were significantly increased after 3, 5 and 10 days of exposure (**Table 1**). This indicates a shift in relative abundance of immune cells, which occurred as early as 3 days of exposure. Unlike e-cigarette exposure, which caused BAL cells and specifically macrophages to decrease as exposure durations increased, these cells significantly increased in CS exposed mice. Also, in contrast to the trends seen following ECIG exposure, greater durations of CS exposure decreased neutrophil, eosinophil, and lymphocyte infiltration, though not significantly (**Fig. 6c - e**). After 10 days of exposure to CS, these populations remained below FA control levels. Interestingly, like e-cigarette exposure, all cell populations, except lymphocytes, had their lowest numbers following 3 days of CS exposure.

Comparing ECIG to CS directly, no significant differences were seen for any cell type after 1, 3, 5 or 10 days (**Fig. 7**). Therefore, in our system, ECIG exposure induced similar levels of inflammation as CS exposure. While the CS responses were dominated by macrophage activity, ECIG induced more immediate granulocyte responses.

Neither ECIG nor CS exposure of any length significantly altered total protein concentrations in BAL significantly (**Fig. 8**).

Lung cytokine gene expression following e-cigarette aerosol exposure

To further investigate the impact e-cigarette exposure on the immunological environment in the lungs, gene expression of key cytokines from lung tissue was quantified via qRT-PCR

(Fig. 9). Of the 6 cytokines evaluated, gene expression was reduced among tested transcripts in lung tissue compared to the FA control group, except for a slight elevation of IL-6 after 1 day of exposure, and IFN β and INF γ after 5 days of exposure. ECIG exposure significantly reduced expression of TNF α after 1, 3 and 10 days (5 days: $P=0.0536$). Similarly, IFN γ expression after 3 days and IL-10 expression after 10 days of ECIG exposure was significantly downregulated.

Gene expression of HO-1, an enzyme involved in oxidative stress which is a potential mediator of toxicity of ECIG, was also investigated (**Fig. 9**). Unlike cytokine gene expression, HO-1 expression was significantly upregulated after 1 day of ECIG exposure and elevated, though not significantly, following longer exposures. Taken together, despite acute immune cell influx into the lungs, significant increases in inflammatory cytokine gene expression are not seen and, in fact, e-cigarette aerosol exposure may dampen cytokine expression.

In vitro cytokine gene expression following exposure to ECIG and CS conditioned media

Due to the significant changes to macrophages following both CS and ECIG exposure, we investigated the impact of 24h exposure of human U37 macrophage-like cells to CS and ECIG conditioned media. To investigate the effects of e-liquid vehicle alone, ECIG conditioned media were created with or without nicotine addition. Compared to untreated control cells, cells exposed to CS and ECIG conditioned media (+ or – nicotine) had reduced cytokine gene expression (**Fig. 10**). Of note, the only slight induction of gene expression was in IL-6 following exposure to ECIG + nicotine conditioned media (**Fig. 10c**). No significant differences were observed between ECIG conditioned media with or without nicotine. TNF α expression was significantly reduced in cells exposed to CS media (**Fig. 10e**). This downregulation was significantly stronger than the modest reductions seen after ECIG conditioned media without

nicotine. Though gene expression was generally reduced, no significant differences were seen in IFN α , IFN β , IFN γ , IL-1 β or IL-6 expression between any of the treatments.

Similarly, exposure to ECIG conditioned media with nicotine reduced cytokine gene expression in human epithelial A549 cells (**Fig. 11**). Relative fold expression of IL-6 was significantly decreased (**Fig. 11c**). This downregulation contrasted with the slight elevation of IL-6 seen after U937 cell exposure to ECIG with nicotine. Though generally reduced, gene expression of IFN α , IFN β , IFN γ , IL-1 β and TNF α were not significantly changed. Taken together, these *in vitro* results corroborate our *in vivo* findings that e-cigarette exposure dampens cytokine gene expression in the lungs.

Discussion:

The goal of this study was to investigate the effects of high nicotine e-cigarette aerosol exposure on immune cell influx and cytokine expression in the lungs and to compare these effects to those resulting from CS exposure. Importantly, our study focused on in-depth characterization of the exposure conditions, which typically have not been discussed in detail in similar studies. Because of the almost endless options of e-cigarettes available to consumers, be it in the device, coil temperature, battery power, nicotine concentration or flavoring of e-liquid, scientific consensus has not yet converged on a single exposure paradigm. Designs for e-cigarette exposures are therefore highly diverse and comparisons across studies should be made with caution. Despite these concerns, it is common to see comparisons made directly between highly different exposure paradigms (e.g. first-generation with non-detectable serum cotinine vs. third-generation with high serum cotinine) without acknowledging of the limitations of such comparisons. This study therefore seeks to provide a detailed accounting of the logic behind our chosen exposure paradigm and the resulting exposure conditions.

To produce a relevant e-cigarette aerosol exposure, careful consideration was given to the type of device, coil, and device settings used. This study uses a third-generation e-cigarette device, also called a tank or mod, which is a refillable, rechargeable device preferred by regular vape users. Tanks/mods are highly customizable and allow consumers to modify or create e-cigarette coils and e-liquids allowing for thousands of different device options that can each be used with countless flavoring and nicotine combinations.

To achieve a high nicotine exposure level, e-liquid with 12 mg/mL of nicotine was used. This represents a commonly used e-liquid formulation that can typically be purchased in many vape stores, though higher nicotine concentrations (approx. 50 mg/mL) are available to

consumers. The e-liquid contained a 50/50 mixture of PG/VG solvents which is also commercially available and appropriate for use with a third-generation device.

Evolv DNA computer chips are embedded in many popular third-generation e-cigarette devices. Evolv chips are regarded as a reliable, high-quality product and therefore are greatly sought after by consumers. Devices with these chips allow users to precisely control the temperature and wattage that their device operates at. They also allow users to monitor puff data in real time via a computer and set different puff profiles that can be used each time the device puffs to achieve desired flavors or vaping experiences. Third-generation devices are often used with sub-ohm coils, defined as a coil with a resistance below 1.0 ohm. In this study, a commercially available FreeMax Mesh Pro sub-ohm tank and coil system operated by a third-generation e-cigarette device with an Evolv DNA 75C chip was used.

To utilize the device's temperature control functions, coils made from metals with higher temperature coefficients of resistance (TCRs) are required. Metals that are ideal for temperature control are nickel (Ni), titanium (Ti), and stainless steel (SS). Coils made from these metals have greater changes in resistance values at different temperatures, allowing the device to deduce the temperature of the coil by measuring the change in resistance from a baseline (room temperature before puffing). In addition to the type of metal a coil may be constructed from, coil configuration is also important. Traditional coils, which come in single, double, triple, or quadruple configurations, have, by comparison to mesh coils, a smaller surface area with which to contact the e-liquid. Mesh coils with increased surface area distribute heat more evenly, therefore limiting burning and allowing for more efficient vaporization of e-liquid. For this reason, mesh coils are preferred by some e-cigarette users. This study utilized a stainless-steel mesh coil (FreeMax M Pro, SS316L) set to operate at, but not above, 450°F. 450°F was chosen

because it is in the middle of the normal range (300-600°F) at which users typically operate temperature controlled sub-ohm e-cigarette devices and is also in the middle of the manufacturer recommended operating temperature range (400-550°F).

One aspect of e-cigarette research, which is not often acknowledged in the literature, is the lack of consistency in aerosol and nicotine output due to inherent variability with commercially available coil functionality. Using the Evolv Escribe software, we were able to test coils before and during use, precisely control exposure parameters, and monitor each puff in real time. Despite this ability, aerosol output was highly variable between daily exposures and between different coils. Coils from the same package performed differently despite identical experimental settings. For example, the first coil used (from days 1-5 of exposure) when set at 450°F and 45W with 5 L/min flow rate, produced an average of approximately 3,000 mg/m³ on the first day of use while the second coil used with the same settings produced nearly 33% (or 1,000 mg/m³) less aerosol on the first day of use. This is despite both coils having a 0.120 ohm set resistance value, and similar peak resistance values during puffing, which would indicate consistent electrical performance. Additionally, the first coil demonstrated decreasing aerosol output over the course of 5 days, while the second coil did not. To our knowledge, neither coil failed in any way which would have been detected by an e-cigarette user (e.g., no dry puffs, no wick burning, no incomplete vaporization of e-liquid or coil flooding were observed).

As we have previously reported, these same coils exhibited high variability of aerosol output at various set temperatures (Li et al., 2021). Large variations in aerosol output represent greater risks for users to be exposed to a wide range of inhaled carbonyl compounds, such as formaldehyde. These data highlight the need for further research into e-cigarette device variability and underscore the need for regulation and quality control of e-cigarette devices.

Though intra-experimental variability is not commonly reported, our lab has observed this variability across several different coil materials, and coil manufacturers. Therefore, it is likely that inconsistency in aerosol generation is present in many third-generation and pod e-cigarette devices, which could further complicate regulatory efforts at harm reduction.

Despite this variability, nicotine absorption, measured via serum nicotine and cotinine, was similar between ECIG and CS exposed mice at all investigated timepoints. This observation is of interest because aerosolized nicotine was significantly higher in ECIG chambers exposures than in CS chambers. It was therefore expected that nicotine absorption and metabolism would be higher in ECIG exposed mice. However, cigarette smoke and e-cigarette aerosols are known to exhibit differential nicotine pharmacokinetics and nicotine delivery efficiencies (Yingst et al., 2019). Indeed, in our study, cotinine to nicotine ratios for CS and ECIG exposed mice were significantly different. Nicotine delivery efficiency from e-liquid can be influenced by many factors including pH, PG/VG ratio, puff duration, or flavors added (Voos, Goniewicz, et al., 2019). E-liquid containing VG, even at PG:VG 1:1 ratio, as was used in the present study, decreases nicotine delivery ratios (Son et al., 2018), which could explain the phenomena we observed. Moreover, studies comparing the efficiency of nicotine delivery to the blood stream between cigarettes and e-cigarettes have reported that despite higher aerosol outputs and nicotine yields per puff, third-generation devices are not able to deliver nicotine as efficiently as cigarettes (Voos, Kaiser, et al., 2019). Taken together, this could explain why higher TPM concentrations were needed to achieve similar serum nicotine levels in mice. This potential inefficiency of nicotine delivery is important to note, as the primary function of these devices is to deliver nicotine. Former smokers, or dual-users of cigarettes and e-cigarettes may take more frequent or longer puffs from a third-generation device to achieve a level of nicotine equivalent

to that of a cigarette. In this study, TPM concentrations were more than 20 times higher in the ECIG chamber than in the CS chamber, while mice had similar nicotine levels in the blood. This difference in nicotine delivery efficiency could potentially increase exposure risks to consumers. Though cigarettes are generally known to contain higher concentrations of harmful chemical byproducts than e-cigarettes (Cunningham et al., 2020), those chemicals are still of concern. For example, we have previously demonstrated (Li et al., 2021) the ability of this exact exposure system to generate formaldehyde to nicotine ratios above those in combustible cigarettes (Farsalinos et al., 2018).

Many pathologies associated with CS exposure, such as COPD, result from sustained macrophage infiltration to the lungs. These macrophages adopt a proinflammatory phenotype. They secrete cytokines and chemokines that further increase immune cell trafficking to the lung. These conditions establish a continued heightened inflammatory environment in the lungs, which can eventually lead to tissue damage and airway obstruction (Lugg et al., 2022). In our model, CS exposure resulted primarily in macrophage infiltration evidenced by significantly increased numbers of BAL macrophages after 5 and 10 days and by significantly increased percentages of BAL macrophages after 3, 5 or 10 days. Similarly, ECIG exposure also induced an increase in number of BAL macrophages after 1 or 5 days of exposure, and an increase in percentages of BAL macrophages after 3 days. ECIG exposure induced significant macrophage cell influx more quickly than CS exposure, however this may not be sustained over longer exposures. Progressive CS exposure demonstrated a more consistent dysregulation of macrophages as a percent of inflammatory cells in the lung. Taken together, we demonstrate that ECIG exposure can induce significant macrophage dysregulation and trafficking to the lungs, which may represent a acute and transient effect compared to the sustained dysregulation seen by CS exposure.

These results are in accordance with another study that reported significantly increased macrophage numbers after 3 days of high nicotine ECIG exposure (Bahmed et al., 2019). A second study also found 3 days of exposure to ECIG from a first-generation e-cigarette device increased total cell and macrophage numbers in BAL, but this was not sustained after 4 weeks of exposure (Glynos et al., 2018). By comparison, in that study they also report 3 days and 4 weeks of CS exposure both resulted in significant macrophage infiltration. Conversely, studies have reported no significant changes to (Lerner et al., 2015; Q. Wang et al., 2019) or significant decreases in macrophage populations following acute and sub-chronic exposures to ECIG. Notably, exposure of female BALB/c mice to 8 weeks of ECIG from a third-generation device caused significant decrease in total cell and macrophage numbers in BAL (Larcombe et al., 2017). These studies represent very different exposure paradigms that used significantly less aerosol and nicotine than the current study, which may explain the discrepancies in acute reactions. Taken together, evidence suggests that compared to CS exposure, acute ECIG exposure induces similar, or at times more significant, macrophage infiltration, but unlike CS exposure, this trend does not continue with further sub-chronic or chronic exposures. However, since long-term health effects of e-cigarettes are unknown, and given the high variability in devices and subsequent exposure risks presented to consumers, more research is needed to determine whether e-cigarette exposure represents a lower risk for developing macrophage related pathologies than CS exposure.

It is known that CS exposure induces neutrophilia (Morrison et al., 1998), even on acute timescales (Botelho et al., 2010; D'hulst et al., 2005). Neutrophil accumulation in the lungs contributes to the development of CS-associated pathologies such as COPD (Jasper et al., 2019). We did not observe significant increases in absolute numbers or frequencies of neutrophils

following any duration of CS exposure. At the TPM concentrations used, it is possible that longer exposures would be needed to induce significant neutrophil activation and recruitment to the lungs. Importantly, neutrophils have been implicated in e-cigarette, or vaping, product use–associated lung injury (EVALI) cases where significant airway neutrophilia is present (Jennifer E. Layden et al., 2020) and are hypothesized to be primary drivers of EVALI tissue damage (Alexander et al., 2020). In our model, absolute numbers of neutrophils were significantly increased following 1 day of ECIG exposure. Similar findings have been reported after 1 or 3 days of e-cigarette exposure from a third-generation device (T. Ma et al., 2021; Q. Wang et al., 2019). This demonstrates the ability of e-cigarette exposure to significantly induce neutrophil infiltration to the lungs which, if persistent, is associated with tissue damage and induction of several disease states. Longer exposure duration studies ranging from 2 to 16 weeks have reported no differences in neutrophil numbers following e-cigarette exposure (Madison et al., 2019; Sussan et al., 2015; Szafran et al., 2020a; Q. Wang et al., 2020a). Similar to macrophages, this indicates an intense acute neutrophil infiltration that does not appear to be sustained long-term. Differences in e-liquid humectants, nicotine concentrations, and flavors may explain why *in vivo* mouse studies do not see prolonged neutrophilia but evidence implicating neutrophils is seen human case studies.

Due to conflicting reports from human studies, *in vivo* animal studies, and *in vitro* studies, to date the impact of e-cigarette exposure on lung inflammation is not well understood. In the present study, overall protein concentrations in BAL were unchanged following any duration of e-cigarette exposure in our study. These results agree with Glynos et al. 2018, who found that total content was not significantly altered following 3 days of exposure to nicotine containing e-cigarette aerosols (Glynos et al., 2018). In our model, despite no effects on global

protein concentrations, proinflammatory cytokine gene expression in lung tissue was significantly reduced after e-cigarette exposure. Most notably, TNF α was downregulated at all investigated timepoints of progressive exposure. One study reported a significant increase in TNF α protein levels in BAL (Lerner et al., 2015), while two studies reported no changes to TNF α gene expression (Husari, Shihadeh, Talih, Hashem, El Sabban, et al., 2016) or protein levels (Glynos et al., 2018) in lung tissue following 3 days of e-cigarette exposure. We report a significant reduction in IFN γ after 3 days of ECIG exposure. However, IFN γ concentrations in BAL have been found to be significantly increased following 3 days of e-cigarette exposure (Q. Wang et al., 2019). Conflicting reports of the effect of e-cigarette exposure on murine pulmonary cytokine production are common for most pro-inflammatory cytokines, particularly IL-6 and IL-1 β (Lerner et al., 2015; Sussan et al., 2015; Q. Wang et al., 2019). Evidence of immune activation following e-cigarette exposure is common (Masso-Silva, Byun, et al., 2021). However, evidence of immunosuppressive effects of e-cigarette aerosols is growing (Hickman et al., 2022; Madison et al., 2019; Martin et al., 2016; Masso-Silva, Moshensky, et al., 2021; Rebuli, Glista-Baker, et al., 2021; Sayed et al., 2021; Sussan et al., 2015). Notably, a recent study showed fourth-generation e-cigarette users had significant immunosuppression evidenced by decreased sputum immune biomarkers (Hickman et al., 2022).

We also report a reduction in cytokine gene expression in human macrophage and alveolar epithelial cells after exposure to ECIG conditioned media. This finding is in line with Ween et. al who reported that 24 hour exposure of THP-1 human macrophages to ECIG conditioned media resulted in a significant decrease in IL-6 and TNF α (Ween et al., 2017b). However, these findings are in the minority. Most reports on *in vitro* models find that proinflammatory cytokines are induced by e-cigarette exposure. For example, IL-6 production

has been shown to be increased by e-cigarette exposure in human bronchial epithelial cells, lung fibroblasts, macrophages and dendritic cells (I. L. Chen et al., 2020; Garcia-Arcos et al., 2016; Lerner et al., 2016; Scott et al., 2018). More research is needed to completely understand the mechanisms by which ECIG disrupts or alters pulmonary immune homeostasis and the effects this has on pulmonary health.

In conclusion, the present study includes an explanation of the logic behind our exposure regimen and provides a detailed report of the resultant exposure conditions to increase transparency in the field, and to facilitate direct comparisons between exposure methods. We demonstrate that ECIG and CS exposures result in differential nicotine metabolism, which could present risks to e-cigarette consumers using the devices for nicotine replacement. In our model, e-cigarette exposure elicited similar levels of inflammation, evidenced by inflammatory cell influx to airways, as CS exposure. Notably, e-cigarette-induced inflammation was more transient and did not increase in severity with longer exposure durations, in contrast to CS-induced inflammation. We also demonstrated the ability of e-cigarette aerosols to dampen pro-inflammatory cytokine gene expression in murine lungs and *in vitro* in macrophage and epithelial cell lines, further adding to the growing body of evidence implicating e-cigarette exposure and immunosuppression. We conclude that e-cigarette use presents a potential health risk to consumers and further research is required to determine the full scope of these risks.

Table 3.1: qRT -PCR Primer Sequences

	Forward Primer	Reverse Primer
β -actin	CCT CTA TGC CAA CAC AGT GC	CCT GCT TGC TGA TCC ACA TC
IL-1 β	GCC CAT CCT CTG TGA CTC AT	AGG CCA CAG GTA TTT TGT CG
IL-10	CCA AGC CTT ATC GGA AAT GA	TTT TCA CAG GGG AGA AAT CG
IL-6	AGT TGC CTT CTT GGG ACT GA	TCC ACG ATT TCC CAG AGA AC
IFN γ	ACT GGC AAA AGG ATG GTG AC	TGA GCT CAT TGA ATG CTT GG
TNF α	AGC CCC CAG TCT GTA TCC TT	CTC CCT TTG CAG AAC TCA GG
HO-1	CAC GCA TAT ACC CGC TAC CT	CCA GAG TGT TCA TTC GAG CA
IFN β	CCC TAT GGA GAT GAC GGA GA	CTG TCT GCT GGT GGA GTT CA

Table 3.2: Bronchoalveolar Lavage Leukocyte Frequencies

	Control	1 day		3 days		5 days		10 days	
	FA	ECIG	CS	ECIG	CS	ECIG	CS	ECIG	CS
Macrophages	98.27 % ± 0.20	98.37% ± 0.36	99.13% ± 0.18	99.47% ± 0.11*	99.45% ± 0.13*	99.10% ± 0.35	99.56% ± 0.12*	98.77% ± 0.24	99.73% ± 0.11**
Neutrophils	0.62% ± 0.12	0.63% ± 0.14	0.33% ± 0.11	0.30% ± 0.15	0.10% ± 0.07	0.27% ± 0.07	0.00% ± 0.00	0.37% ± 0.08	0.10% ± 0.04
Eosinophils	0.32% ± 0.06	0.57% ± 0.21	0.43% ± 0.12	0.07% ± 0.04	0.11% ± 0.08	0.17% ± 0.10	0.20% ± 0.09	0.37% ± 0.13	0.00% ± 0.00
Lymphocytes	0.38% ± 0.07	0.43% ± 0.20	0.10% ± 0.07	0.17% ± 0.11	0.35% ± 0.11	0.47% ± 0.27	0.20% ± 0.00	0.50% ± 0.20	0.13% ± 0.08

Values represent percent (%) of macrophages, neutrophils, eosinophils, or lymphocytes of total BAL cells (cells/mL) in bronchoalveolar lavage after filtered air (FA) exposure or progressive (1, 3, 5 or 10 days) of e-cigarette aerosol (ECIG) or cigarette smoke (CS) exposure and are expressed as mean ± SEM (n=6/group except FA where n=36). Statistical significance determined by two-tailed Student's *t*-test with Welch's correction. *p<0.05, **p<0.01 to FA

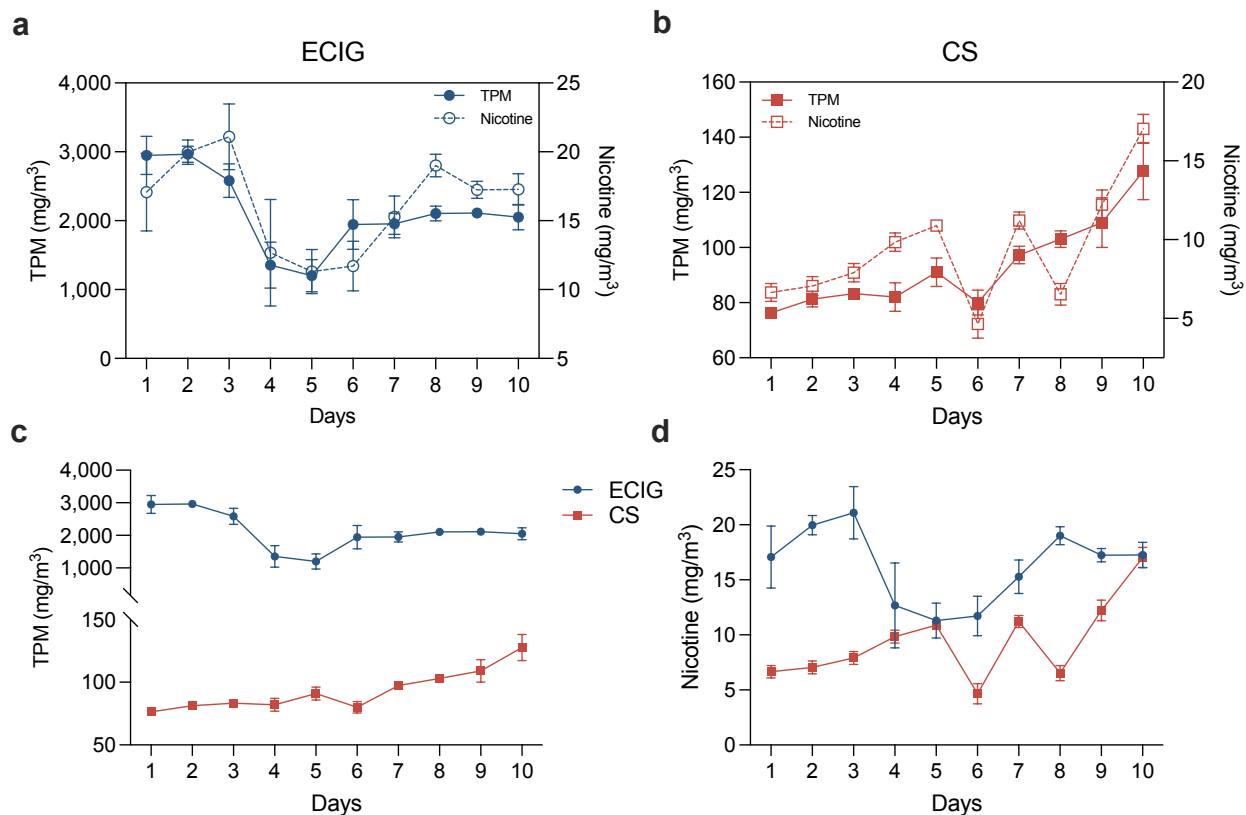


Figure 3.1: Average daily aerosol and nicotine concentrations in ECIG and CS chambers.

(a, b) Once per hour, total particulate matter (TPM) samples were collected gravimetrically from both ECIG (a) and CS (b) chambers. Nicotine samples were collected once per hour in XAD-4 cartridges and analyzed via gas chromatography. (c, d) Average daily TPM (c) and aerosolized nicotine (d) from ECIG and CS chambers were plotted together. Data are presented as mean \pm SEM. n=6-8 per day for ECIG over two independent experiments. n=3 per day for CS.

