MODIFICATION OF RESPIRATORY SYSTEM INNATE DEFENSE MECHANISMS BY FLAVORING AGENTS IN ELECTRONIC CIGARETTES

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ABSTRACT

Phillip W. Clapp: Modification of Respiratory System Innate Defense Mechanisms by Flavoring Agents in Electronic Cigarettes
(Under the direction of Ilona Jaspers)

Electronic cigarettes (e-cigarettes) have emerged as a potentially safer alternative to traditional cigarettes. Because e-cigarettes deliver nicotine without burning tobacco, their use likely confers a lower overall health risk than inhaling combustible tobacco products. However, e-cigarette emissions contain solvents (propylene glycol and glycerol), flavorings, and other additives, which have not been evaluated for inhalation toxicities. The inhalation of flavoring agents, which are frequently aldehydes, poses a significant unknown in regards to the potential health risks of e-cigarette use as many of these chemicals are structurally similar to toxic aldehydes in cigarette smoke. Furthermore, aldehyde flavoring agents are often used at exceedingly high concentrations in e-cigarettes which may lead to high exposure doses. The work presented in this dissertation focuses on evaluating the effects of flavored e-cigarettes, particularly those containing α,β-unsaturated cinnamaldehyde, on respiratory innate defense mechanisms and assessing whether and how these flavorings may alter susceptibility to respiratory infections. In Chapter 2, we report that cinnamon-flavored e-cigarette refill liquids and cinnamaldehyde modify human respiratory innate immune cell defense functions, including neutrophil and alveolar macrophage phagocytosis, neutrophil extracellular trap formation, and natural killer (NK) cell-mediated killing of leukemia cells. Based on these observations we established the cinnamaldehyde exposure concentrations that inhibit normal defense functions (IC_{50}) and induce cytotoxicity (LC_{50}). In Chapter 3, we

report that cinnamon-flavored e-liquids, cinnamon e-cigarette aerosols, and cinnamaldehyde temporarily suppress motile cilia on human bronchial epithelial cells (hBE) and that this response is driven by transient inhibition of mitochondrial respiration and glycolysis. In Chapter 4, we report that cinnamaldehyde, guaiacol, and eugenol elicit a dose-dependent bimodal effect on Nrf2-pathway activation in bronchial epithelial cells (Beas-2B) and cinnamaldehyde exposure significantly depletes cellular glutathione (GSH) levels at low micromolar concentrations. Taken together, the work presented in this dissertation demonstrates that flavoring agents, in the context of e-cigarette use, are capable of dysregulating essential respiratory defense responses, which may increase susceptibility of respiratory infections and lung disease in e-cigarette users. It is our hope that the information provided here will be useful in the development of health-protective policies aimed at reducing or eliminating harmful and potentially harmful chemicals from e-cigarettes.

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%AA	Percent active area
2-DG	2-Deoxy-D-glucose
Abs	absorbance
ADP	Adenosine diphosphate
AHR	Aryl hydrocarbon receptor
ALDH3A1	Aldehyde dehydrogenase 3 family, member A1
ALI	Air liquid interface
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
AP-1	Activator protein 1
APV	Advanced personal vaporizer
ARE/EpRE	Antioxidant/electrophile response element
ATP	Adenosine triphosphate
AUC	Area under the curve
BAL	Bronchial alveolar lavage
Beas-2B	Bronchial epithelial cell line
BEGM	Bronchial epithelial growth medium
BMI	Body mass index
BSO	Buthionine sulphoximine
c3bi	Complement receptor type 3
CaCC	Calcium activated chloride channels
cAMP	Cyclic adenosine monophosphate

CBC	Complete blood count
CBF	Cilia beat frequency
CD11b	Integrin alpha M
CD11c	Integrin alpha X
CD16	Fragment crystallizable fragment of IgG receptor IIIb
CD3-ζ	Cluster of differentiation 3, zeta
CD40	Cluster of differentiation 40
CD94	Cluster of differentiation 94
CDC	Centers for disease control and prevention
CFSE	Carboxyfluorescein succinimidyl ester
CFTR	Cystic fibrosis transmembrane conductance regulator
cGMP	Cyclic guanosine monophosphate
COPD	Chronic obstructive pulmonary disease
CRP	C-reactive protein
CXCL2	Chemokine (C-X-C motif) ligand 2
CYP1A1	Cytochrome P450 family 1, subfamily A, member 1
CYP1B1	Cytochrome P450 family 1, subfamily B, member 1
DAP12	TYRO protein tyrosine kinase-binding protein
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
Duox1	Dual oxidase 1

ECAR	Extracellular acidification rate
EDTA	Ethylenediaminetetraacetic acid
EI	Electron ionization
ELISA	Enzyme-linked immunosorbent assay
ENDS	Electronic nicotine delivery system
eV	Electronvolt
FBS	Fetal bovine serum
Fc	Fragment crystallizable
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Fcε-RIγ	High affinity immunoglobulin epsilon receptor subunit gamma
FEMA	Flavor and extract manufacturers association of the United States
FEV_1	Forced expiratory volume in the first second
fMLP	N-Formylmethionine-leucyl-phenylalanine
FVC	Forced vital capacity
G-CSF	Granulocyte colony-stimulating factor
GC	Gas chromatography
GC/MS	Gas chromatography–mass spectrometry
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GPX1	Glutathione peroxidase 1
GRAS	Generally recognized as safe
GSH	Glutathione
GST	Glutathione s-transferase

HBD-1	Human beta defensin 1
HBD-2	Human beta defensin 2
hBE	Human bronchial epithelial cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Her-2	Human epidermal growth factor receptor 2
HO-1	Heme oxygenase 1
HPLC	High performance liquid chromatography
HPLC-MS/MS	High performance liquid chromatography-tandem mass spectrometry
IC_{50}	The half maximal inhibitory concentration
IFNγ	Interferon gamma
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
IL-1	Interleukin-1
IL-10	Interleukin-10
IL-12	Interleukin-12
IL-13	Interleukin-13
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-6	Interleukin-6
IL-8	Interleukin-8
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif

K562	Human immortalized myelogenous leukemia cell line
KBM	Keratinocyte basal medium
Keap1	Kelch-like ECH-associated protein 1
KGM	Keratinocyte growth medium
KIR	Killer-cell immunoglobulin-like receptor
LDH	Lactate dehydrogenase
Li-ion	Lithium ion
Li-poly	Lithium polymer
LILR	Leukocyte immunoglobulin-like receptors
LiMn	Lithium manganese oxide
LPB	Lipopolysaccharide binding protein
LPS	Lipopolysaccharide
Ly49	Killer cell lectin-like receptor subfamily A
m/z	Mass to charge ratio
MAL	Myelin and lymphocyte protein
MARVEL	MAL protein and related proteins for vesicle trafficking and membrane link
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
MRM	Multiple reaction monitoring
MRP	Multidrug resistance protein
MUC1	Mucin 1, cell surface associated
MUC13	Mucin 13, cell surface associated
MUC16	Mucin 16, cell surface associated

MUC4	Mucin 4, cell surface associated
MUC5AC	Mucin 5AC, oligomeric mucus/gel-forming
MUC5B	Mucin 5B, oligomeric mucus/gel-forming
MyD88	Myeloid differentiation primary response 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinucleotide
NET	Neutrophil extracellular traps
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NiCad	Nickel cadmium
NiMh	Nickel-metal hydride
NIOSH	The National Institute for Occupational Safety and Health
NIST	National Institute of Standards and Technology
NK	Natural killer
NKG2A	Killer cell lectin-like receptor subfamily C, member 1
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
NQO1	NAD(P)H dehydrogenase (quinone 1)
Nrf2	Nuclear factor erythroid 2 (NFE2)-related factor 2
OCR	Oxygen consumption rate
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCD	Primary ciliary dyskinesia
PFA	Paraformaldehyde
PG	Propylene glycol

PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
PMN	Polymorphonuclear leukocyte
ppb	Parts per billion
ppm	Parts per million
PRR	Pattern recognition receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute 1640 medium
SAP	Serum amyloid P component
SAVA	Sisson Ammons Video Analysis
sCD14	Soluble cluster of differentiation 14
SD	Standard deviation
SEM	Standard error of the mean
SFN	Sulforaphane
siRNA	Small interfering RNA
SP-A	Surfactant protein A
SP-D	Surfactant protein D
SPIROMICS	Subpopulations and intermediate outcome measures in COPD study
SRXN1	Sulfiredoxin 1
Tc99m-SC	Technetium 99m sulfur colloid
TCA	Trichloroacetic acid
TCGA	The Cancer Genome Atlas

TEER	Transepithelial electrical resistance
Th1	Type 1 T helper cells
Th17	Type 17 T helper cells
TLR	Toll-like receptor
TLR4	Toll-like receptor 4
TNFα	Tumor necrosis factor alpha
TXNRD1	Thioredoxin reductase 1
US FDA	United States Food and Drug Administration
VEC	Vaped e-liquid condensate
VG	Vegetable glycerin
YRBS	Youth Risk Behavior Surveillance System
ZO-1	Tight junction protein 1
εAdo	N^6-ethenoadenosine
εADP	N^6-ethenoadenosine diphosphate
εAMP	N^6-ethenoadenosine monophosphate
εATP	N^6-ethenoadenosine triphosphate

CHAPTER 1.

INTRODUCTION

1.1 Respiratory system host defense against pathogens and pollutants

Gas exchange is the primary function of the respiratory system where the interface between environment and host is essential for life. Inspired oxygen is transported across alveolar epithelial cells and enters the pulmonary circulation, and carbon dioxide is released from the blood into the external environment. However, this environment/host interface also provides a potential site for injury and infection. An average adult who is moderately active breathes approximately 20,000 liters of air over a 24-hour period. Inevitably, this air contains potentially harmful pathogens, particulate matter, and gasses. Particles and bioaerosols, such as soot, dust, mold, fungi, bacteria, and viruses can deposit on respiratory mucosal surfaces and cause damage to the airways and underlying tissues. Particle deposition in the lung is largely dependent upon size and aerodynamic diameter. Particles greater than 10μm generally deposit in the nasopharynx and oropharynx by impaction where inertial forces carry them out of the airstream and against the posterior pharyngeal wall (2). Particles between 5μm and 10μm enter the conducting airways but generally sediment onto the mucociliary surface. Particles less than 5μm (the size of viral particles and many bacteria) reach the respiratory airways and alveoli. As protection from environmental threats, respiratory tissues have evolved a tightly-regulated immune system which relies on innate and adaptive responses to neutralize and eliminate foreign or dangerous material. Innate immunity is the non-specific first response to inhaled material and is composed of physical

barriers, such as airway mucus, mucociliary clearance, and epithelial tight junctions, soluble antimicrobial molecules, such as surfactant proteins and cytokines, and resident and circulating innate immune cells, such as macrophages and neutrophils (3). Cellular cross-talk during innate immune responses is necessary to establish the appropriate inflammatory response and to direct adaptive immune responses (4, 5). Adaptive immunity is an antigen-specific, cell- and antibody-mediated immune response to eliminate specific invading pathogens (4). The adaptive immune response is mediated by antigen-presenting cells (predominately dendritic cells and macrophages) and T and B lymphocytes, and typically results in immunological memory of a specific pathogen (4, 6). Effective communication between the innate and adaptive immune systems is not only essential for eliminating potential threats, but also for discriminating between potential threats, host tissues, and innocuous substances, and maintaining a state of tolerance to prevent unnecessary inflammation. Disruption of normal respiratory immune function increases susceptibility to respiratory infection, stimulates tissue inflammation, and promotes tissue damage. This can exacerbate existing respiratory conditions, such as asthma (7) and cystic fibrosis (8), and contribute to the development and progression of lung disease, such as pulmonary fibrosis (9) and chronic obstructive pulmonary disease (COPD) (10, 11). The work in this dissertation focuses on the respiratory innate immune system, which will be discussed in more detail in the following sections.

1.2 Innate immunity in the respiratory system

The respiratory innate immune system is an evolutionarily conserved defense system that provides the first line of defense against inhaled pathogens and foreign material. The components of the innate immune system include 1) airway epithelial cells and physical and

mechanical barriers at the luminal epithelial surface, 2) circulating and airway resident leukocytes, and 3) soluble proteins which bind to microbial antigens and facilitate uptake by leukocytes. Each of these components will be described in more detail in the following paragraphs.

1.2.1 Physical and mechanical barriers of respiratory innate immune system

Airway epithelial cells. Airway epithelial cells line the entire respiratory tract and provide a continuous protective barrier against inhaled environmental insults. The type and relative abundance of epithelial cell populating the respiratory tract changes depending on the anatomical region of the airway. The proximal conducting airways which include the nasal passages, pharynx, larynx, trachea, bronchi, and bronchioles, are composed of pseudostratified columnar epithelial cells, goblet cells (mucus-producing secretory cells), serous cells (serous fluid-producing secretory cells in submucosal gland acini), and basal cells (multi-potent stem cells), which function to humidify, warm, and clean inspired air (12-14). As bronchi branch into bronchioles and terminal bronchioles, the pseudostratified epithelium gradually transitions to a simple columnar epithelium and then a simple cuboidal epithelium which is predominately populated with ciliated epithelial cells, club cells (non-ciliated, non-mucus producing secretory cells), and neuroendocrine cells (15). The most distal respiratory airways consist of respiratory bronchioles, alveolar ducts, and clusters of alveoli composed of simple squamous Type I and cuboidal Type II pneumocytes which facilitate gas exchange and secrete surfactant proteins, respectively (16). While epithelial cells of the lower respiratory airways do provide a barrier and generate immune responses to pathogens, the proximal conducting airways play the greatest role in defending the lungs from inhaled foreign material (17).

Airway epithelial cell junctions. An essential feature of airway epithelium is the apical junctional complex that forms between neighboring epithelial cells to regulate paracellular permeability. These complexes are made of apical tight junctions (formed from claudin family proteins, tight junction–associated MAL and related proteins for vesicle trafficking and membrane link (MARVEL) proteins, and IgG-like proteins), which regulate the paracellular transport of ions and certain molecules, and underlying adherens junctions (formed from E-cadherin and nectin family proteins), which initiate and maintain cell-cell adhesion through interactions with the cellular cytoskeleton (18). In addition to creating a nearly impenetrable cell-cell barrier that prevents inhaled pathogens and other environmental insults from injuring the airways, apical junction complexes serve as signaling platforms that regulate gene expression and cell proliferation and are essential for establishing apical–basal epithelial cell polarity (19).

Airway surface liquid. Mucociliary clearance is an important innate defense mechanism that protects the airways from inhaled infectious and toxic substances. Goblet cells in the proximal conducting airways and submucosal gland acini secrete high molecular weight gel-forming mucin glycoproteins (predominately MUC5AC and MUC5B) which confer upon the airway surface liquid the biochemical and biophysical properties necessary for entrapment and transport of foreign material from the lung (20). The airway surface liquid is composed of two layers; an upper layer of viscoelastic mucus which sits above a less viscous periciliary layer. The periciliary layer is composed of a hydrated macromolecular mesh of membrane-spanning mucins (predominately MUC1, MUC4, and MUC16) and large membrane-bound glycoproteins that are densely tethered to cilia, microvilli, and the epithelial surface (21). In the large conducting airways, the periciliary layer is approximately

7μm in height (the length of outstretched cilia) and provides a lubricating environment for cilia beating (17). Effective mucociliary clearance of airway surface liquid depends on the composition of the mucus layer (i.e. the relative abundance of gel-forming mucins) and hydration of the periciliary layer.

Airway cilia. Motile airway cilia are hair-like structures that provide the mechanism by which airway surface liquid containing trapped material is transported from the lung. Ciliated cells account for over 50% of all epithelial cells in the proximal conducting airways and each ciliated cell has approximately 200 to 300 cilia projecting from its apical surface (22). Cilia are microtubule-based organelles, which extend from basal bodies at the apical cell surface. An axoneme, which is the central strand of a cilium, contains a ring of nine microtubule doublets surrounding a central pair, known as a 9+2 configuration. Inner and outer arm dynein ATPases connect the nine microtubule doublets and generate the force needed for cilia motility in an ATP-dependent process. There are thousands of dynein ATPase motors in a single axoneme which require sufficient and sustained levels of ATP to maintain cilia function (23). Although ATP controls basal cilia function, regulation of cilia beat frequency depends on other intracellular and secreted second messengers including cyclic nucleotides (cAMP and cGMP), nitric oxide, intracellular calcium, progesterone, and pH (24-26).

1.2.2 Leukocytes of the respiratory innate immune system

Pulmonary macrophages. Pulmonary macrophages are the most abundant immune cell in the lung under normal homeostatic conditions and provide a variety of specialized functions, including maintenance of pulmonary homoeostasis, removal of cell debris,

immune surveillance, microbial clearance, responses to infection, and resolution of inflammation (27). The remarkable plasticity of these cells can be attributed to differences in their origin, lung microenvironment, and exposure to environmental stimuli. Resident alveolar macrophages (characterized as $CD11b^{low}/CD11c^{high}$) have a high phagocytic capacity and secrete antimicrobial compounds, such as oxygen metabolites, lysozyme, and proteases to eliminate pathogens and debris that have infiltrated the respiratory airways (27, 28). However, alveolar macrophages also actively suppress the induction of adaptive immunity to maintain lung homeostasis (29). When exposed to larger quantities of pathogens or particularly virulent microbes, alveolar macrophages can mount a robust immune response by synthesizing and secreting an array of cytokines (including IL-1, IL-6, and TNF-α), chemokines (including IL-8), and arachidonic acid metabolites (including prostaglandins and leukotrienes) to promote inflammation and recruit neutrophils to the alveoli (30, 31). Less is known about interstitial macrophages (characterized as $CD11b^{high}/CD11c^{low}$), which reside in the lung parenchyma. Recent studies suggest that interstitial macrophages play a significant role in modulating adaptive immune responses by TLR4/MyD88-mediated production of IL-10 and subsequent negative regulation of Th2- and Th17-mediated inflammatory responses (32).

Dendritic cells. Dendritic cells are a heterogeneous population of antigen-presenting cells that bridge the innate and adaptive immune systems. These cells form an elaborate network throughout the conducting airways, lung interstitium, lung vasculature, pleura, and bronchial lymph nodes (33, 34). Myeloid dendritic cells, the predominant respiratory dendritic cell type, are continuously recruited to the lungs to serve as sentinels for pathogens in the respiratory tract. Myeloid dendritic cells localize at the epithelial basal lamina and

extend their cellular processes between airway epithelial cells into the lumen where pattern recognition receptors (PRRs) bind to microbial proteins on pathogens (35, 36). Upon recognition, pathogens are phagocytosed and destroyed in phagosomes; however, this process is much gentler than phagosomal degradation in macrophages and neutrophils, allowing for the preservation of antigen integrity (37). Pathogen proteins are processed into peptide fragments and antigens are presented via major histocompatibility complex (MHC) molecules to T cells at regional lymph nodes (35). In addition to antigens presented on MHC molecules, T cells require co-stimulatory signals (such as CD80/CD86), polarizing signals (such as IL-12 and CCL2), and feedback stimulation (by CD40) to sufficiently mount an adaptive immune response (38). Lack of complementary signaling induces T cell anergy as a mechanism of inactivation and immune tolerance (38).

Neutrophils. Neutrophils, also known as polymorphonuclear cells (PMNs), are the primary effector cell in acute inflammatory responses and actively eradicate pathogens with a variety of killing mechanisms (39). They are continuously produced in the bone marrow by myeloid precursor cells in a process regulated by granulocyte colony stimulating factor (G-CSF) (40). However, recent data suggest that mature neutrophils may proliferate outside of the bone marrow in response to specific signaling molecules, such as serum amyloid A (41). As neutrophils mature, they form primary, secondary, tertiary, and secretory granules, which are filled with proteins necessary for extravasation and the recognition and elimination of pathogens. Primary (azurophilic) granules store toxic antimicrobial mediators, including myeloperoxidase, lysozyme, elastase, defensins, and cathepsins (42). Secondary (specific) granules contain proteins necessary for extravasation and recognition of pathogen-associated and damage-associated molecular patterns, which include collagenase, lactoferrin, matrix

metalloprotease 9, and bacterial protein (fMLP) and complement (c3bi) receptors (43). Tertiary granules are similar to secondary granules and contain matrix metalloproteases and gelatinase (44). Secretory granules/vesicles contain membrane proteins, receptors, and pre-formed cytokines that play an important role in adhesion to endothelial cell membranes and initiation of inflammatory responses, including CD10, CD11b, alkaline phosphatase, plasma proteins, IL-6, IL-12, and CXCL2 (44, 45). Under normal physiologic conditions, neutrophils remain in the systemic circulation while congregating in specific tissues, such as the bone marrow, spleen, liver, and lungs (46, 47). The pulmonary vasculature represents the largest pool of neutrophils in the human body; however there are limited numbers of neutrophils in the airways and alveoli of healthy individuals (48). Conversely, neutrophils are the predominant leukocyte in the nasal passages (49). During acute inflammation, proinflammatory chemokines secreted by pulmonary macrophages, mast cells, and epithelial cells (predominately IL-8), and bacterial-derived chemotactic peptides (such as fMLP), stimulate neutrophils to adhere to capillary walls and extravasate across the thin barrier separating the capillary lumen from the alveolar space (50). These proinflammatory signals result in a rapid and often massive influx of neutrophils into the airways to rapidly eliminate pathogens and suppress infection. Neutrophils eliminate microbes using both intra- and extra-cellular methods. Phagocytosis is the most common method neutrophils use to eradicate pathogens, and neutrophils kill phagosome-encapsulated pathogens with antimicrobial proteins from granules and reactive oxygen species generated by NADPH oxygenase-dependent mechanisms. In instances of more severe infection, primary granules will undergo exocytosis and the toxic contents will be released into the extracellular milieu, thereby eliminating more pathogens than could be cleared by phagocytosis (42). Neutrophils can also

eliminate extracellular organisms through the release of neutrophil extracellular traps (NETs), which are composed of sticky de-condensed chromatin containing antimicrobial histones, toxic granule proteins (including cathepsins, defensins, and lacteoferrin), and enzymes (including lysozyme, neutrophil elastase, and myeloperoxidase) (51). Through this active form of cell death, neutrophils ensnare pathogens in a microbicidal web and, thereby, prevent the spread of infection. While neutrophils are extremely efficient at eliminating pathogens, their antimicrobial actions can also damage host tissues, disrupt the resolution of acute inflammation, and promote chronic inflammation. Aberrant neutrophil activation and accumulation of neutrophils in the airways is associated with the pathogenesis and progression of multiple pulmonary diseases, including asthma, bronchitis, pneumonia, pulmonary fibrosis, and COPD (52). Conversely, aberrant suppression of neutrophil function increases susceptibility to respiratory infection and contributes to the frequent infections associated with chronic granulomatous disease and congenital myeloperoxidase deficiency (53-55).

Natural killer (NK) cells. Natural killer (NK) cells are cytotoxic lymphocytes that play an essential role in innate immune response by identifying and killing tumorigenic and pathogen-infected host cells. Using a repertoire of activating and inhibitory receptors, NK cells circulate through the vasculature, lymphatic system, and peripheral tissues interrogating host cells for the presence or absence of specific surface markers indicative of transformation or infection (56). While NK cells account for approximately 10% of the resident leukocyte population in the lung, they represent nearly 25% of the leukocyte population in the nasal passages (57, 58). However, NK cell numbers rapidly increase in both the lung and nasal passages in response to infection and inflammation. Inhibitory receptors on NK cells, which

include major histocompatibility complex class I receptors, killer cell immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LILRs), Ly49, and CD94-NKG2A, search for ligands on host cells which inhibit NK cell killing (59). The inhibitory receptors share a common cytoplasmic immunoreceptor tyrosine-based inhibitory motif (ITIM) that, when activated, recruit phosphatases to the interface between the NK cell and host cell. Phosphatases subsequently dephosphorylate protein substrates on tyrosine kinases linked to activating receptors which terminates killing mechanisms and NK cell proliferation signals (56). NK cells also contain a vast combinatorial array of activating receptors (including CD16, DAP12, Fcε-RIγ, and CD3-ζ) that work in conjunction with inhibiting receptors to regulate targeted cell killing (60). Activating receptors contain a cytoplasmic immunoreceptor tyrosine-based activating motif (ITAM) that, when stimulated by ligand binding, induces actin cytoskeleton reorganization, NK cell polarization, release of cytotoxic granules containing perforin and granzyme proteins, and transcription of various cytokines and chemokines, including IFNγ, Th2 cytokines (IL-5 and IL-13), and regulatory cytokines such as IL-10 (56, 61). The function of NK cells is intricately regulated through complex signaling pathways, and simultaneous activation of different pairs or combinations of receptors can enhance or suppress effector function (56). Normal NK cell function is critical for maintaining respiratory immune homeostasis as functional impairment is associated with increased cancer risk and enhanced susceptibility of respiratory virus infections (57, 60, 62).

Other cell types. There are other cell types that contribute to innate immunity in the lung or function to bridge the innate and adaptive immune responses. However, they are generally not recognized specifically as cells of the innate immune system. These include mast cells, eosinophils, and basophils. Each of these are granulocytes with specific effector

functions. Mast cells are myeloid-derived cells that reside near epithelial tissue, airway smooth muscle, blood vessels, nerves, and submucosal secretory glands (63). Mast cell granules contain an abundance of proteases including chymase and β-tryptase, as well as cytokines, histamine, heparin, GM-CSF, proteoglycans, and leukotrienes. Activation of mast cells by antigen-specific IgE binding to FcεRI receptors results in degranulation and the rapid onset of allergy-type symptoms (64). In the context of acute respiratory bacterial infections, mast cells are capable of binding and phagocytosing bacteria. Furthermore, they can secrete inflammatory mediators, including TNFα, IL-4, IL-6, IL-10, leukotrienes, and mast cell-specific proteases to modulate innate and adaptive inflammatory responses (63). Eosinophils and basophils represent 1-3% and less than 1% of the circulating leukocyte population, respectively (65). While the specific granule content between these cells varies, they both play a central role in antimicrobial responses to parasitic helminths and RNA viruses. Along with mast cells, eosinophils and basophils mediate allergic and asthmatic responses (65). In the context of innate immunity, eosinophils, but not basophils, are capable of phagocytosing bacteria (66, 67). However, both contain pre-formed cytokines, chemokines, and growth factors, which can be immediately released in response to acute inflammatory signals (65).

1.2.3 Humoral components of respiratory innate immunity

Humoral, or soluble, components in the alveolar and airway lining fluids play an important role in respiratory innate immune responses by directly neutralizing pathogens or facilitating interactions between pathogens and effector cells. Most of these components, including naturally occurring antibodies, defensins, collectins, lactoferrin, and lysozyme, are produced and secreted by resident cells of the respiratory system. However, others, such as C-reactive protein (CRP) and serum amyloid P (SAP), are synthesized in other tissues and

transported to the lungs via the systemic circulation (68). Both CRP and SAP are in the pentaxin family of proteins and function in the innate immune system as opsonins that bind bacteria, fungi, and yeast, to enhance their phagocytosis and killing by macrophages and neutrophils. The airway surface liquid lining the upper airways is rich in protective soluble components, including pentaxins, lysozyme (a glycoside hydrolase enzyme that disrupts glycosidic bonds in the cell walls of some bacteria), lactoferrin (an iron-binding glycoprotein that has bacteriostatic and bactericidal functions), IgA and IgG (naturally occurring antibodies which bind to pathogens, cell debris, and apoptotic cells, and promote pathogen recognition by phagocytes), and defensins (cationic antimicrobial peptides that activate bacterial cell wall lytic enzymes and disrupt virus function) (69-71). The alveolar fluid in the lower respiratory airways is also saturated with soluble immune mediators. These include IgG, complement proteins (opsonins that enhance pathogen recognition and phagocytosis by alveolar macrophages and neutrophils), hydrophilic surfactant proteins SP-A and SP-D (collectin proteins that opsonize bacteria and enhance phagocytosis by alveolar macrophages and neutrophils), sCD14 (a soluble co-receptor for bacterial lipopolysaccharide (LPS) which enhances macrophage recognition of bacteria), and LBP (a protein that binds to shed bacterial LPS to enhance LPS recognition by specific protein receptor complexes on leukocytes) (3, 72-74). These soluble mediators of innate immunity enhance the physical and cellular components of the respiratory innate immune system and provide another layer of protection against inhaled microbes. Compromised humoral immunity, particularly reductions in the levels or functionality of IgG and IgA, has been shown to increase susceptibility to bacterial and viral infections (75, 76).

1.3 Effects of cigarette smoke on respiratory innate immune system

Cigarette smoke causes harm to nearly every organ in the body and smokers have a significantly higher risk for multiple cancers, heart disease, respiratory tract infections, chronic lung disease, Crohn's disease, rheumatoid arthritis, and delayed recovery from injury (77). Some of the constituents of cigarette smoke that cause deleterious health effects include nicotine, 'tar' (resinous, partially combusted particulate matter containing polycyclic aromatic hydrocarbons, hydroquinones, catechol, phenol, benzene, and other substances), aldehydes (including formaldehyde, acetaldehyde, acrolein, and crotonaldehyde), metals (arsenic, cadmium, chromium, nickel, and lead), benzopyrenes, ammonia, carbon monoxide, cyanide, and nitrogen oxides (78). As cigarette smoke enters the respiratory tract, soluble gases are rapidly absorbed in the conducting airways and large particles deposit on airway walls. Small particles remain in the air stream traversing the branching airways until they ultimately settle in the alveoli or are exhaled. Once inhaled, cigarette smoke causes activation or suppression of various respiratory innate immune components.

1.3.1 Effects of cigarette smoke on physical and mechanical innate defenses

Chronic mucus hypersecretion, commonly referred to as excessive phlegm, is a hallmark of chronic bronchitis and is strongly associated with loss of pulmonary function, recurring respiratory infections, and the development of COPD (79). Furthermore, there is a significant association between chronic mucus hypersecretion, reductions in pulmonary function (FEV_1), and death from obstructive lung disease (80). Cigarette smoking stimulates mucus hypersecretion and is the primary etiological factor for COPD. However, exactly how cigarette smoke stimulates mucus hypersecretion is still unclear (81). Healthy smokers with normal lung function as well as cigarette smokers with symptoms of chronic bronchitis and

chronic airflow limitation have been reported to have a greater number of goblet cells and inflammatory cells in the epithelium of peripheral airways as compared to non-smoker controls (82). Haswell and colleagues exposed well-differentiated human bronchial epithelial cells (hBE) to cigarette smoke total particulate matter and various aldehydes present in cigarette smoke, including formaldehyde, acetaldehyde, and acrolein. They found that cigarette smoke particulate matter and acrolein, but not formaldehyde or acetaldehyde, significantly increased the number of MUC5AC positive goblet cells in cultures (83). Borchers and colleagues observed that acrolein and the inflammatory mediators prostaglandin E2 and TNFα, increased MUC5AC transcription in H292 lung carcinoma cells, suggesting that reactive aldehydes, like acrolein, can directly and indirectly (via inflammatory mediators) increase mucin gene expression (84). Other laboratory data suggest that smoking reduces the hydration of airway surfaces and stimulates mucin production, which leads to increased mucin concentrations in the airway surface liquid (85-87). Button and colleagues recently proposed the "two-gel" model of mucociliary transport which predicts that high mucin content in the viscoelastic upper gel layer of airway surface liquid will out-compete the lower periciliary gel layer for hydration and result in failed mucociliary clearance and accumulation of airway mucus (21). Recent data published by Kesimer and colleagues which used the Subpopulations and Intermediate Outcome Measures in COPD Study (SPIROMICS) cohort were consistent with this model (88). The authors of this study found that high total mucin concentrations (both MUC5AC and MUC5B) in smokers were associated with both patient-reported phlegm production and increased mucoid properties of the expectorated material. Moreover, MUC5B was found to be the dominant secreted mucus in smokers and the ratio of total MUC5AC to total MUC5B concentration was higher among

current smokers as compared to never-smoker control subjects (88). Together, these findings indicate that cigarette smoke and its constituent acrolein modify the physical barriers of the respiratory innate defense system and, thereby, contribute to the progression of lung disease.

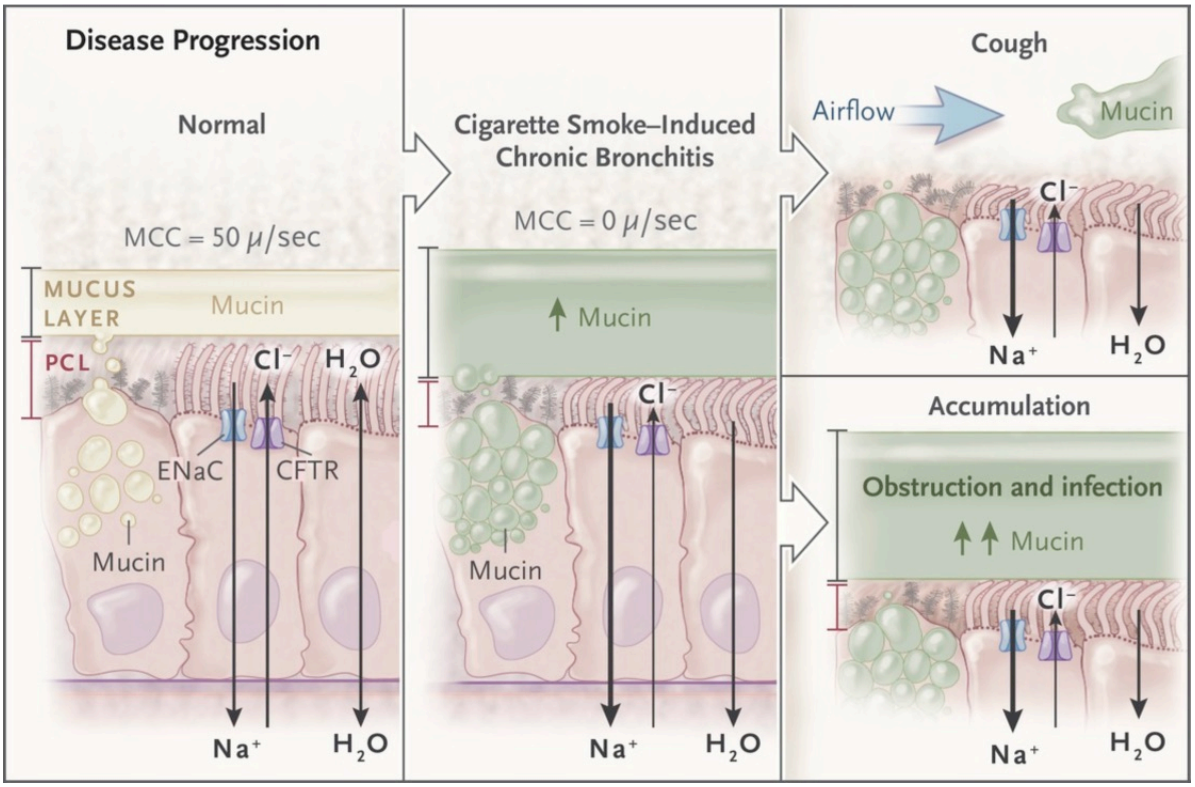

Figure 1.1 The effects of cigarette smoke on airway surface liquid composition and mucociliary transport. Reproduced with permission from Kesimer, M., Ford, A. A., Ceppe, A., Radicioni, G., Cao, R., Davis, C. W., Doerschuk, C. M., Alexis, N. E., Anderson, W. H., and Henderson, A. G. (2017) Airway mucin concentration as a marker of chronic bronchitis. *New England Journal of Medicine* **377**, 911-922., Copyright Massachusetts Medical Society.

Among the earliest observations on the respiratory effects of smoking was that cigarette smoke elicited an acute reduction of airway epithelial cell cilia motility. In 1937, Mendenhall and Shreeve exposed calf tracheas to cigarette smoke and observed reduced cilia motility and impaired mucociliary transport of carmine particles (89). Rakieten and colleagues expanded on this observation in 1952 by exposing respiratory epithelium collected from humans, rabbits, and rats to solutions of nicotine and menthol, as well as smoke from mentholated and non-mentholated cigarettes. Mentholated and non-mentholated cigarettes, but not menthol or nicotine solutions, significantly reduced cilia motility (90). Recent studies have confirmed these early observations using animal and hBE cell culture models (91-93). Histopathology studies have shown that cigarette smoke also causes structural abnormalities of the ciliated airway epithelium. A post-mortem analysis of 1522 Caucasian adults found that bronchial epithelial cells from male and female smokers displayed patches of atypical nuclei and significantly fewer cilia than non-smokers (94). Follow-up studies have determined that these types of abnormalities are proportional to the length of time an individual smokes and occur more frequent in smokers using high-tar/nicotine cigarettes (95). Smokers also have reduced cilia length in the large and small airways as compared to non-smoker controls. Furthermore, this abnormality was more pronounced in smokers with COPD (96). Electron microscopy studies have also noted smoking-related ultrastructural abnormalities in axonemal organization. Lungarella and colleagues observed that airway cilia from patients with chronic bronchitis had structural changes in the axonemal 9 + 2 pattern of organization which are not apparent in non-smokers (97). Many of the direct effects of cigarette smoke on cilia structure and function may be due to the reactive aldehydes in cigarette smoke. Hastie and colleagues exposed rabbit and

porcine tracheal explants to formaldehyde and observed a rapid but reversible reduction in cilia motility. Further, they observed a dose-dependent reduction in ciliary axoneme ATPase activity that was also reversible (98). Sander and Steffens observed that exposure of chicken hatchlings to formaldehyde gas caused rapid ciliostasis and morphologic changes to axonemal organization (99). Acetaldehyde is reported to induce concentration- and time-dependent reductions in CBF and cilia-derived dynein ATPase activity in primary cultures and isolated axonemes of bovine airway epithelial cells (100). Acrolein has been reported to induce ciliostasis at concentrations as low as 0.14ppm (0.14mg/L) (101-104). Data strongly suggest that aldehydes contribute to the increased occurrence of respiratory infections and pathogenesis of lung disease in smokers (105, 106).

Cigarette smoke has been shown to increase mucosal permeability in both animals and humans (107-109). While the exact mechanisms by which cigarette smoke disrupts epithelial barrier integrity have not been elucidated, data indicate that the loss of tight junction integrity is involved (108-110). Oliver and colleagues exposed Calu-3 bronchial epithelial cells to a single exposure of mainstream cigarette smoke and observed a transient reduction in transepithelial electrical resistance (TEER), which persisted for 30 minutes. Inhibition of Rho kinase reduced smoke-induced epithelial permeability, while inhibition of myosin light chain kinase exacerbated the effect of cigarette smoke (111). These data suggest that the smoke-induced reduction in epithelial junction integrity is a regulated process rather than a cytotoxic response. A follow-up study from this group found that mainstream cigarette smoke increased phosphorylation of proteins involved in the regulation of actin polymerization and caused a redistribution of tight-junction proteins from the apical surface to a more basolateral arrangement, further suggesting a regulated response to smoke (112).

