Exploring the Effects of Electronic Cigarette Vape on Pulmonary Immune Cells

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Table of Contents

Abstract

Over the last decade, electronic cigarettes (e-cigarettes) have become the most widely used nicotine delivery device among adolescents. Currently, there are hundreds of vaping devices on the market, and a wide variety of metal atomizers, wicking materials, and flavors being used. Though the ingredients in e-cigarette liquid are considered safe for oral consumption, it is not yet clear how their aerosolized form is tolerated by immune cells in the lungs. Initially introduced as a smoking cessation aid, e-cigarettes have now been linked to lung injury, with over 2,807 hospitalizations, and 68 deaths in the US alone. The recent outbreak of e-cigarette use-associated lung injury (EVALI) has prompted further investigations into the effects of vaping; the results of which have found links to alveolar macrophage (AM) dysfunction, neutrophil impairment and systemic inflammation following e-cigarette use. Such findings are concerning, considering these tissue resident immune cells are the lung's first line of defense and are crucial for maintaining a healthy balance of pro- and anti-inflammatory cytokine signals. Though it has been shown that e-cigarette use disrupts this immune balance in lungs, the pathophysiology of EVALI remains unknown.

The specific aims of this project were (1) to develop a full-body e-cigarette vape exposure system in the laboratory, (2) to examine the effects of acute and chronic e-cigarette vape exposure on immune cell numbers in mice, and (3) to evaluate any differences in male versus female response to e-cigarette vape exposure. Mice were exposed to either full vape (50PG:50VG, 20 mg/ml nicotine, and Razzy3some flavour) or air for 3 days (acute) or 16 days (chronic). Following the final exposure, mice were euthanized and bronchoalveolar lavage (BAL) fluid, lung tissue, and blood were collected for flow cytometry and lipid analysis.

Contrary to our initial hypothesis, we found that vape exposure resulted in decreased innate and adaptive immune cell numbers in the lung tissue and blood, in both acute and chronic vape exposures; however, no changes were seen in immune cell numbers in the BAL. The measured serum cotinine levels indicated that mice were actively inhaling aerosolized vape in our full-body exposure system, and AMs from mice exposed to vape showed excess lipid accumulation compared to air controls after just 3 days of exposure. Interestingly, we did not observe a difference in immune cell numbers, cotinine concentrations, or lipid accumulation when comparing male vs. female vaped mice. Ultimately, our results provide new insight into the effects of vaping on pulmonary immunity and highlight the many variables involved in vape-related lung injuries. Future work will continue to tease apart the effects of e-liquid constituents and coil type to better understand their roles in both innate and adaptive immunity, with the goal of uncovering the mechanisms involved in the progression of EVALI.

Résumé

Durant la dernière décennie, les cigarettes électroniques sont devenues les appareils de consommation de nicotine les plus utilisés auprès des adolescents. Actuellement, des centaines de produits de vapotage sont sur le marché, incluant une grande variété d'atomiseurs de métal, de matériaux pour les mèches et de saveurs. Bien que les ingrédients utilisés dans les cigarettes électroniques soient considérés comme sécuritaires pour la consommation, il n'est pas encore clair si leur forme aérosolisée est tolérée par les cellules immunitaires des poumons. Alors qu'elles avaient été introduites comme étant des produits de cessation tabagique, les cigarettes électroniques sont maintenant reliées à des lésions pulmonaires avec plus de 2 807 hospitalisations et 68 morts aux États-Unis seulement. La récente éclosion de pneumopathie associée au vapotage (PAV) a entraîné d'autres enquêtes plus approfondies sur les effets du vapotage. Les résultats ont trouvé des liens avec le dysfonctionnement des macrophages alvéolaires (MA), l'altération des neutrophiles, et l'inflammation systémique après l'utilisation des cigarettes électroniques. De telles découvertes sont préoccupantes, considérant que ces cellules immunitaires résidentes dans les poumons sont la première ligne de défense et sont cruciales pour maintenir un équilibre sain entre les signaux de cytokines pro et anti-inflammatoires. Bien qu'il ait été démontré que les cigarettes électroniques perturbent cet équilibre immunitaire dans les poumons, la physiopathologie des PAV demeure inconnue.

Les objectifs spécifiques de ce projet étaient (1) de développer un système d'exposition à la vapeur de cigarette électronique sur l'ensemble du corps, (2) d'examiner les effets de l'exposition aiguë et chronique à la vapeur de cigarette électronique sur le nombre de cellules immunitaires chez les souris, et (3) d'évaluer toute différence dans la réponse des hommes par rapport aux femmes à l'exposition à la vapeur de cigarette électronique. Les souris ont été exposées soit à une

dose complète de vapeur de cigarette électronique (50PG :50VG, 20 mg/ml de nicotine avec la saveur Razzy3some), soit à de l'air, pendant 3 jours (exposition aiguë) ou 16 jours (exposition chronique). Après l'exposition finale, les souris ont été euthanasiées et le liquide de lavage broncho-alvéolaire (LBA), le tissu pulmonaire, et le sang ont été prélevés pour la cytométrie en flux et l'analyse des lipides.

Contrairement à notre hypothèse initiale, nous avons constaté que l'exposition à la vapeur de cigarette électronique entraînait une diminution du nombre de cellules immunitaires innées et adaptatives dans les tissus pulmonaires et le sang lors d'expositions aiguës ou chroniques à la vapeur de cigarette électronique. Cependant, aucun changement n'a été observé dans le nombre de cellules immunitaires dans le LBA. Les niveaux de cotinine sérique mesurés ont indiqué que les souris inhalaient activement la vapeur en aérosol à l'intérieur de notre système d'exposition sur l'ensemble du corps. Ainsi, les MA des souris exposées à la vapeur ont démontré une accumulation excessive de lipides par rapport aux souris exposées à l'air après seulement 3 jours d'exposition. Chose intéressante, nous n'avons pas observé de différence dans le nombre de cellules immunitaires, les concentrations de cotinine ou dans l'accumulation de lipides lors de la comparaison entre les souris mâles et femelles exposées. Finalement, nos résultats fournissent de nouvelles informations sur les effets du vapotage sur l'immunité pulmonaire et mettent en évidence les nombreuses variables impliquées dans les lésions pulmonaires liées au vapotage. Les recherches futures continueront de démêler les effets des constituants du e-liquide et du type d'atomiseur pour mieux comprendre leurs rôles dans l'immunité innée et adaptative, dans le but de découvrir les mécanismes impliqués dans la progression des PAV.

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Contribution of Authors

AT conceived the study. AT and JP designed the experiments, and JP performed the experiments with assistance from AK, MM, and FL during the vaping exposures. AK also assisted with cell isolation and flow cytometry experiments. JP and FL isolated alveolar macrophages for lipid staining, and FL provided the images. JP performed the data analysis and wrote the thesis, with help form AT.

List of Abbreviations

ACK	Ammonium-Chloride-Potassium
AM	Alveolar macrophage
ARDS	Acute respiratory distress syndrome
BAL	Bronchoalveolar lavage
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
COPD	Chronic obstructive pulmonary disease
DC	Dendritic cell
DMEM	Dulbecco's modified eagle medium
E-cigarette	Electronic cigarette
E-liquid	Electronic cigarette liquid
ELISA	Enzyme-linked immunosorbent assay
EVALI	E-cigarette or vaping product use-associated lung injury
FMO	Fluorescence minus one
GM-CSF	Granulocyte macrophage-colony stimulating factor
GMP	Granulocyte-monocyte progenitor
IL	Interleukin
KC	Keratinocytes-derived chemokine
LLM	Lipid-laden macrophages
MCP-1	Monocyte chemoattractant protein-1
NET	Neutrophil extracellular trap
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PG	Propylene glycol
PMA	Phorbol 12-myristate 13-acetate
PRR	Pattern recognition receptor

TGF-β	Transforming growth factor-beta
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-alpha
VG	Vegetable glycerin

Chapter 1: Introduction

1.1 Overview of innate immunity in the lungs

Our lungs are an important barrier tissue which come in contact with roughly 11,500 L of air each day [1]. With each breath, we inhale a plethora of small particles such as dust, smoke, and pollen, in addition to potentially harmful pathogens like bacteria, fungi, and viruses. While large particulate will often become trapped in the upper airways and expelled, small particles (≤ 1 μm) can migrate down the lower respiratory tract, all the way to the alveoli, where gas exchange occurs [2]. As such, we have evolved a system of physical barriers, cells, and chemical signals, to protect the lungs from harmful pathogens while tolerating self and inert particulate. The balance between tolerance and immunity in the lungs is especially important given the delicate structures of the alveoli, where unnecessary tissue damage could be lethal.

In terms of cellular response, the lung's first line of defense against harmful pathogens is carried out by tissue-resident, innate immune cells. Innate immunity plays a crucial role in providing an initial barrier to invaders, while simultaneously signaling the recruitment of additional circulating immune cells [1]–[4]. The success of innate immunity relies to a great extent on the expression of pattern recognition receptors (PRRs) on immune cells, such as Toll-like receptors (TLRs), which enable the rapid detection of pathogen-associated molecular patterns (PAMPs) expressed by microbes [5], [6]. PAMPs are ideal targets for PRRs, as they are constitutively expressed and conserved across microbes; therefore, any mutations in PAMPs will likely lead to cell death or greatly reduced function [5]. In addition to the antimicrobial roles of innate immunity, certain cell types are also required for the resolution of inflammation and tissue repair [7]–[9]. Innate immunity is dynamic and involves many different cell populations with

varying functions; however, this project will be focussed on the main effector cells in the lungs: alveolar macrophages (AMs), neutrophils, eosinophils, dendritic cells (DCs), and monocytes.

The predominant immune cells in the lungs of healthy humans and mice are AMs [10]. Distinct from other populations of bone-marrow derived macrophages, AMs are a group of long-lived, self-renewing, phagocytotic cells that patrol the airways of the lungs [11]–[13]. At steady state, these cells play an important role in immunosuppression, by clearing cellular debris and maintaining a proper balance of pro- and anti-inflammatory cytokine secretions. AMs are also important for the maintenance of pulmonary surfactant, a lipid- and protein-rich substance lining the airways, which is essential for proper gas exchange and microbial opsonization [14]. While AMs are generally immunosuppressive, once an invading pathogen or foreign substance is detected through PRRs, AMs shift to a pro-inflammatory phenotype [15]. In this activated state, AMs increase their production of pro-inflammatory cytokines and chemokines (i.e. IL-1, IL-6, IL-12, IL-23, and TNF-α) [16], which ultimately increases their phagocytotic ability and leads to the activation and recruitment of more innate and adaptive immune cells. Importantly, AMs are also crucial for returning the lungs back to steady state following inflammation by clearing short-lived apoptotic cells before they become necrotic, while simultaneously reducing the production of pro-inflammatory cytokines, and increasing the release of anti-inflammatory IL-10 and TGF-β [7], [17]. Given these two opposing roles—i.e., immune activation vs. immunosuppression—AMs require immense plasticity in their phenotype, allowing them to initiate an inflammatory response while minimizing collateral damage to the lungs.

The first cells to respond to chemical signaling and migrate into airways during inflammation are polymorphonuclear leukocytes, such as neutrophils and eosinophils. The rapid recruitment of neutrophils and eosinophils is thought to be due to their strategic positioning

within the lung's vasculature [18]–[20]. These granulocytic cells develop from common myeloid progenitor (CMP) cells, which further differentiate into granulocyte-monocyte progenitor (GMP) cells in the bone marrow. Once they leave the bone marrow, they patrol the vasculature until they are recruited to the airways during infection or undergo programmed cell death. While circulating neutrophils are generally thought to have a short lifespan, they have also been shown to delay apoptosis in response to certain cytokines (e.g. GM-CSF) allowing them to carry out important effector functions during inflammation [7], [21], [22]. Once recruited to the lungs, activated neutrophils have 3 main strategies for killing microbes: 1) phagocytosis; 2) degranulation; and 3) neutrophil extracellular trap (NET) formation [23]. Eosinophils respond in a similar manner to parasites and allergens by secreting cytotoxic granule proteins upon stimulation, while also serving as an important link between innate and adaptive immunity through antigen presentation [9]. Although these strategies are effective in fighting off pathogens, excessive or prolonged neutrophil and eosinophil recruitment can result in tissue damage. That said, similar to AMs, both neutrophils and eosinophils play an important role in inflammatory resolution by releasing anti-inflammatory cytokines and growth factors, to help with tissue remodeling [8], [9]. As such, there is a delicate balance of these cells throughout the body that needs to be maintained to provide adequate protection without causing excessive damage.

In addition to eosinophils, DCs are key antigen-presenting cells that bridge the gap between innate and adaptive immunity. Within the lungs, DCs sample antigens in the alveolar space, and carry them to secondary lymphoid organs where they can interact with naïve T lymphocytes to elicit a more robust and specific immune response [1], [24]. DCs found in the lung are not considered tissue-resident in the strictest sense, because they are replenished from bone marrow

rather than self-replicating, however, pulmonary DCs appear to live longer compared to other non-lymphoid tissues [1]. In addition, like the innate immune cells already mentioned, DCs have multiple phenotypic subsets. These subsets carry out distinct and overlapping roles and can be identified by immunophenotyping their cell surface molecules. For example, $CD103^+$ DCs sample antigens from the alveolar space, and limit allergic inflammation by secreting IL-12, whereas $CD11b^+$ DCs have been shown to play an important role in transporting antigens to lymph nodes, where they interact with T cells [1]. In the context of lung infection, $CD103^+$ DCs are crucial for activating cytotoxic $CD8^+$ T cells, whereas $CD11b^+$ DCs are responsible for generating longer-lived memory $CD8^+$ T cells [25]. Unlike granulocytic cells—i.e., neutrophils and eosinophils—DCs do not release cytotoxic proteins to destroy pathogens, but instead, act as important messengers between the two arms of immunity. As such, changes to their function may not lead to direct tissue damage, but could result in impaired immune response leading to autoimmune, infectious, or allergic diseases [26].

Finally, monocytes are another important bone-marrow-derived cell type which circulate through blood and are recruited to the lungs during inflammation. Again, these cells originate from GMP cells, which further differentiate into mature monocytes in the blood, and finally, macrophages or DCs once they reach infected tissues [24]. In the absence of infection and inflammation, monocytes remain undifferentiated within the lungs, where they take on a homeostatic role and patrol the tissue for antigens [27], [28]. Monocytes generally make up about 5-10% of white blood cells, however the rate of hematopoiesis will change depending on environmental stimuli [24]. For example, during inflammation, more monocytes are recruited to the lungs by AMs, which produce Monocyte Chemoattractant Protein-1 (MCP-1) [2]. Beyond their phagocytotic and antigen presenting roles, monocytes also have an influence on the

adaptive immune system, by establishing tissue-resident memory T-cell populations in the lungs [29] and are important for tissue repair [28].

1.2 Overview of adaptive immunity in the lungs

Working in conjunction with the innate immune response, the lung's second line of defense comes from acquired, or adaptive, immunity. Adaptive immunity is generally delayed, but more efficient at targeting specific pathogens compared to the innate immune response; most notably, upon re-infection. T and B cells originate in the bone marrow from a common lymphoid progenitor (CLP), and then mature in either the thymus (T cells) or bone marrow (B cells) [24]. Once mature, lymphocytes are said to be naïve until they encounter an antigen; at which point they proliferate and differentiate into effector and memory cell subsets [30], [31]. Within the lungs, tissue-resident lymphocytes play an important role in host defense, where they remain suppressed until they encounter an antigen presenting cell [1].

1.3 Lung injury

As outlined above, vertebrates have evolved remarkably efficient and dynamic cellular mechanisms to protect the lungs, as this organ is in constant contact with the outside world. Immune response and inflammation are necessary, protective strategies; however, their dysregulation can lead to severe tissue damage. For example, acute respiratory distress syndrome (ARDS) is a type of serious respiratory failure marked by the rapid influx of immune cells to the lungs causing inflammation, and leading to damaged airway epithelium, increased lung permeability and pulmonary edema [32]. Importantly, it is becoming increasingly clear that activated neutrophils play a central role in provoking the epithelial damage, as they have been shown to release proteases and NETs, resulting in oxidative stress and tissue damage [33]. Considering these circumstances, one might want to reduce the number of neutrophils to limit

tissue damage, but both animal and human studies have shown that neutropenia results in poor pathological outcomes as well [33], likely due to their role in repair and tissue remodeling [8]. Similarly, SARS-CoV-2 results in a large influx of immune cells into the airways, leading to increased production of pro-inflammatory signals, which act as positive feedback and further enhance inflammation [33]. Normally, inflammation is resolved when phagocytotic cells of the innate immune system engulf apoptotic effector cells; a mechanism termed "efferocytosis" [34]. However, if efferocytosis is disrupted, the apoptotic cells will become necrotic, releasing their pro-inflammatory cell contents, and potentially re-releasing pathogens to the surrounding tissue [34], [35]. This type of malfunction can be seen in individuals who smoke and suffer from chronic obstructive pulmonary disease (COPD), as cigarette smoke has been shown to impair efferocytosis in AMs [36]. Thus, lung injury can result from excessive immune cell infiltration in response to both infectious and sterile irritants, leading to chronic inflammation [33], [37]. Of note, in recent years, this type of acute inflammation and lung injury has also been attributed to the use of electronic cigarettes (e-cigarettes) [38], [39].

1.4 Overview of electronic cigarettes

E-cigarettes were first introduced to the north American market in 2007, and have now become the most widely used nicotine delivery device among youth [40]. Initially, e-cigarettes were marketed as a healthier alternative to conventional cigarettes, and were meant to aid with smoking cessation; however, there is little evidence to support their efficacy as such, and worse, there is evidence to suggest they are introducing nicotine to individuals who otherwise would not have started smoking [38], [41]–[43]. In recent years, there has been a significant increase in the use of e-cigarettes among adolescents (<18 years old) and young adults (18-24 years old). According to the American Lung Association, between 2011 and 2019 there was a 1733%

increase in high school students who used e-cigarettes, with close to 2.9 million children who started using e-cigarettes in 2019 alone [44]. One major reason for the popularity of e-cigarettes over conventional cigarettes is the abundance of flavors to chose from. A systematic study in 2018 showed that flavor is the most important factor in initiating e-cigarette use, and that fruit-flavored e-cigarettes are more popular, and are perceived to be less harmful than tobacco-flavored e-cigarettes among adolescents and young adults [43]. In addition to the flavoring, e-cigarette liquid (e-liquid) generally contains a vehicle solvent made of propylene glycol (PG) and vegetable glycerine (VG), as well as varying levels of nicotine. Unfortunately, there is still a lot we do not know in terms of e-cigarette use and its effect on long-term health in humans. This issue is especially concerning considering the growth in vape use among youth, and the ever-evolving landscape of e-cigarette devices and products being used.

There have been several iterations of e-cigarettes on market since their debut in the mid 2000's, starting with the first generation, pre-filled, disposable cig-a-like e-cigarettes. Currently, the most popular devices are the 3rd generation tanks or mods, which are highly customizable and have a refillable tank, and the 4th generation pod-mod devices which are more discrete and use a disposable cartridge [45]. Regardless of the type of device, most e-cigarettes work in a similar manner. Each device uses e-liquid to saturate a wick, which is surrounded by a metal coil. The user heats up the coil with a rechargeable battery and inhales the resulting vaporized e-liquid. While the general principle is the same, there is notable variability between devices due to the different components, which could lead to variable levels of toxicity in the lungs [46]. For example, the wick within the atomizer is made of cotton or silica, and the metal coil can be made of a variety of different metals or alloys such as nickel, chromium, iron, aluminum, or titanium. Unfortunately, these metals have been previously shown to be present in e-cigarette vapor [47]

and have been associated with lung injury, respiratory disease, immunosuppression, and cancer [48]. That said, many studies have not been transparent with the materials used in their wick or atomizer element, making it difficult to know what effects they may have on experimental outcomes.

Newer generations of e-cigarettes may also use batteries with variable voltage and wattage, which the user can manipulate to modify the coil heat and vapor flow [46]. Perhaps not surprisingly, higher coil temperatures and more intensive puffing have been shown to produce more toxic compounds, such as formaldehyde and acetaldehyde [49], [50]. In addition, whether the coil is new or used can impact the level of reactive oxygen species generated during vaping [51], and more frequent coil replacement has been associated with higher nickel concentrations in the urine of e-cigarette users [52]. Thus, depending on the components of the e-cigarette device, and the temperature used to vape, users may be unintentionally exposing themselves to harmful heavy metals and carbonyl compounds in addition to the more obvious aerosolized constituents of the e-liquid, such as nicotine and flavoring.

