The Pulmonary Immunotoxicity of E-Cigarette Aerosols in Murine Models of Airway Disease

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TABLE OF CONTENTS

ABSTRACT

E-cigarettes are battery-powered devices that heat a liquid containing nicotine and flavouring agents to produce an aerosol that is inhaled by the user. Although initially marketed to cigarette smokers as safer alternatives to smoking, e-cigarettes today are predominantly used by youth, including children, adolescents, and young adults. Currently, there are thousands of different devices and flavoured e-liquids available on the market, but limited health information related to their use. This lack of basic safety information is hampered in part because there is no standard preclinical model to study e-cigarette toxicology. Because of this, there is conflicting evidence about the effects of e-cigarette aerosols on pulmonary inflammation, a discrepancy which may be partly due to the different inbred mouse strains used between studies. Furthermore, the possibility that e-cigarette-induced pulmonary inflammation may contribute to the development of chronic respiratory diseases such as allergic asthma has not been investigated. We hypothesized that exposure to e-cigarette aerosols from JUUL, a popular e-cigarette brand, will induce different pulmonary immunophenotypes between the BALB/c and C57BL/6 strains of mice, and that this will alter the host immune response to a respiratory allergen. The Aims of this study are: 1) to compare the pulmonary immunophenotype of male and female BALB/c and C57BL/6 mice after a sub-chronic exposure to e-cigarette aerosols at the cellular, transcriptional, and proteomic levels; and 2) to characterize the pulmonary immunologic effects of e-cigarette aerosol exposure in an ovalbumin (OVA)-induced mouse model of allergic asthma. We first characterized the cellularity of the bronchoalveolar lavage (BAL) fluid and lung tissue through the use of flow cytometry and found there were few changes in innate and adaptive immune cell populations after JUUL exposure in either strain of mouse. However, there were differences in the transcriptional response of BALB/c and C57BL/6 mice to JUUL exposure, with proinflammatory and lung remodelling genes

being significantly upregulated in BALB/c mice but not in C57BL/6 mice. Finally in Aim 1, we quantified the BAL and lung proteome of air- and JUUL-exposed BALB/c and C57BL/6 mice via label-based quantitative mass spectrometry. Within the BAL fluid, 464 proteins were quantified in BALB/c mice and 958 were quantified in C57BL/6 mice. In the lung tissue, 1,316 and 1,226 proteins were quantified in BALB/c and C57BL/6 mice, respectively. Furthermore, sex-dependent differences and JUUL exposure contributed in a strain-dependent manner to the enrichment of various biological processes, including pathways involved in innate immunity. To investigate the effects of inhaled e-cigarette aerosols in an allergic asthma model, C57BL/6 mice were treated with OVA to induce an allergic reaction, or phosphate buffered saline (PBS) as the control. In OVA-treated mice, there were increased eosinophils and serum IgE; however, there was no difference between the air- or JUUL-exposed groups. This study is the first to compare the pulmonary immune response of two widely used inbred strains of mice, the BALB/c and C57BL/6, to inhaled e-cigarette aerosols. These results highlight the unique immunological and proteomic response of two laboratory mouse strains to e-cigarette aerosols, underscoring the need for standardized preclinical research models to study e-cigarette toxicity. Although a prior exposure to e-cigarette aerosols did not significantly alter the allergic immune response in an OVA model, these results add novel information about the potential harms of e-cigarette use.

RESUMÉ

Les cigarettes électroniques sont des appareils à piles qui chauffent un liquide contenant de la nicotine et des aromatisants pour produire un aérosol à vapoter. Initialement, ces appareils ont été commercialisé comme une alternative saine pour la cessation tabagique. Cependant, les cigarettes électroniques sont aujourd'hui principalement utilisées par les jeunes, y compris les enfants, les adolescents et les jeunes adultes. En ce moment, il y a des milliers d'appareils et de saveurs disponible sur le marché, mais il n'y a pas assez de connaissances concernant leurs effets sur la santé. Ceci est à cause d'un manque de modèle préclinique universel pour étudier la toxicologie des cigarettes électroniques. Il y a des informations contradictoires dans la littérature scientifique à propos des effets sur la santé des aérosols contenant de la nicotine car les études tous ont été fait avec des souris de souches différents. De plus, la possibilité que les cigarettes électroniques peuvent contribuer au développement des maladies pulmonaires chroniques comme l'asthme n'a jamais été investigué. L'hypothèse est que les aérosols des cigarettes électroniques populaires, notamment JUUL, vont induire un changement de phénotype immun pulmonaire différents entre deux souches de souris, BALB/c et C57BL/6, et que ces changements vont affecter leur réponse immunitaire à un allergène respiratoire. Les buts de cette étude sont: 1) de comparer les phénotypes immuns pulmonaires des souris mâle et femelle BALB/c et C57BL/6 après un usage sous-chronique des cigarettes électroniques au niveau cellulaire, transcriptionnel et protéomique, et 2) de caractériser les effets immuns pulmonaires des cigarettes électroniques dans le contexte d'un modèle d'asthme allergique induit par l'ovalbumine (OVA). On a commencé par caractériser la cellularité du fluide provenant d'un lavage broncho-alvéolaire en utilisant la cytométrie en flux; on a trouvé qu'il y avait peu de changements dans les populations de cellules innées et adaptives dans les deux souches de souris. Cependant, il y avait des changements transcriptionnels entre les

deux souches de souris: la souche de souris BALB/c, mais pas C57BL/6, a subi une augmentation transcriptionnelle pour les gènes responsables pour l'inflammation et la réponse au stress oxydatif. Finalement pour le premier but, on a quantifié le protéome du liquide provenant du lavage broncho-alvéolaire ainsi que les poumons des souris qui ont vapoté soit de l'air ou de l'aérosol JUUL en utilisant la technique de la spectrométrie de masse étiquetée par le TMT. Dans le liquide broncho-alvéolaire, on a trouvé 464 protéines distinctes dans la souche BALB/c, ainsi que 958 dans la souche C57BL/6. Dans le tissu pulmonaire, on a trouvé 1,316 et 1,226 protéines dans les deux souches, respectivement. De plus, le sexe des souris a influencé les différences de protéines importantes qui ont été notées. Pour investiguer l'effet des aérosols des cigarettes électroniques dans un modèle de l'asthme allergique, les souris C57BL/6 ont d'abord été traités par l'ovalbumine pour induire une réaction allergique, ou de la PBS comme contrôle négatif. Dans les souris traitées par l'ovalbumine, on a noté une augmentation des éosinophiles et de l'IGE dans le sérum; cependant, il n'y avait pas de différences entre les souris qui ont vapoté du JUUL versus de l'air. Cette étude est le premier qui compare la réponse immuno-pulmonaire entre deux souches de souris innés, C57BL/6 et BALB/c, après avoir vapoter l'aérosol d'une cigarette électronique. Les résultats soulignent la réponse unique immunologique entre les souches, ce qui met de l'emphase sur l'importance d'un modèle standard pour évaluer la toxicité des cigarettes électroniques. Bien que les aérosols du JUUL n'ont pas eu un effet important sur la réponse immuno-allergique dans le modèle de l'ovalbumine, les résultats donnent de l'information concernant les effets toxiques potentiels des cigarettes électroniques.

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Contributions of co-authors

This thesis is founded on original work and has been prepared as a manuscript for the purpose of peer-review and submission.

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List of figures and tables

Figures

Tables

Supplementary Figures

Supplementary Figure S2. Innate immune cells in the BAL immediately after a 14-day JUUL aerosol exposure.
Supplementary Figure S3. Innate immune cells in the lung tissue immediately after a 14-day JUUL aerosol exposure.
Supplementary Figure S4. Adaptive immune cells in the lung tissue immediately after a 14-day JUUL aerosol exposure.
Supplementary Figure S5. Gene transcription of Th2 cytokines in the lung tissue immediately after a 14-day JUUL aerosol exposure.

Supplementary Tables
Supplementary Table S1. Total proteins quantified in the BAL fluid of BALB/c and C57BL/6 mice.
Supplementary Table S2. DEPs in the BAL fluid of BALB/c mice.
Supplementary Table S3. DEPs in the BAL fluid of C57BL/6 mice.
Supplementary Table S4. Pathway analysis of DEPs in the BAL fluid of BALB/c mice.
Supplementary Table S5. Pathway analysis of DEPs in the BAL fluid of C57BL/6 mice.
Supplementary Table S6. Total proteins quantified in the lung tissue of BALB/c and C57BL/6 mice.
Supplementary Table S7. DEPs in the lung tissue of BALB/c mice.
Supplementary Table S8. DEPs in the lung tissue of C57BL/6 mice.
Supplementary Table S9. Pathway analysis of DEPs in the lung tissue of BALB/c mice.
Supplementary Table S10. Pathway analysis of DEPs in the lung tissue of C57BL/6 mice.

List of Abbreviations

Abbreviation	*Meaning*
ACE-2	Angiotensin-converting enzyme 2
Actn1	Actinin alpha 1
ADAM10	A Disintegrin and metalloproteinase domain-containing protein 10
AHR	Airway hyperresponsiveness
AMs	Alveolar macrophages
Apcs	Serum amyloid P component
BAL	Bronchoalveolar lavage
BAT	British American Tobacco
Cadm1	cell adhesion molecule 1
Cav1	Caveolin1
CBD	Cannabidiol
CDC	Centers for Disease Control and Prevention
COPD	Chronic obstructive pulmonary disease
Ctsh	Cysteine protease cathepsin H
Cxcl	Chemokine (C-X-C motif) ligand
Cyp450	Cytochrome P450
cysLTs	Cysteinyl leukotrienes
DCs	Dendritic cells
DEPs	Differentially expressed proteins
ENDS	Electronic nicotine delivery systems
EPO	Eosinophil peroxidase
EVALI	E-cigarette or Vaping product Use-Associated Lung Injury
F12	Coagulation factor XII
FDA	Food and Drug Administration
FDR	False discovery rate
Ftl1	ferritin light polypeptide 1
GABA	γ-Aminobutyric acid
Gpx2	Glutathione peroxidase 2
GRAS	Generally recognized as safe
Hbb-b2	Hemoglobin subunit beta-2
HDM	House dust mites
IFN-γ	Interferon gamma
Ig	Immunoglobulin
Igh-1a	Immunoglobulin heavy constant gamma 2A
IL	Interleukin
Il18r1	Interleukin-18 receptor 1
IMs	Interstitial macrophages
Lp1	Lymphocyte proliferation 1
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MBP	Major basic protein
MCC	Mucociliary clearance
Mme	Membrane metalloendopeptidase

MMP-9	Matrix metallopeptidase 9
MS	Mass spectrometry
nAChR	Nicotinic acetylcholine receptor
Nf-κB	Nuclear factor-kappa-light-chain-enhancer of activated B cells
Nqo1	NAD(P)H dehydrogenase quinone 1
Nras	Neuroblastoma RAS viral oncogene
OR	Odds ratio
Orm1	Orosomucoid 1
OVA	Ovalbumin
PBS	Phosphate buffered saline
PG	Propylene glycol
PI3K	Phosphatidylinositol 3-kinase
PM	Particulate matter
PMTA	Premarket Tobacco Product Application
PND	Post-natal day
Pnliprp1	Pancreatic lipase related protein 1
PPS	Puffs per session
Psmc3	Proteasome 26S subunit, ATPase 3
ROS	Reactive oxygen species
S100a9	S100 calcium binding protein A9
TGF-β	Transforming growth factor beta
Th	T helper cell
THC	Tetrahydrocannabinol
TMT	Tandem mass tag
TNF-α	Tumor necrosis factor alpha
T_{RM}	Tissue-resident memory T cells
TVPA	Tobacco and Vaping Products Act
UFP	Ultrafine particulate matter
USA	United States of America
VEA	Vitamin E acetate
VG	Vegetable glycerin
VOCs	Volatile organic compounds

CHAPTER 1: INTRODUCTION

1.1 E-cigarettes

1.1.1 Historical development of e-cigarettes

Electronic cigarettes (e-cigarettes), also called electronic nicotine delivery systems (ENDS), are devices that heat a nicotine-containing liquid into an aerosol to deliver nicotine to the brain. Although the invention of e-cigarettes is often credited to Hon Lik in 2003, the conception of alternative smoking products that heat tobacco, rather than burning it, can be traced back to the early 1960's. In 1963, Herbert A. Gilbert filed a patent for a battery-powered device that heated a flavoured liquid, which he described as a "smokeless non-tobacco cigarette" (1), although Gilbert never found commercial success for his invention. By the 1980's, many tobacco companies, including British American Tobacco (BAT), R.J. Reynolds, and Phillip Morris had begun strategizing to develop more "socially acceptable" cigarette alternatives to combat the growing public concern over the detrimental health effects of tobacco smoking (2, 3). However, none of their products succeeded in test markets. In 2003, citing Gilbert's original 1963 patent, Chinese pharmacist Hon Lik patented a device which could vaporize a nicotine-containing solution (4). This device was marketed by Ruyan, the company that employed Lik, as a "smoking cessation device", despite no evidence to support their claim. In 2007, Ruyan received an international patent to export their device to North America. Since then, the ENDS market has grown into a multibillion-dollar industry, fuelled by technological advancements that gave rise to new devices, novel formulations of e-liquids that contained high concentrations of nicotine, and countless flavouring agents. Today, many of the most popular e-cigarette brands are at least partly owned by tobacco companies, including JUUL (partly owned by Altria, the parent company of Phillip Morris USA) and Vuse (owned by R.J. Reynolds).

1.1.2. Characteristics of e-cigarettes

All e-cigarettes are composed of three basic parts: the battery, the atomizer which contains the wick and metal filament that aerosolize the liquid (also known as e-liquid, or vape juice), and the fluid reservoir which holds the e-liquid. E-cigarette aerosols are produced when the user draws air through the mouthpiece and activates an airflow sensor, causing the filament in the atomizer to heat (5). In some e-cigarette models, the user presses a button on the device to activate the battery. Once the device is activated, the e-liquid in the reservoir is brought to the filament via capillary action of the wick (5). The e-liquid saturated on the wick is aerosolized, and then inhaled by the user. This process is termed 'vaping', from the misclassification of the aerosol as a vapour (6). Depending on the design, model, and brand of the e-cigarette, there are key differences between devices. These differences include that the battery can be of variable voltage and power; the wick in the atomizer may be of different materials (silica, cotton, or ceramic) (7); the filament can be composed of different metals (most common are iron, nickel, or chromium, or a combination of these) and heat to varying temperatures (8); and the fluid reservoir may be refillable, pre-packaged or disposable/replaceable. Thus, the properties of the device, including the battery, atomizer, and fluid reservoir, can influence the end-products produced in the aerosol and inhaled by the user (9).

Since their introduction in the global market over a decade ago, e-cigarette designs have evolved and are broadly defined by four generations of devices (9). The first-generation e-cigarettes (*e.g.,* NJOY, blu) are called "cig-a-like" due to their resemblance to a tobacco cigarette. The original cig-a-like designs featured fixed, low-voltage batteries and were sometimes disposable, depending on the model (9). Second-generation e-cigarettes (*e.g.,* Innokin) known as "clearomizers" have

both a larger battery and fluid reservoir which can be refilled with any liquid (9). Third-generation e-cigarettes (*e.g.,* SMOK) include batteries that allow the user to modify the voltage, wattage, and power of the device and hence are referred to as "mods". Finally, fourth-generation devices are referred to as "pods", contain low-powered batteries and use replaceable liquid cartridges that are either prefilled, refillable, or disposable (7). Pod-style e-cigarettes are the most recent type to enter the market and were popularized by the brand *JUUL* which launched in 2015. JUUL devices resemble USB drives which enabled them to be hidden more effectively, and their sleek designs and appealing liquids were marketed strategically to young people. In 2019-2020, pods were the most frequently used type of e-cigarette among youth (10, 11), and JUUL the most commonly used brand among American middle school and high school students. Recently, disposable e-cigarettes overtook pods as the most popular among teens, owing to the popularity of brands like PuffBar (12, 13).

1.1.3. E-liquid components and toxicity

There is vast array of different e-liquids available to consumers. E-liquids may be sold separately from the e-cigarette device, sold in pre-assembled cartridges/pods, or sold as part of the e-cigarette, as is the case for disposable e-cigarettes. Although it is difficult to estimate how many e-liquids currently exist on the market, and e-liquid accessibility varies by country, current estimates suggest that there are more than 15,000 different flavoured e-liquids on the market (14). Moreover, consumers often make their own e-liquid. Generally, e-liquids contain one or more flavouring agent and varying concentrations of nicotine dissolved in a solvent, which are also referred to as humectants. Furthermore, the thermal degradation of these chemicals results in the production of transformation products, which are not present in the e-liquid itself but are generated when the e-

liquid is heated and aerosolized. Therefore, the toxicity of the inhaled aerosol is dependent on both the composition of the e-liquid and on the aerosolization process.

1.1.3.1. Nicotine

Nicotine is primary pharmacological compound of e-liquids. Nicotine is an alkaloid naturally present in the tobacco plant and is a weak base (15). When inhaled from a combustible cigarette, nicotine reaches the brain in 10-20 seconds, where it has a multitude of rewarding psychoactive effects on mood and cognition by inducing the release of neurotransmitters such as dopamine, norepinephrine, serotonin, and γ-aminobutyric acid (GABA) (16). Chronic nicotine exposure causes upregulation of nicotinic acetylcholine receptors (nAchRs) in the brain, which contributes to the development of tolerance and dependence (17). Nicotine exists as the (S)-nicotine enantiomer with two major sites for protonation to occur; when nicotine is added to more basic e-liquid solvents, it results in a higher proportion of deprotonated nicotine (5). When deprotonated nicotine, or "freebase" nicotine, is aerosolized, it produces an aerosol with a pH of ~8, resulting in a "harsher" and more intense sensation in the throat. Consequently, e-liquid manufacturers add a weak acid such as benzoic acid or citric acid to their formulations; the acid reacts with nicotine to produce a "nicotine salt" which is mono-protonated. By doing so, the pH of the aerosol is lowered to ~4-6 and the resulting aerosol becomes more tolerable to the lungs (18). Nicotine salt formulations thus allow for nicotine concentrations that are between two to ten times greater than those in freebase-nicotine-containing e-cigarettes (19). The use of nicotine salts was implemented by JUUL, which manufactured pods of varying nicotine strength that ranged from 1.5% nicotine by weight (equivalent to approximately 18 mg/mL of nicotine) to 5% (equivalent to 59 mg/mL, or 40 mg of nicotine in a 0.7mL pod) (20). The average tobacco cigarette contains 10-14 mg of

nicotine and delivers approximately 2 mg of nicotine to the smoker (16). This means that one 5% JUUL pod delivers that same amount of nicotine as in between 13-30 cigarettes (21). Although the pharmacology of nicotine is well established, it is unknown whether it can interact with the other constituents of e-liquids and contribute to unknown adverse effects. Furthermore, the addictive potential of nicotine salts in e-cigarettes has not been thoroughly addressed.

1.1.3.2. Propylene glycol and vegetable glycerin (PG/VG)

The largest components of e-liquids by weight are the solvents propylene glycol (PG) and vegetable glycerin (VG). The ratio of PG to VG varies between different e-liquids, although typically they are present in 20/80, 30/70, or 50/50 ratios (22). The FDA classifies PG and VG as 'Generally Regarded As Safe' (GRAS) for oral consumption, although no safety classification exists for their inhalation (23, 24). The thermal degradation of PG/VG during aerosolization of the e-liquid results in the formation of many chemical products with known toxicity, including reactive oxygen species (ROS), aldehydes, and ultrafine particulate matter (UFP). Despite their ubiquitous use in e-cigarette liquids, aerosolized PG/VG may cause toxicity, although controversial findings on the effects of PG/VG on pulmonary health have been reported. For example, exposure of cultured gingival epithelial cells to varying ratios of PG and VG (with or without nicotine) caused a significant decrease in cell viability and an induction of interleukin (IL) 6 (IL-6), IL-8, and matrix metallopeptidase 9 (MMP-9) (25). Aerosolized PG/VG has also inhibited glucose transport in primary human bronchiolar epithelial cells (26). Data from *in vivo* studies in BALB/c and C57BL/6 mice demonstrated that although chronic exposure to aerosolized PG/VG did not alter the cellularity of the BAL fluid, there were effects on pulmonary gene expression, lung lipid homeostasis, and innate and adaptive immunity (27, 28, 29). Increasing evidence supports a

potentially harmful role of aerosolized PG/VG, which may act through multiple mechanisms to disrupt normal lung function. In summary, the widespread use of PG/VG in e-liquids may be associated with significant respiratory toxicity, which underscores the need for further research to investigate the effects of PG/VG inhalation.

1.1.3.3. Flavouring agents

Flavouring agents are commonly added to e-liquids to increase their appeal and mask the harshness of the nicotine-containing aerosol (30, 31); evidence suggests that flavours impact how users perceive and use e-cigarettes (32). Despite thousands of flavouring agents being added to e-liquids, there is relatively little known about the impact of inhaling flavoured aerosols. Indeed, while many flavouring ingredients are labelled as GRAS for oral consumption, they have not been evaluated for inhalation toxicity. It is difficult to know the exact number of flavoured e-liquids available on the market, but it has been estimated that there are over 15,000 flavoured e-liquids (14) and over 200 different flavouring chemicals (33). Flavoured e-liquids can be broadly categorized as candy/dessert flavours, fruity flavours, mint/menthol flavours, and tobacco flavours (34). Some of the most commonly-identified chemical ingredients in flavoured e-liquids are vanillin, ethyl maltol, ethyl butyrate, ethyl acetate, ethyl vanillin, cinnamaldehyde, and menthol (33). Increasing research output has begun to elucidate the potential toxicity of flavoured e-liquids on pulmonary health. Data from *in vitro* studies indicate that flavoured e-liquids disrupts the airway epithelial barrier, induce oxidative stress and activate inflammatory responses (35, 36, 37, 38, 39, 40). Of note is that some flavouring chemicals, such as those found in sweet flavours, mint flavours (*e.g.,* menthol) and cinnamon flavours (*e.g.,* cinnamaldehyde), are cytotoxic and cause DNA damage (41, 42, 43, 44). In addition, flavouring agents have been found to dampen immune cell functions

that protect against pathogens (45). Evidence from animal studies is limited, and conflicting results have been reported. In one such study, female C57BL/6 mice were exposed to PG/VG alone or PG/VG with a French vanilla flavouring for 6 weeks. Immunophenotyping in the lungs revealed a significant increase in frequency of dendritic cells (DCs), $CD4^+$ T cells, and $CD19^+$ B cells, irrespective of the presence of flavour (46). However, exposure to PG/VG alone or to the flavoured PG/VG differentially modulated the expression of genes involved in cellular metabolism and immunotoxicity, and only PG/VG with French vanilla increased levels of immunoglobulin (Ig) G1 in the bronchoalveolar lavage (BAL) fluid (46). These results indicate that flavouring agents may affect the overall toxicity of the aerosol. A similar study that exposed male C57BL/6 mice to a flavoured nicotine-containing aerosol for 3 days or 4 weeks found increased macrophages in the BAL fluid compared to air-exposed mice, as well as an increase in the production of Muc5AC and proinflammatory cytokines IL-1β and IL-6 (47). In a study using flavored JUUL products, exposure of female C57BL/6 mice for 1 and 3 months caused no differences in total leukocyte and neutrophil cell counts in the BAL fluid compared to air-exposed mice (48). However, gene expression profiling using RNA-sequencing (RNA-seq) revealed that this exposure led to significant gene expression changes. Furthermore, mice exposed to JUUL aerosols and challenged with inhaled lipopolysaccharide (LPS) had reduced levels of chemokine (C-C motif) ligand 2 (*Ccl2)* and chemokine (C-X-C motif) ligand (*Cxcl*) 1 (*Cxcl1)* in the BAL compared to air-exposed groups, indicating that JUUL attenuated the inflammatory response to LPS (48). These prior studies suggest that flavouring agents in e-cigarettes have flavour-specific and cell-specific effects on parameters of pulmonary health, as a result of modifying patterns of use and the appeal of e-cigarettes (49). However, assessing the toxicity of flavoured e-cigarettes is hampered in part by the enormous variety of flavoured e-liquids available to consumers and the lack of standard

exposure systems, which makes it difficult to compare exposure models and outcomes across studies (50).

1.1.3.4. Trace metals

Trace chemicals may also be found in e-liquids and the resultant aerosol, such as heavy metals, including chromium (Cr) and nickel (Ni), as well as copper (Cu), zinc (Zn), lead (Pb), iron (Fe), aluminum (Al), cadmium (Cd), magnesium (Mg), cobalt (Co), and arsenic (As) (8, 51, 52, 53). Importantly, the presence and concentration of these metals varies between the liquid and aerosol; Al, Fe, Ni, and Zn are more commonly found in e-liquids while Cr, Cu, and Pd are more consistently found in aerosols and often at higher levels that in e-liquids (54). Four studies reported variable metals in urine and serum from human e-cigarette users (55, 56, 57, 58). The presence of metals in the aerosol comes from the metals leaching out of heating coil during aerosolization of the e-liquid (8). Often, metals are found at concentrations that exceed safe exposure levels, suggesting that chronic exposure may increase the risk of cancer and adverse cardiovascular outcomes (59, 60).

1.1.3.5. Cannabinoids

Cannabis sativa is a plant commonly known as marijuana. Cannabis is the second most smoked product after tobacco. The main pharmacological compounds in cannabis are the cannabinoids tetrahydrocannabinol (THC), responsible for the psychoactive effects of cannabis, and cannabidiol (CBD), the main non-psychoactive cannabinoid (61). In the past decade, alternative methods to consume cannabis have emerged, including vaping. Cannabis vaping is an umbrella term that refers to both heating the dried plant (rather than burning it) and heating highly concentrated THC

or CBD oils/distillates using an e-cigarette (62). Between 2013 and 2020, past-30-day cannabis vaping increased seven-fold among adolescents in Canada and the United States of America (USA) (63). In 2019, the outbreak of a novel acute lung disease linked to the use of THC-containing e-cigarettes raised concerns over the safety of cannabis vaping. Due to issues of legality, limited information exists on if/how vaping cannabis distillates may contribute to lung injury, and there is still insufficient safety data about the health effects of cannabis vaping overall.