Acrolein has been shown to directly disrupt epithelial barrier function. Hales and colleagues exposed dogs to synthetic smoke (carbon particles with a mean particle diameter of 4.3μm) containing acrolein or hydrochloric acid for 10 minutes and monitored extravascular lung water accumulation for 5 hours thereafter. While both acid and acrolein caused epithelial injury, only acrolein caused a dose-dependent increase in peribronchiolar edema (113). The disruption of epithelial junctions provides access for pathogens and allows infection of respiratory tissues, a common ailment among smokers and a contributing factor to the progression of lung disease.

1.3.2 Effects of cigarette smoke on leukocyte function

Cigarette smoking increases the numbers of infiltrating peripheral blood monocytes and alveolar macrophages occupying the respiratory airways several fold (114). Infiltrating macrophages secrete elastase, which can damage host tissues and exacerbate proinflammatory responses (115). The resulting damage to connective tissues and the lung parenchyma may contribute to chronic bronchitis and the pathogenesis of COPD (114). Despite having higher levels of oxygen radicals and myeloperoxidase, the alveolar macrophages from smokers have reduced phagocytic function as demonstrated by studies reporting significantly impaired clearance of *S. aureus* and *L. monocytogenes* bacteria (116, 117). Moreover, alveolar macrophages from smokers have an impaired ability to mount normal proinflammatory cytokine responses to LPS (118). The inability to clear bacterial pathogens and orchestrate immune responses increases susceptibility to lower respiratory infections, which further exacerbates cigarette smoke-induced inflammation.

Pulmonary accumulation of neutrophils is common among active smokers and massive pulmonary neutrophil influx is a typical feature of chronic inflammatory lung diseases, such as COPD (119). Cigarette smoke and nicotine have been reported to impair neutrophil phagocytosis, dysregulate production of a respiratory burst, and significantly reduce the elimination of bacterial pathogens (120). Compromised neutrophil anti-bacterial activity has been proposed as a major cause of increased susceptibility to respiratory infections in smokers (120). Additionally, cigarette smoke-induced neutrophil necrosis- and NETosis-mediated release of proteolytic enzymes, particularly elastase and matrix metalloproteinase, can cause significant damage to host tissue and is believed to be a key mediator of destructive lung diseases (121). Under normal conditions, neutrophils have a short lifespan and undergo caspase-3-dependent spontaneous, as well as phagocytosis-induced, apoptosis (122). Some of the chemical constituents of cigarette smoke, including acrolein and nicotine, have been reported to delay neutrophil spontaneous death (123-126). This is believed to contribute to the accumulation of neutrophils and emphysema in individuals with COPD (127).

NK cells are also impaired by toxicants in cigarette smoke. Ferson et al. investigated whether smoking status altered NK cell function in age- and sex-matched individuals with and without melanoma. Peripheral blood NK cells from all smoking subjects, regardless of melanoma status, were significantly less effective at recognizing and killing cultured melanoma and Chang cells as compared to cells from non-smoker subjects (128). Smokers have also been reported to have significantly fewer circulating NK cells as compared to non-smokers. This is despite overall increases in circulating leukocytes and lymphocytes (129). More recently, Horvath and colleagues investigated whether smoking altered nasal NK cell

responses to live attenuated influenza virus, a cold-adapted surrogate for natural influenza. Their data indicate that nasal NK cells from smokers had significantly lower expression of the activating receptor CD16, as well as significantly lower expression of the cytotoxic serine protease granzyme B, as compared to matched non-smoker controls (130). Taken together, these findings suggest that smoking-induced suppression of NK cell function may result in inadequate surveillance of tumorigenic and virus-infected host cells.

Based on these examples, it is clear that smoking produces various morphological, physiological, biochemical, and enzymatic changes in respiratory leukocytes. In many cases, these changes impair antimicrobial defenses, cellular regulatory activity, and inflammatory responses in the airways, and ultimately contribute to the pathogenesis of lung disease.

1.3.3 Effects of cigarette smoke on humoral immunity

As discussed in the previous sections, cigarette smoke alters the biological activity and function of many respiratory cell types. Furthermore, smoking can alter biological systems throughout the body. It is therefore not surprising that cigarette smoke can alter the composition and function of soluble immune mediators in the airway lining fluid milieu. Several studies have shown that tobacco smoke alters pulmonary surfactant, increases alveolar surface tension, and negatively impacts airway compliance (131-134). The lung lining fluid of smokers has also been reported to have reduced anti-protease activity, which may promote elastase-induced host damage and the progression of emphysema and COPD (135, 136). Cigarette smoke is reported to reduce levels of IgA and IgG in the saliva of smokers which suggests smoking may disrupt local antibody production in the upper airways (137). However, studies of IgA and IgG levels in the bronchial alveolar lavage (BAL) fluid

of smokers indicate no significant difference from non-smokers (138, 139). Gene expression of defensin proteins HBD-1 and HBD-2 are reported to be significantly suppressed in the oral mucosa of smokers (140). Other soluble mediators have been shown to be more abundant in the BAL of smokers, including lysozyme and fibronectin, which is indicative of enhanced inflammation (141, 142). Levels of complement proteins are also elevated in the BAL of smokers; however, complement-receptor-mediated phagocytosis in smokers is impaired (143-147).

1.4 Electronic cigarettes: a safe cigarette or cause for concern?

The smoking epidemic is one of the greatest public health catastrophes of the twentieth century. Between 1900 and 1999, approximately 100 million deaths were directly attributed to smoking (148, 149), and exposure to tobacco smoke (both first- and second-hand) will likely kill 6 million people this year alone (150). The annual costs attributed to smoking in the United States are between $289 billion and $333 billion (151). These estimates include approximately $130 billion for direct medical care, $150 billion for lost productivity due to premature death, and more than $5 billion for lost productivity from premature death due to exposure to secondhand smoke. The Centers for Disease Control and Prevention reports that in 2016, 15.5% of adults identified as current smokers and 10.2% of middle and high school students reported smoking a cigarette in the past 30 days (152, 153). Over one third of U.S. smokers attempt cessation each year. However, the success rate per attempt is low (154). Few smokers seek assistance for cessation and for those that do, the effectiveness of pharmaceutical aids for cessation is limited (155). This has left many smokers enslaved to cigarettes in order to satisfy their nicotine addiction.

Over the last decade, electronic cigarettes (e-cigarettes) have emerged as an alternative to traditional cigarettes. Because e-cigarettes deliver nicotine without burning tobacco or producing smoke, their use likely confers a lower overall health risk than inhaling combustible tobacco products (156). Thus, for both individuals and the public health, e-cigarettes may have the potential to promote smoking cessation among established cigarette smokers, or at least to reduce smokers' exposure to toxicants in tobacco smoke. Two recent meta analyses of randomized control trials for the effectiveness of e-cigarettes as cessation aids reported that e-cigarettes with nicotine improved long-term smoking cessation compared to e-cigarettes without nicotine. However, there were insufficient data to determine if e-cigarettes were more effective than traditional nicotine replacement therapies (157, 158). In addition to providing a new means for complete smoking cessation, replacing conventional cigarettes with e-cigarettes may provide an effective method for harm reduction by reducing tobacco-related health risks at the individual and population levels. If the use of e-cigarettes presents lower health risks compared to combustible tobacco cigarettes, then encouraging use of e-cigarettes as a reduced risk product rather than encouraging complete abstinence could have public health benefits (156). While e-cigarettes have not been theoretically or experimentally proven to be safe, the absence of many known toxicants present in tobacco smoke may make them significantly less harmful than cigarettes in at least some scenarios.

Although there may be great potential for e-cigarettes as cessation aids or cigarette replacements in smoking adults, the surge in e-cigarette popularity among teens and young adults cannot be ignored. E-cigarette use among adolescents and young adults who have never smoked cigarettes is common (156). Past 30-day e-cigarette use in high school students increased from 1.5% in 2011 to a high of 16.0% in 2015 before declining to 11.3% in 2016

(153). Unlike adults who frequently use e-cigarettes to quit or reduce smoking, teens between the ages of 12 and 17 are more likely to cite enjoyment of flavors and social factors as reasons for using e-cigarettes (159, 160). One of the major concerns of adolescent e-cigarette use is that e-cigarettes will provide a gateway to cigarette smoking and increase the number of smoking adults in the years to come (161). Part of this concern lies in the potential for nicotine addiction resulting from e-cigarette use (162). The recently devised catalyst hypothesis proposes several additional processes through which e-cigarette use could lead to smoking. Because vaping may produce positive sensations to the airways, pleasant tastes, and lack aversive effects like lung discomfort, the catalyst hypothesis proposes that vaping may cause adolescents to alter their perceptions about cigarettes and view smoking more favorably (163). Indeed, a recent evaluation of the existing e-cigarette literature by the National Academies of Sciences, Engineering, and Medicine concluded that "There is substantial evidence that e-cigarette use increases risk of ever using combustible tobacco cigarettes among youth and young adults." (156). Furthermore, A recent study found that e-cigarettes were perceived more positively, and usage was more prevalent among adolescents with asthma (12.4%) as compared to their non-asthmatic peers (10.2%) (164). Similarly, data from the 2012 Florida Youth Tobacco Survey indicate that e-cigarette use in Florida high school students was greater in asthmatics than in non-asthmatics (165). This same trend was reported in studies from Canada (166) and South Korea (167). Thus, while e-cigarettes may decrease the use of conventional cigarettes in adults with an existing smoking history, the prevalence of e-cig usage is greater in adolescents, and even greater in asthmatics relative to non-asthmatics, which is cause for concern (168).

Although use of e-cigarettes by adolescents may increase transition to smoking later in life, e-cigarettes might also increase adult smoking cessation and reduce exposures to cigarette smoke. The net public health effect, harm or benefit, of e-cigarettes depends on three factors: 1) their effect on youth transition to tobacco smoking, 2) their effect on adult smoking cessation, and 3) their intrinsic toxicity. If significant numbers of adult smokers abstain from cigarette smoking the benefit to public health could be considerable. If e-cigarettes prove to be an ineffective tool for adult smoking cessation, they would prove detrimental to public health because of the inherent harms of adolescent e-cigarette users transitioning to cigarettes. However, understanding the unique toxicities and risks associated with vaping is an essential step in determining whether e-cigarettes can be considered safer cigarettes or whether they present new concerns for public health.

1.4.1 The emergence of e-cigarettes

While the emergence of e-cigarettes is a recent phenomenon, the concept dates back to 1965 when Herbert Gilbert patented "a smokeless non-tobacco cigarette". In his patent, Gilbert stated that his device was “a safe and harmless means for and method of smoking by replacing burning tobacco and paper with heated, moist, flavored air.” (169). Attempts were made to commercialize Gilbert’s e-cigarette; however, the device was not received well and the idea was abandoned. The modern e-cigarette, which has striking similarities to Gilbert’s design, was developed by the Beijing pharmacist Hon Lik in 2003 (170). Lik’s invention was patented in China in 2003 and by 2004 the e-cigarettes were being marketed by the Chinese company Ruyan. By 2005 Ruyan began exporting out of China and by 2006 the Ruyan e-cigarettes were being marketed in Europe. Ruyan e-cigarettes entered the US market in 2007, which marked the beginning of the e-cigarette boom in the US.

1.4.2 Components of e-cigarettes

Since 2007, the e-cigarette industry has grown tremendously and current e-cigarette products have evolved from the original Ruyan device. Now, the term "electronic cigarette" or "e-cigarette" describes a diverse group of battery-powered devices that deliver nicotine, flavorings, and other constituents to the user by heating flavored e-cigarette liquid (e-liquid) solutions to temperatures sufficient to form aerosols (171). Inhalation of e-cigarette aerosols (commonly referred to as "vapor") provides the sensation of smoking and the desired nicotine effect without combusting tobacco. Despite the different names for e-cigarettes, which include electronic nicotine delivery systems (ENDS), electronic vaporizers, cig-a-likes, e-cigs, vape pens, mods (regulated and unregulated), pod-vapes, e-hookahs, e-cigars, and advanced personal vaporizers (APV), the basic physical components of the devices are the same: a battery, an atomizer, and a tank or cartridge containing e-liquid (162). Each of these components has the potential to affect health outcomes of e-cigarette users.

			
1st Generation "Cig-a-like"	2nd Generation "Vape-pen"	3rd Generation "Box mod"	4th Generation "Temperature Control (TC) Box mod"
• Low price • Similar in size and shape to traditional cigarette • Disposable or rechargeable • Some new models have refillable cartridges • Available at most convenience stores • Power settings not adjustable • Often used by novice vapers or former smokers transitioning from cigarettes	• Low to moderate price • Refillable tank • Rechargeable lithium-ion battery • Some customizability with user-defined voltage (typically 3.0V to 6.0V) • Most have heating coils with >1.0Ω resistance • Often used by novice or intermediate vapers	• Moderate to high price • One or two rechargeable lithium-ion batteries • Highly customizable: user-determines settings for voltage or wattage • Can accommodate a wide variety of refillable tanks, rebuildable atomizers (RBAs), or tankless "dripping" atomizer • Heating coils with <1.0Ω resistance are commonly used with these devices (sub-ohm vaping) • Heating coil temperatures may exceed 300°C with high wattage and low resistance • Generate significantly more aerosol than 1st or 2nd generation devices • Most often used by experienced vapers	• Among most highly priced devices due to more advanced electronics • User can program a maximal heating coil temperature to prevent overheating e-liquids and or burning the wicking material (i.e., a "dry hit") • Temperature control (TC) mode requires stainless steel, nickel, or titanium heating coils • One or two rechargeable lithium-ion or lithium polymer (LiPo) high amperage batteries • Other features and functions similar to 3rg generation box mods • Most often used by experienced vapers

Table 1.1 Examples of common e-cigarette devices. Reproduced with permission from Clapp, P. W., and Jaspers, I. (2017) Electronic Cigarettes: Their Constituents and Potential Links to Asthma. *Curr Allergy Asthma Rep* **17**, 79., Copyright Springer Nature.

Batteries. Most e-cigarette devices are powered by a rechargeable battery (a manufacturer-supplied unit), a non-rechargeable battery, or a replaceable battery (rechargeable or non-rechargeable) (156). Nickelcadmium (NiCad), nickel metal-hydride (NiMh), lithium ion (Li-ion), alkaline and lithium polymer (Li-poly), and lithium manganese (LiMn) batteries are all commonly used in e-cigarettes (172). However, lithium batteries are often preferred because they provide a large amount of energy in a compact size. There have been numerous accounts of e-cigarette fires, explosions, and injuries resulting from "thermal runaway" of lithium batteries (173).

Atomizers. An atomizer is the component of an e-cigarette that converts liquid into aerosol. There are several variants of e-cigarette atomizers, which include cartomizers (plastic cartridges), clearomizers (refillable plastic tanks), glassomizers (refillable glass tanks), and dripping atomizers (no tank). However, they all consist of wicking material which brings e-liquid solutions into contact with a heating coil. The materials used for heating coils and wicks vary significantly among devices. Common wicking materials include silica cord, organic cotton, rayon fibers, hemp, stainless steel mesh, ceramic, fiberglass, and bamboo yarn. Heating coils are typically resistance wires made from various metal alloys, including Kanthal (iron, chromium, and aluminum), stainless steel (iron, carbon, and chromium), and Nichrome (chromium and nickel). However, pure metal coils of nickel and titanium are also popular (168). E-cigarette power settings (battery output), heating coil resistance, the quantity and type of wicking material, and volume of e-liquid determine the temperature of the atomizer, which contributes to the chemical profile of e-cigarette aerosols.

E-liquids. E-liquids (also referred to as e-juices) are typically solutions of propylene glycol (PG) and vegetable glycerin (VG), nicotine, and various flavoring agents. PG and VG

are the primary components of essentially all e-liquids and provide the base to which nicotine and flavoring agents are added. PG and VG are viscous substances that keep nicotine and flavoring agents in suspension, enhance the absorption of wicking materials, and generate plumes of aerosolized particles when heated to sufficient temperatures (168). Additionally, PG and VG are hygroscopic humectants, which produce a dry-mouth or dry-throat sensation when inhaled. This property provides e-cigarette users with a "throat hit" similar to cigarette smoking (174, 175). The PG:VG ratio varies between products and many manufacturers allow customers to choose the PG and VG content of their preference (this is often dependent on the flavor as some flavoring agents are preferentially soluble in PG or VG). Ex-smokers or smokers attempting to quit frequently prefer e-liquids with a high PG content as the throat-hit is reported to be stronger than that of VG (175). Other users prefer e-liquids with a high VG content, which is reported to be less irritating to the throat and capable of generating larger "clouds" of aerosol.

Nicotine is a botanically-derived, parasympathomimetic alkaloid that is readily absorbed by the body through dermal, oral, and inhalational exposures, and easily crosses biological membranes including the blood brain barrier. Inhalation of nicotine increases blood pressure, pulse rate, free fatty acids in the plasma, mobilizes blood sugar, and stimulates the release of norepinephrine and dopamine (176-178). Unlike traditional cigarettes, e-cigarettes allow the user to choose the concentration of nicotine that provides the desired effect. Most suppliers offer any flavored e-liquid in a variety of nicotine concentrations which typically include 6, 12, 18, 24, and 36mg/ml. Additionally, many e-cigarette retailers offer solutions of concentrated nicotine for supplementation to e-liquids. Flavored e-liquids can also be purchased without nicotine. The most recent e-cigarette

devices are using nicotine salts instead of the "free-base" nicotine used in traditional cigarettes and most e-cigarette liquids. Pankow and colleagues recently analyzed the chemical composition of JUUL™ pod pre-filled e-cigarette cartridges and found benzoic acid and nicotine in a 0.97 to 1 molar concentration ratio. When heated during vaping, the nicotine salt, nicotine benzoate, forms and is delivered to the user in the aerosol (179). Users of these products claim that nicotine salt provides an enhanced nicotine effect without the harsh throat-hit typically experienced with free-base nicotine. Whether the inhalation of nicotine salts will have different health outcomes than free-base nicotine is currently unknown.

E-liquids are available in almost any flavor imaginable. However, most e-liquids can be broadly grouped into one of five general categories based on their dominant flavor: tobacco flavors (cigarette, cigar, pipe tobacco, etc.), fruit flavors (blueberry, peach, etc.), minty flavors (menthol, peppermint, etc.), sweet flavors (candy, cookie, cake etc.) and other flavors (coffee, wine, foods, etc.) (180). Flavoring agents are typically food-grade flavoring additives which are mixed in various combinations to produce uniquely-flavored products. Pure flavoring agents (i.e. menthol, cinnamaldehyde, furaneol, limonene, etc.) are used to flavor e-liquids. However, many e-liquid manufacturers purchase pre-mixed flavoring oils, emersions, and blends from various food additive distributors, and blend those further to achieve the desired e-liquid flavor. Flavoring agents are represented by various chemical groups, including alcohols, aldehydes, esters, ketones, lactones, thiols, and terpenes. Many e-liquid manufacturers also use flavor enhancers (i.e. maltol, ethyl maltol, glutamic acid etc.) and stabilizers (i.e. citric acids, ascorbic acid, acetic acid, etc.) to extend the shelf-life of e-liquids.

1.4.3 Flavoring agents pose a significant unknown

The inhalation of flavoring agents poses a significant unknown in regards to the potential risks of e-cigarette use. While the majority of e-cigarette flavoring agents carry the "generally recognized as safe" (GRAS) designation by the United States Food and Drug Administration (US FDA), GRAS substances are only evaluated for safety during oral consumption as a food additive. Other routes of exposure were not considered and, therefore, GRAS designation is not applicable for the inhalation of food additives. Furthermore, there is precedent for inhalation of a GRAS flavoring agent causing irreversible lung disease. Inhalational exposures to high concentrations of aerosolized diacetyl (2,3-butanedione), a diketone with butter-flavor characteristics, were reported to induce acute onset bronchiolitis obliterans, an irreversible obstructive lung disease, in workers at a microwave popcorn manufacturing facility (181). A recent study evaluated the chemical composition of 159 e-liquids and found that diacetyl was present in 110 (69.2%) of the products tested (182). The authors determined that, under normal use, 52 of the e-liquids containing diacetyl would result in exposures greater than the 5ppb recommended 8-hour time-weighted occupational exposure limit established by the National Institute for Occupational Safety and Health (NIOSH) and the Centers for Disease Control (CDC). Twenty-six of the e-liquids tested would result in exposures greater than five times the 5ppb limit and the e-liquid with the highest diacetyl content would result in an exposure 490 times greater than the NIOSH limit (182). However, it is still unclear whether e-cigarette exposures to diacetyl will cause respiratory harm. The Flavor and Extract Manufacturers Association (FEMA) of the USA, a trade association of flavor ingredient manufacturers which evaluates the safety of food flavorings, has identified 1037 food-safe flavoring agents as potential respiratory hazards due

to possible volatility and respiratory irritant properties (183). Common e-cigarette flavoring agents on this list include, but are not limited to: diacetyl, acetoin, 2,3-pentanedione (buttery flavors), camphor and cyclohexanone (minty flavors), benzaldehyde (cherry or almond flavors), cinnamaldehyde (cinnamon flavor), cresol (leathery or medicinal flavor), butyraldehyde (chocolate flavor), and isoamyl acetate (banana flavor) (168, 183). While these flavoring agents are predicted to be respiratory hazards, there is a paucity of data on whether or how they affect the respiratory health of e-cigarette users.

Flavoring agents, by definition, are biologically active compounds. Non-volatile, polar flavoring agents activate receptors on chorda tympani and trigeminal nerve endings in papillae on the tongue to produce taste, astringency, pungency, and cooling sensations. Volatilized aromatic flavoring agents travel from the mouth to the nasal passages where they activate receptors on cilia of olfactory receptor neurons and trigger odorous sensations (184). Indeed, our ability to taste and smell substances relies on the inherent reactivity of flavoring and odorant chemicals. Most flavorings and odorants have thresholds for detection and recognition by taste and odor neurons in the low parts per billion (ppb) range. For example, cinnamaldehyde, the α,β-unsaturated aromatic aldehyde which gives cinnamon its characteristic flavor and odor, is reported to have an odor threshold of 4.45ppb (185). Studies evaluating the cinnamaldehyde content of e-liquids have reported concentrations greater than 1.0M (123,162,000.0ppb) in some products (186, 187). Inhalation of such high concentrations raises concerns for individual dose exposures and the potential for toxicity or altered biological functions. Reactive carbonyls, such as cinnamaldehyde and other aldehyde flavoring agents, share structural similarity with toxic aldehydes in cigarette smoke. Cinnamaldehyde (3-phenylprop-2-enal) is structurally similar to acrolein (prop-2-enal).

Benzaldehyde (phenylmethanal) is structural similarity to formaldehyde (methanal). Acetaldehyde itself is a flavoring agent which has been used to impart the taste of green apples (188). As discussed in previous sections, aldehydes in cigarette smoke are known to have damaging effects on the lung and have varying disruptive effects on respiratory innate immune function. However, whether structurally similar aldehyde flavoring agents in e-cigarettes will have similar effects is unknown.

Acute Exposures

- Increased cough
- Dry throat
- Chest tightness
- Increased mucosal secretions
- Minimal reductions in lung function and fraction of exhaled nitric oxide (FeNO)
- Reactive carbonyls (acrolein, acetaldehyde, formaldehyde) generated by thermal degradation have known respiratory toxicities

Chronic Exposures

- Workplace exposures associated with reduced forced expiratory volume-one second (FEV1) and forced vital capacity (FVC)
- No data for chronic inhalational exposures from e-cig use

Acute Exposures

- Effects are often flavor-specific and dose-dependent
- TRPA1 and TRPV1 agonists, such as cinnamon, mint, and clove, induce strong pulmonary reflex responses
- Very little data on acute inhalational exposures

Chronic Exposures

- Workplace Inhalation of diacetyl, 2, 3-pentanedione, and acetoin (buttery flavorings) caused bronchiolitis obliterans
- Few reported cases of mint allergy and mint-induced asthma
- Occupational asthma reported in workers inhaling cinnamon dust
- No chronic inhalational exposure data on most flavoring agents used to flavor e-cigs

Acute Exposures

- Increased release and metabolism of neurotransmitters
- Increased pulse rate and blood pressure
- Mobilization of blood sugar
- Increased mucus production and mucus viscosity
- Reduced cell proliferation and suppression of apoptosis
- Reduced allergen-induced inflammation in rodents

Chronic Exposures

- Epigenetic reprogramming of rat germline cells resulting in impaired fetal lung development and transgenerational asthma
- Reduced inflammatory responses are associated with increased respiratory viral infections

Figure 1.2 Biological effects of exposure to chemicals in e-cigarette aerosols. Reproduced with permission from Clapp, P. W., and Jaspers, I. (2017) Electronic Cigarettes: Their Constituents and Potential Links to Asthma. *Curr Allergy Asthma Rep* **17**, 79., Copyright Springer Nature.

1.5 Scope of this dissertation

Smoking continues to be the leading cause of preventable death accounting for 12% of all mortalities among adults worldwide. U.S. FDA regulations have limited the sale, marketing, and use of traditional tobacco products in the U.S.; however, regulations on new and emerging tobacco products, such as flavored e-cigarettes, are pending. "Vaping" has gained popularity in the U.S. as e-cigarette usage has increased steadily over the past several years, particularly among teens and young adults. While e-cigarettes are commonly represented as safer alternatives to cigarettes, little is known regarding the health effects of their short- or long-term use. We have previously demonstrated that cigarette smoking significantly affects innate and antiviral host defenses in the context of influenza infections by modifying type I-interferon responses and mucosal immune cell function, thereby enhancing viral entry into epithelial cells. However, whether and how chemicals in flavored e-cigarettes affect respiratory innate immune defenses and, thereby, modify susceptibility to respiratory infections is completely unknown.

The overall objective of this work was to evaluate the effects of flavored e-liquids, particularly those containing α,β-unsaturated cinnamaldehyde, on respiratory innate defense mechanisms, and to assess whether and how these products alter susceptibility to respiratory infections. To achieve this objective, I used tightly-linked human *in vitro* and *ex vivo* translational research approaches to elucidate how flavored e-liquids, and specifically, cinnamaldehyde, affect innate defense mechanisms of the respiratory tract. The hypothesis that flavored e-liquids containing cinnamaldehyde will alter respiratory innate immune mechanisms and impair host defense capabilities was tested by exposing human innate immune cells to e-liquids and cinnamaldehyde alone, and quantifying changes in innate

immune functions (Chapter 2). Additionally, human bronchial epithelial cells were exposed to e-liquids, vaped aerosols, and cinnamaldehyde, and changes in cilia motility and bioenergetic processes were assessed (Chapter 3). Finally, common e-cigarette flavoring agents were evaluated for their ability to activate the Nrf2-antioxidant response element signaling pathway, a major mechanism in the cellular defense against oxidative or electrophilic stress (Chapter 4).

The findings in this dissertation provide compelling evidence that flavored e-liquids and cinnamaldehyde, a common food-safe flavoring agent, have the capacity to alter innate immune defense functions in respiratory tissues. These data indicate that, 1) cinnamaldehyde suppresses neutrophil, alveolar macrophage, and NK cell defense functions in dose-dependent manner at concentrations well below cytotoxic levels, 2) cinnamon-flavored e-liquids (both unheated and vaped) and cinnamaldehyde transiently suppress cilia motility on airway epithelial cells via inhibition of cell mitochondrial and glycolytic functions, and 3) cinnamaldehyde, eugenol, and guaiacol significantly induce Nrf2 pathway activation in bronchial epithelial cells suggesting increased cellular oxidative stress.

The data presented herein provide evidence that flavoring agents in e-cigarettes, particularly electrophilic aldehydes such as cinnamaldehyde, have the capacity to impair respiratory innate defenses, and may place e-cigarette users at a greater risk of respiratory infections. I anticipate that the work in this dissertation will be useful for developing federal regulations aimed at reducing or eliminating harmful and potentially harmful constituents from e-cigarettes.

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CHAPTER 2.

FLAVORED E-CIGARETTE LIQUIDS AND CINNAMALDEHYDE IMPAIR RESPIRATORY INNATE IMMUNE CELL FUNCTION[1]

2.1 Introduction

Smoking continues to be the leading cause of preventable death accounting for 12% of all mortalities among adults worldwide (1). A link between cigarette smoke exposure, suppression of respiratory immune responses, and enhanced susceptibility to respiratory infection is well established (2-8). While tobacco use is steadily declining in the U.S., electronic cigarettes (e-cigarettes) are rapidly gaining popularity, particularly among teens and young adults (9) who often perceive these products as less harmful than traditional cigarettes (10). This perception is in part due to targeted marketing which identifies these products as a "safer alternative" to traditional cigarettes (11); however, little is known regarding the effects of "vaping" on respiratory innate immune cell function. A recent study by Sussan et al. reported that mice exposed to e-cigarette aerosols exhibited impaired bacterial clearance and increased susceptibility to respiratory virus infection (12). Furthermore, lung macrophages from these exposed mice exhibited reduced phagocytic capacity. More recently, Hwang et al. exposed human epithelial cell lines, murine alveolar

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macrophages, and human neutrophils to "e-cigarette vapor extract" and observed dose-dependent cytotoxicity and reduced antimicrobial activity against Staphylococcus aureus (13). The findings in both publications were largely attributed to the immunosuppressive effects of nicotine; however, reactive carbonyls present in these flavored e-liquids and generated by e-cigarette devices may also play a role in the observed immunomodulatory effects (14-16).

E-cigarette devices aerosolize flavored e-liquids, which are typically composed of humectants (propylene glycol and/or vegetable glycerin), chemical flavorings, and nicotine. There are currently more than 7,700 commercially available e-liquids which contain flavorings that have not been evaluated for inhalational toxicity (17). While many of these flavorings are classified as generally recognized as safe (GRAS) for oral consumption by the United States Food and Drug Administration (U.S. FDA), prolonged inhalation of some GRAS flavorings, such as diacetyl, 2,3-pentanedione, and acetoin, can cause irreversible lung disease (18-20). GRAS chemicals commonly used as e-liquid flavoring agents include aliphatic aldehydes (for fruity flavors) and aromatic aldehydes (for sweet and spicy flavors) (21, 22). The Flavor and Extract Manufacturers Association (FEMA) has identified over 1000 GRAS flavorings that may pose a respiratory hazard due to possible volatility and irritant properties (23, 24); however, it is unclear whether these flavorings, in the context of e-cigarette exposures, directly impact respiratory innate immune cells. Previous studies have demonstrated that vanillin and cinnamaldehyde, aromatic aldehydes commonly used as e-liquid flavoring and odorant agents, can suppress macrophage function (25-32). However, most of these studies were conducted in non-human cell lines without direct relevance to the

lung. Consequently, how exposure of innate immune cells patrolling the respiratory tract to these flavoring chemicals induces potential functional changes is unknown.

The testing paradigm we chose to address this knowledge gap was to screen popular e liquids for their potential to alter innate immune cell function, identify common flavoring agents in the e-liquids eliciting a response, and investigate whether exposure to a shared flavoring agent alone could recapitulate the observed response. To this end, we collected alveolar macrophages, peripheral blood neutrophils, and NK cells from healthy, non-smoker volunteers and exposed the cells to varying dilutions of nicotine-free flavored e-liquids and evaluated changes in normal immune functions. The chemical composition of e-liquids which altered immune cell responses was determined, and a shared chemical flavoring, cinnamaldehyde, was investigated for its role in the e-liquid-induced effects on respiratory immune cell function.

2.2 Materials and methods

Subjects

Healthy individuals between the ages of 18-49 were recruited to donate venous blood for the isolation of neutrophils or NK cells or to undergo bronchoscopy and bronchoalveolar lavage for the collection of alveolar macrophages. Subjects donating venous blood were healthy, nonsmoking, nonvaping adults who are not routinely exposed to secondhand smoke. Exclusion criteria included pregnant or nursing females or individuals with a history of egg allergy, allergic rhinitis, aspirin therapy, asthma, immunodeficiency (HIV or other), Guillain-Barre Syndrome, COPD, cardiac disease, any chronic cardiorespiratory condition, or fever/respiratory illness within three weeks prior to entry into study. Subjects undergoing

bronchoscopy received a physical examination, a routine blood panel with complete blood count (CBC) and differential, serum electrolyte, glucose, and liver enzyme testing. Female subjects had to have a negative urine pregnancy test prior to bronchoscopy and all volunteers were required to be free of chronic cardiovascular or respiratory illness and be free of acute respiratory illness within the proceeding three weeks. All subjects undergoing bronchoscopy had forced expiratory volume in one second (FEV_1) and forced vital capacity (FVC) $\geq$ 80% predicted and FEV_1/FVC $\geq$ 80% predicted normal for height and age and were nonvapers and nonsmokers with no smoking history. Subjects were matched for sex, age, and BMI between experimental conditions. Participants in this study were not currently taking immunosuppressive drugs including corticosteroids. Informed consent was obtained from all subjects and all studies were approved by the University of North Carolina at Chapel Hill School of Medicine Institutional Review Board.

Alveolar Macrophage Isolation

Bronchoalveolar lavage (BAL) fluid was collected during bronchoscopy as previously described (33). Briefly, a flexible, fiber-optic bronchoscope was inserted transnasally and wedged into a subsegment of the medial segment of the right middle lobe (fifth or sixth generation). Saline was instilled and immediately aspirated to recover alveolar leukocytes. Recovered BAL fluid was centrifuged and cells (>95% alveolar macrophages) were resuspended in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA, USA).

Neutrophil Isolation

Venous blood was collected into vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA) and allowed to cool to room temperature with gentle rocking prior to

cell isolation. Neutrophils were isolated by density centrifugation of venous blood over Histopaque 1119 (Sigma-Aldrich, St. Louis, MO, USA) and subsequently over a discontinuous Percoll (GE Healthcare Life Sciences, Marlborough, MA, USA) gradient as previously described (34, 35). Isolated neutrophils were re-suspended in RPMI-1640 medium containing 10mM HEPES buffer and 0.5% fetal bovine serum (FBS). Cells were incubated at 37˚C and 5% CO_2 for 30 minutes prior to challenges.

Natural Killer Cell Isolation

Venous blood was collected into vacutainer tubes containing heparin and allowed to cool to room temperature with gentle rocking prior to cell isolation. Blood was diluted 1:4 with PBS and centrifuged over Lymphoprep density gradient medium (STEMCELL Technologies Inc., Vancouver, Canada) to fractionate whole blood components. The plasma and peripheral blood mononuclear cell (PBMC) fractions were harvested and briefly washed with PBS. NK cells were isolated from the PBMC fraction using negative antibody selection with the Dynabeads® Untouched™ Human NK Cell negative selection kit (Thermo Fisher Scientific, Waltham, MA, USA). Purified NK cells were kept at 37˚C and 5% CO_2 in RPMI-1640 medium with 10% FBS prior to challenges.

Flavored E-cigarette Liquids

A panel of 7 flavored, nicotine-free e-liquids was purchased from a local vendor (The Vapor Girl, Chapel Hill, NC, USA). E-liquids were selected for this study based on either a "top-seller" designation ("Menthol Tobacco" and "Solid Menthol") or their likelihood to contain chemical flavorings identified as potential respiratory hazards by FEMA, such as cinnamaldehyde ("Hot Cinnamon Candies", "Kola", and "Sini-cide") and isoamyl acetate

("Banana Pudding" and "Banana"). E-liquids were stored in opaque glass bottles at room temperature and were diluted for use at the time of each experiment. All e-liquids used in this study were reported by the manufacturer to contain the humectants propylene glycol and vegetable glycerin at relative proportions of 55% and 45%, respectively. A mixture of 55% propylene glycol (Thermo Fisher Scientific, Waltham, MA, USA) and 45% vegetable glycerin (Sigma-Aldrich, St. Louis, MO, USA) was used as a vehicle control for all e-liquid experiments.

Stimulation and Quantification of Neutrophil Extracellular Traps (NETs)

Neutrophils were seeded at a density of $1x10^5$ in 100µl volume per well of a cell culture treated 96-well plate and allowed to incubate at 37˚C and 5% CO2 for 30 minutes. Cells were then challenged with a final concentration of 1.0% e-liquid diluted in cell culture medium with or without 25nM phorbol 12-myristate 13- acetate (PMA; Sigma-Aldrich, St. Louis, MO, USA), a protein kinase C activator and potent NET agonist. Wells were assayed for extracellular chromatin every hour during a 4-hour challenge. Chromatin extruded by activated neutrophils was digested with 500mU/ml micrococcal nuclease (Worthington Biochemical, Lakewood, NJ, USA). Micrococcal nuclease activity was quenched with 5mM EDTA following a 10-minute digestion at 37˚C. Chromatin released at each time point was quantified using the Picogreen double stranded DNA kit (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol.

Immunofluorescent Analysis of NETs

Neutrophils were seeded onto 13mm round glass cover slips (0.15mm thick) and challenged with 1.0% e-liquid diluted in cell culture medium with or without 25nM PMA.

Cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich, St. Louis, MO, USA) every hour during a 4-hour challenge and immunolabeling was conducted as described by Brinkmann et al. (34). Neutrophils were blocked (5% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), 1% gelatin from cold water fish skin (Sigma-Aldrich, St. Louis, MO, USA), 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), and 0.05% Tween 20 (Sigma-Aldrich, St. Louis, MO, USA) in PBS (Gibco/Thermo Fisher Scientific, Waltham, MA, USA)) and incubated with a mouse anti-human neutrophil elastase primary antibody (Dako, Carpinteria, CA, USA), which was detected with an Alexafluor 488 conjugated secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Negative controls were conducted using Isotype-matched (IgG1κ) labeling of NETs at each time point. Chromatin was labeled with Hoechst 33342 (Thermo Fisher Scientific, Waltham, MA, USA). Coverslips were mounted onto microscope slides with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Waltham, MA, USA) and the edges sealed with clear fingernail polish. Cells were imaged using a Nikon Digital Eclipse C-1 modular confocal microscope and Plan Apo VC 60x/1.40 oil objective.

Neutrophil and Alveolar Macrophage Phagocytosis

Neutrophils isolated from venous blood were seeded at a density of $1x10^5$ cells per well of a black, clear-bottom 96-well plate in RPMI-1640 media with 10mM HEPES buffer (Sigma Aldrich, St. Louis, MO, USA) and 0.5% FBS (Gibco/Thermo Fisher Scientific, Waltham, MA, USA). Cells were allowed to incubate at 37˚C and 5% CO2 for 30 minutes. Neutrophils were then challenged in triplicate with a final concentration of 1%, 0.5%, or 0.25% e-liquid diluted in cell culture medium for 30 minutes. Opsonized pHrodo™ Red

Staphylococcus aureus BioParticles® (Thermo Fisher Scientific, Waltham, MA, USA) were prepared according to the manufacturer's instructions and added to each well following e-liquid challenge. Neutrophils and BioParticles were incubated at 37˚C and 5% CO_2 for an additional 4 hours to assess e-liquid induced changes in phagocytic capacity.