Currently, there are over 8,000 different e-liquid flavors on the market, many of which use ingredients that are considered safe for oral consumption, but have not been adequately safety tested as inhalants [53], [54]. Along with the immense variety of flavors, research is further complicated by the lack of consistency across products. This issue was illustrated in 2019 by a group in Ontario who compared 5 cherry-flavored products from different manufacturers, and found that some products had as little as 2 flavoring chemicals, while others contained as many as 8 [54]. Building off that, another group in 2020 looked at the chemical profile of 10 flavors of e-liquid, using spectrometry, and then assessed the effects of the flavored vape on macrophage phagocytosis, efferocytosis and cytokine production [55]. Interestingly, they found that flavor

negatively impacted macrophage phagocytotic and efferocytotic capacity, with the most complex flavors—i.e., those made with the most flavoring chemicals—having the greatest effect [55]. It has also been shown that certain flavors of vape can induce IL-8 secretion (a cytokine that is released from lung cells in response to conventional cigarette smoke, and functions as a leukocyte chemoattractant), and that flavors containing sweet or fruit flavors were stronger oxidizers than tobacco flavors [51]. Unfortunately, these harmful flavors are also the most popular among youth and young adults [43], thus could be causing additional damage to the user while simultaneously exposing them to addictive substances such as nicotine.

Nicotine is found in most e-liquids and is a highly addictive, central nervous system stimulant. The concentrations of nicotine can vary widely among e-liquids, however most commercially available, premixed e-liquids range between 16-20 mg/ml. Although the sale of nicotine products is supposed to be tightly regulated, a recent review article found that mislabelled nicotine concentrations of up to 20% were not uncommon, and even some "nicotine-free" e-liquids contained significant amounts of nicotine [56]. Aside from the addictive properties, nicotine has been shown to reduce efferocytosis in AMs [41] and cause endothelial dysfunction in the lungs [57]. Unfortunately, e-cigarettes have opened the door to a new generation of nicotine addiction [43], [56], with consequences that may not be realized for many years.

Finally, propylene glycol (PG) and vegetable glycerin (VG) are humectants used in e-liquid as the principal base solvent. Both PG and VG are generally recognized as safe by the U.S. Food and Drug Administration as food additives; however, there are no controlled, long-term studies in humans to examine their effects on the lungs as heated aerosol [48]. To date, research looking at the effects of PG and VG on lung health have been conflicting. Earlier *in vitro* studies have

shown that exposure to vaporized PG/VG alone led to decreased antimicrobial functions in AMs and neutrophils [58] and reduced NET production in PMA-treated neutrophils [59]. In addition, studies in mice have found that PG/VG vehicle solvent was responsible for increased DC, CD4+ T cell, and B cell numbers in the lungs [60]. However, a newly published paper has now shown that PG/VG reduces IL-8 secretion from epithelial cells and macrophages, and inhibits priming and activation of macrophages in the presence of classical activators [61]. Interestingly, the same group also noted that heating/vaporizing the e-liquid resulted in the immunosuppression, and suggest that it could also be linked to the atomizer and cotton wick used [61]. Depending on the ratio of PG to VG, e-cigarette users can customize the sensation, flavor, and appearance of the aerosol, to fit their personal preference. For example, a higher PG ratio delivers a stronger hit with more flavor, whereas a higher VG ratio results in a milder hit but produces a bigger plume of vapor [62]. The uncertainty on the effects of these components combined with their use in varying ratios warrants further investigation and could provide useful information to improve the safety of vaping.

1.5 E-cigarette use or vaping-related lung injuries

Coinciding with the rapid increase in e-cigarette use among adolescents and young adults, in 2019, there was an outbreak of individuals who presented with E-cigarette or Vaping product use-Associated Lung Injury (EVALI) [63]. EVALI manifests as acute lung injury resulting from the rapid influx of leukocytes into the airways [64]. Patients suffering from EVALI have respiratory and gastrointestinal symptoms that tend to improve upon cessation of e-cigarette use, although it has been hypothesized that EVALI can lead to long-term fibrotic changes to the lung tissue [64]. As of 2020, there have been 2,807 hospitalized cases of EVALI, and 68 deaths in the USA alone [65]. This acute, pulmonary syndrome is seen primarily in young (median age: 24

years old), otherwise healthy male e-cigarette users [53], [64], [66], and has been linked to the use of counterfeit/homemade e-liquids containing tetrahydrocannabinol and vitamin E acetate [67], [68]. Vitamin E acetate has since been banned from vaping products; however, there are likely other variables contributing to vape-related lung injury. For example, a recent study in mice showed that EVALI-like symptoms can be induced without the use of vitamin E products, by simply changing the type of atomizer used in the e-cigarette device [69]. Lung injuries associated to vaping have further been linked to lipid-laden macrophages (LLM) or "foam cells" in the airways [14], [39], [70]. Since EVALI is diagnosed by exclusion, it was previously thought that LLM in the bronchoalveolar lavage (BAL) fluid of patients could be used to test for EVALI; however, more recently it has been shown that LLM are not an adequate biomarker for the disease, but rather represent an array of lung injuries [46], [71], [72]. In an effort to understand EVALI, researchers have used both *in vitro* and *in vivo* studies to show that vaping reduces antimicrobial functions of innate immune cells [58], increases inflammation both in the lungs and systemically [73], and leads to cytotoxicity of both immune and pulmonary epithelial cells [58], [74], [75]; however, the pathophysiology of EVALI, and the substances responsible for lung injury are still not known [46].

1.6 Rationale & aims

Given the novelty of e-cigarettes, there are still many unanswered questions concerning their effects on pulmonary health. Part of this uncertainty stems from the immense variability in e-cigarette devices, e-liquid contents, and vaping protocols used, making it difficult to draw firm conclusions from currently available studies. Additionally, most e-cigarette studies to date have used either male or female mice, with relatively few comparing the two sexes. To gain more insight into the effects of vaping, the aims of this project are: 1) To develop a full-body e-

cigarette vape exposure system in the laboratory; 2) To examine the effects of acute and chronic e-cigarette vape exposure on immune cell numbers in mice; and 3) To explore differences in male versus female response to e-cigarette vape exposure.

Although vaping is generally thought to be a healthier alternative to conventional cigarette smoking, there is a growing consensus that e-cigarettes are also contributing to lung injury; however, the mechanisms and constituents involved remain unknown. Given the current literature, we hypothesize that e-cigarette vape is altering the immunological landscape in the lungs, leading to increased immune cell infiltration, ultimately resulting in tissue damage.

Chapter 2: Materials & Methods

2.1 Mice

Six- to eight-week-old male and female C57BL/6 mice were purchased from Jackson Laboratories and bred in-house. All mice were housed in a sterile animal facility at the Goodman Cancer Centre at McGill University, on a 12-hour light:12-hour dark cycle, in a temperature- and humidity-controlled room. Mice were fed autoclaved rodent feed and water *ad libitum*, except during vape exposures. Mice were age and gender matched for all experiments. All experimental protocols used were approved under the protocol number 2020-8184 by the McGill Faculty of Medicine Animal Care Committee in accordance with Canadian Council on Animal Care guidelines.

2.2 Scireq vaping system & exposure

Mice were randomly assigned to receive either room air or full-vape. The e-liquid used was berry flavour (Razzy3Some) with 70PG/30VG, and 20 mg/ml nicotine, purchased online from Vapours Canada Incorporated. For each exposure, mice were placed into the full-body, Scireq inExpose vaping system, which was attached to a Joytech eVic VTwo Mini e-cigarette (see supplemental **Figure S1**). We used a nickel atomizer and the temperature control mode (TEMP Ni) on the e-cigarette. The temperature control setting is recommended for this type of atomizer and ensures consistent puff quality from one puff to the next. The temperature was set at 245˚C, at 70 watts, with coil resistance of 0.15 ohm. Atomizers were changed after 20 hours of use and were always kept saturated with e-liquid to avoid a dry burn. The e-cigarette was triggered remotely via a the inExpose Base Unit and flexiWare software, which was programmed to deliver an airflow rate of 2 L/min. and an exposure of 2 puffs/min. with a volume of 78 ml per puff. Mice remained in the chamber for 90 minutes/day for either 3 days (acute) or 16 days

(chronic), consecutively. Before each session, both pumps were tested with a rotameter to ensure the system was air-tight and functioning properly.

The flow of vape through the system was as follows: The first pump drew the vape into a buffer chamber, where it mixed with normal air, before being delivered to the full-body exposure chamber; A second pump then pulled the vape/air mixture from the exposure chamber out through a filter chamber, and then out into the fume hood through an exhaust tube. After each exposure, mice were placed back into their home cages, and the vaping system was taken apart, cleaned/disinfected with Lucasol, and wiped dry.

2.3 Blood, BAL, lung tissue, and bone marrow collection

Directly after the final vape exposure, mice were euthanized with CO_2. Cardiac punctures were performed immediately following euthanasia. 200 µl of blood was added to an Eppendorf tube containing 100 µl of heparin (purchased from Sigma-Aldrich) and placed on ice until cell isolation. The remaining blood was placed into a Microtainer® Gel Serum Separator Tube and left at room temperature to coagulate for 1 hour. After 1 hour, the serum tubes were spun at 4000 rpm for 10 minutes at 4˚C. Serum fractions were collected into an Eppendorf tube and frozen at -80˚C until the ELISA was run at a later date.

Next, the trachea of the mice was exposed, and a small incision was made. A 20G catheter attached to a 1 ml syringe was gently inserted into the trachea and secured with forceps. Once secured, 1 ml of 1X PBS (prepared in the lab, see supplemental **Table S1** for protocol) (4˚C) was slowly infused into the lungs and gently removed. This was repeated a total of 4 times. The recovered liquid (BAL) was placed into an Eppendorf tube and kept on ice until cell isolation. Next, the lungs of the mice were removed, rinsed in 1X PBS, placed in a conical tube containing 3 ml of digest buffer (3 mg Collagenase XI [Sigma-Aldrich] and 100 µl Deoxyribonuclease I

[Sigma-Aldrich] mixed into 3 ml of 1X PBS), and kept on ice until cell isolation. Finally, for chronic experiments 1 and 2, the left femur of male mice was removed, and kept on ice in a plate containing 1X PBS until cell isolation.

2.4 Cell isolation and staining

2.4.1 Blood

Blood samples were added to a 50 ml conical tube containing 3 ml of ACK lysis buffer (prepared in the lab, see supplemental **Table S2** for protocol) and left at room temperature for 3 minutes. Samples were then quenched with 10 ml of FACS buffer (1X PBS + 1% BSA [Sigma-Aldrich]) and spun at 2000 rpm for 10 minutes at 4˚C. After being spun down, the supernatant was removed, and ACK lysis was repeated 2 more times. After the final spin, the pellet was resuspended in FACS buffer, split into two (one for each flow panel), and added to the 96-well plate for cell staining.

2.4.2 BAL

Eppendorf tubes containing BAL were placed in a centrifuge and spun at 2000 rpm for 10 minutes at 4˚C. Supernatant was carefully removed using a micropipette, added to a new Eppendorf tube, and then placed in a -80˚C freezer. If pellets appeared red, they were resuspended in 500 µl ACK lysis buffer, and left at room temperature for 3 minutes. Samples with ACK were then quenched with 900 µl of FACS buffer and spun down again. Once the samples were spun down and supernatant was removed, the pellets were resuspended in FACS buffer, and the samples were added to the 96-well plate, and kept on ice, until cell staining.

2.4.3 Lung tissue

Lungs were minced with scissors in their collection tubes. After mincing, the tubes were placed in a shaking incubator at 37˚C for 30 minutes. Digested lungs were then gently pushed

through a 40 µm filter using the plunger of a 1 ml syringe and washed with 10 ml FACS buffer. The samples were then spun down at 2000 rpm for 10 minutes at 4˚C. Supernatant was removed, and the pellet was resuspended in 3 ml ACK lysis buffer to remove red blood cells. After 3 minutes at room temperature, the samples were quenched with 10 ml of FACS buffer and spun at 2000 rpm for 10 minutes at 4˚C. Cell pellets were resuspended in 10 ml of FACS and run through a 40 µm filter into a new conical tube. Finally, the samples were spun down again, resuspended in FACS buffer, split into two (one for each flow panel), and added to the 96-well plate for staining.

2.4.4 Bone marrow

All soft tissues were removed from the femur bone, and the top and bottom of the bone were cut away. The femur was then placed into a PCR tube, containing a small hole in the bottom, which was itself placed inside an Eppendorf tube secured with tape. Eppendorf tubes were then placed in the centrifuge and fast-spun to 5000 rpm. With the cells from the bone marrow now in the Eppendorf tube, the pellet was resuspended in 250 µl ACK lysis buffer. After 3 minutes, the cells were quenched with 1 ml of 1X PBS buffer. The samples were centrifuged at 1500 rpm for 5 minutes. Supernatant was carefully removed using a micropipette, and the pellet was resuspended in FACS buffer and added to the 96-well plate for staining.

2.4.5 Cell staining

After all the samples were added to the 96-well plate (see **Figure S2** in supplemental), the plate was spun down at 2000 rpm for 10 minutes at 4˚C, and the supernatant was discarded. All samples were stained with LIVE/DEAD and incubated on ice for 15 minutes. Following viability stain, BAL, lung, bone marrow and blood cells were stained for 30 minutes on ice with the following antibodies (antibodies from BioLegend): anti-Ly6G BV421, anti-CD11b BV605, anti-

CD45 BV711, anti-MHCII AlexaFluor488, anti-CD11a PerCp-Cy5.5, anti-CD11c APC/Cy7, anti-CD3 PE/Cy7, anti-Siglec F PE, anti-CD117 APC, anti-CD49b PE/Dazzle, and anti-Ly6C BV786. The other blood and lung cells were stained with a different antibody cocktail consisting of the following antibodies (antibodies from BioLegend): anti-CD103 Pacific Blue, anti-CD8 PE/Cy7, anti-CD19 FitC, anti-CD25 PerCp, anti-TCRβ BV605, anti-CD4 APC/Cy7, and anti-CD3e APC. Finally, we did one additional experiment where we examined the effects of chronic vape exposure on hematopoiesis in bone-marrow derived cells from male mice. The antibody cocktail used for the hematopoiesis panel consisted of the following antibodies (antibodies from BioLengend): anti-cKit BV421, anti-Sca 1 PE/Cy7, anti-CD34 FitC, anti-CD16/32 PerCp-Cy5.5, anti-CD115 BV605, anti-F1t3 PE, anti-Ly6C APC, and anti-Streptavidin BV650. For the specific dilutions of each antibody used, please refer to supplemental **Figure S2**. Following staining, the cells were washed with FACS buffer 2X and fixed with 4% PFA. Cells were washed one more time, resuspended in FACS buffer, covered, and kept in the fridge overnight. FMO controls were used when appropriate.

2.5 Flow cytometry

The following morning, samples were spun down, and the supernatant discarded. Cells were resuspended in 180 µl FACS buffer and 20 µl 123count eBeads™ counting beads. Compensations for each fluorophore were made using 200 µl compensation bead solution and 1 µl of antibody conjugate. Samples were run using an LSR Fortessa flow cytometer with UV and analyzed using FlowJo software. Total cell numbers were calculated using the following equation:

$$Absolute\ Count\ (cells/uL) = \left[\frac{(Cell\ Count\ x\ eBead\ Volume)}{(eBead\ Count\ x\ Cell\ Volume)}\right]\ x\ eBead\ Concentration$$

The flow cytometry work was performed in the Flow Cytometry Core Facility for flow cytometry and single cell analysis of the Life Science Complex and supported by funding from the Canadian Foundation for Innovation. Gating strategies can be found in the supplemental information.

2.6 Alveolar macrophage lipid staining

2.6.1 Alveolar macrophage isolation

Alveolar macrophages were separated from a cell suspension using MACS® Magnetic Separator columns from Miltenyi Biotec. Briefly, lungs were collected from mice and placed in 1X PBS. The lungs were then minced with scissors and gently pushed through a 40 µm filter, using the plunger of a 1 ml syringe and washed with 10 ml FACS buffer. The samples were then spun down at 2000 rpm for 10 minutes at 4˚C. Supernatant was removed, and the pellet was resuspended in 3 ml ACK lysis buffer to remove red blood cells. After 3 minutes at room temperature, the samples were quenched with 10 ml of FACS buffer and spun at 2000 rpm for 10 minutes at 4˚C. Supernatant was aspirated completely, and then cells were incubated for 10 minutes at 4 ˚C with Anti-Siglec F Microbeads. Cells were washed, and then resuspended in 500 µl of MS buffer (1X PBS + 0.5% BSA + 2 mM EDTA). Next, a MACS MS column was placed into a magnetic field, and rinsed with 500 µl of MS buffer. The cell suspension was then added to the column to separate the labeled AMs, while the flowthrough was collected and discarded. The column was washed 3X using 500 µl of MS buffer. Following the final wash, the column was carefully removed from the magnetic field, and the cells that were stuck inside the column were flushed out into a sterile collection tube using 1 ml of MS buffer.

2.6.2 LipidTOX staining

The collected cells were centrifuged at 750 G for 10 minutes. Supernatant was aspirated completely, and the pellet was resuspended in DMEM media (37˚C). Coverslips were added to a 24-well plate, and the cell suspension was added to the appropriate wells. 4% PFA was added to the wells and left to incubate at room temperature for 30 minutes. The fixative was removed, and the cells were gently washed with 1X PBS 3 times to remove excess fixative. Once all the liquid was removed, 100 µl of 1X LipidTOX™ Green neutral lipid stain was added to each well and incubated at room temperature for at least 30 minutes before imaging.

2.6.3 Mounting & Imaging

Prolong mounting solution was placed onto a slide. Using forceps, the coverslip was carefully removed from the 24-well plate and any excess liquid was dabbed off using a kimwipe. The coverslip was then added to the slide and left untouched for 24-hours in the fume hood. Imaging was done the following day using a confocal microscope and filter sets appropriate for Alexa Fluor 488 dye or fluorescein.

2.7 ELISA

Serum samples collected from the mice were taken from the -80˚C and brought to room temperature. Serum cotinine levels were measured using the Calbiotech Cotinine Direct ELISA kit, following the manufacturer supplied protocol.

2.8 Statistical analysis

All statistical analysis was performed using GraphPad Prism 9 software. All values are expressed as Mean ± SD. Statistical significance was calculated using one- or two-way ANOVA, or Student's *t*-tests, as appropriate, followed by Tukey's multiple comparisons or Holm-Šídák

correction. Statistical significance was accepted at $\alpha < 0.05$. Data is representative of 3 independent acute and 3 independent chronic experiments, unless otherwise indicated.

Chapter 3: Results

3.1 Serum cotinine levels as a measure of nicotine exposure

Cotinine is a major metabolite of nicotine and is often used to measure nicotine exposure [14], [58], [73], [76]. We measured post-mortem serum cotinine levels in mice, directly after their final vape exposure. Serum cotinine levels were 83.9±2.4 ng/ml and 82.4±0.7 ng/ml (Mean±SD, **Figure 1, A**) for acute and chronic vape exposures, respectively (n = 3-5 mice/group). Serum cotinine levels were also similar between sexes with males and females having concentrations of 82.4±0.7 ng/ml and 81.1±1.5 ng/ml, respectively (Mean±SD, **Figure 1, B**). The serum cotinine concentration of mice in the control group was below the limit of detection, therefore is not shown. Taken together, these results indicate that our vaping system is effectively delivering vape to both male and female mice in acute and chronic treatments.

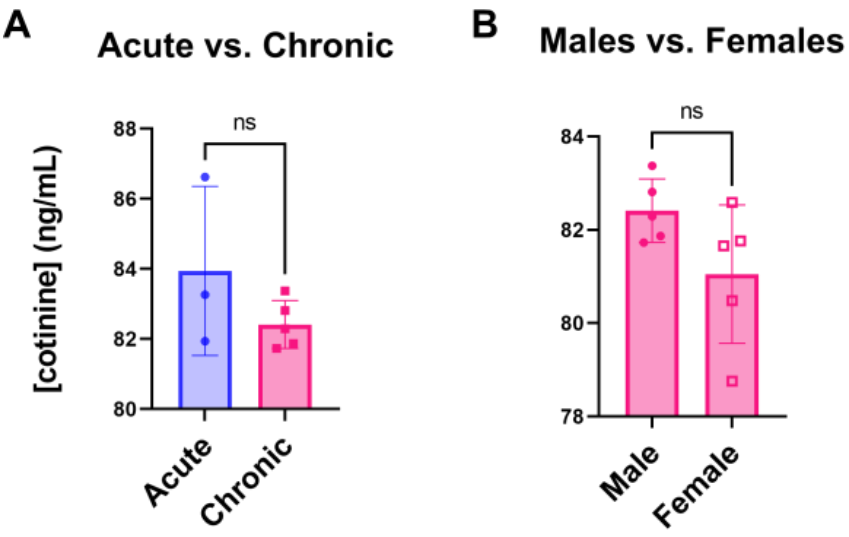


Figure 1. Serum cotinine concentrations were not significantly different between exposures or sexes. Serum samples were taken from mice via cardiac puncture, and cotinine levels were analyzed using ELISA. **(A)** Serum cotinine levels were similar in the acute and chronic exposures. **(B)** Serum cotinine levels from chronic experiments were similar in male and female mice. Data are shown as Mean ± SD. Significance was determined by Student's *t*-tests (2-tailed) with $\alpha<0.05$. Data are representative of 3 independent acute and chronic experiments (n=3-5 mice/group).