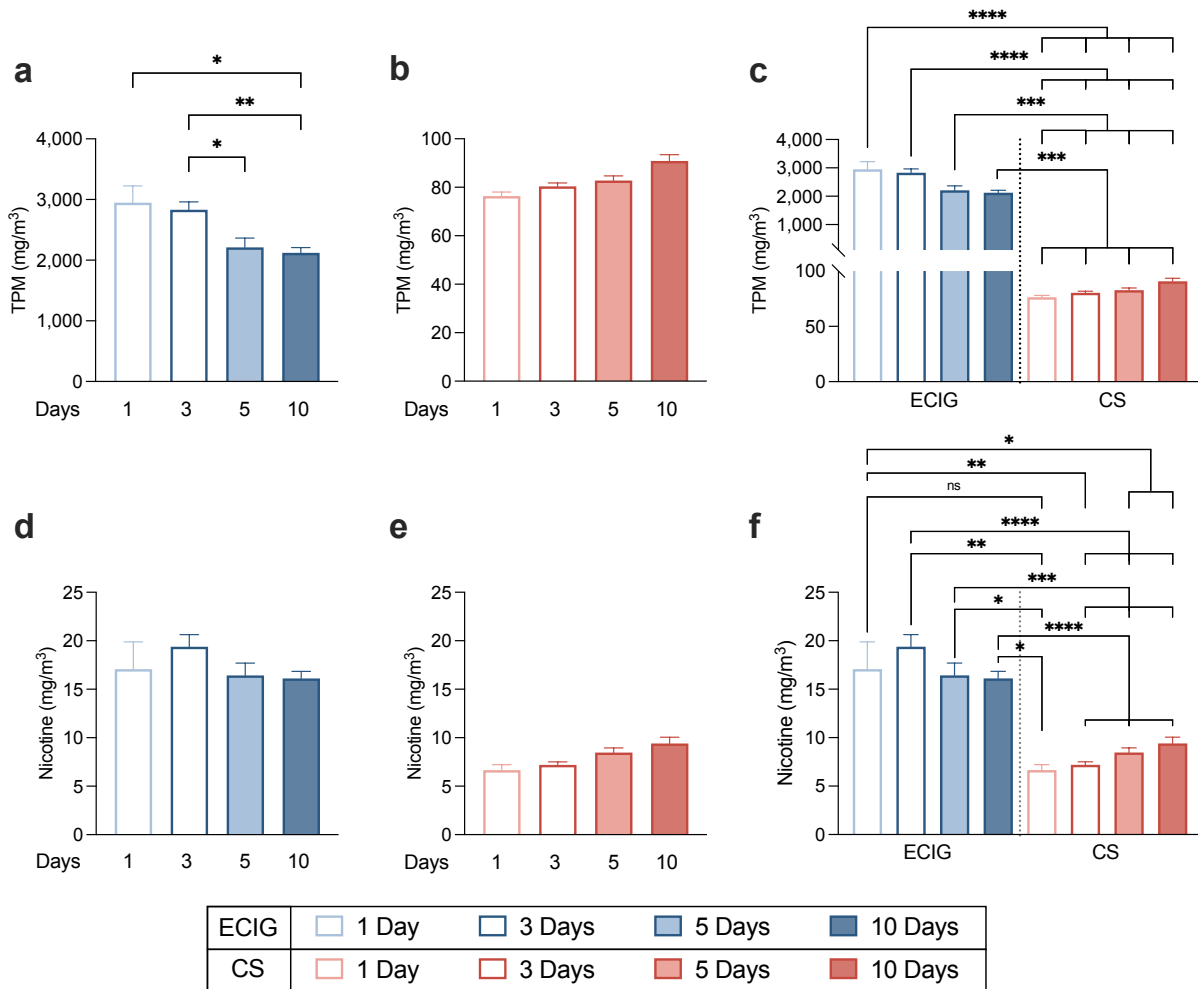


Figure 3.2: Cumulative mean TPM and aerosolized nicotine by exposure group. (a-f) Hourly TPM (a, b) and aerosolized nicotine concentrations (d, e) data, across all exposure days, were aggregated for each progressive exposure group and used to calculate cumulative mean exposure conditions of each group. Cumulative mean TPM (c) and nicotine (f) exposures for ECIG and CS were plotted together. Data are presented as mean \pm SEM. $n=3-6$ for 1 day; $n=9-18$ for 3 days; $n=15-30$ for 5 days; $n=30-62$ for 10 days. Statistical significance determined via one-way ANOVA with Tukey's correction for multiple comparisons. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

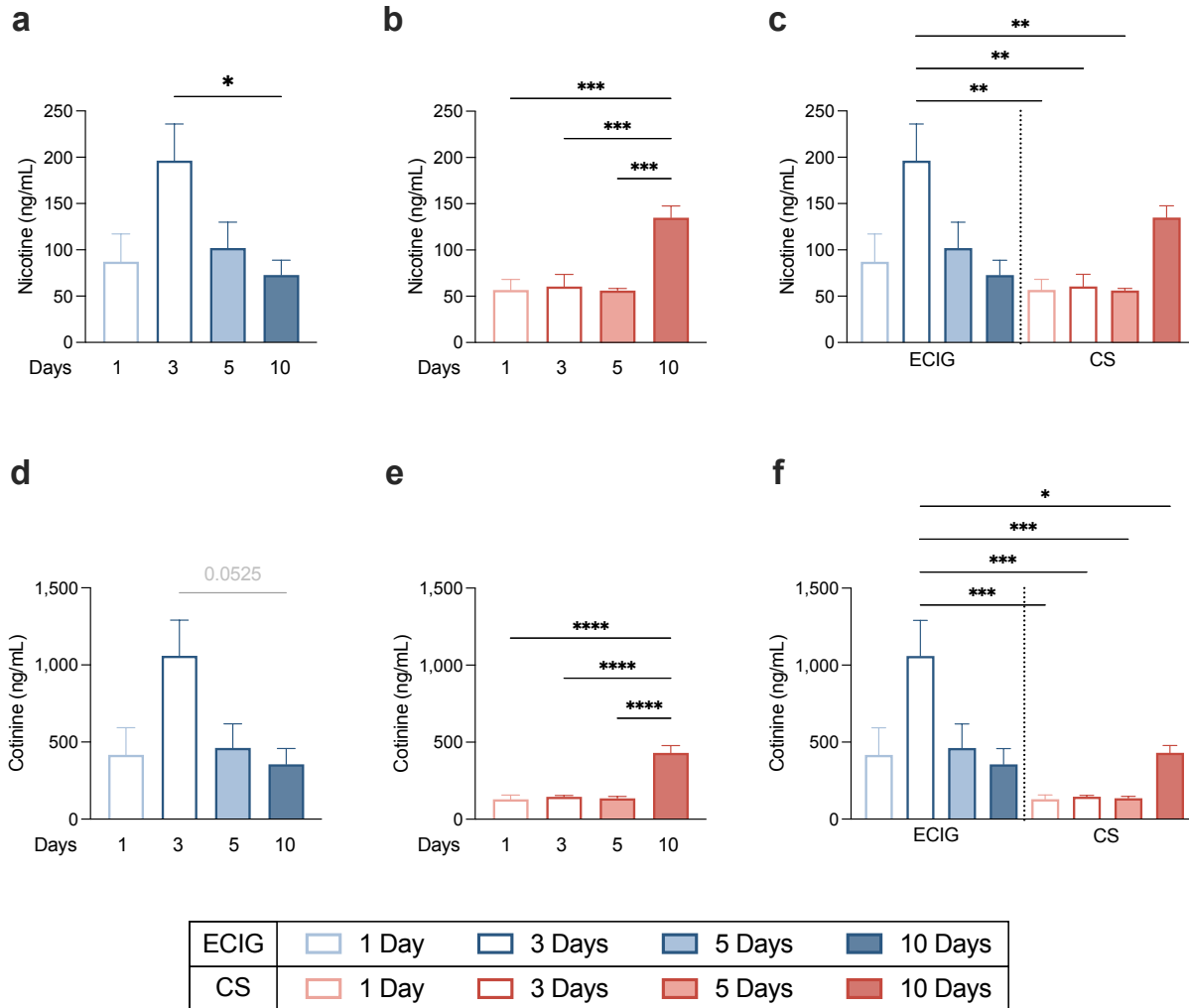


Figure 3.3: Serum nicotine and cotinine following progressive ECIG and CS exposure. (a- f)

Serum was collected immediately following the last day of exposures and analyzed via gas chromatography – mass spectrometry for nicotine (ng/mL; LOD: 20 ng/mL) (**a, b, c**) and cotinine (ng/mL; LOD 40 ng/mL) (**d, e, f**). n=6/group. Data are presented as mean ± SEM. Statistical significance determined via one-way ANOVA with Tukey’s correction for multiple comparisons.

*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

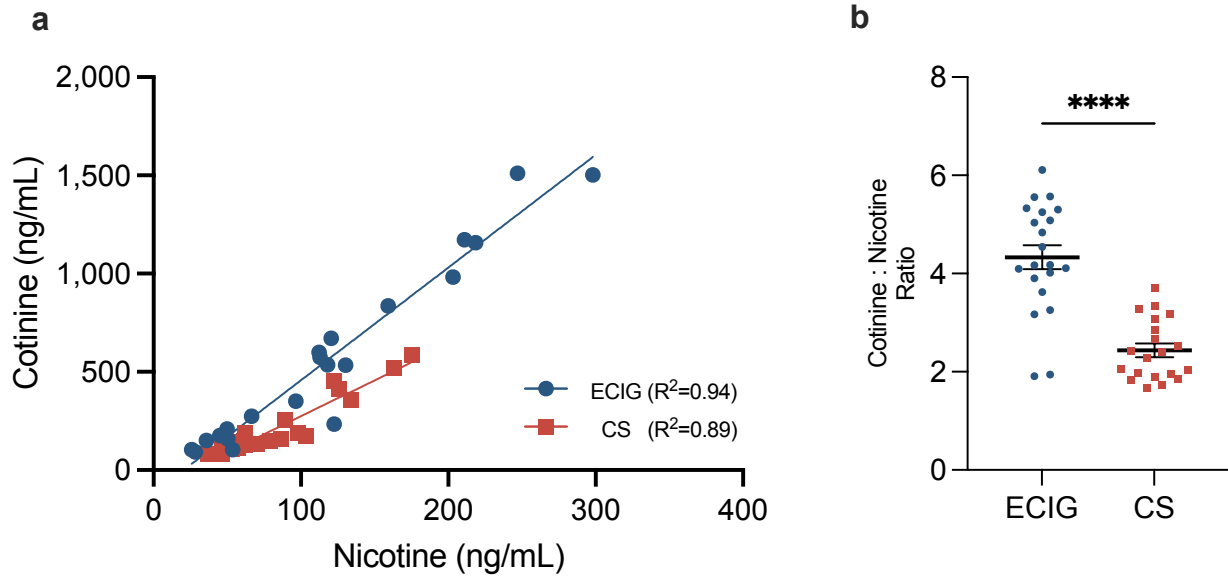


Figure 3.4: Nicotine metabolism following ECIG and CS exposure. (a) Serum nicotine and cotinine values were plotted against each other using linear regression. Each point represents a single mouse. (b) Cotinine and nicotine ratios were calculated. Data are presented as mean \pm SEM. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. $n=24$ per group where all timepoints (1, 3, 5, and 10 days of exposure) were aggregated together to determine ECIG vs. CS effects. **** $p<0.0001$

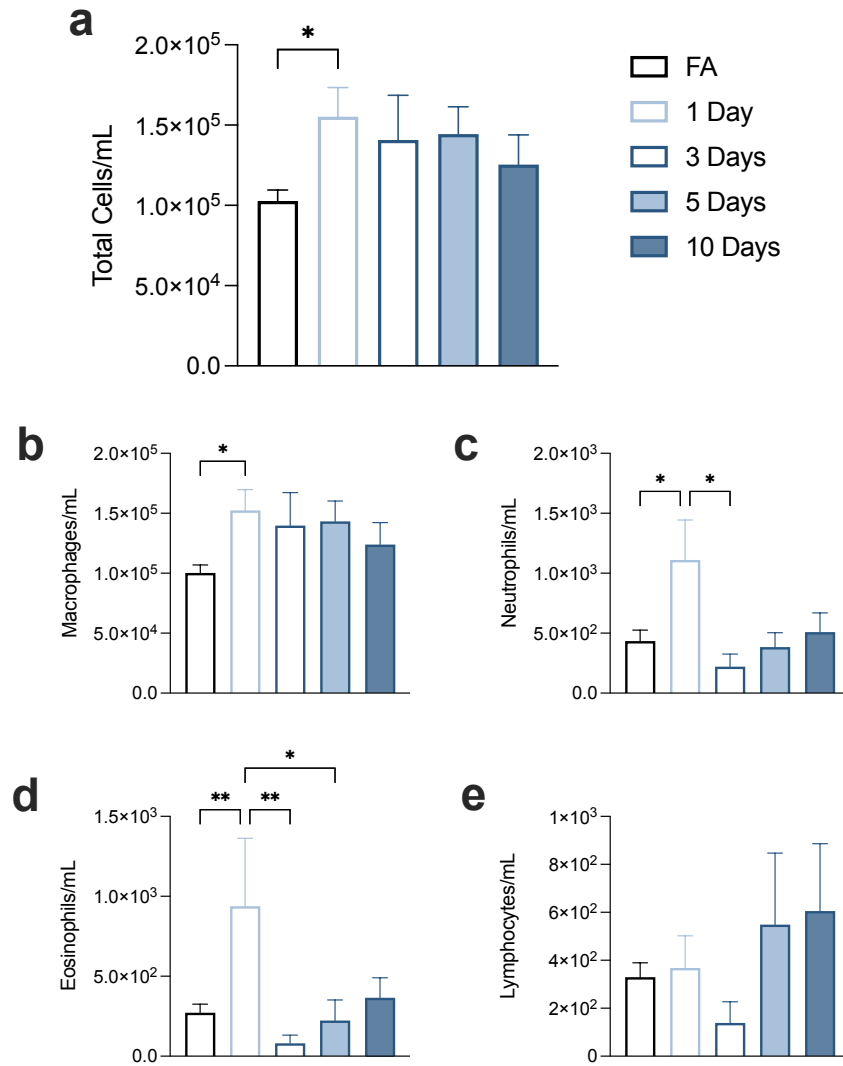


Figure 3.5: Leukocyte influx following progressive ECIG exposure. (a-e) Mice were placed in whole-body exposure chambers and exposed to FA or ECIG for three hours/day for 1, 3, 5 or 10 days. Bronchoalveolar lavage (BAL) was collected immediately following exposure. Data are presented as mean \pm SEM of total BAL cells (a), macrophages (b), neutrophils (c), eosinophils (d) and lymphocytes (e), as assessed by Cytospin. Statistical significance determined by one-way ANOVA with Tukey's correction for multiple comparisons. n=6 for ECIG groups; n=36 for FA. *p<0.05, **p<0.01.

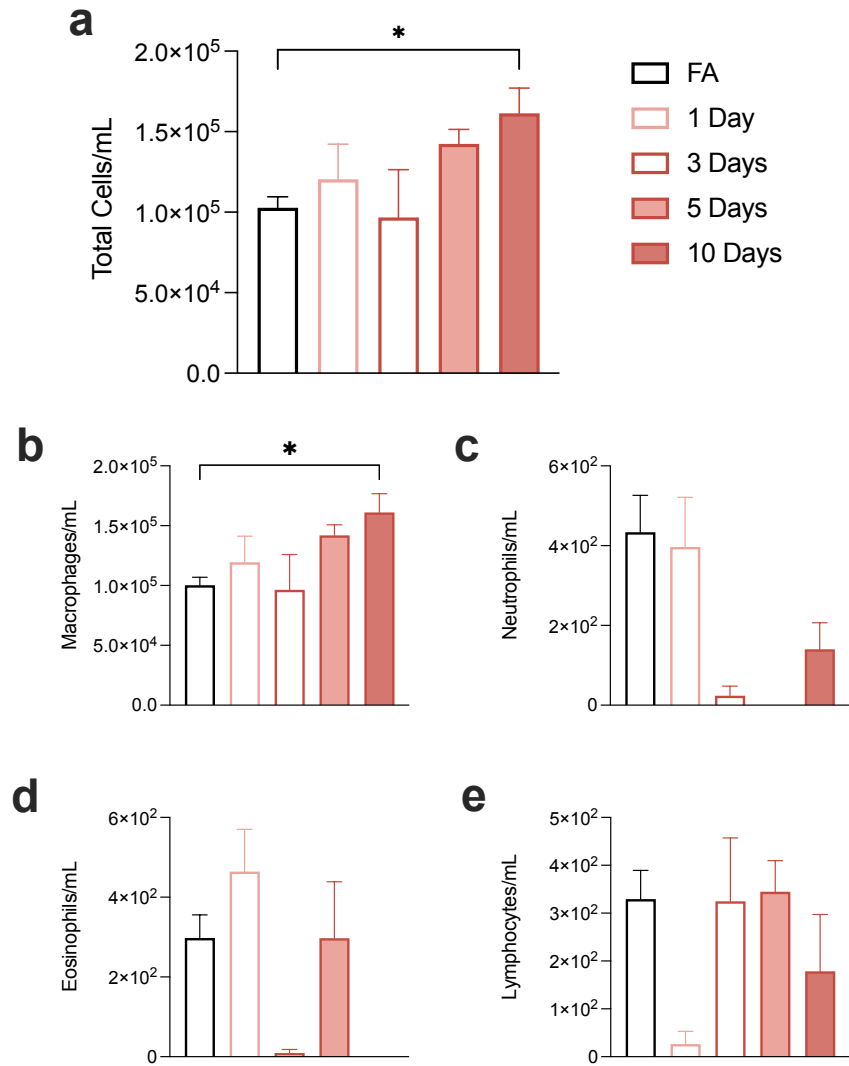


Figure 3.6: Leukocyte influx following progressive CS exposure. (a-e) Mice were placed in whole-body exposure chambers and exposed to FA or CS for three hours/day for 1, 3, 5 or 10 days. Bronchoalveolar lavage (BAL) was collected immediately following exposure. Data are presented as mean \pm SEM of total BAL cells (a), macrophages (b), neutrophils (c), eosinophils (d) and lymphocytes (e), as assessed by Cytospin. Statistical significance determined by one-way ANOVA with Tukey's correction for multiple comparisons. n=6 for ECIG groups; n=36 for FA. *p<0.05.

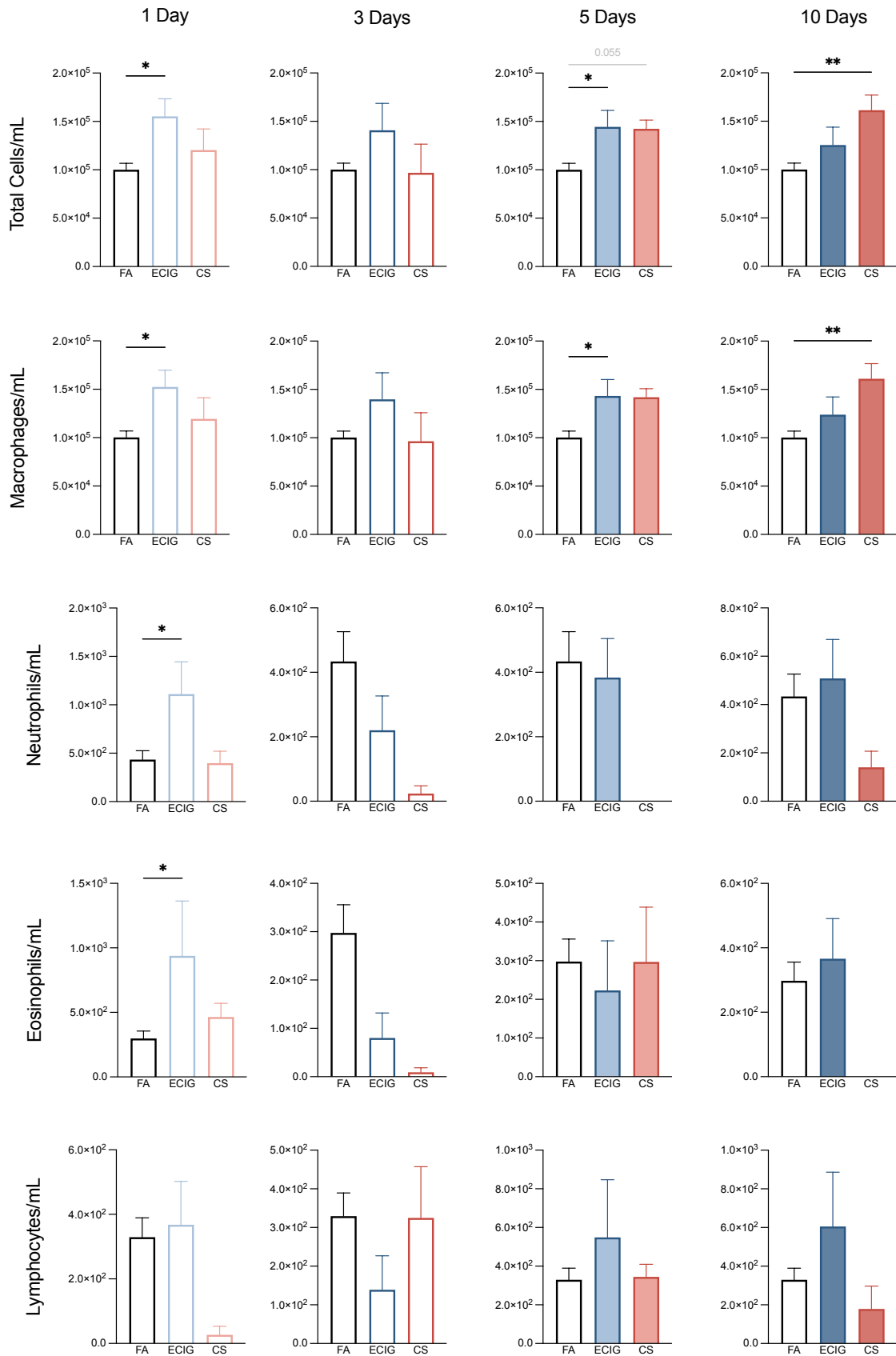


Figure 3.7: Comparison of leukocyte influx following ECIG and CS exposure. Mice were placed in whole-body exposure chambers and exposed to FA, ECIG, or CS for three hours/day for 1, 3, 5 or 10 days. Bronchoalveolar lavage (BAL) was collected immediately following exposure. Data are presented as mean \pm SEM of total BAL cells, macrophages, neutrophils, eosinophils, and lymphocytes, as assessed by cytopsin. Statistical significance determined by one-way ANOVA with Tukey's correction for multiple comparisons. n=6 for ECIG and CS groups; n=36 for FA. *p<0.05, **p<0.01.

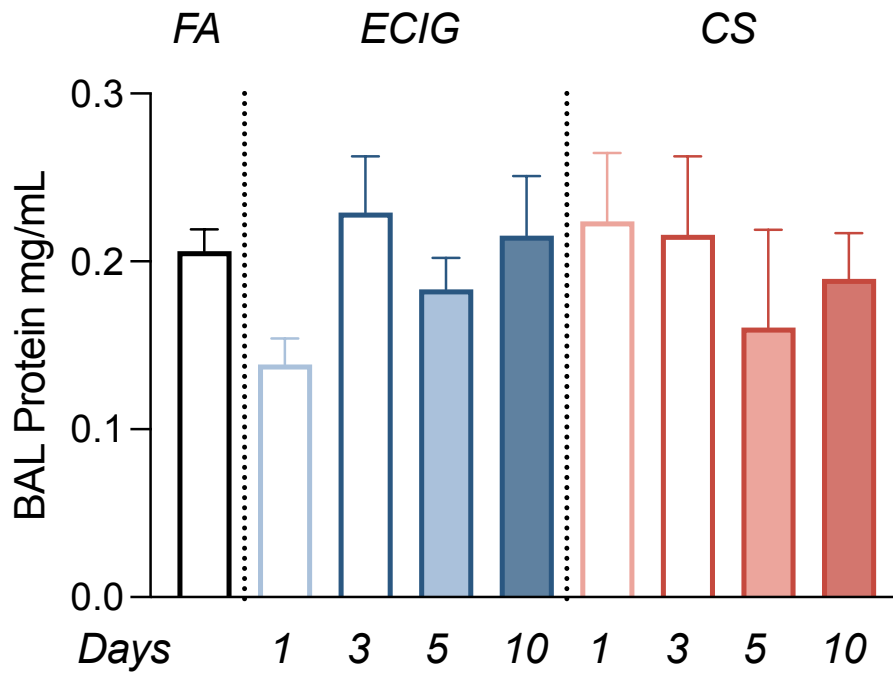


Figure 3.8: Progressive ECIG and CS exposure does not alter total BAL protein concentrations. Bronchoalveolar lavage (BAL) fluid was collected, and cell-free BAL supernatant was analyzed for total protein content by Lowry protein assay. Data are presented as mean \pm SEM. n=6 per group for ECIG and CS, n=36 for FA.

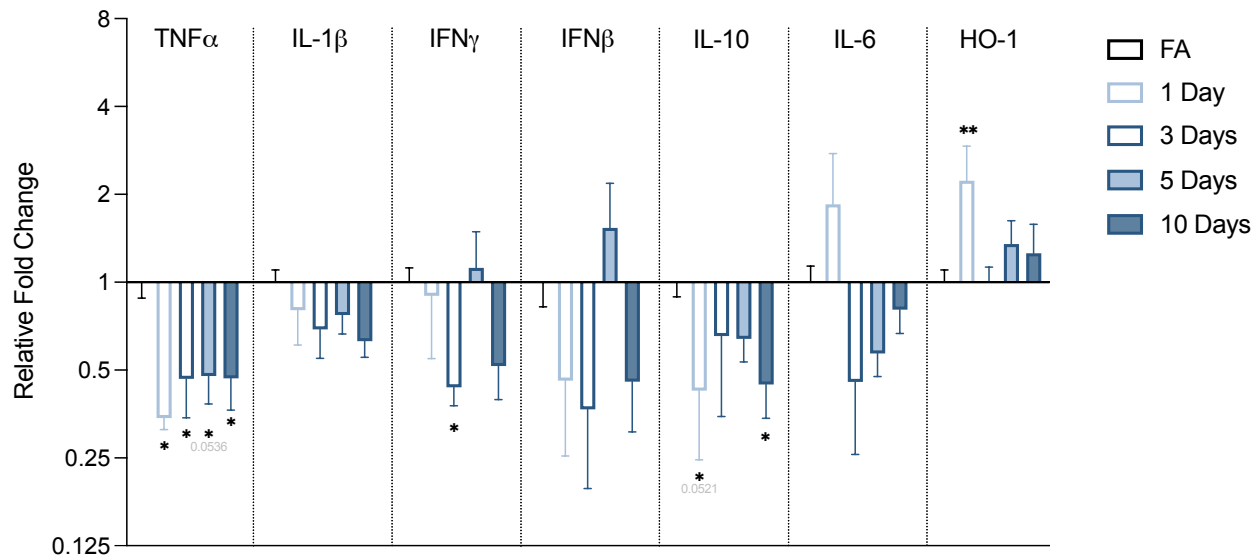


Figure 3.9: Inflammatory cytokine gene expression is significantly decreased in lung tissue following progressive e-cigarette exposure. Right lungs from mice exposed to ECIG for 1, 3, 5, and 10 days were homogenized, and mRNA gene expression was analyzed via RT-qPCR for TNF α , IL-1 β , IFN γ , IFN β , IL-10, IL-6 and HO-1. Data are presented as mean \pm SEM, relative fold change in expression from filtered air (FA) control mice. n=6 per group for all ECIG timepoints, n=24 for FA. Statistical significance determined by one-way ANOVA with Tukey's correction for multiple comparisons. *p<0.05 or as indicated with p value on figure, **p<0.01 from FA control.

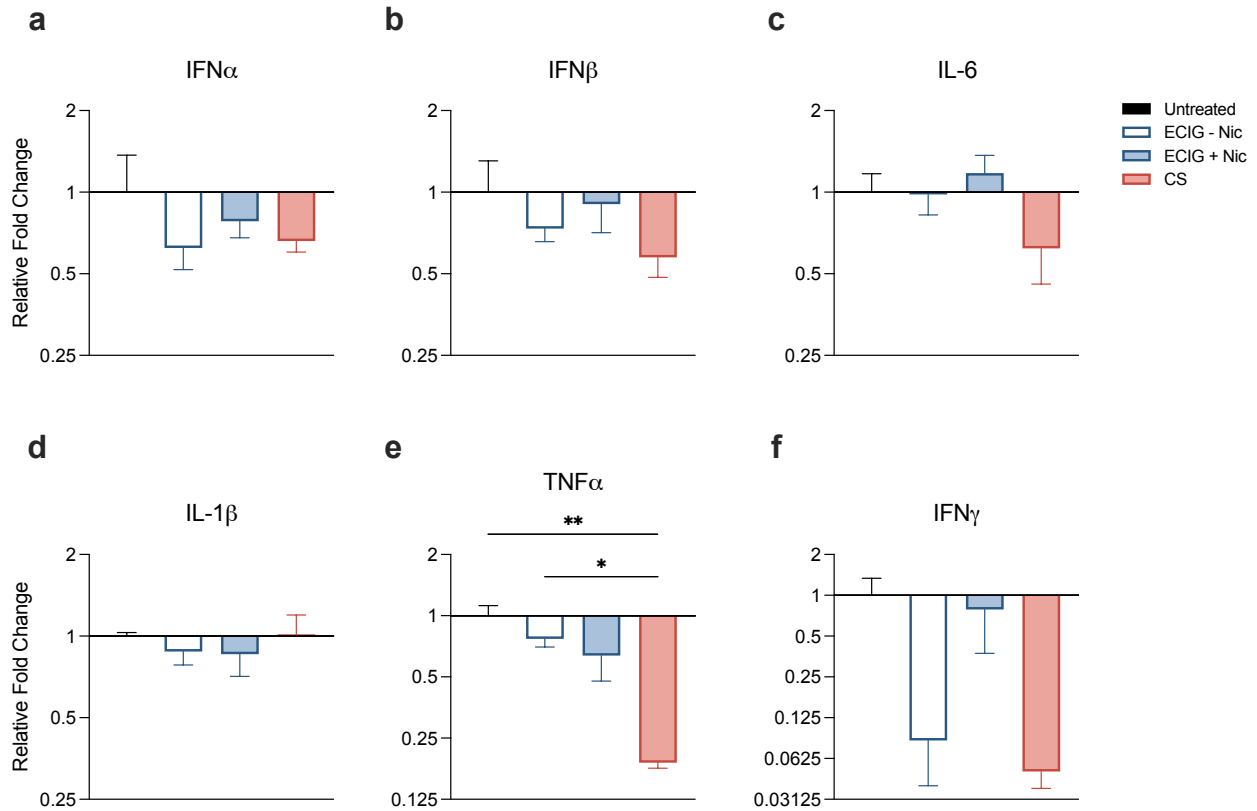


Figure 3.10: Cytokine gene expression in human macrophage-like cells. (a-f) U937 cells were cultured with untreated media, CS-conditioned media, or ECIG-conditioned media with or without nicotine for 24 hours. mRNA expression of IFN α (a), IFN β (b), IL-6 (c), IL-1 β (d), TNF α (e), and IFN γ (f) were analyzed via qRT-PCR. Data are presented as mean \pm SEM of two independent experiments each with n=3/group, relative fold change (\log^2) in expression from untreated controls. Statistical significance was determined by one-way ANOVA with Tukey's correction for multiple comparisons. *p<0.05, **p<0.01.