1.1.3.6. Thermal degradation chemicals

E-cigarettes are viewed as safer alternative to cigarette smoking partly due to the perceived lack of some combustion products present in cigarette smoke. However, e-cigarette devices heat the e-liquid to high temperatures, resulting in the formation of additional chemical products, many of which have known toxicities. The nature of these products depends on multiple characteristics of the device which affect the rate of aerosolization of the e-liquid, including the wick efficiency, material of the coil and heating temperature, the battery voltage, and chemical composition of the e-liquid (5).

The heating temperature of e-cigarette coils ranges from 200-300°C, with JUUL devices heating to approximately 215°C (64). Carbonyl compounds with known deleterious effects, such as formaldehyde, acetaldehyde, and acrolein are found in e-cigarette aerosols (65, 66), and their formation is determined by the temperature of the heating coil (67), the type of device and e-liquid used (68). Additionally, aerosolized PG and VG can be a source of phenols, the formation of which are correlated with the ratio of each solvent (69). E-cigarette aerosols also contain particulate matter (PM) (70) in the ultrafine range. Ultrafine particles (UPFs) are PM with diameter smaller

than 100 nm. When inhaled, UFPs can travel within the lungs and reach the alveoli, where they deposit and enter the bloodstream. Inhaled UFPs contribute to a range of adverse respiratory health effects and exacerbate respiratory symptoms in patients with chronic airway diseases (71).

1.1.4. Trends of e-cigarette use among youth

Over the past decade, e-cigarette use has increased worldwide. Because not all countries record e-cigarette use, it is difficult to estimate the number of e-cigarette users. In a recent review of e-cigarette survey data from 49 countries, the number of e-cigarette users was projected to rise globally to over 86 million users in 2023 (72). Global data on vaping prevalence in youth is more limited, and the majority comes from Canada and the USA.

The earliest reports of youth vaping in Canada date back to 2013, when past-30-day (current) e-cigarette use in Canadians aged 16-24 was estimated to be 0.3% in those who did not smoke cigarettes, and 18% in smokers (73). According to data from the 2017 Canadian Tobacco, Alcohol and Drugs Survey and the 2019 Canadian Tobacco and Nicotine Survey, the prevalence of ever-use in Canadians aged 15-19 and 20-24 was 21.3% and 26.1%, respectively, while the prevalence of past-30-day use was 6.3% and 6.0%, respectively (74). By 2019, there was a near-doubling in vaping among youth: 36% of 15-19-year-olds and 48.2% of 20-24-year-olds reported ever trying e-cigarettes, while current use was 15.1% and 15.2%, respectively (74). Furthermore, in both the 15-19 and 20-24 age groups, never smokers accounted for a significant proportion of e-cigarette users and for most of the increase in past-30-day vaping between 2017 and 2019 (74); this highlights that adolescent and young adult tobacco-naïve recreational users constitute a particularly vulnerable population to vaping initiation. Consistent with these findings, a

comprehensive analysis of cross-sectional data from school-based surveys collected in four Canadian provinces between 2013 and 2019 revealed that the prevalence of current e-cigarette use among high school students increased considerably over three time points: between 2013-2014 and 2014-2015, between 2016-2017 and 2017-2018, and between 2017-2018 and 2018-2019 (75). These results point to the impact of legalization of vaping products in Canada. Prior to 2018, e-cigarettes containing nicotine were not approved for legal sale, despite being widely available (76). With the passing of the Tobacco and Vaping Products Act (TVPA) in May 2018, the federal government legalized the sale of nicotine-containing devices and permitted the advertising and promotion of e-cigarettes (77), which increased their accessibility. The most recent data from the 2021 Canadian Tobacco and Nicotine Survey revealed that 13% of youth aged 15-19 and 17% of young adults aged 20-24 currently use e-cigarettes (78).

The U.S. Centre for Disease Prevention and Control (CDC) and Food and Drug Administration (FDA) have been collecting nationwide youth vaping data since 2011 through the National Youth Tobacco Survey (NYTS), an annual cross-sectional survey of U.S. middle and high school students. In 2012, cigarettes were still the most commonly used tobacco product among youth, and only 2.8% of high school students and 1.1% of middle school students reported using e-cigarettes (79). By 2014, the prevalence of past-30-day vaping surpassed the prevalence of past-30-day cigarette smoking, as 13.4% of high school students and 3.9% of middle school students reported current e-cigarette use (80). Around this time, JUUL was introduced to the North American market, and from 2015-2018, JUUL was the top-selling brand, claiming the majority of the e-cigarette market share (81). JUUL was also the most popular brand among youth due to its sleek designs, flavoured liquids delivering a high concentration of nicotine, and targeted social media

advertising campaigns (81, 82). In 2018, in response to the increasing popularity of nicotine-containing flavoured e-cigarettes among teens and young adults, youth vaping was declared an epidemic by the U.S. Surgeon General and FDA Commissioner (83). By 2019, 27.5% and 10.5% of high school and middle school students, respectively, reported current e-cigarette use – more than half reported JUUL as their usual brand (59.1% of high school students and 54.1% of middle school students) (84). Among exclusive current e-cigarette users, flavoured e-cigarettes were preferred by 72.2% of high school students and 59.2% of middle school students (84). According to the most recent data from the CDC, over 2.55 million youth reported current e-cigarette use in 2022; among these, 84.9% used flavoured e-cigarettes (12). These results emphasize the role of flavours in youth vaping and underscore the need to restrict flavours that appeal to young people. The variety of commercially-available devices and flavours, the perception of reduced harm, and youth-targeted advertising campaigns have been identified as key factors contributing to the rise of e-cigarette use in youth (85). In recent years, regulatory agencies have attempted to restrict access to e-cigarettes and increase public health messaging on the harms of vaping, but the regulatory framework surrounding e-cigarettes remains insufficient.

1.1.5. Review of the legal status of e-cigarettes

In North America, efforts to regulate the sale of e-cigarettes and limit their appeal and availability to youth have been met with minimal success and substantial pushback from lobbyists and e-cigarettes companies. In Canada, e-cigarettes are regulated by federal, provincial, and territorial legislation. Prior to 2018, nicotine-containing e-cigarettes were not legal for sale. With the passage of the TVPA, the federal government enacted a regulatory framework for the manufacturing, sale, labelling and promotion of e-cigarettes (77). In recent years, Health Canada has enacted

regulations to restrict access to certain devices and e-liquids. This included, in 2020, mandating JUUL to stop selling popular flavours in Canada, including the mango, vanilla, fruit and cucumber varieties (86). However, this ban did not require JUUL to remove existing stock from retail outlets; and the flavoured pods continued to be widely available after the ban. In 2021, Health Canada set a maximum nicotine concentration of 20 mg/mL for e-cigarettes marketed in Canada (87). Nevertheless, vaping continues to be popular among Canadian youth, and experts have criticized Health Canada for failing to respond sooner to the crisis (88).

When e-cigarettes appeared in the USA, the FDA attempted to block the import of ENDS by classifying them as unapproved drug delivery devices, but faced push-back from e-cigarette manufacturers (89). Ultimately, a decision by the Supreme Court classified e-cigarettes as tobacco products in 2009; e-cigarettes were thus excluded from FDA oversight, allowing the e-cigarette market in the USA to expand unrestricted. In 2016, the FDA enacted a new rule called the "Deeming Rule", which extended FDA authorization over all e-cigarettes (90). This new rule required e-cigarette companies to comply with the premarket authorization requirements listed in the Federal Food, Drug, and Cosmetic Act. The FDA required alternative tobacco manufacturers to submit a Premarket Tobacco Product Application (PMTA). Although at the time, the FDA set a deadline of 2018 for manufacturers to submit PMTAs, the deadline was subsequently postponed to 2022. This delay prompted a lawsuit by the American Academy of Pediatrics, who called for the FDA to require PMTAs to be submitted by 2020 (91). However, in 2019 a series of novel vaping-related illnesses emerged, predominantly in youth adults. This new disease entity was termed "vaping associated lung illness" (EVALI) and has been partly linked to the unregulated use of vitamin E acetate (VEA) in THC-containing e-cigarettes. This outbreak prompted the FDA to

issue a statement discouraging the use of VEA and provided updated guidance on unauthorized, flavoured cartridge-based ENDS devices. The following year, the FDA banned all flavoured cartridge-based e-cigarettes, including fruit and mint, most popular among younger age groups, but excluding tobacco and menthol (92). This ban applied only to flavoured pre-filled cartridges, or pods; flavoured disposable e-cigarettes were excluded from this ban, causing them to soar in popularity. In 2021, the FDA approved the first marketing authorization to an ENDS product, the e-cigarette manufactured by Vuse (owned by tobacco giant R.J. Reynolds), (93) despite the fact that 10 percent of high school students who used e-cigarettes named Vuse as their usual brand (13). Recently, the FDA issued a marketing denial order to JUUL, forcing them to stop selling and distributing their products in the USA (94). To date, JUUL has been faced with over 5,000 lawsuits in various U.S. states for deceptively marketing its devices to minors and fueling the youth vaping epidemic (95).

Despite efforts to regulate the e-cigarette market, new brands and e-liquids are constantly emerging. For example, disposable e-cigarettes have recently displaced pods as the most commonly-used device among youth, highlighting the urgent need for up-to-date legislation to limit access of these devices and discourage the development of tobacco-based products that are appealing to young people.

1.1.6. Stealth vaping

Smoke-free or clean air policies that prohibit smoking in public areas were implemented to protect non-smokers from hazardous exposure to second hand smoke, prompt current smokers to quit, and discourage children or adolescents from smoking initiation (96). With the emergence of e-

cigarettes, many governments adapted their smoke-free policies to include the prohibition of vaping. As a means to cope with restricted e-cigarette use, some e-cigarette users have turned to 'stealth vaping', which refers to vaping discreetly in areas where it is not allowed (*e.g.,* work, school, bars or nightclubs, restaurants, and movie theatres) (97). This phenomenon is popularized by companies that modify products such as smartwatches and apparel, or produce auxiliary products to conceal e-cigarettes and enable their use where vaping is prohibited (98). Evidence demonstrated that a significant proportion of current e-cigarette users engage in stealth vaping (97, 99). Stealth vaping has been in part attributed to JUUL, whose e-cigarettes were the first to closely resemble USB sticks. A study from 2018 that was performed using an online search of keywords such as 'JUUL' found nearly 150, 000 videos on YouTube, including videos instructing users how to hide their JUUL devices in school (100).

1.2. Clinical evidence of e-cigarette toxicity on the respiratory system

Although e-cigarettes are viewed as safer alternatives to smoking, the health effects of vaping remain largely unknown. The potential harm posed by vaping may be related to numerous factors such as age, cigarette smoking status, and the presence of pre-existing respiratory illness such as asthma. In this regard, youth may be more are risk due to the development of nicotine addiction leading to cigarette smoking initiation, referred to as the 'gateway effect'. Indeed, epidemiological data shows that e-cigarette use is associated with an increased risk of cigarette smoking (101, 102). Moreover, dual use of cigarettes and e-cigarettes is rising (103). In this context, the emerging consensus is that dual use may cause worse adverse pulmonary effects than cigarette smoking alone (104). Another risk to youth is the relatively unknown pulmonary health effects of frequent or daily e-cigarette use. There is evidence that vaping is associated with unique pulmonary

illnesses that are distinct from tobacco-related diseases. An early study found that e-cigarette users exhibited elevated levels of elastase and MMP-9, increased neutrophil granulocyte-related proteins, and altered mucin secretion (105). Multiple mechanisms have been proposed to elucidate the pathophysiology of vaping on the human pulmonary system, including effects on the nasal epithelium, the airways, the bronchial epithelium, and the alveoli (106, 107). In addition to their adverse effects in the respiratory system, e-cigarettes may have additional neurological, cardiovascular (108), and oral/dental toxicity (109), which are outside the scope of this research.

1.2.2. Bronchiolitis obliterans

Bronchiolitis obliterans is a clinical syndrome characterized by dyspnea and irreversible airflow obstruction caused by inflammation and obliteration of the small airways (110). Bronchiolitis obliterans is also known as "popcorn lung disease" due to its occurrence among workers in factories producing microwavable popcorn (111) because of inhalational exposure 2,3-butanedine (diacetyl), the chemical responsible for the buttery flavour of microwave popcorn. Diacetyl is also present in e-liquids, raising concerns that inhaled diacetyl from e-cigarettes may lead to bronchiolitis obliterans in e-cigarette users (112). A case of bronchiolitis obliterans was described in a 17-year-old Canadian male who reported use of flavoured and THC-containing e-cigarettes, although the presence of diacetyl in the patient e-liquids was not confirmed (113).

1.2.3. E-cigarette or vaping product use–associated lung injury (*EVALI)*

The first cases of severe pulmonary illness related to e-cigarette use were reported in the summer of 2019 by the public health departments in the states of Wisconsin and Illinois (114). The majority of patients were young, otherwise healthy individuals, who reported recently using e-cigarettes

containing nicotine, THC, and/or CBD. The CDC subsequently termed this illness e-cigarette or vaping product use–associated lung injury (EVALI) (115) and defined criteria for a 'confirmed' diagnosis as: 1) e-cigarette use in the 90 days prior to symptom onset, 2) pulmonary infiltrates on chest radiograph or CT, 3) absence of pulmonary infection, and 4) no evidence of an alternative plausible diagnosis (116). At the time the CDC stopped monitoring EVALI in February 2020, 2,807 patients had been hospitalized with EVALI, and there were 68 associated deaths (117). In Canada, 20 cases of EVALI were reported between September 2019 and December 2020, and none resulted in death (118). Although EVALI presents as a syndrome of non-specific clinical manifestations, evaluation of the history and clinical symptoms of EVALI patients, radiologic findings and histopathological assessment has shed light on the potential aetiology of this disease. A review of three published case series describing EVALI in a total of 125 patients reported that 98% experienced respiratory symptoms (*e.g.,* dyspnea, cough, and chest pain), 94% experienced constitutional symptoms (*e.g.,* fever), and 86% experienced gastrointestinal symptoms (*e.g.,* nausea) (6). Furthermore, neutrophilic inflammation and lipid-laden macrophages in the BAL fluid of patients with EVALI have been observed (114, 119).

At present, the most probable causative agent contributing to EVALI is VEA, a synthetic form of vitamin E. VEA is highly lipophilic with widespread use in the food and cosmetics industry, and it is considered GRAS as a nutrient and/or dietary supplement (120). VEA is used as a solvent for THC-containing e-liquids that are sold illicitly. Although the mode of action through which VEA exerts its toxic effects is unclear, it is postulated that VEA deposits in the airways in liquid droplets and incorporates into the lipid layer of pulmonary surfactants (121, 122). Additionally, the thermal degradation of VEA results in the production of a noxious ketene gas, ethenone, which further

disrupts the blood-air-barrier and causes epithelial dysfunction and inflammation (123). VEA was identified in over half of the e-cigarettes collected from EVALI patients in New York and Minnesota in 2019 (124, 125). In addition, VEA was identified in the BAL fluid from 48/51 patients hospitalized with EVALI across 16 U.S. states; by contrast, no VEA was detected in the BAL fluid of 99 healthy comparators. Furthermore, 47/50 patients had detectable THC in the BAL fluid or reported vaping THC-containing products in the 90 days prior (126). Nevertheless, the evidence implicating VEA in EVALI is not definitive, and the lack of reported THC-vaping in some EVALI patients underscores that VEA may not be the sole causative agent. In absence of substantiative clinical data from EVALI patients, animal studies provide a preliminary understanding of VEA toxicity. In one such study, transcriptomic analysis of lung RNA derived from mice exposed to individual e-liquid ingredients revealed that PG exposure was associated with lung fibrosis via the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, while VEA exposure was associated with asthmatic airway inflammation via the mitogen-activated protein kinase (MAPK) signaling pathway (127). In a separate study, mice that were exposed to aerosolized VEA prior to influenza inoculation displayed greater body weight loss compared to mice inoculated with influenza alone, although BAL protein concentration and BAL cellularity were similar between the groups (128). These results raise the possibility that e-cigarette-induced lung injury may result in adverse outcomes in respiratory infections. Thus, more research is required to understand the mechanisms of EVALI and inform prevention and treatment strategies.

1.2.4. Respiratory infections and coronavirus disease (COVID-19)

Experimental evidence from *in vitro* and *in vivo* studies indicates that vaping impairs pulmonary immune responses to infection (129, 130, 131). Three cases of severe influenza or *Staphylococcus*

aureus (S. aureus) infections requiring hospitalization in young, otherwise healthy individuals with a daily history of vaping have been reported (132, 133, 134). Of these, two patients were concomitantly diagnosed with EVALI and reported using e-liquids containing THC or CBD.

The coronavirus disease (COVID-19) pandemic raised concerns about the effect of vaping on COVID-19 susceptibility and illness. Two observational studies conducted in 2020 found an association between e-cigarette use and COVID-19. The first study was an analysis of survey data from adolescents and young adults in the U.S., where exclusive past-30-day cigarette users and exclusive past-30-day vapers (individuals who exclusively used cigarettes or e-cigarettes, respectively, during the last 30 days) had similar Odds Ratio (ORs) for a positive COVID-19-diagnosis, and past-30-day dual users were 6.84 time more likely to be diagnosed with COVID-19 compared to never users (135). This supports the hypothesis that dual use of cigarettes and e-cigarettes has synergistic toxicity compared to either one alone. In an ecological study of vaping prevalence statewide, the weighted proportion of e-cigarette users was significantly associated with the number of COVID-19 cases and deaths (136). In contrast with these findings, a cross-sectional study of English adults over 18 years of age did not find a statistically significant association of e-cigarette use with self-reported COVID-19 (137). Concerns over the potential impact of vaping in COVID-19 were reinforced by observations that cigarette smoke affects angiotensin-converting enzyme 2 (ACE-2), the cell surface protein that mediates viral entry of SARS-CoV2, with cigarette smokers having higher airway epithelial cell expression of ACE-2 (138). This effect may be due to activation of α7 subtype of nicotine acetylcholine receptors (α7-nAChR) by nicotine (139). Although emerging evidence from *in vitro* and *in vivo* studies supports that vaping may similarly induce ACE-2 expression in the lungs (140, 141, 142), there are no

conclusive studies in human e-cigarette users. The possibility that vaping may increase susceptibility to COVID-19 remains an area in need of critical research (143). E-cigarette use may therefore constitute an important risk factor for COVID-19. Although the COVID-19 pandemic has been declared over by the World Health Organization, health care providers and public health officials should remain vigilant about the increased vulnerability of youth vapers to COVID-19.

Understanding the clinical impact of vaping-induced respiratory toxicity is in part hampered by the broad spectrum of devices and e-liquids, variability in the frequency and topography of use, dual use of tobacco cigarette, and pre-existing comorbidities. Thus, guidelines are needed to guide researchers and clinicians in collecting vaping history, and to ensure that information such as device type, frequency of use, nicotine concentration, and use of other tobacco or ENDS products is accurately recorded (107). As the long-term effects of e-cigarettes in humans are unknown, and in light of these challenges, there is an urgent need for rigorous preclinical studies to evaluate e-cigarette toxicity.

1.3. Development of pre-clinical models to study e-cigarette toxicity

E-cigarettes have been available to consumers for less than two decades, making the study of e-cigarette toxicology relatively new. There are significant concerns over adverse chronic effects associated with lifetime use, which are compounded by a lack of long-term public health data. Therefore, it is imperative to generate experimental evidence using *in vitro* models and animal studies to assess the health effects of e-cigarettes. However, there is no standard protocol for conducting e-cigarette exposures in animal models, unlike for tobacco smoke (144). Considerations for the development of animal models to study e-cigarette toxicity include the mode of exposure, the type of device, the composition of the e-liquid, and the puff topography. In

addition, special consideration must be given to the choice of animal models; to date, most e-cigarette studies have used mouse models, with some conducted in rats. When working with mice, the choice of inbred strain and sex constitute important biological variables that necessitate careful consideration.

1.3.2. Considerations for in vivo e-cigarette exposures

E-cigarette aerosol exposures should be designed to recapitulate as best as possible vaping behaviours of human users. Key factors to consider in the development of exposure scenarios are the mode of exposure, the length and duration of exposure, the type of e-cigarette device and liquid, puff topography, and frequency (145). Two modes of exposure for inhaled aerosols are whole-body and nose-only. Both types of exposure systems have advantages and disadvantages, and the selection of either one should be guided by considerations such as the number of test animals, the exposure duration, and the availability of the test material (146). Whole-body exposure systems consist of an exposure chamber into which the animals are placed unrestrained, and thus are fully immersed in the aerosol. The advantages of whole-body exposure systems are that the animals in the exposure chamber are unrestrained and therefore less stressed, and animal handling is minimized, which is useful for chronic exposures (146). However, the disadvantage of this approach is that dermal and oral exposure cannot be avoided, which may alter the extent of absorption/response of the aerosol and thus does not reflect human e-cigarette exposure (147). In nose-only exposure systems, animals are placed in restraints that are inserted into a nose-only tower, such that only the muzzle of the animal is exposed to the aerosol. By avoiding oral and dermal routes of exposure, nose-only exposures reduce variability and reflect human use more accurately (146). The disadvantages of this approach are that the animals are restrained and

separated from their cage-mates, which can induce stress. To minimise stress, researchers have limited exposures to 60 minutes and approximately 180 puffs per day (147). In addition, the size of the restraints must be appropriate for the size of the animals. Smaller rodents, particularly younger ones, may attempt to turn around inside the tubes and risk suffocation (148). Furthermore, nose-only exposure systems are more labor intensive, especially when manipulating large numbers of animals. Regarding the length and duration of exposure, it is generally thought than an exposure of up to three days represents an acute exposure, whereas at 60-90 days the exposure becomes chronic, with anything in between being considered as sub-chronic (145).

The international standard cigarette for research purposes is the Kentucky Research Cigarette. In contrast, there is no standard e-cigarette device of e-liquid for research purposes. It is important that researchers are familiar and remain up-to-date with novel types of devices, as well as trends of use among target populations. Research studies should, as much as possible, reflect what people are using (e.g., by using commercially available devices and e-liquids, rather than using e-liquids manufactured in-house).

Puff topography refers to the puff frequency, volume, peak flow, shape, and length. It has been shown that human users adjust their puffing behaviours based on their experience with the device and past smoking history, the type of e-cigarette device used, the nicotine concentration and flavour of the liquid (149, 150). Although limited knowledge exists on how humans use e-cigarette devices, preliminary studies provide a basic understanding of the puffing behaviours of humans, which can help guide the development of puff protocols for animal studies. An early study that characterized puff topography among adult e-cigarette users who were given an e-cigarette

equipped with a wireless personal use monitor found that participants took an average of 225 ± 59 puffs/day, although the total number of puffs varied dramatically from 24 to over 1,000 puffs/day, with a mean puff duration of 3.5 ± 0.39 seconds (151). Similar results were reported in a later study, which also measured an average of 10.2 ± 7.9 puffs per session (PPS) (152). In a separate study, participants were given a second-generation vape pen with flavoured nicotine-containing e-liquids, and their puffing behaviour was monitored for 2 weeks with a wireless monitoring device (153). A "light" session was characterized as 14.7 PPS, while a "heavy" session was characterized as 16.7 PPS. Studies to investigate the effects of e-liquid flavours on puffing behaviours have been conflicting. In one study, it was found that the average puff duration and PPS varied significantly when users used a strawberry flavoured e-liquid compared to a tobacco-flavoured liquid (154). In contrast, a study of young adults who reported JUUL as their usual brand found no significant differences in puff topography when participants used their preferred pod flavour versus a tobacco flavour, although there were differences in subjective measures of experience (155). Using varying puffing topographies, experiments can therefore be designed to relate human use patterns to animal exposure regimes. For example, in one study, mice were exposed to increasing doses of e-cigarette aerosols ranging from very low to high, with a very low dose delivering two 3-second puffs per session, and a high dose consisting of eight 4-second puffs per session (156). Plasma cotinine values were approximately 100 ng/mL after the moderate dose, and 500 ng/mL after the high dose; both were significantly increased compared to the very low dose. These values accurately reflect cotinine levels in humans e-cigarette users, which range from 138-548 ng/mL (157). Thus, serum cotinine can be used to validate puff topographies in preclinical models and relate human use patterns to animal e-cigarette exposure regimes.

1.3.3. Mouse strain

The laboratory mouse has long been used as the model organism to study human diseases because it shares many of the same physiological, immunological, and genetic properties with humans (158). Lineages of laboratory mice are either inbred, in which all individuals within a strain are genetically identical, or outbred, which give rise to genetically diverse individuals. Typically, inbred mouse strains are used for the study of immunology and infectious diseases, while outbred mice are used for toxicological, pharmacological, cancer and aging research (159, 160). The most common inbred strains in respiratory health research are the BALB/c and C57BL/6 strains. The immune response of these strains has been well characterized, and they are known to display differences in both the innate and adaptive immune system. BALB/c mice display a T helper cell (Th) 2-type response, with a tendency to develop airway hyperresponsiveness (AHR), secrete cytokines such as IL-4, IL-5, and tumor necrosis factor alpha (TNF-α), and a elicit a strong humoral response against pathogens (161). For this reason, BALB/c mice are often used in models of infectious diseases and allergic reactions. In contrast, C57BL/6 mice demonstrate a Th1-type response, with high interferon gamma (IFN-γ) production, but less AHR and a weaker humoral response (161). An advantage of using C57BL/6 mice is that they constitute the background strain for most knock-out models. The differences between BALB/c and C57BL/6 mice are attributed to genetic variations (162). The immunological differences between BALB/c and C57BL/6 mice contribute to the diverging response of these mice in experimental models of respiratory diseases. In fact, strain-dependent differences in the susceptibility to disease development have been characterized with regards to respiratory diseases, such as cigarette-smoke-induced emphysema, allergic rhinitis, and asthma (163, 164, 165). However, it is unknown whether BALB/c and

C57BL/6 respond similarly to inhaled e-cigarette aerosols, thereby constituting a significant gap in the literature.