Cells obtained from BAL fluid were seeded at a density of $5x10^4$ cells per well of a black, clear bottom 96-well plate (Corning Life Sciences, Tewksbury, MA, USA) in RPMI-1640 medium with 10% FBS. Cells were allowed to incubate at 37˚C and 5% CO_2 for 2 hours. Non-adherent cells were then aspirated thereby enriching the alveolar macrophage population. Adherent alveolar macrophages were challenged in triplicate with cell culture medium containing 1%, 0.5%, or 0.25% flavored e-liquid for 1 hour. Following e-liquid challenge, opsonized pHrodo™ *S. aureus* BioParticles® were added to the cells and allowed to incubate as described above.

To assess whether nicotine altered e-liquid-induced effects on neutrophil phagocytosis, nicotine (Sigma-Aldrich, St. Louis, MO, USA) was added to the "Kola", "Hot Cinnamon Candies", "Sini-cide", and "PGVG vehicle" e-liquids at concentrations commonly stocked in vape shops (2.4%, 1.8%, 1.2%, and 0.6%). Nicotine- containing e-liquids were then diluted to 0.25% in cell culture medium, neutrophils were challenged as described above, and opsonized pHrodo™ Red *S. aureus* BioParticles® were added to assess phagocytosis.

Phagocytosis of BioParticles® was quantified using a CLARIOstar fluorescent microplate reader (BMG Labtech, Offenburg, Germany) by assessing fluorescence emission at 585nm. Cell-free negative control wells were used to normalize for background

fluorescence resulting from e liquid challenge at 1%, 0.5%, or 0.25%. Data are presented as percent phagocytosis observed in the propylene glycol/vegetable glycerin (PG/VG) vehicle at each dilution tested.

DTT Competition Assay

Alveolar macrophages obtained from BAL fluid were seeded on black, clear-bottom 96-well plates and allowed to incubate as described above. Non-adherent cells were removed and adherent alveolar macrophages were challenged with cell culture medium alone, 500μM cinnamaldehyde, 500μM dithiothreitol (DTT; Sigma-Aldrich, St. Louis, MO, USA), or a challenge, opsonized pHrodo™ *S. aureus* BioParticles® were added to the cells and incubated for an additional four hours. Phagocytosis of BioParticles® was quantified using the CLARIOstar fluorescent microplate reader as described above.

Immunofluorescent Analysis of Macrophage Phagocytosis

Alveolar macrophages were seeded onto 13mm round glass cover slips (0.15mm thick) at $2x10^5$ cells per well of a cell culture-treated 24-well plate. Cells were allowed to incubate at 37˚C and 5% CO_2 for 2 hours and were challenged with 1%, 0.5%, or 0.25% dilutions of flavored e-liquid for 1 hour as described above. Opsonized pHrodo™ *S. aureus* BioParticles® were added to each well according to the manufacturer's protocol and cells were fixed with 4% PFA after 4 hours. Following 30-minute fixation at 4˚C, PFA was aspirated and cells were blocked for 1 hour with 5% normal donkey serum, 1% gelatin from cold water fish skin, 1% bovine serum albumin, and 0.05% Tween 20 in PBS (block buffer). AlexaFluor 488-conjugated phalloidin (Thermo Fisher Scientific, Waltham, MA, USA) and Hoechst 33342 were diluted in block buffer to the manufacturer's recommended

concentrations and applied to the cells for 1 hour. Coverslips were washed with PBS, mounted onto microscope slides with ProLong Gold Antifade Mountant, and the edges sealed with clear fingernail polish. Cells were imaged using a Nikon Digital Eclipse C-1 modular confocal microscope and Plan Apo VC 60x/1.40 oil objective.

NK Cell Killing Assay

$1x10^5$ NK cells were added to duplicate polypropylene flow cytometry tubes with 0.25% e-liquids diluted in cell-culture medium for 1 hour. During e-liquid incubation, K-562 human erythromyeloblastoid leukemia cells (ATCC, Manassas, VA, USA) were labeled with carboxyfluorescein succinimidyl ester (Cayman Chemical, Ann Arbor, MI, USA). Following e liquid challenge, $3.2x10^4$ CFSE-labeled K-562 cells were added to each tube of NK cells and incubated for 4 hours at 37°C and 5% CO_2. The entire cell suspension was then pelleted and stained with LIVE/DEAD® fixable dead cell stain kit (Thermo Fisher Scientific, Waltham, MA, USA), fixed with 0.5% PFA, and data were acquired on a BD™ LSRII Flow Cytometer as described in our previous studies (36).

Enzyme-linked Immunosorbent Assay (ELISA)

Supernatants were harvested from alveolar macrophages and neutrophils after challenge with diluted e-liquids. Specifically, cells collected from BAL were seeded in cell-culture treated 96 well plates as described above. Media containing 1%, 0.5%, or 0.25% flavored e-liquid or PG/VG vehicle was added to each well and supernatants were collected after 24 hours. Neutrophils were seeded at a density of $1x10^5$ cells per well of cell culture-treated 96-well plates in RPMI-1640 media with 0.5% FBS and stimulated with 1%, 0.5%, or 0.25% of each e-liquid flavor for 4 hours, followed by collection of supernatants. Medias

collected post-challenge were stored at -20˚C until analyzed. Interleukin 8 (IL-8) and interleukin 6 (IL-6) were quantified in cell culture supernatants via commercially available ELISA kits (BD Biosciences, San Jose, CA, USA).

Vaped E-liquid Condensate

Vaped e-liquid condensates (VEC) of "PG/VG (vehicle control)", "Kola", "Hot Cinnamon Candies" and "Sini-cide" were generated as described by Olmedo et al (57). Briefly, e-cigarette aerosol was produced by connecting a LAVABOX DNA 200 Box Mod (Volcano e-Cigs, Honolulu, HI, USA) and a SMOK TFV4 Mini Tank with a sub-ohm (0.37Ω) TF-CLP2 Clapton Coil (SMOKtech, Shenzhen, China) to a 16cm length of tubing (4.8mm internal diameter, Masterflex L/S 15, Vernon Hills, IL, USA). A peristaltic pump (drive number 07522-20 and head number 77200-62, Cole-Parmer, Vernon Hills, IL, USA) was used to pull aerosol from the e-cigarette device at a flow rate of one liter per minute. One puff of aerosol was generated every 30 seconds for 10 minutes (20 puffs total) using an output setting of 60 watts. Vaped aerosol was pumped through a series of alternating 250µl pipette tips and Tygon tubing (1.5mm internal diameter, S3 E-3603, Saint-Gobain Corporation, France) to allow the aerosol to cool and condense. VEC for each e-liquid was collected into a sterile 1.5ml microcentrifuge tubes for quantitation of cinnamaldehyde content. Alveolar macrophages and neutrophils were then seeded as described above and challenged with a 0.25% dilution of each VEC or unheated e-liquid to assess whether the vaping process altered e-liquid-induced effects on phagocytic function.

Mass Spectrometry of E-liquids

Qualitative e-liquid analysis was performed on a Bruker Scion 456 gas chromatograph (GC)-triple quadrupole mass spectrometer (Bruker, Billerica, MA, USA) using an Agilent DB-5MS capillary column (30m, 0.25mm ID, 0.25µM film) (Agilent Technologies, Santa Clara, CA, USA). Samples were prepared by diluting 100µL of e- liquid in 1mL of methanol (optima grade) and vortexing for 30 seconds. Splitless injections (2µL) were performed using a Bruker CP-8400 autosampler.

Cinnamaldehyde was quantified in e-liquid and VEC samples using the same GC-MS system and operating conditions. Cinnamaldehyde standard solutions were prepared to concentrations of 7.22mM, 3.61mM,1.44mM, 0.722mM, 0.361mM, and 0.181mM in methanol (Optima grade). A calibration curve was generated using standard addition methods by diluting the "Sini-cide", "Hot Cinnamon Candies", and "Kola" e-liquids to ratios of 1:500, 1:100, and 1:10 respectively in the standard solutions. Dilution factors were selected to ensure that cinnamaldehyde concentrations fell within the linear range of the calibration curve. Analyses were performed in triplicate. Multiple reaction monitoring (MRM) was used for quantitation of the "Kola" e-liquid, as it provided the limit of detection needed for the lower cinnamaldehyde concentration. Two reactions were used: m/z 131 to m/z 77 and m/z 131 to m/z 103. The m/z 77 product ion was used for quantitation with 90% of the scan time, and the m/z 103 product ion was used for qualification with 10% of the scan time to provide increased selectivity. Argon was used as the collision gas at a pressure of 1.5mTorr and the collision energy was 20eV.

The GC oven was programmed to hold the temperature at 50°C for 2 minutes, ramp to 300°C at a rate of 15°C/minute and hold at 300°C for 6.33 minutes for a total runtime of 25 minutes. The transfer line and EI source were held at 250°C throughout the analysis. Compound identification and peak integration was done using AMDIS GC/MS Analysis software based on spectral matching with the NIST Mass Spectral Library (2011).

Statistics

Data were generated from cells isolated from a minimum of n=3 individuals, as noted in the figure legends. When appropriate, data are expressed as a percentage of PG/VG vehicle control. Macrophage and neutrophil data were analyzed using a two-way ANOVA with Fisher's LSD posttest. NK cell-mediated cytotoxicity data were analyzed using a one-way ANOVA with a Dunn's multiple comparison test. All statistical tests were performed using GraphPad Prism Version 7. A p-value of <0.05 was considered to be significant.

2.3 Results

Effects of Flavored E-liquids on Alveolar Macrophages

The effects of flavored e-liquids on alveolar macrophage phagocytosis were assessed by challenging cells with 1%, 0.5%, and 0.25% e-liquids diluted in cell culture medium, followed by incubation with Staphylococcus aureus BioParticles. The BioParticles used for this study emit a minimal fluorescent signal at physiologic pH (approximately 7.4); however, fluorescence intensity increases as particles are phagocytosed and subjected to an acidic pH in phagosomes. Exposure of alveolar macrophages to the highest dilution (1%) of PGVG vehicle alone produced a slight but significant reduction in BioParticle phagocytosis (Figure 2.1A). Of the seven e-liquids tested, only "Kola" (1%) and "Sini-cide" (1%, 0.5%, and

0.25%) significantly suppressed phagocytosis as compared to the PG/VG vehicle control (Figure 2.1B). Fluorescent confocal microscopic analysis of macrophages challenged with 0.25% “Sini-cide” revealed normal alveolar macrophage morphology but loss of phagocytic function and inability to engulf labeled BioParticles (Figure 2.1C). The suppression of macrophage phagocytosis induced by “Sini-cide” was validated by flow cytometry. Flow analysis confirmed that “Sini-cide” at all three dilutions used (1%, 0.5%, and 0.25%) significantly reduced phagocytic function (Figure 2.1D). The reduction in phagocytosis observed with the 1% “Sini-cide” challenge can be attributed to e-liquid-induced cytotoxicity; however, the 0.5% and 0.25% dilutions significantly suppressed phagocytosis without reducing macrophage viability (Figure 2.1E)

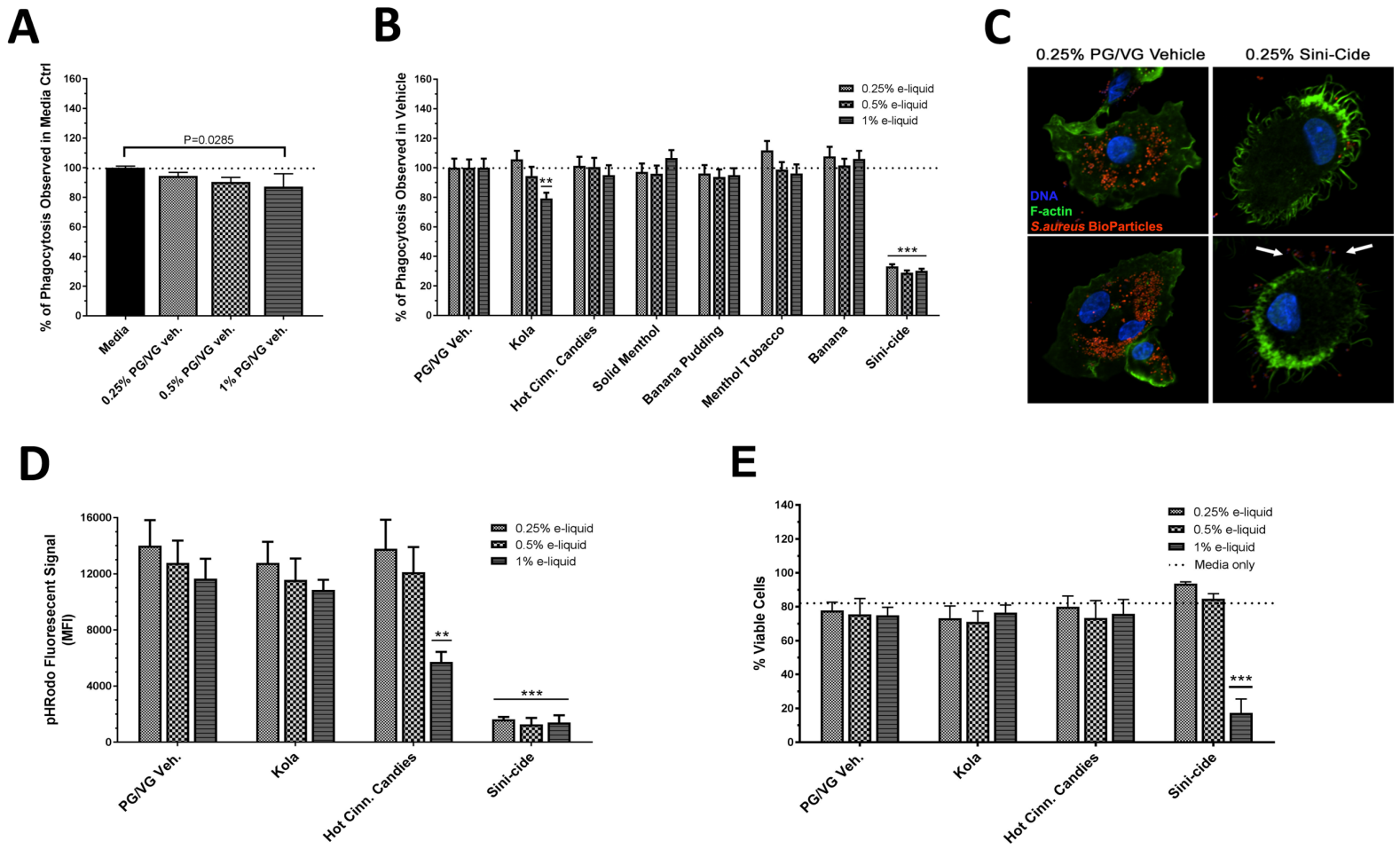

Figure 2.1 Effects of flavored e-liquids on alveolar macrophage phagocytosis. Alveolar macrophages were challenged with 1, 0.5, and 0.25% dilutions of propylene glycol/vegetable glycerin (PG/VG) vehicle (A) or electronic cigarette liquids (e-liquids, B) for 1h before the addition of *Staphylococcus aureus* BioParticles. Phagocytosis was assessed 4h after the addition of Bioparticles (n=10 experiments). C: fluorescent confocal microscopy of alveolar macrophages challenged with PG/VG vehicle (left) or "Sini-cide" e-liquid (0.25%, right). White arrows, non-phagocytosed and extracellular BioParticles. D: e-liquid-induced suppression of phagocytosis was validated by flow cytometry (n=3). E: flow-based analysis of cell viability (n=3). Data are presented as means ± SE. Significance: **P<0.01 and ***P<0.001 compared with vehicle control.

In addition to changes in phagocytic function, e-liquid-induced alterations in alveolar macrophage proinflammatory cytokine production were evaluated. Acute exposure to 1% "Kola" e-liquid significantly increased IL-6 secretion while 0.5% "Sini-cide" challenge significantly suppressed IL-6 secretion relative to the PG/VG vehicle control (Figure 2.2A). "Sini-cide" e-liquid significantly suppressed alveolar macrophage IL-8 secretion across all dilutions tested (Figure 2.2B).

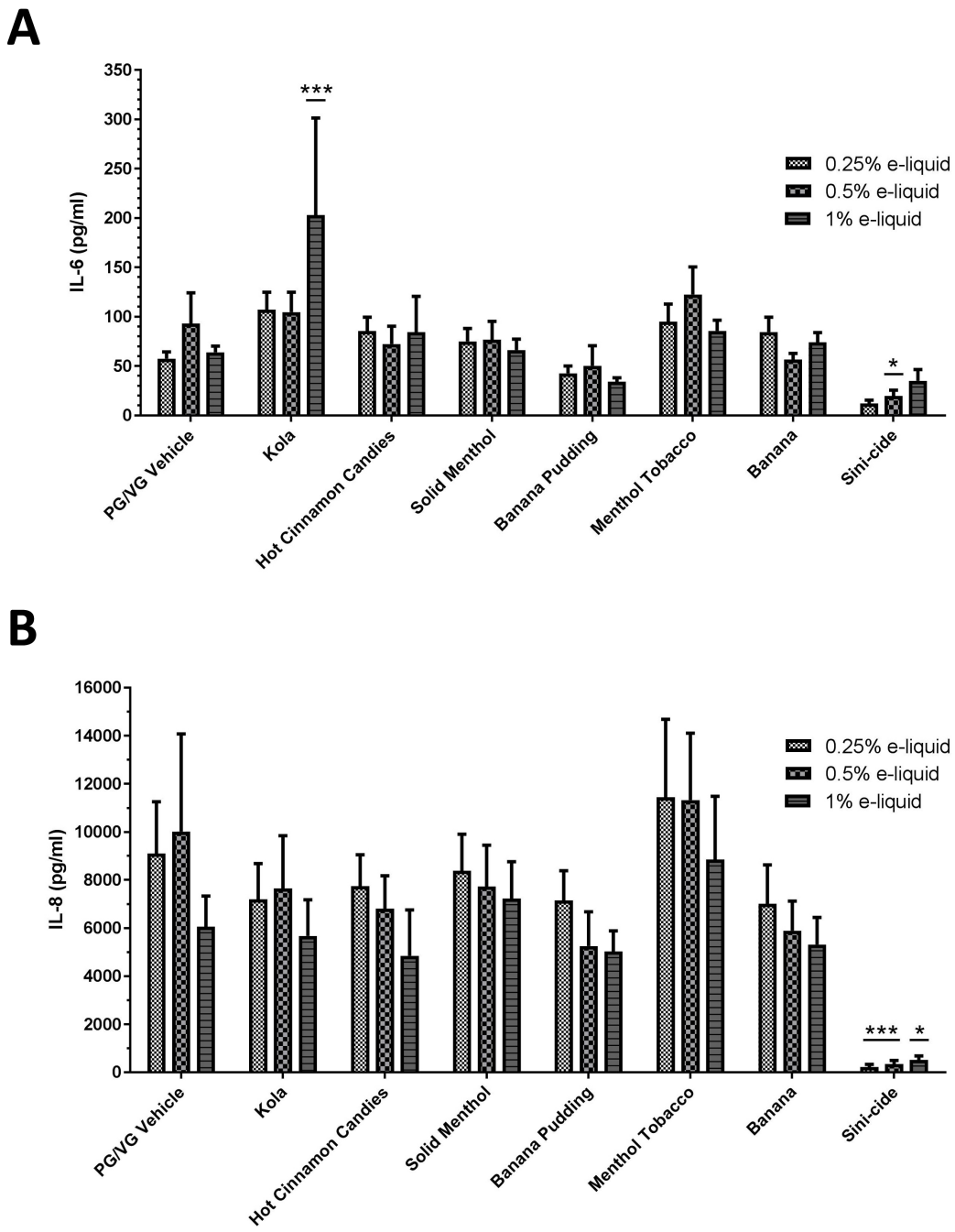

Figure 2.2 Effects of flavored e-liquids on alveolar macrophage cytokine secretion. Alveolar macrophages were challenged with 1, 0.5, and 0.25% e-liquid dilutions for 24h and evaluated for changes in interleukin (IL)-6 (A) and IL-8 (B) protein secretion (n=5). Data are presented as means ± SE. Significance: *P<0.05 and ***P<0.001 compared with vehicle control.

Effects of Flavored E-liquids on Neutrophils

Next, we determined the effects of flavored e-liquids on the phagocytic capacity of human primary neutrophils. Similar to the effect on alveolar macrophages, challenge with the 1% dilution PG/VG vehicle alone significantly reduced neutrophil phagocytosis (Figure 2.3A). Of the seven flavored e-liquids tested, “Hot Cinnamon Candies”, “Banana Pudding”, “Menthol Tobacco”, “Banana”, and “Sini-cide” reduced neutrophil phagocytosis across all dilutions in a dose-dependent manner (Figure 2.3B). While toxicity was observed at higher concentrations for “Kola” (1%) and “Sini-cide” (1%, 0.5%) flavors, none of the flavors showed significant toxicity at 0.25%, suggesting that suppression of phagocytosis was not due to e-liquid-induced cytotoxicity (LDH release assay; data not shown). Interestingly, “Sini-cide” challenge resulted in a dose-dependent change in neutrophil phagocytosis that was opposite of that observed in the other e-liquids. We observed that BioParticle fluorescence intensity (i.e. phagocytosis) increased as the percent “Sini-cide” challenge increased; however, this is likely due to cell lysis, which we only observed with the 0.5% and 1.0% “Sini-cide” challenges. Furthermore, fluorescent confocal microscopic analysis of neutrophils challenged with 0.25% “Hot Cinnamon Candies” and “Sini-cide” revealed normal neutrophil morphology but an inability to engulf labeled BioParticles (Figure 2.3C).

Next, we investigated whether neutrophils exposed to flavored e-liquids exhibited altered proinflammatory cytokine production. All flavored e-liquids, with the exception of “Solid Menthol” and “Sini-cide,” induced a significant increase in neutrophil IL-8 secretion (Figure 2.3D). Specifically, “Kola” (1%, 0.5%), “Hot Cinnamon Candies” (0.5%), “Banana Pudding” (1%, 0.5%, 0.25%), “Menthol Tobacco” (1%), and “Banana” (1%) significantly

increased IL-8 production relative to the PG/VG vehicle control. The “Sini-cide” e-liquid did not stimulate IL-8 secretion; however, this is likely due to the significant cytotoxicity observed with the 1% and 0.5% challenges.

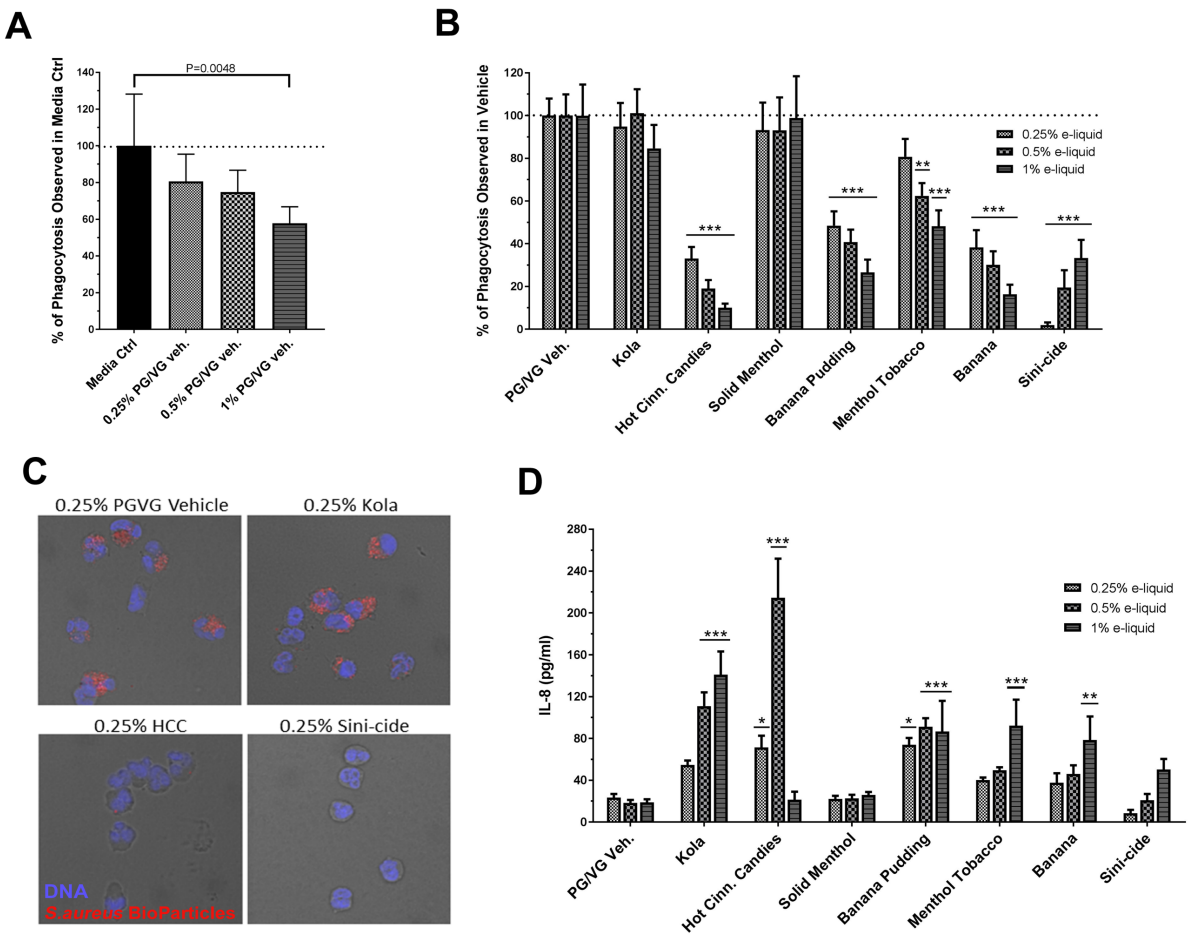

Figure 2.3 Effects of flavored e-liquids on neutrophil phagocytosis. Neutrophils were challenged with 1, 0.5, and 0.25% dilutions of PG/VG vehicle (A) or e-liquids (B) for 30 min before addition of *S. aureus* BioParticles. Phagocytosis was assessed 4h after the addition of Bioparticles (n=5). C: fluorescent confocal microscopy of neutrophils challenged with 0.25% "Kola," "Hot Cinnamon Candies," and Sini-cide. D: neutrophils were challenged with 1, 0.5, and 0.25% e-liquid dilutions for 4h and evaluated for changes in IL-8 secretion (n=3). Data are presented as means ± SE. Significance: *P<0.05, **P<0.01, and ***P<0.001 compared with vehicle control.

In addition to phagocytosis, neutrophils exert their anti-bacterial function via the formation of neutrophil extracellular traps (NETs). To assess the impact of e-liquids on NET generation, primary human neutrophils were incubated with 1% e-liquids diluted in cell culture medium alone or in the presence of 25nM PMA, a PKC agonist and potent activator of NET formation. Challenge of neutrophils with a 1% dilution of PGVG with and without 25nM PMA did not induce or alter NET formation as compared to NET formation generated with media alone and media with 25nM PMA (Figure 2.4A). Similar to PGVG, e-liquid challenges without the addition PMA did not stimulate normal NET formation; however, 1% "Sini-cide" induced chromatin release at all time points (Figure 2.4B). The pattern of chromatin extrusion observed with the "Sini-cide" challenge did not follow the oxidant-driven temporal progression of NET formation (35, 37) or the rapid, oxidant-independent generation of NETs (38), suggesting e-liquid-induced cytotoxicity and cell lysis. To assess whether flavored e-liquids alter normal NET formation, neutrophils were challenged with 1% e-liquid dilutions containing 25nM PMA and NET extrusion was quantified over four hours. Stimulation of neutrophils with 1% "Kola" significantly enhanced chromatin release at 3 hours, while challenge with 1% "Hot Cinnamon Candies" suppressed PMA-induced NET formation at 4 hours (Figure 2.4C). Exposure to 1% "Sini-cide" resulted in a similar pattern of chromatin release observed in the challenge without PMA. To visualize the time-dependent effects of these e-liquids on NET formation, we fixed neutrophils at the various time points and examined cellular morphology and markers of NET formation, such as disintegration of nuclear membranes, nuclear material filling the cytoplasm and mixing with cellular granules, and complete loss of nuclear and granular integrity (39) using confocal microscopy. Figure 2.4D indicates that "Sini-cide" was cytotoxic resulting in chromatin

release by cell lysis within the first hour of exposure, confirming the non-specific release of DNA seen in Figures 2.4B and 2.4C. Interestingly, stimulation with “Kola” in the presence of PMA appeared to expedite the steps associated with NET formation, with complete loss of nuclear and granular integrity seen at 3 hours, rather than 4 hours. In addition, stimulation with “Hot Cinnamon Candies” appeared to block progression of NET formation. These data suggest that stimulation with “Kola” and “Hot Cinnamon Candies” have differential effects on NET formation. Furthermore, the visual illustration in Figure 2.4D largely reflects the quantitative assessment of chromatin release reported in Figures 2.4B and 2.4C.

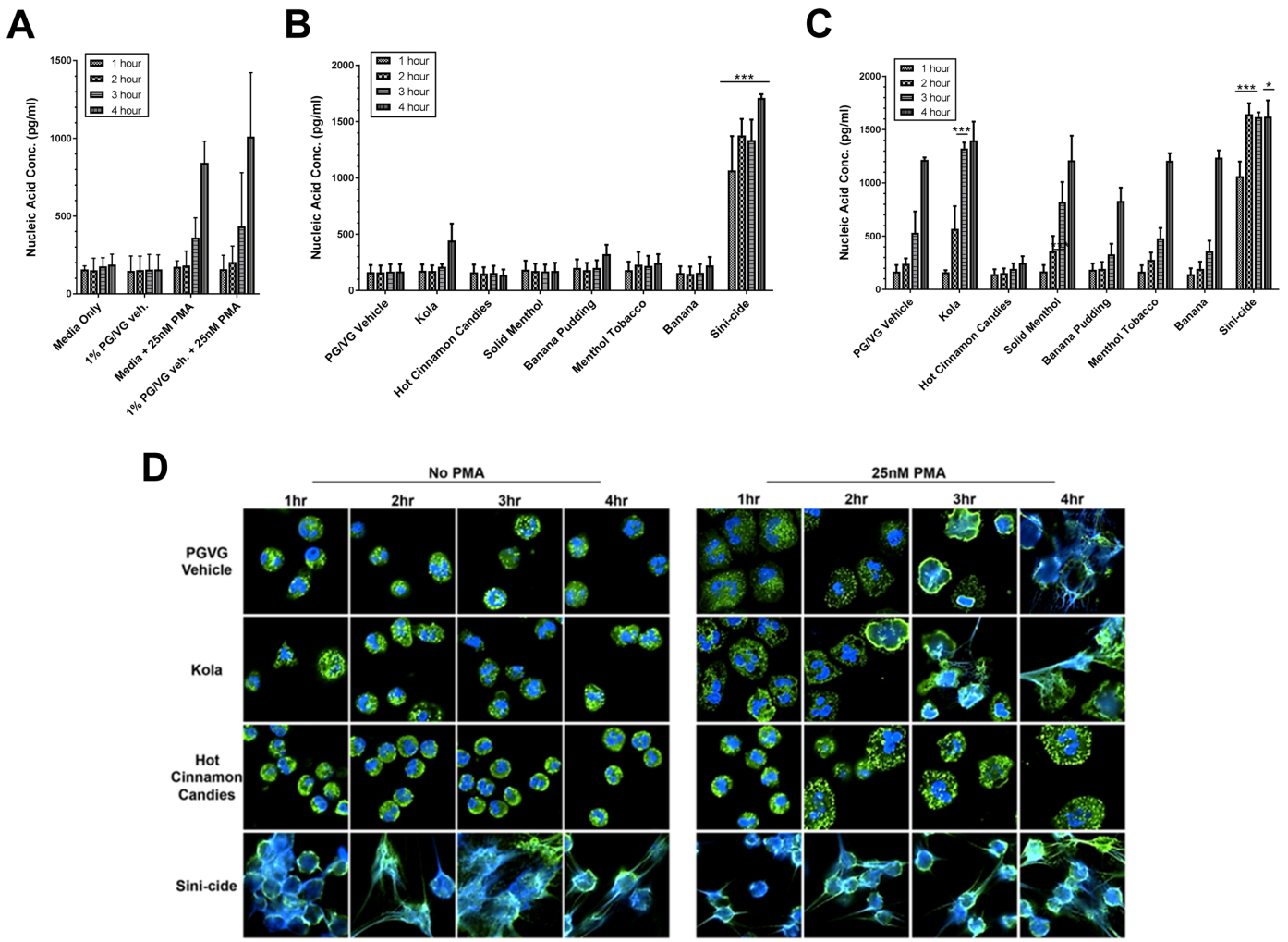

Figure 2.4 Effects of flavored e-liquids on neutrophil extracellular trap (NET) formation. Quantitation of nucleic acid released during challenge with a 1% dilution of PG/VG vehicle (A) or e-liquid challenges with 25nM phorbol 12-myristate,13-acetate (PMA, B) and without 25nM PMA (C) (n=3). D: confocal immunofluorescence microscopy of neutrophils challenged with 1% flavored e-liquids, with and without 25nM PMA. Data are presented as means ± SE. Significance: *P<0.05 and *** P<0.001 compared with vehicle control

Numerous studies have reported that nicotine elicits a variety of immune suppressive responses (13, 40, 41) and prolongs neutrophil survival (42-44). To investigate whether nicotine could alter the observed e-liquid-induced suppression of neutrophil phagocytosis, we added nicotine to the PGVG vehicle, “Kola”, “Hot Cinnamon Candies”, and “Sini-cide” e-liquids at concentrations commonly found in commercially available e-liquids (0.6, 1.2, 1.8, and 2.4 mg/ml). Stimulation with a 0.25% dilution of e-liquids with and without nicotine did not significantly affect neutrophil phagocytosis beyond the effects observed with the different flavored e-liquids alone (Figure 2.5A). In addition, emerging evidence indicates that thermal decomposition of e-liquids during the vaping process can modify the chemical composition of the aerosol mixtures (45). To examine whether and how aerosolization of e-liquids affects the suppressive effects on neutrophil phagocytosis, we prepared vaporized e-liquid condensates (VEC), similar to previously published methods (46). Figure 2.5B shows that stimulation of neutrophils with PGVG vehicle, “Kola”, “Hot Cinnamon Candies” or “Sini-cide” VEC did not significantly alter neutrophil phagocytosis as compared to the unheated e-liquid challenges. These data suggest that vaping and aerosolization of these e-liquids does not significantly modify the ability to suppress neutrophil phagocytosis.

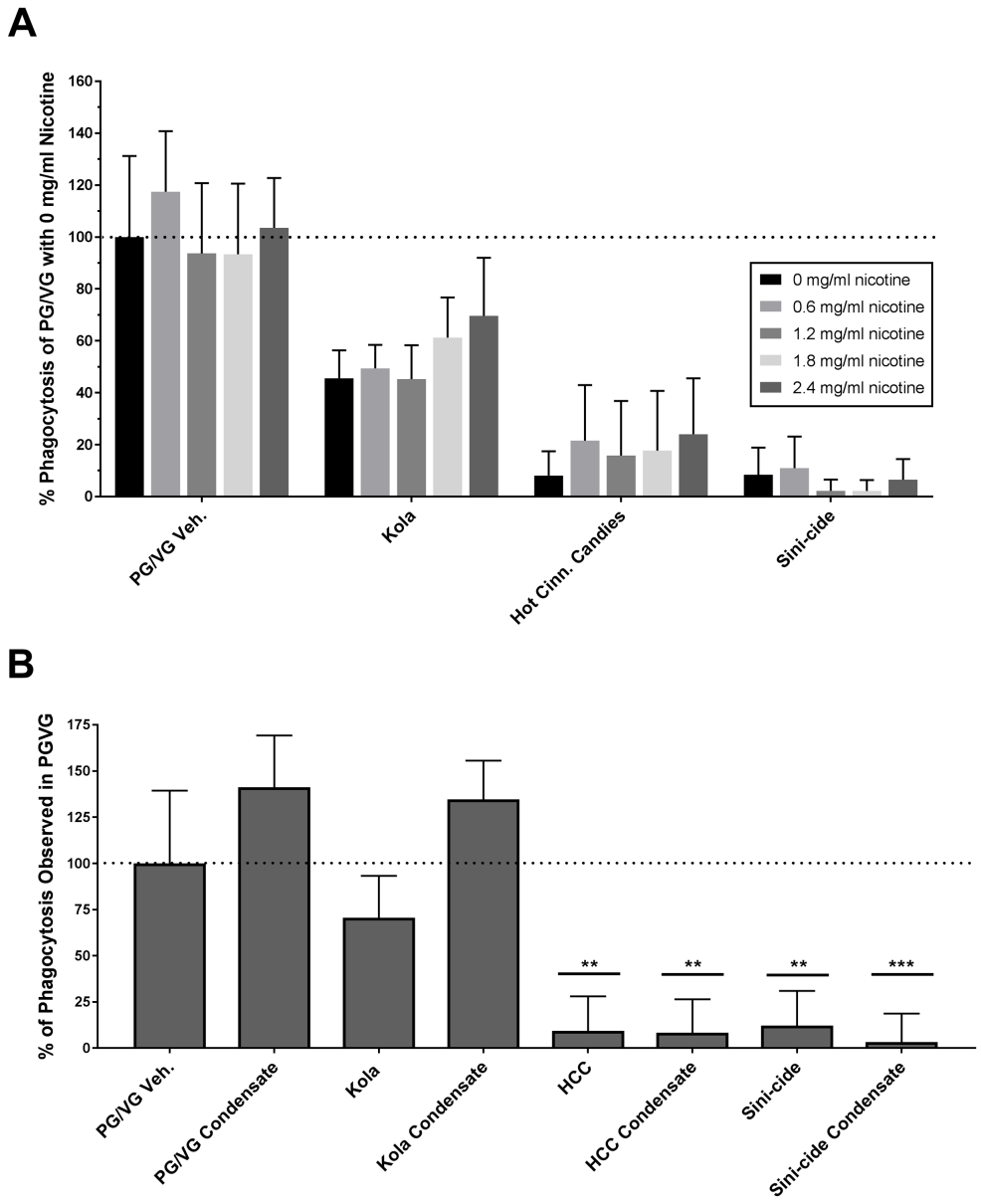

Figure 2.5 Effects of nicotine and vaping on e-liquid-induced changes in neutrophil phagocytosis. Nicotine was added to the Kola, Hot Cinnamon Candies, Sini-cide, and PG/VG vehicle e-liquids at concentrations commonly stocked in vape shops (2.4, 1.8, 1.2, and 0.6%). A: nicotine-containing e liquids were diluted to 0.25% in cell culture medium, and neutrophils were challenged for 30 min before the addition of S. aureus Bioparticles. Phagocytosis was assessed 4h after the addition of Bioparticles (n=4). B: Kola, Hot Cinnamon Candies, Sini-cide, and "PG/VG" e-liquids were "vaped" to generate vaped e-liquid condensates (VECs). Neutrophils were challenged with 0.25% dilutions of Kola, Hot Cinnamon Candies, Sini-cide, and PG/VG e-liquids or VECs for 30min before the addition of *S. aureus* Bioparticles. Phagocytosis was assessed 4h after the addition of Bioparticles (n=3). Data are presented as means ± SE. Significance: **P<0.01 and ***P<0.001 compared with vehicle control.