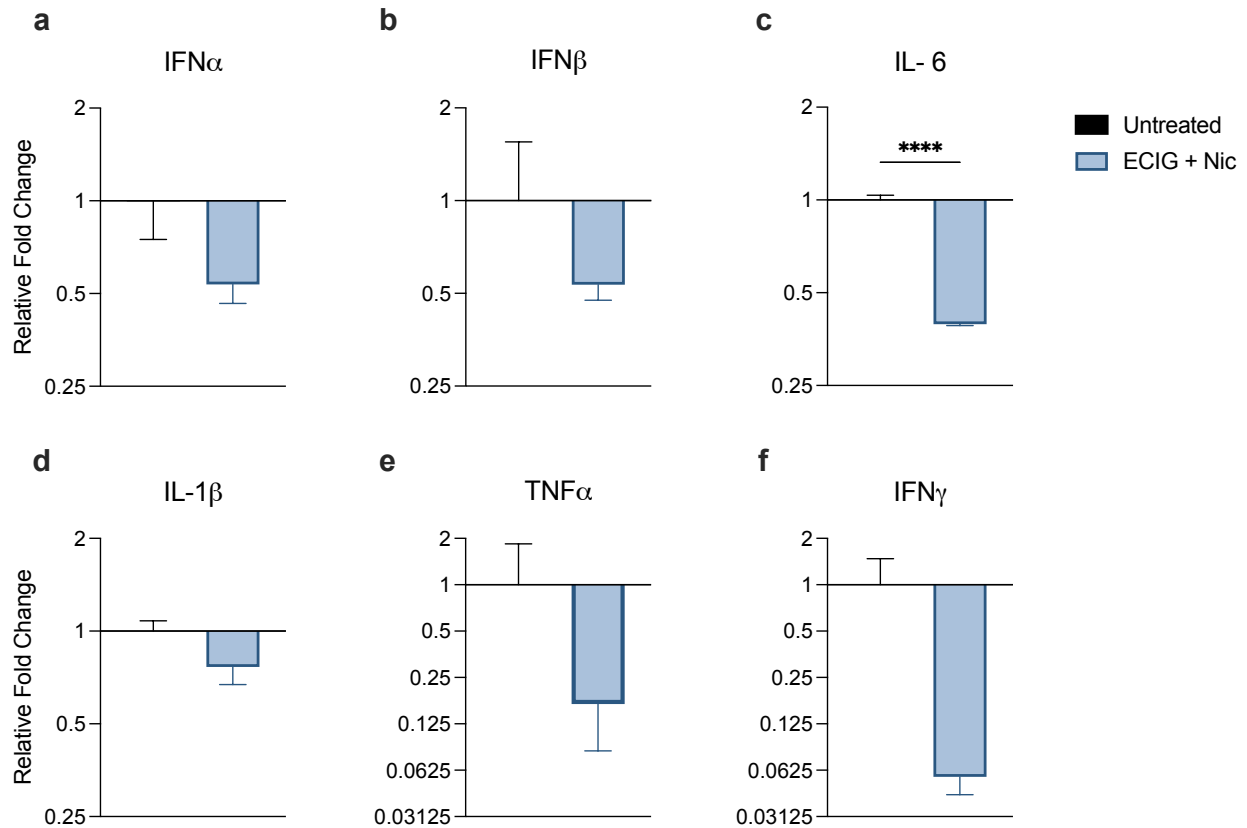


Figure 3.11: Cytokine gene expression in human lung epithelial cells. (a-f) A549 cells were cultured with or without ECIG-conditioned media with nicotine for 24 hours. mRNA expression of IFN α (a), IFN β (b), IL-6 (c), IL-1 β (d), TNF α (e), and IFN γ (f) were analyzed via qRT-PCR. Data are presented as mean \pm SEM of two independent experiments each with n=3/group, relative fold change (\log^2) in expression from untreated controls. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. ****p<0.0001.

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CHAPTER 4:

E-Cigarette Exposure Alters Pulmonary Immunity and Susceptibility to Influenza Infection in Young and Aged Mice

Abstract:

E-cigarettes, electronic nicotine delivery devices (ENDS) continue to gain world-wide popularity, with all age groups. This is despite evidence implicating vaping in numerous adverse health outcomes, such as increasing susceptibility to respiratory infections. In the United States, more than 2,800 hospitalizations and 68 deaths resulted from e-cigarette, or vaping, product use-associated lung injury (EVALI), and vaping status has been associated with higher COVID-19 positivity rates and symptoms. E-cigarette use impacts the pulmonary immune system by dysregulating cytokine and chemokine production and altering immune cell functions, which directly impair host defenses to respiratory viruses. However, the mechanisms of action and the effects of e-cigarette use in the aged remain unclear. To address this gap in knowledge, we developed a mouse model with which we investigated the effects of e-cigarette exposure on the pulmonary immune system and host responses to influenza A viral infection in both young (2-month-old) and aged (12-month-old) C57BL/6 and BALB/c mice. The studies demonstrated that e-cigarette exposure alone causes significant innate immune cell influx into the lungs, while blunting pro-inflammatory cytokine release, an effect enhanced in aged mice. Furthermore, e-cigarette exposure prior to infection, while decreasing mortality and reducing lung viral loads at 3 days post infection (dpi), resulted in delayed viral clearance. These changes correlated with significant shifts in both innate and adaptive lung leukocytes throughout the infection. Particularly striking were significant increases in Natural Killer (NK) cell populations in the lung early after infection, which occurred in both mouse strains and age groups, as well as alterations in the leukocyte and cytokine profiles, changes that persisted after resolution of infection. Together, the study demonstrates that acute e-cigarette exposure significantly increases

pulmonary inflammation to infections, indicating the potential detrimental effects of e-cigarette use on lung health.

Introduction:

E-cigarettes, first invented in 2003 as a smoking cessation aid, are electronic nicotine delivery systems (ENDS) which have gained world-wide popularity among cigarette smokers and non-smokers alike. Though first marketed as a completely safe alternative to traditional tobacco combustion products (cigarettes, cigarillos, and pipes), consumers have reported, and medical case studies have documented numerous adverse health outcomes in many organ systems associated with e-cigarette use (Hua et al., 2020; Tzortzi et al., 2020). The most common respiratory complications reported have been pneumonia and bronchiolitis with variable involvement of macrophages, neutrophils, or more rarely, eosinophils and lymphocytes (Tzortzi et al., 2020). In 2019, following an outbreak of hospitalizations and deaths among both nicotine and THC e-cigarette users, the disease: e-cigarette, or vaping, product use–associated lung injury (EVALI) was defined and characterized. To date, there have been more than 2,800 hospitalizations with EVALI resulting in 68 deaths in the United States. Additionally, during the 2020 COVID-19 pandemic, e-cigarette use was correlated with higher COVID-19 positive rates and increased reporting of symptoms during COVID-19 infection (Gaiha et al., 2020; McFadden et al., 2022). Taken together, e-cigarette use is capable of negatively impacting human health likely through the disruption or modulation of the pulmonary immune response.

Though the mechanisms of action are not fully elucidated, *in vitro* and *in vivo* studies using human cells and murine models reported significant alterations in lung immune cell populations and cytokine/chemokine production in response to e-cigarette exposure (Chatterjee et al., 2019; Crotty Alexander et al., 2018; Ganapathy et al., 2017; Higham et al., 2018; Husari, Shihadeh, Talih, Hashem, El Sabban, et al., 2016; T. Ma et al., 2021; Masso-Silva, Moshensky, et al., 2021; Scott et al., 2018; Vasanthi Bathrinarayanan et al., 2018; Q. Wang et al., 2019,

2020b; Ween et al., 2017a). Both pro- and anti-inflammatory effects have been reported, likely due to differences in exposure conditions. In agreement with the field, we previously showed that 10 days exposure to high nicotine e-cigarette aerosol from a third-generation e-cigarette device resulted in increases in both total leukocytes and specifically macrophages in bronchoalveolar lavage fluid (BALF), as well as decreases in proinflammatory cytokine gene expression in the lungs of young BALB/c mice (Poindexter et al., 2021).

It is still largely unknown how these immune dysregulations alter host responses to respiratory pathogens, but emerging evidence indicates their effect on the susceptibility to, and recovery from, viral and bacterial infections (Agraval et al., 2023; Corriden et al., 2020; Crotty Alexander et al., 2018; Gilpin et al., 2019; Hwang et al., 2016; Madison et al., 2019; Maishan et al., 2023; Rebuli, Brocke, et al., 2021; Schaunaman et al., 2022; Scott et al., 2018; Sivaraman et al., 2021; Sussan et al., 2015; Ween et al., 2017a; Wu et al., 2014). Notably, a recent observational cohort study found that e-cigarette users had a substantial downregulation of critical host defense mediators and reduced IgA antibody levels after live-attenuated influenza virus (LAIV) vaccination (Rebuli, Glista-Baker, et al., 2021). Of concern, flavoring chemicals and additives commonly found in e-cigarette liquid (e-liquid) also have been found to be mediators of inflammation, which can alter susceptibility to viral infection (Day et al., 2023; Langel et al., 2022; Szafran et al., 2020b). However, more research is needed to fully elucidate the potential human health implications of these findings.

Due to concerns that commercialization of vaping specifically targets young children and teens with colorful marketing or social media (Struik et al., 2020) and enticing dessert-like sweet or fruity flavored e-liquids (King, 2020), the majority of research into age, as it relates to vaping, focuses on adolescents. Investigations into the health effects of e-cigarette use in middle-aged or

aging populations are less common. This is despite a growing number of adult e-cigarette users. In the United States, current e-cigarette users aged 25-44 increased 59% from 3.63 million to 5.78 million from 2018 to 2021 (E. Kramarow & Elgaddal, 2023; Villarroel et al., 2020). Over the same period, current e-cigarette users over the age of 45 increased 28% from 2.18 million to 2.79 million. Adults are more likely than adolescents to use e-cigarettes as smoking cessation tools (Dahal et al., 2022), despite a lack of evidence to support their efficacy in this capacity (R. Chen et al., 2022; Hartmann-Boyce et al., 2021; Quigley et al., 2021). Indeed, increasing age has been correlated with decreased harm perception of both e-cigarettes and conventional tobacco cigarettes (Rubenstein et al., 2023). Furthermore, the majority (73%) of EVALI deaths occurred in adults over the age of 35 (median age: 51 years) (Werner et al., 2020). Yet, to date, there is limited information on the pulmonary effects of vaping on middle-aged or aging populations.

The aims of this study are to investigate the effects of e-cigarettes on the pulmonary immune system and to compare, for the first time, how this impacts host responses to influenza A virus (IAV) infection in both young (2-month-old) and aged (12-month-old) C57BL/6 and BALB/c mice. We characterized the immune cell composition of lungs and BAL during infection using flow cytometry to identify 15 immune cell types. We demonstrate that e-cigarettes alone can dysregulate airway immune cell influx and perturb cytokine production in airways, and that these effects are capable of significantly altering pulmonary immune responses and immune cell composition of BAL and lungs during influenza infection.

Materials and Methods:

E-Cigarette Exposures. E-cigarette aerosols were generated into a whole-body exposure chamber using an automated three-port E-cigarette aerosol generator (e~Aerosols LLC, Central Valley, NY, USA) utilizing a second-generation E-cigarette device with E-Smart Clearomizer tanks (KangerTech) and 1.8 Ω NiCr E-Smart coils (KangerTech, Shenzhen, China) operated at 4.7V and 0.85 L/min flow rate through the device to generate 2 puffs (4 seconds/puff) per minute. During exposures, coils were inspected daily for signs of burning from overheating and were replaced every 5 days (following approximately 1,800 puffs). Pure propylene glycol, vegetable glycerin and nicotine (>99% purity, Sigma-Aldrich Inc., St. Louis, MO) were combined to make a 50/50 PG/VG (% w/v) e-liquid containing 24 mg/mL nicotine. The flow rate through the chamber was adjusted to maintain a 400 mg/m³ aerosol concentration (**Fig. 1a**). Aerosolized nicotine concentrations of 2.5 mg/m³ were observed, resulting in 12 ng/mL serum nicotine concentrations in exposed mice (**Fig. 1b**).

Exposure Characterization. Aerosol concentrations (mg/m³) were measured every hour during exposure using a DryTest Meter to measure precise air volumes. Filters were used to collect chamber air samples to gravimetrically determine particulate mass concentrations during sample collection. Nicotine samples were collected every hour during exposure on XAD-4 sorbent tubes (226-30-11-04-GWS, SKC West, Fullerton, CA) then extracted and analyzed via gas chromatography (Varian 3740). Nicotine concentrations (mg/m³) were calculated via area ratio of nicotine/quinoline in each sample.

Animal Protocol. This study was conducted in compliance with regulations set by the University of California, Davis Institutional Animal Care and Use Committee (IACUC) following NIH guidelines. Male and female C57BL/6 and male BALB/c mice (6-8 weeks old or 12 months old) were purchased from Envigo. Animals were housed 3 per cage in a 12-h light/ 12-h dark cycle with Purina 5001 regular laboratory rodent diet (Newco Distributors, Rancho Cucamonga, California) and water provided ad libitum. 3-6 mice of each sex were randomly assigned to each of the treatment and control groups to generate groups of 6-12 animals.

Mice in polycarbonate cages with wire lids were placed in FA or ECIG whole-body exposure chambers for 3 hours/ day, 5 days/ week for 10 days. Mice were euthanized via 0.2 mL intraperitoneal injection of Beuthanasia-D pentobarbital solution (65 mg/kg body weight; Nembutal Cardinal Health, Sacramento, CA). Cardiac puncture was performed on each animal, and blood was collected into a 1.5 mL microcentrifuge tube. The tube was maintained for 20 minutes at room temperature to allow for coagulation, and then centrifuged for 15 minutes at 3,000 rpm. Serum was collected (100 μ L aliquots) and stored at -80 °C.

For gas chromatography – mass spectrometry (GC-MS) analysis of nicotine concentrations in serum (ng/mL), serum samples from mice euthanized immediately following filtered air (FA) or e-cigarette (ECIG) exposure on day 10 of exposure were sent to University of California San Francisco (San Francisco, CA).

Influenza Infections. After anesthetization with isoflurane, mice were intranasally infected with a previously determined sub-lethal dose (10 PFU in 40 μ l PBS) of influenza A/Puerto Rico/8/34 (A/PR8). As previously described (Doucett et al., 2005), virus was grown in hen eggs and each batch was titrated to target <20% weight loss in mice. Beginning at 0 days post infection (dpi),

mice were weighed at the same time daily for the duration of the experiment. A loss of 25% original body weight was established as a humane endpoint.

Bronchoalveolar Lavage. Following cardiac puncture, the trachea was carefully cannulated with a 22-gauge blunt end needle that was sutured in place. Whole lungs were lavaged twice with 0.8 mL of sterile phosphate-buffered saline (PBS, Sigma Aldrich, St.Louis, MO). Each aliquot was intratracheally instilled and recovered 3 times before collecting the bronchoalveolar lavage fluid (BALF). The two BALF samples were combined and centrifuged for 15 minutes at 2,000 rpm at 4°C. BALF supernatant was aliquoted and stored at -80 °C for inflammatory cytokine analysis. The BAL cell pellet was resuspended in 1.5 mL of PBS. Cell counts and cell viability were determined via Trypan Blue using a hemocytometer. Cytospin slides were prepared using an aliquot of 100uL of cell suspension stained with Diff-Quik for cell differentials. The remaining cells were immediately processed into a single-cell suspension for flow cytometry. Following collection of BALF, whole lungs were collected and immediately processed into a single-cell suspension for flow cytometry. Cell counts were determined via Trypan Blue using a hemocytometer.

Flow cytometry. Single-cell suspensions from BALF and whole lungs were enzymatically digested and purified via Percoll gradient centrifugation and then labeled for flow cytometry, as previously described (Doucett et al., 2005). Briefly, following Fc receptor block with anti-CD16/32 (5 mg/ml for 20 min on ice) and Live/dead Fixable Aqua (Thermo Fisher, L34957), BALF and lung samples were adjusted to 2.5×10^7 cells/mL. 25µL of the cell suspension was stained using two antibody-fluorophore conjugate panels, previously optimized to identify most

innate and adaptive immune cell types. Prior to use, reagents were titrated to achieve the highest differential fluorescence intensity between the negative and positive cell fractions. Dilutions varied between reagents and reagent lots, but typically fell between 1:25 and 1:400. Higher concentrated reagents (mostly those made in-house) were kept prediluted at a concentration that allowed a 1:200 dilution at use.

Panel 1(Innate / B cell panel): FITC anti-CD11b (M1/70) (Biolegend, 101206), APC anti-CD11c (N418) (Biolegend, 117310), PE-CF594 anti-CD19 (1D3) (BD Bio, 562291), BV605 anti-CD21/35 (7G6) (BD Bio, 563176), BV711 anti-CD23 (B3B4) (BD Bio, 563987), APC-eFluor780 anti-CD49b (DX5) (Thermo Fisher, 47-5971-82), PE/Cy7 anti-CD317 (ebio927) (Thermo Fisher, 25-3172-82), PE/Cy5 anti-F4/80 (BM8) (Biolegend, 123112), vFluor450 anti-Ly6G (RB68C5) (Tonbo, 75-5931-U100), BV785 anti-Ly6c (HK1.4) (Biolegend, 128041), Alexa 700 anti-MHCII (M5/114.15.2) (Thermo Fisher, 56-5321-82), BV650 anti-CD43 (S7) (BD Bio, 56-5321-82), and PE anti-CD5 (53-7.3) (Biolegend, 100608).

Panel 2: (T cell/NK cell panel): Alexa 700 anti-CD3 (ebio500A2) (Thermo Fisher, 56-0033-82), PE-CF594 anti-CD4 (GK1.5) (Biolegend, 100456), PE anti-CD5 (53-7.3) (Biolegend, 100608), eFluor450 anti-CD8 (53-6.7) (Thermo Fisher, 48-0081-82), FITC anti-CD25 (PC61.5) (Tonbo, 35-0251-U100), BV786, anti-GITR (DTA-1) (BD Bio, 741020), BV711 anti-TCR delta (GL3) (BD Bio, 563994), APC-eFluor780 anti-CD49b (DX5) (Thermo Fisher, 47-5971-82), PE/Cy7 anti-CD44 (IM7) (Biolegend, 103029), and APC anti-CD62L (MEL-14) (Biolegend, 104411).

Data were collected on BD LSR II Fortessa cytometer with BD FACSDiva software and subsequently analyzed using FlowJo v10 software. For each sample, results from the two panels were combined and the absolute number of each cell type per mL of BAL or per lung was

determined. Each sample was verified for accuracy by ensuring the sum frequencies of all identified cell types did not exceed 100% and further verified via cross-referencing of T cell and NK cell frequencies of total white blood cells (WBCs) between the two panels.

Viral Load RT-PCR. At 3- or 7-dpi, mice were euthanized, and lung tissue was harvested, and homogenized in 1 mL PBS using Gentle MACS (Miltenyi Biotech). Lung tissue was pelleted, and supernatant was aliquoted and stored at -80 °C. Viral RNA was purified from lung homogenate supernatants using the QIAamp viral RNA mini-kit (Qiagen) and RNA purity was verified via NanoDrop One (Thermo Fisher). Influenza M gene was detected by RT-PCR gene amplification with AM-151 (5'-CATGCAATGGCTAAAGACAA GACC-3') and AM-397 (5'-AAGTGCACCAGCAGAATAACTGAG-3') primers and primer/probe AM-245 (6FAM-5'-CTGCAGCGTAGAGCTTTGTCCAAAA TG-3'-TAMRA) using TaqPath 1-Step Multiplex Master Mix (Thermo Fisher, A28525) on Applied Biosystems QuantStudio 6 Flex system. For quantification, standards were generated using A/PR8 virus stock (3×10^8 PFU/mL).

Inflammatory Cytokine Quantification. Cytokines in serum and BALF were quantified using the Meso Scale Discovery MULTI-SPOT V-PLEX® Cytokine Assay System Pro-Inflammatory Panel 1 (mouse) Kits (K15048D - Meso Scale Diagnostics LLC, Rockville, MD) per manufacturer protocol. Briefly, serum or BALF and standards for interferon gamma (IFN- γ), interleukin-10 (IL-10), interleukin-12 (IL-12p70 active heterodimer), interleukin-1 beta (IL-1 β), interleukin-2 (IL-2), interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), chemokine C-X-C motif ligand 1 (CXCL1, aka KC/GRO), and tumor necrosis factor alpha (TNF- α) were loaded onto sample plates pre-coated with cytokine capture antibodies. Plates were incubated for

2 hours at room temperature, washed, then detection antibody was added to each well and incubated for 1 hour at room temperature. Lastly, plates were washed, read buffer was added to each well, and plates were analyzed on an MSD QuickPlex SQ 120 instrument (MSD, AI0AA-0). Using linear regression analysis of standard curves, cytokine concentrations were calculated on the accompanying Discovery Workbench (v. 4.0) software.

Statistical Analysis. Statistical analysis was performed with the help of Graph Pad Prism 9 software. ROUT(Q=1%) was used to remove outliers. Analysis between two groups was done using two-tailed Student's t-test with Welch's correction. Analysis between three or more groups was done using one-way ANOVA with multiple comparisons. Unless otherwise specified, data is presented as the mean +/- SEM.

Results:

E-Cigarette exposure results in macrophage recruitment to the lungs and downregulation of key inflammatory cytokines in both young and aged C57BL/6 mice

Compared to FA controls, 10 days of e-cigarette (ECIG) exposure resulted in significant increases in total BALF cells, primarily driven by macrophage infiltration, in young, but not aged mice, as assessed by cell counting and Cytospin analysis (**Fig. 2a, b**). While aged mice experienced a moderate increase in total BALF cells as a result of ECIG exposure, these increases did not reach statistical significance. Numbers of neutrophils and lymphocytes were not measurably affected by ECIG for either age group (**Fig. 2b**). Similarly, relative proportions (percentages) of cell types remained unchanged after ECIG exposure (**Fig. 2c**). Pro-inflammatory cytokines IL-1 β , TNF α , IFN γ , IL-12p70 and IL-5 were significantly reduced in the BAL of both young and aged mice exposed to ECIG compared to FA control mice (**Fig. 2d**). Young ECIG exposed mice also saw significant reductions in IL-6 and IL-4, which were not seen in aged mice. Additionally, young ECIG exposed mice had a significant increase in the anti-inflammatory cytokine IL-10 and a significant decrease in IL-1 β in the serum, suggesting potential systemic effects of ECIG on immune system homeostasis (**Suppl. Fig. 1**).

Taken together, these data indicate that in both young and aged populations ECIG exposure can alter pulmonary immune cell populations in homeostasis, as evidenced by innate immune cell influx to the airways accompanied by overall reductions in local pro-inflammatory cytokines. Of note, both total cell and macrophage recruitment to the lungs as well as suppression of cytokine responses to ECIG were more pronounced in young C57BL/6 mice than in aged mice, indicating that older age alone may not significantly increase health risks associated with vaping.

E-Cigarette exposure prior to infection reduces early viral titers and boosts survival from influenza A virus

To determine if the observed changes to pulmonary inflammation from ECIG exposure could alter anti-viral responses, mice were exposed to ECIG or FA for 10 days prior to i.n. inoculation with a sub-lethal dose (10 PFU) of murine H1N1 influenza (A/PR8) or PBS for mock infections (**Fig. 3a**). FA and ECIG exposed mice of both ages exhibited similar morbidity during infection, evidenced by 13-15% initial body weight loss (**Fig. 3b, c**) despite aged mice weighing significantly more than young mice at the time of infection (mean of 30.3 g vs 23.9 g) (**Fig. 3d**). Both young FA and ECIG exposed mice lost significant weight compared to mock infected controls at 5 dpi, while aged FA and ECIG exposed mice did so at 6 dpi. Although both young and aged ECIG mice exhibited lower lung viral loads at 3 dpi than their FA counterparts, this trend was not statistically significant (**Fig. 3e**). Viral loads were similarly unchanged by prior ECIG exposure at the later 7 dpi timepoint. As expected, young FA mice showed a significant reduction in viral load from 3 to 7 dpi, indicating predicted viral clearance as the infection progressed and adaptive immune responses grew. However, young ECIG exposed mice did not see significant reductions in viral loads over this time. Of note, aged ECIG exposed mice had slightly higher viral loads at 7 dpi than at 3 dpi. Indeed, compared to FA mice, ECIG mice of both ages had higher ratios of viral loads at 7 dpi relative to 3 dpi (**Suppl. Fig. 2**). In young mice, this trend was significant ($p=0.0209$) indicating delayed or stunted viral clearance.

Because young mice had a more pronounced pulmonary response to ECIG alone compared to aged mice, young C57BL/6 mice were used to further investigate if recovery from influenza infection was affected by prior e-cigarette exposure. As before, mice were inoculated with 10 PFU immediately following 10 days of FA or ECIG exposure and weighed before and

daily after infection until 14 dpi. While both groups showed similar weight at the time of infection, ECIG exposed mice weighed significantly more than FA exposed mice at 4 dpi (99.7% vs 95.9%) (**Fig. 4a, c**). Compared to mock infected controls, ECIG exposed mice lost significant weight later and recovered faster than their FA exposed counterparts (**Fig. 4a**). Despite these kinetic differences, the magnitude of weight loss was unchanged by prior ECIG exposure. ECIG exposure prior to infection did minimally boost survival probability (66.7% vs 50% mortality), though this was not statistically significant (**Fig. 4b**). Interestingly, cytokine profiling revealed that after recovery from infection (14 dpi) IL-1 β , TNF α , and IL-10 were significantly elevated in the serum of ECIG exposed mice compared to FA exposed controls (**Fig. 4d**) while local cytokine concentrations in BALF were unchanged (**Fig. 4e**). Taken together, these data indicate that acute exposure to ECIG can modestly affect morbidity, mortality, and viral load during influenza infection and modify systemic cytokine levels up to 2 weeks after the end of exposure.

Because C57BL/6 mice are known to be more resistant to infection with the A/PR8 influenza virus strain than BALB/c mice, we repeated these experiments with the more sensitive BALB/c mouse strain. In contrast to young C57BL/6 mice, 10 days of ECIG exposure in young BALB/c mice caused significant increases in neutrophils in BAL but no changes in macrophage, lymphocyte, or total cell numbers (**Fig. 5**). Aged BALB/c mice exhibited an increase in total BAL cells, primarily driven by macrophage infiltration to the airways, as a result of ECIG exposure (**Fig. 5a, b**). Unlike C57BL/6 mice who had similar morbidity and viral loads regardless of age (**Fig. 4**), aged BALB/c mice had significantly reduced morbidity, mortality and viral loads compared to young BALBc mice. At 7 dpi, aged BALB/c mice lost 4-7% of initial body weight (**Fig. 6b**), whereas young BALB/c mice lost 17-20% (**Fig. 6a**). Aged BALB/c, both FA and ECIG exposed, had 100% survival to 7 dpi whereas young FA exposed BALB/c mice

saw only 50% survival (**Fig. 6c**). As expected, aged BALB/c mice weighed significantly more than their younger counterparts (data not shown) which could explain the significantly reduced severity of infection in that age group.

Despite the clear age-associated discrepancies in disease severity, both young and aged BALB/c mice showed similar trends to C57BL/6 mice as a result of ECIG exposure. Young ECIG exposed BALB/c mice initially gained significant weight in the first three days of infection, causing their morbidity to lag behind FA exposed mice by 2 days (**Fig. 6a**). By 7 dpi, no significant differences in weight loss were seen between young FA and ECIG exposed mice. Interestingly, aged ECIG exposed BALB/c mice never lost significant weight compared to mock infected controls, while FA exposed aged BALB/c mice did so beginning on day 5 post-infection (**Fig. 6b**). Indeed, both young and aged ECIG exposed BALB/c mice had significantly lower lung viral loads at 3 dpi compared to their FA exposed equivalents (**Fig. 6d**). Aged, but not young, BALB/c mice also saw reductions in viral loads at 7 dpi as a result of ECIG exposure ($p=0.0513$). In accordance with the significantly reduced lung viral loads, young ECIG exposed BALB/c mice experienced a significant boost in probability of survival to 7 dpi compared to FA (100% vs 50%, respectively) (**Fig. 6c**).

Together, these data suggest that in BALB/c mice ECIG exposure prior to infection reduces or delays morbidity from influenza infection, correlated with an early reduction in viral titers. For young BALB/c mice the ECIG-induced effect was strong enough to significantly increase survival probability. These trends of delayed morbidity, reduced mortality and reduced early infection lung viral titers were observed for both mouse strains.