3.2 Effects of acute e-cigarette vape exposure

3.2.1 Innate immune cells

We found no changes to immune cell numbers in the airways of vaped mice compared to controls in either sex. Alveolar macrophage, neutrophil, and eosinophil numbers were stable in the BAL fluid, and while there was a slight trend down in total leukocytes and AM numbers in vaped mice, the change was not significant (**Figure 2**). In the lung tissue of male vaped mice, alveolar macrophage numbers were unchanged, total leukocyte and neutrophil numbers trended down, while eosinophil and monocyte numbers decreased significantly after vaping ($p<0.005$ eosinophils; $p<0.05$ monocytes) (**Figure 3, A, B**). Female mice showed similar trends to males, though the effect was only significant in eosinophils ($p<0.005$). DC numbers also decrease after vaping in both sexes, although only significantly in males ($p<0.005$) (**Figure 4, A**). Interestingly, although total AM numbers did not change in the BAL or lung tissue, we did find that the AMs from vaped mice had excess lipid accumulation compared to control mice in both sexes (**Figure 5**). Next, to examine whether the decrease in innate immune cells was systemic, we looked at innate immune cell numbers in the blood. We found that total leukocytes, neutrophils, macrophages, and monocytes all decreased significantly in male mice ($p<0.005$ total leukocytes; $p<0.05$ neutrophils; $p<0.005$ macrophages; $p<0.05$ monocytes) (**Figure 6, A**); however, female mice did not show any significant changes (**Figure 6, B**).

3.2.2 Adaptive immune cells

When we looked at adaptive immune cells in the lung tissue, we found that male vaped mice had a significant decrease in total leukocyte and B cell numbers ($p<0.05$ for both), while CD4+ and CD8+ T cell numbers trended down (**Figure 7, A**). Female vaped mice showed a similar trend of decreased adaptive immune cells in lung tissue, although none of the changes in cell

populations reached significance (**Figure 7, B**). In the blood, we did not see any significant changes to B cell or T cell numbers in either sex, though the frequency of CD4+ T lymphocytes increased significantly in males only ($p<0.05$) (**Figure 8**).

3.3 Effect of chronic e-cigarette vape exposure

3.3.1 Innate immune cells

As we saw with acute exposures, we found no significant changes to innate immune cell numbers (alveolar macrophages, neutrophils, eosinophils) or total leukocyte numbers in the airways of chronically vaped mice (**Figure 9**). In lung tissue, all innate immune cell types except for AMs went down significantly in both sexes (Males: $p<0.0005$ neutrophils; $p<0.0005$ eosinophils; $p<0.0001$ monocytes. Females: $p<0.05$ neutrophils; $p<0.0001$ eosinophils; $p<0.05$ monocytes) (**Figure 10, B**), which therefore resulted in a significant increase in AM relative frequency ($p<0.0001$, both sexes) (**Figure 10, C**). Both CD11b high and CD11b low DCs decreased significantly in male vaped mice ($p<0.005$ CD11b high; $p<0.005$ CD11b low) (**Figure 11, A**); however, in females, only CD11b low DCs decreased significantly ($p<0.0001$) (**Figure 11, B**). In the blood, we found a significant decrease in total leukocytes ($p<0.05$), neutrophils ($p<0.05$), macrophages ($p<0.05$), and monocytes ($p<0.05$) in male vaped mice (**Figure 12, A**), and a similar but less pronounced trend in the female mice (**Figure 12, B**). Since our acute experiments showed that males seemed to respond more strongly to the vape, we also decided to look at neutrophil numbers in the bone marrow of chronically vaped male mice only. We found

that total leukocyte numbers and neutrophil numbers trended down in the bone marrow of males vaped mice; however, the effect was not significant (**Figure 13**).

3.3.2 Adaptive immune cells

In the lung tissue, we found that total leukocyte numbers, B cells, CD4+ T cells, and CD8+ T cells all decreased significantly after chronic vaping in both sexes (Males: $p<0.005$ total leukocytes; $p<0.005$ B cells; $p<0.05$ CD4+ T cells; $p<0.05$ CD8+ T cells. Females: $p<0.005$ B cells; $p<0.005$ CD4+ T cells; $p<0.005$ CD8+ T cells) (**Figure 14**). In the blood, males showed a significant decrease in B cells after vape exposure ($p<0.05$), while female vaped mice showed a similar trend, though not significant (**Figure 15**). Both sexes also showed an increase in the relative frequency of CD4+ T cells after chronic vaping ($p<0.0001$ males; $p<0.05$ females) (**Figure 15, C**).

3.3.3 Hematopoiesis in the bone marrow of male mice

Since we continued to see significant decreases in granulocytic cell populations in the lung tissue and blood of vaped mice, we added an additional panel to our last chronic experiment to examine common myeloid progenitor (CMP), megakaryocyte-erythroid progenitor (MEP), and granulocyte-monocyte progenitor (GMP) cell numbers in the bone marrow of male mice (**Figure 16, A**). We found that mice exposed to chronic e-cigarette vape had significantly fewer GMP cell numbers ($p<0.05$), indicating that the vape could be inhibiting granulocyte proliferation processes in the bone marrow.

3.4 Examining the effects of the full-body chamber and PG/VG alone

For all previous experiments, the air-treated mice were kept in their home cages while the vaped mice were moved into the vaping chamber for their 90-minute exposure each day. To determine whether the immune-suppression effect we were seeing was due to stress of the vaping

chamber, we performed another acute experiment where we put control mice into a separate, full-body chamber that received air only. We also added a second control group of mice which received vape that contained the vehicle solvent alone (i.e., PG/VG without nicotine or flavoring). We weighed the mice each day and found there were no differences in body weight between groups in either sex (**Figure 17**), indicating that short-term vaping does not have a significant effect on weight gain or loss in mice. In both the lung tissue and blood, we did not see significant changes in innate immune cell numbers; however, we did see similar trends of decreased immune cells following vaping (**Figure 18**), suggesting that the observed effect was not due to stress of the vaping chamber.

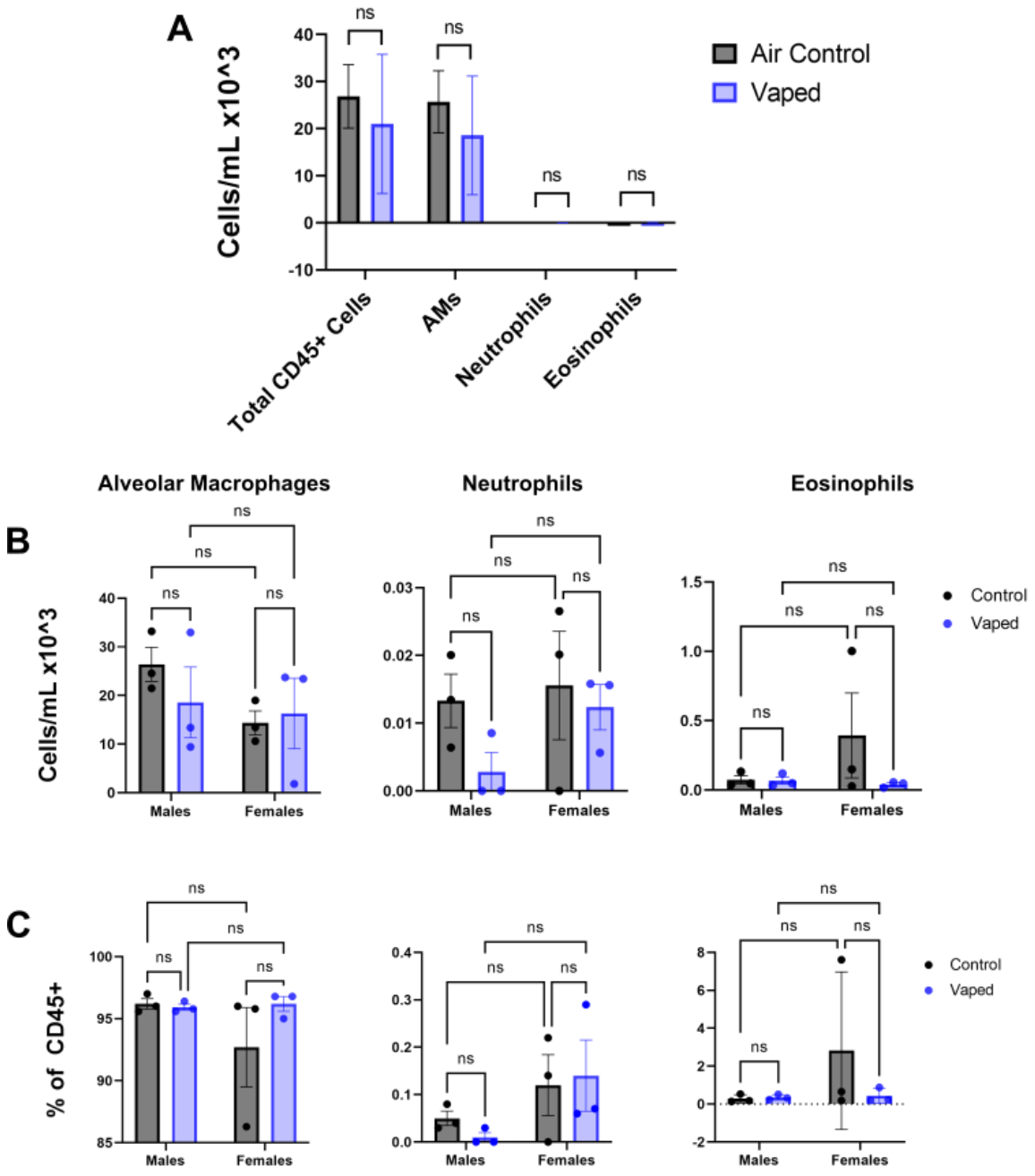


Figure 2. Innate immune cell populations are unchanged in the airways following acute vape exposure. After vaping mice for 3 days, BAL was collected to examine immune cell populations in the airways. **(A)** No significant changes to immune cell numbers in BAL of male vaped mice compared to air control, although total leukocyte and AM numbers trend down. Similarly, there were no significant changes to cells numbers **(B)** or cell frequencies **(C)** in the BAL of vaped mice in either sex compared to controls. Data are shown as Mean ± SD, n=3 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05. Data are representative of 3 independent acute experiments.

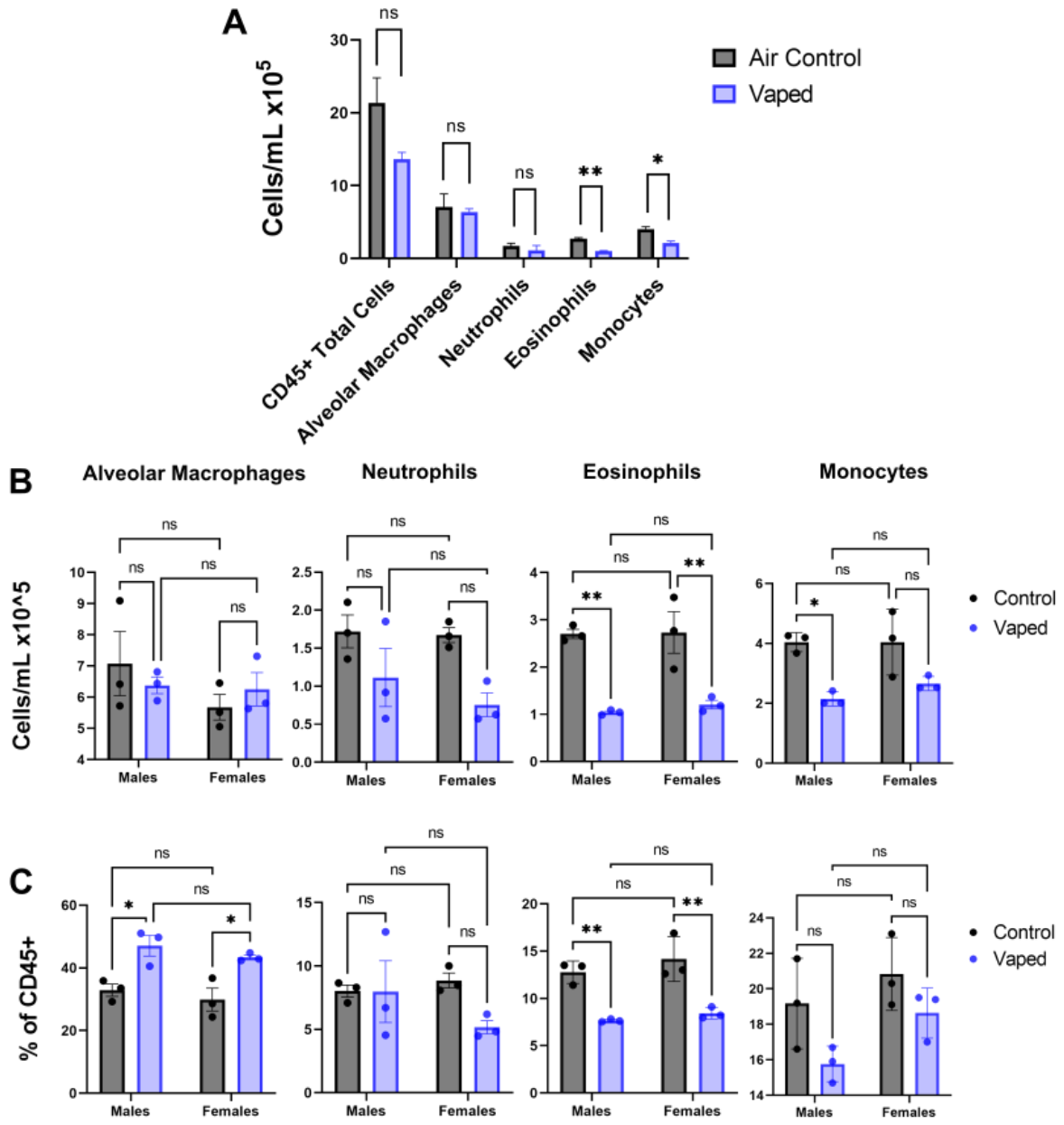


Figure 3. Eosinophil and monocyte cell numbers decreased in the lung tissue of acutely vaped mice. (A) Vaping mice for 3 days led to a significant decrease in eosinophil and monocyte numbers in the lungs of male mice. **(B)** Both sexes showed no change in AM numbers, a downward trend in neutrophil numbers, and a significant decrease in eosinophil numbers in lung tissue following acute vape exposure. Monocyte numbers also decreased significantly in male vaped mice, while they trended down in females. **(C)** With the numbers of neutrophils, eosinophils, and monocytes decreasing, the relative frequency of AMs increased significantly in the lung tissue following vape exposure. Data are shown as Mean ± SD, n=3 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05, **p<0.005. Data are representative of 3 independent acute experiments.

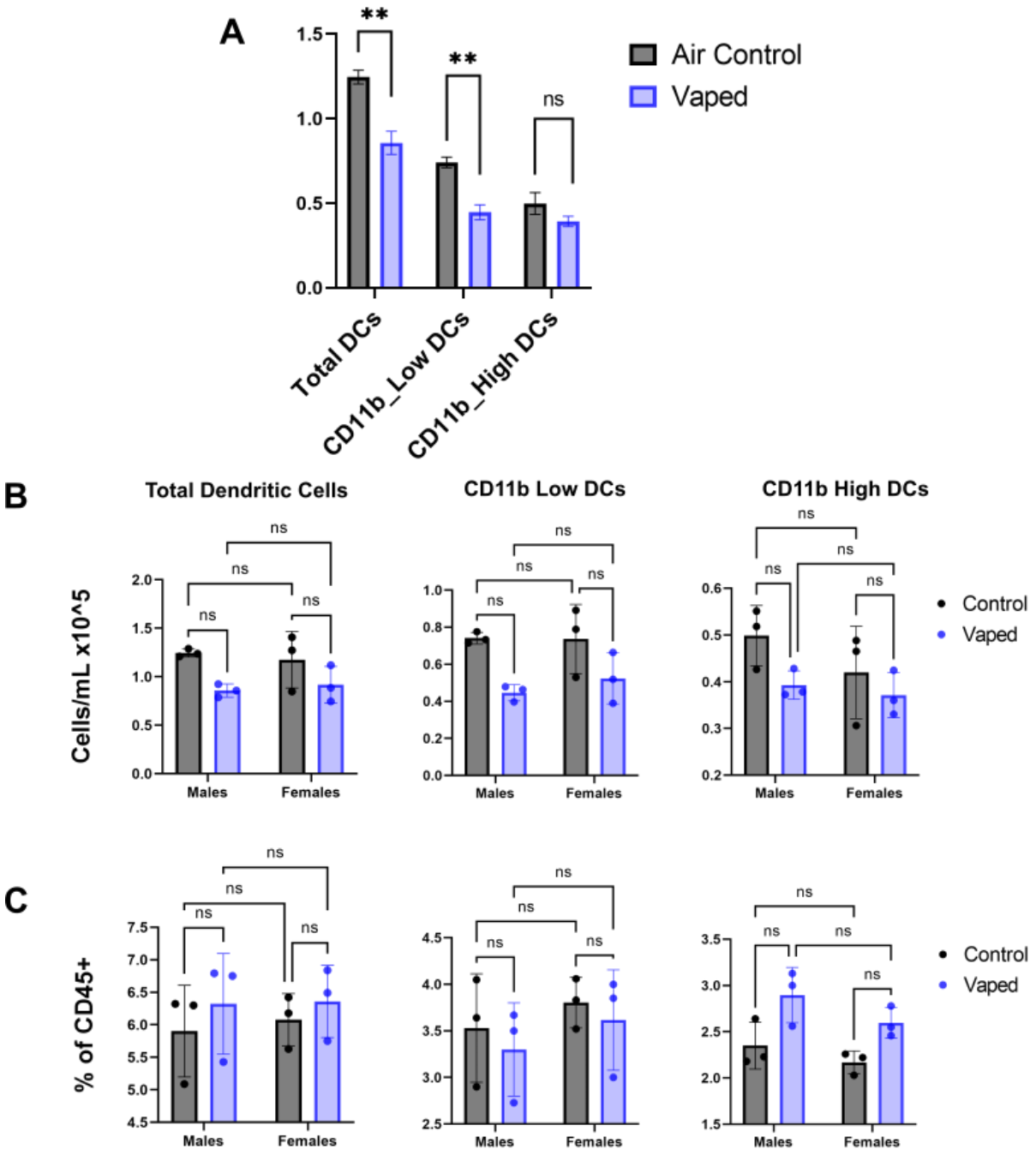


Figure 4. Dendritic cell populations appear to decrease in lung tissue following acute vape exposure. (A) Total DC numbers and DCs expressing low levels of the cell surface marker CD11b decreased significantly in male vaped mice. **(B)(C)** Female vaped mice show similar trends in DCs numbers in the lung tissue; however, their changes were not significant. Data are shown as Mean ± SD, n=3 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). **p<0.005. Data are representative of 3 independent acute experiments.