Effects of Flavored E-liquids on NK Cells

To evaluate the impact of flavored e-liquids on normal NK cell function, we exposed NK cells to flavored e- liquids in cell-culture medium prior to incubation with fluorescently-labeled K562 leukemia target cells. Based on the previous results in macrophages and neutrophils, we focused our analysis on the 0.25% e-liquid dilution. Following 4-hour incubation, the ability of the NK cells to eliminate target cells was determined using flow cytometry (Figure 2.6A). Killing of target cells was suppressed by cinnamon-flavored e-liquids. Specifically, 0.25% “Hot Cinnamon Candies” and 0.25% “Sini-cide” reduced the killing efficiency of NK cells as compared to the PG/VG vehicle control (Figure 2.6A, 2.6B). Reductions in NK cell killing capacity were not due to a loss of NK cell viability (data not shown).

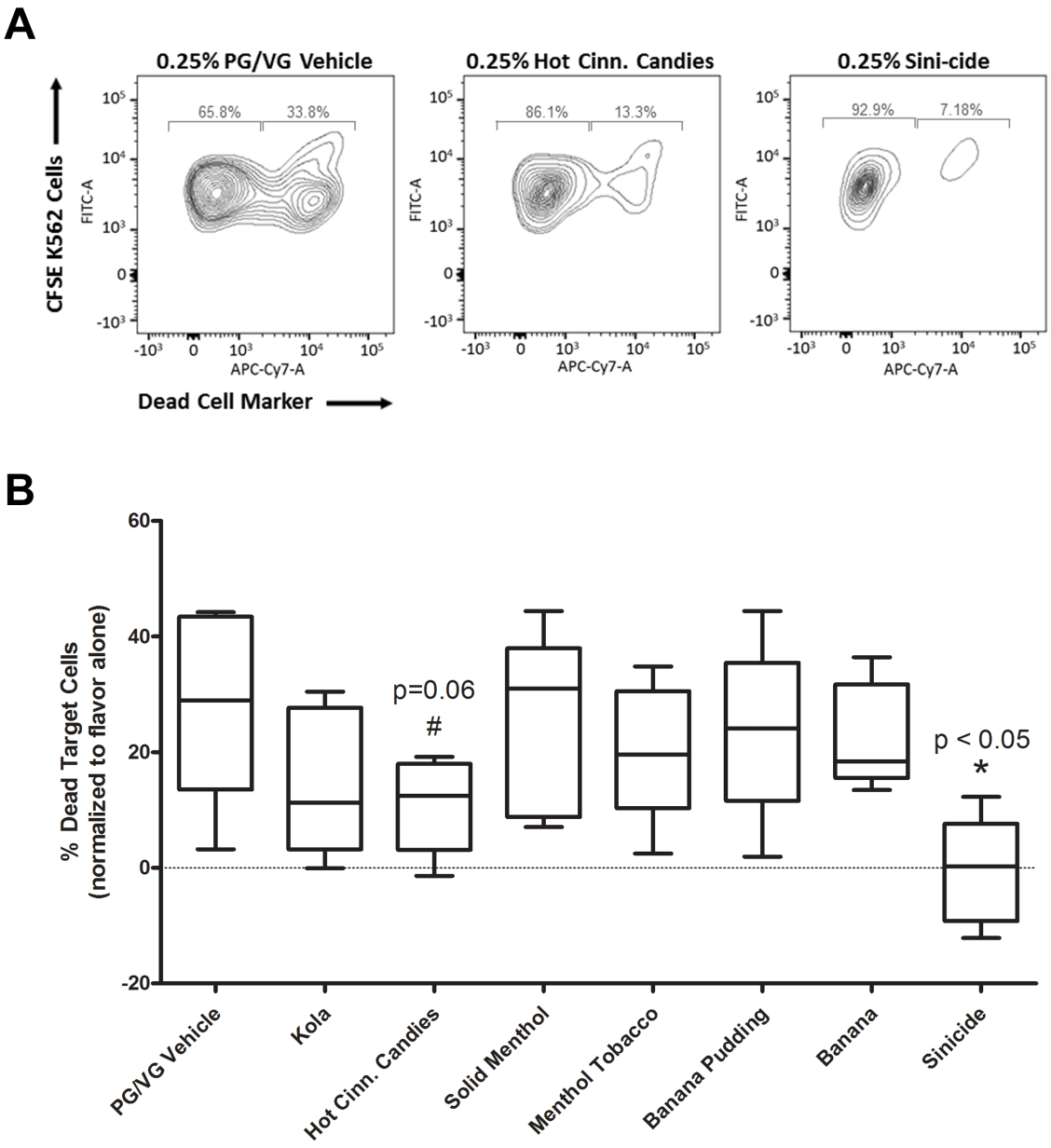

Figure 2.6 Effects of flavored e-liquids on natural killer (NK) cell-mediated cytotoxicity. NK cells were incubated for 1 h with 0.25% e-liquids and then combined with carboxyfluorescein succinimidyl ester (CFSE)-labeled K562 target cells for 4 h. A: flow cytometry analysis of CFSE bright target cells counter-labeled with a far-red live/dead indicator. B: quantitation of NK cell-mediated killing of K562 leukemia target cells (n=5). Data are presented as means ± SE. Significance: *P<0.05 compared with vehicle control.

Chemical Composition of Flavored E-liquids

Gas chromatography-coupled mass spectrometry was used to determine the qualitative chemical composition of the 7 flavored e-liquids used for this study. Spiro[1,3-dioxolane 2,2'(1'H)-naphthalene], 3',4'-dihydro-, vinyl trans-cinnamate, and cinnamaldehyde were present in the "Kola", "Hot Cinnamon Candies", and "Sini-cide" flavored e-liquids (Table 2.1). Quantitative mass spectrometry analysis for cinnamaldehyde content in these flavored e-liquids revealed that the "Kola," "Hot Cinnamon Candies," and "Sini-cide" contained 0.896mM ± 0.360mM, 39.328mM ± 3.414mM, and 1.131M ± 0.0199M, respectively (Table 2.2). Based on these measured concentrations of cinnamaldehyde in the neat e-liquids, we extrapolated the concentrations of cinnamaldehyde in the 1%, 0.5%, and 0.25% dilutions of "Kola", "Hot Cinnamon Candies", and "Sini-cide" used to challenge cells. In addition, we determined whether aerosolization of these e-liquids modified the cinnamaldehyde content. As shown in Table 1, cinnamaldehyde was present in the VEC derived from "Kola", "Hot Cinnamon Candies", and "Sini-cide", but at lower concentrations, likely eflecting wall-loss caused by the system generating the condensate.

Kola	Hot Cinn. Candies	Solid Menthol	Banana Pudding	Menthol Tobacco	Banana	Sini-cide
Propylene Glycol	Propylene Glycol	Propylene Glycol	Propylene Glycol	Propylene Glycol	Propylene Glycol	Propylene Glycol
Dipropylene Glycol	Dipropylene Glycol	-	Dipropylene Glycol	Dipropylene Glycol	Dipropylene Glycol	Dipropylene Glycol
-	Glycerin	Glycerin	Glycerin	Glycerin	Glycerin	Glycerin
Dimethyl ether	-	-	Dimethyl ether	Dimethyl ether	Dimethyl ether	Dimethyl ether
-	Vanillin	-	Vanillin	Vanillin	Vanillin	Vanillin
-	Eugenol	-	Eugenol	-	Eugenol	Eugenol
Triacetin	Triacetin	Triacetin	-	-	Triacetin	-
-	Glycerol 1-monoacetate	Glycerol 1-monoacetate	-	Glycerol 1-monoacetate	-	-
Spiro[1,3-dioxolane-2,2'(1'H)-naphthalene], 3',4'-dihydro-	***Spiro[1,3-dioxolane-2,2'(1'H)-naphthalene], 3',4'-dihydro-***	-	-	-	-	***Spiro[1,3-dioxolane-2,2'(1'H)-naphthalene], 3',4'-dihydro-***
Cinnamaldehyde, (E)-	***Cinnamaldehyde, (E)-***	-	-	-	-	***Cinnamaldehyde, (E)-***
Vinyl trans-cinnamate	***Vinyl trans-cinnamate***	-	-	-	-	***Vinyl trans-cinnamate***
2-Propenoic acid, 3-phenyl-, ethyl ester	-	-	-	-	-	2-Propenoic acid, 3-phenyl-, ethyl ester
-	-	-	Benzyl alcohol	-	-	Benzyl alcohol
-	-	-	-	Decane, 3,7-dimethyl-	-	Decane, 3,7-dimethyl-
-	-	-	-	Estragole	-	Estragole
1-Propanol, 2-(2-hydroxypropoxy)-	-	-	1-Propanol, 2-(2-hydroxypropoxy)-	-	-	-
-	Maltol	-	Maltol	-	-	-
-	-	-	Ethyl Maltol	-	Ethyl maltol	-
-	-	-	Isoamyl acetate (banana oil)	-	Isoamyl acetate (banana oil)	-
-	-	-	Pentanoic acid, 1,1-dimethylpropyl ester	-	Pentanoic acid, 1,1-dimethylpropyl ester	-
-	-	-	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	-	2,6-Octadien-1-ol, 3,7-dimethyl-, acetate, (Z)-	-
-	-	-	Ethyl Vanillin	Ethyl Vanillin	-	-
-	-	Menthol	-	Menthol	-	-
α Terpineol	-	-	-	-	-	-

Kola	Hot Cinn. Candies	Solid Menthol	Banana Pudding	Menthol Tobacco	Banana	Sini-cide
1,2,3-Propanetriol, monoacetate	-	-	-	-	-	-
1,3-Dioxolane, 4-methyl-2-phenyl-	-	-	-	-	-	-
Acetic acid, methyl ester	-	-	-	-	-	-
Benzaldehyde	-	-	-	-	-	-
Benzene, 1-methyl-3-(1-methylethyl)-	-	-	-	-	-	-
Benzeneacetic acid, methyl ester	-	-	-	-	-	-
beta.-Pinene	-	-	-	-	-	-
D-Limonene	-	-	-	-	-	-
Eucalyptol	-	-	-	-	-	-
S-(+)-5-(1-Hydroxy-1-methylethyl)-2-methyl-2-cyclohexen-1-one	-	-	-	-	-	-
-	Benzyl Benzoate	-	-	-	-	-
-	Cinnamyl acetate	-	-	-	-	-
-	-	4H-Pyran-4-one, 2-ethyl-3-hydroxy-	-	-	-	-
-	-	Propanedioic acid, (2-phenylethyl)-, diethyl ester	-	-	-	-
-	-	Propanedioic acid, diethyl ester	-	-	-	-
-	-	Pyridine, 3-(1-methyl-2-pyrrolidinyl)-, (S)-	-	-	-	-
-	-	-	1,2,3-Propanetriol, diacetate	-	-	-
-	-	-	1,2-Cyclopentanedione, 3-methyl-	-	-	-
-	-	-	1,3-Dioxolane, 2-(4-methoxyphenyl)-4-methyl-	-	-	-

Kola	Hot Cinn. Candies	Solid Menthol	Banana Pudding	Menthol Tobacco	Banana	Sini-cide
-	-	-	2(3H)-Furanone, 5-ethyldihydro-	-	-	-
-	-	-	3-Acetyl-2,5-dimethyl furan	-	-	-
-	-	-	4-Methoxycarbonyl-4-butanolide	-	-	-
-	-	-	Benzaldehyde, 4-methoxy-	-	-	-
-	-	-	Benzenemethanol, 4-methoxy-	-	-	-
-	-	-	Hemineurine	-	-	-
-	-	-	Veratraldehyde propylene glycol acetal	-	-	-
-	-	-	-	5-Methyl-2-phenyl-2-hexenal	-	-
-	-	-	-	Cyclohexanol, 5-methyl-2-(1-methylethyl)-, (1α,2β,5α)-	-	-
-	-	-	-	Phenol, 2-methoxy-	-	-
-	-	-	-	-	2-Octynoic acid, methyl ester	-
-	-	-	-	-	3,3-Dimethyl-1,2-epoxybutane	-
-	-	-	-	-	3-Hexen-1-ol	-
-	-	-	-	-	-	2,6-Piperidinedione, 3-phenyl-
-	-	-	-	-	-	Anethol
-	-	-	-	-	-	Carbamic acid, methyl-, 3-methylphenyl ester

Table 2.1 Composition of flavored e-liquids used for this study

	Kola	Hot Cinnamon Candies	Sini-cide
Neat E-liquid	0.896mM ± 0.360mM	39.32mM ± 3.41mM	1131mM ± 19.9mM
1% Dilution†	8.96µM	393.28µM	11.31mM
0.5% Dilution†	4.48µM	196.64µM	5.66mM
0.25% Dilution†	2.24µM	98.32µM	2.83mM
Vaped E-liquid Condensate	0.742mM ± 0.035mM	9.807mM ± 0.743mM	183.9mM ± 4.089mM

Table 2.2 Quantitation of cinnamaldehyde in e-liquids and vaped e-liquid condensates. Values are means ± SD. †Cinnamaldehyde in e-liquid dilutions is extrapolated from neat e-liquid measurements.

Effects of Cinnamaldehyde on Alveolar Macrophage, Neutrophil, and NK cell Function

Mass spectrometry analysis revealed that cinnamaldehyde was a major flavoring component in "Sini-cide," "Hot Cinnamon Candies," and "Kola," and each of these e-liquids impaired normal immune cell function in our study. To determine the specific contribution of cinnamaldehyde to the observed immunosuppressive effects, we challenged alveolar macrophages, neutrophils, and NK cells with various doses of cinnamaldehyde diluted in cell culture medium and assessed changes in functional endpoints. Alveolar macrophages were incubated with cinnamaldehyde for one hour prior to the addition of Staphylococcus aureus BioParticles. Changes in phagocytosis were assessed after four hours. A significant reduction in phagocytosis was observed with cinnamaldehyde concentrations at and greater than 312.5μM, while cinnamaldehyde-induced cell death was only apparent at the 10mM concentration (Figure 2.7A). Similarly, neutrophils were challenged for 30 minutes with a broad range of cinnamaldehyde concentrations and phagocytosis of BioParticles was assessed. Neutrophil phagocytosis was significantly inhibited at cinnamaldehyde concentrations of 10μM and greater. Viability was significantly reduced with 5mM and 10mM cinnamaldehyde exposures (Figure 2.7B). To assess the direct effects of cinnamaldehyde on NK cell killing of target leukemia cells, NK cells were challenged with a broad range of cinnamaldehyde concentrations for one hour prior to incubation with fluorescently-labeled K562 target cells. Flow cytometry analysis revealed that NK cell killing efficiency was significantly reduced with cinnamaldehyde challenges between 78μM and 1mM. Exposure of target cells to cinnamaldehyde concentrations greater than 1.25mM caused significant cell death independent of NK cell activity. Cinnamaldehyde significantly reduced NK cell viability at concentrations of 625μM and greater (Figure 2.7C).

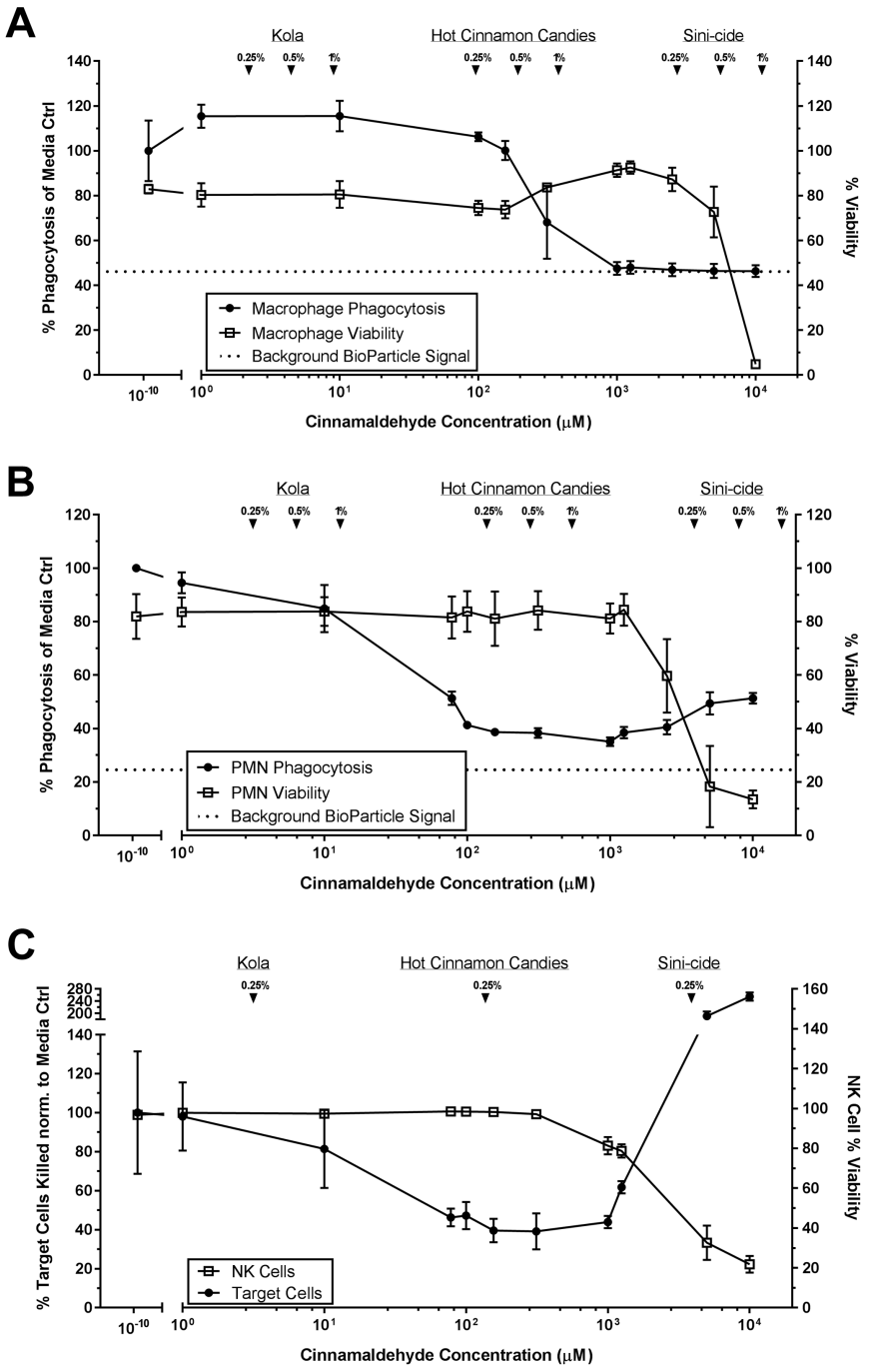

Figure 2.7 Effects of cinnamaldehyde on alveolar macrophage, neutrophil, and NK cell function. Alveolar macrophages (A) (n=3) and neutrophils (B) (n=3) were challenged with a broad range of cinnamaldehyde concentrations. Cinnamaldehyde-induced changes in Bioparticle phagocytosis and cell viability were evaluated by flow cytometry. C: NK cells (n=3) were challenged with a broad range of cinnamaldehyde concentrations, and changes in cell-mediated killing of K562 leukemia cells and NK cell viability were quantified using flow cytometry. Data are presented as means ± SE. Extrapolated e-liquid concentrations of cinnamaldehyde for each dilution tested (1, 0.5, and 0.25%) are indicated within each panel.

The inhibitory (IC_{50}) and cytotoxic (LC_{50}) concentrations for cinnamaldehyde on each endpoint in the respective cell type tested are reported in Table 2.3.

	Alveolar Macrophages	Neutrophils	NK Cells
IC_{50} (mM)	0.243 ± 0.0593	0.0324 ± 0.0129	0.0205 ± 0.0054
LC_{50} (mM)	6.984 ± 0.942	3.352 ± 0.838	3.133 ± 0.732

Table 2.3 The inhibitory and cytotoxic potential of cinnamaldehyde on human primary alveolar macrophages, neutrophils, and NK cells.

Cinnamaldehyde is a potent electrophile that likely reacts with free sulfhydryl groups on cysteine residues on proteins (47). However, it is unclear whether the sulfhydryl-modifying activity of cinnamaldehyde contributes to the observed suppression of immune cell function. To investigate this potential mechanism, we conducted competition experiments using the small-molecule reducing agent dithiothreitol (DTT). Cinnamaldehyde- mediated suppression of alveolar macrophage phagocytosis was reversed by co-exposure with cinnamaldehyde and DTT (Figure 2.8), suggesting protein thiol modification as a potential mechanism for cinnamaldehyde-induced immune cell dysfunction.

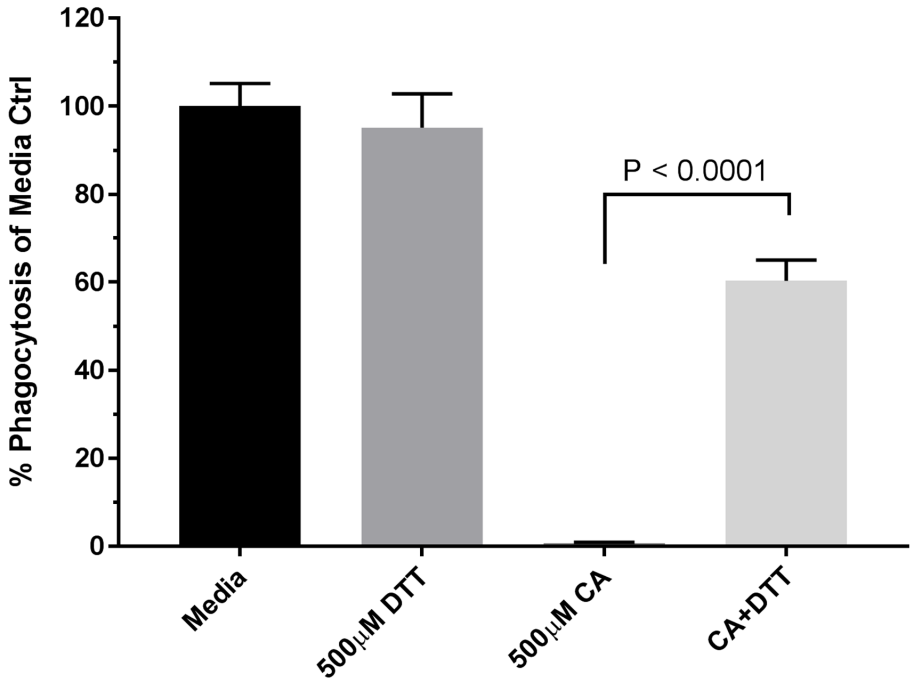

Figure 2.8 Coexposure of alveolar macrophages with cinnamaldehyde and DTT reverses cinnamaldehyde-induced suppression of phagocytosis. Alveolar macrophages were challenged with cell culture medium, 500uM DTT, 500uM cinnamaldehyde, or 500uM DTT and 500uM cinnamaldehyde, and changes in BioParticle phagocytosis were evaluated (n=3). Data are presented as means ± SE.

2.4 Discussion

Vaping e-cigarettes is frequently viewed as a safer alternative to cigarette smoking based on studies that have reported substantially lower levels of known toxicants and carcinogens commonly found in cigarette smoke (48-51). Recent publications have highlighted the potential risk for exposure to reactive chemicals (52-57) and toxicants (45, 58, 59), such as flavoring agents, not present in traditional cigarette smoke. Furthermore, we recently demonstrated that vaping e-cigarettes is associated with decreased expression of immune genes in the nasal mucosa of human volunteers (60). However, the manner by which these genomic alterations may affect respiratory immune responses is not known. In the present study, we evaluated the effects of seven flavored e-liquids on respiratory innate immune cell function by exposing human alveolar macrophages, neutrophils, and NK cells to varying dilutions of e-liquid and VEC. In addition to identifying flavor-specific and dose-dependent decreases in functional endpoints across all three human primary immune cell types, our data demonstrate that cinnamaldehyde-containing e-liquids had the most immunosuppressive effects across the different endpoints and cell types examined. Exposure of alveolar macrophages, neutrophils, and NK cells to a broad range of cinnamaldehyde spanning seven orders of magnitude established a dose- response relationship for cinnamaldehyde-induced suppression of human immune cell function, supporting a direct cause-and-effect relationship between exposure to cinnamaldehyde-containing e-liquids and suppression of respiratory immune cell function. Furthermore, addition of the thiol reducing agent DTT inhibited the effects cinnamaldehyde on alveolar macrophages, suggesting cinnamaldehyde-induced thiolation as a potential mechanism mediating the observed immune suppressive effects.

Cinnamon-flavored e-liquids are popular among e-cig users and are often identified as “Best Sellers” by online e-liquid retailers. However, some users have reported mouth, throat, and respiratory irritation after vaping some cinnamon e-liquids (61) and strong cinnamon-flavored e liquids have been reported to erode plastic tanks used on many second generation vaporizers (https://www.reddit.com/r/electronic_cigarette/comments/1bnuo7/why_do_some_eliquids_crack_plastic_tanks/). Several recent studies have illustrated the potential for cinnamaldehyde to cause harm in the context of e- cigarette and e-liquid exposures. Behar et al. evaluated the effects of eight cinnamon-flavored refill fluids on human embryonic stem cells and human adult pulmonary fibroblasts and found that most of the e-liquids tested were cytotoxic (61). Furthermore, they specifically attributed the observed e-liquid-induced cytotoxicity to cinnamaldehyde and 2-methoxycinnamaldehyde. More recently, Lerner et al. showed that human lung fibroblasts exhibited cellular stress responses, morphological changes, increased IL-8 secretion, and ultimately cell death in response to challenge with a cinnamon flavored e liquid (62). Our data reveal that both cinnamon- flavored e-liquids and cinnamaldehyde suppress immune cell function in each cell type tested in a dose-dependent manner at concentrations below levels causing cytotoxicity (Table 2.2). Thus, our data establish a direct link between a specific flavoring chemical used in e-liquids and suppression of respiratory immune cell function.

Innate immune cells of the respiratory system, which predominately consist of alveolar and airway macrophages, neutrophils in the perialveolar vasculature, and NK cells in the nasal passages and sinuses (63), play an essential role in eliminating inhaled pathogens and damaged host cells to prevent infection and injury. Toxicants that impair the innate

immune functions of these cell types, by either aberrant activation or suppression, would therefore disrupt respiratory immune system homeostasis and alter susceptibility to respiratory infections. Hwang et al. recently reported that e-cigarette vapor extract reduced both macrophage and neutrophil antimicrobial activity against *Staphylococcus aureus* (13). However, the effect was attributed to nicotine, propylene glycol, and glycerin rather than specific flavoring agents. We report here that acute exposure of human alveolar macrophages and neutrophils to various flavored e-liquids reduced phagocytosis of Staphylococcus aureus; however, the impaired clearance we observed was flavor-specific with phagocytosis being suppressed more broadly in neutrophils (Figures 2.1 and 2.3). Moreover, the addition of nicotine to our e-liquid challenges did not significantly alter the suppression of phagocytosis observed with exposure to nicotine-free e-liquids (Figure 2.5). While nicotine, at sufficient concentrations, may independently suppress innate immune cell activation and impair the clearance of Staphylococcus aureus, our data illustrate that chemical flavorings likely contribute to the suppression of key respiratory immune cell functions. Indeed, previous work by Kim et al. found that cinnamaldehyde alters immune responses in monocyte and macrophage cell lines by thiolation of target cysteine residues, supporting the notion that a more direct mode of action, such as protein modification, mediates the cinnamaldehyde-induced suppression of macrophage immune function (27). To investigate whether the sulfhydryl-modifying activity of cinnamaldehyde mediates suppression of respiratory innate immune cell function, we conducted competition experiments using the small-molecule reducing agent dithiothreitol (DTT). The observed cinnamaldehyde-mediated suppression of alveolar macrophage phagocytosis was reversed by co-exposure with DTT, suggesting that the sulfhydryl-modifying activity of cinnamaldehyde correlates with cinnamaldehyde-

induced reductions in phagocytic capacity (Figure 2.8). While these data strongly suggest that interactions between cinnamaldehyde and susceptible nucleophilic residues of proteins are driving cinnamaldehyde-mediated impairments of immune cell function, further work is needed to determine which proteins are adducted and which cellular pathways are affected.

Neutrophils play an essential role in protecting the airways from bacterial infection by common inhaled pathogens including *Streptococcus pneumoniae* (64), *Klebsiella pneumoniae* (65), *Legionella pneumophila* (66), and *Staphylococcus aureus* (67). In healthy individuals, phagocytosis of bacterial pathogens stimulates the production of reactive oxygen species (ROS), which in turn facilitates the release of microbicide granule components into phagosomes. Tobacco smoke impairs respiratory burst activation and thereby compromises the anti-bacterial function of neutrophils (68). Furthermore, recent studies of neutrophil function (including cell migration, ROS generation, degranulation, phagocytosis and NET generation) in COPD patients indicate impaired responses that predispose towards reduced bacterial clearance and increased inflammation (69). Acrolein, an α,β-unsaturated aldehyde found in cigarette smoke, impairs neutrophil function by depleting glutathione levels and suppressing NADPH oxidase activity by the direct alkylation of cysteine residues in proteins involved in NADPH oxidase activation (68). While levels of acrolein in e-liquids and e-cigarette aerosols are reported to be substantially lower than those present in cigarette smoke (49), α,β unsaturated aldehyde flavorings, such as cinnamaldehyde, may impair neutrophil function by a similar mechanism. Our results indicate that cinnamaldehyde-containing e-liquids suppress neutrophil phagocytosis of Staphylococcus aureus in a dose-dependent manner (Figure 2.3A and 2.3B) and subsequent challenge with cinnamaldehyde alone recapitulated the effect (Figure 2.7B). Moreover, cinnamaldehyde-induced inhibition of

phagocytosis was observed at low micro molar concentrations (IC_{50} = 32.4μM ± 12.9μM), more than two orders of magnitude lower than those eliciting cytotoxicity (LC_{50} = 3.35mM ± 0.84mM) (Table 2.3).

Neutrophils also eliminate bacterial pathogens by expelling extracellular traps composed of chromatin and anti- microbial peptides originating from intracellular vesicles (35, 69, 70). NET formation is initiated by stimulation of surface receptors including Toll-like receptors (TLRs), cytokine receptors, and fragment crystallizable (Fc) receptors resulting in activation of protein kinase C (PKC) and the NADPH oxidase complex (69, 71). While the exact role of NETs in respiratory disease remains unclear, accumulation of NETs has been implicated in the pathogenesis of numerous non-infectious diseases, inflammatory disorders, and autoimmune disorders, including cystic fibrosis, asthma, and COPD (72). Less is known about the consequences of impaired NET formation; however, the developmental syndrome of neonatal neutrophil dysfunction is characterized by compromised NET generation and increases susceptibility for neonatal septicemia and other infectious complications in human neonates (73). Our finding that 1% Kola (8.96μM cinnamaldehyde) significantly increased the rate of Phorbol 12-myristate 13-acetate (PMA)-induced NET formation, while 1% Hot Cinnamon Candies (393.28μM cinnamaldehyde) significantly decreased the rate of PMA-induced NET formation, suggests that cinnamaldehyde may elicit a concentration-dependent, bimodal response (Figure 2.4). Aberrant NET activation, such as we observed during the 1% Kola e liquid exposure, may increase NET-mediated tissue damage and respiratory dysfunction. Alternatively, inhibition of NET formation, such as we observed with exposure to the 1% dilution of Hot Cinnamon Candies e-liquid, may allow for enhanced dissemination of bacterial pathogens in the lungs and increase the risk of respiratory infections.

NK cells are a subset of cytotoxic lymphocytes that serve as sentinels of the immune system by recognizing and killing virus-infected and tumor cells. Unlike other lymphocytes, NK cells do not require previous sensitization to recognize compromised cells; rather, they utilize activating and inhibiting receptors to interrogate host cells for molecular changes indicative of damage or infection (74). NK cells lyse host cells presenting abnormal surface antigens via exocytosis of cytotoxic perforin and granzymes. Studies indicate that smoking is associated with reduced numbers of circulating NK cells (75) and cigarette smoke reduces NK cell-mediated killing of cancer cells in vitro (76). Furthermore, our group reported that NK cells are a major immune cell type in human nasal passages and influenza-induced cytotoxic NK cell responses are suppressed in smokers (63). Here, we expanded on the current literature by evaluating the effects of flavored e-liquids and cinnamaldehyde flavoring on NK cell function. Our data indicate that exposure of NK cells to 0.25% dilutions of two cinnamon-flavored e liquids in our panel suppressed NK cell cytotoxic function, as measured by the ability to kill leukemia target cells (Figure 2.5). Exposure to cinnamaldehyde alone recapitulated this effect, inhibiting NK cell killing at low micromolar concentrations (IC_{50} = 20.5μM ± 5.4μM) (Figure 2.7 and Table 2.3). To our knowledge, we are the first to report that flavored e-liquids and cinnamaldehyde, a common flavoring agent, can suppress NK cell mediated killing of target cells, which could have implications for their ability to kill both tumorigenic and virus-infected host cells.

Flavoring agents, which were banned from use in traditional cigarettes by the U.S. FDA in 2009, are broadly used in the manufacture of e-cigarettes and refill e-liquids (17) and contribute to the surging popularity of these products (77). A precedent for food-safe chemical flavorings causing inhalational injury was established in May 2000 when eight

workers at a microwave popcorn-processing facility developed bronchiolitis obliterans, an irreversible obstructive disease of the bronchioles, as a result of exposure to the aerosolized butter flavoring diacetyl (78). A recent study published by Allen et al. screened 51 unique, flavored e-liquids for the presence of diacetyl, acetoin, and 2-3 pentanedione (52) and revealed that 47 of 51 e-liquids contained at least one of the potentially hazardous butter-flavoring chemicals. In addition to the butter flavorings, recent in vitro studies report that the cytotoxic effects of e-liquids were largely due to chemical flavorings (79, 80). Bahl el al. evaluated the cytotoxicity of 41 e-liquids on human pulmonary fibroblasts, human embryonic stem cells, and mouse neural stem cells. They report that cytotoxicity varied by product; however, cytotoxicity did not result from nicotine exposure but rather correlated with the number and concentration of chemicals used to flavor fluids (81). More recently, Gerloff et al. evaluated lung epithelial cell and fibroblast inflammatory responses following exposure to various e-cigarette flavoring chemicals. Their data indicate that ortho-vanillin, acetoin, and maltol stimulate proinflammatory cytokine (IL-8) secretion in lung epithelial cells and fibroblasts. Furthermore, the authors report that exposure of airway epithelial cells to diacetyl, coumarin, acetoin, maltol, and cinnamaldehyde resulted in a significant and persistent loss of epithelial barrier function (55). Taken together, previous reports and our data shown here demonstrate a clear dose-response relationship between levels of cinnamaldehyde occurring in commercially available e-liquids and suppressed function of human respiratory immune cells. These findings add to the concern that flavored e-liquids may contain potentially hazardous chemicals and illustrate the need to investigate the respiratory effects of common flavorings used in the manufacture of e-liquids.

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CHAPTER 3.

CINNAMALDEHYDE IN FLAVORED E-CIGARETTES SUPPRESSES CILIARY MOTILITY ON BRONCHIAL EPITHELIAL CELLS BY DYSREGULATION OF MITOCHONDRIAL RESPIRATION AND GLYCOLYTIC FUNCTION

3.1 Introduction

Mucociliary clearance is the primary physical airway defense that prevents inhaled pathogens and debris from injuring the airway epithelium and underlying tissues. Particles deposited on the epithelial surface during inspiration become trapped within airway mucus and motile airway cilia beat in coordinated metachronal waves to propel mucus-particle aggregates towards the larynx (1). Airway cilia are complex biochemical engines that are essential to effective mucus transport. Substances that interfere with normal cilia motility can impede mucociliary clearance and increase the residence time of inhaled pathogens, thereby enhancing susceptibility to respiratory infection. Reduced mucociliary clearance is a functional abnormality in smokers which often precedes or co-occurs with the development of chronic bronchitis and obstructive lung disease (2). Cigarette smoke alters mucus composition, airway surface hydration, cilia structure, intercellular cilia coordination, and reduces cilia beat frequency (CBF) (3-9). Many of the direct effects of cigarette smoke on cilia structure and function are believed to be due to highly reactive aldehydes produced when smoking. Hastie and colleagues exposed rabbit and porcine tracheal explants to formaldehyde, a known toxin in cigarette smoke, and observed a rapid but reversible

reduction in cilia motility (3). Further, they observed a dose-dependent reduction in ciliary axoneme ATPase activity that was also reversible. Acetaldehyde is reported to induce concentration- and time-dependent reductions in CBF and cilia-derived dynein ATPase activity in primary cultures and isolated axonemes of bovine airway epithelial cells (4). Acrolein is a known pulmonary hazard in cigarette smoke which alters mucus composition, irreversibly modifies glutathione, and induces ciliostasis at concentrations as low as 0.14ppm (0.14mg/L) (5-8). These and other aldehydes in cigarette smoke contribute to the increased occurrence of respiratory infections and pathogenesis of lung disease in smokers (9, 10).

While smoking remains the number one cause of preventative death in the U.S., the prevalence of cigarette smoking among U.S. adults has steadily declined since 1965 (11). As the use of traditional tobacco products has fallen in recent years, the popularity of new and emerging tobacco products, such as e-cigarettes, has surged. There are currently thousands of flavored e-cigarette products on the market (12). Over the last decade, e-cigarette devices have evolved from simple, disposable "cig-a-likes" to complex, highly-modifiable devices with advanced electronics. E-liquids are becoming increasingly customizable in terms of nicotine and propylene glycol/glycerine content, and there are countless flavor offerings available. These products are not only popular with current smokers attempting to quit but have rapidly gained popularity with adolescents and young adults who have never smoked (13-15). E-cigarettes have been heavily marketed as safer alternatives to traditional cigarettes and effective cessation aids to reduce smoking (16). This representation has led to the perception by many that e-cigarettes are harmless (17). Numerous scientific studies have reported that e-cigarettes produce substantially lower levels of known toxicants and carcinogens commonly found in cigarette smoke, including reactive aldehydes. However,

recent publications have highlighted the potential risk for exposure to toxicants (18), metals (19, 20), and reactive chemicals, such as flavoring agents (21), which are not present in traditional cigarette smoke.