E-Cigarette exposure significantly alters the immunocompetent cellular composition of lungs and BAL

To further characterize the ECIG-induced alterations to immune cell populations during infection, airways and lung parenchyma were analyzed by flow cytometry to determine the frequency of 15 immune cell populations – composed of 7 innate cell subsets, 3 B cell subsets, and 5 T cell subsets (**Table 1**) at 3 and 7 dpi (**Fig. 7**). Gating strategies outlined in Fig. 8 identify macrophages, neutrophils, monocytes, NK cells, eosinophils, pDCs, cDCs, follicular B cells, NKB cells, and B1 cells (**Fig. 8a, b**) and $\gamma\delta$ T cells, NKT cells, Tregs, CD4⁺ T cells, and CD8⁺ T cells (**Fig. 8a, c**). Results are summarized for each compartment and for major cell populations in **Figs. 9-18**. In addition, we analyzed the cell compositions of bronchoalveolar lavage (mean \pm SEM) and generated statistical comparisons between FA and ECIG exposed groups of young and aged mice (**Tables 2 and 3**). Complete cell compositions of lung (mean \pm SEM) and statistical comparisons between FA and ECIG exposed groups of young and aged mice are described in **Tables 4 and 5**, respectively.

Those data showed strain differences between naïve C57BL/6 and BALB/c mice in both airway and lung compartments. Notably, BALB/c mice had significantly more neutrophils in both airways and lungs than C57BL/6 mice (**Fig. 9b, d** and **Fig. 10b, d**), consistent with the Cytospin findings (**Fig. 2b** and **Fig. 5b**). BALB/c mice also had significantly fewer total B cells in both airways and lungs (**Fig. 15b** and **Fig. 16b**), and significantly fewer classical dendritic cells (cDC) in airways than C57BL/6 mice (**Fig. 13a, Fig 14a, and Tables 2-5**).

As expected, after infection with influenza virus, cellular influx to BAL was significantly increased compared to mock infected controls (**Fig. 9a, c** and **Fig. 10a, c**). Innate cells, primarily macrophages and neutrophils, were most abundant early in infection peaking at 3 dpi and

contracting significantly by 7 dpi. Lymphocytes, primarily CD8⁺ T cells, rapidly expanded by 7 dpi such that they comprised the majority of cells in the airways at that timepoint (**Fig. 9b, d** and **Fig. 10b, d**). In young C57BL/6 mice, ECIG exposure prior to infection significantly increased total BAL cell counts at 3 dpi (**Fig. 9a**). Additionally, as expected, in young mice cellular influx to lungs was significantly increased during infection, peaking at 7 dpi (**Fig. 11a, c**). This trend was not consistently seen in aged mice (**Fig. 12a, c**). Cellular profiles of lung parenchyma leukocytes were more stable during infection than those in airways. However, similar early macrophage and neutrophil expansions followed by lymphocyte expansions were seen. ECIG exposure prior to infection did not affect total lung cell counts except in aged C57BL/6 mice where it caused a significant increase in total lung leukocytes at 3 dpi and a decrease at 7 dpi (**Fig. 12a**).

ECIG exposure alone resulted in few changes to total cell counts. However, we identified 62 significant shifts in absolute cell numbers and 87 significant shifts in cell population frequencies when comparing the 15 identified cell types between FA and ECIG exposed groups in BAL and lungs from young and aged C57BL/6 and BALB/c mice at both 3 and 7 dpi (**Figs. 13-18**). Almost all observed changes varied by age, mouse strain, and cellular compartment. Consistently, however, NK cells were significantly increased in ECIG exposed mice, regardless of age or mouse strain, in both airways and lung tissue at 3 dpi (**Fig. 19**). Moreover, monocyte populations were increased in both BAL and lungs at 7 dpi following ECIG exposure. Young C57BL/6 mice saw the most consistent increase in monocytes into airways and lungs, which was observed at 3, 7 (**Fig. 13a, b** and **Fig. 14a, b**) and 14 dpi (data not shown). Though a relatively small population of cells, conventional dendritic cells (cDCs) were significantly increased in lung tissue at 3 and 7 dpi in young BALB/c mice (**Fig. 14a, b**). In both

airways and lung tissue at 7 dpi, B-1 cells were significantly increased in ECIG exposed mice compared to those exposed to FA. This trend was more pronounced in young mice of both backgrounds (**Fig. 15a, b** and **Fig.16a, b**). Compared to FA exposed controls, both young and aged ECIG BALB/c mice saw a trend of increased total T cells in BAL and lungs at 7 dpi, though this trend did not reach significance. Conversely, C57BL/6 mice of both ages exposed to ECIG prior to infection tended to have fewer T cells at 7 dpi. In aged C57BL/6 mice, this trend was significant in both BAL and lung tissue (**Fig. 17a, b** and **Fig.18a, b**). NKT cell populations were significantly expanded in young C57BL/6 and BALB/c mice at 7 dpi in both BAL and lungs (**Fig. 17a, b** and **Fig.18a, b**). Indeed, changes in T cell populations at 7 days post-infection were more common in young mice, both C57BL/6 and BALB/c, than in aged mice. Here, total CD8⁺ T cell counts in BAL (**Fig. 20a**) and lungs (**Fig. 20b**) at 7 dpi were unchanged by ECIG exposure. However, significant increases in effector CD8⁺ T cells in BAL (**Fig. 20c, e**) and lungs (**Fig. 20d, f**) of BALB/c but not C57BL/6 mice were observed.

Taken together, these data demonstrate that even short-term, acute exposure to ECIG significantly alters myeloid and lymphoid cell compositions of airways and lung tissue during infection in both young and aged C57BL/6 and BALB/c mice.

Discussion:

This study compares the effects of e-cigarette-induced pulmonary inflammation and its subsequent impacts on disease susceptibility to influenza virus infection in young and aged mice. We demonstrate that acute exposure to e-cigarette aerosol significantly impacts IAV mortality and delays viral clearance, and that alterations in immune cell populations and serum cytokine profiles persist beyond resolution of infection. E-cigarette exposure especially enhanced NK cell recruitment during early infection in both age groups, indicating their sensitivity to e-cigarette aerosol-induced lung changes. Thus, our findings provide clear evidence of e-cigarette exposure-mediated pulmonary immune system changes and provides insight into the potential health consequences of those disruptions.

The observed e-cigarette-induced increases of immune cell influx to the lungs, while local and systemic cytokine production appeared blunted, strongly indicates immune system disruption. Recruitment of macrophages and neutrophils to the lungs following e-cigarette exposure has been well documented (Bahmed et al., 2019; Glynos et al., 2018; Sussan et al., 2015; Taha et al., 2020; Q. Wang et al., 2020b) and our study corroborates these findings. As is the case with cigarette smoke (CS), e-cigarette use has anti-inflammatory or immunosuppressive effects on lungs (Stämpfli & Anderson, 2009). As shown previously, pulmonary cytokines are dampened and immune responses to respiratory illnesses are blunted in humans and mice as a result of both acute and chronic e-cigarette exposure (Hickman et al., 2022; Madison et al., 2019; Martin et al., 2016; Masso-Silva, Moshensky, et al., 2021; Rebuli, Glista-Baker, et al., 2021; Sayed et al., 2021; Sussan et al., 2015) and our results are consistent with those findings. Notably, substantially more immune gene downregulation was observed in nasal epithelial cells of e-cigarette users than cigarette users, when compared to non-smoking controls (Martin et al.,

2016). A recent study also found immunosuppression in the sputum of fourth-generation e-cigarette users (Hickman et al., 2022). Using a high-nicotine e-cigarette exposure model, we have also previously reported downregulations of pro-inflammatory cytokine gene expression *in vivo* (murine lung tissue) and *in vitro* (human lung epithelial and macrophage cell lines). Our current findings support the possibility that e-cigarette use may dampen or suppress certain aspects of pulmonary immunity. Despite these findings, many publications have also reported that e-cigarette use increases inflammation, evidenced by increased pulmonary cytokine concentrations or gene expression (Masso-Silva, Byun, et al., 2021). Like cigarette use, e-cigarette use likely results in myriad effects which are both pro- and anti-inflammatory, the sum of which determine the overall outcome (Stämpfli & Anderson, 2009). Variations in exposure regimen, e-cigarette device type, and e-liquid composition further complicate direct comparisons between studies. More research is needed to understand the precise mechanisms by which e-cigarette aerosols dysregulate the pulmonary immune system.

The pulmonary effects of e-cigarette exposure on aged mice have, to our knowledge, not previously been reported. Although, one study using aged mice has reported greater e-cigarette-induced metabolic changes in older mice (Crawford et al., 2021). In our model, age did not consistently increase e-cigarette-induced effects. Compared to young C57BL/6 mice, aged mice had more moderate increases in macrophage infiltration and more moderate reductions in local cytokines. Because e-cigarette and cigarette aerosols share many of the same chemical compounds, their effects on the lung should be compared. In a cigarette smoke (CS) exposure model with C57BL/6 mice, increasing age did not enhance neutrophil or macrophage recruitment to BAL (S. Zhou et al., 2013), which is consistent with our results. This study also reported a

slightly blunted gene expression of CS-induced inflammatory mediators in aged mice, which was also consistent with our cytokine data.

Conversely, we observed that compared to young BALB/c mice, aged mice had significantly enhanced macrophage influx to airways, indicating more sensitivity to e-cigarette exposure. Young BALB/c mice did experience significant neutrophil influx to the lungs following e-cigarette exposure, a unique inflammatory response which was not present in aged animals. Age-dependent increases in CS-induced macrophage influx have also been previously described (John-Schuster et al., 2016; Moriyama et al., 2010; Rashid et al., 2018). Termed “inflammaging” (Dugan et al., 2023), age is generally known to increase baseline pulmonary inflammation; however, the effects of e-cigarette exposure on aged lungs specifically have not been clearly elucidated. Indeed, age-associated responses to cigarette smoke are still debated despite many decades of research (Jaramillo-Rangel et al., 2023). The present study demonstrates e-cigarette exposure causes significant immune activation in older lungs, which could be enhanced by aging. More research will be required to determine exactly how aging and e-cigarette exposure interact to affect the pulmonary immune system.

It is well documented that increasing age is associated with increased susceptibility to IAV infection and poorer resultant disease outcomes in humans (Torrelles et al., 2022). Additionally, most *in vivo* studies in mice find that aged mice have impaired viral clearance, increased weight loss, and decreased resistance to IAV infection (Harpur et al., 2021). However, similarly to our results from e-cigarette exposure alone, aging alone did not consistently increase susceptibility to IAV infection in our model. No differences in morbidity or lung viral load were observed between young and aged C57BL/6 mice of either exposure group. Surprisingly, aged BALB/c mice of both exposure groups exhibited reduced morbidity, mortality, and day 3 viral

titers compared to young BALB/c mice. Notably, recent studies have reported aged C57BL/6 mice have an increased resistance to the A/PR8 strain of IAV, characterized by decreased morbidity, mortality, and early infection viral titers (Lu et al., 2018; Pillai et al., 2016; Smith et al., 2019), which is in line with this study's findings. In these studies, a small volume of viral inoculum (under 40 μ L) was found to correlate with improved outcomes in aged mice; when given a larger volume of inoculum, the trend reversed and aged mice experienced more severe morbidity and mortality (Smith et al., 2019). Therefore, given that our study inoculated mice with 40 μ L, it is somewhat surprising that we still observed an increased resistance to IAV in aged mice. Of note, these studies used C57BL/6 mice while we observed this trend only in BALB/c mice. Genetic background, as well as other possible experimental model differences, such as type of anesthesia or route of infection, could explain these discrepancies.

Previous studies have reported both increases, decreases and no changes in viral titers as a result of e-cigarette exposure, indicating a lack of consensus in the field. Treatment of human lung slices directly with e-liquid *in vitro* has been shown to increase viral loads of both influenza and rhinovirus (Agraval et al., 2023; Wu et al., 2014). These results, however, were not replicated by Schaunaman et al. who found similar IAV RNA levels in infected e-cigarette exposed and unexposed small airway epithelial cell cultures (Schaunaman et al., 2022). Rebuli et al. reported a statistically non-significant decrease in viral load in human e-cigarette users 2 days after vaccination with LAIV. Sussan et al. found that e-cigarette exposed mice had significantly higher lung viral titers at 4 dpi. However, Maishan et al. reported that e-cigarette exposed mice had a statistically non-significant decrease in viral load at 3 dpi. Given the conflicting reports, our current finding that e-cigarette exposure prior to infection significantly decreases viral load at 3 dpi contributes to the emerging understanding of viral kinetics after e-cigarette exposure, as

it may point to enhanced innate immune cell activation, which depending on the pathogenicity of the influenza virus causing the infection can increase viral clearance and/or result in enhanced lung pathology.

E-cigarette-induced innate immune cell influx, driven by macrophages or neutrophils, prior to infection in our model may be responsible for the reduction in lung viral titers early on in infection wherein the lungs are inadvertently primed for rapid activation of the innate immune system. Indeed, total BAL cell counts and macrophages were found via Cytospin analysis to be increased at 3 dpi in e-cigarette exposed mice (data not shown). In contrast, the previous study by Sussan et al., which reported increased lung viral titers at 4 dpi, did not find any simultaneous changes to BAL cell counts. Therefore, sustained innate immune cell influx may explain the seemingly contradictory viral load findings.

Our flow cytometry analysis confirms significant alterations in innate immune cells in both BAL and lung tissue at 3 days post-infection as a result of e-cigarette exposure for both C57BL/6 and BALB/c mice of both ages. Notably, NK cells were significantly increased in BAL and lungs in all e-cigarette exposed groups compared to their FA counterparts. Monocytes and dendritic cells were also significantly increased, though not universally so across all groups. Little is known about the effects of e-cigarettes on NK cell populations, but two reports have shown increases in NK cells in the lungs of mice and in the blood of human e-cigarette users (Kelesidis et al., 2020; Szafran et al., 2020b). Cigarette smoking has also been noted to increase NK cell populations (Eriksson Ström et al., 2018; Stolberg et al., 2014) and increase NK cell activation in response to poly (I:C) (Motz et al., 2010). The early increases in NK cells seen in both BAL and lungs in our model are in line with these reports. NK cells are known to be important homeostatic mediators of viral control and disease severity which are protective from

low to medium dose influenza infections (Mooney et al., 2020; Vashist et al., 2018; G. Zhou et al., 2013). The increased trafficking of NK cells to the lungs during early infection could be partly responsible for decreased lung viral titers. As NK cell populations did not remain increased through the duration of infection, e-cigarette exposed mice did not experience increased disease severity, morbidity, or mortality, which is possible with unchecked NK cell activation (L. Ma et al., 2021; Scharenberg et al., 2019; G. Zhou et al., 2013). Though NK cell enhancement possibly promoted control of the infection in this model, sustained exposure-induced NK cell activation is not without risk. For example, NK cells have been implicated in Chronic Obstructive Pulmonary Disease (COPD) pathology (Rao et al., 2021). For this reason, more research is needed to understand how e-cigarette use may alter NK cell responses, particularly NK cell functions, which were outside of the scope of this study. E-cigarettes have been shown to increase reactive oxygen species production by NK cells and decrease NK cell-mediated cytotoxicity (Clapp et al., 2017; Kelesidis et al., 2020). E-cigarette use is also known to modify the functional capacity of many cells including macrophages and neutrophils, further bolstering the potential for e-cigarette exposure to alter host defenses against viral pathogens (Corriden et al., 2020; Hickman et al., 2019; Jasper et al., 2021; Madison et al., 2019; Sinha et al., 2022; Snoderly et al., 2023; Ween et al., 2017a).

In the current study, e-cigarettes were found to delay viral clearance. To our knowledge, one study has reported similar findings in mice (Maishan et al., 2023). Delayed viral clearance has also been observed in cigarette exposed mice (Mebratu et al., 2016). Despite an early boost in the control of viral replication, our e-cigarette exposed mice were unable to clear the virus by 7 dpi as efficiently as FA controls. This was further evidenced by the observed increases in serum IL-1 β , TNF α , and IL-10 and lung monocytes at 14 dpi. Though the mechanism is unclear,

we did observe a consistent decrease in follicular B cells in both lungs and BAL at 7 dpi in e-cigarette exposed mice, which could signal a deficient humoral immune response.

Immunoglobulin production has been reported to be disrupted by e-cigarette use. Influenza-specific IgA induction was significantly reduced in the nasal lavage of e-cigarette users after vaccination with LAIV (Rebuli, Glista-Baker, et al., 2021) and decreased IgA gene expression was observed in e-cigarette exposed mice (Masso-Silva, Moshensky, et al., 2021). These observations of delayed viral clearance are important to note even though they did not have a significant detrimental impact on morbidity, mortality, or recovery from infection in our model. Delays in viral clearance have been associated with increased mortality and poorer health outcomes in more severe influenza infections in humans (To et al., 2010; Y. Wang et al., 2018).

Despite the perturbations in viral loads during infection, we did not observe significant shifts in morbidity as a result of e-cigarette exposure. However, weight loss is an imperfect measure of disease severity as its direct relationship to viral load and lung injury is not always clear in animal models (Maishan et al., 2023; Myers et al., 2021). Histological analyses, coupled with weight loss data, would need to be done to determine whether disease severity was truly unchanged by prior e-cigarette exposure.

Further complicating the use of weight as a direct measure of disease severity in our model is the observation that e-cigarette exposed mice gained significant weight above uninfected controls in the first 3 days of infection, while FA exposed mice did not. The initial weight gain could be a result of exposure-induced stress which modestly stunted normal weight gain in these young mice. Though the data are incomplete, we did note a delayed weight gain during exposure in young ECIG exposed mice, below FA exposed controls. In agreement with our observations, weight loss as a result of e-cigarette exposure was documented in at least one

other study (Scieszka et al., 2023). We posit that mice were able to begin gaining weight normally for a few days between inoculation which took place on the same day as the last e-cigarette exposure and when the infection progressed significantly. This early addition of weight likely caused the observed modest delay in weight loss seen in ECIG exposed mice.

Two murine studies have reported increased mortality from IAV infection as a result of prior e-cigarette exposure (Madison et al., 2019; Sussan et al., 2015). One study has shown increased mortality in a coronavirus mouse model (Sivaraman et al., 2021). Somewhat surprisingly we found no significant changes in mortality in young or aged C57BL/6 and aged BALB/c mice, and significantly decreased mortality in young BALB/c mice, a result of e-cigarette exposure. In our model, young BALB/c mice were the most susceptible to IAV evidenced by the fact that they experienced the highest viral titers and weight loss during infection of all groups in this study. The second most susceptible group was the young C57BL/6 mice who also showed a decrease in mortality as a result of e-cigarette exposure, though this trend was not statistically significant. To our knowledge, increased survival to viral challenge in e-cigarette exposed animals has not previously been reported. Our experiments followed young BALB/c mice to the peak of infection at day 7, but not through infection recovery to day 14. This is a notable limitation of our study findings. Therefore, it is not possible to determine if e-cigarette exposed mice would have maintained a survival advantage at later infection timepoints. For example, in our young C57BL/6 mouse recovery experiment, FA and ECIG survival was 50% and 67% at day 14, respectively; survival was 83% vs 100% at day 7. Therefore, more experimentation is needed to confirm these findings. Even still, our study highlights the potency of acute e-cigarette exposure and its ability to modulate viral infection recovery, which coupled

with reports of increased infections in humans who vape, underscores the need for future research into the mechanisms through which e-cigarettes may alter pulmonary immunity.

As discussed above, enhanced early control of viral replication and expansion of NK cells in e-cigarette exposed animals could be responsible for the decreased mortality in these mice. Additionally, young BALB/c mice were the only group which had significantly increased Effector CD8⁺ T cells at 7 dpi as a result of e-cigarette exposure. Young BALB/c mice also had the most consistent enhancement in conventional dendritic cell (cDC) influx to lungs at 3 dpi and to both lungs and BAL at 7 dpi. Pulmonary cDCs are required for sufficient activation of CD8⁺ T cell responses and depletion of these cells significantly increases mortality from IAV (Ng et al., 2018). Together this could explain the significant increase in survival seen in these mice, as effective cytotoxic T cell responses are necessary for infection control and resolution (Grant et al., 2016).

In conclusion, the present study is the first to report aging alone can increase inflammation associated with e-cigarette use, which warrants further research into whether age amplifies risks associated with vaping. Importantly, we demonstrate that e-cigarette exposure prior to infection in both young and aged mice resulted in a reduction in lung viral loads at day 3 coupled with a lack of viral clearance by day 7 and a decrease in mortality without significant changes to morbidity. E-cigarette exposure caused significant shifts throughout infection in both innate and adaptive immune cell populations, in particular NK cells, which persisted after resolution of infection, underscoring the potential health risks associated with e-cigarette use, and the need for further research.

Table 4.1: Identification of Leukocytes by Flow Cytometry

	Cell Population	Cell Surface Markers
Innate cells		
	Macrophages	CD11b ⁺⁺ F4/80 ^{+/-} or CD11b ⁺ F4/80 ⁺⁺
	Neutrophils	Ly6G ⁺⁺ CD11b ⁺⁺
	Monocytes	CD11b ⁺ Ly6C ^{+/++}
	NK cells	CD49b ⁺
	Eosinophils	CD11b ⁺ SSC-hi
	pDCs	Ly6C ⁺⁺ CD317 ⁺
	cDCs	MHCII ⁺ CD11c ⁺
B cells		
	Follicular B cells	CD19 ⁺
	NKB cells	CD19 ⁺ CD49b ⁺
	B1 cells	CD19 ⁺ CD43 ⁺
T cells		
	CD4 ⁺ T cells	CD5 ⁺ CD4 ⁺ GITR ^{+/-}
	CD8 ⁺ T cells	CD5 ⁺ CD8 ⁺
	NKT cells	CD5 ⁺ CD49b ⁺
	Tregs	CD5 ⁺ CD4 ⁺ CD25 ⁺ GITR ⁺⁺
	$\gamma\delta$ T cells	TCRg/d ⁺

Cell surface marker expression per cell type as determined by the flow cytometry gating scheme seen in Figure 7 where ⁻ indicates negative, ⁺ indicates intermediate or low and ⁺⁺ indicates high expression. NK, natural killer; pDCs, plasmacytoid dendritic cells; cDCs, classical dendritic cells; Tregs, Regulatory T cells.

Table 4.2: Cell Composition of 2-month-old Bronchoalveolar Lavage during IAV Infection

	<i>mock infection</i>		<i>3 days post-infection</i>				<i>7 days post-infection</i>			
	C57BL/6	BALB/c	C57BL/6		BALB/c		C57BL/6		BALB/c	
			FA	ECIG	FA	ECIG	FA	ECIG	FA	ECIG
Macrophages	6,876 ± 1,247	5,369 ± 1,707	119,708 ± 32,279	200,354 ± 38,851	185,736 ± 21,718	138,279 ± 51,473	78,158 ± 11,721	74,620 ± 11,534	42,981 ± 8,826	63,459 ± 9,113
Neutrophils	525 ± 162	3,751 ± 818	147,148 ± 32,680	208,181 ± 25,660	660,861 ± 145,250	709,267 ± 57,178	93,404 ± 22,187	67,544 ± 13,239	46,311 ± 7,879	61,113 ± 10,279
Monocytes	200 ± 57	136 ± 30	8,442 ± 2,075	20,154 ± 4,103*	39,788 ± 4,131	33,170 ± 4,568	16,176 ± 2,318	24,038 ± 4,624	31,504 ± 6,453	43,276 ± 5,757
NK cells	1,337 ± 259	691 ± 144	6,967 ± 1,179	31,321 ± 5,139**	30,309 ± 4,930	62,490 ± 10,739*	29,923 ± 6,900	45,269 ± 8,205	70,903 ± 3,226	56,768 ± 8,364
Eosinophils	1,257 ± 282	899 ± 204	1,988 ± 514	1,735 ± 298	5,041 ± 662	3,957 ± 719	2,229 ± 329	3,108 ± 576	2,291 ± 492	2,786 ± 312
pDCs	32 ± 10	42 ± 21	701 ± 179	2,046 ± 481*	3,649 ± 867	2,956 ± 1,301	4,837 ± 984	5,441 ± 867	23,692 ± 9,758	25,230 ± 3,907
cDCs	1,099 ± 229	240 ± 46	1,213 ± 450	844 ± 152	205 ± 38	106 ± 51	932 ± 143	1,087 ± 132	582 ± 94	1,107 ± 120**
Follicular B cells	682 ± 193	701 ± 124	1,419 ± 235	550 ± 83*	1,454 ± 134	1,083 ± 250	2,599 ± 342	2,070 ± 377	2,235 ± 1,173	1,799 ± 320
NKB cells	115 ± 42	19 ± 19	7 ± 7	0 ± 0	0 ± 0	0 ± 0	14 ± 5	140 ± 46*	57 ± 23	12 ± 8
B1 cells	95 ± 33	57 ± 17	102 ± 24	144 ± 40	254 ± 82	286 ± 99	1,120 ± 193	2,573 ± 427**	2,080 ± 1,066	5,416 ± 917*
CD4 ⁺ T cells	2,081 ± 458	1,005 ± 190	3,334 ± 331	3,303 ± 451	5,223 ± 824	4,471 ± 450	23,511 ± 3,648	22,301 ± 4,165	43,395 ± 10,985	49,722 ± 10,005
CD8 ⁺ T cells	415 ± 91	150 ± 48	8,036 ± 1,445	10,192 ± 3,079	6,252 ± 1,196	4,556 ± 831	193,103 ± 34,520	188,482 ± 31,239	190,379 ± 33,399	222,297 ± 29,693
NKT cells	207 ± 54	78 ± 27	959 ± 91	1,640 ± 416	866 ± 160	670 ± 76	1,385 ± 301	4,456 ± 647***	3,165 ± 646	12,626 ± 2,248**
Tregs	220 ± 64	576 ± 213	407 ± 104	1,379 ± 367*	794 ± 101	1,059 ± 190	6,860 ± 1,167	8,606 ± 1,329	23,243 ± 4,245	23,518 ± 3,096
γδ T cells	261 ± 142	74 ± 38	4,417 ± 1,378	31,991 ± 16,185*	34,294 ± 4,786	4,816 ± 857**	3,419 ± 922	3,978 ± 1,195	5,493 ± 778	12,009 ± 3,090
<i>Total Innate cells</i>	11,327 ± 1,863	11,211 ± 2,523	286,167 ± 63,430	464,633 ± 53,071	925,589 ± 154,685	969,001 ± 72,423	225,659 ± 36,498	221,106 ± 36,547	218,682 ± 33,216	253,738 ± 36,201
<i>Total B cells</i>	1,031 ± 244	776 ± 139	1,533 ± 249	694 ± 108*	1,708 ± 191	1,369 ± 313	3,733 ± 504	4,934 ± 748	4,372 ± 2,219	7,227 ± 1,222
<i>Total T cells</i>	3,059 ± 621	1,883 ± 361	17,151 ± 2,637	48,505 ± 19,552	47,427 ± 6,016	15,572 ± 1,344*	228,279 ± 39,058	227,822 ± 37,459	265,675 ± 48,426	320,173 ± 43,168

Values represent cell counts (cells/mL) in bronchoalveolar lavage from 2-month-old mice after mock infection or on day 3 or 7 post-infection with 10 PFU A/PR8 and are expressed as mean ± SEM (n=6-13). Statistical significance determined by two-tailed Student's *t*-test with Welch's correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. FA, filtered air; ECIG, E-Cigarette; NK, natural killer; pDCs, plasmacytoid dendritic cells; cDCs, classical dendritic cells; Tregs, Regulatory T cells.