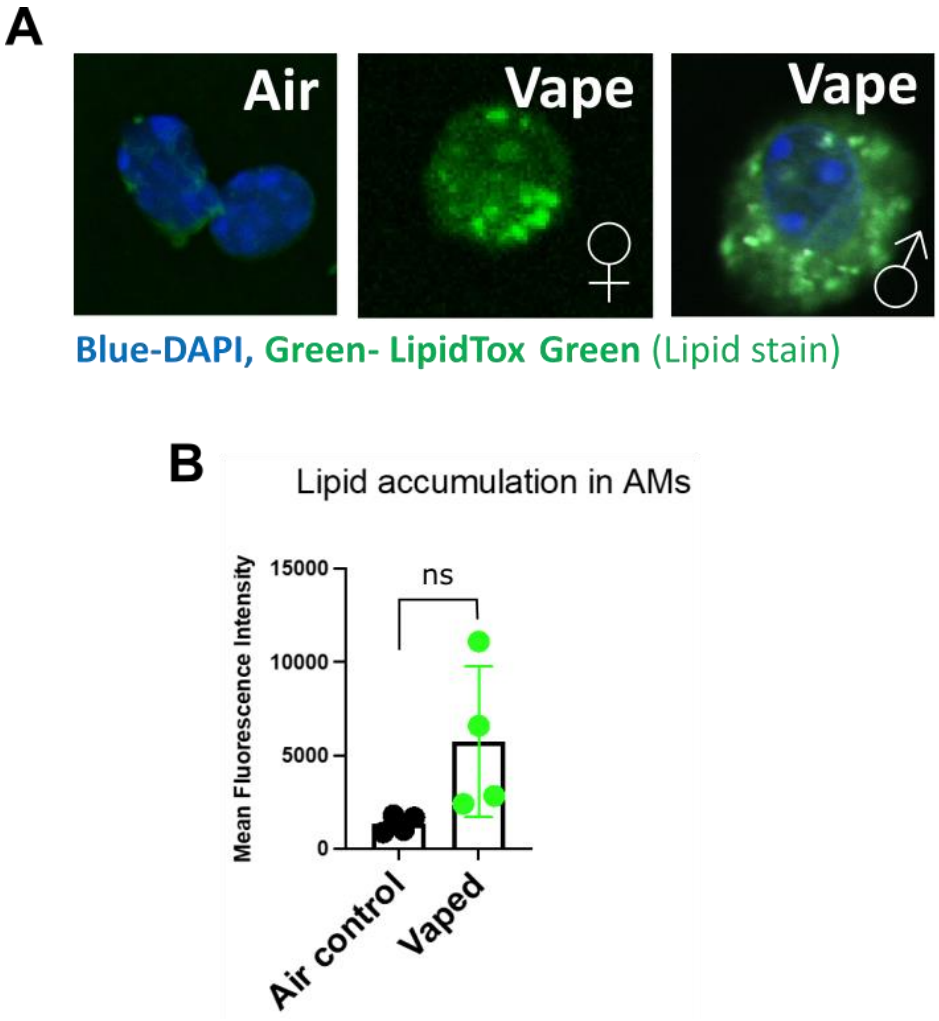


Figure 5. Acute vaping led to excess lipid accumulation in the AMs of vaped mice. AMs were isolated from the lung tissue of air control and vaped mice using anti-Siglec F antibody conjugated to magnetic beads. The isolated AMs were then stained with LipidTox Green lipid stain and imaged using a confocal microscope. **(A)** Both male and female mice showed increased lipid accumulation following 3 days of vaping compared to control; however, as shown in panel **(B)**, the mean fluorescence intensities were not significantly different. Data are shown as Mean ± SD, n=4 mice/group. Significance was set at $\alpha < 0.05$ and determined using a Student's *t*-test.

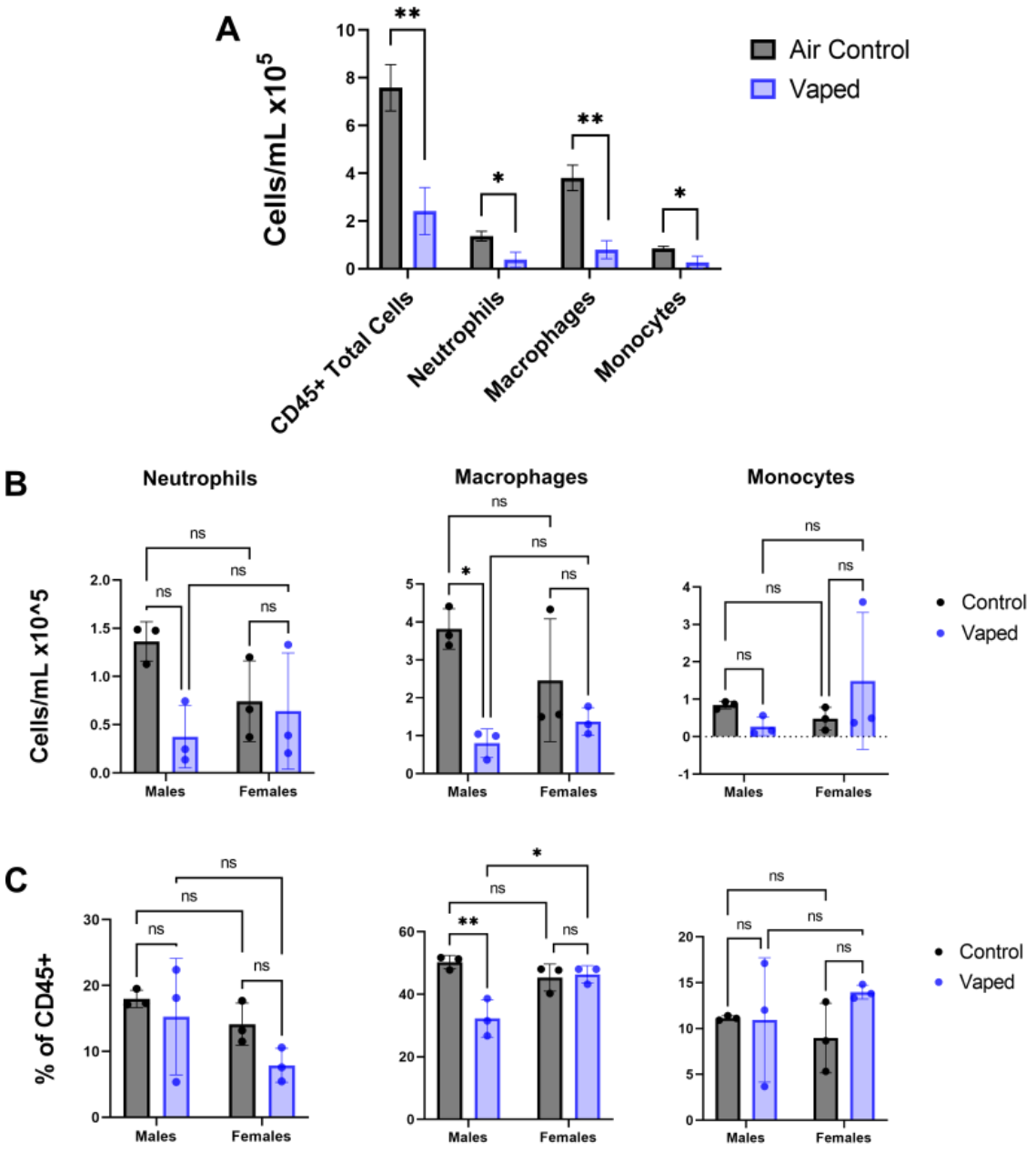


Figure 6. Male vaped mice show a significant decrease in innate immune cell numbers in the blood following acute vape exposure. (A) Following 3-days of vaping, male mice showed a significant decrease in total leukocyte, neutrophil, macrophage, and monocyte numbers systemically. **(B)** The decrease in innate cell populations in the blood of male vaped mice is more pronounced than in female mice. **(C)** The relative frequencies of innate cell populations are mostly unchanged in both sexes, except for a significant decrease in the frequency of macrophages in male mice. Data are shown as Mean ± SD, n=3 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05, **p<0.005. Data are representative of 3 independent acute experiments.

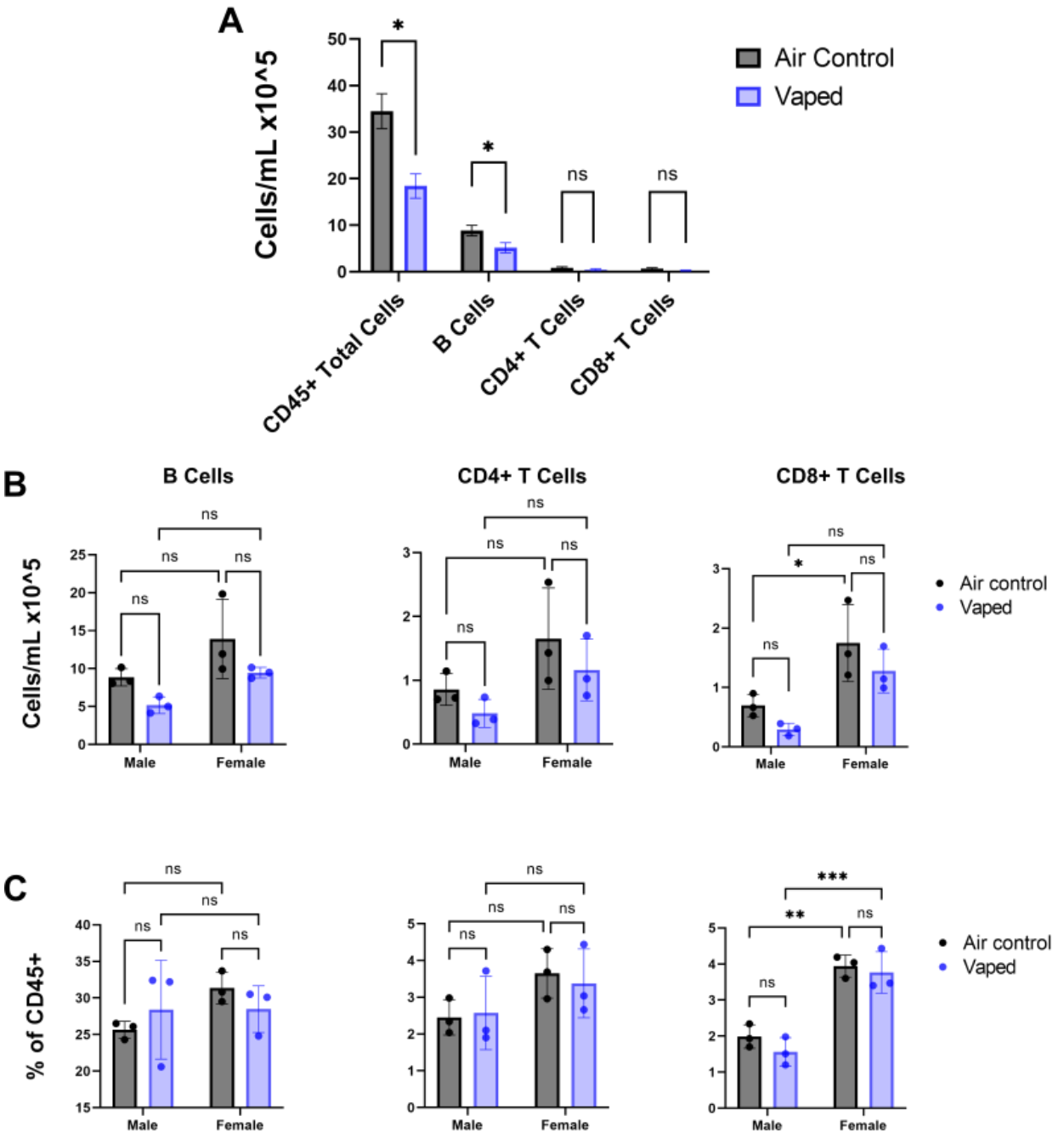


Figure 7. Male mice show a significant decrease in B cell numbers in lung tissue follwing acute vape exposure. (A) Total leukocyte and B cell numbers decreased significantly in the lung tissue of male vaped mice. **(B)** Adaptive immune cell populations trended down in the lung tissue of mice following acute vape exposure, though not significantly. **(C)** Relative frequencies of B and T cells in lung tissue were unchanged. Data are shown as Mean ± SD, n=3 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05, **p<0.005, ***p<0.0005. Data are representative of 3 independent acute experiments.

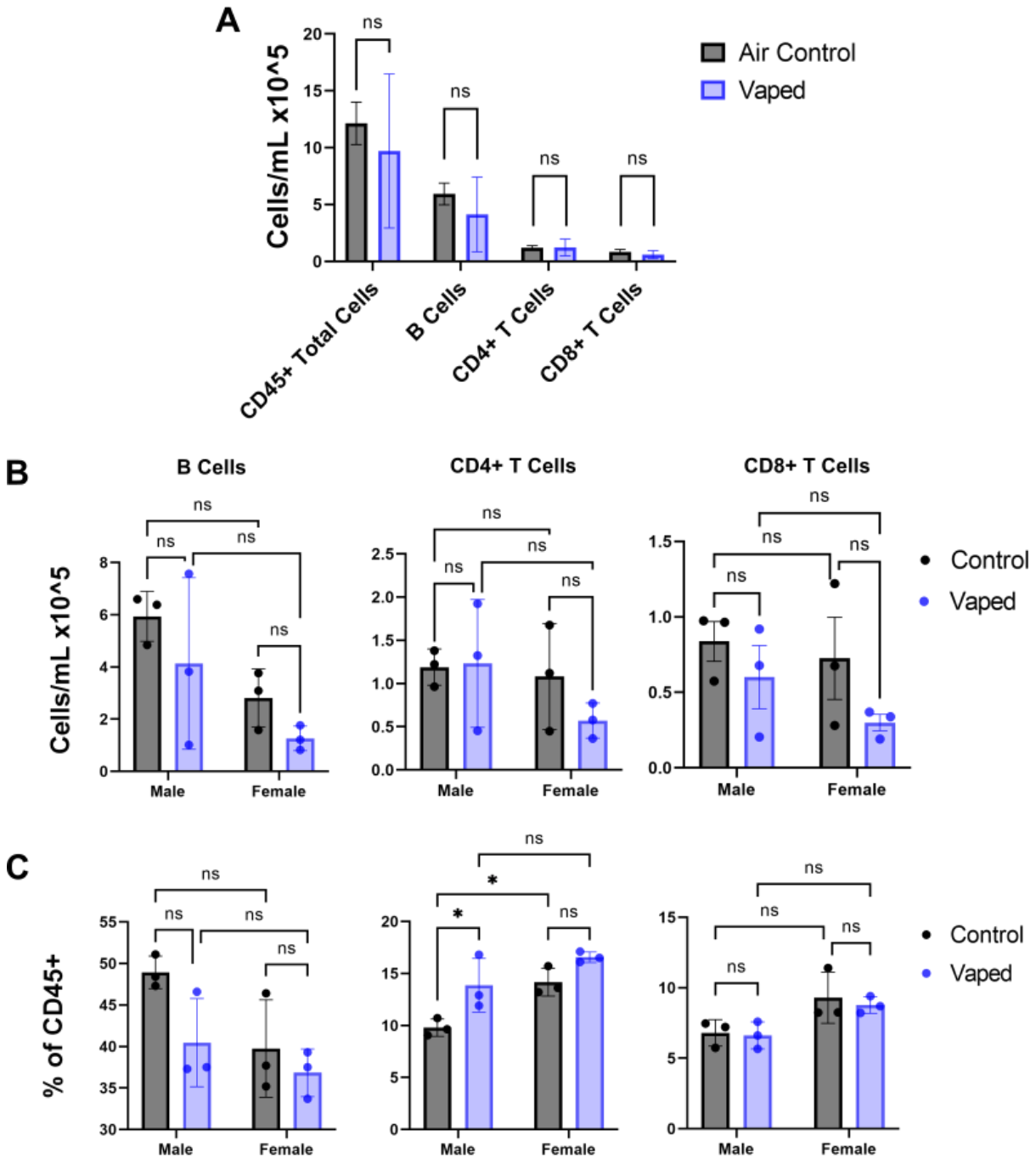


Figure 8. Acute vape exposure did not change adaptive cell numbers in the blood. (A) Male vaped mice did not show a significant change to total leukocyte, B cell, or T cell numbers. **(B)** B cells, CD4+ T cells and CD8+ T cells remained relatively stable in the blood of both sexes following acute vape exposure. **(C)** The relative frequency of B cells trended down in male mice, while the frequency of CD4+ T cells increased significantly. Data are shown as Mean ± SD, n=3 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05. Data are representative of 3 independent acute experiments.

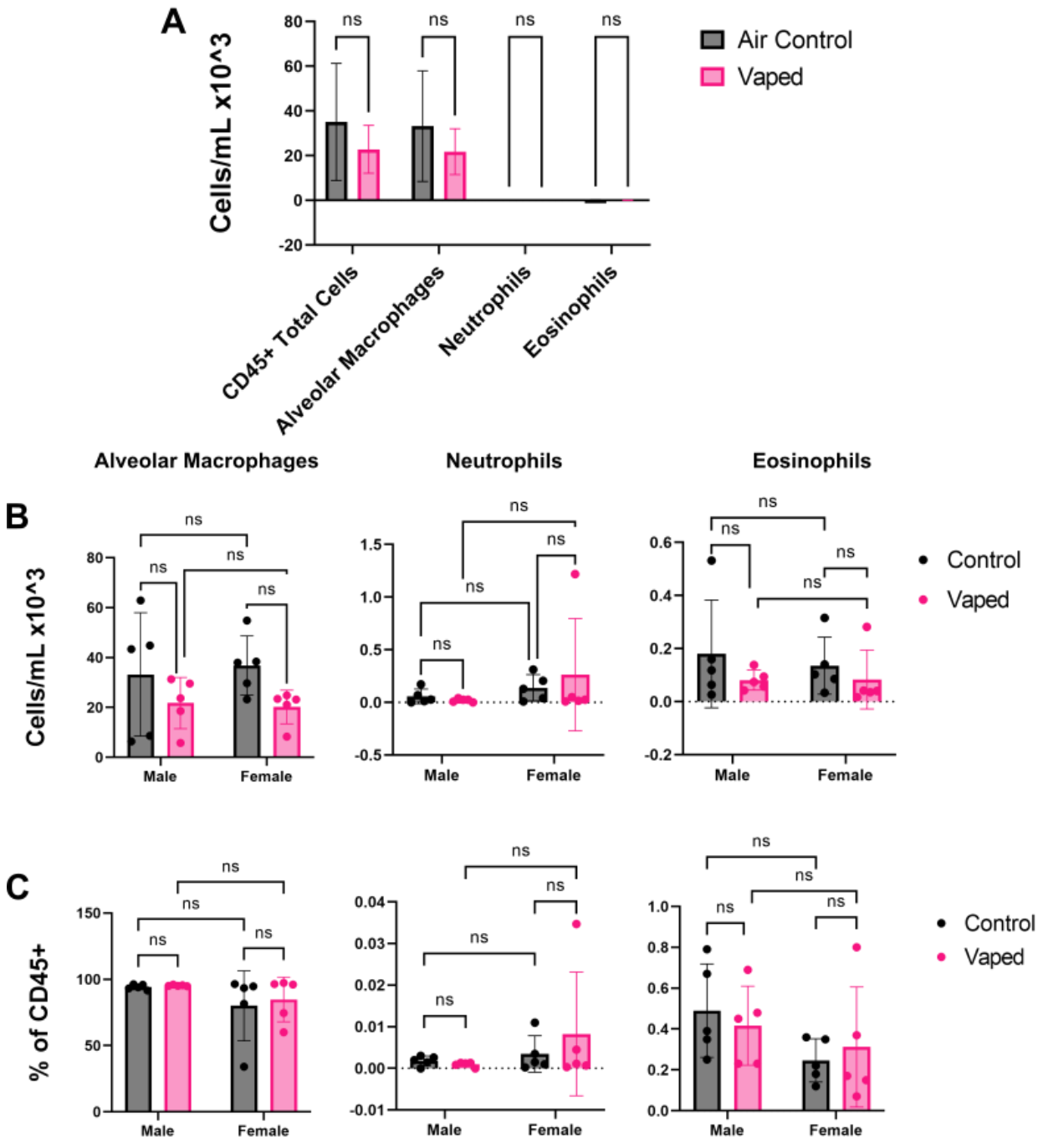


Figure 9. Chronic vape exposure does not lead to immune cell influx into the airways of mice. (A) After 16 days of vape exposure, we found no significant changes to immune cell numbers in BAL of male vaped mice compared to air control. Similarly, there were no significant changes to cells numbers **(B)** or cell frequencies **(C)** in the BAL of vaped mice for either sex compared to controls. Data are shown as Mean ± SD, n=5 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05. Data are representative of 3 independent chronic experiments.