Most flavorings agents used in e-cigarettes are identified as "generally recognized as safe" (GRAS) by the U.S. Food and Drug Administration (FDA). However, this designation only pertains to oral exposures and not exposures by inhalation. Prolonged inhalation of some GRAS flavorings, including diacetyl, 2,3-pentanedione, and acetoin, is well documented to cause irreversible lung disease (22-24). The Flavor and Extract Manufacturers Association (FEMA) of the USA, a trade association of flavor ingredient manufacturers which evaluates the safety of food flavorings, has identified 1037 GRAS flavoring agents as potential respiratory hazards due to possible volatility and respiratory irritant properties, however most of these compounds still lack any toxicologic evaluation for inhalation exposures (17). Recent work by Sears and colleagues found that most current e-cigarette users perceive flavoring agents in e-cigarettes as safe to inhale, and the majority of college students they surveyed believed the FDA's GRAS designation extended to safety of inhalation exposures (16). These findings are cause for concern as electrophilic flavoring agents, such as aldehydes and ketones, are commonly used to flavor e-cigarettes and there is currently no regulation governing the quantities used in these products.

While the effects of aldehydes in cigarette smoke on airway cilia and mucociliary clearance are known, it is unclear whether reactive aldehyde flavorings in e-liquids will cause a similar response. Several recent studies have evaluated the concentrations of aldehyde flavoring agents in commercially available e-liquids. Behar and colleagues identified cinnamaldehyde in 20 of 39 e-liquids tested with concentrations ranging from 1.7×10^{-5}M to

1.1M (25). Recent work by Kosmider et al. quantified benzaldehyde in aerosol generated from 145 flavored e-liquids (26). One hundred eight of these e-liquids contained benzaldehyde, which ranged in concentrations from 5.129μg/30 puffs to 141.2μg/30 puffs. A study by Tierney and colleagues identified and measured flavorings in 30 e-liquids and found six unique aldehydes with concentrations that varied between e-liquids: vanillin ($6.5x10^{-4}$M to 0.22M), ethyl vanillin ($1.8x10^{-3}$M to $5.1x10^{-2}$M), benzaldehyde ($5.7x10^{-3}$M to 0.20M), *p*-tolualdehyde (0.023M), cinnamaldehyde ($6.1x10^{-3}$M), and piperonal ($2.0x10^{-3}$M to $4.7x10^{-3}$M) (27). Our group recently evaluated a panel of seven e-liquids and identified numerous aldehyde flavorings, including vanillin, ethyl vanillin, benzaldehyde, 4-anisaldehyde (4-methoxybenzaldehyde), cocoa hexenal (5-methyl-2-phenyl-2-hexenal), and cinnamaldehyde (28). The concentration of cinnamaldehyde in the e-liquids we tested ranged from 8.9×10^{-4}M to 1.13M (28). Many of the aldehydes listed here have structural similarity to toxic aldehydes in cigarette smoke. We have previously shown that cinnamon e-liquids and cinnamaldehyde suppresses respiratory immune cell function at concentrations significantly lower than those resulting in cell death (28). However, whether cinnamon-flavored e-liquids and/or cinnamaldehyde disrupt normal hBE cell function, such as airway cilia motility or bioenergetic pathways, is completely unknown. To address this knowledge gap, we exposed hBE cells to cinnamaldehyde-containing e-liquids, vaped e-liquid aerosol, and cinnamaldehyde alone, and quantified changes in cellular functions, including cilia motility, mitochondrial respiration, and glycolysis.

3.2 Materials and methods

Primary hBE Cell Culture.

Human lung tissue was procured under an Institutional Review Board-approved protocol, and hBE cell harvest and culture was performed using established procedures previously described in detail (29). Briefly, hBE cells were enzymatically dissociated with 0.01% protease XIV (Sigma-Aldrich, St. Louis, MO, USA) and 0.001% DNase (Sigma-Aldrich, St. Louis, MO, USA) in minimum essential medium (Sigma-Aldrich, St. Louis, MO, USA) from the trachea, main stem, and lobar bronchi of 6 non-smoker lung donors described in Table 1. Primary hBE cells were plated at a density of 2-6×10^6 per 100mm collagen-coated (Advanced BioMatrix, San Diego, CA, USA) cell culture plate (Corning Life Sciences, Tewksbury, MA, USA) in BEGM medium (prepared in house). At 70-90% confluence, cells were passaged by trypsin (Sigma-Aldrich, St. Louis, MO, USA) dissociation, counted, and plated on human placental type IV collagen (Sigma-Aldrich, St. Louis, MO, USA)-coated 12mm Transwell permeable supports (Corning Life Sciences, Tewksbury, MA, USA) at a density of 3x10^5 cells per Transwell. Upon confluence, apical growth medium was removed and cultures were differentiated at an air-liquid interface (ALI) for greater than 40 days. Basolateral ALI growth medium (prepared in house) was replaced 3-times per week and cultures were washed with PBS (Sigma-Aldrich, St. Louis, MO, USA) once each week to prevent accumulation of apical mucus and debris. Cell were cultured at 37°C with 95% humidity and 5% carbon dioxide prior to all experiments.

Donor	Age,yr	Sex	Race	Smoking	Cause of Death
1	57	F	Cau	NS	Intracranial hemorrhage
2	46	F	Cau	NS	Head trauma
3	14	M	Cau	NS	Head trauma
4	27	M	Blk/AA	NS	Head trauma
5	26	M	Hisp	NS	Head trauma
6	23	M	Cau	NS	Head trauma

M, male; F, female; Cau, Caucasian; Blk/AA, Black/African American; Hisp, Hispanic; NS, nonsmoker. Non-cystic fibrosis (CF) lungs were obtained from organ donors whose lungs were unsuitable for transplant due to acute injury or lack of a matching recipient.

Table 3.1 Demographic data for lung donors.

Beas-2B Bronchial Epithelial Cell Culture

Beas-2B cells were obtained from ATCC (Manassas, VA, USA) and cultured in serum-free keratinocyte growth medium (KGM; Lonza, Walkersville, MD, USA) under standard airway epithelial cell growth conditions (37°C, 95% humidity, and 5% carbon dioxide). All studies were performed using Beas-2B cells between passage 40 and passage 60, with passage 1 defined as the thawed cells from the supplier. All experiments using Beas-2B cells were repeated 4 times (N=4) with 3 technical replicates for each experimental condition.

Flavored E-cigarette Liquids and Cinnamaldehyde

Three cinnamaldehyde-containing, nicotine-free e-liquids (“Kola”, “Hot Cinnamon Candies”, and “Sinicide”) were purchased from a local vape shop (The Vapor Girl, Chapel Hill, NC, USA). E-liquids were stored in opaque glass bottles at room temperature and were diluted for use at the time of each experiment. All e-liquids used in this study were reported by the manufacturer to contain the humectants PG and VG at relative proportions of 55% and

45%, respectively. A mixture of 55% PG (Thermo Fisher Scientific, Waltham, MA, USA) and 45% VG (Sigma-Aldrich, St. Louis, MO, USA) was used as a vehicle control for all e-liquid and vape experiments. Food grade trans-cinnamaldehyde (≥98% pure) was purchased from Sigma Aldrich (St. Louis, MO, USA) and stored at room temperature in the opaque glass bottle provided by the manufacturer. Cinnamaldehyde was diluted for use at the time of each experiment.

CBF and Percent Active Area (%AA)

Twenty-four hours prior to CBF analysis, 150µl of PBS was applied to the apical surface of well-differentiated (>40 day old) hBE cell cultures for 20 minutes to loosen mucus and debris. Apical PBS and basolateral medium were removed by vacuum aspiration and 2.5ml of warm (37°C) ALI medium was added to the basolateral compartment. Twenty minutes prior to CBF analysis, the apical compartment of ALI cultures was briefly rinsed again with 150µl of PBS. For each CBF experiment, a well-differentiated hBE cell culture was placed on Nikon Diaphot inverted microscope equipped with a 20x phase contrast objective (Nikon, Melville, NY, USA) and CBF for a single 20x field of view was observed. Cilia motion was recorded with a high speed digital camera (Basler AG, Ahrensburg, Germany) and analyzed with software designed to quantify beat frequency and the percent of motion in the field of view (SAVA; Ammons Engineering, Mt. Morris, MI, USA)(30). Baseline whole-field CBF and %AA were evaluated at 30 second intervals for 5 minutes. Following baseline CBF and %AA assessment, cultures were challenged apically with 150µl of 1% (v/v) flavored e-liquid, 1% PG/VG vehicle, cinnamaldehyde (1mM, 5mM, 10mM, 15mM), or ALI growth medium alone (media control). Whole-field CBF and %AA were quantified at 30 second intervals for 115 minutes following the apical addition of treatments.

Apical challenges were not removed for the duration of the experiment. All cilia beat measurements were conducted at room temperature (approximately 24°C).

The threshold of detection for CBF was 2.5Hz and whole-field analysis data with less than 100 points of information collected were omitted from analysis. CBF and %AA data for each experiment were plotted as a function of time using GraphPad Prism version 7. Cell cultures from 4 donors (N=4) were used for CBF and %AA analysis. To determine whether any e-liquid or cinnamaldehyde treatment significantly altered CBF and %AA, the area under the curve (AUC) for each CBF and %AA plot was calculated and analyzed using a one-way ANOVA and Holm-Sidak multiple comparisons test. All statistical analyses were carried out using GraphPad Prism version 7. Differences with a P value ≤ 0.05 were considered significant.

Vaped Aerosol Exposures

Baseline whole-field CBF and %AA were evaluated in well-differentiated hBE cell cultures at 30 second intervals for 5 minutes as described above. Following baseline CBF and %AA assessment, cultures were placed into the center of a 3-liter acrylic exposure chamber and exposed to Sinicide or PG/VG aerosol, similar to previous studies conducted by Carson et al (31). E-cigarette aerosol was generated by connecting a LAVABOX DNA 200 Box Mod e-cigarette (Volcano e-Cigs, Honolulu, HI, USA) with a SMOK TFV4 Mini Tank and sub-ohm (0.37Ω) TF-CLP2 Clapton Coil (SMOKtech, Shenzhen, China) to the exposure chamber with a 10-cm length of tubing (4.8 mm ID; Masterflex L/S 15, Vernon Hills, IL, USA). Similar to previous studies, a vacuum pump connected to a flow meter was used to pull aerosol from the e-cigarette device at a flow rate of 3L/min (31). One 5-second puff of

aerosol was generated every 30 seconds for 5 minutes (10 puffs total) using an e-cigarette device setting of 70 watts. E-cigarette tank vents were fully opened to supply maximal airflow to the heating coil. Mouthpiece vents were completely closed to prevent dilution of aerosol. Thirty seconds after the final puff, the cell culture was removed from the exposure chamber and immediately placed on the microscope for CBF analysis. To assess particle deposition at the airway surface during vape exposures, 12mm glass coverslips were inserted into empty transwells and exposed to e-cigarette aerosols at the same time as hBE cultures. Immediately following vape exposures, glass coverslips were removed and weighed on a microbalance. Initial coverslip weights were subtracted from post-exposure weights do determine the mass of aerosol deposition. The mean deposition of aerosol was determined to be 828.7±78.5µg. Whole-field CBF and %AA were measured at 30-second intervals for 55 minutes following the aerosol exposure. CBF and %AA data was plotted and AUC values for each experiment were calculated as described above. Cell cultures from 3 donors (N=3) were used for vape experiments. An unpaired *t*-test was used to determine whether there was a statistically significant difference between the effect of Sinicide and PG/VG. All statistical analyses were carried out using GraphPad Prism version 7. P value ≤ 0.05 was considered significant.

Analysis of Mitochondrial Respiration and Glycolysis

Live-cell metabolic assays were conducted using the Seahorse XFe24 Analyzer (Agilent Technologies, Santa Clara, CA, USA) and the Seahorse XF Cell Mito Stress Test kit (Agilent Technologies, Santa Clara, CA, USA). Beas-2B cells were plated at $3x10^4$ cells per well in XF24 cell culture plates. Twenty-four hours after plating, Beas-2B growth medium was aspirated and replaced with keratinocyte basal medium without supplements or growth

factors (KBM; Lonza, Walkersville, MD, USA). Cells were analyzed 24 hours after plating in KBM medium. All cultures were >90% confluent at the time of analysis. XFe24 sensor cartridges were hydrated overnight with XF Calibrant at 37°C prior to analysis. XF Cell Mito Assay Media (XF Base Media with 10mM glucose, 1mM sodium pyruvate, and 2mM glutamine) was prepared fresh on the day of assay and adjusted to pH 7.4. KBM medium was aspirated from Beas-2B cells and replaced with warm (37°C) XF Cell Mito Assay Media. Cells were incubated at 37°C without CO_2 for 1 hour prior to Seahorse analysis. Stock concentrations of oligomycin (Sigma-Aldrich, St. Louis, MO, USA), FCCP (Sigma-Aldrich, St. Louis, MO, USA), rotenone (Sigma-Aldrich, St. Louis, MO, USA), and Antimycin A (Sigma-Aldrich, St. Louis, MO, USA) were prepared in DMSO (Sigma-Aldrich, St. Louis, MO, USA) and stored at -20°C. Stock solutions were diluted to working concentrations in XF Cell Mito Assay Media with final DMSO concentrations less than 0.1%. Assay injections were ordered as follows: A) Vehicle or cinnamaldehyde (0.05-5.0mM adjusted to pH 7.4); B) 1μM oligomycin; C) 1μM FCCP; D) 0.5μM rotenone and 0.5μM Antimycin A. Sequential mix-wait-measurement times were set at 3 minutes-2 minutes-3 minutes. Parameters for mitochondrial and glycolytic function were calculated as recommended by the instrument manufacturer (Agilent Technologies). Data were collected from four independent experiments (N=4). A one-way ANOVA and Dunnett's multiple comparisons test were used to determine whether the effects of cinnamaldehyde were significantly different from the vehicle control. All statistical analyses were carried out using GraphPad Prism version 7. P value ≤ 0.05 was considered significant.

ATP, ADP, and AMP Quantification

Nucleotide extraction from hBE cells

Well-differentiated hBE cells cultures were briefly washed with PBS and 150µl of 10mM cinnamaldehyde (diluted in ALI growth medium) was added to the apical surface. Cultures were incubated with cinnamaldehyde for 15 minutes, 120 minutes, or 24 hours, followed by a brief PBS wash of both the apical and basolateral surfaces to remove excess cinnamaldehyde. To assess intracellular nucleotide content, Transwell membranes were immediately excised with a #11 scalpel and placed, cells facing downward, into a 24-well plate containing ice-cold trichloroacetic acid (TCA; 5% in HPLC-grade water). Membranes were incubated on ice for 30 minutes to provide optimal nucleotide recovery. TCA solution was collected from each well and TCA was extracted from the solution with ethyl ether (10 washes with 2ml). Residual ether remaining after aspiration of the final wash was removed by evaporation with nitrogen gas.

Derivatization of Adenosine and Adenine Nucleotides

Adenosine and Adenine nucleotides were derivatized using a modification of the protocol described by Lazarowski et al. (32). Briefly, 200µl aliquots of the TCA-extracted samples (diluted 1:10 in PBS) were added to microcentrifuge tubes containing 100µl derivatization buffer (62mM citric acid, 38mM Na_2POH_4; pH 4.0) followed by the addition of 10µl of 50% chloroacetaldehyde (v/v in HPLC grade water)(Sigma-Aldrich, St. Louis, MO, USA). Samples were heated at 80°C for 40 minutes, cooled to 4°C on ice, and analyzed by HPLC within 72 hours of derivatization.

HPLC Analysis

Identification and quantification of ethenylated species were performed with an automated Waters HPLC apparatus equipped with a fluorescence detector (excitation, 307nm; emission 410nm). Derivatized samples were transferred to 0.7-ml plastic vials (Sun-SRi, Duluth, GA, USA) and kept at 4°C in the sample injector rack. A 100µl sample aliquot was injected into a Chromolith® reversed-phase column (RP-18e, 100-3mm; Merck KGaA, Darmstadt, Germany). The mobile phase (1.0ml/min, isocratic) was composed of 200mM NaH_2PO_4-H_2O, 0.2M Na_2HPO_4-$2H_2O$, and 5mM tetrabutyl NH_4 Bromide (pH 6.1). Typical elution times (in minutes) of authentic etheno (ε) standards are as follows: εAMP, 2.09; εAdo, 2.61; εADP, 3.10; and εATP, 4.96). HPLC data was collected using Waters Empower Pro software build 1154. Individual nucleotide content was calculated as a percent of the total nucleotide counts. Data was collected from four independent experiments (N=4). A one-way ANOVA and Dunnett's multiple comparisons test was used to determine whether cinnamaldehyde-induced changes in nucleotide content at each time point was significantly different from untreated (0 minute) control. All statistical analyses were carried out using GraphPad Prism version 7. Differences with a P value ≤ 0.05 were considered significant.

Chemical Inhibition of Glycolysis and Mitochondrial Respiration in hBE Cells for CBF and %AA Analyses

The glycolytic function of well-differentiated hBE cells was inhibited by replacing growth medium with a modified Ringers solution consisting of 115mM NaCl, 20mM Hepes buffer, 5mM K_2HPO_4, 2mM $MgSO_4$, 1mM $CaCl_2$, 2mM glutamine, and 10mM 2-deoxyglucose (33). hBE cell cultures were washed with PBS and baseline CBF and %AA

were determined by taking 10 random SAVA measurements (20x magnification) throughout each culture. Following baseline measurements, 500µl of the modified Ringers solution was added to the basolateral compartment and 150µl was added apically. CBF and %AA measurements were taken again at 15 minutes and 30 minutes after the addition of modified Ringers by randomly sampling 10 random fields. After the 30-minute measurements were recorded, the apical and basolateral modified Ringers solution was aspirated and replaced with 500µl of ALI growth medium in the basolateral compartment and 150µl of ALI medium apically. CBF and %AA was assessed 15, 30, and 90 minutes after the addition of normal growth medium by sampling 10 random fields as described above.

Inhibition of hBE cell mitochondrial respiration (but not glycolysis) was accomplished by replacing the 10mM 2-deoxyglucose in the modified Ringers solution with 10mM glucose and supplementing the Ringers solution with 10µM Antimycin A, a potent mitochondrial complex III inhibitor. hBE cell cultures were washed with PBS and CBF and %AA measurements were collected as described above.

Inhibition of both glycolysis and mitochondrial respiration was achieved by using the modified Ringers solution (glucose free) with 10mM 2-deoxyglucose and 10µM Antimycin A. Measurements of CBF and %AA were collected as described above. CBF and %AA data for each experiment were plotted as a function of time using GraphPad Prism version 7. Well-differentiated hBE cell cultures from 4 donors (N=4) were used. Differences in CBF and %AA from baseline were determined by one-way ANOVA and Holm-Sidak multiple comparisons test. P value ≤ 0.05 was considered significant.

Cytotoxicity Assays

hBE and Beas-2B cell viability was determined by measuring lactate dehydrogenase (LDH) release into the cell culture medium using a commercially available colorimetric (490/650 nm) assay kit following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). One hundred microliters of culture medium were used for analysis. The absorbance data for each sample was normalized to 100% cell death (maximal LDH release caused by lysis with 1.0% Triton X-100 in cell culture medium) and baseline LDH release (untreated cells) using the following formula: corrected viability = 100 – [[Abs(X) − Abs(Y)]/[Abs(MAX) − Abs(Y)]*100] where Abs(X) is the absorbance of the sample, Abs(Y) is the absorbance of media from the untreated control cells, and Abs(MAX) is the absorbance of media collected from cells exposed to 1.0% Triton X-100. Data for cinnamaldehyde toxicity in Beas-2B cells were collected from 4 independent experiments (N=4). Data for cinnamaldehyde toxicity in well-differentiated hBE cells were conducted using cell cultures from 3 donors (N=3). Differences in viability following cinnamaldehyde exposures were evaluated by one-way ANOVA and Holm-Sidak multiple comparisons test. P value ≤ 0.05 was considered significant.

3.3 Results

Temporal analysis of CBF and %AA during flavored e-liquid exposures

We exposed well-differentiated hBE cells to PG/VG vehicle or e-liquids containing different concentrations of cinnamaldehyde. The effects of exposure on CBF and %AA were recorded over 120 minutes (Figure 3.1). Apical addition of the Kola and Hot Cinnamon Candies (HCC) e-liquids did not significantly alter CBF or %AA as compared to the PG/VG

vehicle (Figure. 3.1A-C). However, exposure to the Sinicide e-liquid rapidly reduced both CBF and %AA (Figure 3.1D). Interestingly, the complete ciliostasis was transient with %AA being fully recovered by 90 minutes. CBF began to recover by 50 minutes but did not reach baseline levels by 120 minutes. Exposure of aerosolized Sinicide e-liquid inhibited CBF and %AA similar to what was observed with apical e-liquid exposures (Figure 3.1E). Both the Sinicide e-liquid and aerosolized Sinicide e-liquid exposures resulted in a significant reduction in CBF and %AA as compared to the PG/VG vehicle (Figure 3.2).

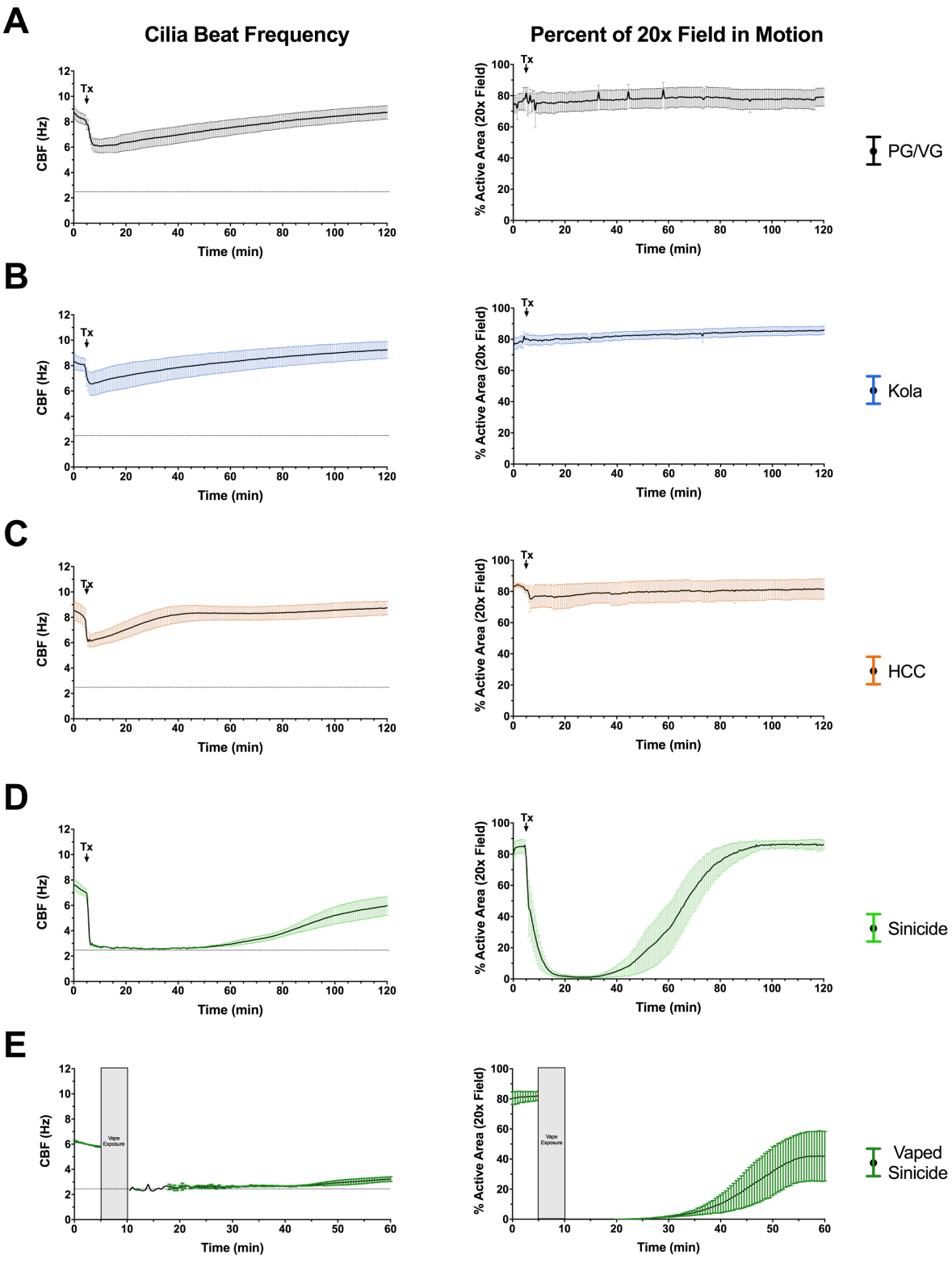
A
Cilia Beat Frequency
Percent of 20x Field in Motion
CBF (Hz)
% Active Area (20x Field)
Time (min)
Tx
PG/VG
B
Kola
C
HCC
D
Sinicide
E
Vape Exposure
Vaped Sinicide

Figure 3.1 Effects of cinnamaldehyde-containing e-liquids on airway cilia motility. A-D) Following 5-minute baseline measurements, well-differentiated primary hBE cells were exposed to 1% dilutions of e-liquids (v/v) in cell culture medium and cilia beat frequency (CBF; left panels) and percent of the 20x field of view in motion (%AA; right panels) were recorded for 120 minutes (N=4). E) 5-minute baseline measurements were recorded prior to exposing well-differentiated primary hBE cells to vaped Sinicide e-liquid aerosols for 5 minutes. CBF and %AA were recorded for 50 minutes following exposures (N=3). Data presented as mean ± SEM.

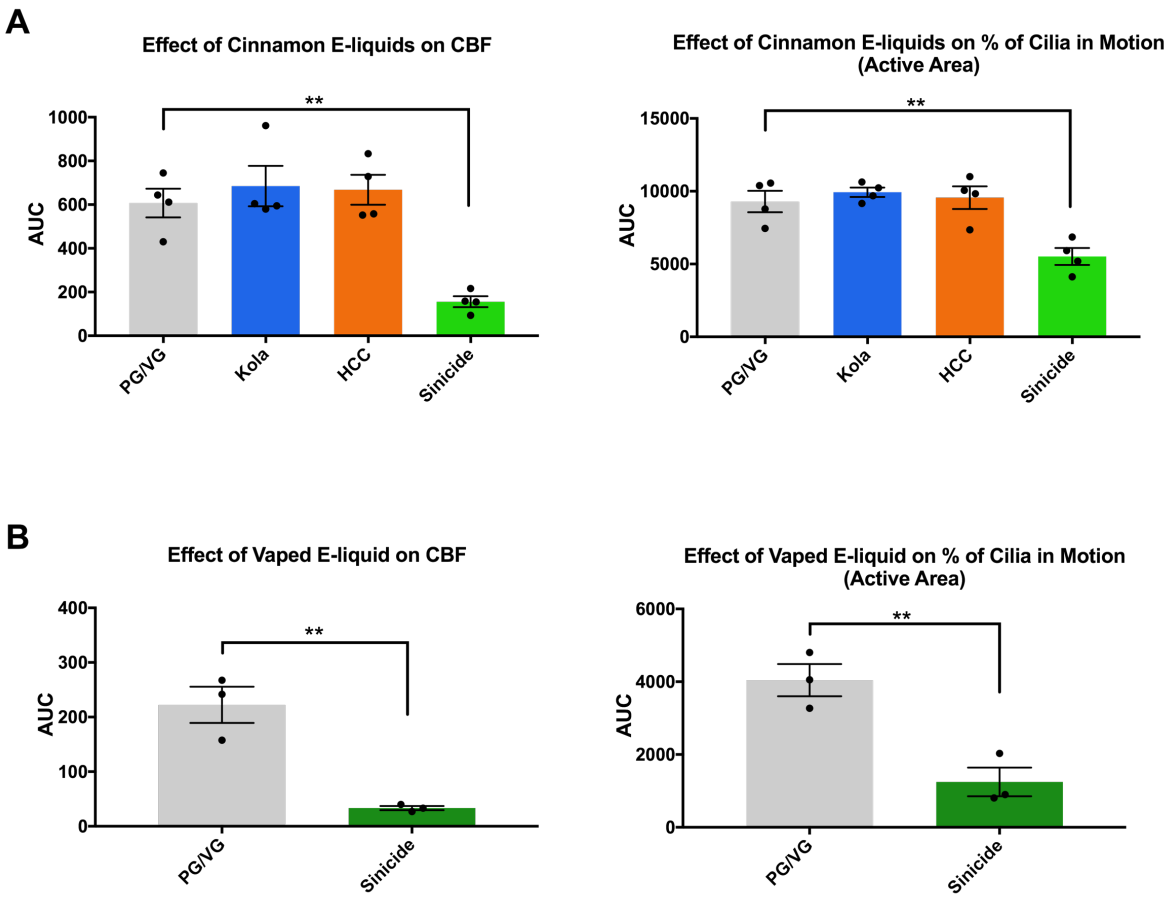

Figure 3.2 Quantification of e-liquid-induced effects on cilia motility. Area under the curve (AUC) was calculated for both CBF and %AA data collected from A) e-liquid exposure experiments (N=4), and B) vaped Sinicide e-liquid experiments (N=3). Significant differences from the propylene glycol/vegetable glycerin (PG/VG) control were determined by one-way ANOVA and Holm-Sidak multiple comparisons test. Data presented as mean ± SEM. Significance represented as ** $p<0.01$, *** $p<0.001$ as compared to vehicle control.

Temporal analysis of CBF and %AA during flavored cinnamaldehyde exposures

Previously published work from our group quantified cinnamaldehyde in the e-liquids used for this study: Kola, 0.896mM ± 0.360mM; 'Hot Cinnamon Candies', 39.32mM ± 3.41mM; and Sinicide, 1,131mM ± 19.9mM (34). Our finding that exposure of hBE cells to a 1% dilution of the Sinicide e-liquid (11.31mM cinnamaldehyde content based on quantification of neat Sinicide e-liquid) resulted in rapid, but transient, ciliostasis led us to investigate whether cinnamaldehyde alone, at concentrations approximate to those in the 1% Sinicide e-liquid, would produce a similar response. Apical exposures to 1mM, 5mM, 10mM and 15mM cinnamaldehyde produced dose-dependent reductions in both CBF and %AA (Figure 3.3) with 10mM and 15mM exposures significantly reducing CBF and %AA as compared to vehicle control (Figure 3.4). Similar to the effects of Sinicide on cilia motility, 10mM and 15mM cinnamaldehyde caused a rapid reduction in cilia function that recovered over time. The time to recover was also dose-dependent with the higher exposure doses requiring more time for recovery. As observed with the Sinicide exposures, CBF recovery after 10mM and 15mM cinnamaldehyde exposures did not recover to the baseline frequency by 120 minutes. However, normal cilia beat was apparent 24 hours after exposure (data not shown).

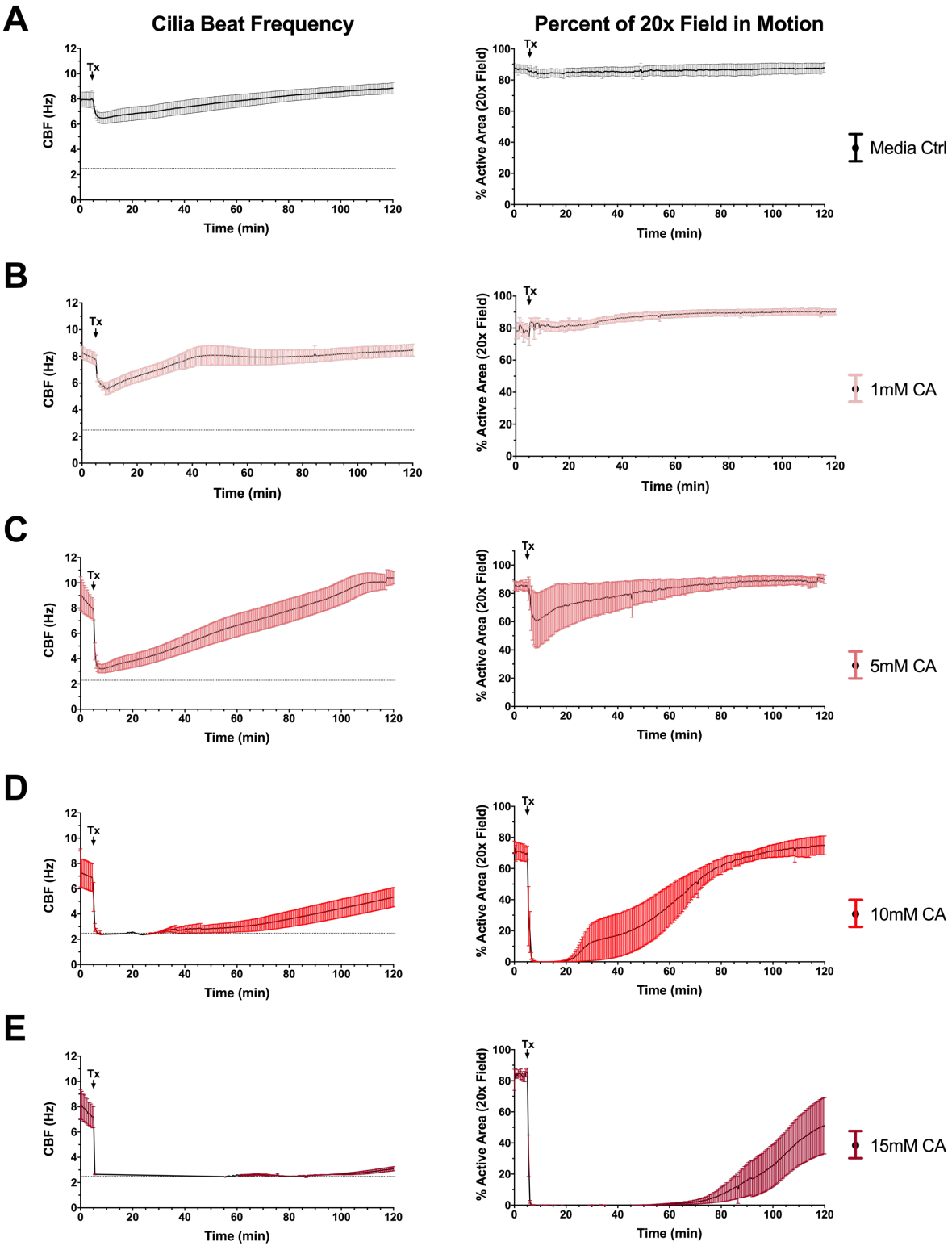
A
Cilia Beat Frequency
Percent of 20x Field in Motion
CBF (Hz)
% Active Area (20x Field)
Time (min)
Tx
Media Ctrl
B
1mM CA
C
5mM CA
D
10mM CA
E
15mM CA

Figure 3.3 Effects of cinnamaldehyde on airway cilia motility. Following 5-minute baseline measurements, well-differentiated primary hBE cells were exposed to either A) vehicle (cell culture medium) control, or B-E) 1mM, 5mM, 10mM, or 15mM cinnamaldehyde. Cilia beat frequency (CBF; left panels) and percent of the 20x field of view in motion (%AA; right panels) were recorded for 120 minutes (N=4). Data presented as mean ± SEM.

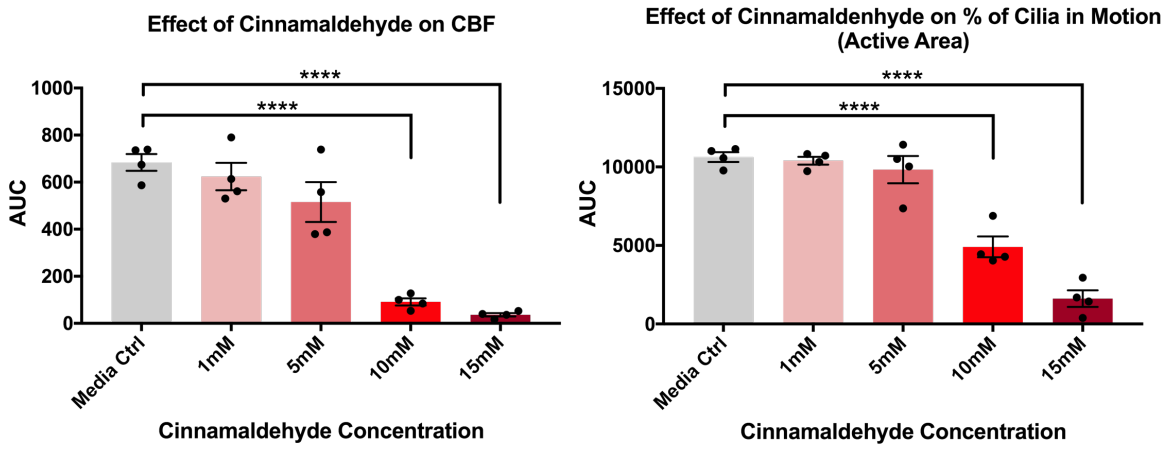

Figure 3.4 Quantification of cinnamaldehyde-induced effects on cilia motility. Area under the curve (AUC) was calculated for both CBF and %AA data collected from each independent experiment (N=4). Significant differences from the vehicle (cell culture medium) control were determined by one-way ANOVA and Holm-Sidak multiple comparisons test. Data presented as mean ± SEM. Significance represented as **** $p<0.0001$.

Effects of cinnamaldehyde on bronchial epithelial cell mitochondrial respiration and glycolysis

Motile cilia require sufficient ATP to maintain the energy requirements of ciliary proteins, including the molecular motors dynein and kinesin. Reductions in intracellular ATP levels can reduce motor protein ATPase activity leading to reduced cilia motility (35). To determine whether cinnamaldehyde alters ATP production in bronchial epithelial cells, we utilized the Seahorse XFe24 Analyzer and Seahorse XF Cell Mito Stress Test to assess bioenergetic function following exposure to various concentrations of cinnamaldehyde. The Seahorse XF Cell Mito Stress Test evaluates various parameters of mitochondrial and glycolytic function by injection of specific bioenergetic modulators. Cinnamaldehyde (or vehicle) was injected first for this assay to determine if exposure altered baseline mitochondrial respiration. Oligomycin, a potent ATP Synthase inhibitor, was injected after cinnamaldehyde (or vehicle) to measure changes in oxygen consumption (OCR) associated with mitochondrial ATP production and proton leakage across the mitochondrial membrane. In addition to reducing mitochondrial OCR, inhibition of ATP synthase forces the cell to rely on glycolysis for ATP production and allows for quantification the maximal glycolytic capacity by measuring changes in the extracellular acidification rate (ECAR). Following oligomycin, the protonophore FCCP is injected to collapse the proton gradient at the inner mitochondrial membrane. Disruption of the proton gradient maximizes mitochondrial OCR allowing for quantification of maximal mitochondrial respiration and spare respiratory capacity. The final injection of rotenone and Antimycin A, inhibits mitochondrial Complex I and III respectively. These inhibitors prevent mitochondrial electron transport and

completely inhibit mitochondrial oxygen consumption allowing for quantification of the OCR linked to non-mitochondrial respiration processes.