Table 4.3: Cell Composition of 12-month-old Bronchoalveolar Lavage during IAV Infection

	<i>mock infection</i>		<i>3 days post-infection</i>				<i>7 days post-infection</i>			
	C57BL/6	BALB/c	C57BL/6		BALB/c		C57BL/6		BALB/c	
			FA	ECIG	FA	ECIG	FA	ECIG	FA	ECIG
Macrophages	7,086 ± 2,112	10,849 ± 3,626	151,794 ± 11,579	136,364 ± 62,584	84,473 ± 22,265	255,282 ± 59,096*	70,020 ± 13,091	57,094 ± 9,923	75,703 ± 25,527	43,426 ± 5,543
Neutrophils	521 ± 280	6,036 ± 1,964	166,396 ± 13,371	181,892 ± 84,506	678,648 ± 201,403	948,289 ± 192,352	98,750 ± 25,348	64,376 ± 17,490	143,371 ± 30,935	98,311 ± 16,939
Monocytes	117 ± 14	180 ± 60	11,026 ± 2,806	11,878 ± 5,600	10,913 ± 3,335	46,169 ± 12,173*	18,404 ± 2,851	16,915 ± 3,370	29,572 ± 8,149	25,439 ± 4,617
NK cells	1,474 ± 344	1,200 ± 376	4,712 ± 854	9,268 ± 4,044	7,563 ± 1,095	60,452 ± 14,021*	25,550 ± 7,194	13,919 ± 3,394	48,419 ± 11,309	48,686 ± 10,321
Eosinophils	2,047 ± 484	2,253 ± 747	1,981 ± 876	1,475 ± 478	2,302 ± 706	4,585 ± 1,649	2,686 ± 564	3,169 ± 658	1,892 ± 564	2,057 ± 409
pDCs	82 ± 27	259 ± 116	761 ± 256	789 ± 381	1,762 ± 538	8,543 ± 2,337*	4,772 ± 842	3,224 ± 610	12,071 ± 2,077	17,473 ± 3,719
cDCs	2,001 ± 535	572 ± 154	962 ± 306	1,104 ± 277	335 ± 23	195 ± 46*	1,459 ± 288	1,195 ± 225	588 ± 144	1,016 ± 156
Follicular B cells	1,433 ± 273	515 ± 171	513 ± 52	308 ± 32*	1,061 ± 244	1,540 ± 384	3,867 ± 982	2,083 ± 436	3,058 ± 470	1,455 ± 333*
NKB cells	609 ± 170	0 ± 0	6 ± 6	0 ± 0	7 ± 7	96 ± 50	78 ± 19	360 ± 88*	40 ± 17	294 ± 82*
B1 cells	147 ± 54	187 ± 61	167 ± 95	371 ± 186	419 ± 102	541 ± 143	2,359 ± 490	2,567 ± 673	1,857 ± 327	2,952 ± 554
CD4 ⁺ T cells	2,439 ± 872	3,622 ± 1,830	5,664 ± 1,803	3,855 ± 1,093	6,298 ± 755	7,341 ± 1,798	44,922 ± 5,802	18,421 ± 4,663**	38,672 ± 7,560	39,300 ± 11,945
CD8 ⁺ T cells	1,088 ± 364	1,162 ± 770	17,767 ± 4,197	9,680 ± 2,979	5,749 ± 426	7,180 ± 1,837	276,378 ± 31,695	149,737 ± 38,532*	61,621 ± 12,946	78,462 ± 17,778
NKT cells	188 ± 95	615 ± 292	890 ± 108	1,368 ± 179	686 ± 140	546 ± 115	5,775 ± 1,112	3,824 ± 826	2,769 ± 650	3,972 ± 708
Tregs	153 ± 43	408 ± 237	654 ± 265	474 ± 117	675 ± 188	3,443 ± 913*	6,920 ± 1,707	5,090 ± 1,543	13,838 ± 2,896	17,343 ± 4,730
γδ T cells	487 ± 375	884 ± 209	759 ± 212	6,720 ± 3,219*	1,642 ± 529	8,619 ± 3,426	7,331 ± 1,236	843 ± 454***	2,155 ± 293	3,422 ± 904
<i>Total Innate cells</i>	13,328 ± 3,366	21,348 ± 5,894	337,632 ± 23,594	342,769 ± 156,132	785,997 ± 220,814	1,323,515 ± 266,812	221,641 ± 47,541	159,891 ± 31,898	475,553 ± 194,628	236,409 ± 28,394
<i>Total B cells</i>	2,189 ± 400	702 ± 231	588 ± 41	624 ± 198	1,488 ± 332	2,176 ± 471	6,304 ± 1,448	5,010 ± 1,062	4,955 ± 794	4,701 ± 782
<i>Total T cells</i>	4,354 ± 1,384	6,691 ± 3,276	25,734 ± 5,858	22,096 ± 6,622	16,761 ± 2,755	27,128 ± 6,257	341,327 ± 38,932	177,914 ± 44,096*	119,057 ± 23,155	142,499 ± 33,685

Values represent cell counts (cells/mL) in bronchoalveolar lavage from 12-month-old mice after mock infection or on day 3 or 7 post-infection with 10 PFU A/PR8 and are expressed as mean ± SEM (n=6-13). Statistical significance determined by two-tailed Student's *t*-test with Welch's correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. FA, filtered air; ECIG, E-Cigarette; NK, natural killer; pDCs, plasmacytoid dendritic cells; cDCs, classical dendritic cells; Tregs, Regulatory T cells.

Table 4.4: Cell Composition of 2-month-old Lungs during IAV Infection

	<i>mock infection</i>		<i>3 days post-infection</i>				<i>7 days post-infection</i>			
	C57BL/6	BALB/c	C57BL/6		BALB/c		C57BL/6		BALB/c	
			FA	ECIG	FA	ECIG	FA	ECIG	FA	ECIG
Macrophages	663,177 ± 67,900	698,165 ± 104,476	1,474,201 ± 175,503	1,424,635 ± 230,823	1,389,217 ± 95,360	1,406,198 ± 130,275	2,592,865 ± 158,085	2,494,493 ± 201,894	1,948,808 ± 163,368	2,074,968 ± 159,055
Neutrophils	62,992 ± 8,882	303,171 ± 43,201	230,781 ± 36,672	248,457 ± 42,679	945,557 ± 40,785	830,836 ± 96,254	382,339 ± 30,303	472,635 ± 53,352	768,066 ± 120,949	734,755 ± 89,421
Monocytes	131,736 ± 13,929	95,993 ± 9,031	126,471 ± 14,621	142,897 ± 6,316	123,888 ± 10,114	136,576 ± 8,830	148,299 ± 12,819	231,434 ± 18,433**	178,889 ± 10,340	192,425 ± 14,711
NK cells	50,272 ± 4,300	155,385 ± 20,817	56,638 ± 9,413	209,787 ± 27,166**	96,155 ± 7,134	225,413 ± 16,899**	141,735 ± 23,612	198,100 ± 29,092	314,220 ± 80,891	245,483 ± 26,050
Eosinophils	9,409 ± 1,154	12,988 ± 1,775	13,231 ± 3,689	12,155 ± 3,753	5,080 ± 683	7,101 ± 876	5,982 ± 484	11,351 ± 1,774*	6,406 ± 1,653	5,736 ± 872
pDCs	111,679 ± 13,598	56,054 ± 6,860	31,055 ± 3,614	30,751 ± 3,880	73,147 ± 7,353	72,848 ± 8,336	118,782 ± 16,550	96,749 ± 13,257	182,345 ± 33,634	232,729 ± 19,733
cDCs	9,434 ± 1,151	7,336 ± 841	10,327 ± 642	8,951 ± 1,662	4,851 ± 498	7,388 ± 591**	9,570 ± 751	17,674 ± 2,703*	7,026 ± 628	12,201 ± 1,097**
Follicular B cells	966,387 ± 108,989	403,556 ± 44,238	839,245 ± 140,047	1,017,303 ± 200,648	424,782 ± 34,728	537,282 ± 79,639	448,124 ± 38,994	287,493 ± 34,832**	417,524 ± 79,958	358,913 ± 46,457
NKB cells	1,849 ± 174	2,684 ± 340	1,218 ± 220	2,859 ± 314**	917 ± 124	2,947 ± 520*	872 ± 103	21,163 ± 6,308**	1,897 ± 496	853 ± 103
B1 cells	91,016 ± 9,300	55,504 ± 5,438	67,209 ± 9,318	67,888 ± 10,426	65,637 ± 5,122	50,323 ± 5,383	66,968 ± 5,186	110,869 ± 13,414**	44,372 ± 9,121	53,835 ± 7,747
CD4 ⁺ T cells	243,788 ± 27,321	609,290 ± 67,382	149,922 ± 11,286	173,778 ± 23,327	264,990 ± 10,227	377,663 ± 52,428	135,931 ± 13,414	105,620 ± 17,648	319,503 ± 59,839	308,380 ± 33,873
CD8 ⁺ T cells	164,688 ± 19,683	192,199 ± 22,454	117,770 ± 10,916	137,033 ± 16,645	80,031 ± 4,291	148,842 ± 28,202	403,243 ± 45,909	386,707 ± 61,284	297,969 ± 23,317	334,839 ± 52,457
NKT cells	5,239 ± 638	11,599 ± 1,585	8,720 ± 1,215	9,718 ± 2,627	3,935 ± 484	8,258 ± 994**	4,736 ± 800	17,033 ± 3,659**	6,970 ± 1,171	36,487 ± 4,411***
Tregs	3,198 ± 456	20,548 ± 1,651	1,536 ± 372	3,071 ± 1,348	3,514 ± 390	5,759 ± 1,215	22,148 ± 1,748	25,527 ± 3,879	38,845 ± 3,508	46,746 ± 7,574
γδ T cells	14,001 ± 1,903	15,329 ± 1,750	86,591 ± 16,720	115,121 ± 44,383	174,719 ± 22,326	28,972 ± 3,506**	27,903 ± 4,063	38,073 ± 9,625	40,538 ± 5,101	41,234 ± 6,395
<i>Total Innate cells</i>	1,038,699 ± 96,884	1,329,091 ± 183,799	1,942,705 ± 208,397	2,077,633 ± 274,527	2,637,895 ± 116,919	2,686,360 ± 232,767	3,400,218 ± 192,774	3,522,437 ± 245,309	3,405,758 ± 207,876	3,498,298 ± 283,708
<i>Total B cells</i>	1,059,252 ± 117,563	461,744 ± 49,290	907,672 ± 149,226	1,088,050 ± 210,821	491,335 ± 39,726	590,552 ± 85,215	515,964 ± 43,546	406,371 ± 25,693*	463,793 ± 87,766	413,601 ± 54,010
<i>Total T cells</i>	430,915 ± 47,931	848,964 ± 93,885	364,540 ± 38,074	438,722 ± 69,504	527,188 ± 29,974	569,493 ± 78,002	600,453 ± 52,879	572,960 ± 89,762	703,823 ± 63,430	767,686 ± 92,281

Values represent cell counts (cells/lung) in lung tissue from 2-month-old mice after mock infection or on day 3 or 7 post-infection with 10 PFU A/PR8 and are expressed as mean ± SEM (n=6-13). Statistical significance determined by two-tailed Student's *t*-test with Welch's correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. FA, filtered air; ECIG, E-Cigarette; NK, natural killer; pDCs, plasmacytoid dendritic cells; cDCs, classical dendritic cells; Tregs, Regulatory T cells.

Table 4.5: Cell Composition of 12-month-old Lungs during IAV Infection

	<i>mock infection</i>		<i>3 days post-infection</i>				<i>7 days post-infection</i>			
	C57BL/6	BALB/c	C57BL/6		BALB/c		C57BL/6		BALB/c	
			FA	ECIG	FA	ECIG	FA	ECIG	FA	ECIG
Macrophages	850,839 ± 127,543	1,620,765 ± 202,723	1,369,334 ± 225,382	1,916,217 ± 247,742	1,224,362 ± 158,187	1,257,338 ± 70,133	2,326,770 ± 225,489	1,297,900 ± 182,293**	1,655,434 ± 157,130	1,779,704 ± 202,252
Neutrophils	154,236 ± 11,737	672,629 ± 131,193	332,863 ± 83,938	413,757 ± 91,492	531,274 ± 110,594	631,591 ± 85,886	466,452 ± 74,892	370,511 ± 91,618	691,943 ± 177,697	540,999 ± 112,443
Monocytes	97,757 ± 7,600	66,110 ± 6,191	99,565 ± 16,081	148,056 ± 25,867	78,663 ± 8,024	89,910 ± 9,539	159,351 ± 8,764	126,917 ± 12,340	148,900 ± 14,324	136,508 ± 14,209
NK cells	24,876 ± 1,748	317,429 ± 32,151	29,669 ± 5,533	150,192 ± 34,521*	58,398 ± 6,070	163,740 ± 13,826***	100,475 ± 12,784	66,730 ± 17,093	212,155 ± 28,501	168,594 ± 10,336
Eosinophils	8,050 ± 908	28,844 ± 8,835	5,929 ± 1,228	11,243 ± 2,567	4,600 ± 657	4,899 ± 858	8,001 ± 1,031	8,318 ± 967	2,993 ± 201	7,483 ± 744**
pDCs	36,194 ± 7,229	63,410 ± 9,725	25,087 ± 9,155	26,876 ± 1,816	36,401 ± 4,833	47,778 ± 4,727	124,311 ± 9,308	28,613 ± 3,149****	161,611 ± 12,978	161,678 ± 24,847
cDCs	8,365 ± 901	15,428 ± 1,411	8,551 ± 1,764	15,158 ± 3,136	6,376 ± 804	6,267 ± 601	13,073 ± 1,309	8,702 ± 795*	7,713 ± 780	13,391 ± 1,610*
Follicular B cells	1,284,593 ± 112,066	674,585 ± 186,910	596,149 ± 112,638	1,502,281 ± 295,269*	397,706 ± 57,841	751,601 ± 77,969**	426,046 ± 58,892	244,907 ± 74,955	613,327 ± 93,864	688,876 ± 112,092
NKB cells	1,850 ± 247	3,361 ± 774	1,262 ± 209	6,037 ± 1,359*	991 ± 250	3,921 ± 443***	1,240 ± 274	26,208 ± 4,166**	1,545 ± 229	2,468 ± 432
B1 cells	93,890 ± 6,361	82,955 ± 25,650	54,252 ± 6,140	55,455 ± 7,136	64,105 ± 8,486	61,474 ± 5,960	71,227 ± 14,470	100,609 ± 15,092	81,578 ± 12,862	95,291 ± 19,883
CD4 ⁺ T cells	157,155 ± 15,364	576,556 ± 104,130	83,360 ± 5,751	188,551 ± 29,672*	311,065 ± 43,994	498,970 ± 95,101	108,213 ± 9,175	57,985 ± 9,556**	392,153 ± 57,730	372,430 ± 55,285
CD8 ⁺ T cells	191,595 ± 22,562	169,618 ± 27,202	84,612 ± 10,886	244,114 ± 50,667*	152,076 ± 45,460	184,697 ± 27,128	435,647 ± 58,820	282,964 ± 86,345	158,750 ± 20,824	168,096 ± 23,636
NKT cells	1,872 ± 345	9,741 ± 1,950	5,050 ± 1,140	9,930 ± 3,505	6,111 ± 653	6,460 ± 1,453	8,791 ± 1,991	5,820 ± 599	10,143 ± 2,507	19,242 ± 5,644
Tregs	1,928 ± 416	4,489 ± 422	2,214 ± 544	4,662 ± 713*	2,406 ± 356	3,732 ± 763	19,535 ± 1,405	12,109 ± 2,010*	22,387 ± 5,911	27,845 ± 6,861
γδ T cells	10,624 ± 1,450	38,192 ± 11,374	25,165 ± 13,054	47,549 ± 15,424	37,987 ± 7,505	11,831 ± 2,791*	51,805 ± 10,300	4,095 ± 1,220***	27,674 ± 3,641	28,913 ± 8,393
<i>Total Innate cells</i>	1,180,316 ± 131,564	2,784,615 ± 283,345	1,870,998 ± 305,137	2,700,858 ± 392,556	1,940,073 ± 240,410	2,201,522 ± 131,899	3,180,229 ± 295,520	1,907,690 ± 296,891*	2,846,938 ± 324,057	2,808,356 ± 294,423
<i>Total B cells</i>	1,380,333 ± 118,320	760,900 ± 212,968	651,664 ± 118,063	1,563,772 ± 301,643*	462,802 ± 64,641	816,996 ± 82,211**	491,292 ± 71,698	371,724 ± 86,548	696,192 ± 106,882	786,635 ± 131,341
<i>Total T cells</i>	363,172 ± 37,809	901,851 ± 191,364	200,400 ± 26,101	494,805 ± 81,301*	509,645 ± 89,981	705,690 ± 125,202	643,806 ± 73,078	362,972 ± 85,212*	609,994 ± 83,969	616,525 ± 82,495

Values represent cell counts (cells/lung) in lung tissue from 12-month-old mice after mock infection or on day 3 or 7 post-infection with 10 PFU A/PR8 and are expressed as mean ± SEM (n=6-13). Statistical significance determined by two-tailed Student's *t*-test with Welch's correction. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. FA, filtered air; ECIG, E-Cigarette; NK, natural killer; pDCs, plasmacytoid dendritic cells; cDCs, classical dendritic cells; Tregs, Regulatory T cells.

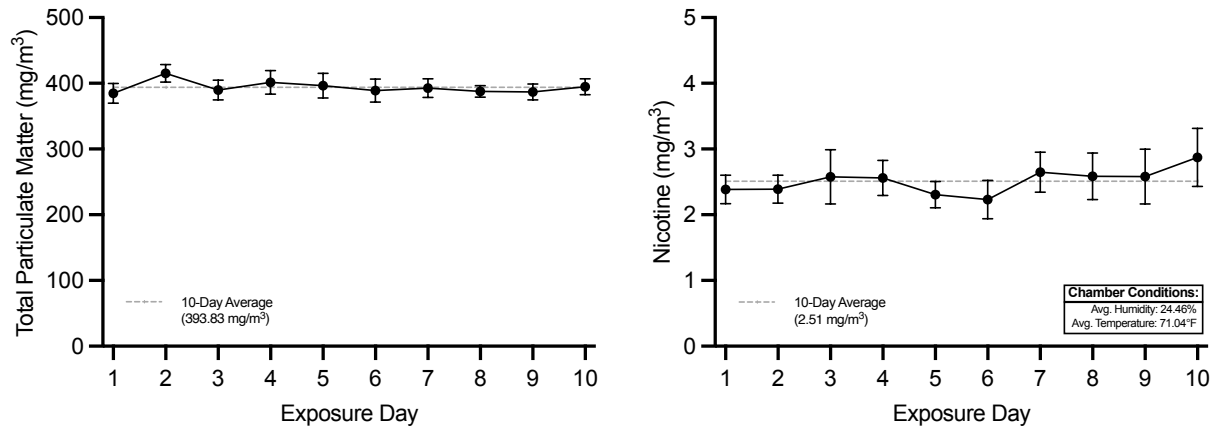


Figure 4.1: E-cigarette chamber aerosol and nicotine concentrations. (a) Total particulate matter (TPM) (mg/m^3) in whole-body exposure chambers was measured gravimetrically once per hour during e-cigarette exposures. (b) Aerosolized nicotine samples (mg/m^3) were collected once per day via XAD-4 cartridges, then analyzed via gas chromatography. Data presented as daily mean \pm SEM over 46 exposure days. Grey dashed line indicates the cumulative 10-day average.

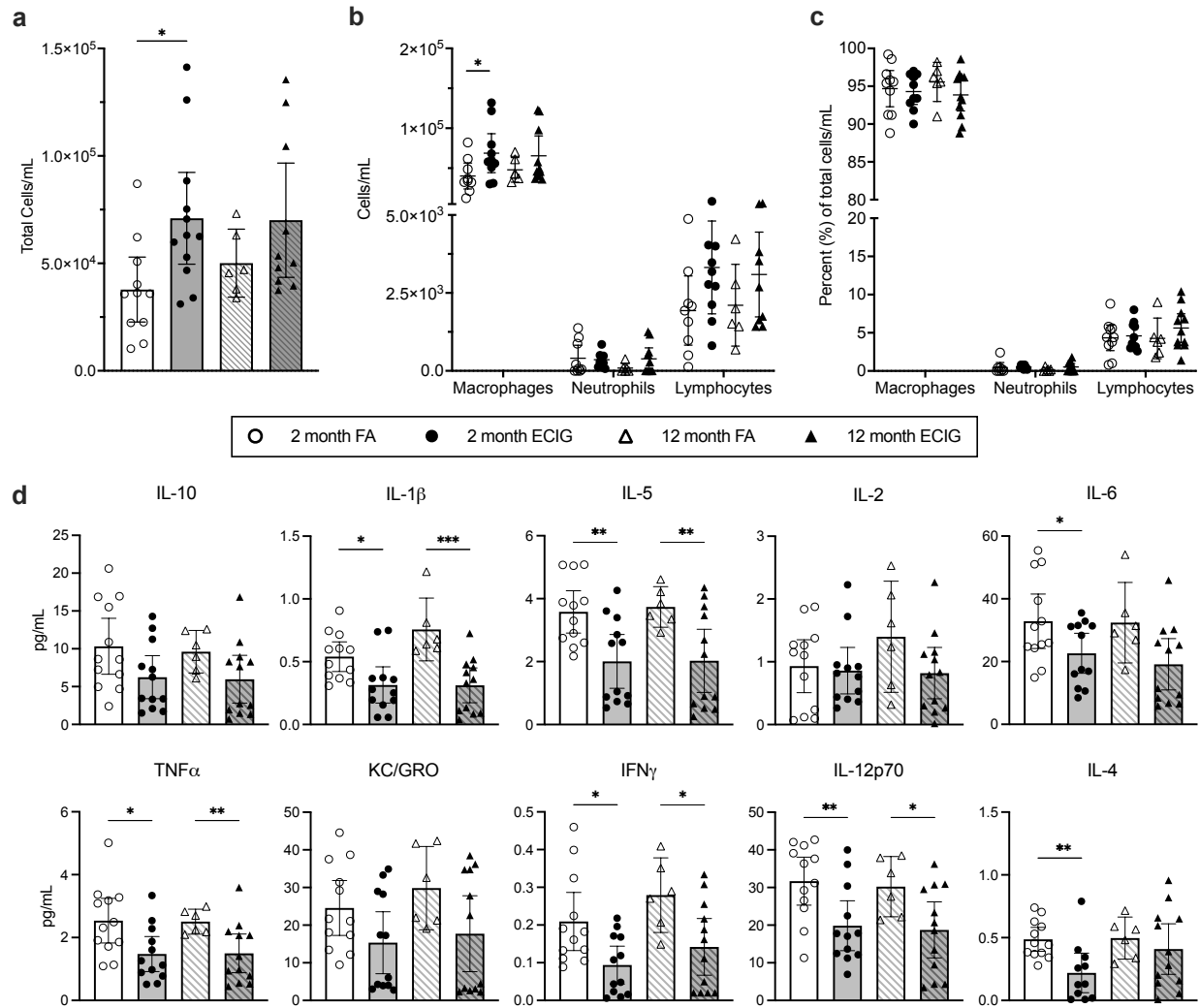


Figure 4.2: E-cigarette exposure increases leukocyte influx to lungs and suppresses local cytokine production. (a-d) 2- and 12-month-old C57BL/6 mice were placed in whole-body exposure chambers and exposed to filtered air (FA) or e-Cigarette aerosols (ECIG) for three hours/day for 10 days. (a, b, c) Bronchoalveolar lavage (BAL) and serum were collected immediately following exposure on day 10. Total BAL cells (a) were counted and analyzed via Cytospin to determine absolute numbers (b) and proportions (c) of leukocyte populations. (d) Cytokines in BAL were quantified. n=6-12 per group. Data are presented as mean \pm 95% CI. Statistical significance determined by two-tailed Student's *t*-test with Welch's correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

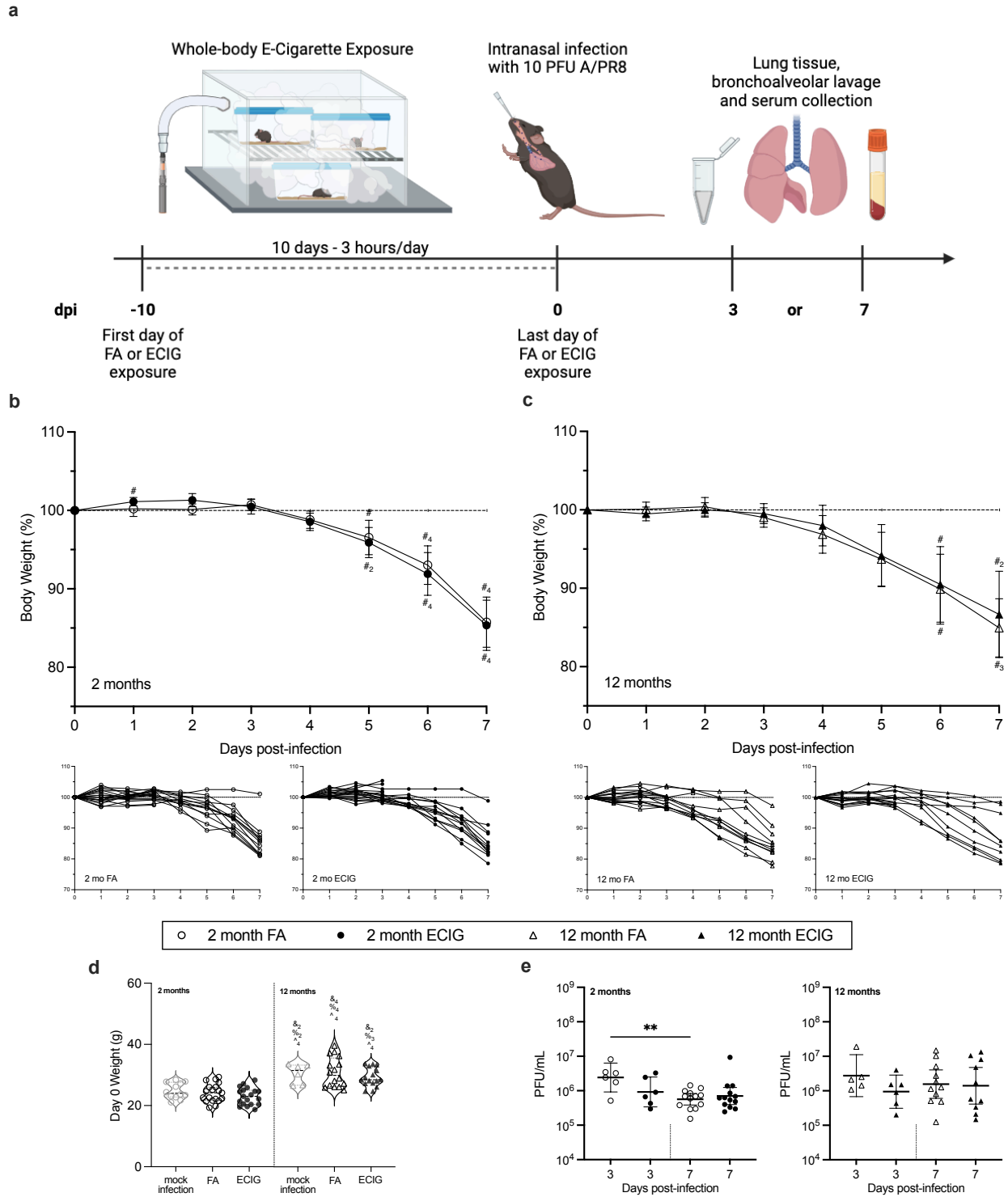


Figure 4.3: E-cigarette exposure enhances early, but not later, viral clearance. (a) 2- and 12-month-old C57BL/6 mice were exposed to FA or ECIG for 10 days prior to intranasal inoculation with 10 PFU of A/PR8 murine influenza, or PBS for mock infections. (b, c) 2- (b) and 12-month-

old (c) mice were weighed daily over the course of infection. Percent of Day 0 body weight data is presented as an average (top) and individually (bottom). Data are reported as mean \pm 95% CI. (d) Violin plot of Day 0 weights in grams. (e) Viral loads from lung homogenate were determined by RT-qPCR at 3 and 7 dpi. n=6-19 per group. Statistical significance determined by one-way ANOVA with Tukey's correction for multiple comparisons (b-d) or two-tailed Student's *t*-test with Welch's correction (e). **p<0.01; #p<0.05, #₂p<0.01, #₃p<0.001, or #₄p<0.0001 to mock infection; &₂p<0.01, or &₄p<0.0001 to 2-month mock infection; %₂p<0.01, %₃p<0.001 or %₄p<0.0001 to 2-month FA; ^₄p<0.0001 to 2-month ECIG.