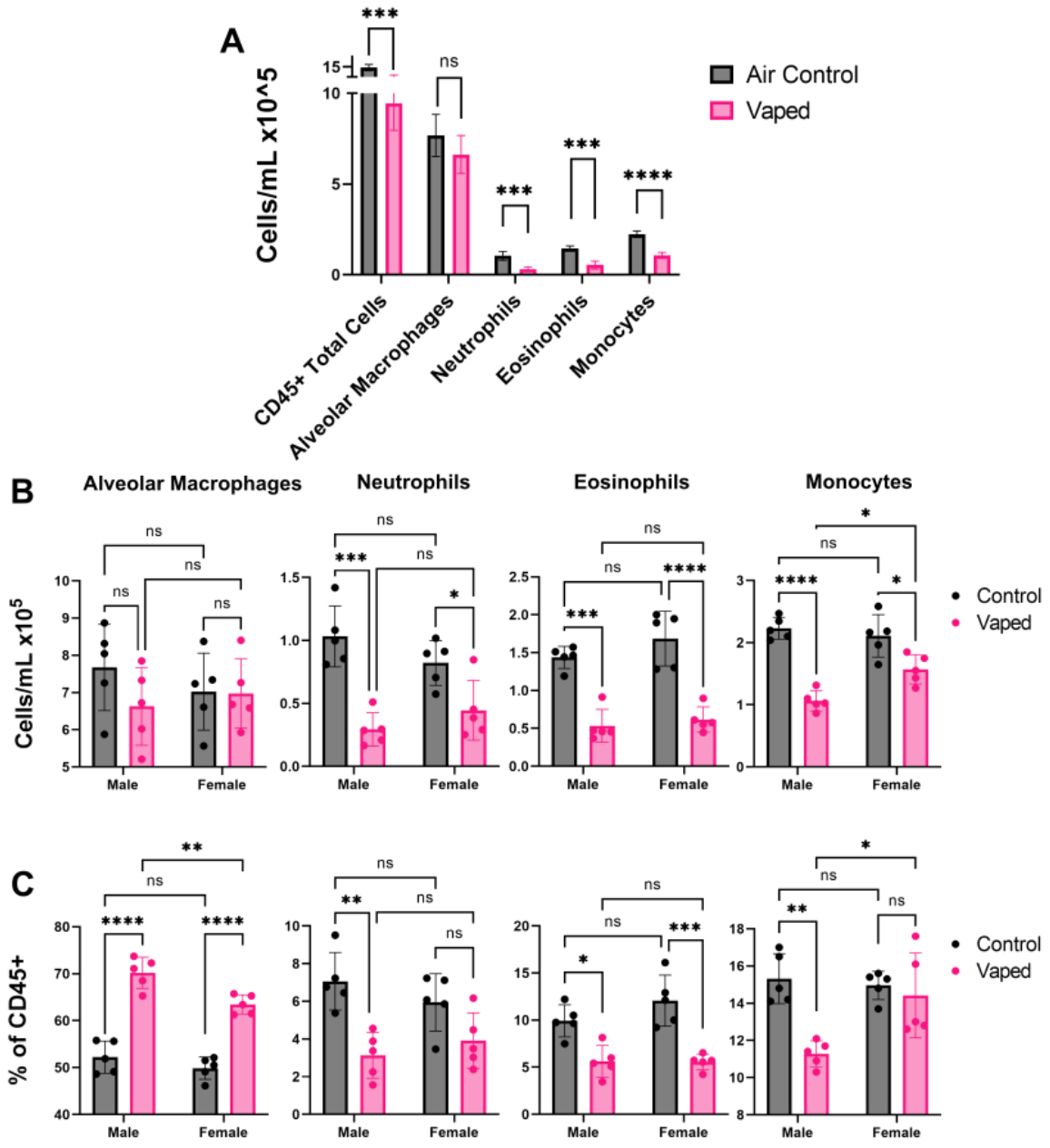


Figure 10. All innate immune cells, except alveolar macrophages, decrease significantly in lung tissue following chronic vape exposure. Chronic exposure to vape resulted in significant decreases in total leukocyte, neutrophil, eosinophil, and monocyte numbers in the lung tissue. **(A)** Total numbers of cell populations in the lung tissue of male mice decrease significantly, except for AMs. **(B)** Both sexes show significant decreases in neutrophil, eosinophil, and monocyte numbers. **(C)** The relative frequency of alveolar macrophages in the lung tissue increased significantly in vaped mice as the other innate immune cells decreased. Data are shown as Mean ± SD, n=5 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001. Data are representative of 3 independent chronic experiments.

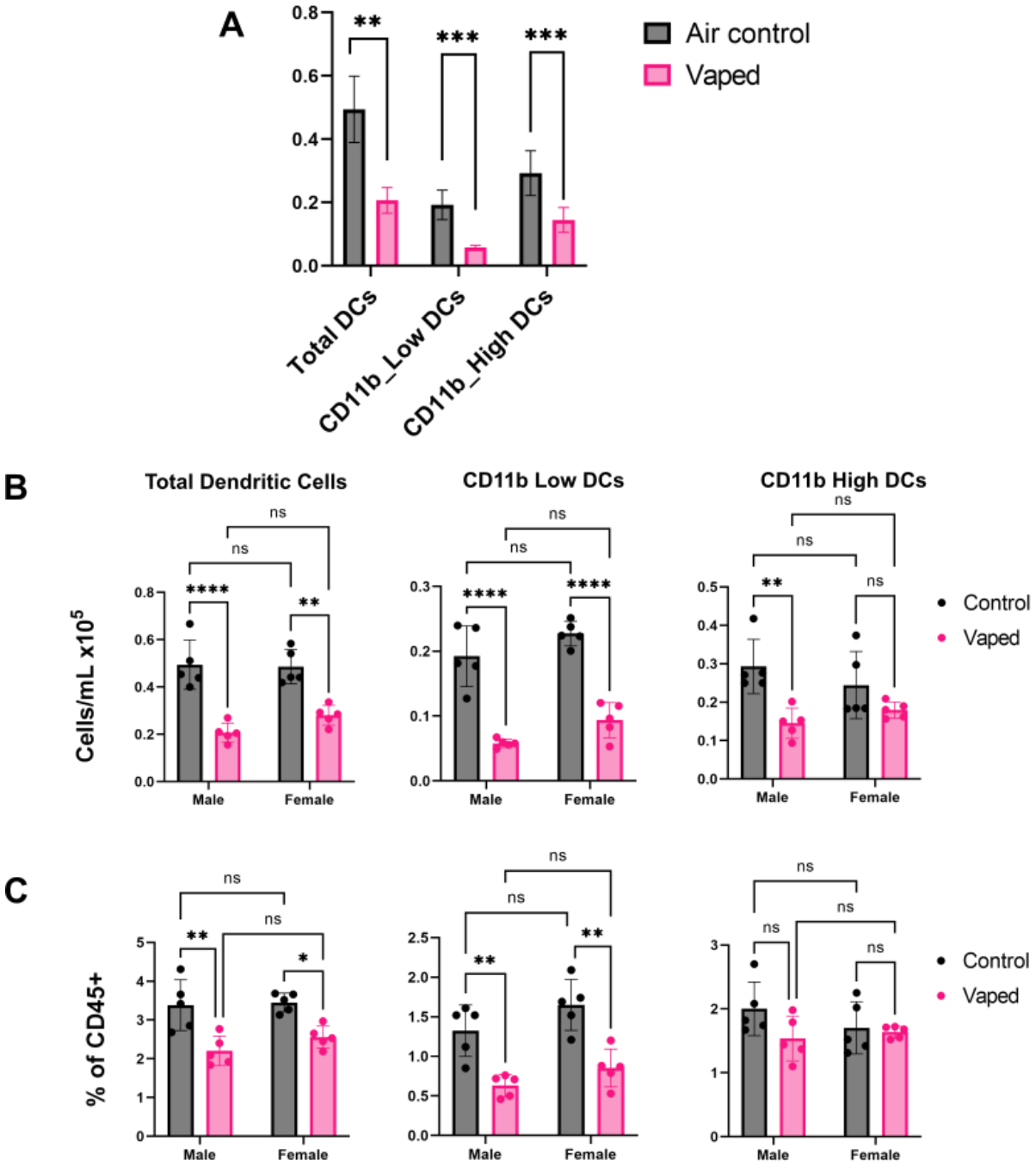


Figure 11. DCs decrease significantly in the lung tissue of both sexes following chronic vape exposure. (A) Male mice showed a significant decrease in both CD11b low and CD11b high DCs following 16 days of vaping. **(B)(C)** Both sexes showed a significant decrease in CD11b low DCs in response to vaping, while CD11b high DC numbers went down in male mice but were not significantly different in females. Data are shown as Mean ± SD, n=5 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05, **p<0.005, ***p<0.0005, ****p<0.0001. Data are representative of 3 independent chronic experiments.

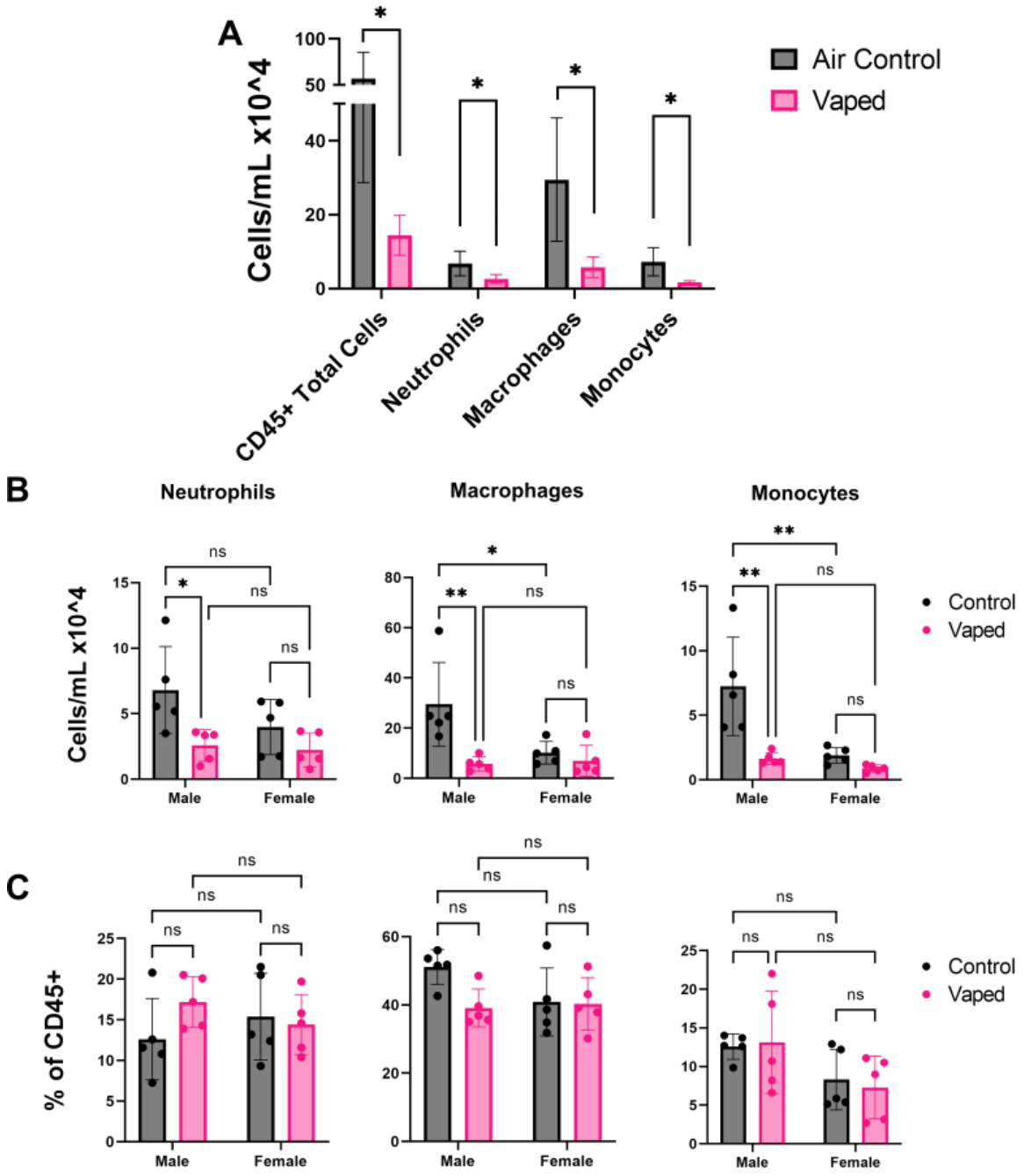


Figure 12. Chronic vaping leads to decreased innate immune cells in the blood of male mice. (A) Total leukocyte, neutrophil, macrophage, and monocyte numbers decrease significantly in the blood of male vaped mice. **(B)** Male control mice have significantly more macrophages and monocytes compared to female controls. Female vaped mice do not show a significant change in absolute number **(B)**, or relative frequency **(C)** of neutrophils, macrophages, or monocytes in the blood following vaping. Data are shown as Mean ± SD, n=5 mice/group. Significance was determined by multiple t-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05, **p<0.005. Data are representative of 3 independent chronic experiments.

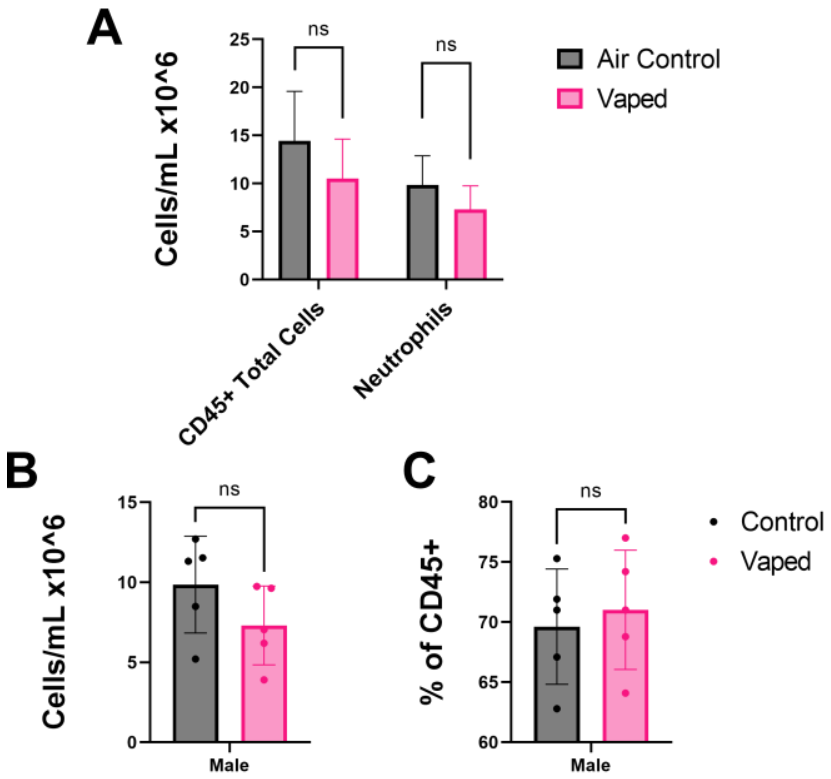


Figure 13. Neutrophil numbers in bone marrow trend down, but do not change significantly after chronic vape exposure. Since male mice appeared to have a slightly stronger response to vaping in the acute exposures, we decided to look at numbers of neutrophils in the bone marrow following chronic vape exposure. **(A)(B)** There was a trend down in the total number of leukocytes and neutrophils; however, the relatively frequency of neutrophils did not appear to be affected **(C)**. Data are shown as Mean ± SD, n=5 mice/group. Significance was determined by Student's *t*-tests (2-tailed) with $\alpha < 0.05$. Data are representative of 2 independent chronic experiments.

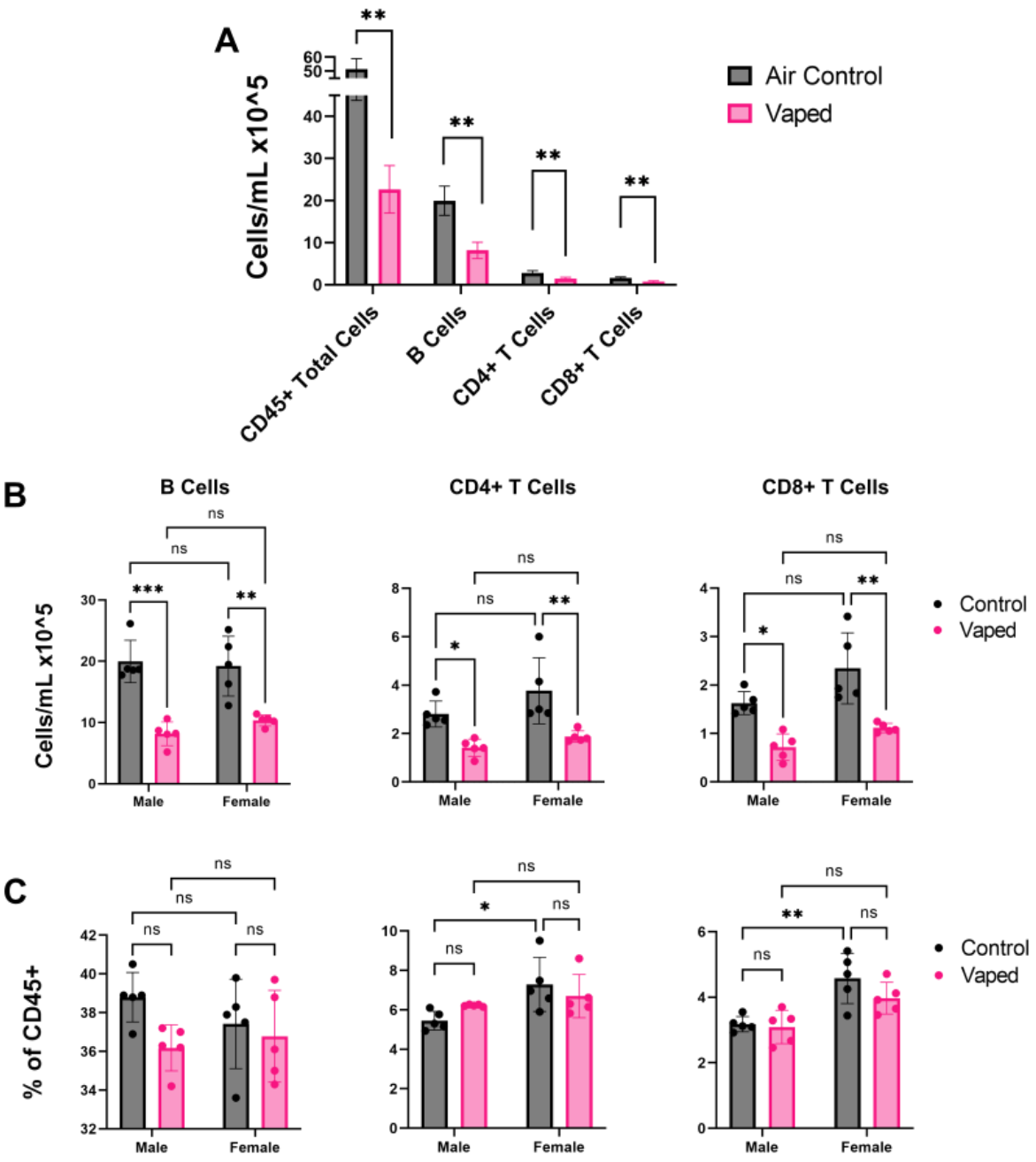


Figure 14. Adaptive immune cells decrease in lung tissue following chronic vape exposure. To examine whether vape-induced immunosuppression systemically in adaptive immune cells, we collected blood via cardiac puncture and determined B and T cell populations using flow cytometry. **(A)** Male mice showed a significant decrease in total leukocyte, B cell, CD4+ T cell, and CD8+ T cell population following chronic vape exposure. **(B)** Both sexes showed a significant decrease in B cells, CD4+ T cells and CD8+ T cells after chronic vaping. **(C)** Cell frequencies were unchanged in both male and female mice. Data are shown as Mean ± SD, n=5 mice/group. Significance was determined by multiple t-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05, **p<0.005, ***p<0.0005. Data are representative of 3 independent chronic experiments.