1.3.4. Sex as a biological variable

The consideration of sex as a biological variable in experimental design is critical because male and female mice display genetic variability which results in anatomical and physiological sex-dependent differences. For instance, male mice are typically larger than female mice, and they have reported differences in immunity, partially due to the influence of sex hormones on the immune system (166, 167).

Sex-dependent differences in the effects of nicotine have been investigated: male and female mice have different sensitivities to nicotine, in that female mice self-administer more nicotine but are less sensitive to its antinociceptive and anxiolytic effects (168, 169). In studies using female ICR and C57BL/6 mice, female mice have higher concentration of plasma nicotine following exposure to e-cigarette aerosols compared to male mice (170, 171).

Sex differences related to inflammation have also been explored, with female mice being more susceptible to the development of allergic airway inflammation (172) due to the influence of estrogen on acute lung inflammation (173). Recent work from our lab showed that both acute and chronic exposures to flavoured JUUL aerosols differentially modulated the inflammatory response of male and female C57BL/6 mice (174, 175). Sex differences were also reported in BALB/c and C57BL/6 mice exposed to e-cigarette aerosols, where exposure to e-cigarettes increased lung mRNA and protein levels of Ace-2 in male mice, but not in females (141, 176).

Finally, in murine models of asthma with BALB/c and C57BL/6 mice, males and females have different levels of airway hyperresponsiveness and lung mechanics (177). This is relevant because the prevalence of asthma is different between males and females, but the mechanisms responsible for these differences are not well understood.

1.4. Asthma

1.4.1 Prevalence and phenotypes of asthma

Asthma is an inflammatory disease of the airways. It is the most prevalent chronic illness in youth, affecting 850,000 children under the age of 14 in Canada and 6.4 million children in the USA (178, 179). Asthma is more common in boys during childhood, but more common in women in adulthood (180). Asthma is characterized by reversible airway obstruction, bronchial hyperreactivity, and variable airway inflammation (181). However, increasing awareness of its heterogeneity has led to the evolution of the asthma diagnosis from a single disease to a disease that encompasses multiple diseases with varying clinical manifestations (*i.e.,* phenotypes), and distinct underlying immunological mechanisms (*i.e.,* endotypes) (182, 183). Phenotypes of asthma can be broadly grouped in Th2- and Non-Th2-mediated processes; the former group encompasses allergic asthma, late-onset eosinophilic asthma, exercise-induced asthma, and aspirin-exacerbated respiratory disease while the latter includes obesity-associated asthma, asthma with fixed airflow limitation, and neutrophilic asthma (181, 182). Early-onset allergic asthma is the most commonly recognized phenotype that typically appears during childhood, is more common in males than females, and it is often associated with other allergic diseases such as atomic dermatitis or allergic rhinitis. Patients with allergic asthma often have sputum eosinophilia (indicative of eosinophilic

airway inflammation) and respond well to inhaled corticosteroid treatment (182, 184). Non-Th2 asthma phenotypes typically affect adult women with a range in severity (182).

1.4.2. Immunological mechanisms of asthma

Efforts to characterize phenotypes of asthma based on clinical manifestation have been accompanied by a need to better understand the immunological pathways (endotypes) driving each phenotype. Immunological responses are often divided into Th1- and Th2-type processes. The Th1 response is typically activated by viral infection, while Th2-type processes occur in response to allergic stimuli and parasitic *Helminth* infections. Asthma has long been considered a Th2-type immune response, particularly allergic asthma (Figure 1.1). During the initial response to allergen, DCs in the airways present inhaled allergens to naïve $CD4^+$ T cells, stimulating their differentiation into $CD4^+$ T helper 2 (Th2) cells. Th2 cells release cytokines such as IL-4, IL-5, and IL-13. IL-4 and IL-13 act in conjunction to induce the release of IgE from B cells, while IL-5 activates eosinophils to induce their degranulation. Eosinophils release several inflammatory mediators, including IL-13, major basic protein (MBP), eosinophil peroxidase (EPO), cysteinyl leukotrienes (cysLTs) and transforming growth factor beta (TGF-β), which act on the cells of the respiratory epithelium and of the bronchial smooth muscle to promote inflammation and bronchoconstriction (182, 183). IgE binds the high-affinity receptor for the Fc region of IgE, also known as FcεRI, on mast cells, causing the release of prostaglandin D2 (PGD2) , which recruits Th2 cells, and histamine and leukotrienes, which induce further bronchoconstriction, increased vascular permeability, and mucus production (185). A large proportion of asthma are likely caused by non-Th2 mechanisms, and are instead mediated by the interaction of multiple immune pathways, such as those regulated by Th1 and Th17 $CD4^+$ T cells (182) .

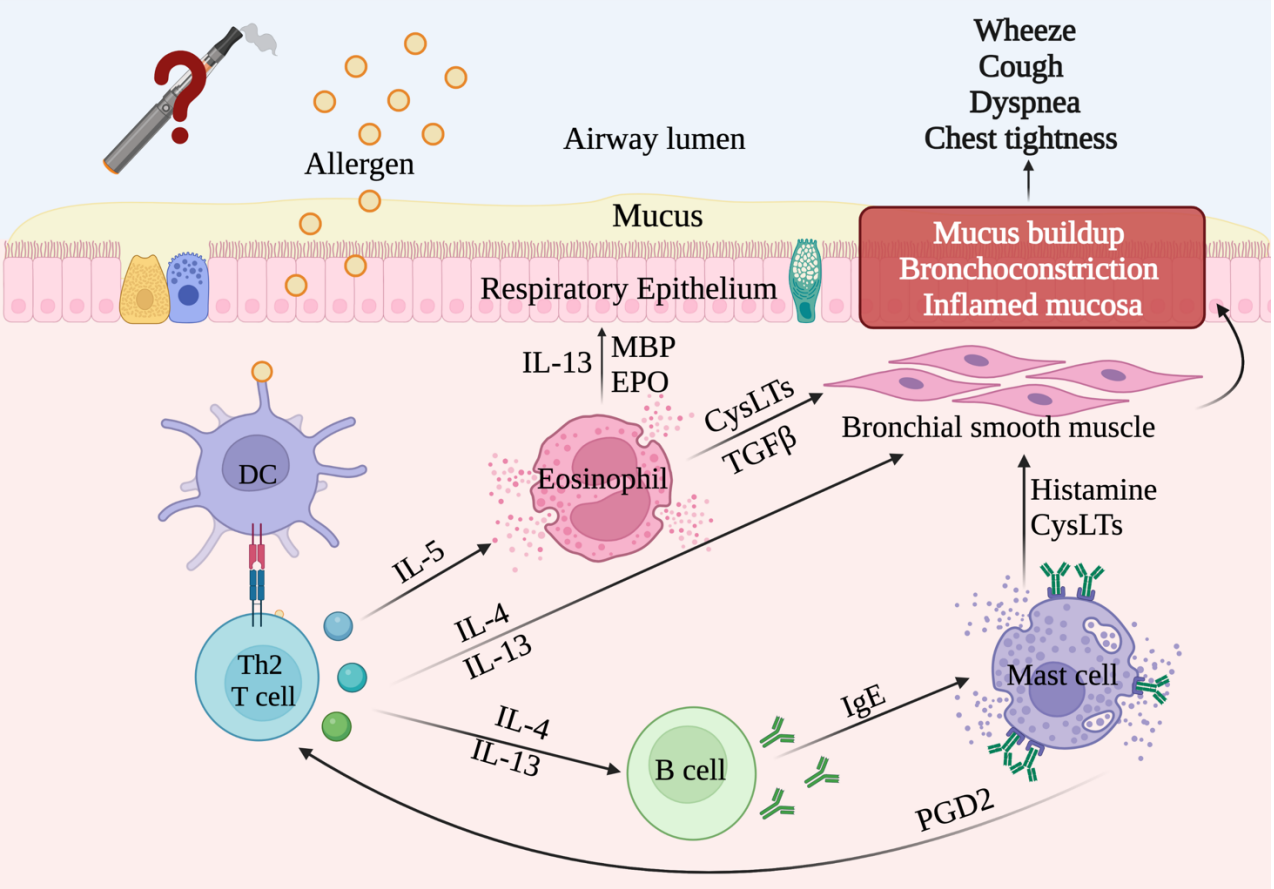


Figure 1.1 Immunological processes in allergic asthma. Upon infiltration of allergen into the airways, dendritic cells engulf and present allergen to $CD4^+$ T cells, stimulating their differentiation into Th2 cells. Activated Th2 cells release IL-4, IL-5, and IL-13, which promote allergic inflammation, resulting in bronchoconstriction, increased vascular permeability, and mucus production.

Several environmental factors can alter the underlying immunoinflammatory processes of asthma phenotypes, particularly cigarette smoke. Former or current smoking is a risk factor for developing asthma and current smoking is associated with adverse clinical outcomes, including increased mortality and decreased quality of life (186). In children, second hand exposure (SHS) exacerbates allergic asthma symptoms (187). The mechanisms by which cigarette smoking impacts asthma are multifactorial and involve cellular and structural changes that arise from the activation of allergic and non-allergic inflammatory and oxidative stress pathways, resulting in airway inflammation,

epithelial remodelling, and an impaired host response (188). At present, it is unknown whether e-cigarette aerosols affect asthma susceptibility or modify the immune response. Despite epidemiological data supporting a link between e-cigarette use and asthma, few studies have investigated the effects of e-cigarettes in animal models of allergic asthma, underscoring the need for increased research efforts to better understand the potential harms of e-cigarettes for youth with asthma.

1.4.3. E-cigarette use among asthmatic youth

Asthmatic youth (*i.e.,* children and adolescents) constitute a vulnerable population at risk of the harms potentially associated with e-cigarette use. Although limited, observational data in this age demographic supports a significant association of e-cigarette use with respiratory disorders such as asthma, independent of cigarette smoking. An early study that evaluated vaping among high school students with asthma found that asthmatic students were nearly twice more likely to use e-cigarette than those without asthma (189). However, this study was based on a small dataset from the 2013 Ontario Student Drug Use and Health Survey. Given the increased popularity of e-cigarettes among Canadian youth in recent years, more research is needed to understand evolving patterns of e-cigarette use nationwide in young Canadians with asthma. Two studies using U.S. statewide survey data of high school students in Florida and Hawaii concluded that e-cigarette use was associated with having asthma and with more asthma exacerbations compared to non-users (190, 191). Emerging data supports that e-cigarette use is also associated with worse symptoms. Moreover, this association may be unique to certain asthmatic phenotypes. Survey data collected between 2015 and 2016 from a nationally representative sample of adolescents in the U.S. was evaluated for associations between e-cigarette use and respiratory manifestations (192). Compared

to non-e-cigarette users, past-year-e-cigarette users were more likely to have experienced wheezing and dry cough at night (192). There was no association between past-year-e-cigarette use and exercise-induced wheezing, indicating that not all asthma phenotypes are affected by e-cigarette use (192). In contrast with these findings, a cross-sectional analysis of data collected between 2018-2020 from teens in California and Connecticut did not find a statistically significant difference of ever-history of asthma or asthma exacerbations in frequent e-cigarette users compared to non-users (193). Nevertheless, use of multiple types of devices (pods, vape pens, mods, and disposables) in past-30-day users was associated with asthma exacerbations compared to primarily pod users, potentially due to more frequent use per month among multiple-device users (193). Overall, numerous published studies have demonstrated significant association of e-cigarette use with asthma (194, 195, 196, 197). However, observational data cannot provide conclusive evidence for a causal relationship between e-cigarette use and asthma.

1.4.4. E-cigarettes in experimental models of allergic asthma

Animal models are used to provide mechanistic evidence of disease pathogenesis. Multiple animal models have been developed to recapitulate different phenotypes of asthma, allowing researchers to elucidate the pathophysiological mechanisms responsible and identify potential therapeutic targets. Although many animal species are used to study asthma, mice are advantageous due to a combination of scientific and economic reasons (198). The most commonly used mouse strains in experimental asthma models are the BALB/c and C57BL/6 strains because their immune response is well-characterized (198). In addition to the choice of mouse strain, the allergen used and the sensitization and challenge protocol influence the outcome (199). One of the most commonly used allergens in mouse models of allergic asthma is ovalbumin (OVA), a protein derived from chicken

eggs. The OVA-mouse model includes a sensitization phase, in which the animal is exposed twice intraperitoneally to OVA with an adjuvant, such as aluminum hydroxide (Alum) (200). Subsequently, the animal is repeatedly challenged with a lower dose OVA delivered to the airways via intranasal or intratracheal injection, with airway inflammation observed over the following 24-48 hours (201). Other commonly used allergens include house dust mites (HDM), cockroach, ragweed, and *Aspergillus*.

To date, only a few studies have investigated the effects of e-cigarettes using animal models of allergic asthma. In one study, BALB/c mice were treated with OVA and concurrently exposed to an unflavoured, nicotine e-cigarette aerosol for 4 weeks (202). Compared to air-exposed mice treated with OVA, e-cigarette exposed mice displayed significantly more neutrophils in the BAL fluid (202). In a related study, male and female BALB/c mice were exposed to flavoured e-cigarette aerosols with and without nicotine, and concurrently treated with HDM to induce an asthmatic phenotype (203). Immunophenotyping of the BAL fluid revealed a flavour-dependent effect of nicotine-free aerosols on HDM-induced airway inflammation; the Black Licorice and Banana Pudding flavoured-aerosols increased total leukocytes, eosinophils, and macrophage cell counts, whereas Cinnacide decreased these cell types. In contrast, nicotine-containing aerosols dampened airway inflammation irrespective of flavour (203). Finally, a recent study investigated whether *in utero* exposure to mint-flavoured JUUL aerosols aggravated HDM-induced pulmonary inflammation in adult offspring (204). BALB/c dams were exposed to JUUL aerosols during gestation, and offspring were evaluated at post-natal day (PND) 1 or treated with HDM and assessed at 11 weeks of age. Several key proinflammatory genes were upregulated in both dams and offspring exposed to JUUL. In addition, offspring exposed to JUUL aerosol *in utero* followed

by HDM treatment as adults exhibited an exaggerated neutrophilic (in male offspring) and mixed neutrophilic and lymphocytic (in females) response compared to air-exposed, HDM-treated offspring (204).

In summary, evidence from combined experimental models of e-cigarette exposure and allergic asthma suggest that inhaled aerosols may increase susceptibility to allergen-induced pulmonary inflammation. However, these studies highlight how different factors such as e-cigarette flavour, mouse strain and sex, and allergen type can influence the response and must thus be carefully considered in the interpretation of the results. In absence of standardized models to study e-cigarette toxicity in allergic asthma, there is a need for increased efforts to understand the complex mechanisms by which e-cigarette aerosols modulate pulmonary inflammation.

CHAPTER 2: HYPOTHESIS AND AIMS

2.1 Hypothesis

Exposure to inhaled flavoured JUUL e-cigarette aerosols will induce pulmonary inflammation in BALB/c and C57BL/6 mice and alter the immune response in a mouse model of allergic asthma.

2.2 Aims

1. Compare airway and pulmonary inflammation in BALB/c and C57BL/6 mice after a sub-chronic exposure to a JUUL e-cigarette aerosol.

2. Characterize the pulmonary immunologic effects of JUUL exposure in an OVA-induced C57BL/6 mouse model of allergic asthma.

CHAPTER 3: MATERIALS AND METHODS

3.1. Animals

BALB/c and C57BL/6 mice were purchased from the Jackson Laboratory and bred in-house. For Aim 1, BALB/c and C57BL/6 mice (6-7 weeks of age) were used. For Aim 2, C57BL/6 mice (5-9 weeks of age) were used. This age range represents adolescence in mice (205). For all experiments, equal numbers of male and female mice were used. Mice were housed in regular cages (3-5 mice per cage) with *ad libitum* access to food and water, under a regular diurnal light cycle. All procedures were approved by the McGill University Animal Care Committee and performed in accordance with the Canadian Council on Animal Care Committee.

3.2. E-cigarette exposures

Mice were randomly allocated to one of three exposure groups: air, PG/VG, or JUUL. The air group was placed in the exposure system but were exposed to air only. The PG/VG group was exposed to a flavourless aerosol derived from a liquid composed of a 30:70 ratio of PG and VG purchased from Fusion Flavours (fusionflavors.ca), which was loaded into a refillable cartridge compatible with JUUL devices. The JUUL group was exposed to an aerosol derived from a JUUL mint flavoured liquid containing 1.5% nicotine, equivalent to 18 mg/mL. JUUL devices and pods were purchased at local retailers. All e-cigarette aerosol exposures were performed using the SCIREQ® inExpose™ inhalation system equipped with a nose-only tower and an ENDS extension compatible with JUUL devices. The aerosols were delivered according to a pre-established e-cigarette puffing profile which was programmed with the flexiWare™ software, consisting of a 70 mL puff volume, and 3.3 second puff duration (206). A 2 L/minute bias flow provided the mice uninterrupted air in between puffs. For Aim 1, puffs were delivered at a rate of 2 puffs/minute for

30 minutes per session, two sessions per day, for 14 consecutive days (Figure 3.1). The two daily exposure sessions were 5 hours apart, and mice were euthanized 12 hours after the last exposure. For Aim 2, puffs were delivered at a rate of 4 puffs/minute for 30 minutes per session, once per day, for 14 consecutive days (Figure 3.2).

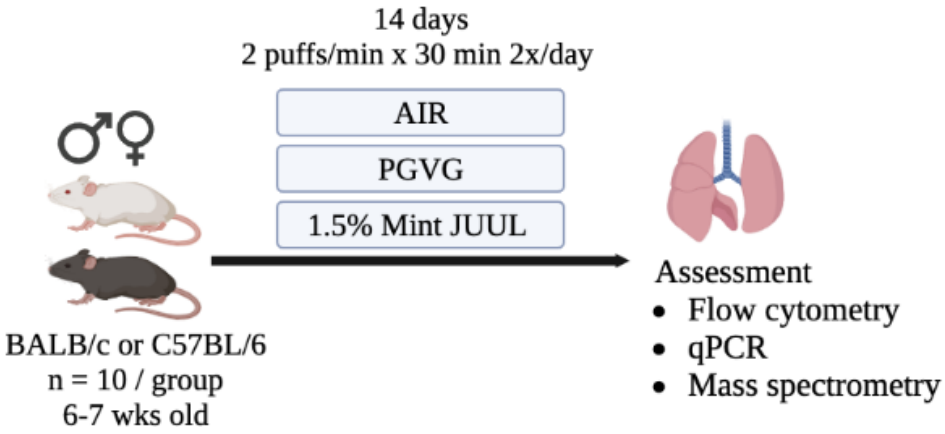


Figure 3.1. E-cigarette exposure model. In Aim 1, BALB/c and C57BL/6 mice were exposed to e-cigarette aerosols twice per day, for 14 consecutive days. BAL fluid and lung tissue samples were collected to assess markers of inflammation using flow cytometry, qPCR, and mass spectrometry.

3.3 Allergic asthma model

Following e-cigarette aerosol exposures for 14 days, on Day 0 and Day 7, mice were sensitized with OVA administered via intraperitoneal injection (i.p.). First, 5 mg of OVA (Sigma-Aldrich, St. Louis, MO, USA) were diluted in 5 mL of phosphate buffered saline (PBS) for a final concentration of 1 mg/mL OVA. Then, 3000 µl of the working solution was added with 750 µL of Imject® Alum adjuvant (ThermoFisher Scientific, Rockford, IL, USA) to 2250 µL of PBS. A solution of PBS was prepared as follows: 750 µL Imject® Alum was diluted in 5250 µL of PBS. Both tubes were agitated for one hour at room temperature prior to administration to mice. Mice

were injected with 0.2 mL of the final solution (100 µg of OVA). On Day 14, 15, and 16, mice were challenged with 10 µg of OVA diluted in 10 µL of PBS, administered via intranasal injection under light anesthesia with isoflurane. PBS-treated mice were euthanized 24 hours after the last treatment, and OVA-treated mice were euthanized 72 hours after the last challenge. This protocol was adapted from a previous publication (207) (Figure 3.2).

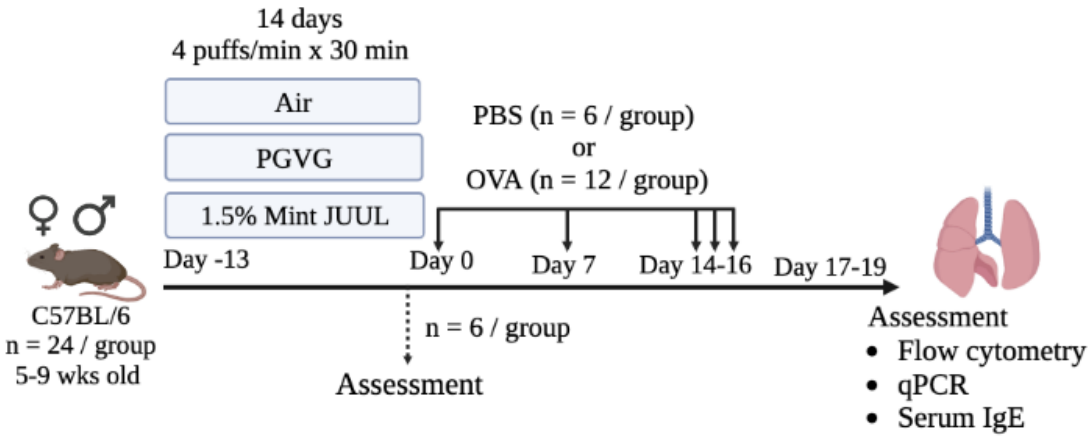


Figure 3.2. Allergic asthma model. C57BL/6 mice were exposed to e-cigarette aerosols once per day for 14 consecutive days. Six mice per group were euthanized immediately after the end of the 14-day aerosol exposure. Remaining mice were subsequently sensitized and challenged with OVA or PBS. BAL fluid, lung tissue, and serum samples were collected to assess the immunologic effects of the prior JUUL exposure.

3.4 Lung tissue harvest and BAL fluid collection

After the exposures**,** mice were anesthetized with Avertin (2,2,2-tribromoethanol, 250 mg/kg i.p.; Sigma-Aldrich, St. Louis, MO, USA). Blood samples were collected by intracardiac puncture. To collect the BAL fluid, the lungs were lavaged twice using 0.5 mL of cold PBS. The right lobe was used to prepare single cell suspensions and the left lobe was frozen in liquid nitrogen for storage at -80°C. The blood was centrifuged for 10 mins at 10,000 rpm and the serum was separated from red blood cells. The BAL fluid was centrifuged for 5 minutes at 5,000 rpm at 4°C. The cell-free supernatant of each sample was transferred into a microcentrifuge tube and stored at -80°C. The

remaining cell pellets were resuspended with 50 µL of ACK lysis buffer (ThermoFisher Scientific, Grand Island, NY, USA) and incubated for 2 minutes to lyse red blood cells. Then, BAL cells were resuspended in cold FACS buffer prepared as follows: 5 g of bovine serum albumin (BSA) (GE Healthcare Life Sciences, Logan, UT, USA) and 4 mL of 0.5 M ethylenediamine tetra acetic acid (EDTA) (ThermoFisher Scientific, Vilnius, Lithuania) dissolved in a final volume of 1 L of PBS.

3.5 Flow cytometry

For analysis in Aim 1, lungs were manually sheared and enzymatically digested with collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) for 1 hour at 37°C. Tissue samples were passed through 70 mm cell strainers (CellTreat, Pepperell, MA, USA) to generate single-cell suspensions. After centrifugation at 1500 rpm for 10 minutes at 4°C, cell pellets were resuspended and incubated for 10 minutes in 2 mL of ACK Lysis Buffer (ThermoFisher Scientific, Grand Island, NY, USA) to lyse red blood cells. Cells were counted using the AcT differential cell counter (Beckman Coulter) and resuspended in FACS buffer prepared in house following a previously published protocol (208). Lung tissue and BAL cells were transferred to 96-well round bottom plates at 1 million cells per well. Cells were stained with the viability dye eFluor^{TM} 506 (ThermoFisher Scientific, Carlsbad, CA, USA) for 30 minutes at 4°C, washed twice with FACS buffer, and subsequently incubated with a blocking buffer prepared by diluting 0.5 µL of anti-CD16/32 antibody (BioLegend, San Diego, CA, USA) in 70 µL FACS buffer per well for 15 minutes at 4°C. Afterwards, lung tissue and BAL cells were stained with a mix of fluorochrome-conjugated antibodies (Table 3.1). Innate immune cells were identified as follows: neutrophils (CD11b^{+}, Ly6G^{+}), eosinophils (Siglec-F^{+}, CD11c^{-}), macrophages (MerTK^{+}, CD64^{+}), resident alveolar macrophages (Siglec-F^{+}, CD11b^{-}), interstitial macrophages (Siglec-F^{-}, CD11b^{+}), dendritic cells

(CD11c^{+}, MhcII^{+}), and monocytes (MerTK^{+}, Ly6C$^{+/-}$) (Figure 3.3). Adaptive immune cells were identified in the lung tissue as follows: B cells (CD19^{+}, CD3^{-}), CD4^{+} T cells (CD3^{+}, CD4^{+}), and CD8+ T cells (CD3^{+}, CD8^{+}). Fluorescence compensation for each fluorochrome was set with single-stained UltraComp eBeads^{TM} compensation beads (Invitrogen, Eugene, OR, USA). Lung tissue and BAL cells stained with the innate immune cell panel were acquired on the LSR Fortessa X-20 cytometer (BS Biosciences), and lung cells stained with the adaptive immune cell panel were acquired on the FACSCanto II cytometers (BD Biosciences). All data was analyzed on FlowJo (v10.8.2; FlowJo^{TM} Software, BD, USA).