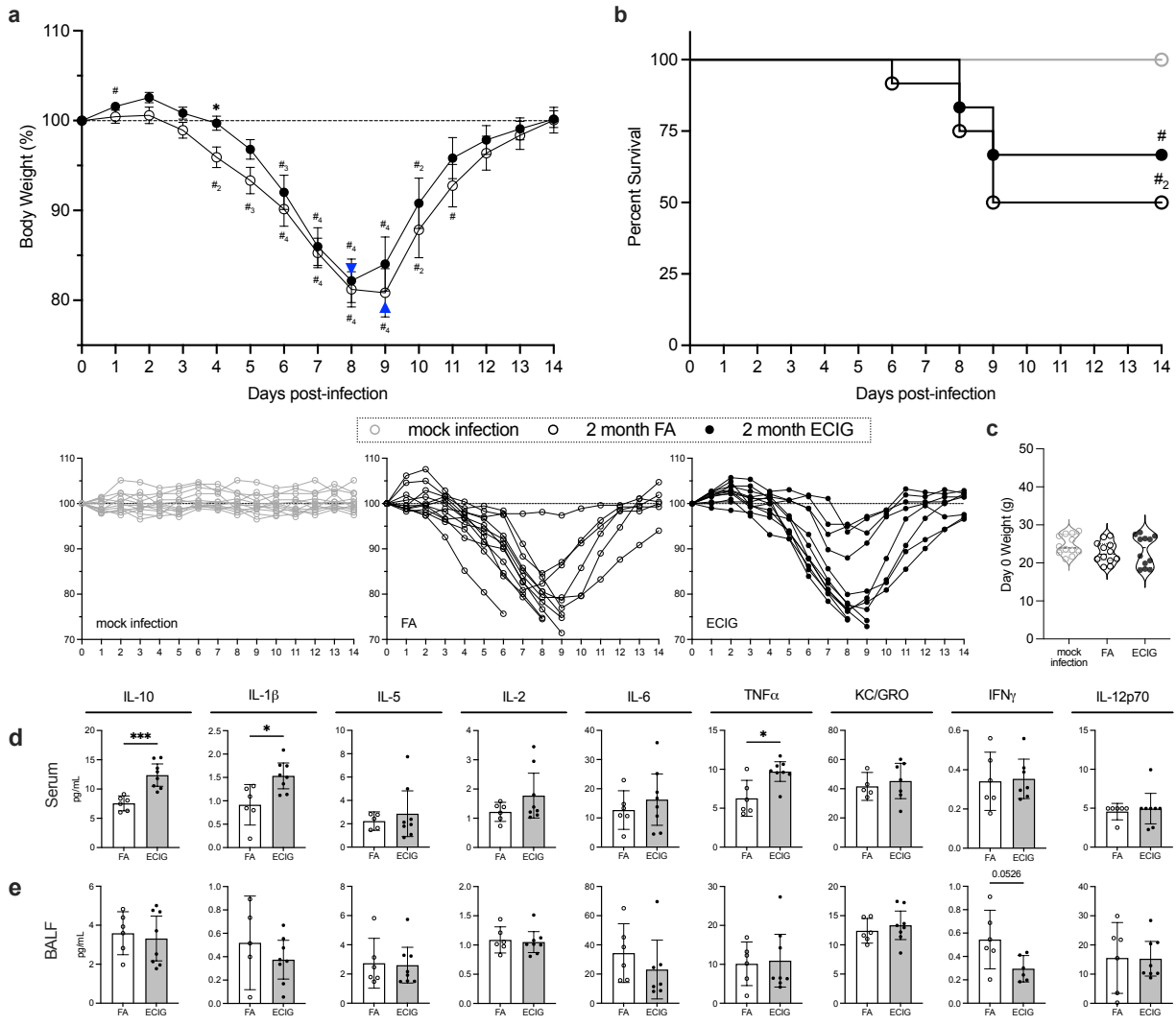


Figure 4.4: E-cigarette exposure prior to influenza infection alters systemic cytokines after recovery. (a-e) 2-month-old C57BL/6 mice were exposed to FA or ECIG for 10 days prior to intranasal inoculation with 10 PFU of A/PR8 IAV, or PBS for mock infections; n=12 per group. (a) Mice were weighed daily over the course of infection. Percent of Day 0 body weight data is presented as an average (top) and individually (bottom). Weights from mice that reached the humane endpoint were included in analysis. Blue triangles indicate day of peak weight loss. Data are reported as mean \pm SEM. (b) Probability of survival to 14 dpi was plotted. Statistical significance was determined by log rank (Mantel-Cox) test. (c) Violin plot of Day 0 weights in

grams. **(d, e)** Serum **(d)** or BALF **(e)** was collected at 14 dpi and cytokine concentrations were quantified. n=6-8 per group. Data are presented as mean \pm 95% CI. Statistical significance determined by one-way ANOVA with Tukey's correction for multiple comparisons **(a)**, log rank (Mantel-Cox) test **(b)**, or two-tailed Student's *t*-test with Welch's correction **(d, e)**. * $p < 0.05$, *** $p < 0.001$ comparison to FA or as indicated by bars; # $p < 0.05$, #₂ $p < 0.01$, #₃ $p < 0.001$, or #₄ $p < 0.0001$ to mock infected control.

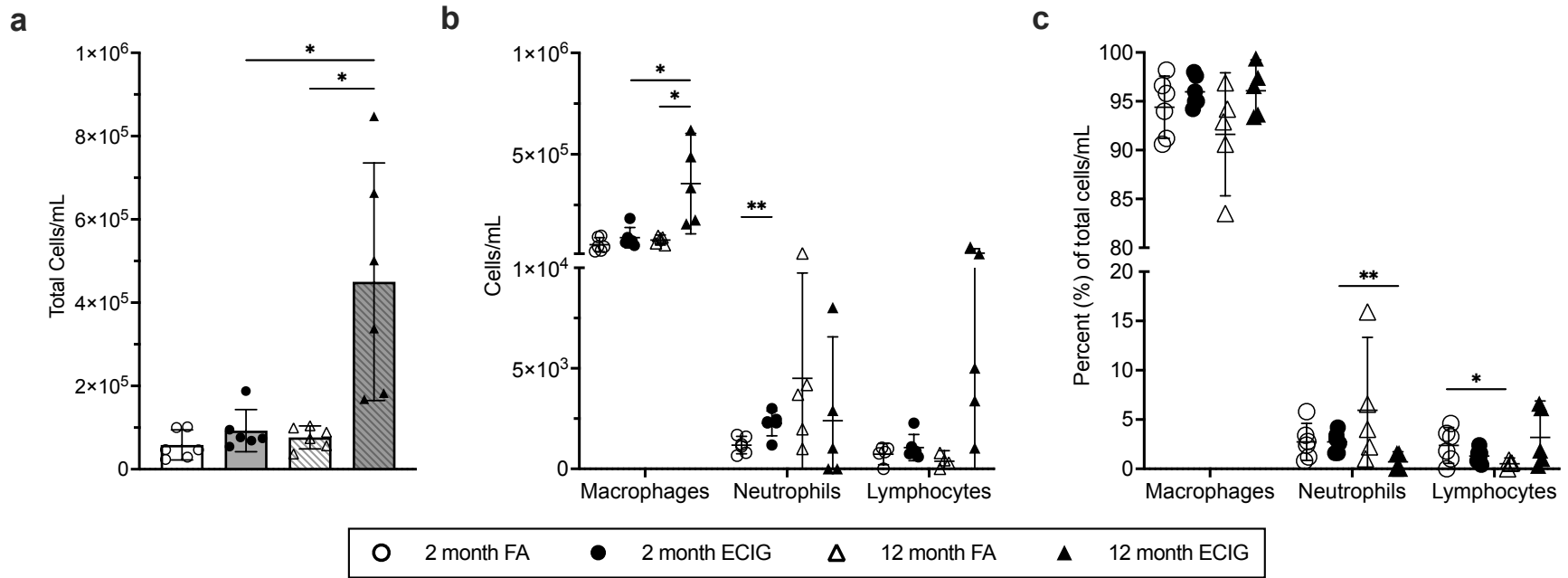


Figure 4.5: E-cigarette exposure differentially impacts lung leukocyte influx in young and aged BALB/c mice. (a-c) 2- and 12-month-old BALB/c mice were placed in whole-body exposure chambers and exposed to filtered air (FA) or e-cigarette aerosols (ECIG) for three hours/day for 10 days. Bronchoalveolar lavage (BAL) was collected immediately following exposure on day 10. Total BAL cells were counted (a) and analyzed via Cytospin to determine absolute numbers (b) and proportions (c) of leukocyte populations. n=6 per group. Data are presented as mean \pm 95% CI. Statistical significance determined by two-tailed Student's *t*-test with Welch's correction. * $p < 0.05$, ** $p < 0.01$.

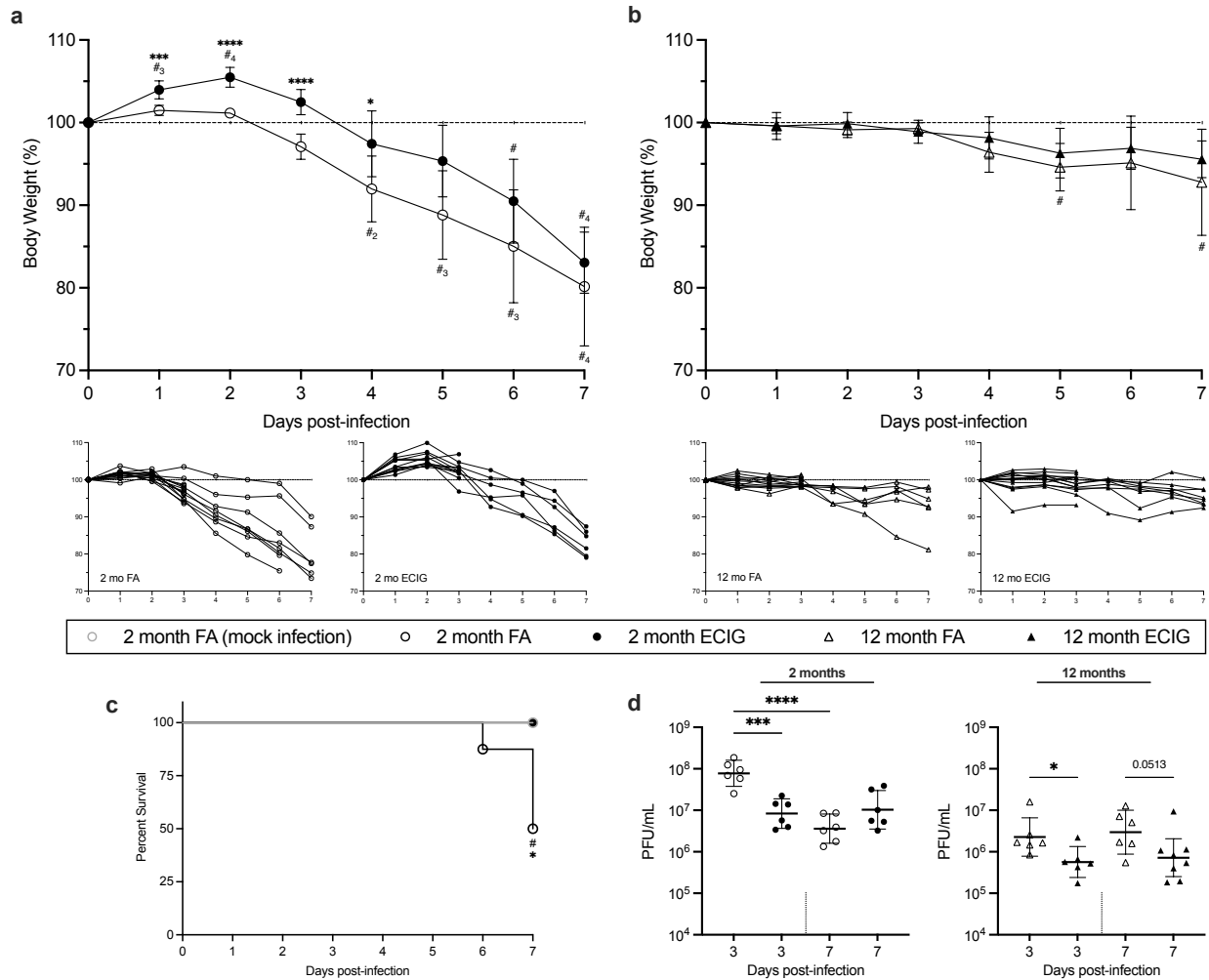


Figure 4.6: E-cigarette exposure prior to infection reduces viral titers and promotes survival.

(a-d) 2- and 12-month-old BALB/c mice were exposed to FA or ECIG for 10 days prior to intranasal inoculation with 10 PFU of A/PR8 IAV or PBS for mock infections. **(a, b)** 2- (a) and 12-month-old (b) mice were weighed daily over the course of infection. Percent of Day 0 body weight data is presented as an average (top) and individually (bottom). Data are reported as mean \pm 95% CI. **(c)** Probability of survival to 7 dpi for 2-month-old BALB/c mice was plotted. 12-month-old BALB/c survival was 100% for all groups (data not shown). **(d)** Viral loads from lung homogenate were determined by RT-qPCR at 3 and 7 dpi. n=6-9 per group. Statistical significance determined by one-way ANOVA with Tukey's correction for multiple comparisons (**a, b**) or log

rank (Mantel-Cox) test (**c**) or two-tailed Student's t-test with Welch's correction (**d**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ to ECIG or as indicated by bars; # $p < 0.05$, #₂ $p < 0.01$, #₃ $p < 0.001$, or #₄ $p < 0.0001$ to mock infected control.

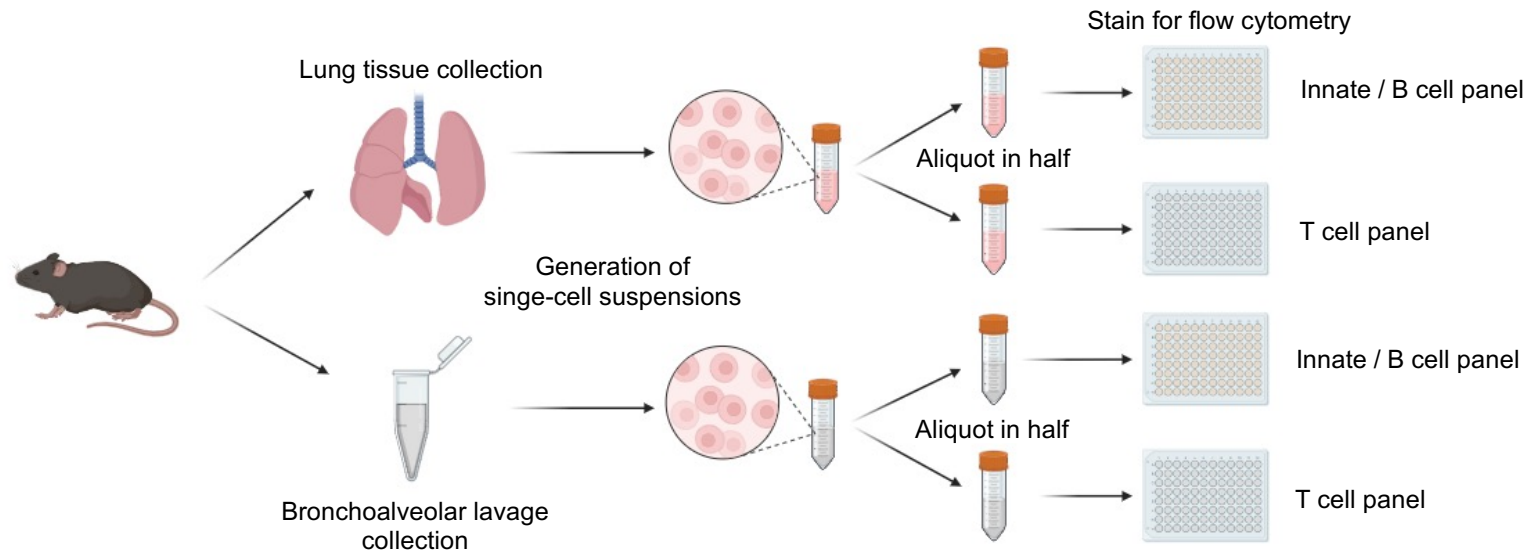


Figure 4.7: Diagram of tissue collection, processing, and staining for flow cytometry. BAL and lung tissue were processed into single-cell suspensions and then were stained with two antibody-fluorophore conjugate panels for quantification of 15 immune cell populations using sequential gating.

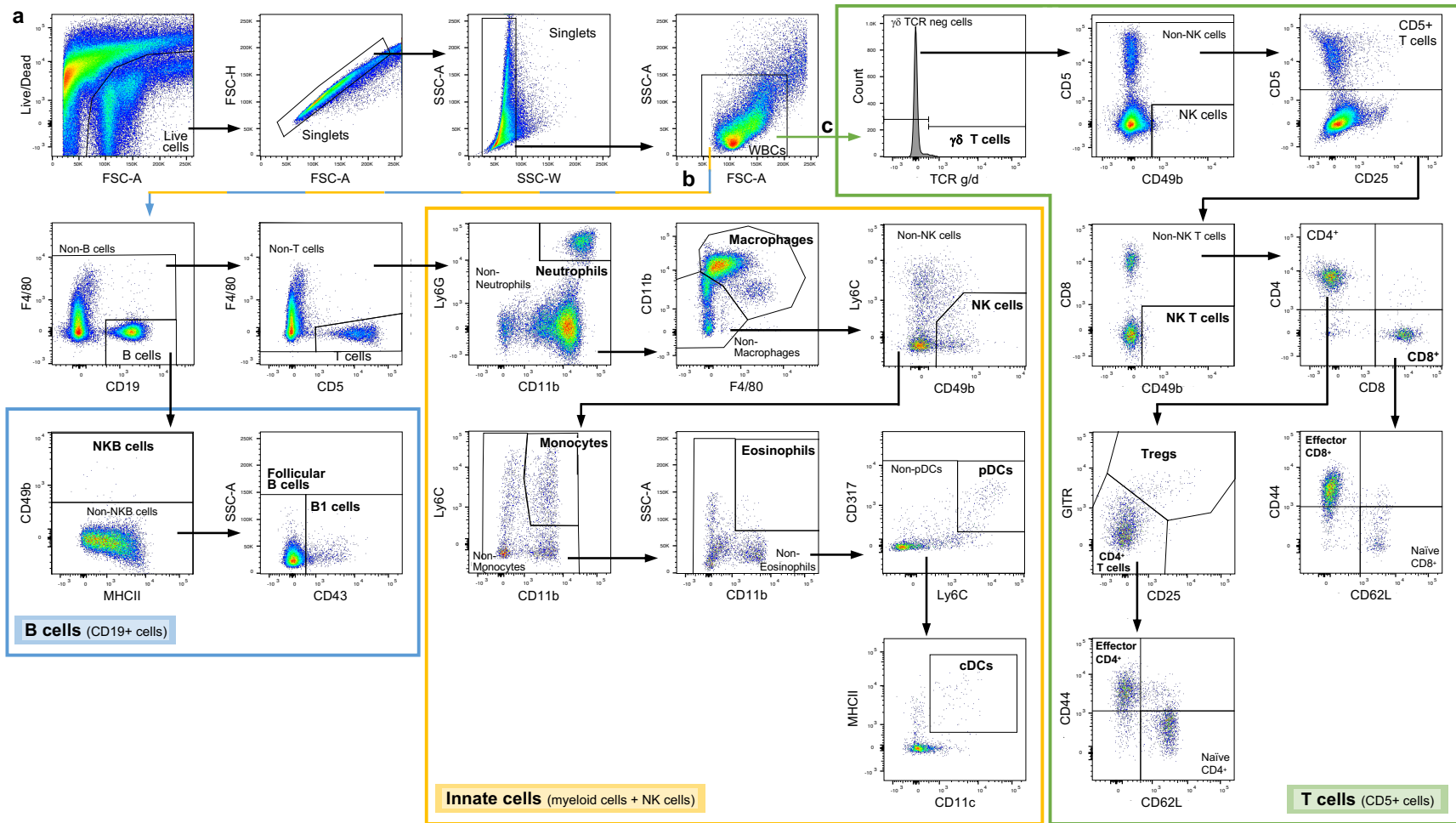


Figure 4.8: Flow cytometry gating strategies for investigation of immune cells in murine BAL and lungs. (a-c) Representative flow plots for identification of 15 immune cell types (bolded on plots) as described in Table 1, where all BAL and lung samples were first gated on live, singlet WBCs (a) as seen in the first 4 panels. Then either cells stained for innate and B cell markers were identified

(b) or cells stained for T cell markers were identified (c). Striped yellow and blue line indicates all subsequent plots result from the innate and B cell marker panel. Green line indicates all subsequent plots result from the T cell panel.

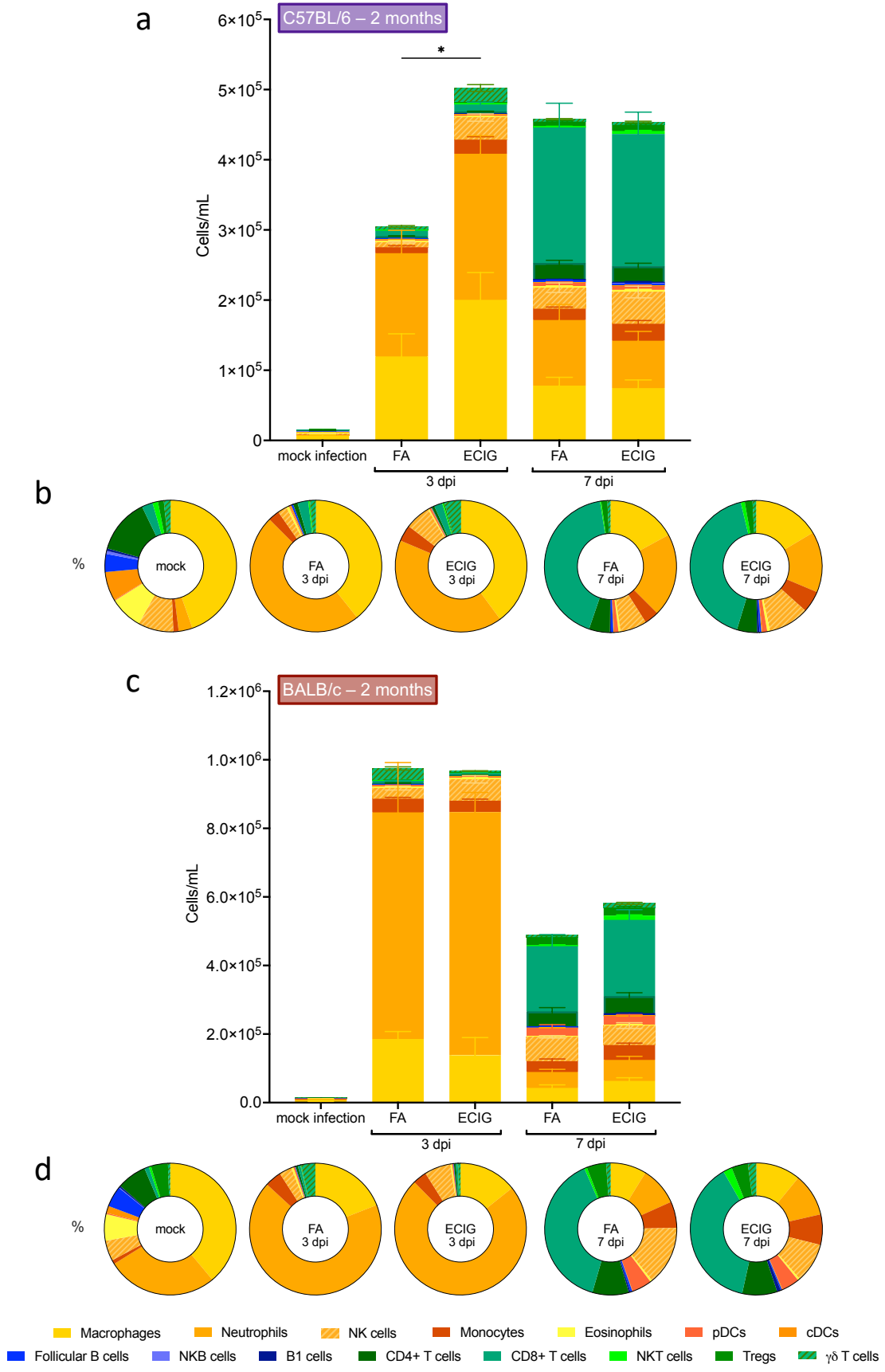


Figure 4.9: Immune cell populations in bronchoalveolar lavage from 2-month-old mice during influenza infection. (a-d) 2-month-old C57BL/6 or BALB/c mice were infected with 10 PFU A/PR8 or mock infected with PBS following 10 days of exposure to either FA or ECIG aerosol. At 3 or 7 dpi, bronchoalveolar lavage (BAL) fluid was collected, processed into a single-cell suspension, and stained with two antibody-fluorophore conjugate panels for quantification of 15 immune cell populations using the sequential gating strategy seen in **Figure 8a-c**. Cell population surface markers are defined in **Table 1**. Cell counts shown in stacked bar graphs (**a, c**) are presented as mean \pm SEM. Mean cell population proportions (as % of total BAL cells) are shown in donut graphs (**b, d**). Comparison of total BAL cells are indicated by bars. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. *p<0.05.

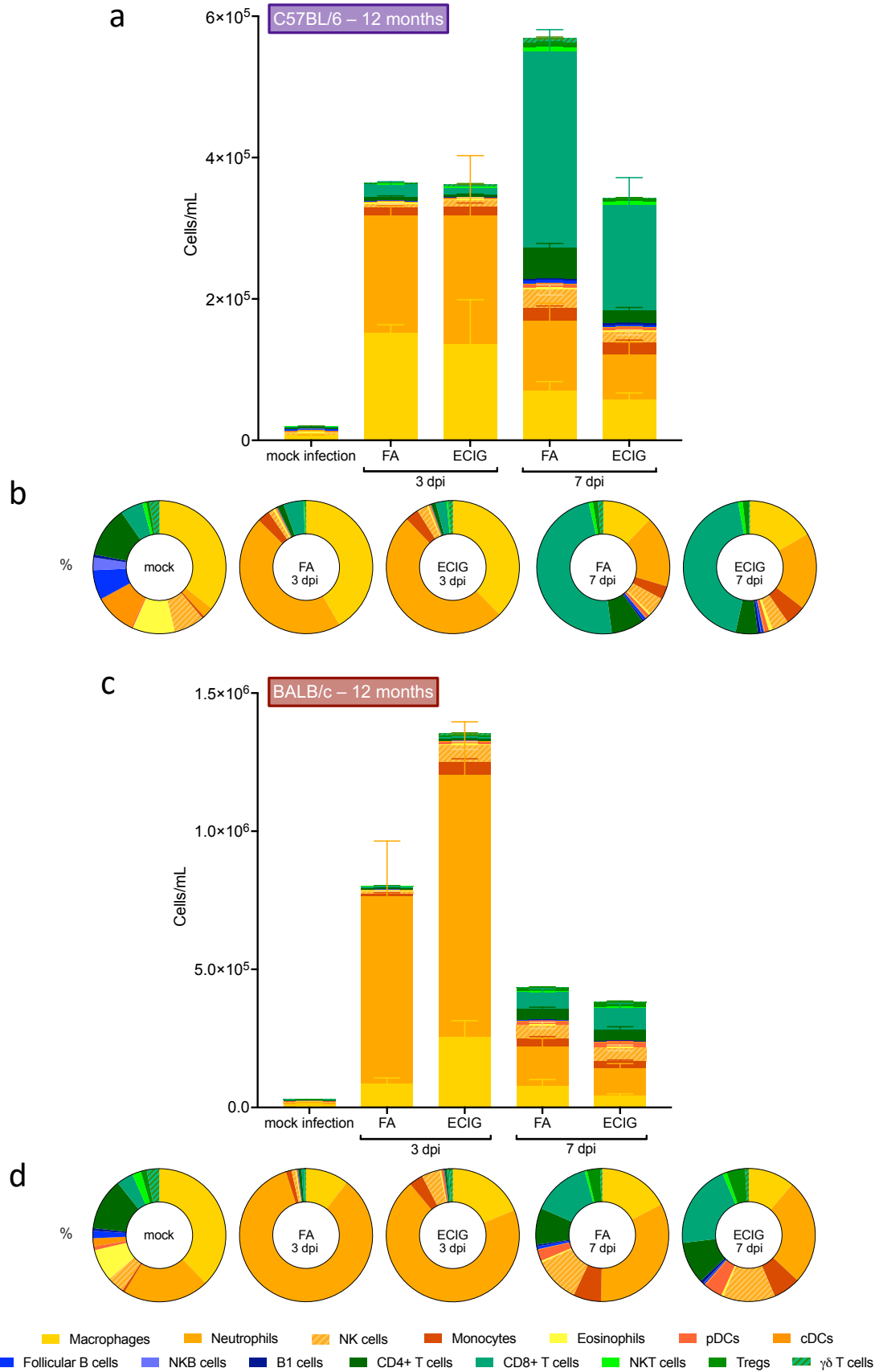


Figure 4.10: Immune cell populations in bronchoalveolar lavage from 12-month-old mice during influenza infection. (a-d) 12-month-old C57BL/6 or BALB/c mice were infected with 10 PFU A/PR8 or mock infected with PBS following 10 days of exposure to either FA or ECIG aerosol. At 3 or 7 dpi, bronchoalveolar lavage (BAL) fluid was collected, processed into a single-cell suspension, and stained with two antibody-fluorophore conjugate panels for quantification of 15 immune cell populations using the sequential gating strategy seen in **Figure 8a-c**. Cell population surface markers are defined in **Table 1**. Cell counts shown in stacked bar graphs (**a, c**) are presented as mean \pm SEM. Mean cell population proportions (as % of total BAL cells) are shown in donut graphs (**b, d**). Comparison of total BAL cells are indicated by bars. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group.