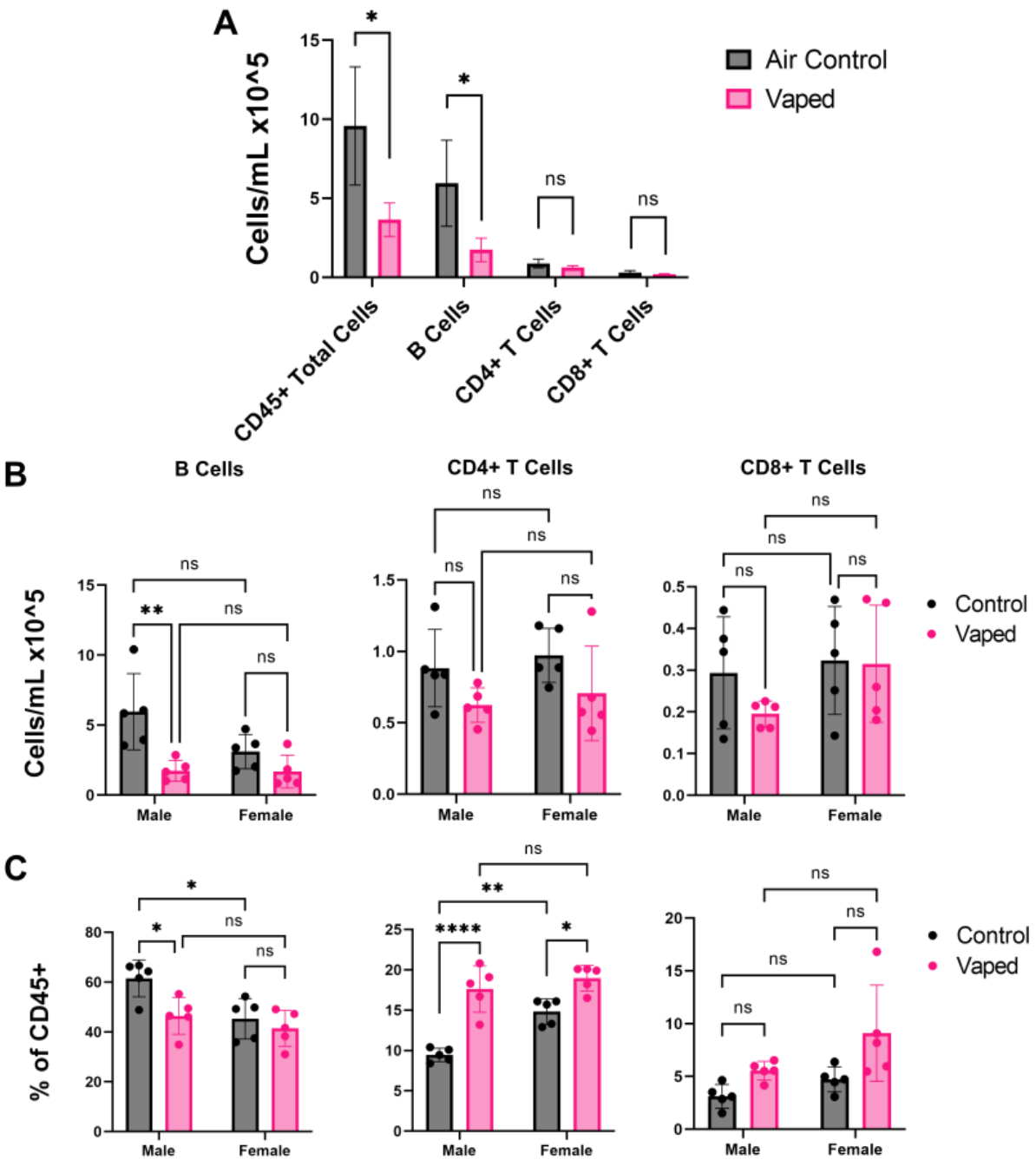


Figure 15. Adaptive immune cell numbers are altered in the blood following chronic vape exposure. Blood was collected via cardiac puncture from mice following 16 days of consecutive vape or air exposure. **(A)** Male mice showed a decrease in total leukocyte numbers as well as B cells. **(B)** B cell numbers decrease significantly in male mice only; however, the blood of male and female mice exposed to vape showed an increase in the relative frequency of CD4+ T cells **(C)**. Data are shown as Mean ± SD, n=5 mice/group. Significance was determined by multiple *t*-tests with Holm-Šídák correction (A), or 2-way ANOVA with Tukey's multiple comparisons (B, C). *p<0.05, **p<0.005, ****p<0.0001. Data are representative of 3 independent chronic experiments.

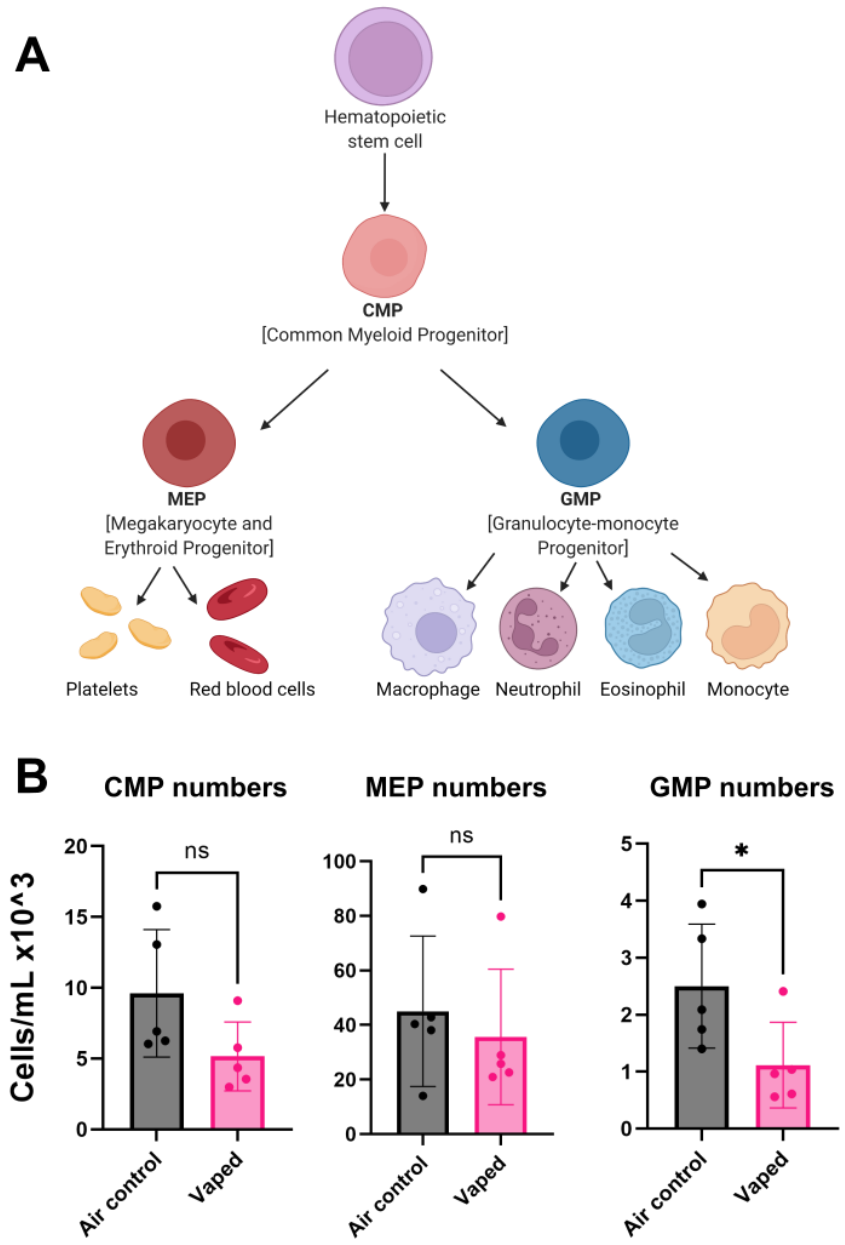


Figure 16. Chronic exposure to e-cigarette vape led to decreased GMP numbers in bone marrow of male mice. Since we continued to see decreased numbers of granulocytic cell populations following vape exposure, we wondered if vaping was affecting hematopoiesis in the bone marrow. On our last chronic experiment, we isolated cells from the bone marrow of male mice and stained them with antibodies which allowed us to isolate CMP, MEP, and GMP cell types. **(A)** Flow chart depicting the cells of interest and their progenitors. Created using BioRender. **(B)** We found a significant decrease in GMP numbers only, following vape exposure. Data are shown as Mean ± SD, n=5 mice/group. Significance was determined by 2-tailed unpaired *t*-test. *p<0.05.

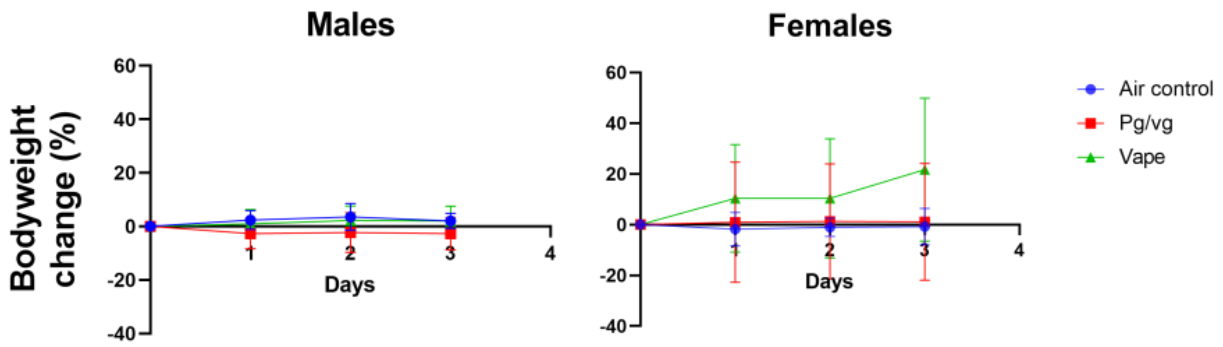


Figure 17. Acute exposure to vape does not alter bodyweight in mice. Male and female mice were place in full-body chambers for 90 minutes/day for 3 days, and exposed to either air, PG/VG alone, or full vape. Bodyweight was measured each day before exposure, and the percent change was calculated based on the bodyweight at baseline (before the first exposure). No significant differences were observed between groups at any time point. Data are shown as Mean ± SD, n=4-5 mice/group.

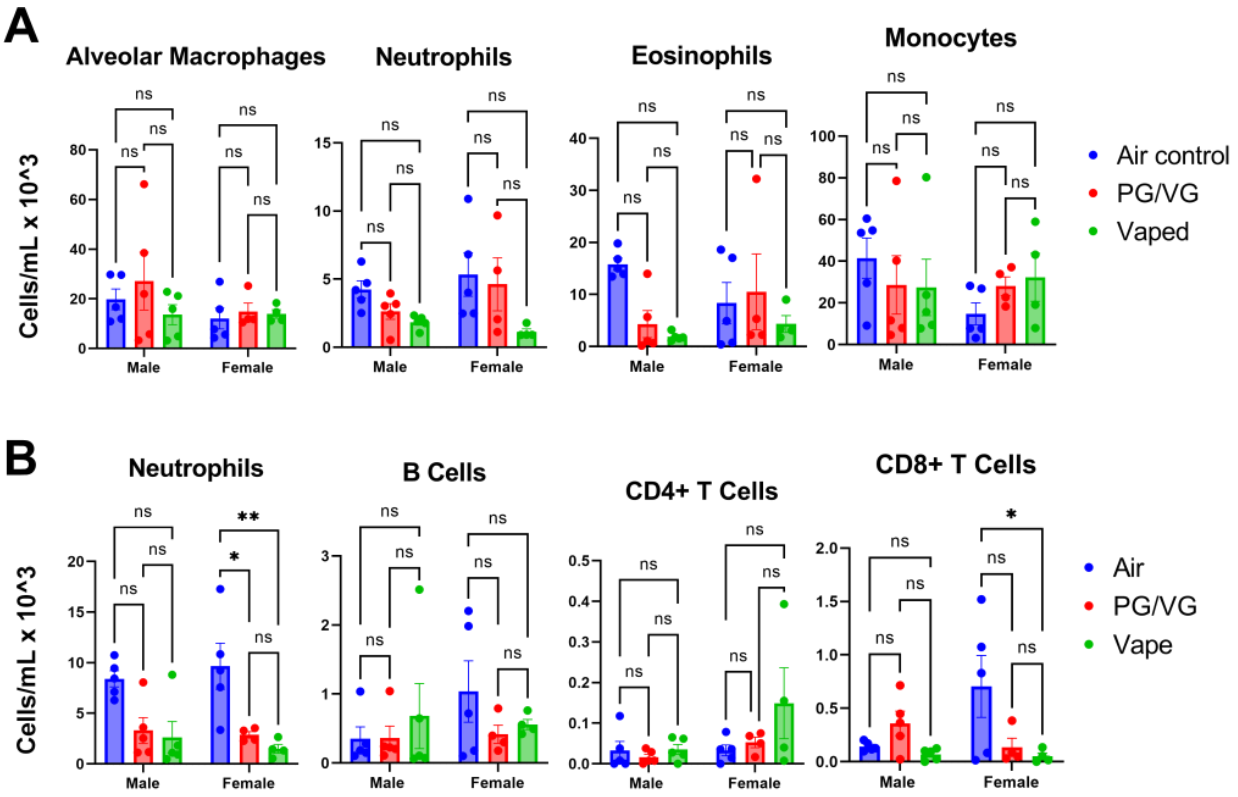


Figure 18. Immunosuppressive trends are seen following vape exposure, even when air control mice are placed in full-body chambers. To ensure the decrease in immune cells we were seeing was due to the vape and not an effect of the vaping chamber, male and female mice were place in full-body chambers for 90 minutes/day for 3 days, and exposed to either air, PG/VG alone, or full vape. **(A)** There were no significant changes to immune cell numbers in the lung tissue, although eosinophils showed a trend down in males, and neutrophils trended down in females. **(B)** Neutrophils and CD8+ T cells decreases significantly in the blood of female mice. Data are shown as Mean ± SD, n=4-5 mice/group. Significance was determined by 2-way ANOVA with Tukey's multiple comparisons. *p<0.05, **p<0.005.

Chapter 4: Discussion

4.1 Vaping resulted in reduced myelocyte and lymphocyte numbers in lung tissue and blood

In this study, we sought to develop a full body e-cigarette exposure system to examine the effects of acute and chronic vape exposure on pulmonary immune cells in male and female mice. Based on the current e-cigarette literature, we hypothesized that both short- and long-term exposure to vape would cause an influx of pro-inflammatory cells into the lungs, ultimately causing unnecessary tissue damage. To our surprise, we observed the opposite effect: That is, we found a significant decrease in both innate and adaptive cell populations in the lung tissue and blood of vaped mice, and no change to immune cell numbers in the airways (BAL). At present, we have no explanation for the sudden loss of immune cells in response to vaping; however, there are several key aspects to our study to consider while interpreting our results.

One explanation for the discrepancy between our results and the results of other studies is the immense variability among vaping devices, exposure settings, and experimental setups used in e-cigarette research. *In vitro* studies have provided insights into the impact that vaping can have on pulmonary immune cells, and have shown that exposing alveolar macrophages, neutrophils and pulmonary epithelial cells to e-cigarette vape can lead to cytotoxicity, the release of pro-inflammatory cytokines, and functional impairment [58], [77]. While aerosolized vape appears to have an impact on immune cells under laboratory conditions, we cannot extrapolate that the same outcomes will be seen in the complex system of a living organism. As such, for our experiments we used an animal model to create an e-cigarette exposure system that would closely mimic the vaping experience in humans.

There have been a growing number of animal studies looking at the effects of vaping using nose-only or full-body exposure systems, with each type of exposure having its advantages and disadvantages. The main advantage of using a nose-only system is that the vape is delivered directly to the respiratory system of the mice, minimizing certain confounding factors, such as e-liquid absorption through dermal contact, or ingestion due to grooming. That said, such a level of control comes at the cost of stress on the animal [78], which could lead to other confounding effects, such as skewing the immune system towards a pro-inflammatory phenotype [79]. While there is not yet a systematic study directly comparing the outcomes of e-cigarette exposure in mice using whole-body vs. nose-only systems, there is a recently published paper comparing the two exposure types using cigarette smoke [80]. The authors found that mice exposed to nose-only cigarette smoke had higher stress response compared to whole-body, indicated by decreased bodyweight. The group also found that both nose-only and whole-body cigarette exposure led to increased numbers of total free lung cells in the BAL compared to air controls, but among the two cigarette exposures, nose-only mice had higher cell counts than whole-body for all cell types analyzed: namely, total lymphocytes, macrophages, and neutrophils [80]. While this does not explain the decreased immune cells numbers we observed in our vaped mice compared to air controls, it does highlight the impact that the exposure systems can have on end results. As mentioned, this direct comparison between nose-only and whole-body exposure is still

Nose-only mesh restraint from SciReq

needed for e-cigarette-related research and would aide in interpreting our results, as well as results across multiple experiments.

Besides the type of exposure system, another source of variability that could be resulting in our decreased immune cell numbers is the type of vaping device, and the coil used in our system. Unfortunately, many studies looking at the effects of vaping do not clearly state important parameters, such as the type of coil used or the temperature at which they are aerosolizing the e-liquid. These parameters could have major effects on the outcomes of experiments, as high temperatures have been shown to decompose both PG and VG, resulting in the formation of carbonyl compounds (e.g. formaldehyde, acetaldehyde, acrolein and acetone) with toxic properties [81]. In addition, it has been shown that the type of coil used can have dramatic affects on pathological outcomes. Recently, a group in California were interested in the effects of e-cigarette vapor on rat cardiovascular physiology and pathology. They began using a device with a stainless-steel atomizer, but during their experiments, they were forced to switch to a nichrome atomizer for the remainder of their exposures. Interestingly, although they kept all other parameters the same, once they switched to a nichrome atomizer, 14 of the 18 rats developed acute respiratory distress, resembling EVALI-like symptoms [69]. Their findings were important for two reasons: 1) They demonstrated the impact that coil composition can have on experimental results; and 2) They showed that EVALI can occur independent of THC or vitamin E oil, which had previously been closely linked to the illness [67], [68], [82].

For our experiments, we used a temperature-controlled atomizer with a nickel coil. The temperature-control setting ensures consistent puff quality for the duration of the 90-minute exposure. To our surprise, we have not been able to find other examples in the literature of researchers who explicitly state that they used a nickel coil in their vaping system. As such, we

have questioned whether the nickel in the coil could be influencing our immune-dampening results. To date, there have been a few studies looking at the respiratory effects of nickel, though very few in the context of vaping. For example, a study in 2003 showed that nickel suppressed T-cell function and promoted immunosuppressive macrophage phenotypes in rats by inhibiting the production of pro-inflammatory TNF-α, and increasing the production of anti-inflammatory IL-10 in a dose-dependent manner [83]. In the same study, the authors state that the range of plasma nickel concentrations required to induce immunosuppression ranged between 209-585 ng/ml. The question then becomes: How much nickel is being transferred from our heated coil to the vaporized e-liquid; and of that, how much is being absorbed by the animals?

It has already been demonstrated that metals from the coil and tin from the joints of vaping devices end up in the aerosol produced by e-cigarettes [47], [52], [84], [85]. In a study which sampled e-liquid and aerosol from 56 devices of daily e-cigarette users, researchers found that nickel concentrations in the aerosol can reach up to 233 µg/kg, though the compositions of the coils were not specified [47]. Importantly, another study looked at non-invasive metal biomarkers in 64 regular e-cigarette users, and found strong evidence to support that the metals in the aerosol are being absorbed by the users [52]. Further, they also noted that an earlier time to first vape and more frequent coil replacement were associated with higher urine nickel concentrations in users. These results demonstrate that the coil of an atomizer is not inert, and thus could be influencing experimental outcomes. Since our vaping device and coil are unique, a closer look into the nickel concentrations in the aerosolized vapor, and biomarkers in exposed vs. control mice is warranted to see if increased nickel levels from the coil could be associated to the immune cell loss.

Another obvious source of variability among studies is the composition of e-liquid used. For our experiments, we wanted to closely mimic human vape exposures, therefore we used

commercially available vape, with a 70PG:30VG vehicle solvent ratio, 20 mg/ml of nicotine, and a popular mixed berry flavor [86]. Additionally, since our e-liquid contained nicotine, we were able to confirm that the mice were inhaling vape by examining their serum cotinine levels. We found that our results were similar to what has been shown in human e-cigarette users [76], though others have reported serum cotinine levels in mice that are much lower [51], [87], or much higher [88]–[90]. The wide range of serum cotinine concentrations likely reflects the short half-life of this metabolite in mice, combined with varied cotinine measurement protocols; however, our results nonetheless indicate that there was systemic uptake of the aerosolized e-liquid by our mice.

In terms of e-liquid constituents, many studies have used a roughly 50:50 ratio of PG:VG ± nicotine, without flavor [14], [58], [59], [73], [88], while others chose to include flavoring as well [51], [60], [91]–[93]. Again, with so much variety in the ratio of vehicle solvents used, the concentration of nicotine, and the presence or absence of flavoring, it is difficult to tease apart which components could be contributing to pro- or anti-inflammatory results, if any. While our e-liquid is comparable to other studies, one slight change is that we used a higher ratio of PG to VG. Interestingly, in a study from 2017, researchers were looking at the effects of e-cigarette exposure in mice with either PG or VG, and found that the mice exposed to PG with nicotine had significantly fewer macrophages and total cell numbers in the airways compared to air controls [92]. Though they saw a decrease in innate immune cells like us, an important difference is that they saw a drop in macrophage numbers, while we saw decreases in neutrophils, eosinophils, and monocytes. Of note, PG has been shown to be an irritant in human subjects as well [48]. To better understand the effects of the e-liquid components in our experimental setup, further testing is required with differing levels of PG, VG, flavoring, and nicotine.