For analysis in Aim 2, lungs were dissociated using the gentleMACS™ Octo Dissociator (Miltenyi Biotec) in a solution of enzymes prepared as follows: 10 units of DNAse I (ThermoFisher Scientific, Carlsbad, CA, USA) and 0.4 units of Collagenase D (Millipore Sigma, Mannheim, Germany) diluted in HBSS (ThermoFisher Scientific, Grand Island, NY, USA) to a final volume of 2.5 mL per sample. Single-cell suspensions of lung tissue cells were prepared as described above. Mice which were euthanized immediately after the e-cigarette exposure were stained with the panels described in Table 3.1. The OVA- and PBS-treated mice were stained using the panel in Table 3.2, with innate immune cells being identified as follows: neutrophils (CD11b^{+}, Ly6G^{+}), eosinophils (Siglec-F^{+}, CD11c^{-}), macrophages (MerTK^{+}, CD64^{+}), resident alveolar macrophages (Siglec-F^{+}, CD11b^{-}), interstitial macrophages (Siglec-F^{-}, CD11b^{+}) (Figure 3.3). Lung adaptive immune cells were identified as follows: CD4^{+} T cells (CD3^{+}, CD4^{+}), and CD8^{+} T cells (CD3^{+}, CD8^{+}). All samples were acquired on the LSR Fortessa X-20 cytometer and FACSCanto II cytometer, and data was analyzed on FlowJo™.

Table 3.1. Panels of fluorochrome-conjugated antibodies for flow cytometry

Innate immune cell panel					
Fluorochrome	**Marker**	**Company**	**Cat#**	**Clone**	**Volume**
APC-Cy7	CD45	BD Bioscience	557659	30-F11	0.5 μL
APC	CD11b	BD Bioscience	553312	M1/70	0.5 μL
BV711	CD11c	BD Bioscience	563048	HL3	0.5 μL
PE	Ly6C	BioLegend	128007	HK1.4	0.5 μL
PE/Cy7	Ly6G	BioLegend	127618	1A8	0.5 μL
AlexaFluor 700	mhcII	Invitrogen	56-5321-82	M5/114.15.2	0.5 μL
BV421	merTK	BioLegend	151510	2B10C42	0.5 μL
BV605	Siglec-F	BD Bioscience	740388	E50-2440	0.5 μL
PerCP/Cy5.5	CD64	BioLegend	139308	X54-5/7.1	0.5 μL
Adaptive immune cell panel					
Fluorochrome	**Marker**	**Company**	**Cat#**	**Clone**	**Volume**
APC-Cy7	CD45	BD Bioscience	557659	30-F11	0.5 μL
BV421	CD19	BioLegend	115538	HIB19	0.5 μL
FITC	CD3	Invitrogen	11-0032-82	17A2	0.5 μL
PE	CD4	BioLegend	100408	GK1.5	0.5 μL
APC	CD8	BioLegend	100712	53-6.7	0.5 μL

Table 3.2. Panels of fluorochrome-conjugated antibodies for flow cytometry

Innate immune cell panel					
Fluorochrome	**Marker**	**Company**	**Cat#**	**Clone**	**Volume**
APC-Cy7	CD45	BD Bioscience	557659	30-F11	0.5 μL
APC	CD11b	BD Bioscience	553312	M1/70	0.5 μL
FITC	CD11c	BD Bioscience	553801	HL3	0.5 μL
PE/Cy7	Ly6G	BioLegend	127618	1A8	0.5 μL
BV421	merTK	BioLegend	151510	2B10C42	0.5 μL
PE	Siglec-F	BD Bioscience	552126	E50-2440	0.5 μL
PerCP/Cy5.5	CD64	BioLegend	139308	X54-5/7.1	0.5 μL
Adaptive immune cell panel					
Fluorochrome	**Marker**	**Company**	**Cat#**	**Clone**	**Volume**
APC-Cy7	CD45	BD Bioscience	557659	30-F11	0.5 μL
FITC	CD3	Invitrogen	11-0032-82	17A2	0.5 μL
PE	CD4	BioLegend	100408	GK1.5	0.5 μL
APC	CD8	BioLegend	100712	53-6.7	0.5 μL

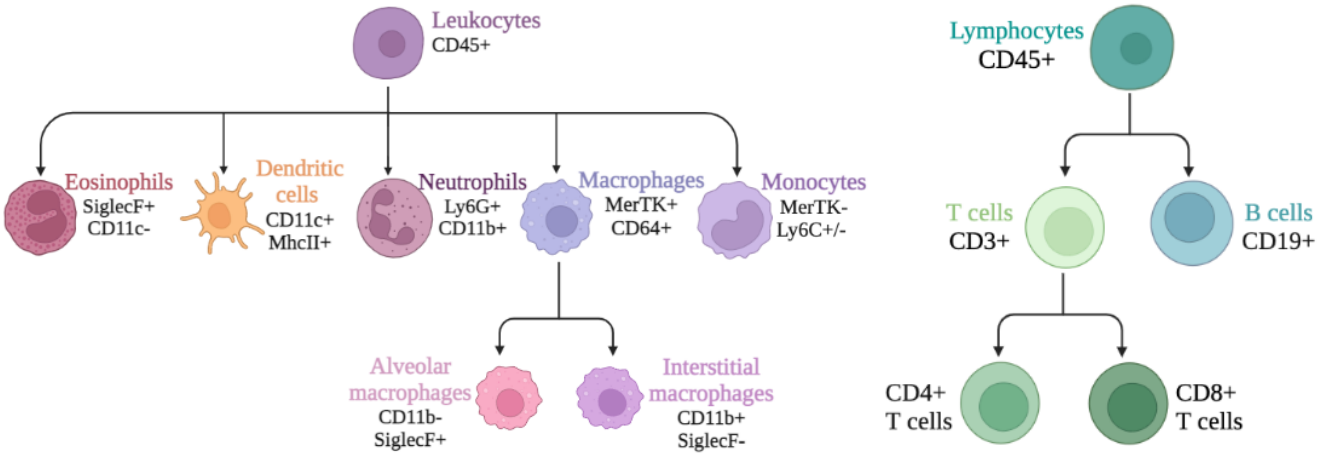


Figure 3.3. Immune cells identified with flow cytometry. To identify innate and adaptive immune cells in the lung tissue and BAL fluid, we designed two panels of fluorochrome-conjugated antibodies specific for mouse cell-surface markers. Innate immune cells (*left*) were identified as follows: all immune cells ($CD45^+$), eosinophils (Siglec-F^+, $CD11c^-$), dendritic cells ($CD11c^+$, $MhcII^+$), neutrophils ($Ly6G^+$, $CD11b^+$), macrophage ($MerTK^+$, $CD64^+$), alveolar macrophages ($CD11b^-$, Siglec-F^+), interstitial macrophages ($CD11b^+$, Siglec-F^-), monocytes ($MerTK^+$, $Ly6C^{+/-}$). Adaptive immune cells (*right*) were identified as follows: all immune cells ($CD45^+$), T cells ($CD3^+$), $CD4^+$ T cells ($CD3^+$, $CD4^+$), $CD8^+$ T cells ($CD3^+$, $CD8^+$), and B cells ($CD19^+$). The complete gating strategy is available in Supplementary Figure S1.

3.6 Quantitative RT-PCR (RT-qPCR)

Total mRNA was extracted from whole lung tissue using the Aurum miniKit (Bio-Rad, Mississauga, ON, CA) as per the manufacturer's instructions. RNA was quantified using an Infinite M200 plate reader (Tecan Trading AG, Switzerland) and reverse transcribed into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, CA). Custom primers for genes of interest were designed and synthesized from Integrated DNA Technologies (Toronto, ON, CA). Quantitative PCR (qPCR) to measure levels of mRNA of each gene of interest was performed by combining 1 µL cDNA and 0.5 µM primers with 3.5 µL SSoFast EvaGreen Supermix (Bio-Rad, Mississauga, ON, CA) and sterile H_20. Amplification was measured using a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories, Canada). The thermal cycling protocol started at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 10 seconds and annealing at

various temperatures for 30 seconds. All genes were normalized to 18s rRNA and expressed as relative fold-change using the $2^{-\Delta\Delta Ct}$ method. Primer sequences and annealing temperatures are listed in Table 3.3.

Table 3.3. List of primer sequences and annealing temperatures for genes of interest

Gene	**Forward primer sequence** **Reverse primer sequence**	**Annealing temperature (°C)**
18s rRNA	aggaattgacggaggggcac ggacatctaagggcatcaca	55.7
Tnfα	ccaaagggatgagaagttcc ctccacttggtggtttgcta	52
Il4	aggtcacaggagaagggacgc atgcgaagcaccttggaagcc	57
Il5	ctgttgacaagcaatgagacga ccccacggacagtttgattc	52
Il1β	tgccacctttgacagtgatgaga tgttgatgtgctgctgcgaga	52
Il13	aaggcccccactacggtct atgcccagggatggtctctc	52
Ptgs2	tgcctggtctgatgatgtatgcca agtagtcgcacactctgttgtgct	63.3
Nrf2	atacgcaggagaggtaagaataaagtc agagagtattcactgggagagtaagg	63.3

Sod2	ccaccgaggagaagtaccacg tagcctccagcaactctcctttg	56
Muc5b	ctcatggtgtggccagcagaga cccgcagtgtgaagagaagac	57
Col3a1	cctatgttaactacctcaactggtcag ctccttcaaattcctgctctatagtct	57.7

3.7 Mass spectrometry (MS)

BAL fluid and lung tissue samples of air- and JUUL-exposed mice were analyzed using Tandem Mass Tag (TMT)-labelled mass spectrometry. Samples were treated with TMT-16plex reagents (ThermoFisher Scientific Scientific) according to the manufacturer's instructions. Labelled peptides were fractionated using Pierce™ High pH Reversed-Phase Peptide Fractionation Kit into 8 fractions. Each fraction was re-solubilized in 0.1% aqueous formic acid and 2 μg of each was loaded onto a Thermo Acclaim Pepmap (Thermo, 75 μM ID X 2cm C18 3 μM beads) precolumn. Then, fractions were loaded onto an Acclaim Pepmap Easyspray (Thermo, 75 μM X 15cm with 2 μM C18 beads) analytical column for separation using a Dionex Ultimate 3000 uHPLC at 250 nL/min with a gradient of 2-35% organic (0.1% formic acid in acetonitrile) over three hours, running at the default settings for MS3-level SPS TMT quantitation (209). Fractions were run on an Orbitrap Fusion instrument (ThermoFisher Scientific Scientific) operated in DDA-MS3 mode. MS1 scans were collected at 120,000 resolution, scanning from 375-1500 m/z, collecting ions for 50ms or until the AGC target of 4e5 was reached. Precursors with a charge state of 2-5 were included for MS2 analysis, which were isolated with an isolation window of 0.7 m/z. Ions were collected for up to 50 ms or until an AGC target value of 1e4 was reached and fragmented using CID at 35% energy; these were then read out on the linear ion trap in rapid mode. Subsequently, the top 10 (height) sequential precursor notches were selected from MS2 spectra for MS3 quantitative TMT reporter ion analysis, isolated with an m/z window of 2 m/z, and fragmented with HCD at 65% energy. Resulting fragments were read out in the Orbitrap at 60,000 resolution, with a maximum injection time of 105 ms or until the AGC target value of 1e5 was reached.

To translate .raw files into protein identifications (SeQuest) and TMT reporter ion intensities,

Proteome Discoverer 2.3 (ThermoFisher Scientific Scientific) was used with the built-in TMT Reporter ion quantification workflows. Default settings were applied, with Trypsin as enzyme specificity. Spectra were matched against the mouse protein fasta database obtained from Uniprot (2023). Dynamic modifications were set as Oxidation (M) and Acetylation on protein N-termini. Cysteine carbamidomethyl was set as a static modification, together with the TMT tag on both peptide N-termini and K residues. All results were filtered to a 1% False discovery rate (FDR) for protein identification.

3.8 Proteomics analysis

To obtain a list of quantified proteins with relative abundances, all proteins identified by mass spectrometry were filtered to a 1% Protein FDR Confidence. No imputation of missing values was performed. Relative protein abundances were calculated for the following comparisons: JUUL versus Air (in male mice), JUUL versus Air (in female mice), Male versus Female (in air-exposed mice), and Male versus Female (in JUUL-exposed mice). A $-\text{Log10}(p) > 1.3$ ($p < 0.05$) was used to identify all differentially expressed proteins (DEPs) (both up- and down-regulated proteins). Proteomics data were analyzed for differences due to the strain (BALB/c or C57BL/6), exposure (JUUL or Air), or the biological sex of the mice (male or female). Venn diagrams of all DEPs were generated using an online Venn diagram generator (VENNY 2007. available at: https://bioinfogp.cnb.csic.es/tools/venny/index.html.). Enrichment analysis of DEPs using Reactome pathways was performed on Metascape (210).

3.9 Serum IgE

Serum IgE in PBS- and OVA-treated mice was measured via single plex assay by Eve Technologies (EveTechonologies, Calgary, AB, Canada).

3.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (v.9.5.1; GraphPad Software Inc, USA). One-way or two-way analysis of variance (ANOVA) with Tukey's multiple comparisons tests were performed to identify statistically significant changes. Where indicated, Grubb's outliers test was performed to identify outliers. A p value < 0.05 was considered statistically significant.

CHAPTER 4 RESULTS

4.1. Inhaled PG/VG and JUUL aerosols differentially affect pulmonary immune cell composition between inbred strains of mice.

To assess whether e-cigarette aerosols differentially induce pulmonary inflammation, we first characterized innate and adaptive immune cells in the BAL fluid and lung tissue of BALB/c and C57BL/6 mice. In the BAL fluid, JUUL exposure significantly decreased the frequency of total immune cells only in BALB/c mice (Figure 4.1A). In both PG/VG- and JUUL-exposed mice, the frequency of $CD45^+$ cells were lower in BALB/c mice compared to C57BL/6 mice (Figure 4.1A). Although there was a trend towards a sex-dependent difference in neutrophils in BALB/c mice, this did not reach statistical significance; neutrophils were also unaffected by JUUL exposure (Figure 4.1B). In air-exposed mice, BAL eosinophils were significantly higher in C57BL/6 mice compared to BALB/c mice, but were unaffected by JUUL exposure (Figure 4.1C). Macrophages constituted approximately 80 per cent of all BAL $CD45^+$ cells (Figure 4.1D), and there was a significant difference in alveolar macrophages in JUUL-exposed C57BL/6 mice compared to BALB/c mice (Figure 4.1E). Dendritic cells were not detected in the BAL fluid of either strain (Figure 4.1F) and monocytes were not affected by JUUL exposure (Figure 4.1G).

Next, we profiled innate immune cells in the lung tissue, which included neutrophils, eosinophils, macrophages/alveolar macrophages, dendritic cells, and monocytes (Figure 4.2). Overall, JUUL exposure had minimal impact on innate immune cells within the lung tissue, although there were some strain-dependent differences. Here, neutrophils were significantly higher in air-exposed BALB/c mice compared to C57BL/6 mice (Figure 4.2B). The frequencies of eosinophils and macrophages were unchanged (Figure 4.2C-D) whereas alveolar macrophages were significantly

lower in JUUL-exposed C57BL/6 mice compared to BALB/c (Figure 4.2E). Dendritic cells were lower in air- and PG/VG-exposed BALB/c mice compared to C57BL/6 mice (Figure 4.2F). Finally, monocytes were higher in BALB/c mice compared to C57BL/6 mice irrespective of exposure (Figure 4.2G). Within the lung tissue, there were also changes in the frequency of adaptive immune cells (Figure 4.3). Here, B cells were significantly lower in air- and PG/VG-exposed BALB/c mice compared to C57BL/6, although there was no difference in JUUL-exposed mice between the strains (Figure 4.3B). In contrast, $CD4^+$ T cells were significantly higher across all exposures groups in BALB/c mice compared to C57BL/6 mice (Figure 4.3C). Finally, compared to air-exposed mice, JUUL exposure decreased the frequency of $CD8^+$ T cells only in C57BL/6 mice (Figure 4.3D). Overall, these data highlight that there are strain-dependent differences in the cellular composition of the pulmonary immune system in response to e-cigarette aerosols.

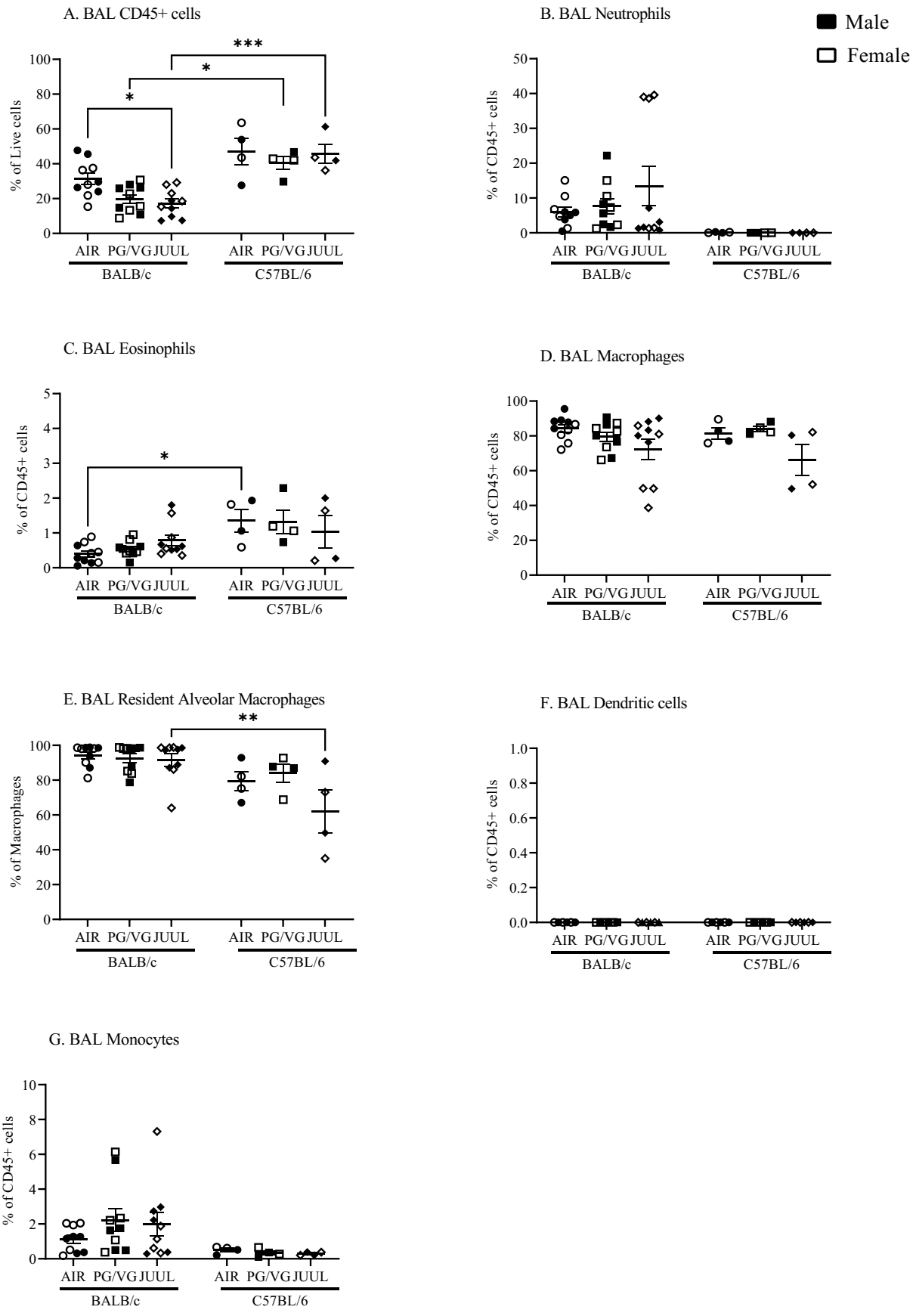

A. BAL CD45+ cells
% of Live cells
***
*
*
AIR PG/VG JUUL
AIR PG/VG JUUL
BALB/c
C57BL/6
B. BAL Neutrophils
% of CD45+ cells
Male
Female
C. BAL Eosinophils
% of CD45+ cells
*
D. BAL Macrophages
% of CD45+ cells
E. BAL Resident Alveolar Macrophages
% of Macrophages
**
F. BAL Dendritic cells
% of CD45+ cells
G. BAL Monocytes
% of CD45+ cells

Figure 4.1. Differential changes in the percentages of BAL immune cells between BALB/c and C57BL/6 mice after JUUL exposure. The frequency of total immune cells (A), neutrophils (B), eosinophils (C), macrophages (D), resident alveolar macrophages (E), dendritic cells (F), and monocytes (E) are shown. JUUL exposure decreased the frequency of total immune cells only in BALB/c mice. There were strain-dependent differences in the frequency of eosinophils and alveolar macrophages among air- and JUUL-exposed mice. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

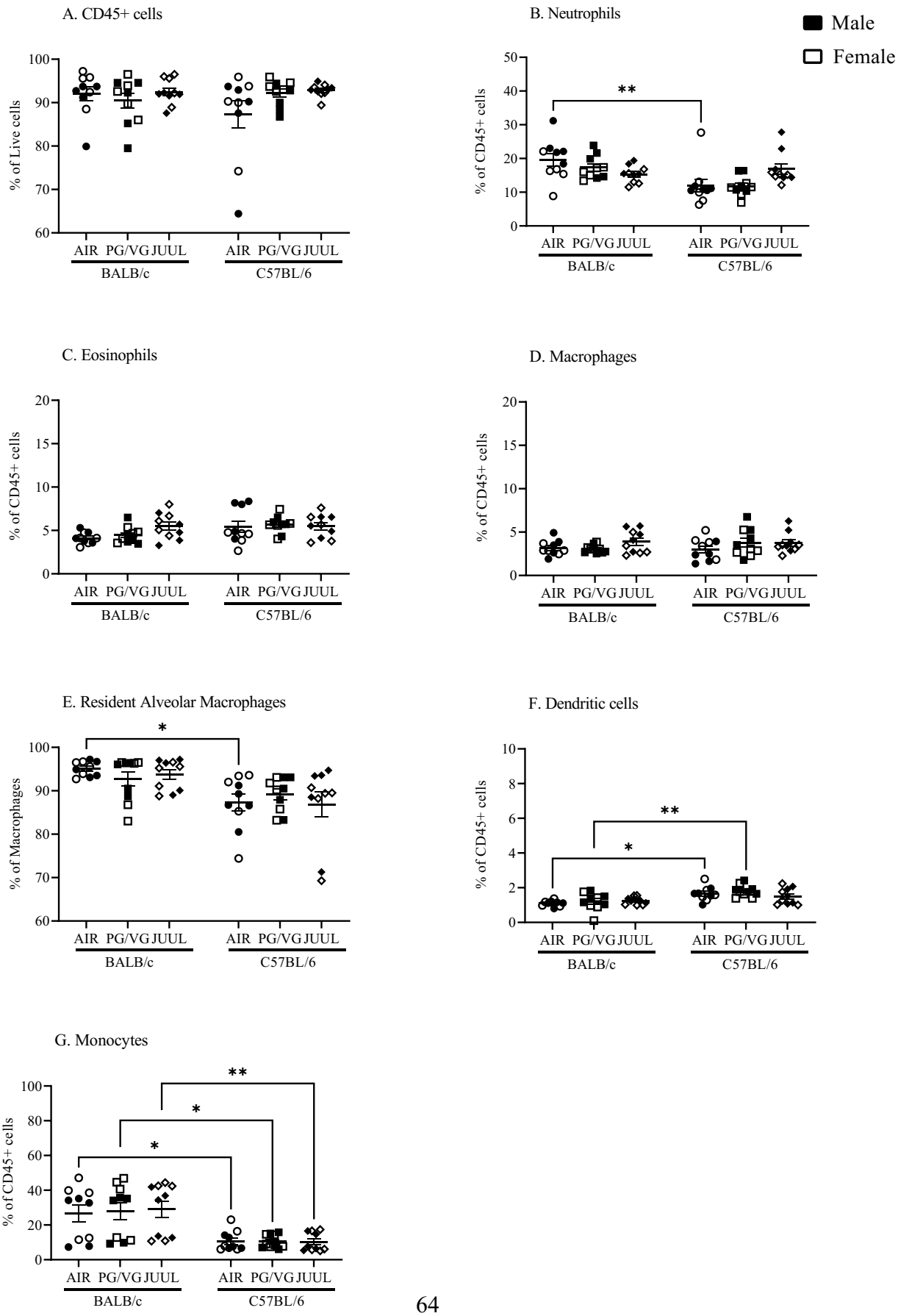

A. CD45+ cells
% of Live cells
100
90
80
70
60
AIR PG/VG JUUL
BALB/c
AIR PG/VG JUUL
C57BL/6
B. Neutrophils
Male
Female
% of CD45+ cells
50
40
30
20
10
0
**
AIR PG/VG JUUL
BALB/c
AIR PG/VG JUUL
C57BL/6
C. Eosinophils
% of CD45+ cells
20
15
10
5
0
AIR PG/VG JUUL
BALB/c
AIR PG/VG JUUL
C57BL/6
D. Macrophages
% of CD45+ cells
20
15
10
5
0
AIR PG/VG JUUL
BALB/c
AIR PG/VG JUUL
C57BL/6
E. Resident Alveolar Macrophages
% of Macrophages
100
90
80
70
60
*
AIR PG/VG JUUL
BALB/c
AIR PG/VG JUUL
C57BL/6
F. Dendritic cells
% of CD45+ cells
10
8
6
4
2
0
**
*
AIR PG/VG JUUL
BALB/c
AIR PG/VG JUUL
C57BL/6
G. Monocytes
% of CD45+ cells
100
80
60
40
20
0
**
*
*
AIR PG/VG JUUL
BALB/c
AIR PG/VG JUUL
C57BL/6

Figure 4.2. Innate immune cells in the lung tissue of BALB/c and C57BL/6 mice after JUUL exposure. The frequency of total immune cells (A), neutrophils (B), eosinophils (C), macrophages (D), resident alveolar macrophages (E), dendritic cells (F), and monocytes (G) are shown. In air-exposed mice, there were significant differences between inbred strains in the frequency of neutrophils, alveolar macrophages, dendritic cells, and monocytes. There were also strain differences in dendritic cells and monocytes among PG/VG- and JUUL-exposed mice. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$).