Exposure of Beas-2B cells to various concentrations of cinnamaldehyde resulted in a dose-dependent reduction in mitochondrial oxygen consumption (Figure 3.5A-B). Exposures of 0.25mM, 0.5mM, and 5.0mM cinnamaldehyde significantly reduced ATP production, reserve capacity, proton leak, and maximal respiration (Figures 3.5C-F, respectively). Interestingly, exposures of 0.25mM, 0.5mM, and 5.0mM cinnamaldehyde also significantly reduced the ECAR (Figure 3.6A-B) indicating an effect on glycolytic function. Stimulation of glycolysis by addition of oligomycin revealed that 0.5mM and 5.0mM exposures of cinnamaldehyde significantly reduced glycolytic capacity (Figure 3.6C). Taken together, these data indicate that cinnamaldehyde exposure causes a dose-dependent reduction in both mitochondrial respiration and glycolytic function (Figure 3.7).

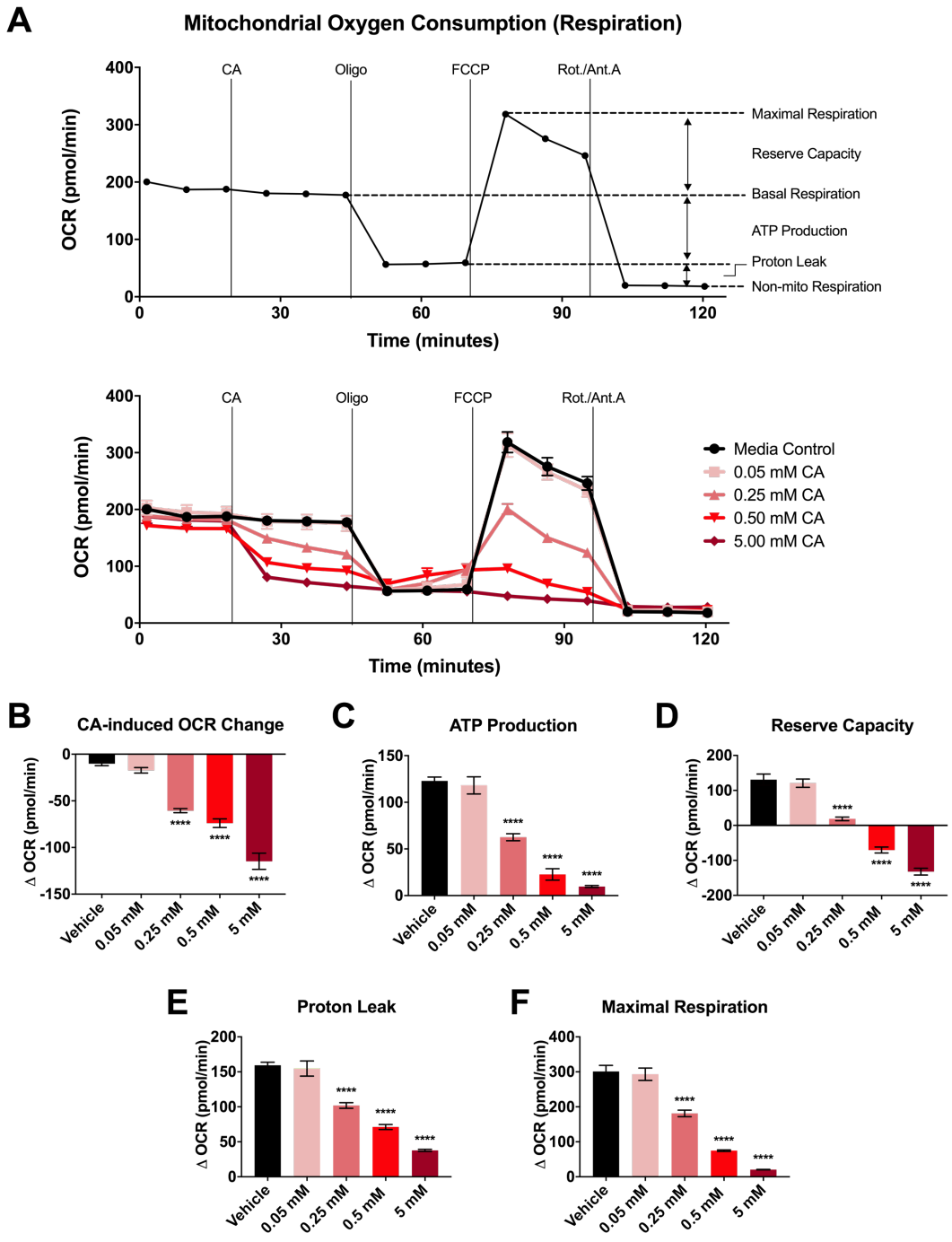

Figure 3.5 Cinnamaldehyde induces a concentration-dependent reduction in bronchial epithelial cell mitochondrial respiration. A) Beas-2B cells were exposed to 0.05mM, 0.25mM, 0.5mM, and 5.0mM concentrations of cinnamaldehyde and changes in mitochondrial oxygen consumption rate (OCR) were evaluated using a Seahorse bioanalyzer (N=4). Exposures of 0.25mM, 0.5mM, and 5mM cinnamaldehyde significantly suppressed B) basal OCR, C) ATP production, D) reserve capacity, E) proton leak, and F) maximal respiration, in a concentration-dependent manner. Data presented as mean ± SEM. Significance represented as **** $p<0.0001$.

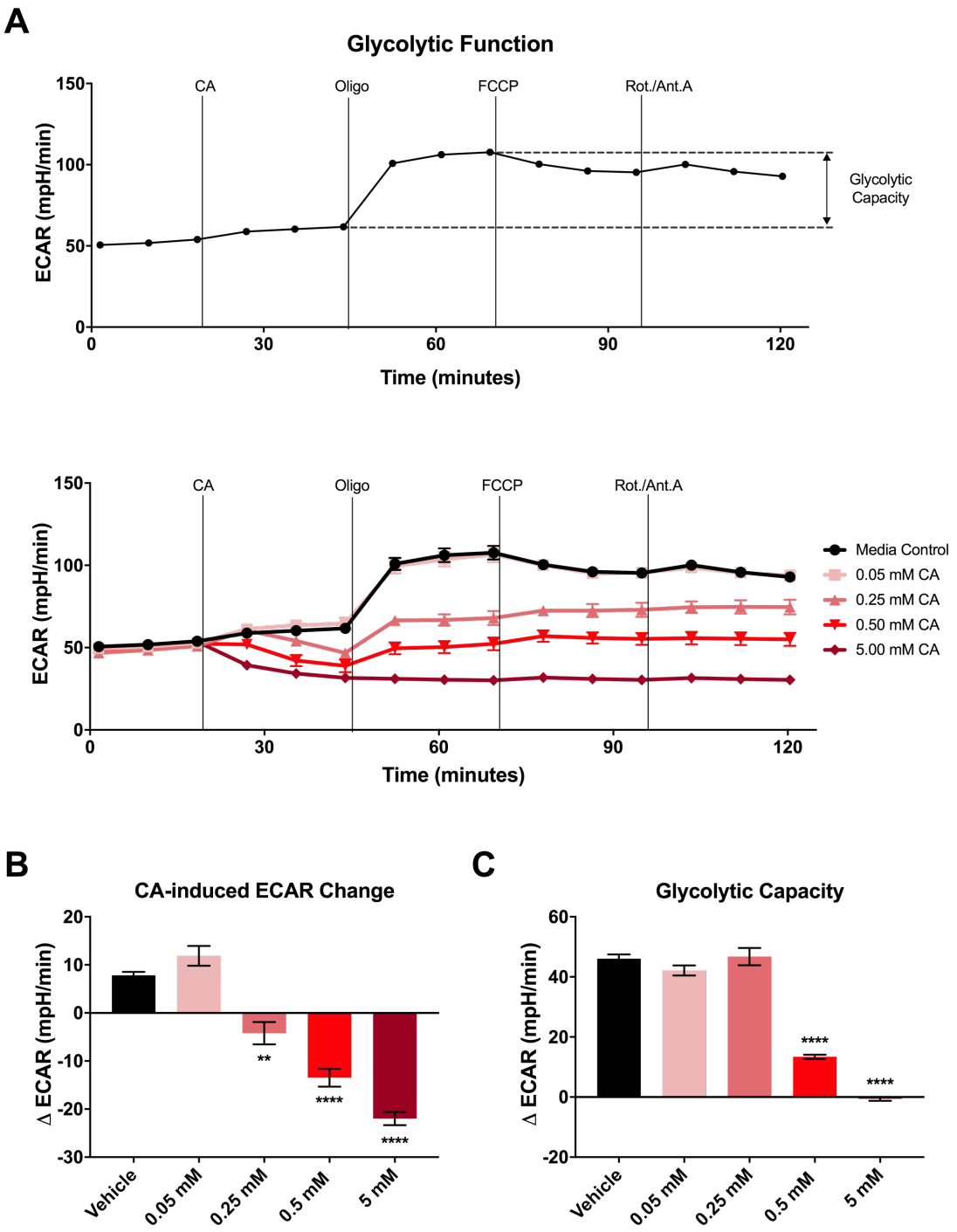

Figure 3.6 Cinnamaldehyde reduces bronchial epithelial cell glycolytic function. A) Beas-2B cells were exposed to 0.05mM, 0.25mM, 0.5mM, and 5.0mM concentrations of cinnamaldehyde and the rate of extracellular acidification (ECAR) was measured using a Seahorse bioanalyzer (N=4). B) Exposures of 0.25mM, 0.5mM, and 5mM cinnamaldehyde significantly suppressed basal ECAR, while C) 0.5mM, and 5mM cinnamaldehyde significantly reduced Beas-2B cell glycolytic capacity. Data presented as mean ± SEM. Significance represented as ** $p<0.01$ and **** $p<0.0001$.

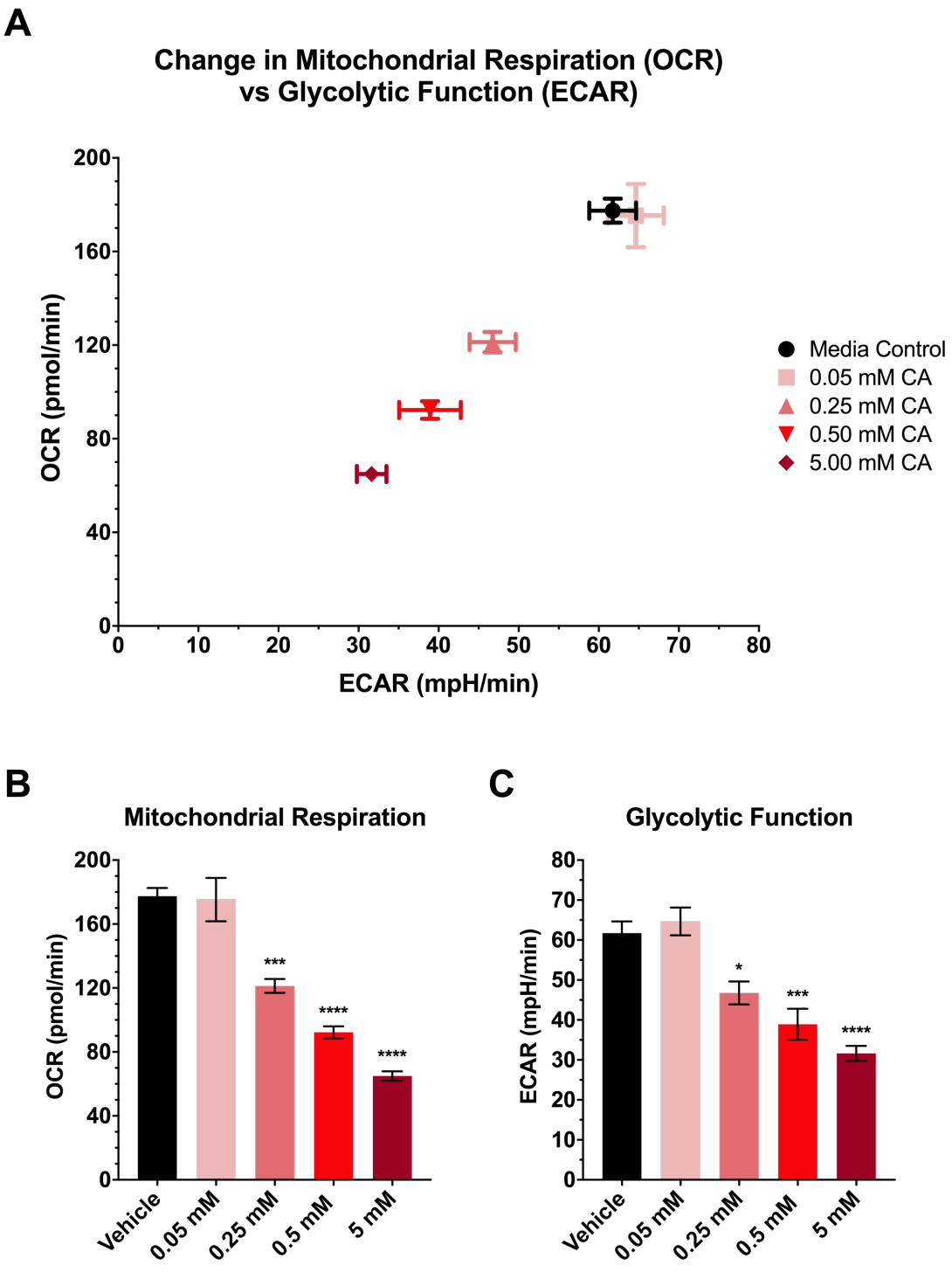

Figure 3.7 Cinnamaldehyde inhibits the basal energetic state of Beas-2B cells. A) Comparison of cinnamaldehyde-induced changes in mitochondrial OCR and glycolytic ECAR for 0.05mM, 0.25mM, 0.5mM, and 5mM exposures. Cinnamaldehyde causes an acute concentration-dependent reduction in both B) mitochondrial OCR and C) glycolytic ECAR. Data presented as mean ± SEM. Significance represented as * $p<0.05$ *** $p<0.001$ and **** $p<0.0001$.

Quantitation of intracellular ATP levels in well-differentiated hBE cells exposed to cinnamaldehyde

Our observation that cinnamaldehyde rapidly impairs cellular bioenergetic pathways suggests that cells experience a concomitant reduction in intracellular ATP levels following exposure. To confirm cinnamaldehyde-induced reductions in intracellular ATP levels, well-differentiated hBE cells were exposed to 10mM cinnamaldehyde for various times and adenosine nucleotide (ATP, ADP, and AMP) levels were quantified. Apical addition of 10mM cinnamaldehyde significantly reduced hBE cell ATP levels after 15 minutes (Figure 3.8A). 120-minute and 24-hour exposures did not significantly alter ATP levels as compared to the untreated controls, indicating that ATP levels are restored by the later time points although the cinnamaldehyde challenge is not removed. Notably, ADP and AMP levels were significantly elevated after 15-minute cinnamaldehyde exposure providing evidence that the reduction in ATP was due to hydrolysis by cellular activity and not exocytotic release or leakage at the cell membrane (Figure 3.8B-C).

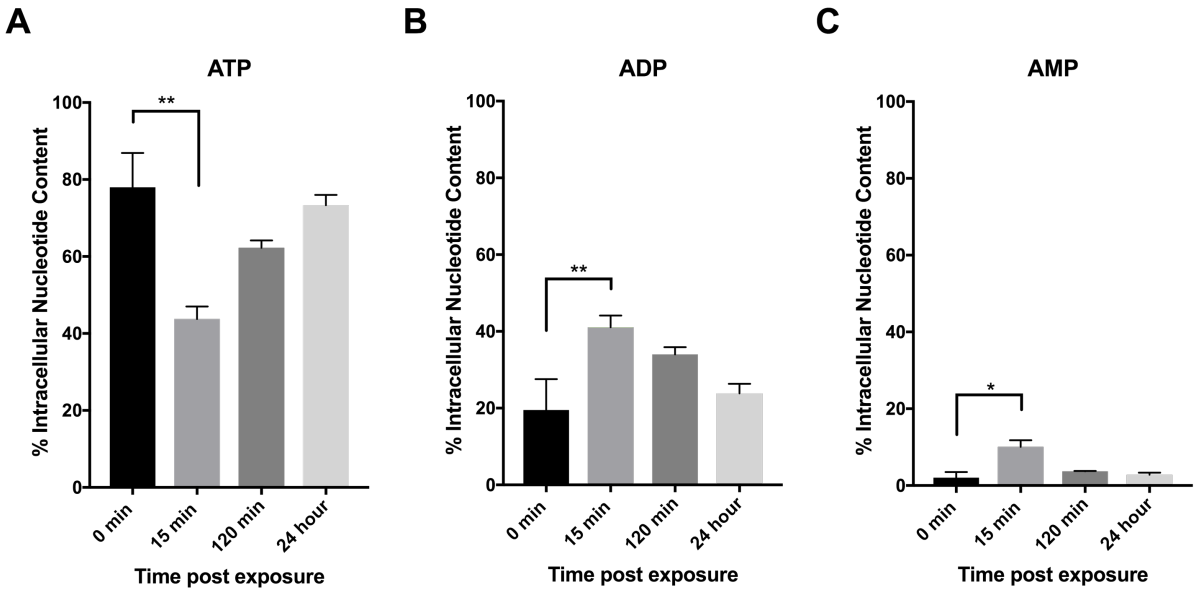

Figure 3.8 Exposure of well-differentiated hBE cells to cinnamaldehyde causes a rapid but temporary reduction in intracellular ATP levels. Well-differentiated hBE cells were exposed to 10mM cinnamaldehyde for 15 minutes, 120 minutes, and 24 hours, and the effect on intracellular adenosine nucleotides was quantified by HPLC (N=4). Exposure caused A) a significant reduction in ATP levels after 15 minutes that correlated with increased levels of B) ADP and C) AMP. Data presented as mean ± SD. Significance represented as * $p<0.05$ and ** $p<0.01$.

Inhibition of cellular bioenergetic activity and temporal analysis of hBE cell cilia motility

Various aldehydes are reported to suppress airway cilia motility and impede mucociliary transport, including formaldehyde, acetaldehyde, acrolein, methacrolein, and crotonaldehyde (3, 4, 36-39). Many of these aldehydes have been identified as mitochondrial toxins and inhibitors of glycolytic activity (40-43). However, with the exception of some bacterial studies, there are very few reports which link toxin-induced impairments of bioenergetic pathways with altered cilia motility (33, 44). To better understand how targeted inhibition of mitochondrial respiration and glycolysis effects human airway cilia motility, we exposed well-differentiated hBE cells to the mitochondrial complex III inhibitor Antimycin A, the glycolytic pathway inhibitor 2-deoxy-D-glucose, and a combination of both to assess changes in CBF and %AA. hBE cells exposed to 2-deoxy-D-glucose experienced a significant increase in CBF but not %AA as compared to baseline levels (Figure 3.9A). Repletion of glucose at 30 minutes further increased CBF without affecting %AA. Exposures to Antimycin A caused a significant reduction in both CBF and %AA which was partially restored when the Antimycin a-containing medium was replaced with recovery medium (Figure 3.9B). Cells treated with glucose-free medium containing both 2-deoxy-D-glucose and Antimycin A experienced a significant and rapid reduction in CBF and %AA with complete ciliostasis occurring 30 minutes post exposure (Figure 3.9C). Replacing the inhibitors with recovery medium restored CBF to baseline levels by 60 minutes; however, %AA did not fully recover by 120 minutes.

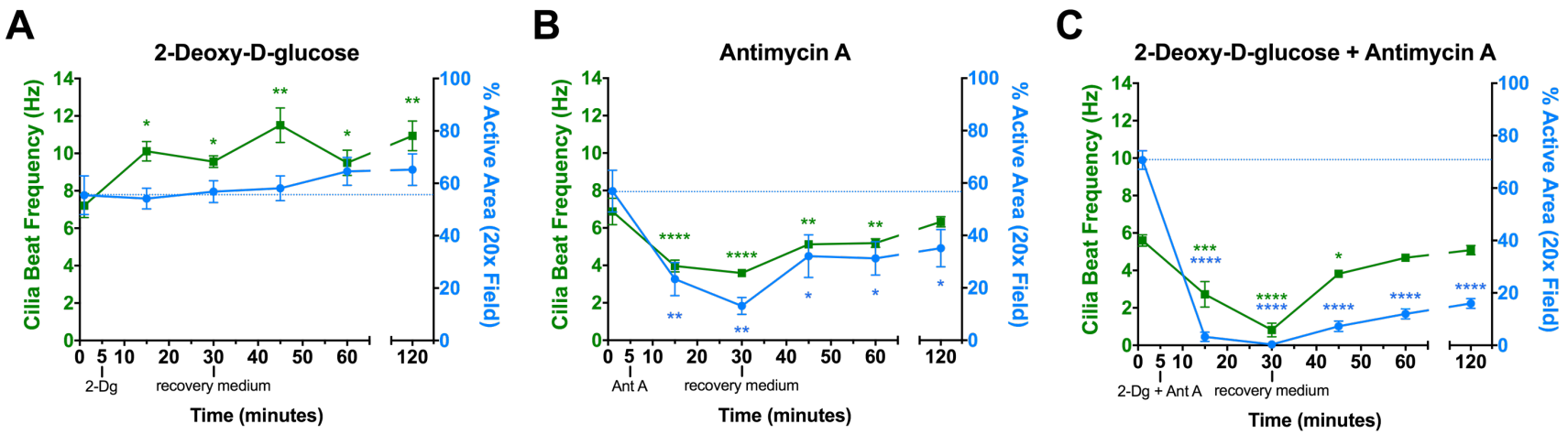

Figure 3.9 Chemical inhibition of well-differentiated hBE cell glycolytic function and mitochondrial respiration significantly alters cilia motility. A) Inhibition of glycolytic function with 2-DG significantly increased CBF while B) inhibition of mitochondrial respiration with Antimycin A caused a significant reduction in CBF and %AA. C) Addition of both chemical inhibitors produced a greater reduction in CBF and %AA. However, Cilia motility was restored by removing inhibitors and supplementing cultures with fresh growth medium. (N=4). Data presented as mean ± SEM. Significance represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.00001$.

3.4 Discussion

In the present study, hBE cells were collected from healthy, non-smoker donors, differentiated to a mucociliary phenotype, and used to evaluate temporal changes in cilia motility subsequent to a single acute exposure to cinnamon-flavored e-liquid, vaped cinnamon e-liquid, or cinnamaldehyde flavoring. The data presented here demonstrate that cinnamaldehyde, at concentrations relevant to e-cigarette exposures, is capable of causing rapid ciliostasis that varies in time to recovery dependent upon the exposure does. Reductions in cilia motility correlated with a significant, but temporary reduction in intracellular ATP levels and cinnamaldehyde exposures of Beas-2B cells resulted in a dose-dependent reduction in mitochondrial respiration and glycolysis. While cinnamaldehyde suppressed bioenergetic activity, reduced ATP levels, and impaired cilia motility, exposures did not significantly reduce cell viability (Figure 3.10) or have long-lasting effects of cilia motility. Together, these data indicate that exposure to e-cigarette flavoring chemicals can have significant impact on essential airway epithelial cell physiology without causing overt cytotoxicity.

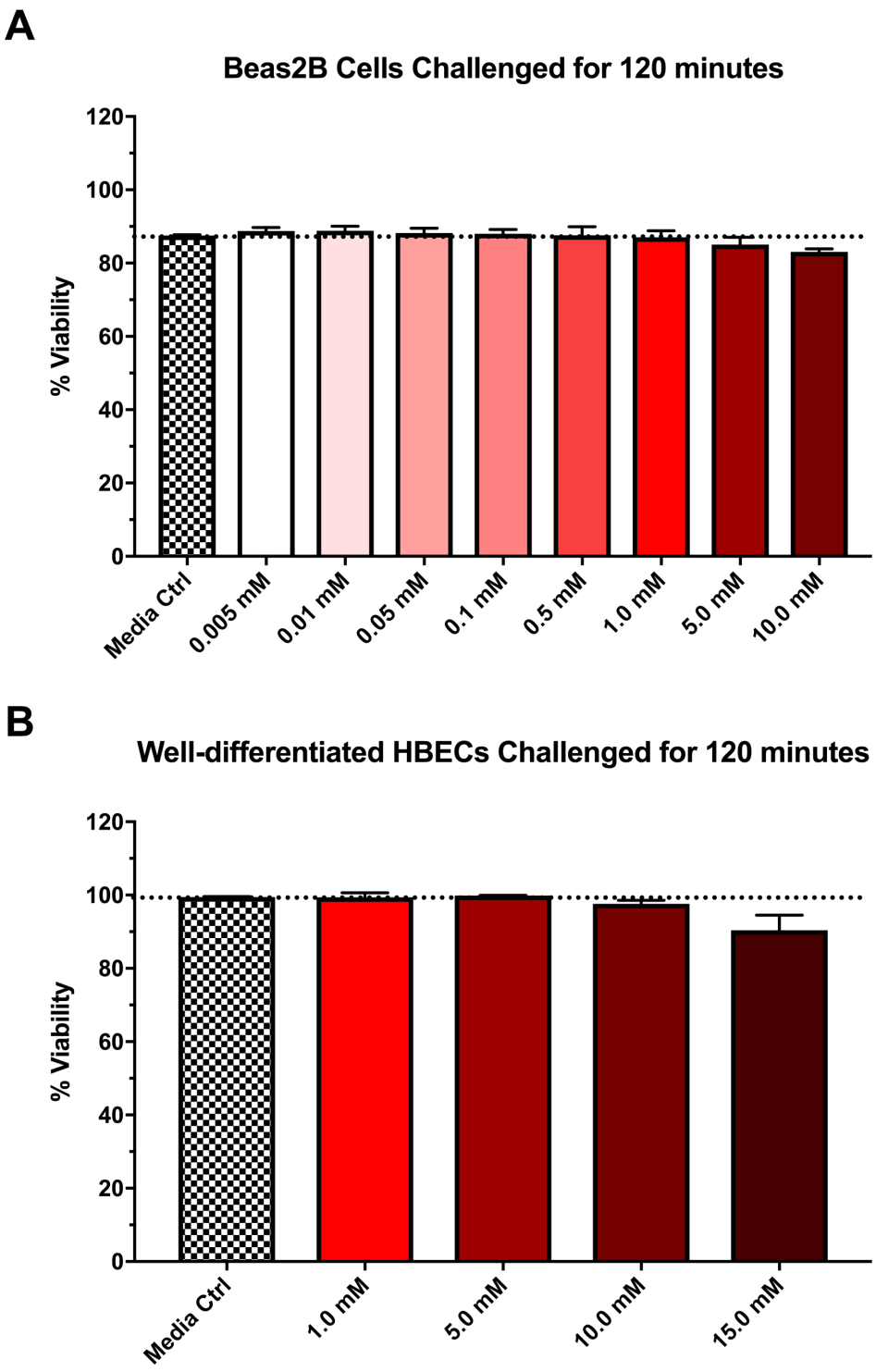

Figure 3.10 Effects of cinnamaldehyde on bronchial epithelial cell viability after acute exposures. A) Beas-2B cells (N=4) and B) well-differentiated hBE cells (N=3) were exposed to various concentrations of cinnamaldehyde and cytotoxicity was determined by quantification of LDH release after 120 minutes. Data presented as mean ± SEM.

Several studies have identified aldehydes at various concentrations in e-liquids and e-cigarette aerosols (19, 45-49). Furthermore, recent work has shown that formaldehyde, acetaldehyde, and acrolein can be generated by the thermal degradation of propylene glycol and glycerin in e-cigarettes. While much of the current e-cigarette research has focused on identifying and quantifying known toxic aldehydes generated by e-cigarettes, fewer studies have investigated whether common aldehyde flavoring agents may also be toxic to respiratory tissues. Bahl and colleagues evaluated the effects of 29 e-liquids on human embryonic stem cells (hESCs), mouse neural stem cells (mNSEs), and human pulmonary fibroblasts (hPFs), and observed that a cinnamon-flavored e-liquid exhibited the most cytotoxicity across all cell types (50). Follow-up studies by Behar et al. attributed the cytotoxic effect to cinnamaldehyde and 2-methoxycinnamaldehyde (51). Moreover, sub-cytotoxic cinnamaldehyde exposures resulted in depolymerization of microtubules, reduced proliferation, impaired cell migration, and increased DNA double strand breaks (25). Gerloff et al. recently reported that ortho-vanillin, but not cinnamaldehyde, stimulated IL-8 release by Beas-2B bronchial epithelial cells and HLF-1 human lung fibroblasts. Additionally, they observed that cinnamaldehyde impaired epithelial barrier function in 16-HBE human bronchial epithelial cells (52). We have previously shown that three cinnamaldehyde-containing e-liquids impaired human neutrophil and alveolar macrophage phagocytosis, altered PMA-induced neutrophil extracellular trap (NET) formation, and suppressed the ability of natural killer (NK) cells to eliminate leukemia target cells (34). Exposures of these cells types to cinnamaldehyde alone recapitulated the effects of the cinnamon-e-liquids indicating that the altered immune cell function directly resulted from the bioactivity of this

aldehyde flavoring agent. These studies strongly indicate that inhalation of cinnamaldehyde will likely induce adverse health effects at several levels.

The reactivity of aldehydes stems from their electrophilic carbonyl group which can form a chemical bond with electron-donating nucleophiles, such as amine groups on protein lysine residues (53). α,β-unsaturated aldehydes, such as acrolein, are generally considered more reactive than saturated aldehydes as nucleophilic additions can occur at either the carbonyl group (1,2-addition) or β-carbon (1,4-addition). Nucleophilic addition at the β-carbon (i.e., Michael addition) results in the formation of a stable, covalent addition. Weak bases, including alcohols, thiols, primary amines, and secondary amines can readily form Michael adducts with α,β-unsaturated aldehydes (54, 55). By these mechanisms, reactive aldehydes can deplete the cellular glutathione pool, modify DNA, and form aldehyde-protein adducts (56, 57). These actions can produce a variety of cellular and biochemical responses including altered protein function, increased oxidative stress, inflammatory responses, apoptosis, and immunogenicity, which may be the underlying mechanisms of the various adverse effects described in the context of cinnamaldehyde exposures.

Data shown here expand on our previous work by demonstrating that cinnamaldehyde dysregulates airway cilia motility, a critical component of effective mucociliary clearance and essential respiratory innate defense mechanism. Deficits in cilia motility which contribute to defective mucociliary clearance have been described in a number of inherited and acquired respiratory disorders. Primary ciliary dyskinesia (PCD), an autosomal recessive disorder of motile cilia, causes abnormalities in ciliary dynein structure and function which results in immotile cilia or impaired CBF (58). Cilia motility in PCD is dyskinetic, which results in a loss of the normal metachronal wave form and significantly impairs mucociliary

transport. This was clearly demonstrated in a study by Regnis et al. which found that PCD patients had prolonged retention of particles in their large airways at 24 hours as compared to healthy control subjects (59). The clinical respiratory manifestations of PCD are directly linked to the failure of mucociliary transport and include nasal congestion, chronic sinusitis, recurrent respiratory tract infections, and bronchiectasis (1). Similarly, patients with cystic fibrosis, an inherited disorder affecting the cystic fibrosis transmembrane conductance regulator (CFTR) gene, display abnormal mucociliary clearance which results in chronic infections, excessive inflammatory responses, bronchiectasis, and respiratory failure (60). The impaired mucociliary clearance in cystic fibrosis results from the abnormal biophysical properties of airway mucus rather than impaired airway cilia motility (61, 62). However, cilia from patients with cystic fibrosis do commonly exhibit structural anomalies similar to those in patients with chronic bronchitis (e.g., atypical arrangement of microtubule doublets, compound cilia, excess cytoplasmic matrix) (63). COPD, an acquired respiratory disease that most frequently occurs in chronic smokers, is characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases (64). Cigarette smoke significantly reduces cilia length and this phenomenon is more pronounced in COPD (65, 66). These structural defects impair mucociliary clearance and contribute to frequent respiratory infections, bacterial colonization of the lower respiratory tracts, chronic inflammation, oxidative stress, and severe tissue damage (64). The loss of ciliary motility and subsequently mucociliary transport is clearly associated with increased susceptibility to respiratory infections and negative health outcomes. Our finding that acute exposures to aerosolized cinnamon e-liquid and cinnamaldehyde induces rapid ciliostasis of airway epithelial cell CBF

is cause for concern as it demonstrates a potential risk for e-cigarette users inhaling cinnamaldehyde. While further studies are needed to address e-cigarette aerosol deposition in the lungs to ultimately understand exposure doses associated with vaping, these data add to the growing body of evidence that inhalational exposures of food-safe flavorings may have unintended and potentially harmful respiratory effects.

Cinnamaldehyde is a potent antimicrobial agent and has been used extensively in the agriculture and food industries to prevent microbial growth in a broad range of foods and beverages (67). Studies by Gill and Holley which investigated the mechanisms of bactericidal action of cinnamaldehyde against *L. monocytogenes* and *E. coli* found that cinnamaldehyde rapidly reduced intracellular ATP levels and inhibited ATP synthesis (44). Follow-up studies confirmed that impaired ATP synthesis was mediated by cinnamaldehyde-induced suppression of membrane bound ATPase activity (68, 69). These findings are similar to our observation that cinnamaldehyde reduces intracellular ATP concentrations in hBE cells. It is entirely unknown whether the ability of cinnamaldehyde to suppress bacterial ATP synthesis may alter the lung microbiome. Based on our data and the existing data on the antimicrobial functions of cinnamaldehyde, additional studies investigating acute, sub-chronic, and chronic exposures on various ATP-dependent antimicrobial processes are warranted.

ATP is the canonical energy carrier that powers cellular machinery, fuels metabolic reactions, and serves as a building block for nucleic acids. Depletion of ATP has widespread effects on many essential cellular systems. Molecular motor proteins, including dynein, kinesin, and myosin ATPases, function by hydrolyzing ATP. Hydrolysis of ATP converts chemical energy into mechanical motion by conferring a small conformational change on the

globular motor domain of the protein (70). Reductions in intracellular ATP levels beyond the minimal requirements for normal activity dysregulate motor protein functions, including ciliary motility and intracellular transport (33, 71). Additionally, the alveolar epithelium relies on basolateral Na^+/K^+-ATPases to actively transport Na^+ out of epithelial cells and generate a transepithelial osmotic gradient which causes the movement of fluid out of the alveolar airspace (72). The active transport of Na^+ and K^+ across the cell membrane to maintaining an ionic gradient consumes approximately 40% of the ATP in mammalian cells (73). Studies on the respiratory effects of hypoxia have shown that hypoxia-induced ATP depletion inhibits Na^+/K^+-ATPase function by downregulation of active Na^+ pumps at the membrane, which impairs fluid clearance from the alveolar space but conserves ATP levels (74). Furthermore, the net gain of intracellular solutes drives an isosmotic accumulation of intracellular water, resulting in cell swelling and dilation of the endoplasmic reticulum (75). These data suggest that cinnamaldehyde-induced reductions in ATP could have significant impact on the function of alveolar epithelial cells.

In addition to being an essential energy source, ATP is also released by airway epithelial cells where it, and its metabolite adenosine, serve as secreted second messengers which regulate multiple cellular processes via autocrine/paracrine activation of purinergic receptors (76). These processes include stimulation of CFTR-dependent and CFTR-independent (CaCC-mediated) chloride channel activity, regulation of Na^+ absorption, regulation of mucin secretion, and regulation of CBF (76). More recently, ATP-mediated activation of purinergic receptors and subsequent activation of Duox1 has been shown to play an important role in airway epithelial cell migration and repair following injury (77). There are very little data on whether or how depletion of ATP alters purinergic receptor

activation and downstream cellular responses. It is plausible that ATP depletion would reduce receptor activation and dysregulate the downstream effects. Thus, cinnamaldehyde-induced reductions of hBE cell intracellular ATP levels may affect ATP-mediated signaling dynamics and downstream cell functions.

The sustained beating of airway cilia demands a tremendous amount of energy. A significant part of the ATP needed to fuel ciliary dynein ATPases is supplied by mitochondria massed at the base of each cilium (78). Inhibition of mitochondrial respiration disrupts ATP production, reduces the amount of available ATP for ciliary dynein, and in turn, impairs cilia motility (33). Reactive aldehydes present in cigarette smoke have been shown to impair mitochondrial function by various mechanisms. Formaldehyde has been reported to inhibit dinitrophenol-stimulated ATPase activity and reduce the rate of oxidative phosphorylation (40). Acetaldehyde inhibits oxidative phosphorylation and respiratory control through reactions with glutamate, P-hydroxybutyrate, and α-ketoglutarate (79). Acrolein is reported to cause a dose-dependent inhibition of NADH- and succinate-linked mitochondrial respiration, form protein carbonyls, and alter mitochondrial permeability (41). Aromatic aldehydes have also been reported to impair mitochondrial function. Anisaldehyde, benzaldehyde, *p*-tolualdehyde, which have all been identified in e-liquids and e-cigarette aerosols, were shown to inhibit pyruvate/malate- and succinate-mediated mitochondrial respiration in isolated rat liver mitochondria at concentrations ranging from 0.5mM to 1.0mM (80). Exposures to 300μM benzaldehyde inhibited pyruvate/malate-mediated state 3 mitochondrial respiration by 50% suggesting that no additional functional group or metabolism to another species was required for the inhibitory effects. Hence, many different aldehydes potentially inhaled by aerosolizing flavored e-liquids have known significant

effects on mitochondrial function. Similarly, cinnamaldehyde was also reported to impair mitochondria isolated from rat liver by uncoupling the electron transport chain and significantly inhibiting NAD(P)H oxidase (complex I) activity (81). To investigate whether cinnamaldehyde produced similar inhibition of mitochondrial function in airway epithelial cells, we exposed bronchial epithelial cells to various concentrations of cinnamaldehyde and evaluated changes in the rate of mitochondrial oxygen consumption. The reduction in bronchial epithelial cell mitochondrial respiration we observed supported the existing data describing cinnamaldehyde-induced suppression rat liver mitochondria function. In contrast to the previous report, we did not observe increases in basal oxygen consumption following the addition of cinnamaldehyde, indicating that cinnamaldehyde did not uncouple the electron transport chain. Interestingly, addition of cinnamaldehyde rapidly reduced the rate of extracellular acidification, which is an indicator of cellular glycolytic function. The suppression of both mitochondrial respiration and glycolysis supports our finding that cinnamaldehyde rapidly reduces intracellular ATP levels. It was beyond the scope of this study to identify specific molecular mechanisms by which cinnamaldehyde impaired bioenergetic processes. Acrolein, an electrophilic α,β-unsaturated aldehyde, is reported to readily react with thiolate groups on redox-active cysteine residues to form covalent additions (82, 83). However, covalent bonds between acrolein and cysteine are somewhat unstable at physiologic pH and temperature, which results in a transition to Schiff base adducts with the ε-NH_2 group of lysine and free N-terminal α-NH_2 groups (83). Cinnamaldehyde is also an α,β-unsaturated aldehyde and would likely generate covalent and Schiff base additions to reactive cysteine residues, similar to acrolein. Hence,

cinnamaldehyde-induced modification of mitochondrial and glycolytic pathway proteins may be involved in the loss of bioenergetic functions and reduction of intracellular ATP.