Finally, although we did not see an influx of immune cells into the lungs of vaped mice—a hallmark of inflammation—we did see some evidence of AM dysfunction and lung injury in agreement with other reports. For example, in 2019, a group used a similar full-body exposure system with mice to directly compare the pulmonary effects of chronic e-cigarette use to conventional cigarettes [14]. In line with our results, Madison et al. found that chronic vape exposure did not alter immune cell numbers in the airways or cause pulmonary inflammation, but it did result in lipid filled AMs, disrupted lipid homeostasis, and immune impairment. After only 3 days of vape exposure, we saw similar trends in lipid-filled AMs, while immune cell numbers in the airways were unchanged; however, further examination into the function of our lipid-filled AMs is needed to determine whether they too show disrupted lipid homeostasis and immune impairment. Additionally, other studies have found no changes to immune cell numbers in the airways following vape exposure, but they do see other signs of injury, such as increased production of pro-inflammatory cytokines, or higher albumin concentrations in the BAL [51], [58], [91]. Given our two endpoints for measuring inflammation—i.e., influx of pulmonary immune cell populations and lipid accumulation in AMs—it is difficult to say how our results fit into the literature, though our findings provide us with opportunities to explore inflammation more deeply in the future.

4.2 Sex differences in response to vaping

Currently, there are insufficient studies comparing the effects of vaping between sexes, despite having evidence to show that sex hormones impact lung inflammation [94], and that EVALI is more commonly seen in young male patients [95]. Most e-cigarette work in mice have used male or female mice exclusively, though more recently the influence of sex on vaping is gaining some attention [87], [88], [96]. Perhaps not surprisingly, evidence thus far supports sex dimorphism in

mouse immunity. For example, one study showed that female mice had a significant influx of neutrophils and CD8T+ lymphocytes into the airways after a 3-day exposure to PG + nicotine, while male mice did not show the same acute inflammatory response [87]. Our results did not show increased CD8+ T cells numbers in vaped mice compared to air controls, but we did find that females had a significantly higher frequency of CD8+ T cells compared to males, both in control (acute and chronic) and vape (acute) treatments. More recently, Moshensky et al. analyzed systemic metabolites in male and female mice, and found that exposure to vape led to distinct metabolic profiles for each sex, though they fail to provide information on what the different metabolic signatures could mean in terms of vaping pathology [96]. Surprisingly, our results did not show striking differences when comparing the vaped male vs. vaped female immune cell numbers. In lung tissue of chronically vaped mice, we saw significantly higher numbers of monocytes in females compared to males, which could be indicative of a stronger immune response, but again, compared to the control mice, both sexes show significant decreased cell numbers. In the blood, we found that control male mice had higher numbers of macrophages and monocyte than control females; however, when we compare the two sexes after vaping, the cell populations were not significantly different.

4.3 Future directions

As is often the case, the findings of our experiments have sparked many more questions that remain unanswered. This study was designed as a starting point for examining the effects of vape on pulmonary immune cells and has provided us with many experiments to pursue moving forward.

Firstly, as mentioned earlier, we focussed on treating mice with full vape (vehicle solvent, nicotine, and flavoring) or air. We did one acute experiment looking at the effects of PG/VG alone

compared to air control and full-vape and found similar results to that of full-vape, but further work is needed to include treatments that contain vehicle solvent alone, vehicle + flavor without nicotine, and vehicle + nicotine without flavoring, to explore the effects of each constituent separately. Secondly, because injury can be measured in several ways, a clear next step will be to measure the levels of certain cytokines in the BAL and lung tissue of vaped mice. Markers of inflammation such as IL-6, TNF-α, and KC, could shed light onto whether AMs are functioning normally, or if the vape is altering cytokine signalling. Additionally, we could look at lung histology, albumin concentrations in the BAL, or signs of oxidative stress in the lung tissue to better understand the impact of acute vs. chronic vaping between sexes.

Since we saw such dramatic cell loss in lung tissue and blood of vaped mice compared to other studies, it would be interesting to run the same experiments, but with coils made of different metals (i.e., stainless steel or titanium). Controlled experiments comparing the different types of vaping coils are absent in the field and would provide insight into whether certain atomizers are more dangerous than others. Since we saw significant decreases to granulocytic cell populations specifically, we wanted to examine whether there was a link between vape exposure and hematopoiesis. We carried out one experiment where we tested whether there were changes to granulocyte-monocyte progenitor (GMP) numbers in the bone marrow and found a significant decrease in GMP numbers specifically. In the future, the same experiment should be replicated and tested under other conditions (i.e., varied e-liquid constituents, and different coils) in order to gain further insight into the effects of vaping on hematopoiesis.

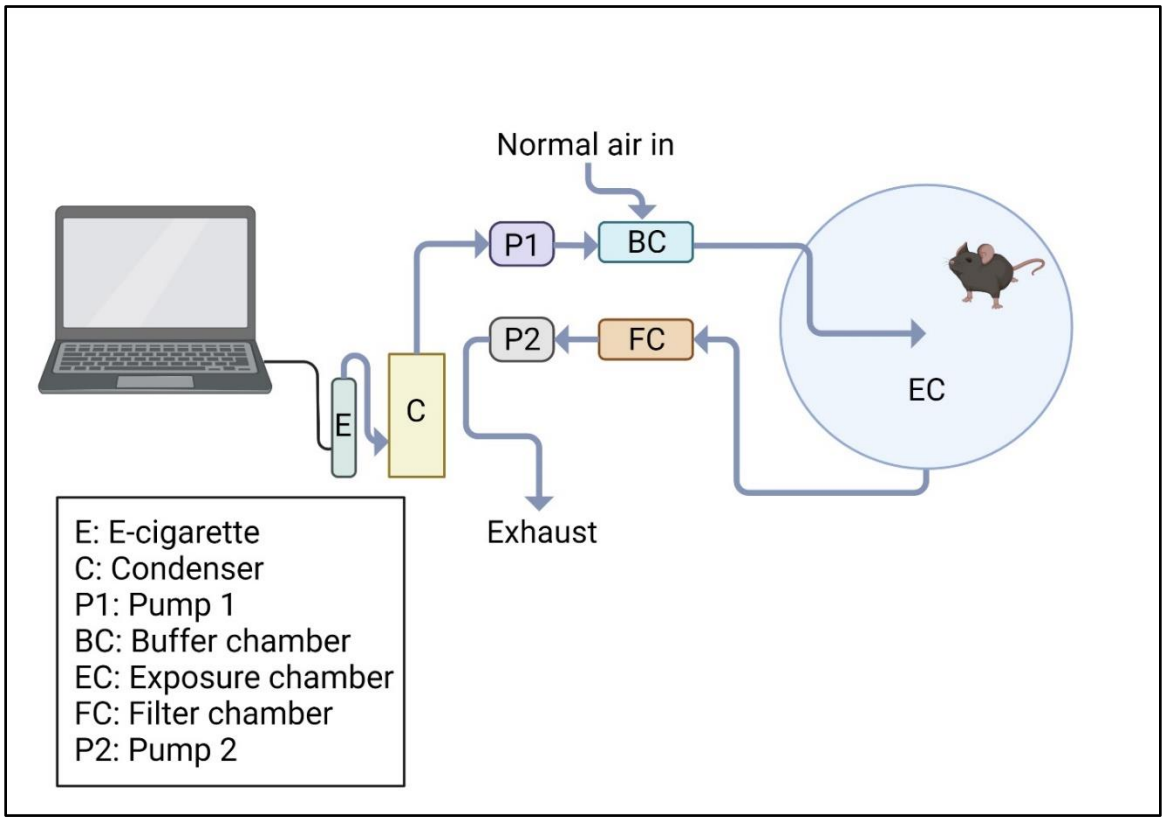
Finally, given unique loss of hematopoietic immune cells, it would be interesting to see how the mice respond to infection, post-vape treatment. Since infection induces hematopoiesis, we

would expect to see increased immune cell numbers at the site of infection, though if there is an interaction involving hematopoiesis and vaping, we may not see an immune cell response.

4.4 Conclusions

Overall, our study provides new and unanticipated results to e-cigarette-related research. Unlike most other studies, we found that both acute and chronic vape exposure led to significant decreases in innate and adaptive immune cell populations. In agreement with previous reports, we also found there was no change to airway cell populations, but that AMs began to show increased lipid accumulations after just 3 days of vape exposure. While male mice showed more instances of significant cell loss, the post-vape numbers in both sexes were relatively stable. Our results did not agree with our original hypothesis, that vaping would lead to immune cell infiltration in the lungs, but instead provided us with more questions to explore. Ultimately, this research underscores the importance of having standardized vaping protocols and transparency in terms of device, e-liquid, coil, and temperature used, so that research can be compared across studies with the goal of understanding the effects of short- and long-term e-cigarette use.

Supplemental Information



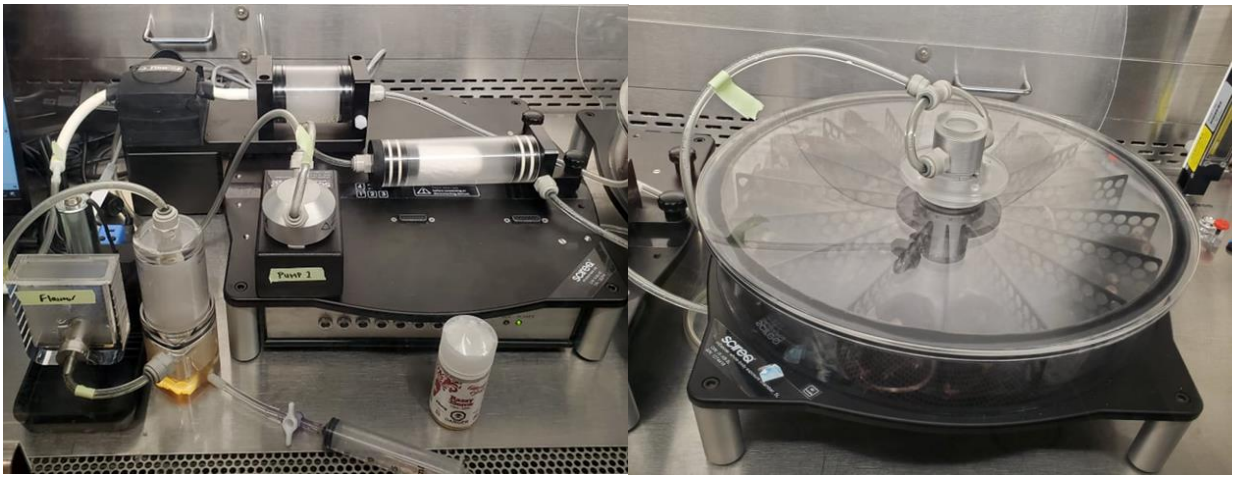
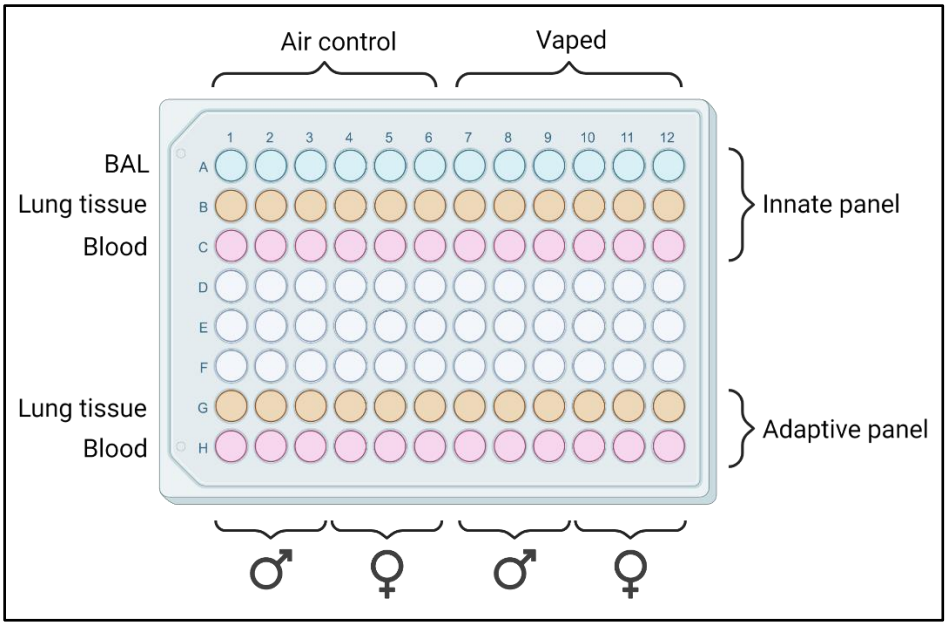


Figure S1. Full-body vaping system. Top: Representation of our full-body, Scireq inExpose vaping system, created using BioRender. The e-cigarette (E) was connected to a laptop and triggered remotely using flexiWare software. Once triggered, vape was pulled into the condenser (C), and then into the buffer chamber (BC), where it was mixed with normal air. From there, the vape mixture entered the exposure chamber (EC) where the mice were placed in separators. Vape was then pulled out through a filter chamber, and out through an exhaust tube. Bottom: A picture of our mice being exposed to vape. The entire system was set up in a fume hood.



Innate Flow Panel

BUV395	(L/D)	1:1000
BV421	(Ly6G)	1:350
BV605	(CD11b)	1:350
BV711	(CD45)	1:300
FITC	(MHCII)	1:400
PerCP-Cy5.5	(CD11a)	1:400
APC/Cy7	(CD11c)	1:350
PE/Cy7	(CD3)	1:350
PE	(Siglec F)	1:400
Alexa Fluor647	(CD117)	1:350
PE-Dazzle	(CD49b)	1:250
BV786	(Ly6C)	1:100

Adaptive Flow Panel

BUV395	(L/D)	1:1000
PE	(CD1d Tetramer)	1:100
BV421	CD103	1:100
PE/Cy7	CD8	1:200
BV711	CD45	1:350
FitC	CD19	1:300
PerCp	CD25	1:100
BV605	TCRβ	1:100
APC/Cy7	CD4	1:200
APC	CD3e	1:200

Bone Marrow Flow Panel

BUV395	(L/D)	1:1000
PE	(F1t3)	1:100
BV421	(c-kit)	1:100
PE/Cy7	(Sca-1)	1:100
FitC	(CD34)	1:50
PerCp-Cy5.5	(CD16/32)	1:100
BV605	(CD115)	1:50
APC	(Ly6C)	1:100
BV650	(Streptavidin)	1:350

Figure S2. Layout of 96-well plate and innate/adaptive flow panels. After cell isolation, lung and blood samples were split in half so they could be stained with two separate antibody panels: one to identify innate cell populations of interest (i.e., neutrophils, macrophages, eosinophils, monocytes, and dendritic cells) and another to identify adaptive cells populations of interest (i.e. B and T lymphocytes). BAL and bone marrow samples were only stained with the innate panel, apart from one experiment where bone marrow samples were stained with "Bone Marrow Flow Panel" to examine hematopoiesis. The top figure was created using BioRender.

Reagent	Quantity (for 1 L of 10X)
NaCl (Sigma-Aldrich)	80 g
$Na_2HPO_4 \cdot 2H_2O$ (Sigma-Aldrich)	17.8 g
KH_2PO_4 (Sigma-Aldrich)	2.4 g
KCl (EMD Millipore)	2.0 g

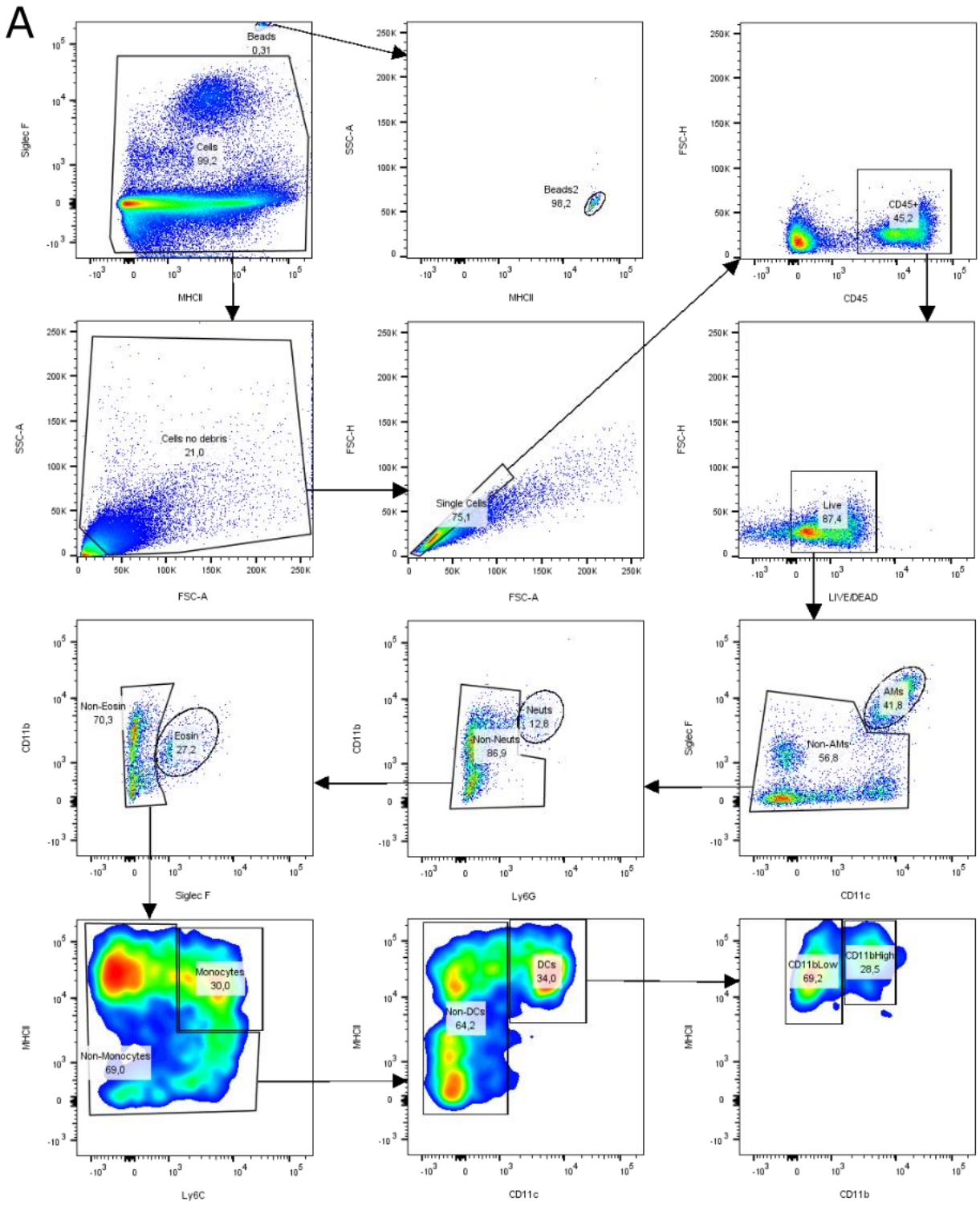
All reagents were dissolved in 850 mL of H_2O. The pH was adjusted to 7.2-7.4, and then H_2O was added to 1000 mL.

Table S1. Phosphate buffered saline (PBS) protocol.

Reagent	Quantity (for 1 L)
NH_4Cl (Sigma-Aldrich)	8.02 g
$KHCO_3$ (Sigma-Aldrich)	1.0 g
Na_2EDTA (EMD Millipore)	37.2 mg

All reagents were dissolved in 850 mL of H_2O. The pH was adjusted to 7.2-7.4, and then H_2O was added to 1000 mL.