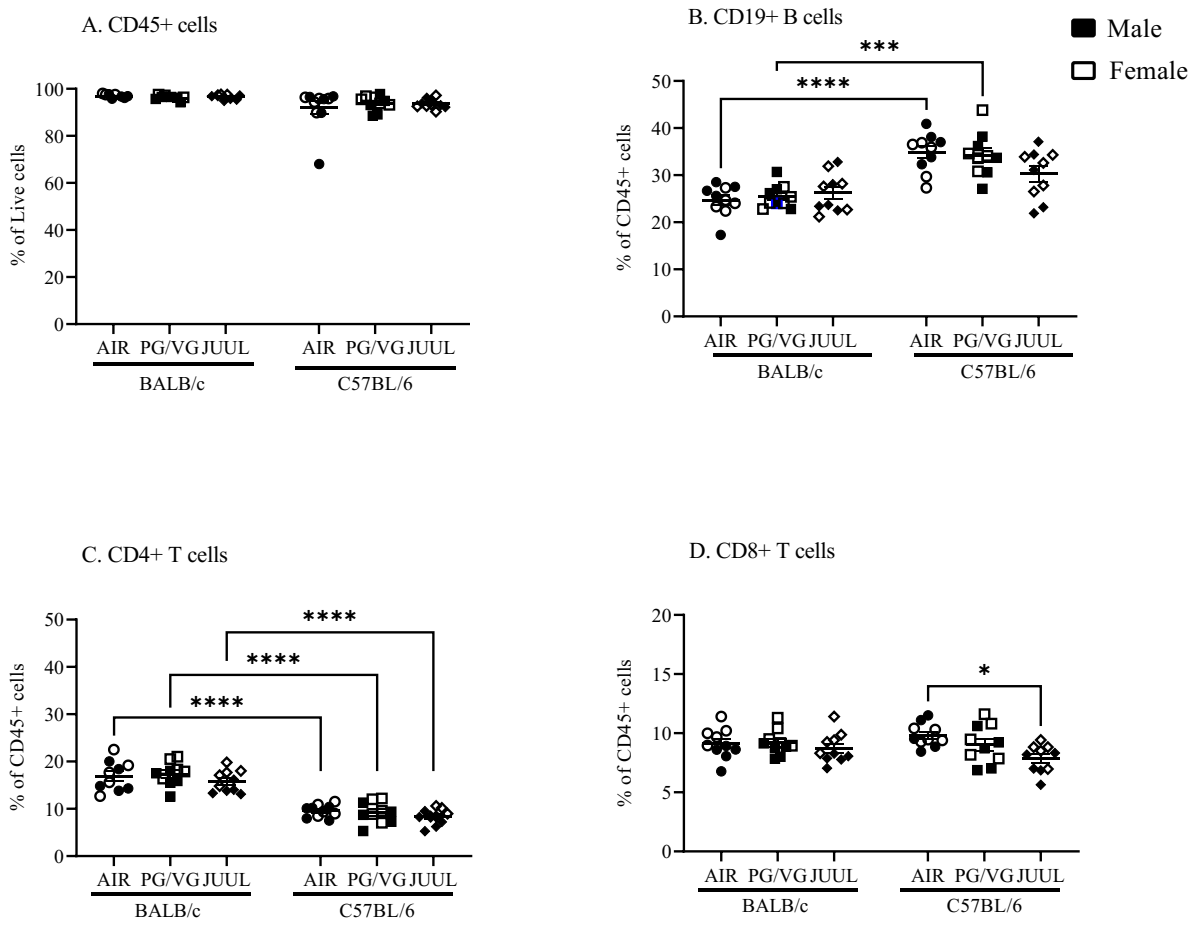


Figure 4.3. Differential effects on adaptive immune cell populations in the lung tissue of BALB/c and C57BL/6 mice after JUUL exposure. The frequency of total immune cells (A), B cells (B), $CD4^+$ T cells (C), and $CD8^+$ T cells (D) are shown. B cells were higher among air- and PG/VG-exposed C57BL/6 mice compared to BALB/c mice. $CD4^+$ T cells were higher among all BALB/c mice. $CD8^+$ T cells were decreased by JUUL in C57BL/6 mice. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA (* $p \leq 0.05$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.2. JUUL upregulates pulmonary gene expression in BALB/c but not C57BL/6 mice.

To next understand the effects of JUUL exposure on gene expression in the lungs, we measured the mRNA level of genes that are associated with inflammation (*Tnfα, Il1β, Il4, Il13,* and *Ptgs2*), oxidative stress (*Nrf2* and *Sod2*), and tissue remodeling (*Col3a1,* and *Muc5b*) (Figure 4.4). Here, there emerged a distinct pattern of expression between JUUL-exposed BALB/c and C57BL/6 mice. In this regard, JUUL exposure significantly increased the expression of *Tnfα* (Figure 4.4A), *Col3a1* (Figure 4.4H) and *Muc5b* (Figure 4.4I) only in BALB/c mice; in contrast, the expression of these genes was unaffected by JUUL exposure in C57BL/6 mice. The interleukins *Il1β, Il4,* and *Il13* (Figure 4.4B-D), *Ptgs2* (Figure 4.4E), *Nrf2* and *Sod2* (Figure 4.4F-G) were unchanged by JUUL exposure in either strain. These results highlight the differential impact of JUUL exposure on gene expression between inbred strains of mice.

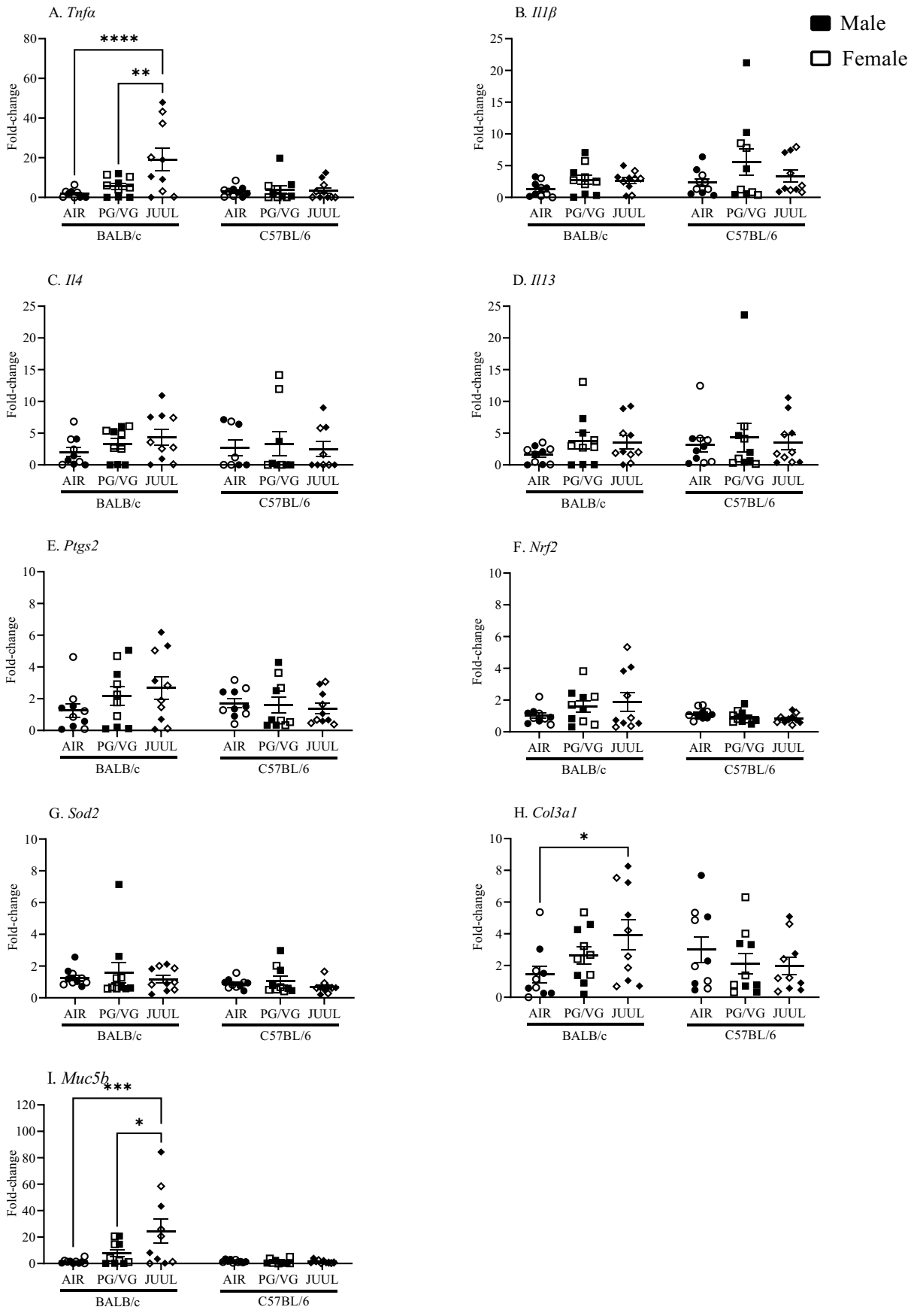

A. Tnfα
B. Il1β
C. Il4
D. Il13
E. Ptgs2
F. Nrf2
G. Sod2
H. Col3a1
I. Muc5b
Male
Female
Fold-change
AIR
PG/VG
JUUL
BALB/c
C57BL/6
****
**
***
*

Figure 4.4. Differential effects of JUUL exposure on gene expression in the lung tissue of BALB/c and C57BL/6 mice. Expression of *Tnfα* (A), *Il1β* (B), *Il4* (C), *Il13* (D), *Ptgs2* (E), *Nrf2* (F), *Sod2* (G), *Col3a1* (H), *Muc5b* (I) was evaluated. There was significant upregulation of *Tnfα*, *Col3a1*, and *Muc5b* only in JUUL-exposed BALB/c mice. There were no significant changes in the expression of these gene in C57BL/6 mice. Gene expression was normalized to 18s rRNA. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

4.3. Proteomic analysis reveals distinct sex-dependent pathways driven by JUUL exposure in BALB/c and C57BL/6 mice.

The previous data revealed subtle but distinct changes in pulmonary immune cell composition and gene expression in response to JUUL exposure. To more comprehensively understand the extent to which JUUL exposure affects the respiratory system, we performed quantitative proteomics on the cell-free BAL fluid and lung tissue of air- and JUUL-exposed BALB/c and C57BL/6 mice using isobaric chemical labeling with tandem mass tags (TMT). TMT are isobaric chemical tags, which are identical in mass but dissociate to yield reporter ions of distinct mass (211). The main advantage of TMT quantitative proteomics is that it allows for the accurate determination of the relative protein abundance of many different samples at the same time through multiplexed protein quantification (211). This therefore allowed us to quantitatively assess for differences between exposures, sex, and inbred strains of mice. One of the most striking initial observations was the difference in the total number of proteins quantified in the BAL fluid between BALB/c and C57BL/6 mice: 464 proteins were quantified in BALB/c mice, whereas more than double (958) were quantified in C57BL/6 mice (Figure 4.5). Of these, 51 proteins were quantified only in BALB/c (including the cytochrome P450 (Cyp450) enzyme Cyp2b19, neuroblastoma RAS viral oncogene (Nras), hemoglobin subunit beta-2 (Hbb-b2), and caveolin 1 (Cav1). A total of 413 proteins were quantified in both strains, and 545 proteins were quantified only in C57BL/6 mice. The list of total proteins quantified in each strain is available in Supplementary Table S1.

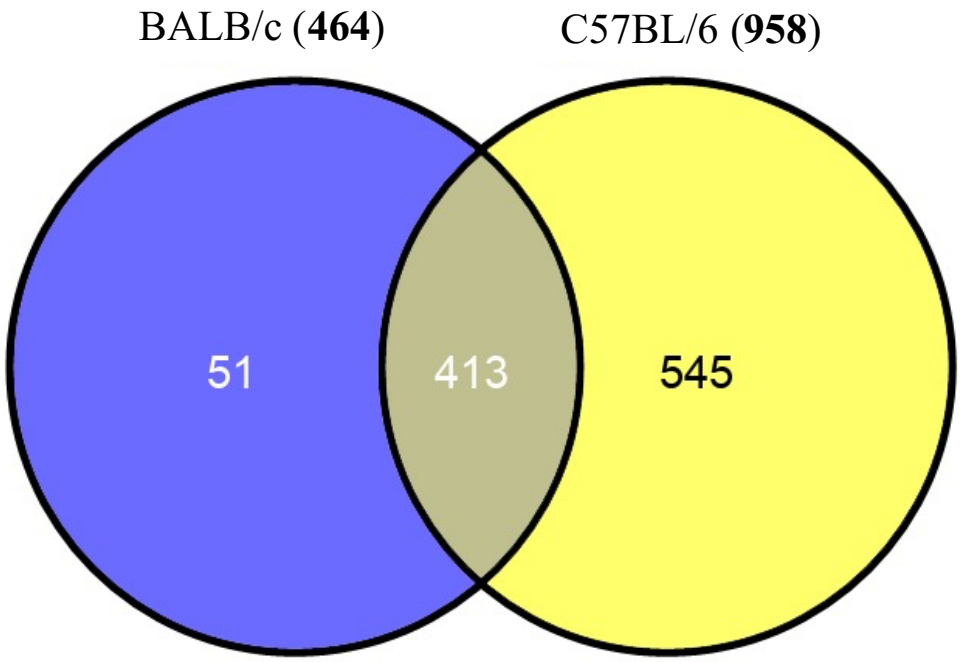


Figure 4.5. Venn diagram of total proteins quantified in the BAL fluid of BALB/c and C57BL/6 mice. The total number of proteins quantified in each strain is indicated in bold. In BALB/c mice, 464 proteins were quantified in the BAL fluid. In C57BL/6 mice, 958 proteins were quantified in the BAL fluid; 413 proteins were common to both strains.

To next understand the extent to which JUUL exposure affected protein levels in the BAL fluid of BALB/c and C57BL/6 mice, we separately compared the proteomes of male (Group 1) and female (Group 2) mice exposed to room air only or JUUL (Figure 4.6). These first two comparison groups reflect how JUUL exposure affects protein expression. Then, to assess the effect of sex-specific differences, we compared proteomic changes between air-exposed male and female mice (Group 3); this group thus evaluates intrinsic sex-dependent differences in the absence of exposure (Figure 4.6). Finally, to identify sex-specific differences in the pulmonary proteomic response to JUUL exposure, we compared changes between JUUL-exposed male and female mice (Group 4). These last two comparison groups evaluate the effect of sex as a biological variable. We applied these four comparisons to the BAL fluid and lung tissue proteomic data and analyzed BALB/c and C57BL/6 mice separately.

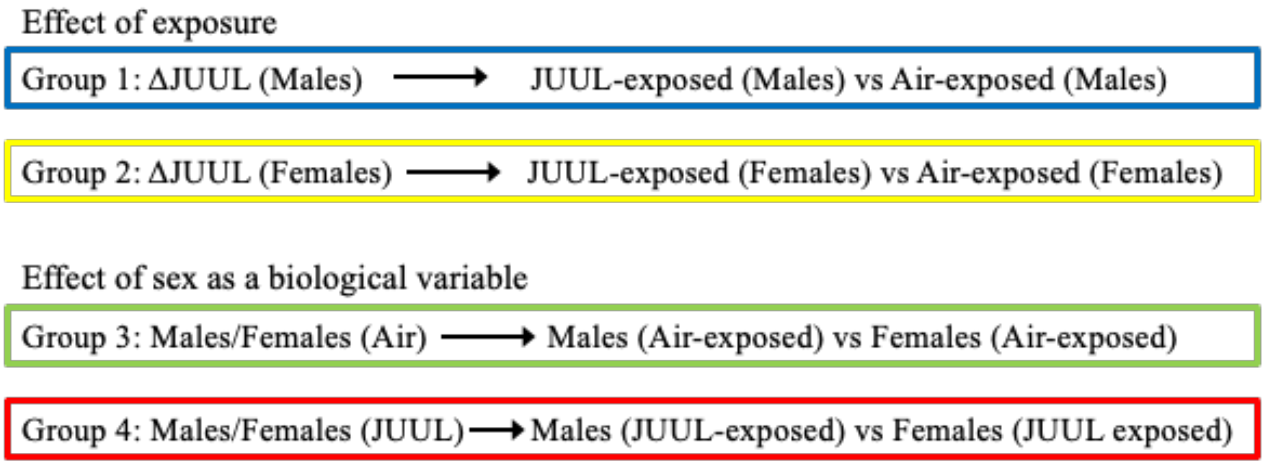


Figure 4.6. Nomenclature for the four comparison groups. To understand the extent to which JUUL exposure affect the BAL fluid and lung tissue proteomic profiles of BALB/c and C57BL/6 mice, we compared changes in protein expression between JUUL- and air-exposed male (Group 1) and female (Group 2) mice. To understand the extent to which sex-specific differences affect protein expression, we compared the proteomic profiles between male and female mice exposed to air only (*i.e.,* at baseline) (Group 3). Separately, we compared male and female mice exposed to JUUL aerosol (Group 4).

We first compared the differentially expressed proteins (DEPs) within the BAL fluid of BALB/c (Figure 4.7A) and C57BL/6 (Figure 4.7B) mice. Among male BALB/c mice, there were 3 DEPs in the ΔJUUL group (Group 1); of these, two were unique in this group and thus did not overlap with other comparisons. These two proteins were immunoglobulin heavy constant gamma 2A (Igh-1a), a variant heavy chain of immunoglobulins, and orosomucoid 1 (Orm1), a key acute-phase plasma protein involved in regulating inflammation and metabolism (Figure 4.7A). Among female BALB/c mice, there were 20 DEPs in the ΔJUUL group (Group 2). Of these, two proteins were only differently-expressed in this group; these two proteins were serum amyloid P component (Apcs), an acute-phase protein involved in complement activation and modulation of viral processes, and actinin alpha 1 (Actn1), a protein involved in actin filament bundle assembly (Figure 4.7A). When considering differences between air-exposed BALB/c mice, 42 proteins were differentially-expressed in the males compared to females (Group 3). Of these, 21 proteins were only present in this group, including serine protease inhibitor ('serpin') proteins Serpina3n and Serpind1, interleukin-18 receptor 1 (Il18r1), and a cysteine protease cathepsin H (Ctsh) (Figure 4.7A). Among JUUL-exposed BALB/c mice, 32 proteins were differentially-expressed in the males compared to females (Group 4). Of these, 11 proteins were only differentially-expressed in this group; these included S100 calcium binding protein A9 (S100a9), cell adhesion molecule 1 (Cadm1), Cxcl15, Serpina6, and Serpina3m (Figure 4.7A). The complete list of DEPs in BALB/c mice is available in Supplementary Table S2. In summary, these results indicate that many of the differentially-expressed proteins in BALB/c mice are involved in the immune response, particularly in the acute phase response, and the perturbations in the BAL fluid proteome are driven predominantly by sex-dependent differences between male and female mice.

Next, we compared the BAL fluid proteomic profile in C57BL/6 mice. Among male C57BL/6 mice, there were 34 DEPs in the ΔJUUL group (Group 1). Of these, 18 proteins were unique in this group (Figure 4.7B). Among female C57BL/6 mice, there were 18 DEPs in the ΔJUUL group (Group 2), of which 11 were only present in this group (Figure 4.7B). Among air-exposed C57BL/6 mice, 28 proteins were differentially expressed in the males compared to females (Group 3), 4 of which were unique (*i.e.,* pancreatic lipase related protein 1 (Pnliprp1), ferritin light polypeptide 1 (Ftl1), proteasome 26S subunit, ATPase 3 (Psmc3), and lymphocyte proliferation 1 (Lpl)) (Figure 4.7B). Among JUUL-exposed C57BL/6 mice, 31 proteins were differentially expressed in the males compared to females (Group 4), of which 10 were only present in this group (Figure 4.7B). The complete list of DEPs in C57BL/6 mice is available in Supplementary Table S3. Overall, these results suggest that JUUL exposure in C57BL/6 mice significantly changed the expression of more proteins than sex-dependent differences.

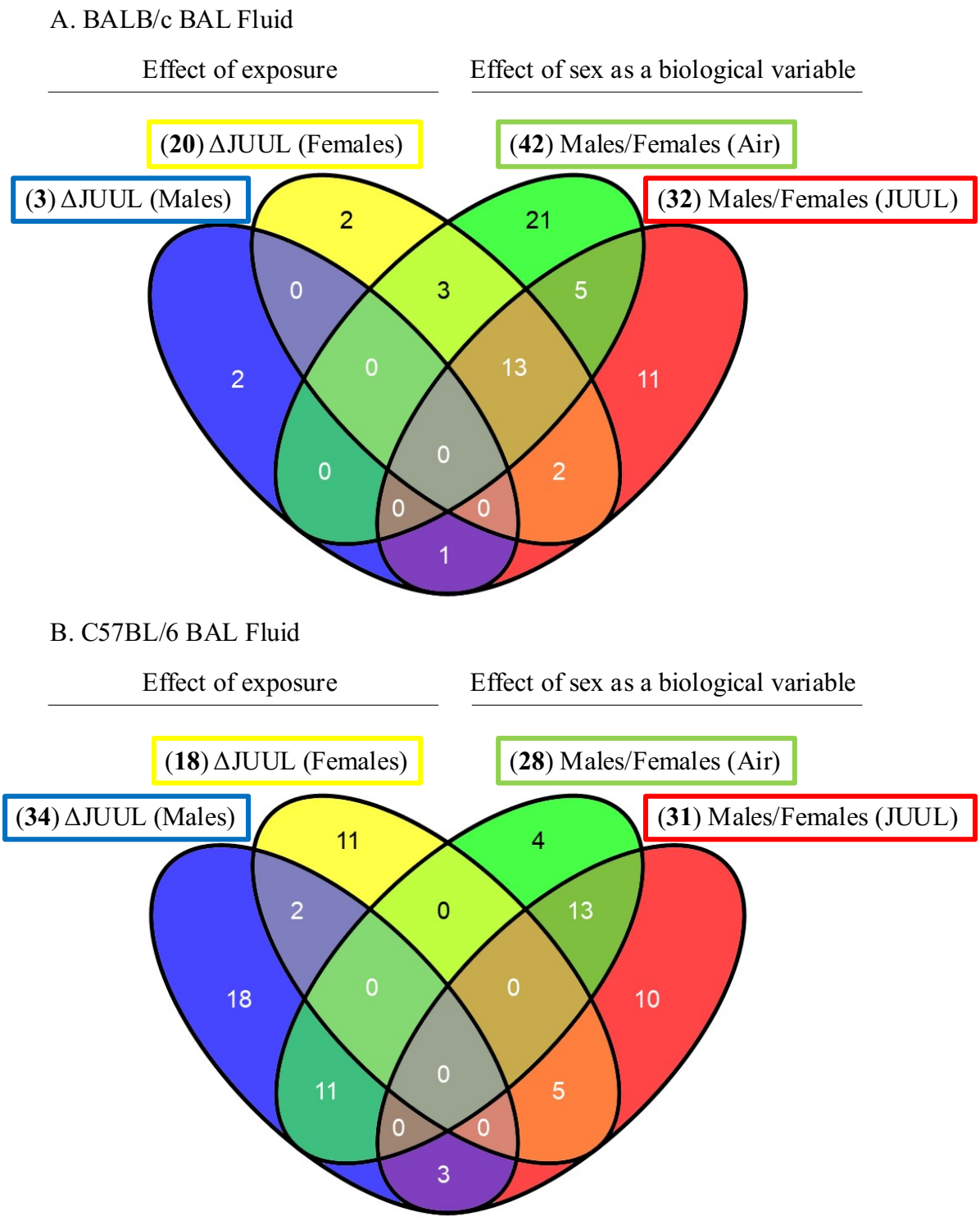


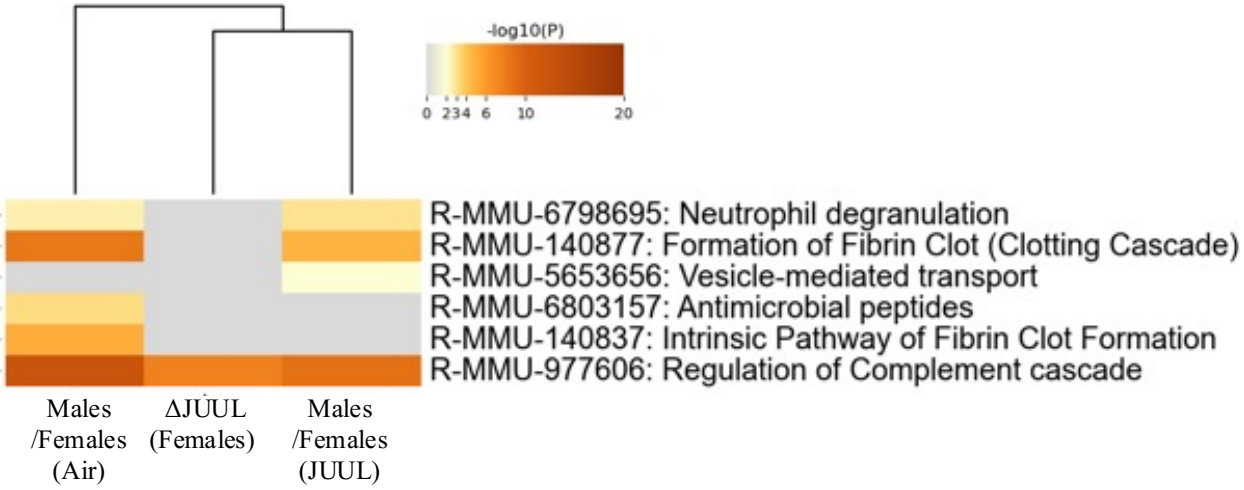
Figure 4.7. Venn diagrams of DEPs in the BAL fluid of BALB/c and C57BL/6 mice. The total number of DEPs within each comparison group is indicated in bold. In BALB/c mice, changes in the BAL fluid proteome are driven predominantly by sex-dependent differences between male and female mice (A). In contrast, in C57BL/6 mice JUUL exposure caused significant changes in the expression of several proteins, more so than sex-dependent differences (B).