Taken together, the data presented here clearly demonstrate the capability of cinnamaldehyde to alter normal bronchial epithelial cell function by suppressing airway cilia motility, which is an essential respiratory mucosal defense mechanism. The cellular responses we observed following cinnamaldehyde exposure are similar to those described in previous studies using toxic aliphatic aldehydes found in cigarette smoke. This raises concern for inhalational exposures to aromatic aldehyde flavoring agents, such as cinnamaldehyde, in the context vaping. Based on our findings, we conclude that cinnamaldehyde, at sufficient exposure concentrations, disrupts bronchial epithelial cell mitochondrial respiration and glycolytic function, reduces intracellular ATP levels, and impairs cilia motility. Further work is necessary to determine how inhalation of cinnamaldehyde aerosols generated by e-cigarettes recapitulates our *in vitro* findings in e-cigarette users *in vivo*, and whether exposures could lead to increased susceptibility to respiratory infections and enhanced risk of obstructive lung disease. Ultimately, understanding the biological effects of exposures to flavoring agents and concentrations at which these effects occur will be an essential component to evaluating the safety of e-cigarettes.

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CHAPTER 4.

COMMON E-CIGARETTE FLAVORING AGENTS ELICIT DOSE-DEPENDENT BIMODAL EFFECTS ON NRF2-PATHWAY ACTIVATION IN BRONCHIAL EPITHELIAL CELLS

4.1 Introduction

Cellular responses to environmental stresses are orchestrated by tightly-regulated protein networks which detect signals from the environment, transduce signals to gene expression machinery, and activate transcription factors to upregulate the necessary stress-response genes. These responses must be precisely coordinated to effectively alleviate stress and maintain cell homeostasis (1-4). Among environmental stresses, oxidants and electrophiles are broadly recognized as etiologic factors for a host of human diseases including chronic inflammation, cancer, diabetics, rheumatoid arthritis, atherosclerosis, myocardial infarction, cardiovascular disease, respiratory disease, ischemic reperfusion injury, stroke, septic shock, aging and neurodegenerative diseases (5-7). To protect against oxidative and electrophilic stresses, vertebrates have evolved various defense mechanisms, which include the Nuclear factor erythroid-derived 2-like 2 (Nrf2)- Kelch-like ECH-associated protein 1 (Keap1) antioxidant pathway (1, 8-10). Nrf2 is a basic leucine zipper protein transcription factor which regulates stress-dependent expression of numerous cytoprotective genes including NAD(P)H quinone oxidoreductase 1 (NQO1), glutathione s-transferase (GST), heme-oxygenase 1 (HO-1), thioredoxin reductase 1 (TXNRD1),

sulfiredoxin 1 (SRXN1), glutamate-cysteine ligase, and multidrug resistance-associated proteins (MRPs) (11, 12). Transcription of these targets is mediated by Nrf2s interaction with a *cis*-acting antioxidant/electrophile responsive element (ARE/EpRE) located in the promoter of cytoprotective genes (13-15).

In the absence of oxidative or electrophilic stress, homodimeric Keap1 sequesters Nrf2 into the cytoplasmic Cul3-containing E3 ubiquitin ligase complex where it undergoes polyubiquitin conjugation and subsequent degradation by the 26S proteasome (16). However, an alternate model in which Keap1 transiently engages in nucleocytoplasmic shuttling and targets Nrf2 for ubiquitylation in the nucleus has been proposed (15). The rapid proteasomal degradation of Nrf2 and its short half-life (approximately 20 minutes) limits Nrf2 activity and, thereby, regulates cellular redox homeostasis. In addition to sequestering Nrf2 and facilitating its degradation under homeostatic conditions, Keap1 acts as a molecular sensor for electrophilic chemicals. Each human Keap1 protein contains 27 cysteine residues (17). Importantly, 10 of these cysteines are adjacently located to positively-charged amino acids. The proximity of cysteine residues to positively-charged amino acids decreases the pKa of the cysteine sulfhydryl group and thus stabilizes the reactive thiolate anion (17). Both endogenous and exogenous electrophiles readily react with thiolate anions on Keap1 cysteine residues, resulting in a conformation change of Keap1, release and stabilization of Nrf2, and subsequent transcription of cytoprotective genes (17). However, if oxidant and electrophile exposures overwhelm the protective antioxidant responses of Nrf2 pathway activation, cell death and tissue damage can occur.

Cigarette smoke is a toxic mixture of harmful substances, which causes damage to virtually every part of the body. In the lungs, cigarette smoke induces oxidative and

electrophilic stress, which contributes to the pathogenesis of cancer and chronic obstructive pulmonary disease (COPD) (18, 19). Cigarette smoke is a potent pro-oxidant, both by way of free radicals produced when combusting tobacco and also by way of generating intracellular oxidant species from smoke-induced disruption of normal cellular processes, such as mitochondrial respiration (20, 21). While the exact mechanisms underlying how components in cigarette smoke impair antioxidant defenses continue to be investigated, it is likely that chronic exposures to free radicals, reactive oxygen and nitrogen species, and aldehydes, which disrupt cellular redox homeostasis, play a central role in the pathogenesis and progression of cigarette-smoke-induced lung disease. Glutathione (GSH) is the most abundant non-protein thiol-containing antioxidant compound present in living organisms and plays an important role in protecting cells from oxidants and reactive electrophiles (22). α,β-unsaturated carbonyls, such as acrolein, crotonaldehyde, and methyl vinyl ketone, are major toxicants in cigarette smoke and have been shown to irreversibly modify GSH (23). This modification, which is typically a covalent Michael addition to the thiol group of the cysteinyl moiety on GSH, depletes the intracellular pool of free GSH and thereby increases susceptibility to oxidant and electrophilic damage. Thus, depletion of GSH by cigarette smoke plays a significant role in the development and progression of oxidant-induced lung diseases including emphysema and COPD.

In recent years, e-cigarettes have emerged as an alternative to traditional cigarettes. While the health effects of e-cigarette use remain unclear, studies of e-cigarettes aerosols have confirmed that their emissions are less complex than cigarette smoke and contain significantly lower levels of known toxicants, including free radicals, reactive oxygen species, and aldehydes (24, 25). A recent report from Public Health England concluded that

e-cigarettes are 95% less harmful than tobacco cigarettes, and when supported by a smoking cessation service, are effective at helping most people quit smoking (26). This view was supported by the Royal College of Physicians which stated that although harm from long-term use of e-cigarettes cannot be excluded, it is likely to be substantially less than conventional cigarettes (27). However, e-cigarette aerosols remain poorly characterized complex mixtures of inert and reactive chemicals, and it is unclear how these products will affect respiratory health. Moreover, the lack of regulation in this industry creates considerable variability in the chemical composition of these products between manufacturers, precluding accurate estimations of e-cigarette safety.

The use of food-grade flavoring agents in e-cigarettes represents a significant unknown in the context of oxidative lung damage. Many flavoring agents are reactive carbonyls and inherently bioactive at low concentrations (28). Analyses of e-cigarette refill liquids have identified reactive carbonyl flavoring agents at concentrations greater than 1.0M, which may be cause for concern (29-32). Several studies have shown that flavored e-cigarette aerosols contain reactive oxidants and induce oxidative and electrophilic stress responses in *in vitro* and animal *in vivo* models (33-37). There are conflicting data indicating that e-cigarettes, when used at lower temperatures, do not generate significant oxidants or induce cellular oxidative stress (38-40). Some common flavoring agents have been reported to have prooxidant effects on rat hepatocytes and generate reactive oxygen species in the presence of transition metals (41-43). Other common e-cigarette flavoring agents have been identified as nutraceuticals for their ability to activate Nrf2 antioxidant responses and upregulate cytoprotective gene expression (44, 45). However, it is unclear if flavoring agents, as components of e-cigarettes, will alter oxidative stress responses in respiratory tissues. In

order to better understand whether and how common e-cigarette flavoring agents affect oxidative stress response pathways in bronchial epithelial cells, we identified eight common e-cigarette flavoring agents in e-liquids which induced HEK293 cell cytotoxicity in high-throughput screening assays, exposed bronchial epithelial cells containing an Nrf2/ARE luciferase promoter reporter with various concentrations of these flavoring chemicals, and quantified Nrf2 antioxidant pathway activation and cytotoxicity at each flavoring concentration. Furthermore, we investigated whether intracellular GSH levels modify flavoring-induced Nrf2 pathway activation. These data provide additional insights into the potential health effects of e-cigarette use.

4.2 Materials and methods

Selection of Flavoring Chemicals for Analysis

Flavoring chemicals were selected for analysis using the University of North Carolina Center for Tobacco Regulatory Science and Lung Health database for e-liquid toxicity and chemical compositions (www.eliquidinfo.org). E-liquids were ranked according to their reported toxicity in HEK293T cells (lowest LC50 values to highest) and the chemical compositions of the 50 most cytotoxic e-liquids were compared. Isoamyl acetate, menthol, limonene, cinnamaldehyde, guaiacol, benzaldehyde, eugenol, and linalool were the most common flavoring agents in the 50 e-liquids we compared (Table 4.1). Because these flavoring agents were identified in multiple highly-cytotoxic e-liquids, they were selected for further investigation in this study.

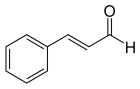
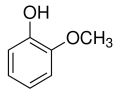
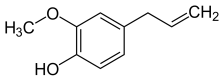
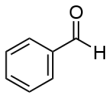
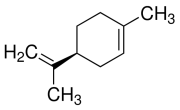
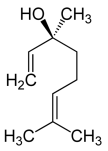
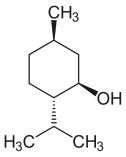
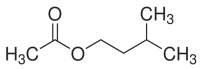
	Structure	Chemical Class	Characterizing Flavor	Times Identified
Cinnamaldehyde		aromatic aldehyde (α,β-unsaturated)	cinnamon/spicy	8/50
Guaiacol		monomethyl ether of catechol	wood smoke	8/50
Eugenol		allyl-chain substituted guaiacol	clove	3/50
Benzaldehyde		aromatic aldehyde	cherry/almond	4/50
Limonene		cyclic monoterpene	citrus	9/50
Linaloool		terpene alcohol	floral	3/50
Menthol		cyclic monoterpene alcohol	minty/cool	12/50
Isoamyl Acetate		carboxylic acid ester	banana	14/50

Table 4.1 E-liquid flavoring agents used for this study. Comparison of the chemical constituents of e-liquids with the lowest LC_{50} (greatest toxicity) in HEK293T cells revealed common flavoring agents from various chemical classes. Data on e-liquid-induced cytotoxicity was obtained from University of North Carolina Center for Tobacco Regulatory Science and Lung Health database for e-liquid toxicity and chemical compositions (www.eliquidinfo.org).

Beas-2B Bronchial Epithelial Cells

Beas-2B bronchial epithelial cells were obtained from ATCC (Manassas, VA, USA) and cultured in serum-free keratinocyte growth medium (KGM; Lonza, Walkersville, MD, USA) under standard airway epithelial cell growth conditions (37°C, 95% humidity, and 5% carbon dioxide). All studies were performed using Beas-2B cells between passage 40 and passage 60, with passage 1 defined as the thawed cells from the supplier.

Beas-2B ARE Promoter Luciferase Reporter Cells and Flavoring Exposures

Beas-2B cells were transduced with a lentiviral vector containing tandem repeats of the human Nrf2 consensus DNA-binding site (ARE) linked to the firefly luciferase gene and a constitutively expressed green fluorescent protein (GFP) reporter (kind gift from Dr. Steve Simmons, National Health and Environmental Effects Research Laboratory, U.S. EPA, Durham, NC) at a multiplicity of infection (MOI) of 20. Stably transduced Beas-2B ARE-luciferase reporter cells were plated in white 96-well plates (Corning Inc, Corning, NY, USA) at $6.0x10^4$ cells per well in KGM and cultured overnight at 37°C prior to experimentation. For flavoring exposure experiments, growth medium was aspirated and cells were washed briefly with 1x PBS (Sigma-Aldrich, St. Louis, MO, USA). Cinnamaldehyde, guaiacol, eugenol, isoamyl acetate, limonene, linalool, menthol, and benzaldehyde (Sigma-Aldrich, St. Louis, MO, USA) were diluted in 37°C KGM to 10.0, 1.0, 0.1, 0.01, 0.001, 0.0001, and 0.00001mM, and cells were incubated with 200µl of diluted flavorings or KGM alone for 24 hours (37°C, 95% humidity, and 5% carbon dioxide). Following exposures, cell culture medium was collected for cytotoxicity assays, cells were lysed with 1x Passive Lysis buffer (Promega, Madison, WI, USA), and cell lysates were

analyzed for GFP expression and luciferase activity on a BMG CLARIOstar microplate reader (BMG Labtech, Ortenberg, Germany). Luciferase activity was normalized to GFP expression and reported as percent Nrf2 activation observed in cells exposed to cell culture medium alone. Differences in luciferase activity following flavoring exposures were evaluated by one-way ANOVA and Holm-Sidak multiple comparisons test. Differences with a P value ≤ 0.05 were considered significant.

Cytotoxicity Assays

Beas-2B cell viability following 24-hour flavoring exposures was determined by measuring lactate dehydrogenase (LDH) release into the cell culture medium using a commercially available colorimetric (490/650 nm) assay kit following the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA, USA). One hundred microliters of culture medium were used for analysis. The absorbance data for each sample was normalized to 100% cell death (maximal LDH release caused by lysis with 1.0% Triton X-100 in cell culture medium) and baseline LDH release (untreated cells) using the following formula: corrected viability = 100 – [[Abs(X) − Abs(Y)]/[Abs(MAX) − Abs(Y)]*100] where Abs(X) is the absorbance of the sample, Abs(Y) is the absorbance of media from the untreated control cells, and Abs(MAX) is the absorbance of media collected from cells exposed to 1.0% Triton X-100. Data for cinnamaldehyde toxicity in Beas-2B cells were collected from 3 independent experiments (N=3). Differences in viability following flavoring exposures were evaluated by one-way ANOVA and Holm-Sidak multiple comparisons test. Differences with a P value ≤ 0.05 were considered significant.

Buthionine-sulfoximine (BSO) Inhibition of Glutathione (GHS) Synthesis

A 1M stock solution of BSO (Sigma-Aldrich, St. Louis, MO, USA) was prepared by dissolving BSO in sterile cell culture grade water (Thermo Fisher Scientific, Waltham, MA, USA) and vortexing for 2 minutes. BSO was further diluted to working concentrations of 1.0, 0.5, 0.25, 0.125, 0.0625, 0.0313, 0.0158, 0.0078, or 0.0039mM in 37°C KGM. Beas-2B cells were incubated with BSO in KGM, cinnamaldehyde in KGM, or KGM alone for 24 hours and intracellular reduced GSH levels were quantified using a commercially available fluorescence-based kit following the manufacturer's instructions (Abcam, Cambridge, United Kingdom).

Inhibition of GSH Synthesis in Beas-2B ARE promotor luciferase reporter cells Prior to Cinnamaldehyde Exposures

Beas-2B ARE-luciferase reporter cells were plated in white 96-well plates at $2.5x10^4$ cells per well in KGM and cultured overnight at 37°C prior to experimentation. Cells were briefly washes with 1x PBS and incubated with 10μM BSO for either 6 hours or 24 hours prior to the addition of cinnamaldehyde (10.0, 1.0, 0.1, 0.01, 0.001, 0.0001, and 0.00001mM). After the addition of cinnamaldehyde, cells were incubated for an additional 24 hours (37°C, 95% humidity, and 5% carbon dioxide). Following exposures, cell culture medium was collected for cytotoxicity (LDH) assays and cells were lysed for GFP expression and luciferase activity as described above. Luciferase activity was normalized to GFP expression and reported as percent Nrf2 activation observed in cells exposed to cell culture medium alone. Differences in luciferase activity following flavoring exposures were

evaluated by one-way ANOVA and Holm-Sidak multiple comparisons test. Differences with a P value ≤ 0.05 were considered significant.

4.3 Results

Nrf2 Pathway Activation by E-liquid Flavoring Agents

Beas-2B cell Nrf2 pathway activation was flavor-specific and concentration dependent (Figure 4.1). Cinnamaldehyde (0.01mM), guaiacol (0.01, 0.1, 1.0mM), and eugenol (0.01, 0.1mM) significantly increased Nrf2 pathway activation. However, this effect was bimodal as greater concentrations of cinnamaldehyde (0.1, 1.0, 10.0mM), guaiacol (10.0mM), and eugenol (10.0mM) significantly suppressed Nrf2 pathway activation as compared to their media only controls. The highest concentration of linalool (10.0mM) and menthol (10.0mM) also significantly suppressed Nrf2 pathway activation although lower concentrations had no significant effect. Benzaldehyde, limonene, and isoamyl acetate had no effect on Nrf2 pathway activation at any of the concentrations investigated.

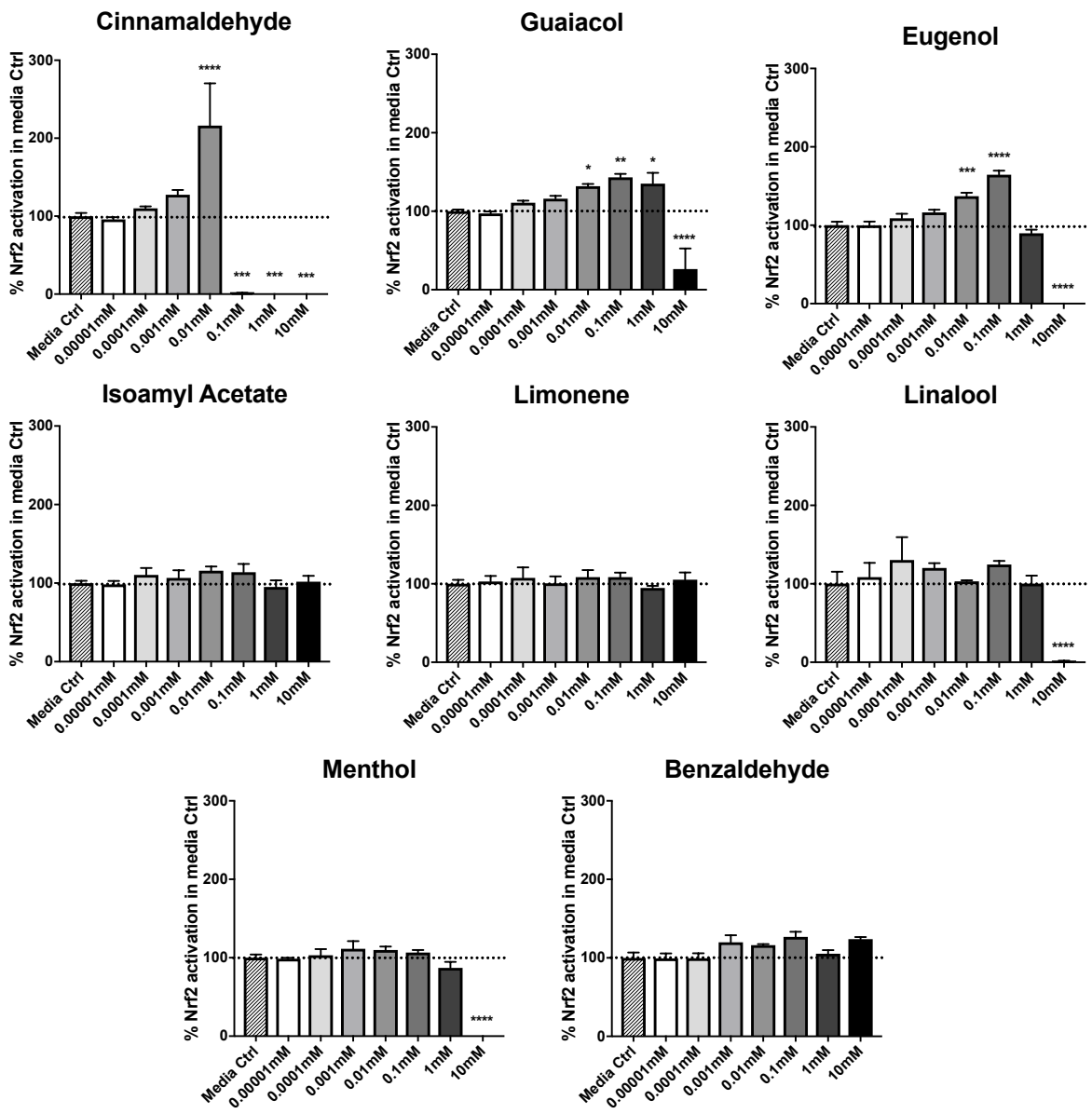


Figure 4.1 Nrf2 antioxidant pathway activation is flavor-specific and concentration-dependent. Cinnamaldehyde (0.01mM), guaiacol (0.01mM, 0.1mM, and 1.0mM), and eugenol (0.01mM and 0.1mM) significantly increased Nrf2 pathway activation as compared to the media control (N=3). High concentrations of cinnamaldehyde (0.1mM, 1.0mM, and 10.0mM), guaiacol (10.0mM), eugenol (10.0mM), linalool (10.0mM), and menthol (10.0mM) significantly suppressed Nrf2 pathway activation as comparted to the media only controls. Data presented as mean ± SEM. Significance represented as * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.

Effects of Flavoring Agents on Cell Viability

The effect of flavorings on Beas-2B cell viability was also flavor-specific and concentration dependent (Figure 4.2). Cinnamaldehyde (10mM), eugenol (10.0mM), limonene (10.0mM), linalool (10.0mM), and menthol (10.0mM) significantly reduced cell viability to 78.9%, 62.3%, 75.7%, 71.7%, and 61.5%, respectively. Guaiacol, benzaldehyde, and isoamyl acetate did not significantly affect cell viability at any of the concentrations tested.

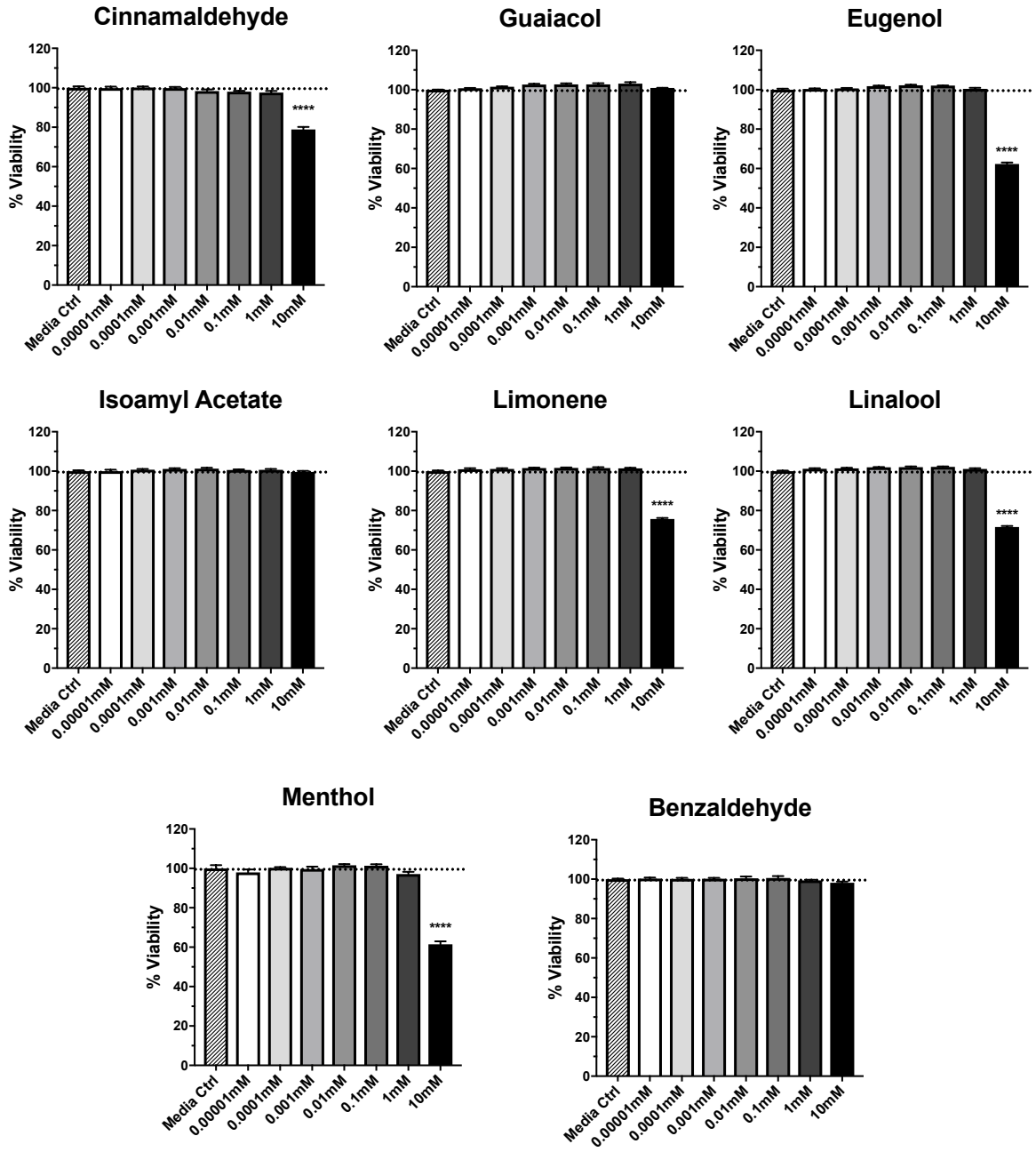


Figure 4.2 Cytotoxicity is flavor-specific and concentration-dependent. The highest concentrations of cinnamaldehyde (10mM), eugenol (10.0mM), limonene (10.0mM), linalool (10.0mM), and menthol (10.0mM) induced significant cytotoxicity, as determined by LDH released following 24-hour exposures (N=3). Data presented as mean ± SEM. Significance represented as **** $p<0.0001$.

Effect of BSO and Cinnamaldehyde on Intracellular GSH Levels

Twenty-four-hour exposure of Beas-2B cells to BSO significantly reduced intracellular GSH levels in a concentration-dependent manner (Figure 4.3A). Reductions in GSH ranged from 41.8% (3.9μM BSO) to 20.6% (1mM BSO) of control levels. Reduced GSH levels did not significantly activate Nrf2 pathway activation (Figure 4.3B) or decrease Beas-2B cell viability (Figure 4.3C) at any of the concentrations tested. Previous studies investigating mechanisms of cinnamaldehyde toxicity in rat isolated hepatocytes report an immediate reaction between cinnamaldehyde and GSH which resulted in depleted intracellular GSH levels (46, 47). To determine if non-cytotoxic concentrations of cinnamaldehyde altered GSH levels in bronchial epithelial cells, we exposed Beas-2B cells to cinnamaldehyde for 24 hours and then quantified GSH. Cinnamaldehyde depleted GSH in Beas-2B cells over a concentration range of 0.5mM to 0.016mM (Figure 4.4). All cinnamaldehyde exposures eliciting a significant reduction decreased GSH by approximately 25% from GSH levels observed in media controls.

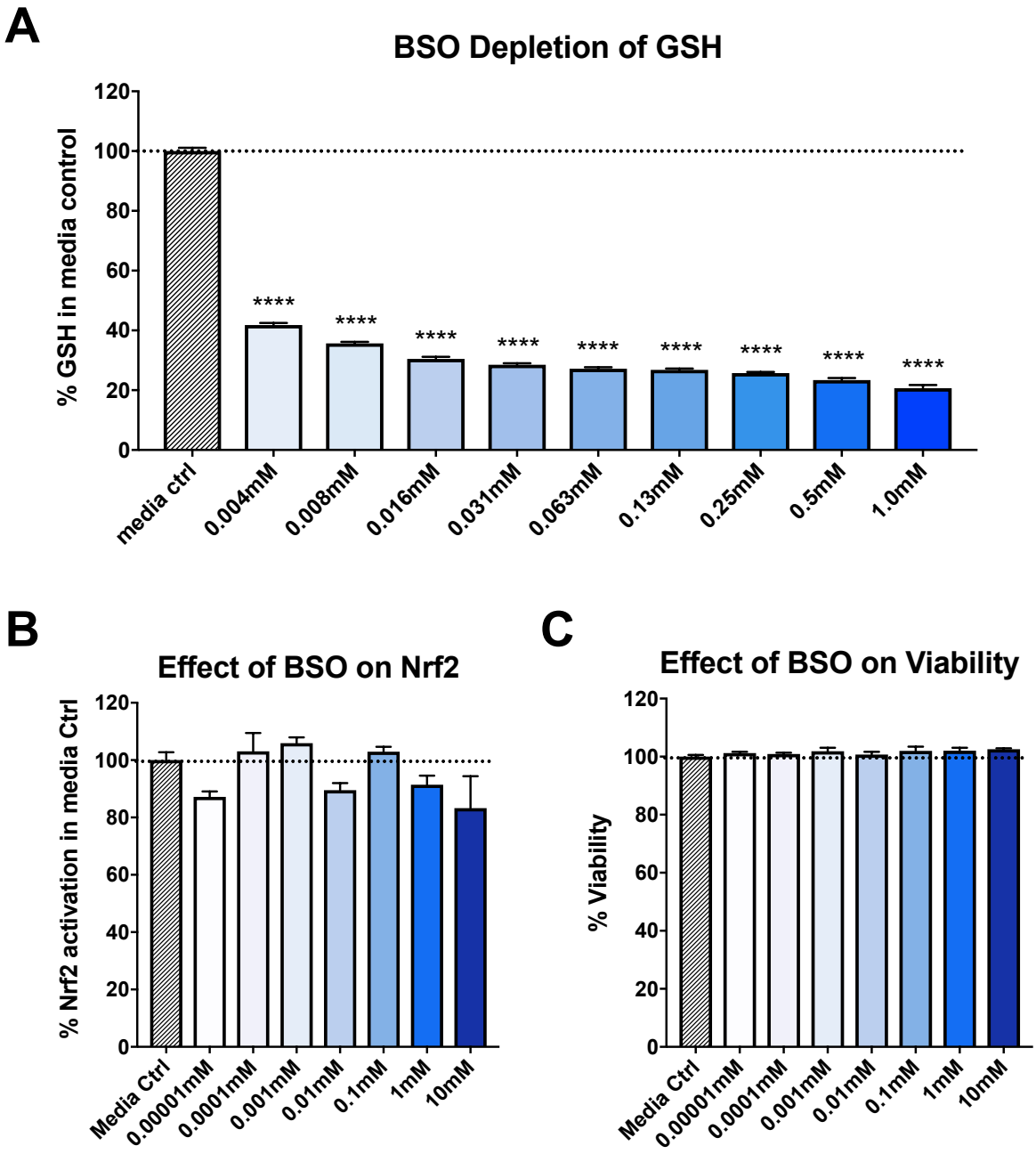


Figure 4.3 Buthionine-sulfoximine (BSO) depletion of glutathione (GSH) does not activate Nrf2 responses or cause cell death. A) BSO is a potent inhibitor of GSH synthesis in Beas-2B cells as indicated by a significant reduction in intracellular GSH levels at all concentrations tested (0.004mM to 1.0mM). B) Depletion of GSH did not significantly affect Nrf2 signaling or C) reduce cell viability, as determined by LDH release (N=3). Data presented as mean ± SEM. Significance represented as **** $p<0.0001$.

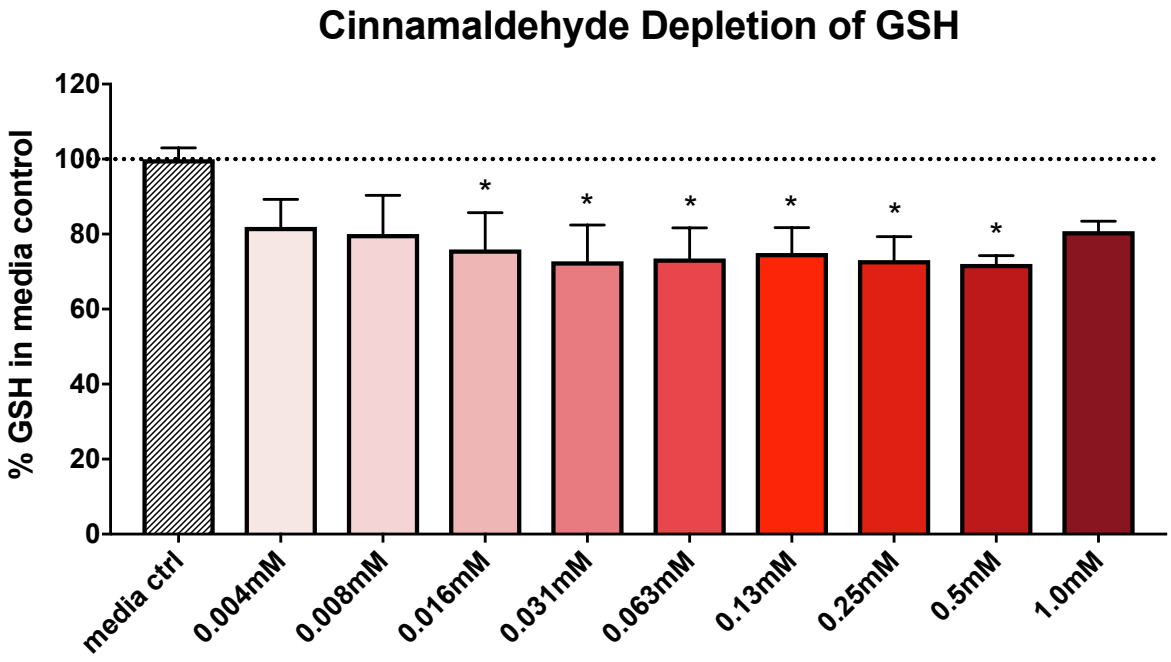


Figure 4.4 Cinnamaldehyde reduces Beas-2B cell GSH levels. Beas-2B cells were exposed to various concentrations of cinnamaldehyde (0.004mM to 1.0mM) for 24 hours and intracellular GSH levels were quantified (N=3). Cinnamaldehyde concentrations from 0.016mM to 0.5mM significantly reduces intracellular levels of GSH (N=3). Data presented as mean ± SEM. Significance represented as * $p<0.05$.

Effect GSH depletion on Cinnamaldehyde-induced Nrf2 Pathway Activation

To investigate the role GSH plays in Nrf2 pathway activation by cinnamaldehyde, Beas-2B cells were pretreated with 10mM BSO for 6 hours or 24 hours to deplete GSH levels prior to cinnamaldehyde exposures. BSO depletion of GSH significantly enhanced cinnamaldehyde-induced Nrf2 pathway activation for 0.01mM, 0.001mM, 0.0001mM, and 0.00001mM exposures (Figure 4.5). However, BSO depletion of GSH did not alter the concentration-dependent bimodal effect on Nrf2 pathway activation as 0.1mM, 1.0mM, and 10.0mM cinnamaldehyde suppressed Nrf2 pathway activity as observed with cinnamaldehyde alone.

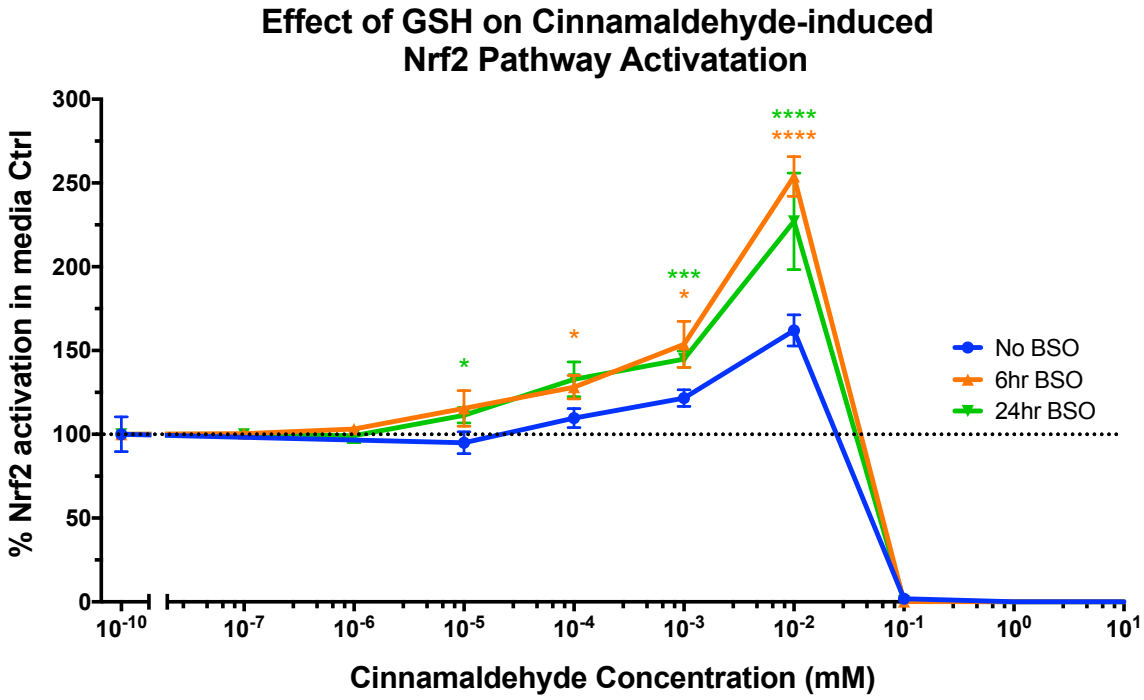


Figure 4.5 BSO depletion of Beas-2B cell GSH increases sensitivity to cinnamaldehyde-induced activation of the Nrf2 pathway. Beas-2B cells were pretreated with 10μM BSO for either 6 hours or 24 hours before the addition of cinnamaldehyde. Depletion of GSH significantly enhanced cinnamaldehyde-induced activation of Nrf2 pathway activation with 0.00001mM, 0.0001mM, 0.001mM, and 0.01mM exposures (N=3). Data presented as mean ± SEM. Significance represented as * $p<0.05$, *** $p<0.001$, and **** $p<0.0001$.