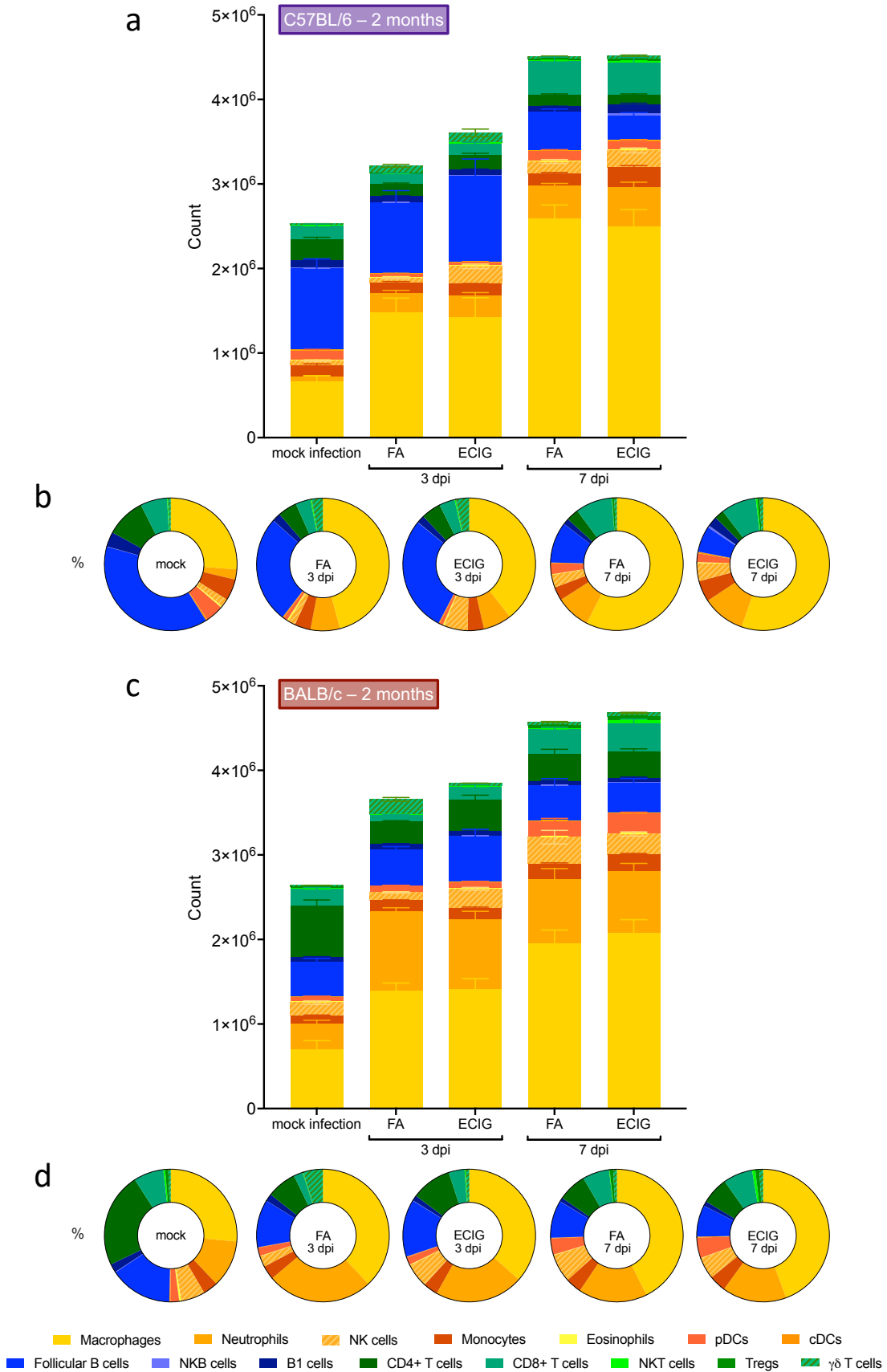


Figure 4.11: Immune cell populations in lung tissue from 2-month-old mice during influenza infection. (a-d) 2-month-old C57BL/6 or BALB/c mice were infected with 10 PFU A/PR8 or mock infected with PBS following 10 days of exposure to either FA or ECIG aerosol. At 3 or 7 dpi, lung tissue was collected, processed into a single-cell suspension, and stained with two antibody-fluorophore conjugate panels for quantification of 15 immune cell populations using the sequential gating strategy seen in **Figure 8a-c**. Cell population surface markers are defined in **Table 1**. Cell counts shown in stacked bar graphs (**a, c**) are presented as mean \pm SEM. Mean cell population proportions (as % of total lung cells) are shown in donut graphs (**b, d**). Comparison of total lung cells are indicated by bars. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. *p<0.05.

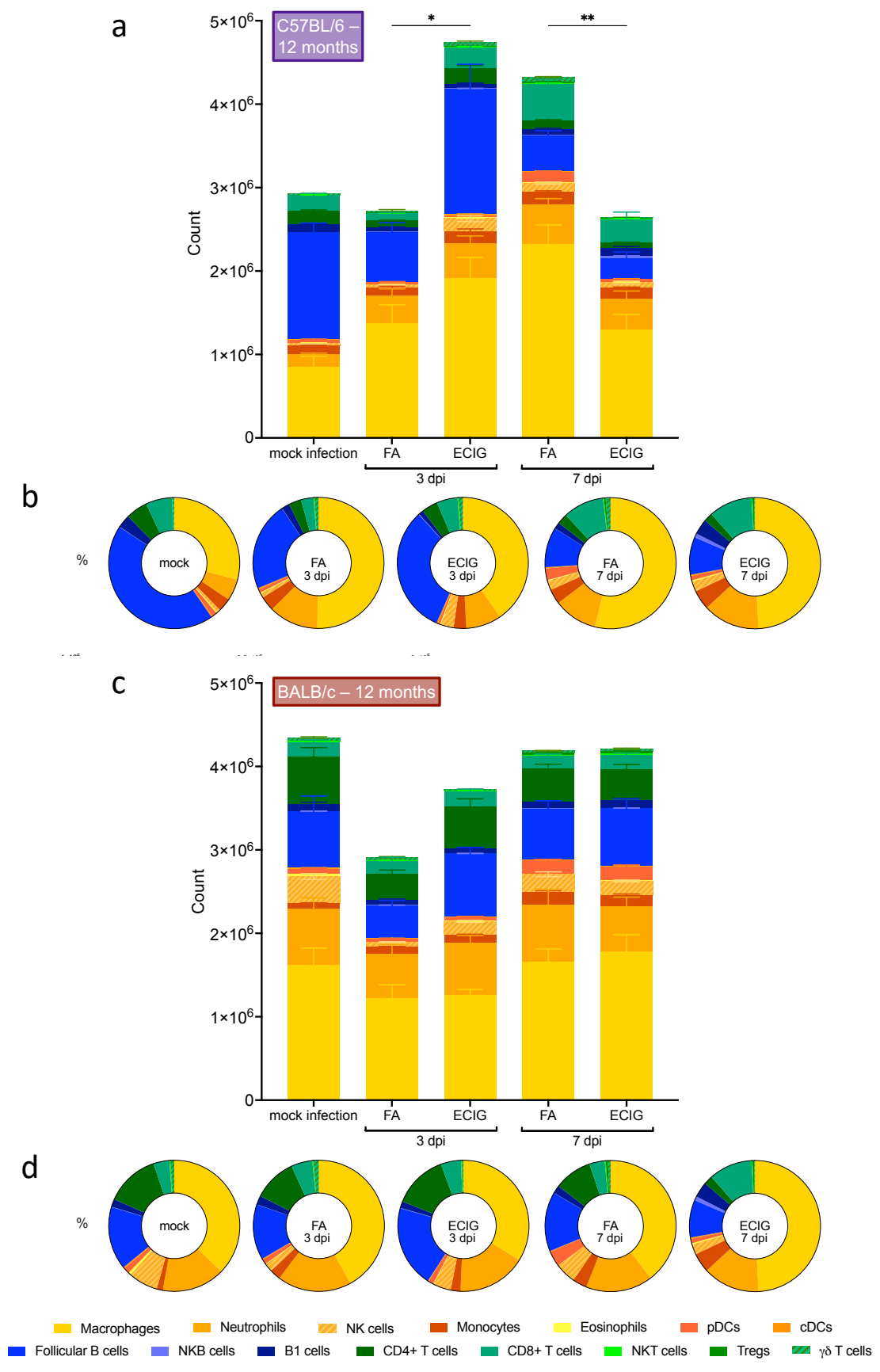


Figure 4.12: Immune cell populations in lung tissue from 12-month-old mice during influenza infection. (a-d) 12-month-old C57BL/6 or BALB/c mice were infected with 10 PFU A/PR8 or mock infected with PBS following 10 days of exposure to either FA or ECIG aerosol. At 3 or 7 dpi, lung tissue was collected, processed into a single-cell suspension, and stained with two antibody-fluorophore conjugate panels for quantification of 15 immune cell populations using the sequential gating strategy seen in **Figure 8a-c**. Cell population surface markers are defined in **Table 1**. Cell counts shown in stacked bar graphs (**a, c**) are presented as mean \pm SEM. Mean cell population proportions (as % of total lung cells) are shown in donut graphs (**b, d**). Comparison of total lung cells are indicated by bars. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. *p<0.05, **p<0.01.

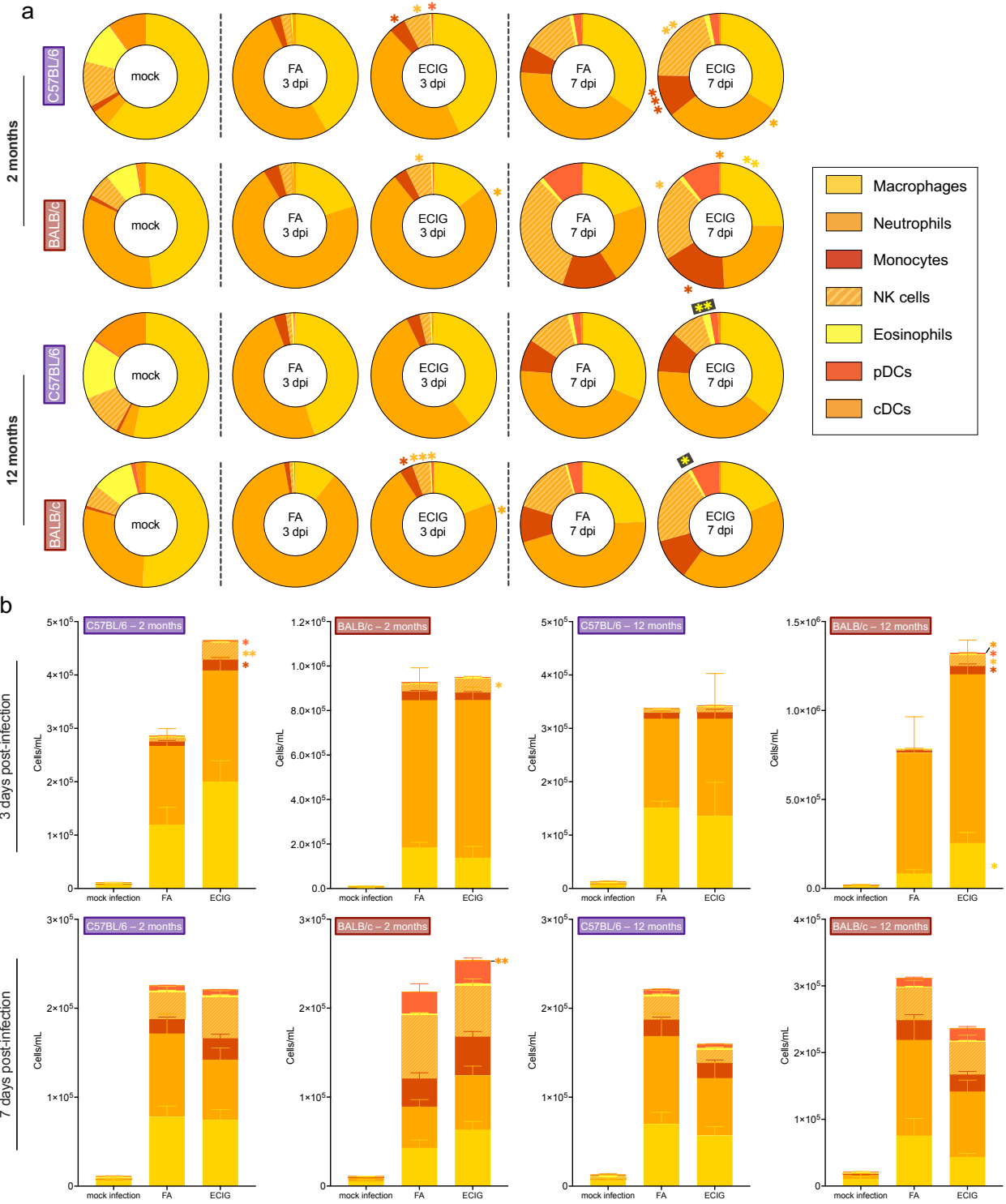


Figure 4.13: Innate cell populations in bronchoalveolar lavage during influenza infection.

Macrophages, neutrophils, Natural Killer (NK) cells, monocytes, eosinophils, plasmacytoid dendritic cells (pDCs), and classical dendritic cells (cDCs) were quantified in bronchoalveolar

lavage from 2- or 12-month-old C57BL/6 and BALB/c mice by flow cytometry using sequential gating as seen in **Figure 8a, b**. Cell population surface markers are defined in **Table 1**. **(a)** Innate cell proportions, as percent of total innate cells, in BAL. Data are presented as mean. **(b)** Composition of total innate cells in BAL. Data are presented as mean \pm SEM. **(a, b)** Colored symbols in donut graphs or to right of stacked bar graph indicate significance for like-colored individual cell types; comparison of FA to ECIG. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

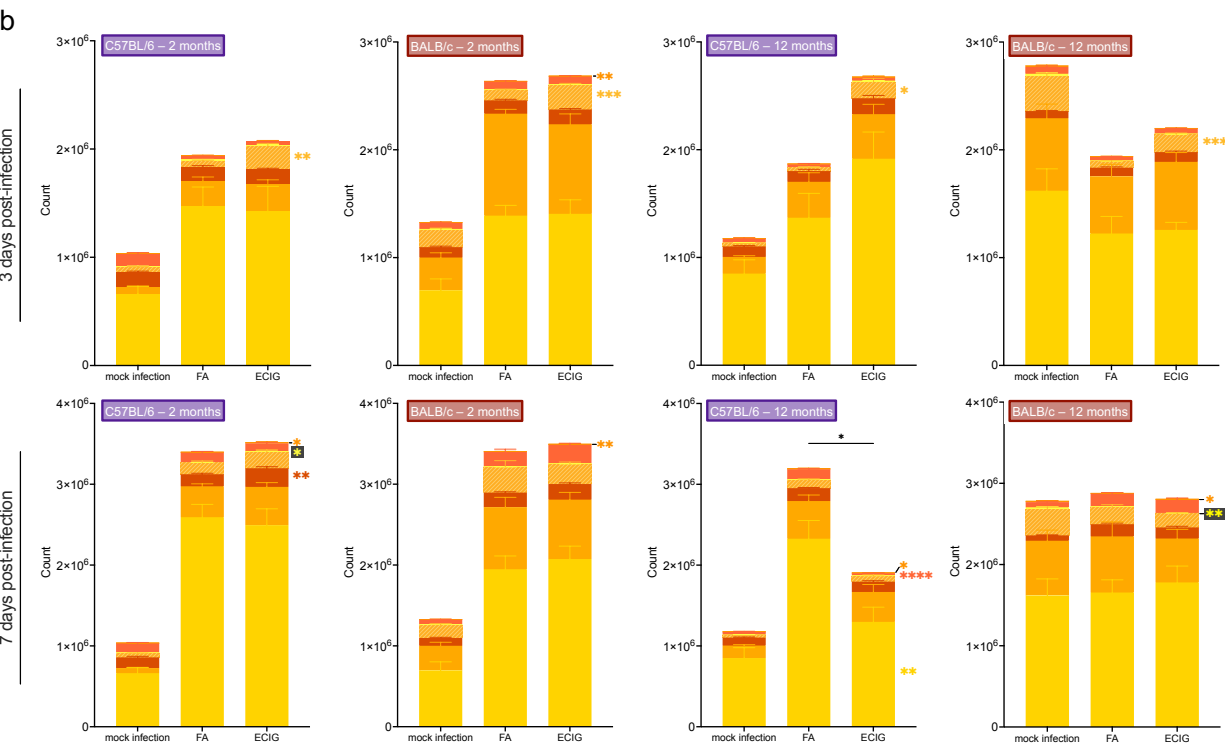
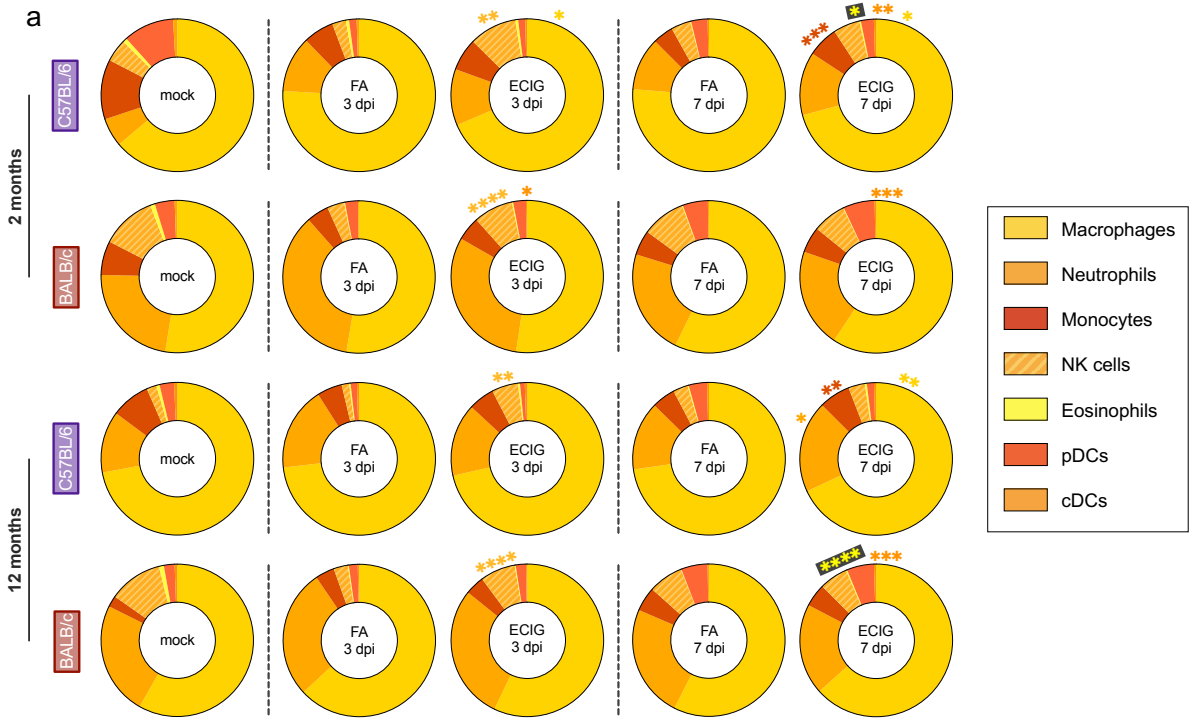


Figure 4.14: Innate cell populations in lung tissue during influenza infection. Macrophages, neutrophils, Natural Killer (NK) cells, monocytes, eosinophils, plasmacytoid dendritic cells (pDCs), and classical dendritic cells (cDCs) were quantified in lung tissue from 2- or 12-month-

old C57BL/6 and BALB/c mice by flow cytometry using sequential gating seen in **Figure 8a, b**. Cell population surface markers are defined in **Table 1**. **(a)** Innate cell proportions, as percent of total innate cells, in lungs. Data are presented as mean. **(b)** Composition of total innate cells in lungs. Data are presented as mean \pm SEM. Black bars indicate statistical significance of total innate cells; comparison of FA to ECIG. **(a, b)** Colored symbols in donut graphs or to right of stacked bar graph indicate significance for like-colored individual cell types; comparison of FA to ECIG. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

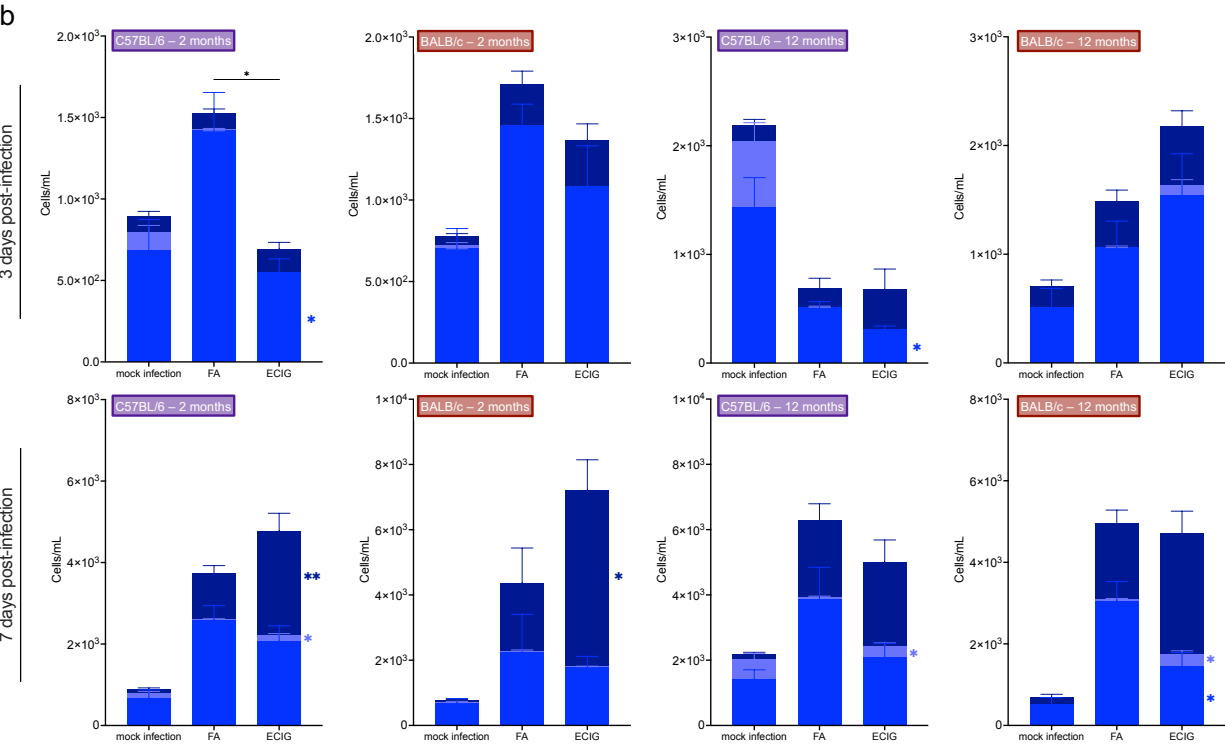
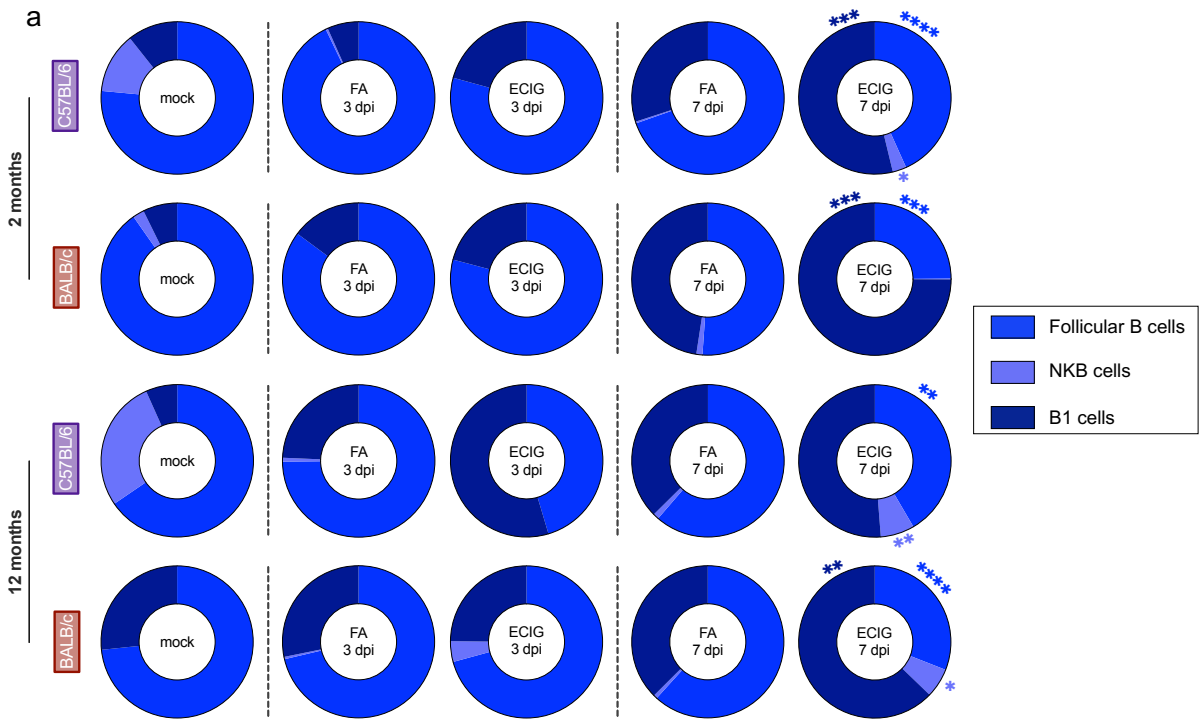


Figure 4.15: B cell populations in bronchoalveolar lavage during influenza infection.