We next performed pathway analysis of DEPs to understand which biological processes were affected by the changes within the BAL fluid. Overall, this analysis shown that DEPs in the BAL fluid of BALB/c and C57BL/6 mice are associated with different biological pathways (Figure 4.8). Because there were insufficient DEPs in the BALB/c ΔJUUL (Males) group to perform pathway analysis, this group does not appear on the figure (Figure 4.8A). In BALB/c mice, there were two pathways which were significantly enriched in the males versus females irrespective of exposure; these were 'Neutrophil degranulation' (included the protein S100a9) and 'Formation of fibrin clot (clotting cascade)' (included the protein Serpind1) (Figure 4.8A). These two pathways were enriched in both Air and JUUL exposure groups, thereby underscoring that these pathways are driven by sex-dependent differences. One pathway, 'Vesicle-mediated transport', was only enriched in JUUL-exposed males compared to females (Group 4). The 'Antimicrobial peptides' pathway and 'Intrinsic Pathway of Fibrin Clot Formation' pathway (including the proteins Serpind1 and coagulation factor XII (F12)) were only enriched in air-exposed males compared to females (Group 3). Finally, the 'Regulation of complement cascade' pathway was significantly enriched across all comparison groups. Complete enrichment analysis results for BALB/c mire are available in Supplementary Table S4.

In C57BL/6 mice, the proteomic changes that occurred in the BAL fluid included biological processes that were both similar and distinct from BALB/c mice (Figure 4.8B). The only common pathway between BALB/c and C57BL/6 mice was 'Neutrophil degranulation', which included the proteins membrane metalloendopeptidase (Mme) and Serpin Family B Member 12 (Serpinb12)); however, this pathway was not enriched in female mice exposed to ΔJUUL (Group 2). Two pathways were enriched only in males exposed to ΔJUUL (Group 1); these were the 'Cellular

response to chemical stress' (including the antioxidant enzymes glutathione peroxidase 2 (Gpx2) and NAD(P)H dehydrogenase quinone 1 (Nqo1)) and 'Post-translational protein phosphorylation' (Figure 4.8B). The 'Programmed cell death' pathway was enriched only in JUUL-exposed female mice (Group 2). Finally, the 'Terminal pathway of complement' was similarly enriched in male mice exposed to both air only and JUUL (compared to female mice), indicating that enrichment of this pathway is driven by sex (Figure 4.8B). Complete enrichment analysis results for C57BL/6 mice are available in Supplementary Table S5. In summary, pathway enrichment analysis of DEPs in the BAL fluid revealed that sex and JUUL exposure uniquely modulate the proteomic profiles of two inbred strains. In BALB/c mice, sex-dependent differences drove changes in proteins involved in immunity and clot formation. In C57BL/6 mice, JUUL exposure induced changes in proteins involved in cellular response to stress, protein phosphorylation, and cell death, whereas sex-dependent differences drove changes in pathways involved in immunity. A notable similarity in both strains was the enrichment of the 'Neutrophil degranulation' pathway.

A. BALB/c BAL Fluid



B. C57BL/6 BAL Fluid

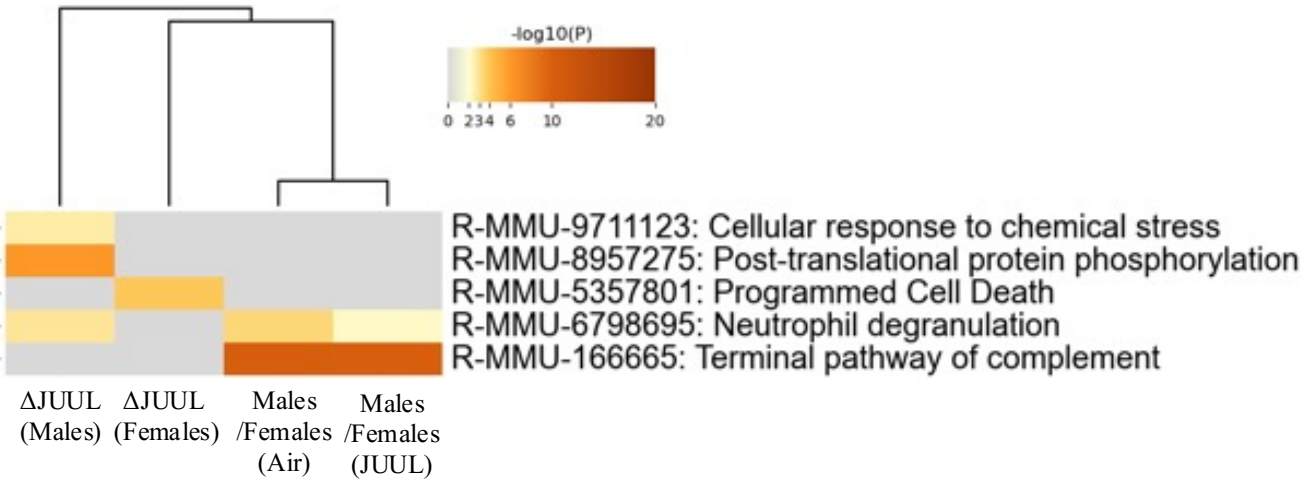


Figure 4.8. Pathway analysis of DEPs within the BAL fluid. There was significant pathway enrichment in the BAL fluid of BALB/c mice, including enrichment of pathways involved in immunity and clot formation. There were insufficient DEPs in ΔJUUL (Males) group to perform pathway analysis, thus this group does not appear on the figure (A). In C57BL/6 mice, there was enrichment of pathways involved in cellular response to stress, protein phosphorylation, cell death, and the immune system (B).

We also quantified proteins in the lung tissue of BALB/c and C57BL/6 mice and similarly evaluated for exposure- and sex-dependent differences. Within the lung tissue, 1,316 proteins were quantified in BALB/c and 1,226 proteins were quantified in C57BL/6 mice (Figure 4.9). Of these, 369 proteins were only in BALB/c mice, 947 proteins were common between both strains, and 279 proteins were unique to C57BL/6 mice (Figure 4.9). The list of total proteins quantified in each strain is available in Supplementary Table S6.

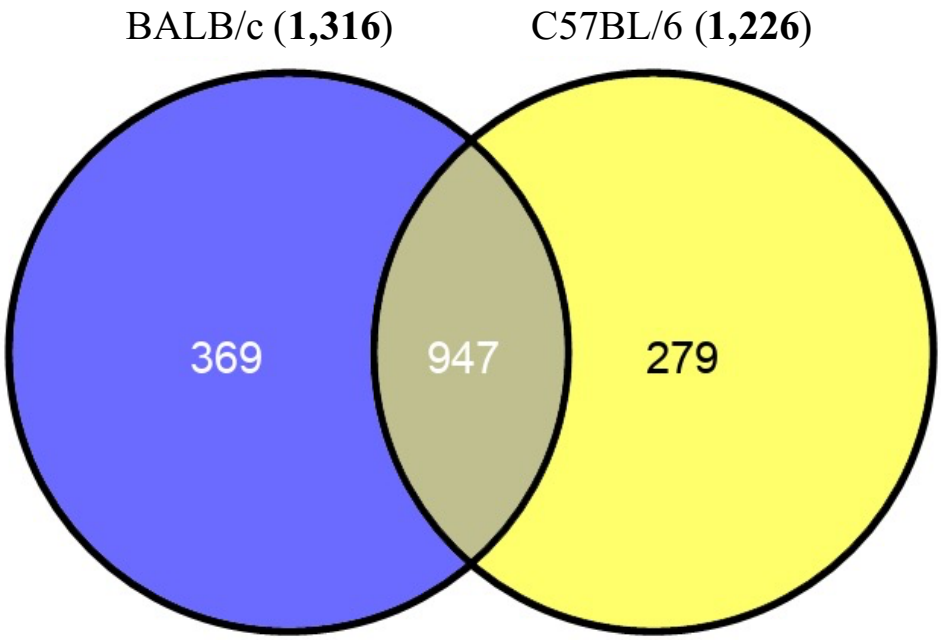


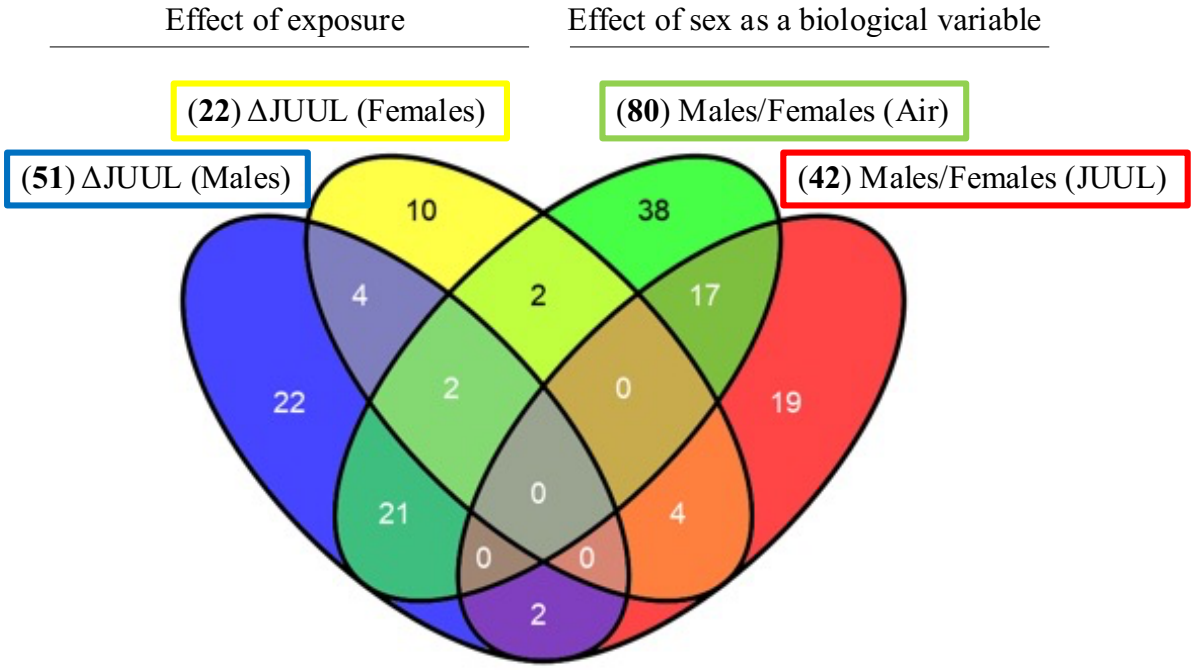
Figure 4.9. Venn diagram of total proteins quantified in the lung tissue of BALB/c and C57BL/6 mice. The total number of proteins quantified in each strain is indicated in bold. A total of 1,316 and 1,226 proteins were quantified in BALB/c and C57BL/6 mice, respectively, and 947 proteins were in common to both strains.

We then compared the DEPs in the lung tissue between the four comparison groups. Among male BALB/c mice in the ΔJUUL group (Group 1), 51 proteins were differentially expressed, of which 22 were only in this group (Figure 4.10A). Among female BALB/c mice in the ΔJUUL group (Group 2), there were 22 DEPs, ten of which were only in this group and included A Disintegrin and metalloproteinase domain-containing protein 10 (Adam10). A notable sex-dependent difference was observed among air-exposed BALB/c mice (Group 3), as indicated by the differential expression of 80 proteins in males compared to females at baseline (*i.e.,* after exposure to air only). Of these 80 proteins, 38 were differentially expressed only in this group. Among JUUL-exposed BALB/c mice, 42 proteins were differentially expressed in males compared to females (Group 4), of which 19 proteins were differentially expressed only in this group (Figure 4.10A). The complete list of differentially expressed lung proteins in BALB/c mice is available in Supplementary Tables S7. These results indicate that sex-dependent differences at baseline affect the expression of more proteins in the lung tissue than JUUL exposure. Moreover, when assessing the effect of JUUL exposure compared to air, male mice had more uniquely differentially-expressed proteins than female mice.

Among male C57BL/6 mice, there were 69 DEPs in the ΔJUUL group (Group 1), approximately half (34) of which were differentially-expressed only in this group (Figure 4.10B). Among female C57BL/6 mice in the ΔJUUL group (Group 2), there were 22 DEPs and ten of these were only differentially-expressed this group (Figure 4.10B). Among air-exposed C57BL/6 mice (Group 3), 54 proteins were differentially-expressed in males compared to females; of these, 16 proteins were unique to this group. Among JUUL-exposed C57BL/6 mice (Group 4), 45 proteins were differentially expressed in males compared to females, of which 23 proteins were unique to this

group (Figure 4.10B). The complete list of differentially expressed lung proteins in C57BL/6 mice are available in Supplementary Table S8. Overall, these results highlight that that JUUL exposure induces more unique changes in protein expression in the lung tissue than the effect of sex. Additionally, as in BALB/c mice, JUUL exposure induced more unique changes in male C57BL/6 mice than in females.

A. BALB/c Lung Tissue



B. C57BL/6 Lung Tissue

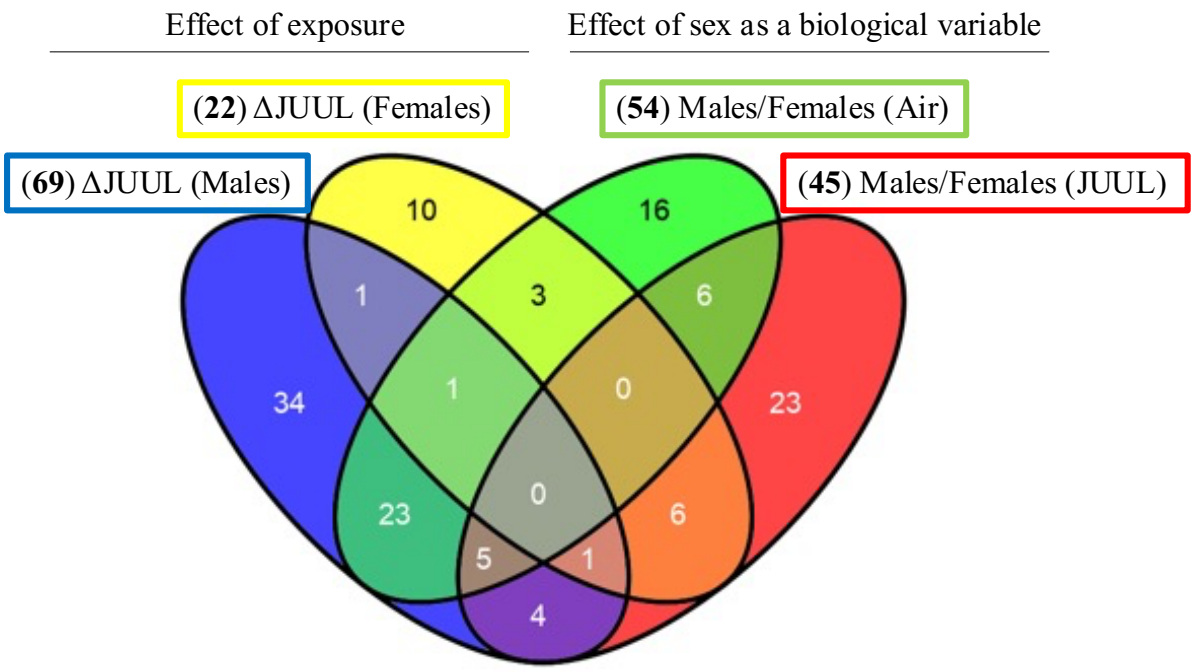


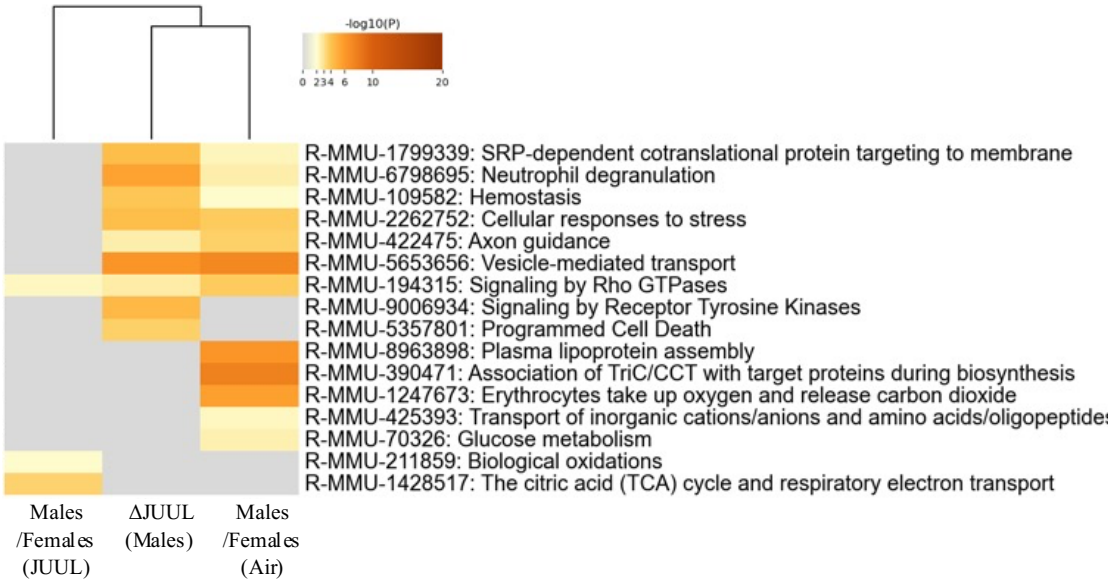
Figure 4.10. Venn diagrams of DEPs in the lung tissue of BALB/c and C57BL/6 mice. The total number of DEPs within each comparison group is indicated in bold. In BALB/c mice, sex-dependent differences at baseline affected the expression of more proteins in the lung tissue than JUUL exposure (A). In contrast, JUUL exposure in C57BL/6 mice induced more protein unique changes than the effect of sex (B). In both strains, the effect of JUUL exposure compared to air caused a greater number of DEPs in males than in females.

We then performed pathway enrichment analysis to understand which biological processes were affected by the lung protein differences caused by JUUL exposure (Figure 4.11). Note that there were insufficient DEPs within the BALB/c ΔJUUL (Females) group for pathway analysis, so this comparison group does not appear on the figure (Figure 4.11A). In BALB/c mice, there was a striking similarity in the enriched pathways between JUUL- versus air-exposed male mice, and male versus female air-exposed mice (*i.e.,* ΔJUUL (Males) and Males/Females (Air)). These pathways included 'SRP-dependent cotranslational protein targeting', 'Neutrophil degranulation', 'Homeostasis', 'Cellular response to stress', 'Axon guidance', 'Vesicle-mediated transport', and 'Signaling by Rho GTPases' (Figure 4.11A). The latter pathway was also significantly enriched in the Males/Females (JUUL) comparison group (Group 4). Two pathways, 'Signaling by Receptor Tyrosine Kinases' and 'Programmed cell death' were only enriched in the ΔJUUL (Males) comparison group (Group 1), indicating that these pathways are driven by JUUL exposure. In contrast, several pathways were enriched between male versus female air-exposed mice (Group 3), indicating that this effect is driven by sex rather than JUUL exposure. These pathways were 'Plasma lipoprotein assembly', 'Association of TriC/CCT with target proteins', 'Erythrocyte oxygen uptake', 'Transport of inorganic ions and amino acids', and 'Glucose metabolism'. Finally, two pathways were uniquely enriched between males and females exposed to JUUL (Group 4); these were 'Biological oxidation' and 'TCA cycle' (Figure 4.11A). Complete enrichment analysis results for BALB/c mire are available in Supplementary Table S9.

In the lung tissue of C57BL/6 mice, one pathway was enriched in females exposed to ΔJUUL (Group 2), which was 'Membrane trafficking' (Figure 4.11B). The 'Metabolism of nucleotides' pathway was enriched in both the ΔJUUL (Females) and the Males/Females (Air) groups. Two

pathways, ‘Plasma lipoprotein assembly’ and ‘Respiratory electron transport chain’, were enriched only in the Males/Females (Air) group (Group 3). In contrast, three pathways, ‘Rho-associated GTPases cycle’, ‘Metabolism of RNA’ and ‘Apoptotic cleavage of cellular proteins’ were only enriched in the Males/Females (JUUL) group (Group 4). Similar to BALB/c mice, there was similarity in the enriched pathways between JUUL- versus air-exposed male mice, and male versus female air-exposed mice (*i.e.,* ΔJUUL (Males) and Males/Females (Air) on Figure 4.11B) and included the ‘Pentose phosphate pathway’, ‘Metabolism of carbohydrates’, ‘Cellular response to chemical stress’, ‘Heme degradation’, ‘Biological oxidations’, and ‘Phase I – functionalization of compounds’. Of note, ‘Neutrophil degranulation’ was enriched in all C57BL/6 mice, regardless of exposure and sex. However, the level of enrichment was higher in males exposed to ΔJUUL (Group 1) as indicated by the -log10(P) value (Figure 4.11B). Finally, three pathways, ‘Cellular response to heat stress’, ‘Metabolism of amino acids’ and ‘Metabolism of lipids’, were enriched only in males exposed to ΔJUUL (Figure 4.11B). Complete enrichment analysis results for C57BL/6 mire are available in Supplementary Table S10. In summary, DEPs in the lung tissue of BALB/c mice are involved in vesicle-mediated transport, signaling by Rho GTPases, and programmed cell death. In C57BL/6 mice, DEPs are involved in membrane trafficking, biological oxidation, cellular response to stress, and metabolism. A striking similarity between both strains is that male mice were more susceptible to JUUL-induced proteomic changes than female mice, which included changes in proteins involved in neutrophil degranulation.

A. BALB/c Lung Tissue



B. C57BL/6 Lung Tissue

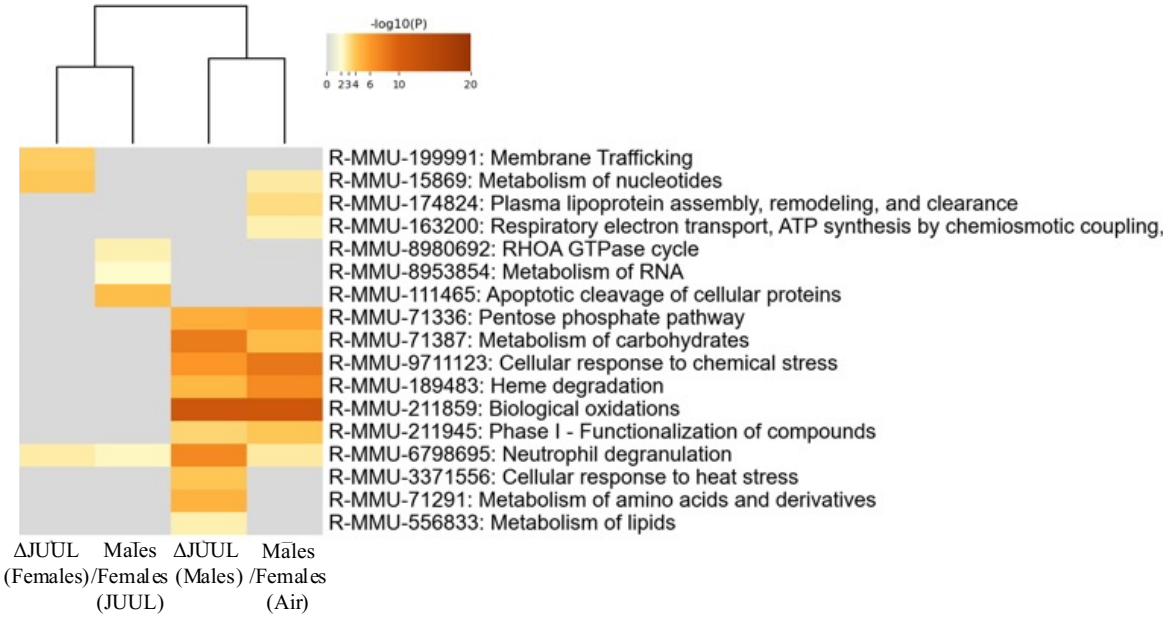


Figure 4.11. Pathway analysis of DEPs in the lungs. There was significant pathway enrichment in the lung tissue of BALB/c and C57BL/6 mice. In BALB/c mice, enriched pathways were involved in many biological processes, including vesicle-mediated transport, signaling by Rho GTPases, and programmed cell death. Note that there were insufficient DEPs in the ΔJUUL (Females) group to perform pathway analysis, thus this group does not appear on the figure (A). In C57BL/6 mice, there was enrichment of pathways involved in membrane trafficking, biological oxidation, cellular response to stress, and metabolism (B).

4.4. A prior exposure to inhaled JUUL aerosols does not affect lung immune cell composition during allergic airway inflammation.