Table S2. ACK lysis buffer protocol.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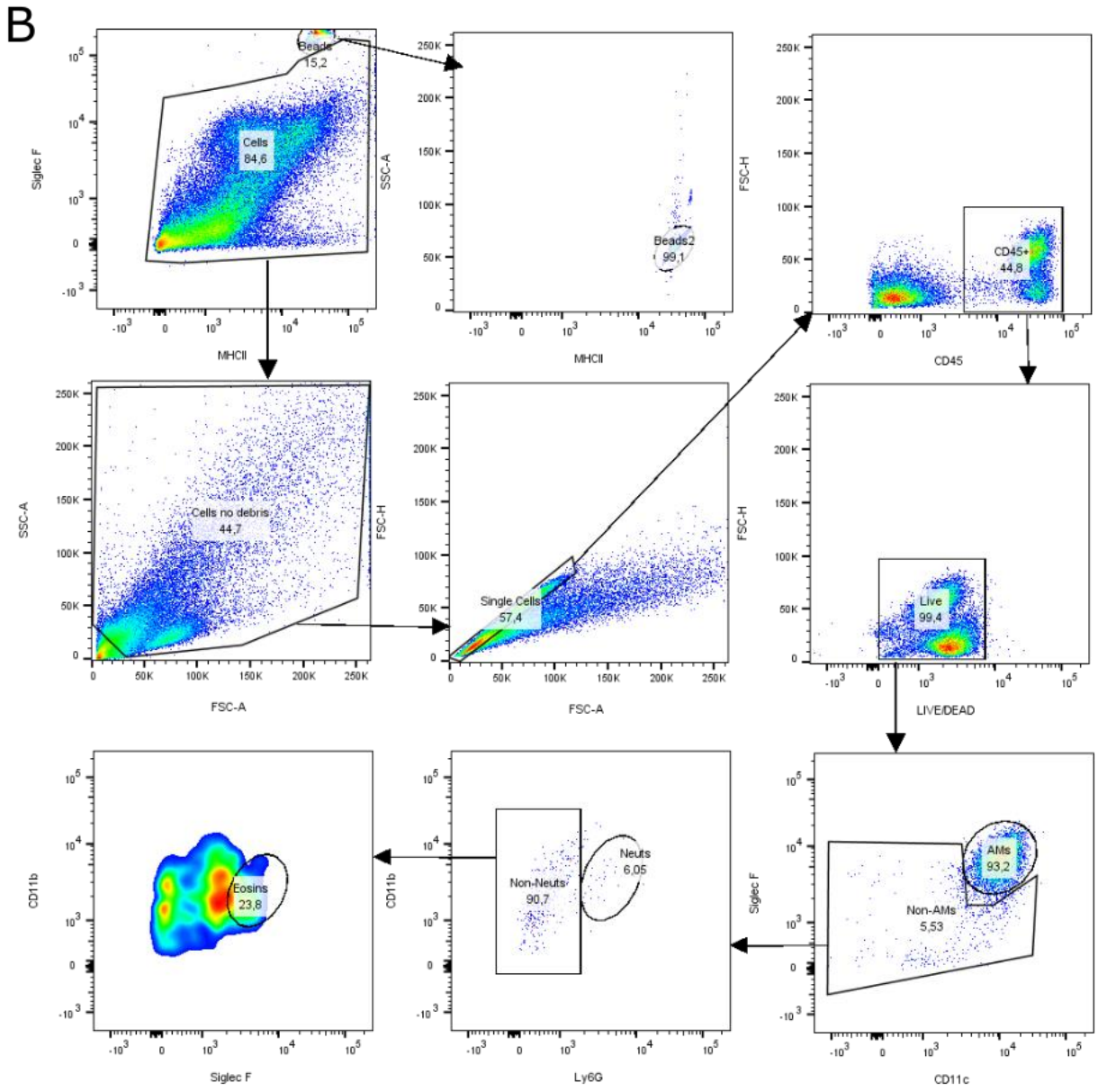
Cells
99,2
Siglec F
MHCII
SSC-A
Beads2
98,2
FSC-H
CD45+
45,2
CD45
Cells no debris
21,0
FSC-A
Single Cells
75,1
Live
87,4
LIVE/DEAD
Non-Eosin
70,3
Eosin
27,2
CD11b
Siglec F
Neuts
12,8
Non-Neuts
86,9
Ly6G
AMs
41,8
Non-AMs
56,8
CD11c
Monocytes
30,0
Non-Monocytes
69,0
Ly6C
DCs
34,0
Non-DCs
64,2
CD11bLow
69,2
CD11bHigh
28,5


B
Beads
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84,6
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FSC-H
FSC-A
Single Cells
57,4
FSC-H
FSC-A
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LIVE/DEAD
CD11b
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CD11c

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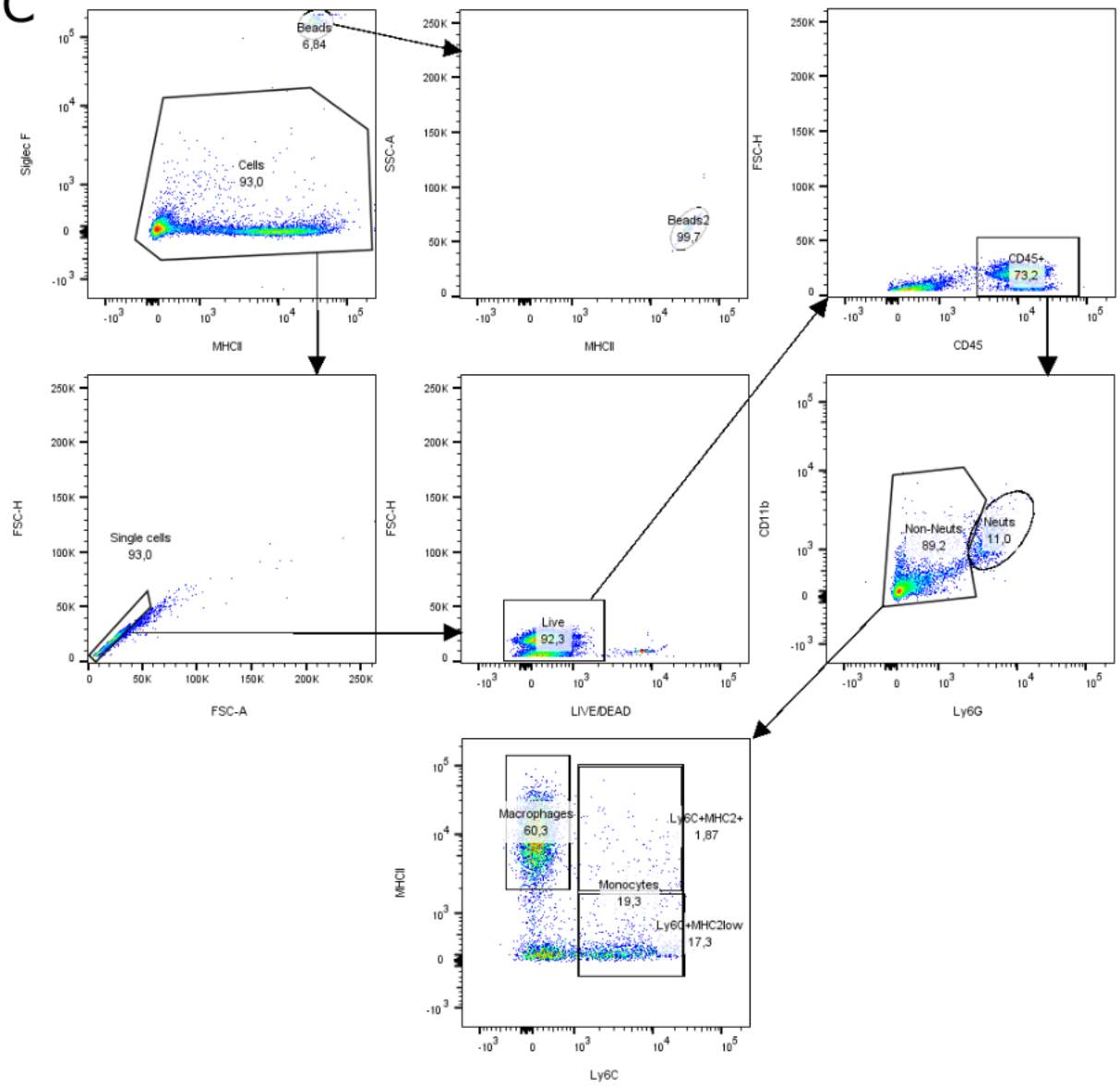
Beads
6,84
Cells
93,0
Siglec F
MHCII
SSC-A
Beads2
99,7
FSC-H
CD45+
73,2
CD45
Single cells
93,0
FSC-A
FSC-H
Live
92,3
LIVE/DEAD
CD11b
Non-Neuts
89,2
Neuts
11,0
Ly6G
Macrophages
60,3
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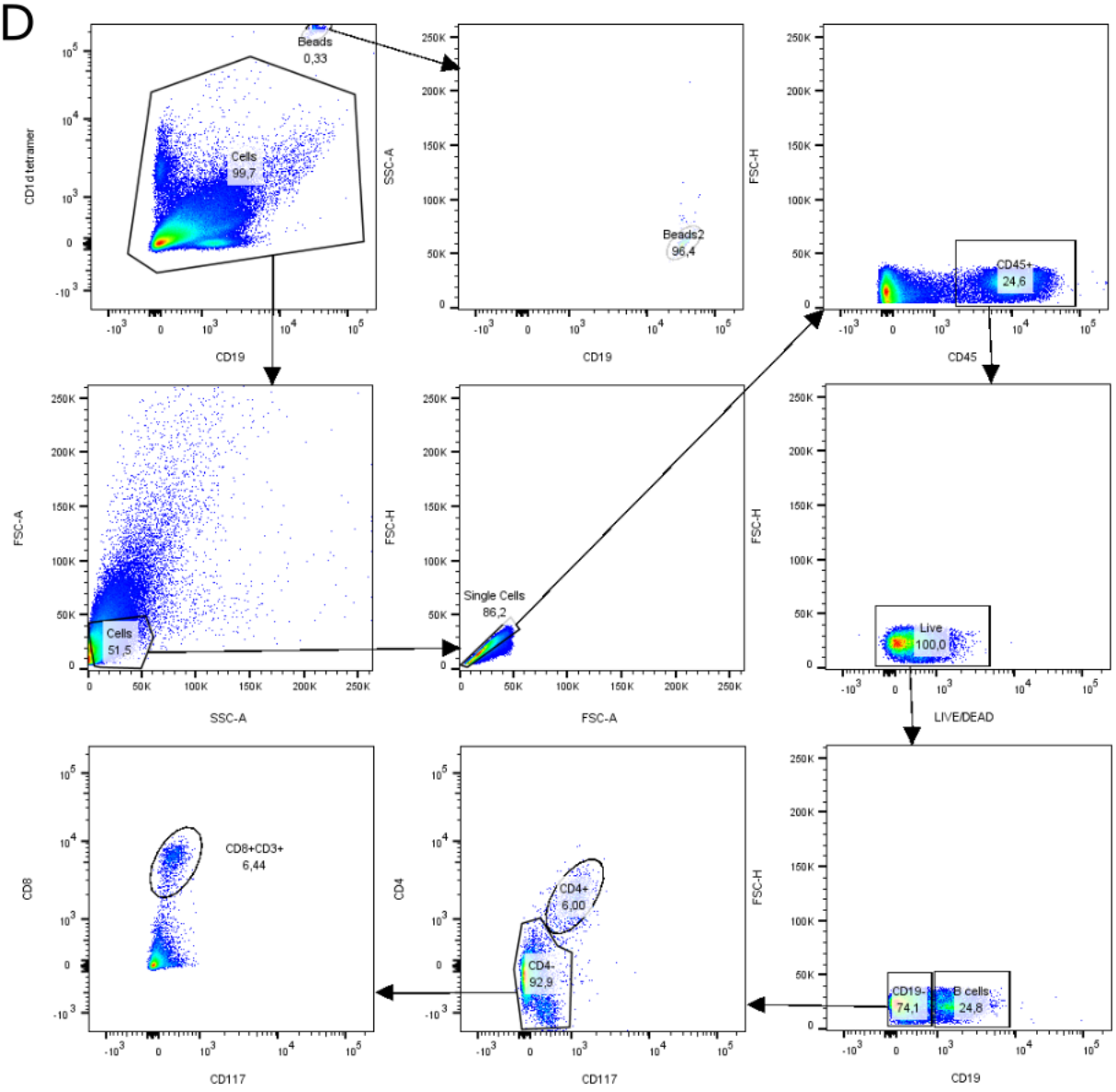
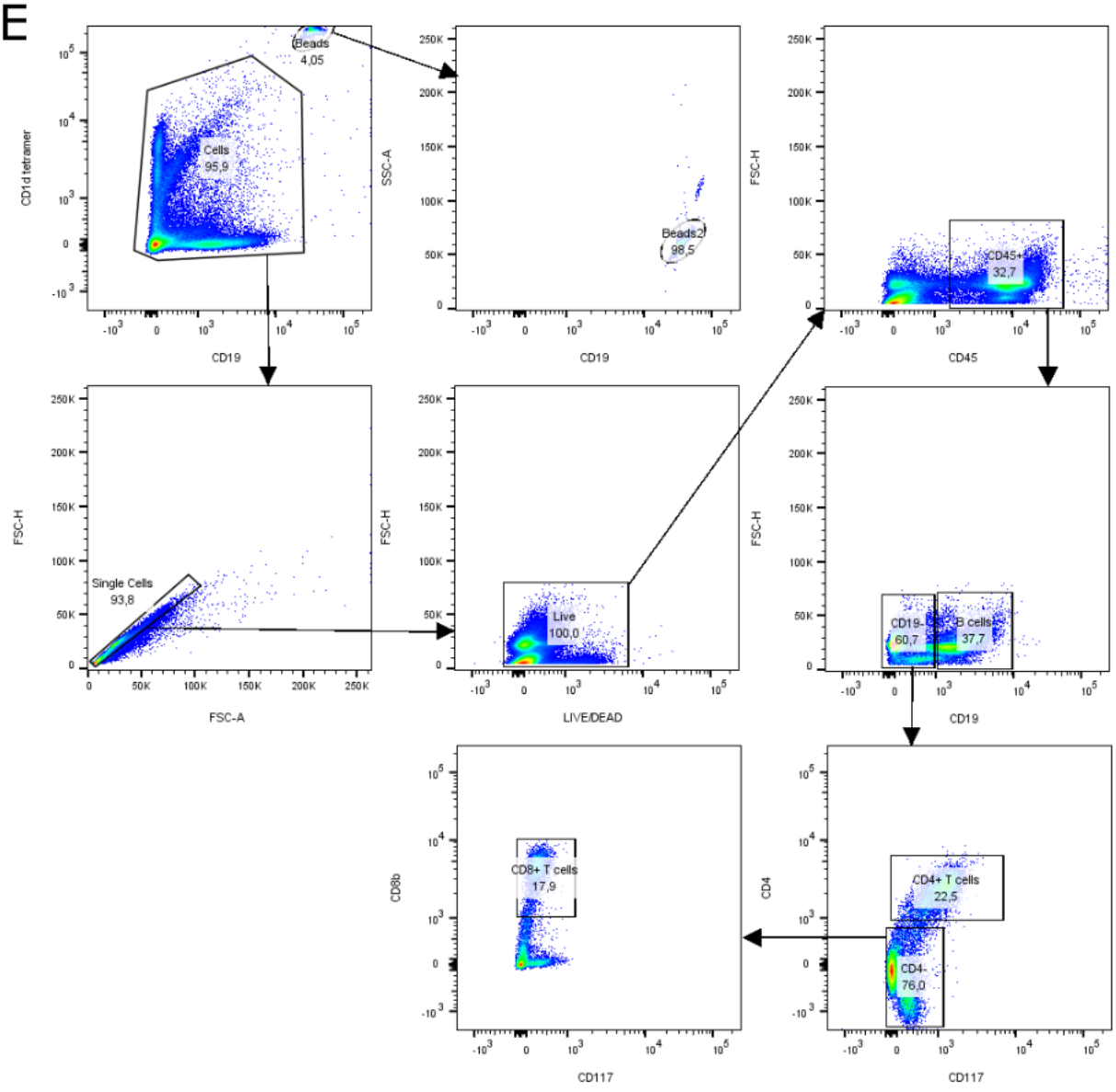
Monocytes
19,3
Ly6C+MHC2low
17,3
MHCII
Ly6C

D


Beads
0,33
Cells
99,7
CD1d tetramer
CD19
SSC-A
Beads2
96,4
CD19
FSC-H
CD45+
24,6
CD45
FSC-A
Cells
51,5
SSC-A
FSC-H
Single Cells
86,2
FSC-A
FSC-H
Live
100,0
LIVE/DEAD
CD8
CD8+CD3+
6,44
CD117
CD4
CD4+
6,00
CD4-
92,9
CD117
FSC-H
CD19-
74,1
B cells
24,8
CD19

E


Beads
4,05
Cells
95,9
CD1d tetramer
CD19
SSC-A
Beads2
98,5
CD19
FSC-H
CD45+
32,7
CD45
FSC-H
Single Cells
93,8
FSC-A
FSC-H
Live
100,0
LIVE/DEAD
FSC-H
CD19-
60,7
B cells
37,7
CD19
CD8b
CD8+ T cells
17,9
CD117
CD4
CD4+ T cells
22,5
CD4-
76,0
CD117

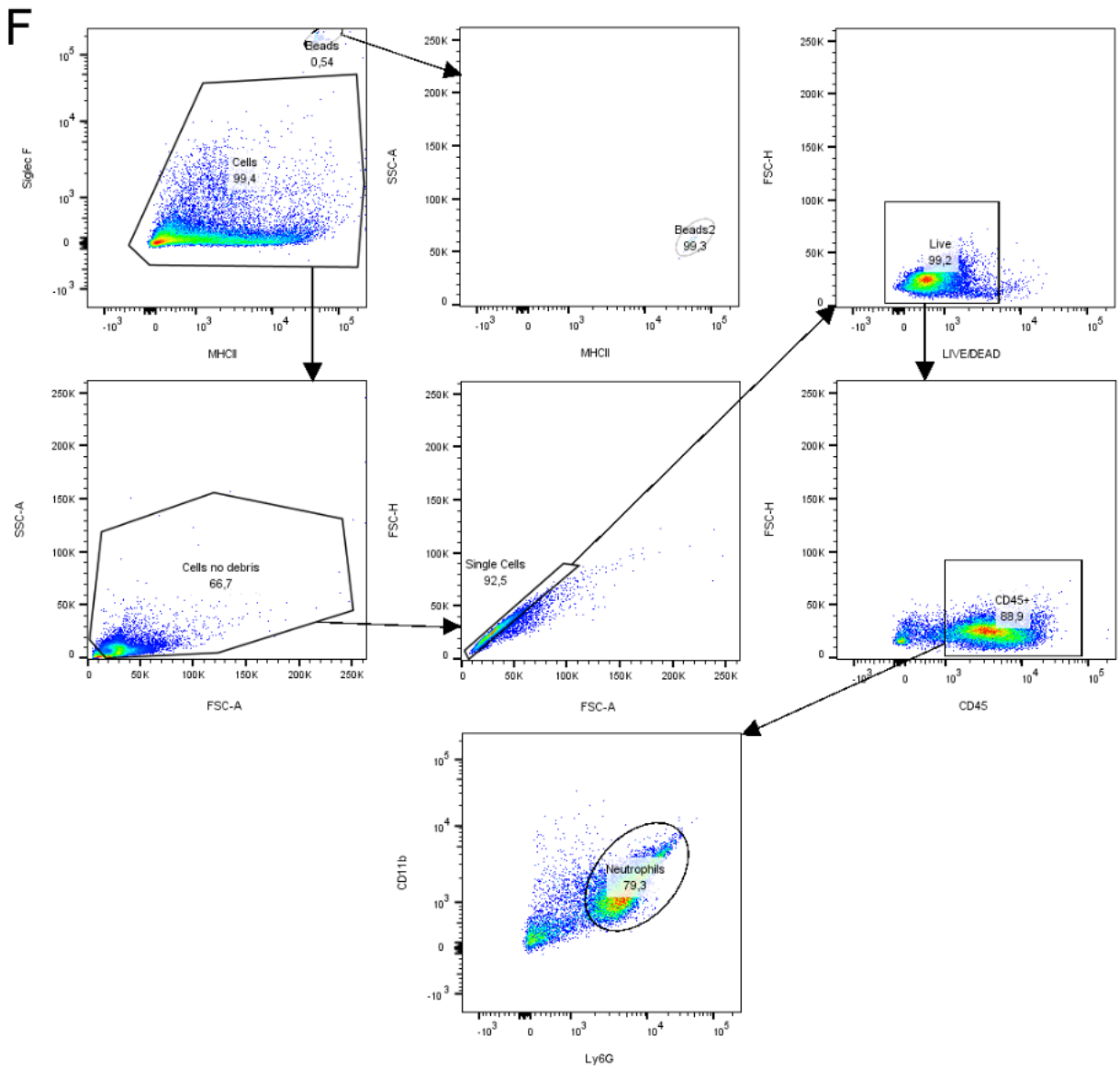


Figure S3. Representative gating strategies. (A) Lung tissue (innate panel). (B) BAL. (C) Blood (innate panel). (D) Lung tissue (adaptive panel). (E) Blood (adaptive panel). (F) Bone marrow (innate panel).

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