Follicular B cells, NKB cells, and B1 cells were quantified in bronchoalveolar lavage from 2- or 12-month-old C57BL/6 and BALB/c mice by flow cytometry using sequential gating as seen in

Figure 8a, b. Cell population surface markers are defined in **Table 1.** **(a)** B cell proportions, as percent of total B cells, in BAL. Data are presented as mean. **(b)** Composition of total B cells in BAL. Data are presented as mean \pm SEM. Black bars indicate statistical significance of total B cells; comparison of FA to ECIG. **(a, b)** Colored symbols in donut graphs or to right of stacked bar graph indicate significance for like-colored individual cell types; comparison of FA to ECIG. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

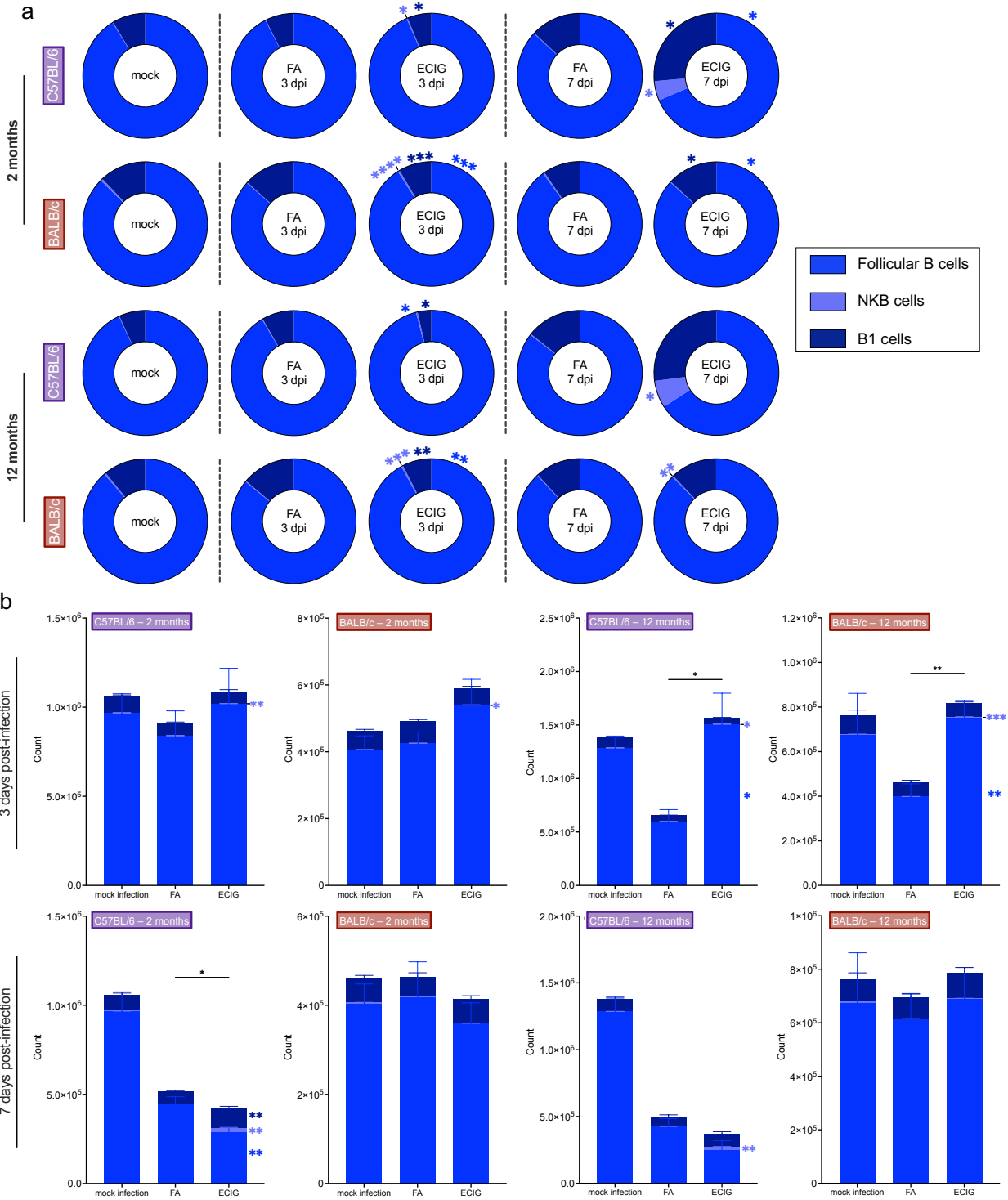


Figure 4.16: B cell populations in lung tissue during influenza infection. Follicular B cells, NKB cells, and B1 cells were quantified in lung tissue from 2- or 12-month-old C57BL/6 and BALB/c mice by flow cytometry using sequential gating seen in **Figure 8a, b**. Cell population

surface markers are defined in **Table 1**. **(a)** B cell proportions, as percent of total B cells, in lungs. Data are presented as mean. **(b)** Composition of total B cells in lungs. Data are presented as mean \pm SEM. Black bars indicate statistical significance of total B cells; comparison of FA to ECIG. **(a, b)** Colored symbols in donut graphs or to right of stacked bar graph indicate significance for like-colored individual cell types; comparison of FA to ECIG. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

as seen in **Figure 8a, b**. Cell population surface markers are defined in **Table 1**. **(a)** T cell proportions, as percent of total T cells, in BAL. Data are presented as mean. **(b)** Composition of total T cells in BAL. Data are presented as mean \pm SEM. Black bars indicate statistical significance of total T cells; comparison of FA to ECIG. **(a, b)** Colored symbols in donut graphs or to right of stacked bar graph indicate significance for like-colored individual cell types; comparison of FA to ECIG. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

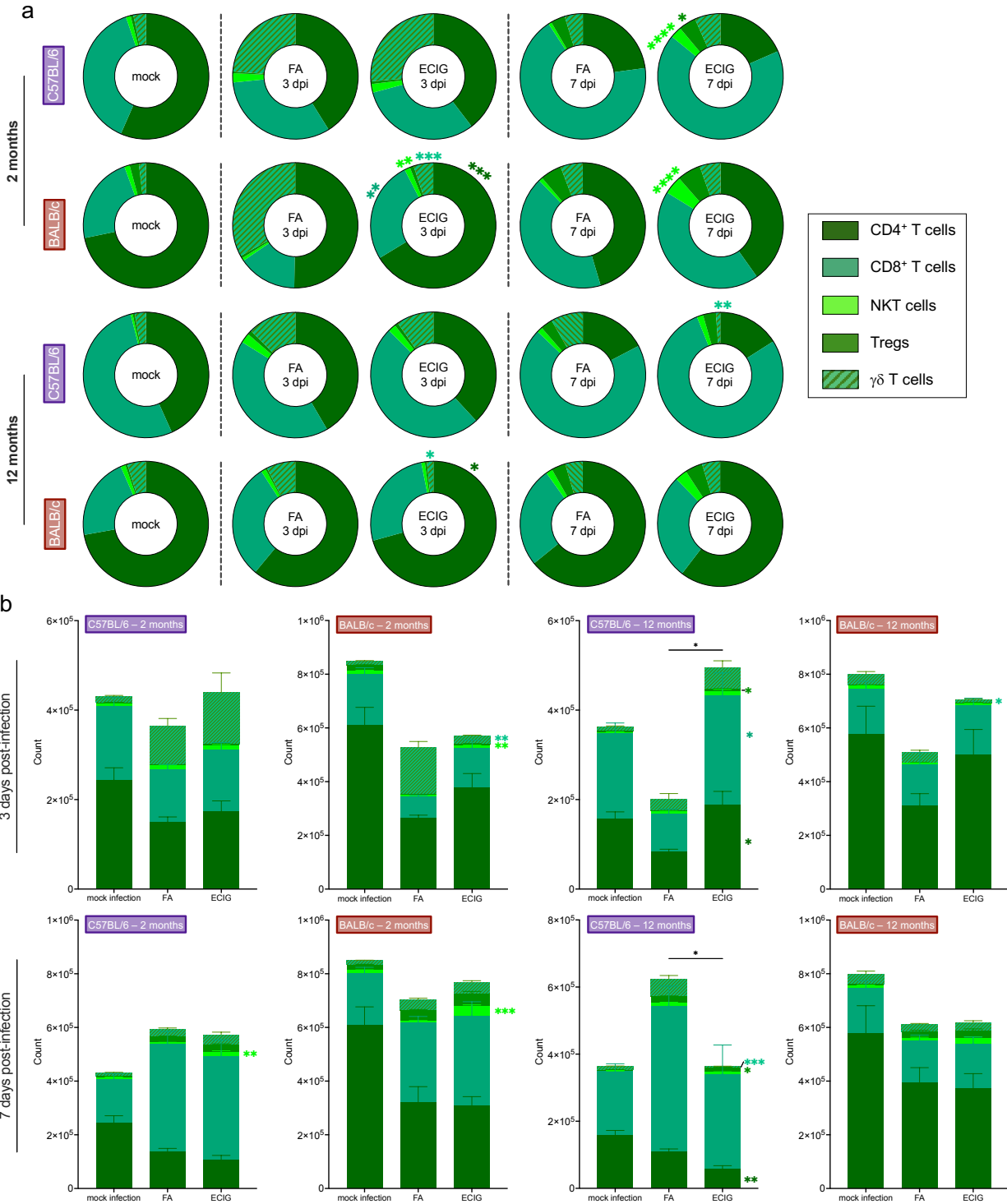


Figure 4.18: T cell populations in lung tissue during influenza infection. CD4⁺ T cells, CD8⁺ T cells, NKT cells, Tregs, and γδ T cells were quantified in lung tissue from 2- or 12-month-old C57BL/6 and BALB/c mice by flow cytometry using sequential gating as seen in **Figure 8a, b**.

Cell population surface markers are defined in **Table 1**. **(a)** T cell proportions, as percent of total T cells, in lungs. Data are presented as mean. **(b)** Composition of total T cells in lungs. Data are presented as mean \pm SEM. Black bars indicate statistical significance of total T cells; comparison of FA to ECIG. **(a, b)** Colored symbols in donut graphs or to right of stacked bar graph indicate significance for like-colored individual cell types; comparison of FA to ECIG. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

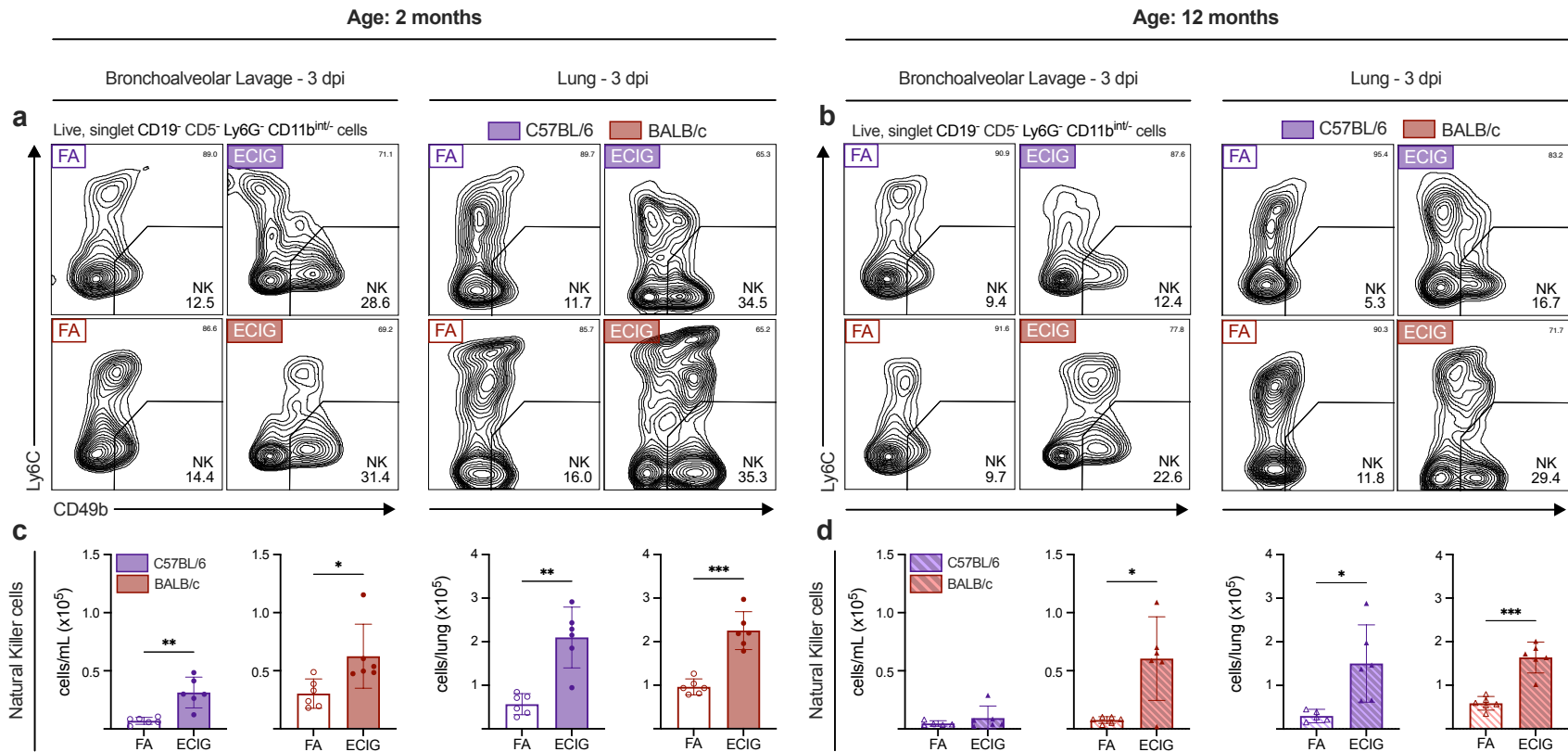


Figure 4.19: E-cigarette exposure increases Natural Killer cell infiltration to airways and lungs at 3 days post-infection.

(a, b) Representative flow cytometric contour plots of Natural Killer (NK) cells identified in bronchoalveolar lavage and lungs from 2-month-old (a) or 12-month-old (b) C57BL/6 and BALB/c mice exposed to 10 days of FA or ECIG prior to infection with influenza (10 PFU). BAL and lungs collected at 3 days post-infection. Frequencies of NK cells are indicated in bottom right-hand corner of each plot. (c, d) Absolute numbers of NK cells represented in (a, b) from each compartment are shown. Data are mean \pm 95% CI. Statistical

significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

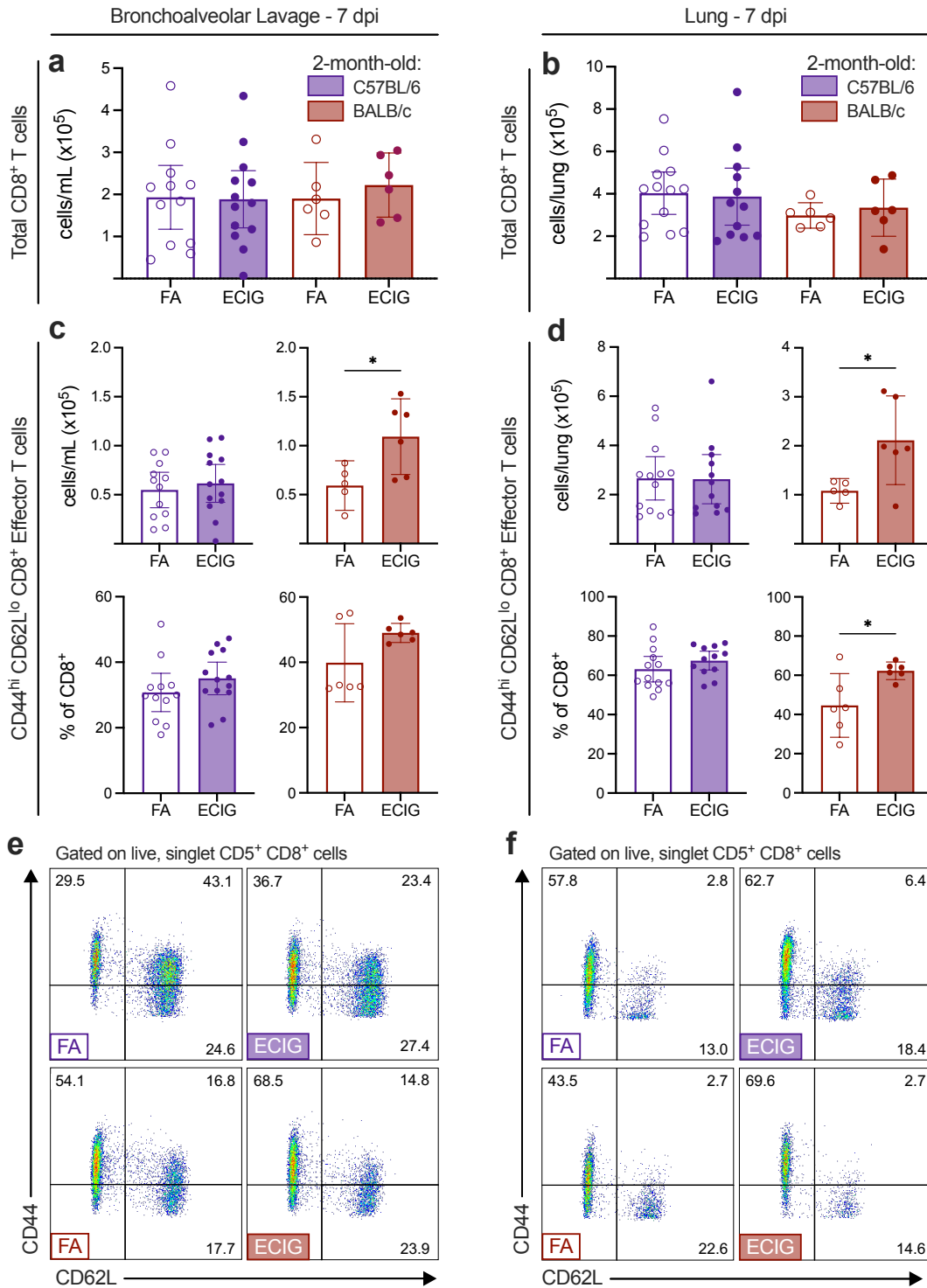


Figure 4.20: E-cigarette exposure prior to infection results in increased effector, CD8+ T cells populations in airways and lungs of BALB/c mice (a, b) Absolute numbers (cells/mL or cells/lung) of total CD8⁺ T cells (live, singlet, TCRgd⁻ CD49b⁻ CD5⁺ CD8⁺) identified by flow

cytometry from BAL (**a**) or lung tissue (**b**) at 7 dpi from 2-month-old C57BL/6 or BALB/c mice exposed to 10 days of FA or ECIG prior to infection with influenza (10 PFU). (**c**, **d**) Absolute numbers (cells/mL or cells/lung) and frequencies of effector CD8⁺ T cells (live, singlet, TCRgd⁺ CD49b⁻ CD5⁺ CD8⁺ CD44^{hi} CD62L^{lo}) as a percent of total CD8⁺ T cells in BAL (**c**) or lung (**d**) as identified by flow cytometry. **e**, **f** Representative flow plots of BAL (**e**) or lung (**f**) showing frequency of effector CD8⁺ T cells. Data (**a-d**) are mean ± 95% CI. Statistical significance was determined by two-tailed Student's *t*-test with Welch's correction. n=6-13 per group. *p<0.05

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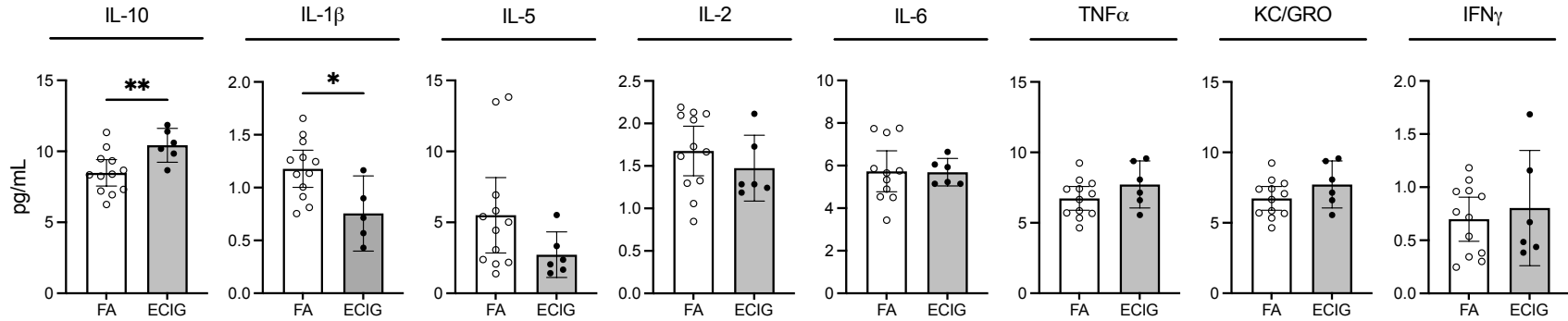
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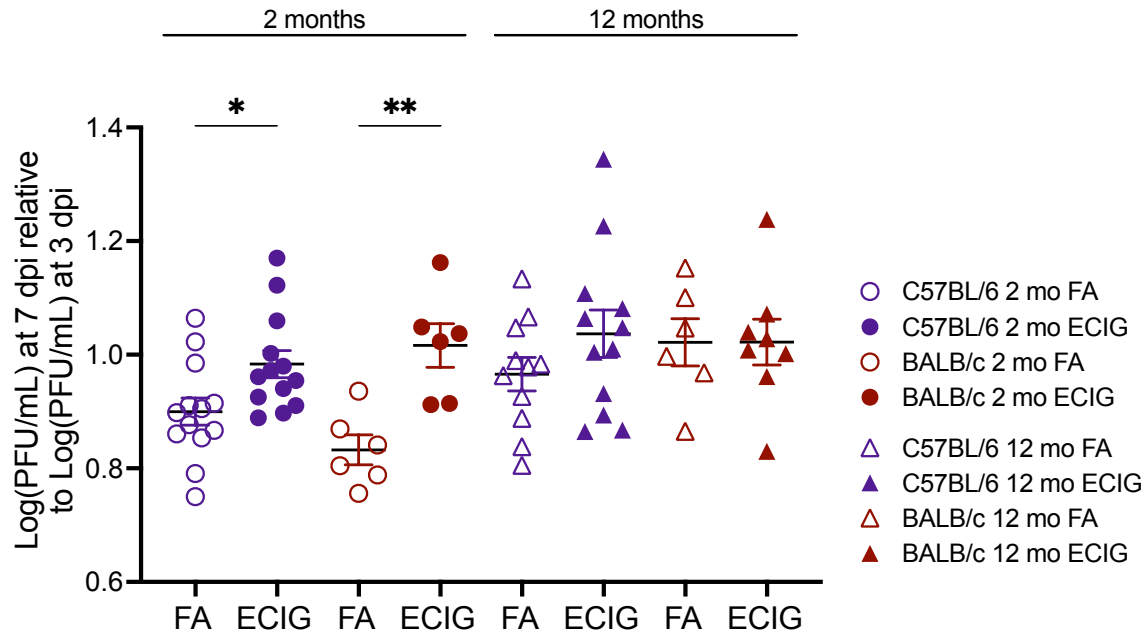
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Supplemental Figure 4.1: Serum cytokine concentrations following ECIG exposure 2- month-old C57BL/6 mice were placed in whole-body exposure chambers and exposed to FA or e-cigarette aerosols (ECIG) for three hours/ day for 10 days. Serum was collected immediately following exposure on day 10 for cytokine analysis. n=6 or 12 per group. Data are presented as mean ± 95% CI. Statistical significance determined by two-tailed Student's t-test with Welch's correction. *p<0.05, **p<0.01



Supplemental Figure 4.2: E-cigarette exposure prior to IAV infection delays viral clearance.

2- and 12-month-old C57BL/6 or BALB/c mice were exposed to FA or ECIG for 10 days prior to i.n. inoculation with 10 PFU of PR8 murine influenza. Viral loads from lung homogenate were determined by RT-qPCR at 3 and 7 dpi. The ratio of viral load at 7 dpi to viral load at 3 dpi is shown. Data are mean \pm SEM. n= 6-13 per group. Statistical significance determined by two-tailed Student's *t*-test with Welch's correction. * $p < 0.05$, ** $p < 0.01$.

CHAPTER 5:

Conclusions

Conclusions:

In this dissertation, we reviewed the maternal and fetal health outcomes associated with pre-natal or early-life e-cigarette exposures. Vaping during pregnancy is not risk free. Overall, the data clearly show that *in utero* exposures to e-cigarette aerosols confers significant risks to the developing fetus. As shown in experimental models, significant harm can be done to fetal lungs, heart, and other organs and these effects can last far beyond birth into the postnatal life. There are still many knowledge gaps related to the effects of early life exposures to e-cigarette aerosols on the health of the offspring. Thus, further investigations are necessary to better understand the health effects of e-cigarette usage on vulnerable populations such as pregnant women and their unborn children.

Women of reproductive age, who are planning to conceive, pregnant women, and women who are breastfeeding, should be made aware of the potential adverse effects of e-cigarette aerosol exposures during this critical and sensitive period of fetal and neonatal development. E-cigarette devices may not be a ‘safe’ alternative to cigarette smoking, or even for cessation or temporary switching, for this vulnerable population. Health care providers such as obstetrician-gynecologists are helpful resources that should disseminate knowledge about e-cigarettes and their harm during pregnancy. Nonetheless, more clinical, epidemiological, and experimental research are needed to better understand both the maternal and fetal health effects induced by *in utero* e-cigarette aerosols exposures both with and without nicotine content.

We then investigated how e-cigarette coil temperature impacts the chemical composition of e-cigarette aerosols and whether this results in changes to pulmonary inflammation. Third-generation e-cigarette users can precisely control the power and temperature settings of their devices to achieve a desired experience (improved taste, larger vape cloud, etc.). Regulation of

device capabilities, such as laws setting a maximum temperature a device can operate at, are non-existent in the U.S. This is likely because health consequences of vaping at higher temperatures are not well characterized. However, chemical composition studies suggest that vaping at high temperatures increases aerosol generation, which can increase concentrations of potentially harmful carbonyl compounds. We found that direct sampling from our e-cigarette device confirms that as temperature increases, total aerosol and carbonyl concentrations per puff increase. Interestingly, we found that when total aerosol concentrations were constant, vaping at a higher coil temperature resulted in a significant reduction in thermal carbonyls such as formaldehyde and acetaldehyde. The reason for this is largely unknown and requires further research by analytical chemists. Our research shows that vaping at higher temperatures can dampen pulmonary immune responses and enhance cytokine gene downregulation. This indicates a potentially increased risk to e-cigarette users who choose to vape at higher temperatures. Our research underscores the need for government oversight and regulation of e-cigarette device power and temperature settings to minimize health risks to consumers.

Next, we investigated progressive exposure to e-cigarettes and compared it to traditional cigarette smoke exposure in mice. We found that nicotine metabolism from e-cigarettes and cigarettes was significantly different in mice, evidenced by serum cotinine to nicotine ratios, indicating a different accumulation of nicotine metabolites depending on the nicotine source. This highlights the need for more research into nicotine processing as this could impact many organ systems of the body. The new trend of e-cigarette companies using nicotine salts to further maximize nicotine delivery underscores the critical need for more research in this field.

Progressive e-cigarette exposure was found to induce a unique, acute inflammatory response compared to that seen after progressive cigarette exposure in mice. This study also

found a consistent immunosuppressive effect of e-cigarettes on pulmonary cytokine expression which was replicated in human macrophage and epithelial cell lines. Like the previous study in this dissertation, this data demonstrates that e-cigarettes can dampen pulmonary immune responses. Alterations in pulmonary immunity can have important consequences for susceptibility to infections, especially in immunologically vulnerable populations.

To investigate this possibility, lastly, we exposed young and aged mice to e-cigarette aerosols then infected them with influenza A virus. This study is, to our knowledge, the first to report that aging alone can increase inflammation associated with e-cigarette use, which warrants further research into whether age amplifies risks associated with vaping. Importantly, we demonstrated that e-cigarette exposure can significantly alter mortality from influenza and delay viral clearance. E-cigarette exposure was also able to alter immune cell populations and serum cytokine concentrations which remained after resolution of infection. Importantly, we produced a detailed report of how e-cigarette exposure altered the lung and bronchoalveolar lavage immune cell compositions during influenza infection. We demonstrate that both innate and adaptive leukocyte populations are impacted by e-cigarette exposure prior to infection. Of note, we reported that e-cigarette exposure prior to infection consistently enhanced NK cell recruitment during early infection in both age groups. Together, our findings help to further the understanding of how e-cigarette exposure disrupts the pulmonary immune system and provides insight into the potential health consequences of those disruptions.

In conclusion, this dissertation helps further our current scientific understanding of the impacts of e-cigarette aerosols and e-cigarette device settings on pulmonary immune homeostasis. In addition, our data helps further elucidate how e-cigarette-mediated immune dysregulation can impact susceptibility to viral infections. Together, this data underscores the

need for continued research in this field and highlights the need for potential government action to regulate e-cigarette devices and e-liquid sales to minimize negative public health outcomes.

E-cigarette use is unlikely to drastically decline in the near future. If history is any indicator, e-cigarette devices will continue to evolve and will remain a moving target for scientists seeking to understand the health consequences of their use. In the last 10 years, e-cigarette research has lagged behind e-cigarette user trends such that papers are still being published using first- and second-generation devices, which fell out of fashion more than 10 years ago around 2011. Future research should, therefore, try to use the most up-to-date e-cigarette devices such that published data can remain relevant to real-world e-cigarette users.

At present, e-cigarette research is complex due to the vast number of different devices, e-liquid formulations, and experimental parameters to choose from. Direct comparisons across studies is difficult due to lack in reporting of experimental conditions which are critical to understanding and contextualizing published results. Future e-cigarette research therefore would benefit from increased publication of experimental set-up and methodological data to facilitate meaningful comparisons. Adoption of exposure regimen guidelines across the field would also help to minimize confounding data.

Future research into the effects of e-cigarettes on the pulmonary immune system, and their impact on host pathogen interactions will benefit from both of these critical approaches.