The results above show strain- and exposure-specific differences in lung immune cell composition, as well as gene and protein expression. Notable though is the lack of change in the percentage of immune cells or changes in gene expression in the lungs of JUUL-exposed C57BL/6 mice, an inbred mouse strain that is Th1-biased, despite profound alterations in lung proteins associated with immunological function. A common condition associated with dysregulated immune function is allergic asthma, yet, in this regard, there is limited information on e-cigarettes. To now investigate whether a prior JUUL exposure impacts the composition of the pulmonary immune system during allergic asthma, we exposed C57BL/6 mice to PG/VG or JUUL aerosols for 14 consecutive days; immediately after this exposure, there were no significant changes in the lungs (Supplementary Figures S2-S5). After the aerosol exposure, mice were sensitized and challenged to OVA using our established protocol (207). In the BAL fluid, there were no statistically significant differences in immune cell composition between PBS- or OVA-treated mice (Figure 4.12) and neutrophils were not detected (Figure 4.12B). Although there was a trend towards increased BAL eosinophils in OVA-treated mice, this did not reach statistical significance (Figure 4.12C). Additionally, neither PG/VG nor JUUL exposure significantly affected the frequency of eosinophils. There was also no significant change in the percentage of macrophages (Figure 4.12D); alveolar macrophages constituted 100 per cent of BAL macrophages (Figure 4.12E), and no interstitial macrophages were detected (Figure 4.12F).

In lung tissue, a prior exposure to JUUL did not affect the composition of innate or adaptive immune cells. However, there was a difference in the percentage of total $CD45^+$ cells in the PG/VG

exposure group, which was higher among OVA-treated mice compared to PBS-treated mice (Figure 4.13A). The frequency of neutrophils was unchanged and thus not affected by OVA or JUUL exposure (Figure 4.13B). Among air-exposed mice, eosinophils were significantly higher in the OVA group compared to the PBS control (Figure 4.13C) whereas the percentage of macrophages, including resident alveolar macrophages and interstitial macrophages, was unchanged (Figure 4.13D-F). Although $CD4^+$ T cells were unchanged by JUUL exposure (Figure 4.14B), compared to the PBS control, there was a significant increase in the frequency of $CD8^+$ T cells in OVA-treated mice previously exposed to PG/VG. However, there was no difference with JUUL exposure (Figure 4.14C). Overall, these findings suggest that a sub-chronic JUUL exposure prior to sensitization and challenge with OVA does not significantly affect the frequency of immune cells within the BAL fluid and lung tissue in C57BL/6 mice.

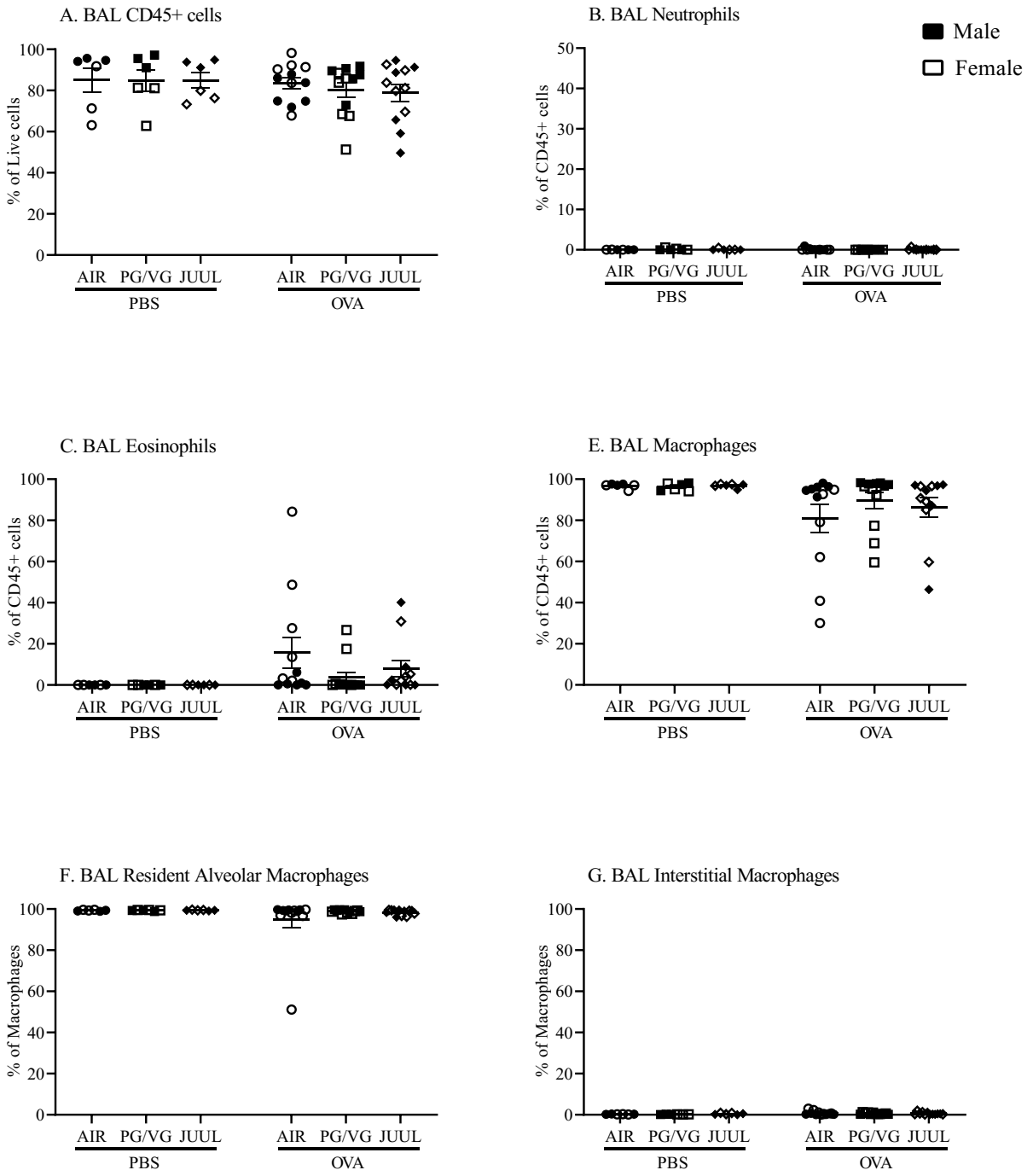


Figure 4.12. BAL fluid cellularity of OVA-treated mice previously exposed to JUUL. There were no statistically significant changes in total immune cells (A), neutrophils (B), eosinophils (C), macrophages (D), resident alveolar macrophages (E), and interstitial macrophages (E). Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA.

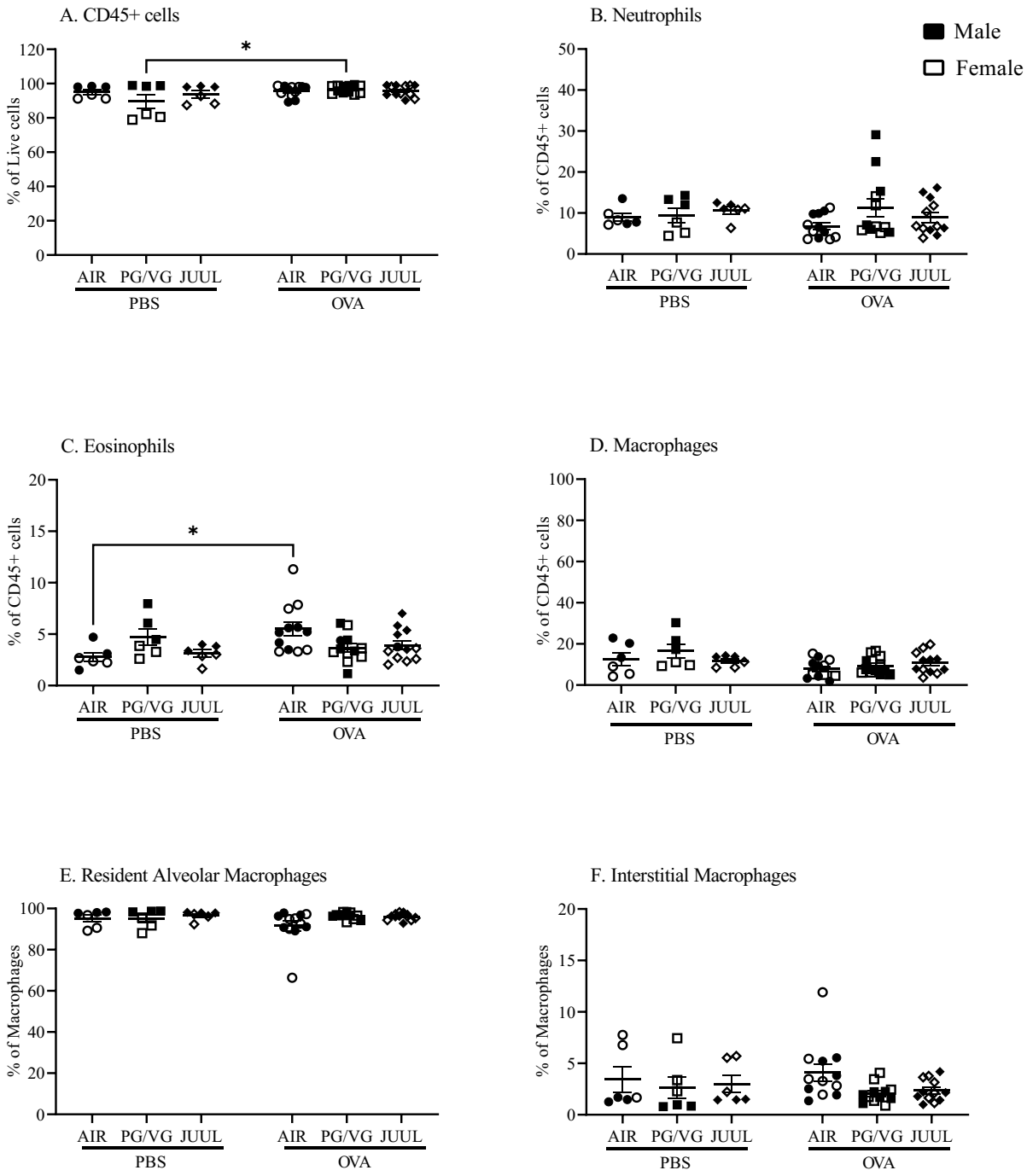


Figure 4.13. Innate immune cells in the lung tissue of OVA-treated mice previously exposed to JUUL. The frequency of total immune cells (A), neutrophils (B), eosinophils (C), macrophages (D), resident alveolar macrophages (E), interstitial macrophages (F) are shown. In the OVA group, the frequency of total immune cells and eosinophils was higher in PG/VG- and air-exposed mice, respectively, but no other immune cell was altered. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA (* $p \leq 0.05$).

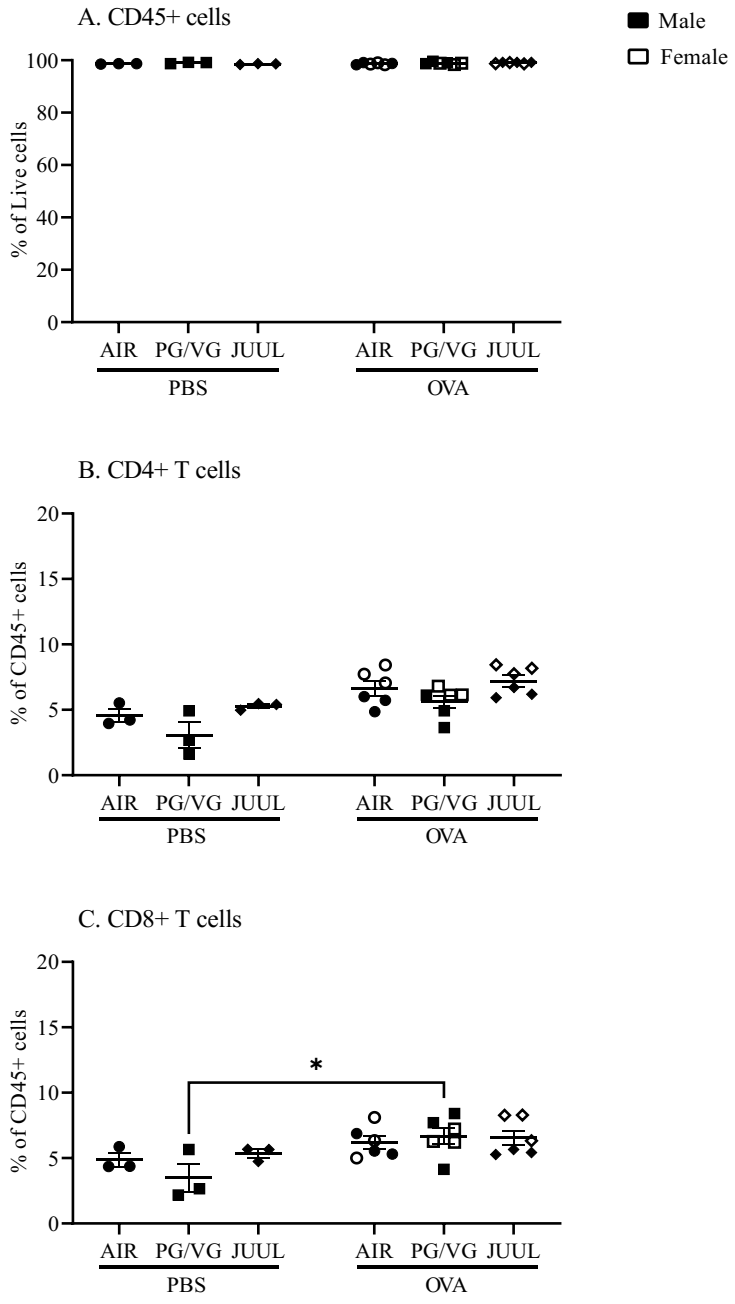


Figure 4.14. Adaptive immune cells in the lung tissue of OVA-treated mice previously exposed to JUUL. The frequency of total immune cells (A), $CD4^+$ T cells (B), and $CD8^+$ T cells (C) are shown. $CD8^+$ T cells were significantly increased in PG/VG-exposed OVA-treated mice. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA (* $p \leq 0.05$).

4.5. The expression of Th2 cytokines in the lungs is not significantly altered by JUUL.

In absence of significant changes in airway and pulmonary immune cells, we aimed to determine whether the expression of two key Th2 cytokines (*Il4* and *Il5*) was changed by JUUL exposure in a mouse model of allergic inflammation. Here, the mRNA expression of *Il4* (Figure 4.15A) and *Il5* (Figure 4.15B) was unchanged in either PBS- or OVA-treated mice, and there were no significant differences between the exposure groups. Thus, a prior sub-chronic JUUL exposure in a C57BL/6 model of allergic asthma did not affect the mRNA expression of two Th2 cytokines.

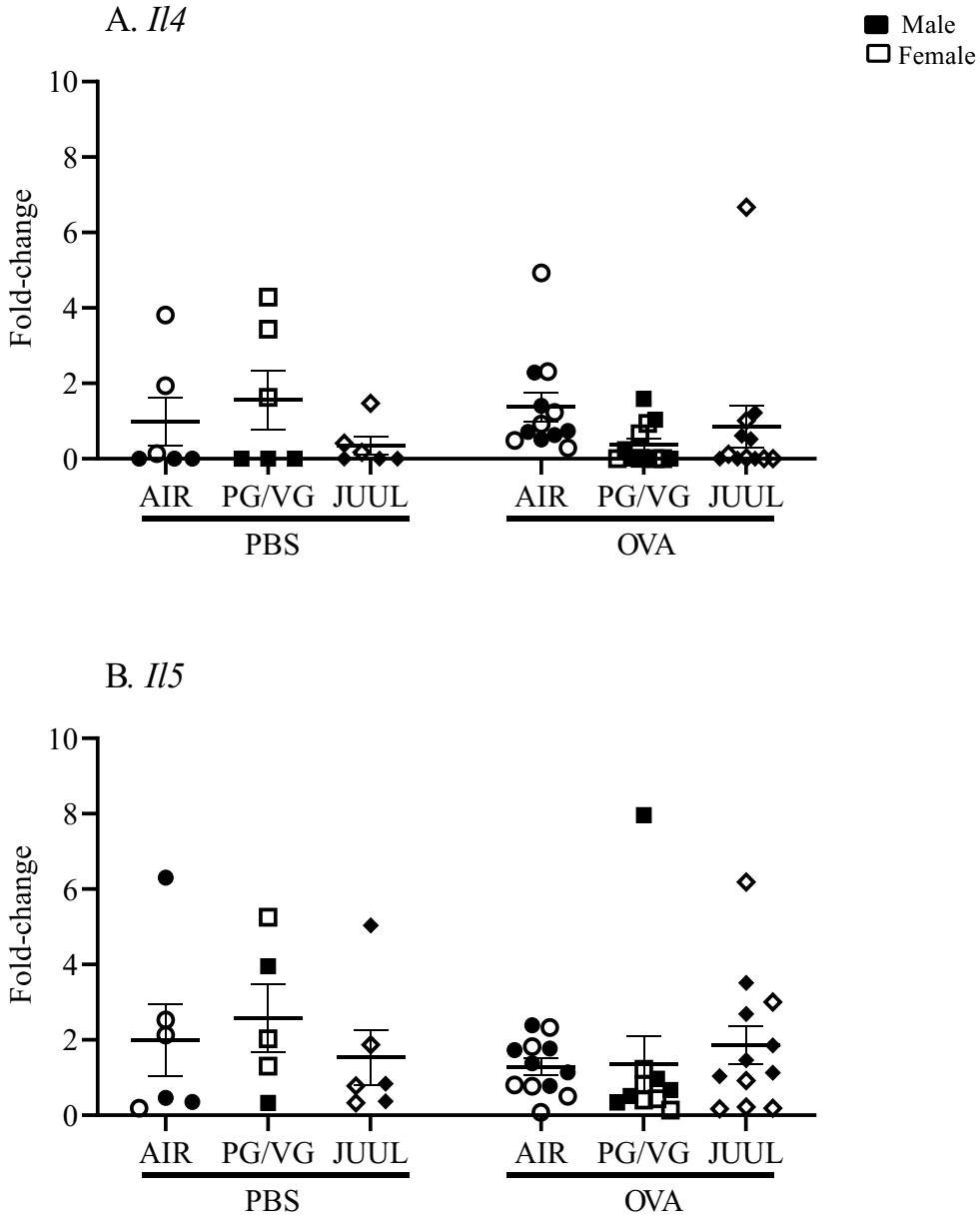


Figure 4.15. Gene transcription of Th2 cytokines in the lung tissue of OVA-treated mice previously exposed to JUUL. There were no differences in mRNA expression of *Il4* (A) and *Il5* (B) among PBS- and OVA-treated mice previously exposed to air, PG/VG, or JUUL. Grubb's outlier test was used to remove 3 outliers removed in *Il5*. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA.

4.6. A prior JUUL exposure does not increase circulating immunoglobulin E in an OVA-induced model of allergic airway inflammation.

Finally, we aimed to determine whether JUUL would affect the humoral response in an allergic asthma mouse model. We measured the concentration of IgE, a key immunoglobulin in allergic asthma that induces degranulation of mast cells. Overall, serum IgE was higher in OVA-treated mice compared to the PBS control groups (Figure 4.16). The mean concentration of IgE in the OVA group was 12,615 ng/mL in air-exposed mice, 11,395 ng/mL in PG/VG-exposed mice, and 15,328 ng/mL in JUUL-exposed mice. In contrast, in the PBS group, the mean concentration was 3,145 ng/mL, 4,678 ng/mL, and 7,909 ng/mL in mice exposed to air, PG/VG, and JUUL, respectively. In both PBS- and OVA-treated mice, there was a trend of increased IgE with JUUL exposure, but this did not reach statistical significance. Thus, a sub-chronic JUUL exposure prior to OVA treatment did not significantly alter circulating IgE in a C57BL/6 mode of allergic asthma.

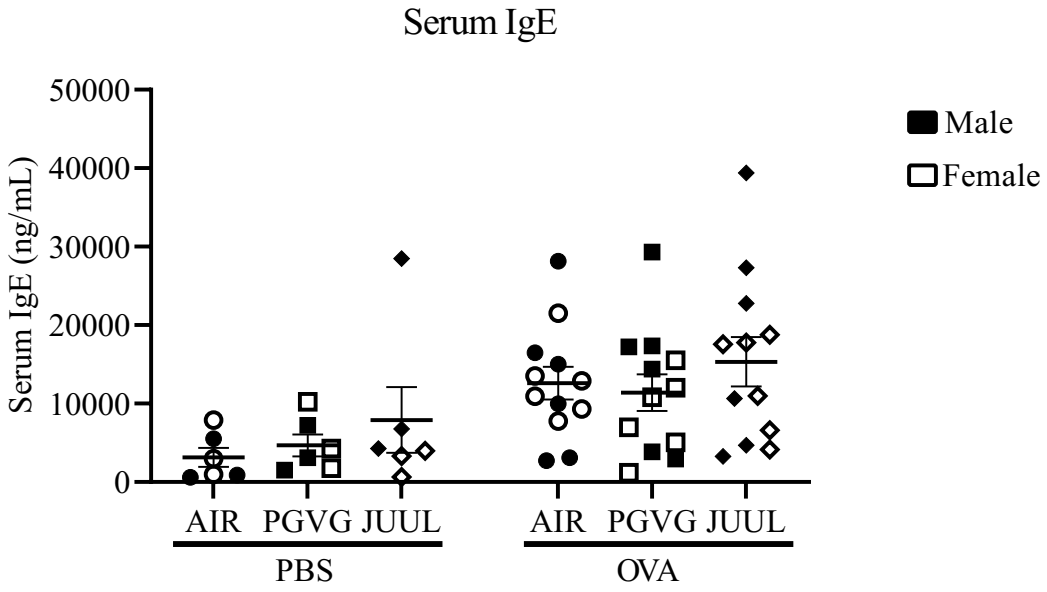


Figure 4.16. Serum IgE concentrations in OVA-treated mice previously exposed to JUUL. OVA-treated mice had higher serum IgE compared to PBS-treated mice, but there were no statistically significant changes associated with JUUL exposure. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by two-way ANOVA.

CHAPTER 5 DISCUSSION

When first introduced to the North American market, e-cigarettes were touted as safer alternatives to tobacco smoking, largely based on the absence of many combustion products found in cigarette smoke. However, e-cigarette aerosols contain a myriad of chemicals with known respiratory toxicity, and the safety of vaping is still under debate (212). The popularity of flavoured e-cigarettes such as JUUL among youth has raised many concerns that these products may not only contribute to nicotine addiction, but may also be associated with adverse pulmonary effects caused by long-term use, particularly in youth with pre-existing respiratory illness such as asthma (213). In absence of long-term public health data, experimental evidence is required to better understand the effects of vaping on respiratory health. Data from *in vitro* and *in vivo* experimental studies of e-cigarette toxicity provide some information about the pulmonary health effects of vaping, but much remains unknown. Overall, our results demonstrate that exposure to mint-flavored JUUL aerosols dysregulates the expression of key genes and proteins involved in the maintenance of lung health, including many of which are indicative of airway inflammation even without overt changes in the frequency of pulmonary immune cell populations.

The pulmonary system is the first to be exposed to inhaled chemicals and particles, such as those in e-cigarette aerosols. Upon exposure to such inhaled toxicants, the first line of defense is provided by the mucus barrier lining the airways, which traps inhaled substances and clears them from the lungs via mucociliary clearance (MCC). Herein, we showed for the first time that JUUL exposure significantly upregulates the expression of *Mucb5*, a major mucin glycoprotein required for MCC (214). Elevated mRNA expression of *Muc5b* suggests an increased production of mucin proteins, a potentially protective mechanism aimed at clearing the inhaled particulates present in

e-cigarette aerosols. These results are in line with those of others, including *in vitro* studies which have shown that e-cigarettes increased the expression of Muc5a in human bronchial epithelial cells (215, 216, 217). These findings have implications for lung health, as excessive mucin production can lead to mucus hypersection and thickening of the mucosal layer. This can subsequently result in impaired MCC, and be associated with airway obstruction and remodelling, all of which may increase susceptibility to respiratory infections, impair lung function, and contribute to the exacerbation of respiratory diseases such as asthma (218, 219). Therefore, increased *Muc5b* mRNA expression by JUUL exposure suggest that e-cigarette aerosols induce mucus hypersection with the potential to negatively impact lung health if used long term.

When inhaled toxicants are not successfully cleared by MCC, an important second line of defense in the lungs is provided by neutrophils. Upon exposure to agents that cause inflammation, neutrophils sequester from the pulmonary capillaries and enter the alveolar space (220). Neutrophils contribute to clearing inhaled particulates by secreting a variety of products during degranulation (221). Our quantitative proteomic analysis highlighted that one of the main biological processes affected by JUUL exposure in both strains of mice was neutrophil degranulation. In this regard, JUUL exposure significantly altered the expression of multiple proteins involved in neutrophil degranulation in a strain- and sex-dependent manner. For example, in the BAL fluid of male C57BL/6 mice, JUUL exposure increased BPI fold containing family A, member 1 (BPIFA1), a protein widely expressed in the respiratory epithelium that regulates neutrophil recruitment to the airways (222), and lactoferrin (Ltf), a protein with antimicrobial activity secreted from the secondary granules of neutrophils during degranulation. Other products of neutrophil degranulation are neutrophil serine proteases (NSPs), which include neutrophil

elastase (NE), myeloblastin (proteinase 3), and cathepsin G (223). Through proteolytic degradation of the surrounding lung tissue, NSPs contribute to host defence against infections and maintenance of lung tissue homeostasis. In health, the activity of serine proteases is tightly regulated by inhibition by serine protease inhibitors ('serpins'). However, under pathological conditions, an imbalance of protease-antiprotease activity results in excessive or deleterious proteolysis (223). We found that in BALB/c female mice, JUUL exposure decreased the expression of SerpinA1 and SerpinA3, two serpin proteins that inhibit the activity of NSPs (NE and Cathepsin G, respectively). The downregulation of these proteins by JUUL may be caused by the ROS present in the e-cigarette aerosol, as it is known that ROS in cigarette smoke inactivate SerpinA1 in smokers (224). Decreased serpin activity may have deleterious effects as it can result in unregulated activity of serine proteases, resulting in excessive degradation of the lung tissue. Indeed, decreased activity of SerpinA1 and SerpinA3 have been implicated in the development of emphysema/COPD, due to the unregulated proteolytic activity of NE (225).

In addition to neutrophils, resident and recruited macrophages contribute to innate immune defense in the lungs. E-cigarettes have been shown to affect several macrophage functions, for example by decreasing phagocytosis and increasing ROS and cytokine secretion (226, 227). One of the main cytokines secreted by macrophages is Tnfα, a potent inflammatory cytokine that amplifies inflammation by promoting the release of other pro-inflammatory cytokines and stimulating the expression of adhesion molecules that promote adhesion and recruitment of immune cells (228). Herein, we observed an increase in the lung mRNA expression of *Tnfα* in BALB/c mice only, which may be a result of macrophage activation. The notion that there is Tnfα production from e-cigarette-induced activation of macrophages is not without precedent, although there is conflicting

evidence about the magnitude of this effect. For example, flavoured e-liquids increased Tnfα production from activated THP-1 macrophages *in vitro* (229). In other studies, exposure of activated THP-1 macrophages to nicotine-containing e-cigarettes decreased Tnfα production in a dose-dependent manner (226, 230). From separate studies utilizing BALB/c mice, exposure to e-cigarettes significantly increased lung protein levels of Tnfα*,* irrespective of nicotine concentration (231, 232), indicating that our results are in line with published findings. However, the lack of *Tnfα* upregulation in JUUL-exposed C57BL/6 mice in our study is in fact in contrast with previous findings from our lab. We previously found that exposure of C57BL/6 mice to JUUL aerosols upregulated *Tnfα* only when mice were acutely exposed to a high dose (*i.e.,* 4 puffs/minute) whereas a low dose (*i.e.,* 1 puff/minute) did not induce transcriptional changes in the lungs (174). One possible reason for the discrepancy between our past findings and our present results is that the low-to-moderate dose of exposure in our model (*i.e.,* 2 puffs/minute) was also insufficient to induce transcriptional activation of *Tnfα* transcription in C57BL/6 mice.

Under conditions of chronic inflammation, additional immune protection is provided by adaptive immune mechanisms. Despite the important roles of adaptive immunity in providing host defense, little is known about the effects of e-cigarette exposure on lung tissue-resident lymphocytes, including B cells and $CD4^+$ and $CD8^+$ T cell subsets. In our results, there were strain-dependent differences in the frequency of lung tissue-resident B cells and $CD4^+$ T cells, but there were no differences in the frequency of these cells when mice were exposed to JUUL. Of note though, JUUL exposure decreased the frequency of $CD8^+$ T cells in C57BL/6 mice only. These results are in contrast with those reported in a previous study, where C57BL/6 mice exposed to a vanilla flavoured aerosol displayed an increased frequency of B cells and $CD4^+$ T cells, and no differences

in $CD8^+$ T cells (46). $CD8^+$ T cells are crucial for providing protection to intracellular pathogens such as respiratory viruses, and generating immunological memory for long-term protection (233). The lungs harbor tissue-resident memory (T_{RM}) $CD8^+$ lymphocytes that rapidly respond to invading pathogens, with the number of $CD8^+$ T_{RM} cells determining the efficacy of the immune response to influenza (234, 235). Thus, a decrease in $CD8^+$ T cells after JUUL exposure may impair immunological memory against viral pathogens. With fewer $CD8^+$ T cells in the lungs, e-cigarette users may be more susceptible to recurrent respiratory viral infections. Future studies aimed at investigating the effects of JUUL on susceptibility to the influenza infection are thus warranted.

While an acute inflammatory response is needed for protection against pathogens, inflammation that becomes chronic in nature is linked to a number of diseases including lung cancer and COPD. Mechanistically, in the presence of chronic inflammation, inflammatory mediators released by epithelial and immune cells activate fibroblasts, the main connective tissue cell in the pulmonary interstitium, and stimulate their differentiation into myofibroblasts. Both fibroblasts and myofibroblasts produce collagen fibers (*e.g.,* type III and type I) which provide structural integrity and normal mechanical properties of the lungs (236). In our results, JUUL exposure significantly upregulated *Col3a1*, the gene that encodes the alpha 1 chain of type III collagen. This is a novel finding, as little is currently known about the effects of e-cigarettes on collagen synthesis. Increased *Col3a1* suggests activation of fibroblasts and subsequent production of collagen type III fibers. Importantly, although collagen production under homeostatic control provides essential tissue repair, excessive or dysregulated accumulation of collagen in chronically inflamed airways results in tissue remodelling and stiffening of the airways (236). In asthma, deposition of type III

collagen is well established, and is known to be associated with disease severity (237). Therefore, increased *Col3a1* mRNA expression by JUUL exposure suggest that e-cigarette aerosols may result in abnormal airway collagen deposition.

In the first part of our study, we showed that JUUL exposure affects multiple mechanisms that contribute to the pathogenesis of asthma, including changes in the functions of innate immune cells and airway remodelling. Our results thus contribute to the growing evidence that vaping may make individuals more susceptible to asthma triggers (227, 238). To next investigate the effect of JUUL in the context of allergic airway inflammation, we combined a sub-chronic e-cigarette exposure that was designed to mimic youth vaping patterns (*i.e.,* use of young mice that were exposed sub-chronically to a commercially available e-cigarette) with an OVA-induced asthma model. To validate our model, we assessed BAL fluid eosinophilia, which is a characteristic feature of allergic airway inflammation in murine asthma models. Although there was a significant increase in lung tissue eosinophils in OVA-challenged mice, there was a non-significant trend of increased BAL eosinophils. These results are in contrast with those reported by previous studies in which OVA induced a significant increase in BAL eosinophils in C57BL/6 mice (165, 239). A potential explanation for the lack of significant eosinophilia in the BAL of air-exposed C57BL/6 mice is that we collected the BAL fluid 72 hours after the last OVA challenge. Thus, we may have missed the time-frame of peak eosinophil induction, as the half-life of circulating eosinophils is 8-18 hours (240). Indeed, one study showed that the level of BAL eosinophils induced by OVA challenge in C57BL/6 mice returned to baseline after a period of recovery (241). We also found no significant effects of JUUL exposure on lung tissue mRNA levels of *Il4* or *Il5*, and JUUL did not affect circulating concentrations of serum IgE. Overall, the lack of change suggests that a

preceding JUUL exposure does not further boost the Th2 immune response during a window of susceptibility. Our results can help guide future experimental design aimed at better understanding the impact of vaping in asthmatic youth. There is need for a future experiment to investigate the pulmonary immunologic effects of mice that are first sensitized to OVA and subsequently challenged while simultaneously being exposed to e-cigarette aerosols; this experimental design would represent vaping in individuals already susceptible to allergic airway inflammation (*e.g.,* adults with a prior diagnosis of allergic asthma during childhood). Our data also support a role for e-cigarette-induced dysregulation of neutrophil degranulation, which could have important implications for neutrophilic asthma, an asthmatic phenotype that can be caused by certain types of occupational or recreational exposures (*e.g.,* chlorinated swimming pools). Thus, future research should examine the effects of e-cigarettes in mouse models of neutrophilic asthma.

In summary, we are the first to characterize the pulmonary immune response of BALB/c and C57BL/6 mice to a sub-chronic flavoured e-cigarette aerosol using JUUL, a popular brand of e-cigarette used by youth and young adults. We also showed that a prior e-cigarette exposure does not aggravate inflammation in an OVA-induced model of allergic asthma. The use of e-cigarettes is increasing worldwide, particularly among youth, and there is an urgent need for rigorous preclinical studies to evaluate the safety or harms of e-cigarettes. Increased research efforts are needed to match the evolving landscape of e-cigarettes, and to understand how vaping may affect the pathophysiology of respiratory diseases, such as asthma. This important research may provide critical knowledge to inform public health policies aimed at regulating the ENDS market and reducing youth vaping.

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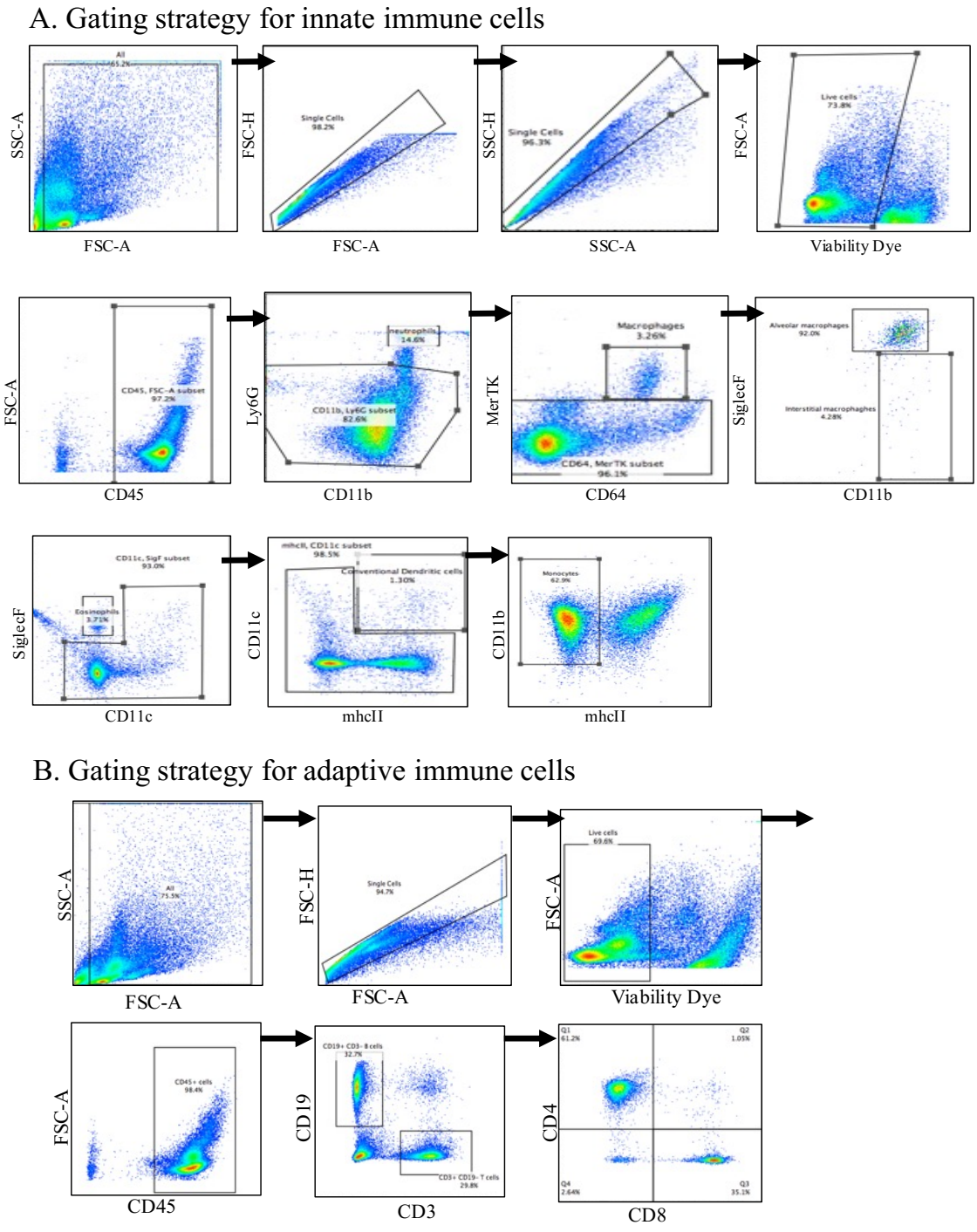
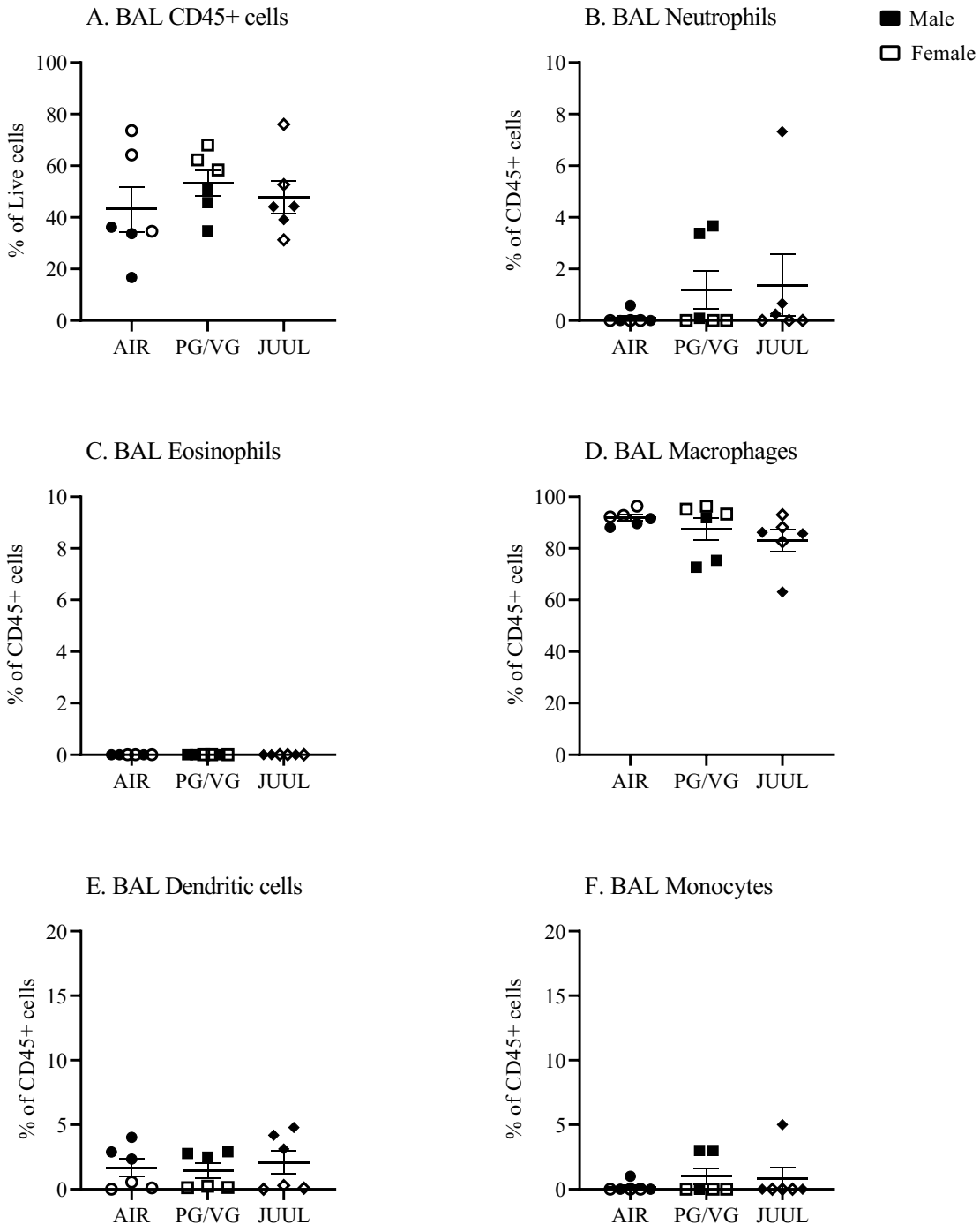
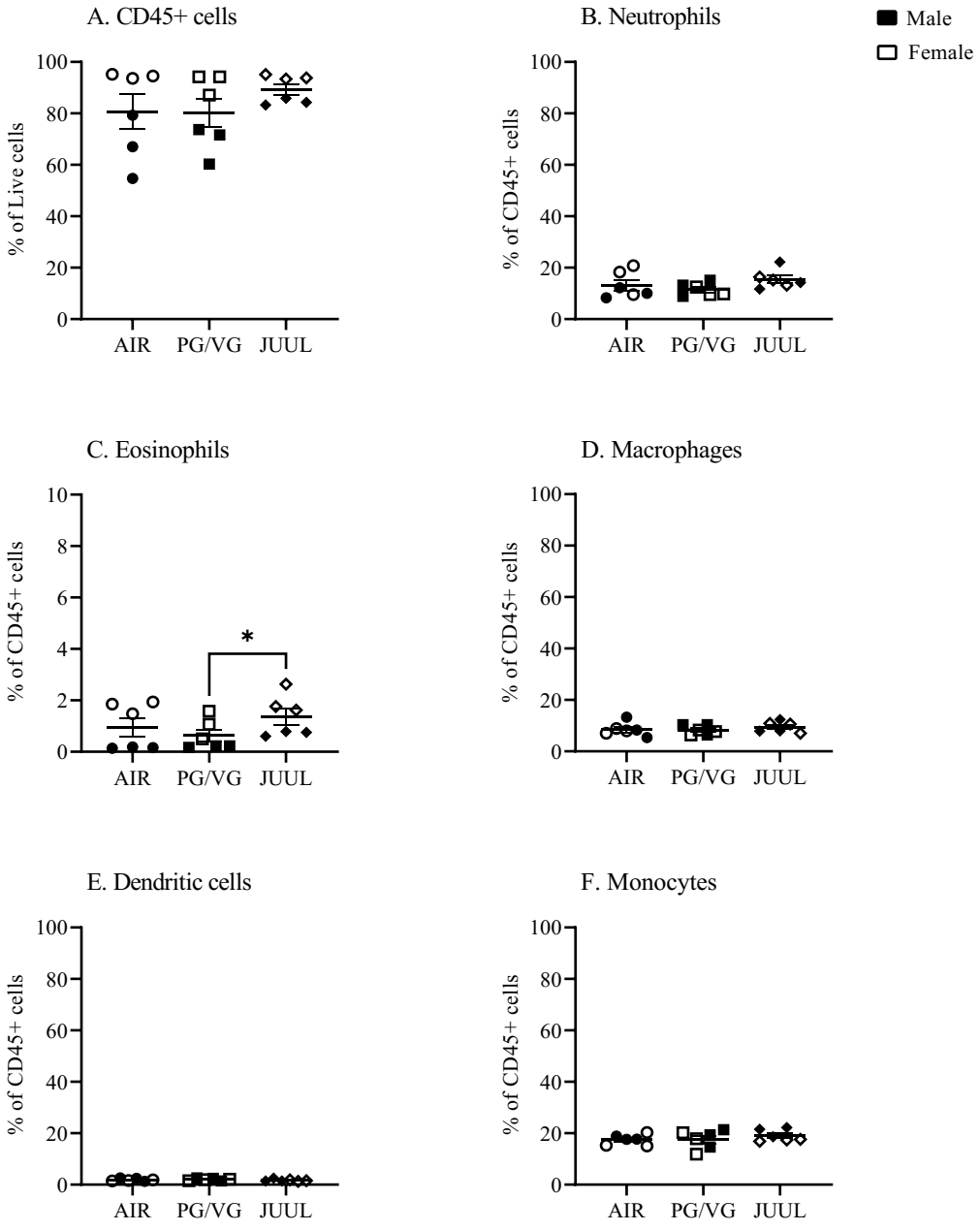
APPENDIX



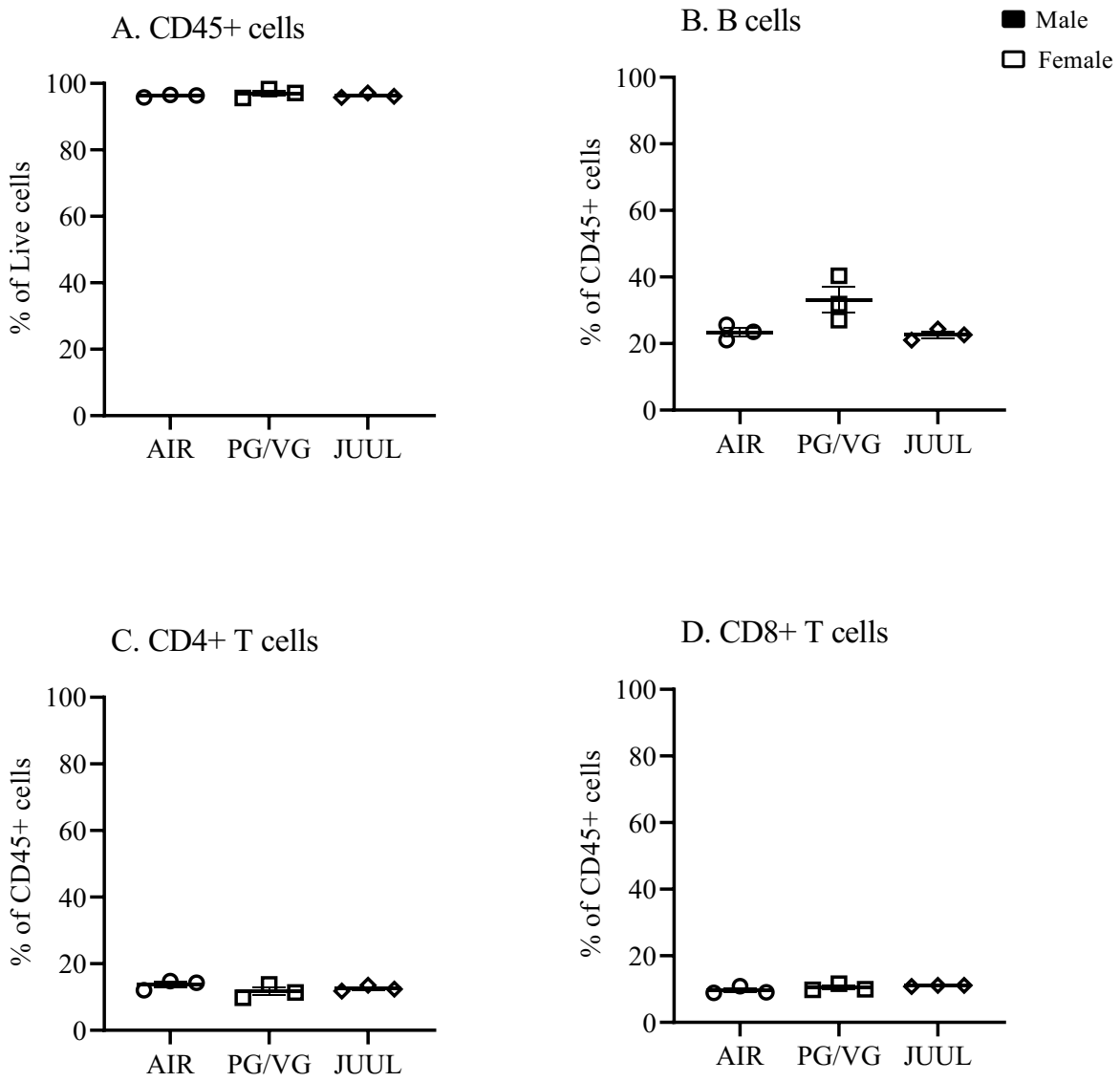
Supplementary Figure S1. Gating strategy for the identification of immune cells in BAL fluid and lung tissue. The gating strategies for innate immune cells (A) and adaptive immune cells (B) are shown.



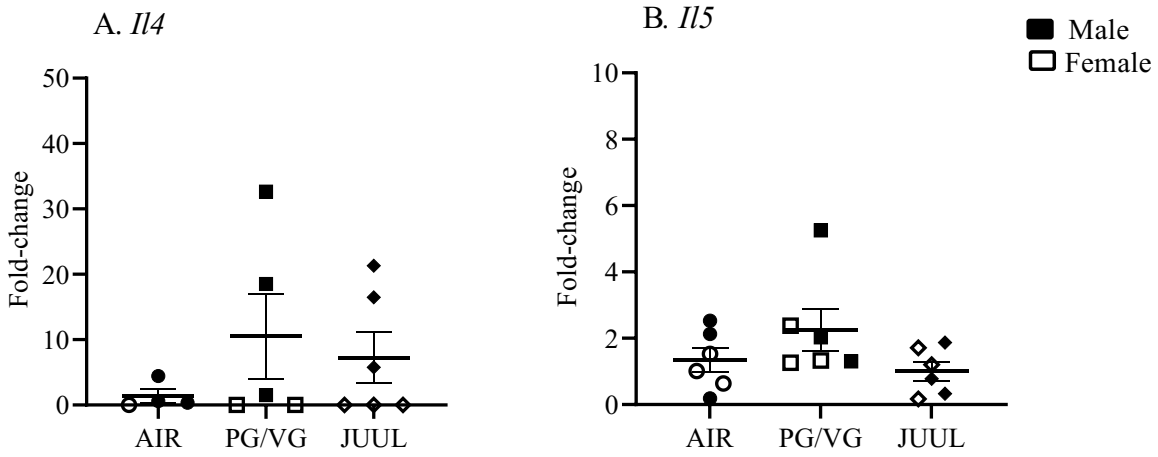
Supplementary Figure S2. Innate immune cells in the BAL fluid immediately after a 14-day JUUL aerosol exposure. The frequency of total BAL immune cells (A), neutrophils (B), eosinophils (C), macrophages (D), dendritic cells (E), and monocytes (F) are shown. There were no statistically significant changes in any immune cell population. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by one-way ANOVA.



Supplementary Figure S3. Innate immune cells in the lung tissue immediately after a 14-day JUUL aerosol exposure. The frequency of total immune cells (A), neutrophils (B), eosinophils (C), macrophages (D), dendritic cells (E), and monocytes (F) are shown. Eosinophils were significantly increased in JUUL-exposed mice, compared to PG/VG. There were no other statistically significant changes in any immune cell population. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by one-way ANOVA (* $p \leq 0.05$)



Supplementary Figure S4. Adaptive immune cells in the lung tissue immediately after a 14-day JUUL aerosol exposure. There were no differences between total immune cells (A), $CD19^+$ B cells (B), $CD4^+$ T cells (C), and $CD8^+$ T cells (D). Data are expressed as mean ± SEM. Differences were analyzed by one-way ANOVA.



Supplementary Figure S5. Gene transcription of Th2 cytokines in the lung tissue immediately after a 14-day JUUL aerosol exposure. There were no differences between exposure groups in the expression of *Il4* (A) or *Il5* (B). Gene expression was normalized to 18s rRNA. Data represent pooled samples from two independent experiments. Data are expressed as mean ± SEM. Differences were analyzed by one-way ANOVA.