4.4 Discussion

Here, we investigated whether common food-safe flavoring agents in e-cigarette refill liquids had the capacity to modulate normal Nrf2 antioxidant pathway activity in bronchial epithelial cells and whether exposures to cinnamaldehyde, a reactive α,β-unsaturated aldehyde flavoring agent, altered bronchial epithelial cell GSH levels. Our data indicate that cinnamaldehyde, guaiacol, and eugenol have concentration-dependent effects on Nrf2 pathway activation with lower concentrations significantly increasing ARE-luciferase activity and the highest concentrations significantly suppressing luciferase activity as compared to the media only controls. The observation that 0.01mM cinnamaldehyde augments Nrf2 pathway activation supports findings from previous studies. Chew and colleagues observed increased Nrf2/ARE-luciferase reporter activity in colon carcinoma HCT 116 cells exposed to 0.01mM cinnamaldehyde, which decreased with exposures to higher concentrations (48). Wang et al. report that 0.01mM cinnamaldehyde induced human umbilical vein endothelial cell (HUVEC) Nrf2 expression, promoted Nrf2 nuclear translocation, and increased HO-1, NQO1, catalase, and glutathione peroxidase 1 (GPX1) expression under high glucose conditions (49). Kim et al. exposed human dental pulp cells (hDPCs) to 0.02mM cinnamaldehyde and observed increased Nrf2 nuclear translocation and upregulation of HO-1 protein expression, which correlated with reduced levels of intracellular reactive oxygen species and protected the hDPCs from H_2O_2-induced oxidative stress (50). Similarly, Hiroshi et al. exposed human keratinocytes to 0.025mM cinnamaldehyde and observed a significant induction of Nfr2 activation and HO-1 expression, which correlated with a reduction in benzo[a]pyrene-induced oxidative stress (51). Interestingly, the authors noted that cinnamaldehyde potently suppressed AHR nuclear

translocation following benzo[a]pyrene exposure. Altogether, these findings suggest that low micro-molar concentrations of cinnamaldehyde activate the Nrf2 pathway and upregulate cytoprotective gene expression in a variety of cell types without negatively impacting cell viability.

Other studies have reported that higher concentrations of cinnamaldehyde enhance oxidative stress. Raveendran and colleagues orally exposed rats to 73.5 mg/kg cinnamaldehyde for 30 days and observed reduced GSH and ascorbic acid levels, as well as reduced catalase and glucose-6-phosphate dehydrogenase activity in rat livers (41). Additionally, the authors observed elevated levels of thiobarbituric acid reactive substances, indicating oxidative damage. Ka et al. report that exposure of human promyelocytic leukemia HL-60 cells to 0.04mM cinnamaldehyde rapidly decreased intracellular GSH and significantly induced reactive oxygen species-mediated mitochondrial permeability transition and release of cytochrome c, resulting in increased cell death (52). Our findings and the existing literature support the idea of a potential bimodal response pattern for cinnamaldehyde, where exposure concentrations at or below 0.01mM may initiate cytoprotective gene expression and enhance cellular responses to environmental stresses. However, cinnamaldehyde-induced inhibition of Nrf2 pathway activation at high concentrations would likely have the opposite effect and enhance oxidative and electrophilic damage, which could exacerbate the progression of respiratory diseases such as emphysema and COPD.

Our observation that guaiacol significantly enhanced Nrf2 pathway activation contradicts a previous study by Senger and colleagues which found that chemicals with structural similarity to catechol, including guaiacol (0.03mM), did not significantly activate

the Nrf2 pathway or induce expression of Nrf2 target genes in human dermal microvascular endothelial cells after 24-hour exposure (53). One possible explanation for this discrepancy could be inherent differences in the endogenous peroxide/peroxidase content of Beas-2B bronchial epithelial and dermal microvascular endothelial cells. Numerous studies have shown that guaiacol can be oxidized by peroxidases in the presence of H_2O_2 resulting in the formation of various reactive quinones (54, 55). Indeed, a colorimetric assay of peroxidase activity using guaiacol is a common analytical method for quantifying enzymatic activity based on the change in absorbance at 470nm (56). Quinones are oxidative, electrophilic Michael acceptors known to activate Nrf2 antioxidant response through their interactions with reactive cysteine residues on Keap1 (57, 58). Thus, it is plausible that the addition of guaiacol in our study resulted in the generation of quinone species, which subsequently induced Nrf2 pathway activation.

Eugenol (4-allyl-2-methoxyphenol) is an allyl-chain substituted guaiacol, which is the characteristic odor and flavor of clove. Eugenol is widely used as a flavoring additive in various foods and beverages, and has been investigated as a pharmacological agent (59). Studies have indicated that eugenol has both antioxidant and prooxidant properties, which are dependent on exposure concentrations. At low micro-molar concentrations, eugenol is reported react with 2,2-diphenyl-1-picrylhydrazyl (DPPH) and shows high DPPH free radical-scavenging activity (60-62). The reported IC_{50} values for DPPH scavenging by eugenol range from 98μM to 138μM (62, 63). Additionally, eugenol has ferric ion (Fe^{3+}) reducing ability and electron donor properties for neutralizing free radicals by forming stable products (62). While there is substantial literature on the radical-scavenging activity of eugenol, there is a paucity of data on whether eugenol effects Nrf2 pathway activation. Han

and colleagues observed that MCF 7 breast cancer cells exposed to low micro-molar concentrations of eugenol (5, 10, or 20μM) had concentration-dependent inhibition of cytochrome p450 1A1 and 1B1 (CYP1A1 and CYP1B1) activity and reduced AHR nuclear translocation (64). Furthermore, 18-hour treatment with eugenol significantly induced ARE-luciferase activity, Nrf2 nuclear translocation, and NQO1 expression, indicating that, similar to cinnamaldehyde, eugenol suppresses AHR nuclear translocation and activates the Nrf2 antioxidant pathway. Our findings that eugenol induces ARE-luciferase activity in bronchial epithelial cells is in agreement with the data reported by Han. However, the exact mechanism by which eugenol activates Nrf2 remains unclear. Similar to guaiacol, eugenol may undergo oxidation by cellular enzymes to form quinone methides. These electrophilic Michael acceptors may react with thiolate anions on Keap1, thereby releasing Nrf2 and stimulating antioxidant responses.

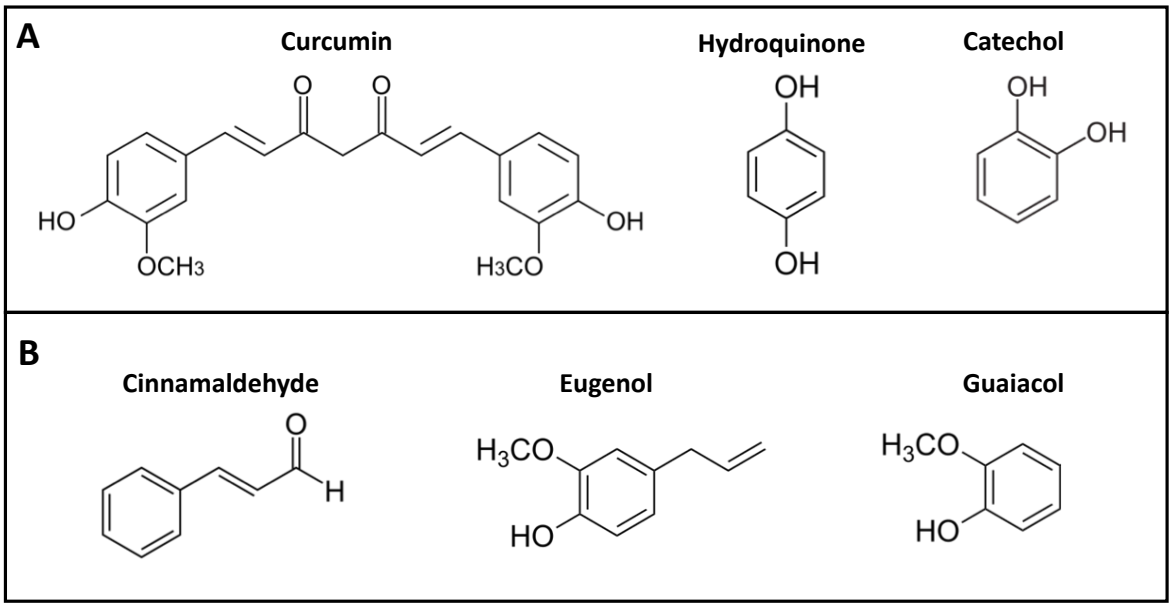


Figure 4.6 Chemical structures of Nrf2 pathway activators. A) Known activators of the Nrf2 antioxidant pathway. B) Common e-cigarette flavoring agents shown here to activate the Nrf2 pathway.

Susceptibility of the lung to oxidative and electrophilic injury, such as that originating from cigarette smoke exposure, depends largely on induction of cytoprotective gene expression (18, 65). Animal studies have shown that disruption of the *Nrf2* gene and subsequent reductions in *Nrf2*-dependent gene expression in mice lead to earlier-onset and more severe cigarette smoke-induced emphysema than observed in wild-type littermate controls (66). The emphysema in these *Nrf2*-deficient mice was associated with more pronounced bronchoalveolar inflammation, enhanced markers of oxidative stress, and greater endothelial cell and type II pneumocyte death as compared to *Nrf2*-sufficient animals. Alveolar macrophage expression of antioxidant and antiprotease genes was also significantly attenuated in *Nrf2*-deficient mice, which contributed to the severity of lung injury and elastase-provoked emphysema (67). Transplantation of wild-type bone marrow into *Nrf2*-deficient mice significantly reduced elastase-induced lung injury and delayed the onset of emphysema clearly demonstrating the importance of Nrf2 in protecting against cigarette smoke-related respiratory insults. Further demonstrating this point, we have shown that consumption of broccoli sprout homogenates containing the Nrf2 activating agent sulforaphane (SFN) enhanced NQO1 expression in nasal lavage cells and significantly reduced virus quantity and virus-induced markers of inflammation in healthy smokers (68). Moreover, our data indicate that SFN-induced Nrf2 pathway activation significantly reduces susceptibility to influenza infections by inhibiting viral entry (69). Furthermore, rodent studies demonstrate that SFN-induced Nrf2 pathway activation augments endogenous antioxidant defense responses and improves airway epithelial barrier integrity in HIV-infected animals (70). Taken together, these data demonstrate that Nrf2 status plays an important role in health and disease.

Cinnamaldehyde is known to covalently modify cellular proteins. Indeed, allergic reactions to cinnamaldehyde have been related to its Michael reactivity and the ability to form stable adducts with proteins (71). However, little is known regarding the effects of cinnamaldehyde on cellular GSH levels. Gowder and colleagues exposed rats to cinnamaldehyde by oral gavage for 10, 30, and 90 days, and observed dose-dependent reductions in ascorbic acid, alpha-tocopherol, and GSH in rat kidney tissues (72). Liao et al. exposed a human endothelial cell line to 100μM cinnamaldehyde and observed a rapid reduction in GSH levels followed by a significant increase in GSH levels after 9-hour exposures. This increase was attributed to the induction of Nrf2-mediated glutamylcysteine synthetase gene expression, which suggests a potential temporal component to cinnamaldehyde-induced changes in GSH levels (73). To our knowledge, there are no studies evaluating the effects of cinnamaldehyde on GSH levels in human respiratory tissues. To address this knowledge gap, we exposed Beas-2B cells to various concentrations of cinnamaldehyde for 24 hours and quantified changes in intracellular GSH content. Cinnamaldehyde (0.016, 0.31, 0.63, 0.125, 0.25, and 0.5mM) reduced GSH levels in Beas-2B cells by approximately 25%. While cinnamaldehyde did significantly reduce GSH levels, we did not observe a concentration-dependent effect, which has been reported with aldehyde exposures in rodent studies. Meacher and Menzel exposed adult rat lung type II alveolar cells to various low-molecular-weight aldehydes and observed concentration-dependent reductions in GSH (74). However, this study exposed cells to aldehydes for 20 minutes prior to GSH analysis. Based on the data reported by Liao et al., GSH levels may rapidly decrease and recover following aldehyde exposures (73). Twenty-four-hour exposures in our study may reflect this recovery phase. The data presented here also demonstrate the role for GSH in

buffering the Nrf2 antioxidant pathway against activation by electrophiles. Inhibition of GSH synthesis with BSO augmented cinnamaldehyde-induced activation of the Nrf2 pathway, significantly increasing activation at the 0.00001mM cinnamaldehyde exposure and increasing the maximal response (0.01mM cinnamaldehyde) by approximately 50%. These observations indicate that dysregulation of antioxidant/electrophile defenses, such as depletion of glutathione, enhances the biological activity of cinnamaldehyde and potentially other electrophilic flavoring agents.

There is a growing body of evidence suggesting that many flavoring compounds have nutraceutical value and that ailments stemming from dysregulation of cellular redox homeostasis may be alleviated by the bioactivity of these substances (75-78). Much of the existing literature attributes the potential health benefits of nutraceuticals to the induction of antioxidant genes and upregulation of phase II detoxifying enzymes as a means for eliminating environmental stresses (79, 80). While the vast majority of these studies have been done in the context of oral exposures, the emergence of e-cigarettes and the ubiquitous use of flavoring agents with these devices creates a necessity for understanding the biological effects of inhalational exposures. We have previous shown that cinnamon-flavored e-liquids and cinnamaldehyde inhibited neutrophil, alveolar macrophage, and NK cell innate immune functions in a concentration-dependent manner (29). Moreover, inhibition of immune functions occurred well below cytotoxic concentrations of cinnamaldehyde. More recently, we reported that vaped cinnamon e-liquid aerosol and cinnamaldehyde caused temporary stasis of human bronchial epithelial cell motile cilia by transient suppression of mitochondrial respiration and glycolysis (Chapter 3). The data we report here demonstrate that cinnamaldehyde, guaiacol, and eugenol, common e-cigarette flavoring agents, have a

dose-dependent bimodal effect on Nrf2-pathway activation in bronchial epithelial cells. The bimodal response we observed may present cause for concern as sufficiently high concentrations of these compounds may impair antioxidant responses in vapers and promote oxidative and electrophilic lung damage; pathologic changes commonly observed in smoking-related lung diseases. On the other hand, our data suggest that low concentrations of these flavoring agents stimulate Nrf2 pathway activation, which may have potential therapeutic benefits. However, e-cigarette aerosols have also been shown to contain reactive electrophiles (acrolein, formaldehyde, crotonaldehyde, etc.) metals, and reactive oxygen species, which may disrupt cellular defenses such as free GSH and exacerbate oxidative and electrophilic damage (33, 34, 81, 82). Thus, further work is necessary to determine, in the context of complex e-cigarette aerosols and repeated vaping exposures, how flavoring agents will affect lung health.

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CHAPTER 5.

PERSPECTIVES, FUTURE DIRECTIONS, AND CONCLUDING REMARKS

5.1 Perspectives

5.1.1 Flavorings drive youth initiation on e-cigarettes

The e-cigarette industry has seen tremendous growth over the last decade. E-cigarette sales in the U.S. more than doubled between 2012 and 2013, from $273.6 million to $636.2 million, respectively (1). In 2014, U.S. e-cigarette sales reached approximately $2.5 billion and estimates for 2016 sales are approximately $3.3 billion (2). The rapid expansion of the e-cigarette industry reflects the rise in popularity of these products among teens and adolescents (3). The 2015 National Youth Tobacco Survey reported that 27.1% of middle and high school students had tried e-cigarettes, and 4.3% of middle school students and 11.3% of high school students reported using e-cigarettes within the past 30 days (4). Similar findings have been reported by the Monitoring the Future (MTF) and Youth Risk Behavioral Surveillance (YRBS) cross-sectional surveys (5, 6). While e-cigarette use in this demographic is strikingly high, it remains unclear what, precisely, youth are vaping. Substantial proportions of youth report using non-nicotine electronic cigarettes. The MTF survey found that nearly two-thirds of ever e-cigarette users reported vaping "just flavoring" at last use (5). A recent study by Harrell and colleagues found that at initiation, the majority of Texas school-going youth (98%), Texas young adult college students (95%), and young

adults (71.2%) nationwide said their first e-cigarettes were flavored to taste like something other than tobacco, compared to 44.1% of older adults nationwide (7). Furthermore, the authors report that fruit and candy flavors predominated for all groups; and, for youth, flavors were an especially salient reason to use e-cigarettes. The indication that many youths are using e-cigarettes specifically to inhale flavorings is concerning because 1) many flavoring agents are reactive compounds which have structural similarities to known respiratory irritants and toxicants, 2) there is a dearth of information on the health effects of inhaling reactive food flavoring agents, and 3) there is currently no regulation on the breadth or quantity of flavoring agents used in these products.

5.1.2 The perception of e-cigarette flavoring agents as harmless

The vast majority of flavoring agents used in the manufacture of e-cigarette liquids have been designated as "generally recognized as safe" (GRAS) by the United States Food and Drug Administration (U.S. FDA). With the emergence of e-cigarettes, the applicability of the GRAS designation has often been misconstrued. In 1958, the U.S. FDA established the Food Additives Amendment to the 1938 Food, Drugs, and Cosmetic Act, which stated that any substance intentionally added to food is a food additive and is subject to pre-market approval by U.S. FDA, unless the use of the substance is generally recognized as safe (8). Substances would be exempt from pre-market evaluation as a food additive if there was consensus among a panel of scientific experts (which may be from the food industry) that the substance was generally recognized as safe under the conditions of its intended use. This provision allowed common substances, such as table salt, to be used in food preparation without being required to undergo extensive and costly pre-market evaluation. The caveat to this designation is that it is only applicable to substances added to foods and intended for oral

consumption. This entirely precludes the notion of GRAS substances in e-cigarettes, as inhalation does not fall within the intended use of food additives. Nonetheless, early e-cigarette products were sometimes marketed with the U.S. FDA GRAS logo printed on the bottle or displayed on websites. This misrepresentation/misunderstanding may have contributed to a perception that inhalation of e-cigarette flavorings is harmless (9). The idea that inhalational and oral exposures of the same substance may have inherently different biological effects reflects a fundamental principle of toxicology; the route of exposure for a xenobiotic can influence toxicological responses.

5.1.3 The route of exposure makes the poison

Reactive aldehydes are natural constituents of foods and are commonly used as flavoring or aroma additives to improve the attractiveness of dietary products (10). The most common aldehydes in food are furfural (present in apples, cherries, carrots, potatoes and coffee, which has the highest concentration of 2.34mM [0.025%]), anisaldehyde (present in apricots, cranberries, alcoholic beverages, and anise, which has the highest concentration of 183.6mM [2.5%]), vanillin (present in fruits, asparagus, spices, and vanilla, which has the highest concentration of 152.5mM [2.32%]), trans-2-hexenal (the aroma of bananas, which contain 0.8mM [0.0076%]), and cinnamaldehyde (approximately 90% of cinnamon oil) (11). Cinnamaldehyde is often added to candies and chewing gum at concentrations of approximately 5.0mM [0.07%] and 37.1mM [0.49%], respectively (11). The alimentary canal has evolved substantial defense mechanisms to neutralize, process, and eliminate reactive chemicals, such as dietary aldehydes. This detoxification begins with saliva in the oral cavity. Human saliva contains significant quantities of enzymes, including peroxidase, catalase, super oxide dismutase, glutathione peroxidase, alcohol dehydrogenase, and aldehyde

dehydrogenase (12-14). Additionally, there is an abundance of antioxidants, including GSH and its precursors cysteine, cysteinylglycine and homocysteine, uric acid, and vitamins E and C (14, 15). Aldehyde dehydrogenase 3A1 (ALDH3A1), the predominate aldehyde dehydrogenase in saliva which converts aldehydes to less reactive carboxylic acids, is highly active towards aromatic and long aliphatic aldehydes, including cinnamaldehyde, benzaldehyde, anisaldehyde, vanillin, and 4-hydroxy-2-nonenal (11, 13). ALDH3A1 is considered an essential defense mechanism against food aldehydes as individuals with insufficient ALDH3A1 activity are predisposed to cancers of the oral cavity (11). Aldehydes that persist beyond the oral cavity can be rapidly oxidized by ALDH1A1 isoforms expressed in esophageal, stomach, and intestinal epithelial cells (11). The combination of aldehyde dehydrogenase enzymes and electrophile sinks, such as glutathione, actively protects the alimentary canal from oxidative and electrophilic damage. However, despite these robust defenses, high concentrations of and prolonged exposures to some common food aldehydes are associated with cancers of the gastrointestinal tract (10).

As discussed in Chapter 1, the lungs have evolved effective defense mechanisms, including a protective airway surface liquid milieu, mucociliary clearance, epithelial tight junctions, and resident leukocyte populations, to protect the body against common environmental toxicants. However, it is unclear how well these defenses will withstand new and emerging insults, such as those produced when vaping. The inhalation of reactive aldehyde flavoring agents presents a significant concern based on the known detrimental effects of environmental aldehydes on the respiratory system. This concern is compounded by data reporting that flavoring agents in e-liquids, including cinnamaldehyde, exceed concentrations used in foods and beverages (11, 16, 17).

5.1.4 The known respiratory effects of flavoring agents

Perhaps the most well-known example of a food-safe flavoring causing severe inhalation toxicity is diacetyl-induced lung disease. In May, 2000, eight persons who had formerly worked at a microwave popcorn production facility were reported to the Missouri Department of Health to have bronchiolitis obliterans, an irreversible obstructive lung disease (18). The etiologic agent in all eight cases was determined to be the GRAS butter-flavoring agent diacetyl (2,3-butanedione). Follow-up studies in rats and mice confirmed the inhalation toxicity of diacetyl with sub-chronic exposures causing necrotizing rhinitis, necrotizing laryngitis, and bronchitis, emphysema and hyperemia, and death (19). Electron transfer and the subsequent generation of reactive oxygen species (ROS) has been proposed as a mechanism of diacetyl-mediated respiratory toxicity (20).

Eugenol and cinnamaldehyde have both been identified as potent skin sensitizers in humans, and there are data linking these sensitizers to asthma. A 2015 case report describes a 34-year-old professional cleaner who developed cough, dyspnea, and maculopapular erythema after inhalation and dermal exposures to an industrial cleaning agent containing eugenol (21). Bronchial challenge with eugenol significantly reduced pulmonary function and induced cough and dyspnea. Based on these responses, the patient was subsequently diagnosed with eugenol-induced occupational asthma. A study investigating the health effects of cinnamon dust exposures in 40 Sri Lankan cinnamon workers found that 20 workers (50%) experienced skin irritation, 15 workers (37.5%) had increased cough, and 9 workers (22.5%) were asthmatic (22). The number of asthmatics in this study was reported to be disproportionately higher than other Sri Lankan industries with comparable working conditions and dust exposures. However, more evidence is needed to directly link

inhalational exposures of cinnamaldehyde and the development or exacerbation of asthma. There are also reports of mint flavoring agents causing or exacerbating respiratory conditions. Several case studies describe wheezing, bronchospasm, dyspnea, or asthma exacerbation in individuals following exposures to mint-containing products, including toothpaste, chewing gum, and candies (23-25). These reports indicate that inhalation exposure to common food-flavorings can affect biological functions in the lungs.

5.1.5 Cinnamon e-liquids and cinnamaldehyde suppress respiratory defenses

The focus of this dissertation work was to investigate whether flavored e-liquids and specific flavoring agents alter innate immune functions of the respiratory system as a means to assess the potential health effects of e-cigarette use. Our data indicate that cinnamaldehyde-containing e-liquids suppress neutrophil and alveolar macrophage phagocytosis of *S.aureus* bioparticles, neutrophil extracellular trap formation, natural killer (NK) cell targeted cell killing of leukemia cells (Chapter 2), and human bronchial epithelial (hBE) cell cilia motility (Chapter 3). Furthermore, the e-liquid-induced effects on neutrophil and macrophage phagocytosis, NK cell killing, and cilia motility were recapitulated following exposure to cinnamaldehyde alone suggesting that the e-liquid-induced suppression was driven by cinnamaldehyde. All of the observed effects were dose/concentration dependent and dose-responses varied between cell types. Neutrophils and NK cells were the most susceptible to the effects of e-liquid and cinnamaldehyde exposures, with IC_{50} values for phagocytosis and targeted cell killing of 0.0324mM and 0.0205mM, respectively. The IC_{50} for alveolar macrophage phagocytosis was 0.243mM. Exposure of hBE cells to 10mM and 15mM cinnamaldehyde significantly suppressed cilia motility. Taken together, these data indicate that the components of the respiratory innate defense system

have varying degrees of susceptibility to acute exposures of cinnamaldehyde. These data are meaningful because they suggest that different exposure doses of cinnamaldehyde may elicit different health outcomes. If e-cigarette users receive inhalation exposures to cinnamaldehyde of 20-30μM, neutrophil phagocytosis and NK cell killing functions may be suppressed. This could potentially increase susceptibility to respiratory infections, enhance dissemination of pathogens in the lungs, and promote inflammation. Compromised neutrophil anti-bacterial activity has been proposed as a major cause of increased susceptibility to respiratory infections in smokers (26). However, other innate defense mechanisms, such as macrophage phagocytosis and cilia motility, would likely maintain normal defense functions during exposures to 20-30μM cinnamaldehyde and may effectively compensate for neutrophil and NK cell dysfunction. E-cigarette users receiving cinnamaldehyde exposures at 300μM may lose the phagocytic functions of neutrophils and alveolar macrophages, which would likely pose a greater risk for respiratory infections and inflammation. Additionally, our data indicate that cinnamaldehyde exposures greater than 0.1mM significantly suppress Nrf2 pathway activation and reduced GSH levels in hBE cells. The loss of these antioxidant responses could exacerbate oxidative damage resulting from normal cellular processes or infections and significantly enhance inflammation in the lungs. Exposures sufficient to impair cilia motility (≥10mM cinnamaldehyde) would likely have severe effects on respiratory health as impaired mucociliary clearance in smokers is associated with increased occurrence of respiratory infections and obstructive lung diseases (27, 28). Moreover, we observed that 10mM cinnamaldehyde was highly cytotoxic to immune cells. Exposures at this concentration could lead to an abundance of necrotic immune cells releasing toxic enzymes into the airways. Cigarette smoke-induced neutrophil

necrosis- mediated release of proteolytic enzymes, particularly elastase and matrix metalloproteinase, cause significant damage to host tissue and are believed to be key mediators of destructive lung diseases (29). Our *in vitro* data suggests that e-cigarette users inhaling sufficiently high concentrations of cinnamaldehyde may have compromised innate defense responses, which could promote respiratory infections and increase oxidative stress in the lungs.

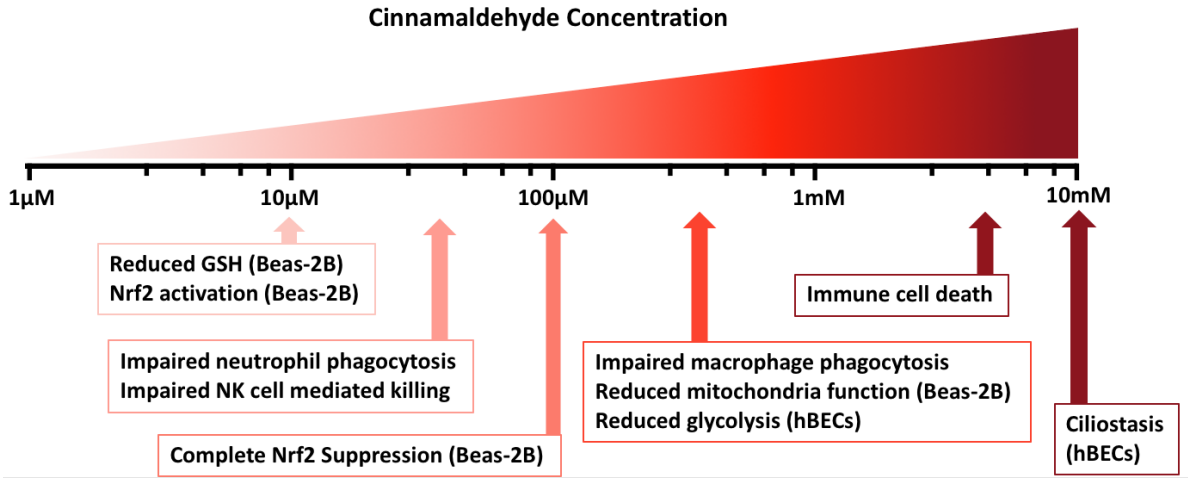

Figure 5.1 Summary of the cinnamaldehyde-induced effects reported in Chapters 2, 3, and 4.

5.1.6 Potential concerns for cinnamaldehyde exposures and cancer

Much of the existing literature on cinnamaldehyde has focused on its potential use as a chemotherapeutic agent. For example, cinnamaldehyde has been reported to induce *in vitro* and *in vivo* melanoma cell death through the inhibition of NF-κB and AP-1 (30). Additionally, human cervical cancer cells (SiHa) treated with cinnamaldehyde exhibited reduced proliferation and migration, down regulation of Her-2 expression, and increased apoptosis which was mediated by elevations in intracellular calcium levels (31). Imai and

colleagues observed that mice fed a diet containing 5000 ppm [37.8mM] cinnamaldehyde daily for 26 weeks had significantly lower rates of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-induced lung tumors as compared to mice without cinnamaldehyde (32). More recently, polyphenolic compounds constructed on a cinnamaldehyde scaffold were shown to cause cell cycle arrest at the G2/M phase and induce apoptosis in cisplatin-resistant A2780/Cis human ovarian cancer cells (33). All of these, as well as other studies, suggest that cinnamaldehyde may have potential as a chemotherapeutic agent or work synergistically with other chemotherapeutic agents. However, our observation that cinnamaldehyde, at low micro molar concentrations, impairs NK cell function raises concern that e-cigarette users may have impaired surveillance and elimination of tumorigenic cells. NK cell activity is impaired in smokers and NK cell cytotoxic and secretory functions are commonly disrupted in lung cancer patients (34-36). Additionally, our observation that low micro molar concentrations of cinnamaldehyde increase hBE cell Nrf2 pathway activation may have implications for cancer. While Nrf2 pathway activation is a mechanism to restore cellular redox homeostasis, aberrant activation of Nrf2 is a common feature of non-small cell lung cancers (37). The Cancer Genome Atlas (TCGA) indicates that approximately 1/3 of squamous cell lung cancers display Keap1/Nrf2/Cullin3 pathway alterations (38). Hu et al. examined Nrf2 sequences of 103 patients with non-small cell lung cancer and found that the Nrf2 mutation rate in ever-smokers was significantly higher than that in never-smokers (39). In accordance with Hu, Sasaki et al. sequenced Nrf2 in 262 surgically resected lung tumors and confirmed that Nrf2 activating mutations were significantly more common in smokers and squamous lung cancers (40). Other studies have shown that biallelic inactivation of *Keap1* is a common event in non-small cell lung cancers (41). It has been suggested that

aberrant Nrf2 activation in lung cancer leads to increased expression of drug efflux pumps, thereby providing a mechanism for resistance to platinum-based chemotherapy agents (41, 42). In support of this idea, a recent study showed that siRNA-mediated inhibition of Nrf2 augmented carboplatin-induced tumor growth inhibition in a non-small cell lung cancer xenograft mouse model (42). Taken together, the inhibition of NK cell functions and enhanced Nrf2 pathway activation achieved with low micro molar concentrations of cinnamaldehyde, may be physiologic changes that promote the development or progression of lung cancer in e-cigarette users. This may be particularly important for long-term smokers with dysplastic changes or early stage cancer who transition to e-cigarettes, as impaired NK cell functions and enhanced Nrf2 pathway activation may accelerate tumor growth. Additional sub-chronic and chronic cinnamaldehyde inhalation studies in animals would be useful to assess whether 1) the *in vitro* findings of cinnamaldehyde-induced NK cell dysfunction and Nrf2 pathway activation occur *in vivo*, 2) whether low dose exposures increase the rates of non-small cell lung cancers, and 3) whether cinnamaldehyde exposure accelerates cancer progression resulting from exposures to other carcinogens, such as those in cigarette smoke.

5.1.7 Cinnamaldehyde-induced suppression of bioenergetic functions

One of the most interesting findings we report here is that cinnamaldehyde elicits dose-dependent reductions in hBE cell mitochondrial respiration and glycolytic activity, as determined by reductions in the cellular oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). We validated this observation by exposing hBE cells to 10mM cinnamaldehyde and quantifying ATP levels 15 minutes, 120 minutes, and 24 hours after exposure. The addition of 10mM cinnamaldehyde caused a significant reduction in ATP

levels 15 minutes post exposure. However, ATP levels recovered by 120 minutes and reached baseline levels by 24 hours. These data demonstrate that cinnamaldehyde-induced dysfunction of bioenergetic pathways in hBE cells significantly reduces ATP, but only temporarily. In the context hBE cell defense functions, we show that this reduction in ATP causes transient ciliostasis, which may disrupt mucociliary clearance and increase the risk of infections in respiratory tissues. However, cinnamaldehyde-induced disruption of bioenergetic functions may have broad implications involving other cellular processes and various cell types. Countless cellular processes are dependent upon the hydrolysis of ATP. ATPase enzymes convert ATP to ADP and phosphate, releasing energy which is used to facilitate chemical reactions which otherwise would not occur. Transmembrane ATPases facilitate the transport of solutes into and out of the cell. Molecular motor proteins, including cytoskeletal motors (myosins, kinesins, dyneins), nucleic acid motors (helicases and topoisomerases), polymerization motors (actin ATPase), and rotary motors (F_oF_1-ATP synthase), utilize ATP hydrolysis to facilitate molecular movement. Reduction in ATP below threshold activation levels would disrupt the function of these proteins and disrupt normal cellular activity.

Phosgene (carbonyl chloride, $COCl_2$) is a toxic gas which forms acyl linkages with amino, hydroxyl and sulfhydryl groups on proteins (43). Phosgene-protein modifications lead to inhibition of enzymes involved in energy metabolism, reduced cellular ATP levels, loss of alveolar epithelial barrier integrity, and significant pulmonary edema (43). The pulmonary edema associated with phosgene exposure is believed to result from insufficient ATP to power Na^+/K^+-ATPases (which typically account for approximately 40% of cellular ATP consumption) (44, 45). Additionally, phosgene exposure dysregulates actin polymerization

and disrupts epithelial tight junctions, which may result from insufficient ATP (46, 47). The acute effects on tight junction integrity have been quantified by rapid reductions in transepithelial electrical resistance (TEER) (48). Based on this information, it is plausible that cinnamaldehyde-induced reductions in hBE cell ATP levels may have similar effects on Na^+/K^+-ATPase functions and reduce epithelial barrier integrity in the airways of e-cigarette users. Indeed, we have preliminary data that indicate a rapid reduction in well-differentiated hBE cell TEER and disruption of tight junction (zonula occludens-1; ZO-1) protein expression following acute exposure to cinnamaldehyde (data not shown). Additional investigation of cinnamaldehyde effects on Na^+/K^+-ATPase activity would also be useful.

In Chapter 2, we report that cinnamaldehyde impairs respiratory immune cell phagocytosis and reduces NK cell killing of transformed cells. However, potential mechanisms underlying this dysregulation were not identified. Based on the observation of cinnamaldehyde-induced dysregulation of bioenergetic functions, it is possible that reductions in intracellular ATP played a role in the altered immune cell functions. Recent studies have shown that stimulation of neutrophil formyl peptide receptors (FPRs) increases mitochondrial membrane potential and triggers a rapid burst of ATP to be released from neutrophils (49, 50). ATP-mediated autocrine activation of purinergic receptors on neutrophil membranes boosts Ca^{2+} signaling, amplifies mitochondrial ATP production, and initiates functional neutrophil responses (49). Disruption of this process by depletion of ATP or impaired mitochondrial function could prevent neutrophil activation and phagocytosis of pathogens. Macrophages are also reported to utilize ATP-purinergic receptor signaling for subsequent activation and ablation of ATP may cause a similar inhibition of function (51). Additionally, hypoxia-induced depletion of ATP in alveolar macrophages significantly

reduced phagocytosis of glutaraldehyde-fixed red blood cells, demonstrating a clear link between available ATP and phagocytic capacity (52). NK cells require the ATP-dependent molecular motor myosin II for lytic granule fusion with the cell membrane and subsequent killing of target cells (53). Reduced intracellular ATP levels could impair myosin activity and limit NK cell killing ability. Furthermore, ATP depletion has been reported to cause cytoskeletal derangements (54). Neutrophils, alveolar macrophages, and NK cells require tightly-controlled regulation of cytoskeletal rearrangement to effectively carry out their immune functions. Further work is needed to determine if cinnamaldehyde exposures reduce intracellular ATP levels in immune cells similar to the levels observed in hBE cells.

5.2 Future directions

While the work presented here adds to the general knowledge of how a common aldehyde flavoring agent, in the context of e-cigarette use, may affect respiratory innate defense responses, it has also highlighted significant knowledge gaps and potential avenues for future research. Some of these future directions will be discussed the following section.

5.2.1 E-cigarette aerosol deposition in the lung

We, and others, have shown that some chemical constituents of e-cigarettes have significant *in vitro* and *in vivo* toxicities. However, it is still unclear whether e-cigarette users will reach exposure doses resulting in toxicity. Dosimetry models can be used to predict doses of inhaled material; however, these models require several parameters including particle size distribution. Aerosols generated by e-cigarettes are concentrated, chemically complex, and temporally dynamic as they contain semi-volatile compounds which change in particle size with dilution (55). These characteristics have led to considerable variability in

reports of particle size distribution (55-60). Moreover, many of the studies investigating e-cigarette particle size distribution have used disposable first-generation "cig-a-likes", which does not reflect the devices typically used by adolescent and young adult e-cigarette users. Moving forward, it will be essential to understand the aerosol characteristics of third-generation e-cigarette devices and how different vaping temperatures affect particle size distribution. This information, when incorporated with real-world puff topography and use frequency, will be extremely useful for establishing dose exposures for chemical constituents in e-cigarettes and accurately integrating our *in vitro* findings into risk assessments for e-cigarette use.

5.2.2 Cinnamaldehyde and mucociliary clearance in healthy e-cigarette users

The data presented in Chapter 3 clearly indicate that cinnamon-flavored e-liquids, cinnamon e-liquid aerosols, and cinnamaldehyde alone have the capacity to rapidly induce hBE cell ciliostasis. This observation is striking and may have serious health implications if response translates to impaired mucociliary clearance in e-cigarette users. *In vivo* mucociliary clearance rates can be quantified in humans assuming that a labeled and inhaled marker deposited on the airway surface is cleared from the lung at the same rate as the airway secretions in which it is immersed. The most common technique for measuring mucociliary clearance *in vivo* is to use inhaled, radiolabeled Technetium-99m sulfur colloid (Tc99m-SC) particles that, upon deposition in the airways, can be tracked by gamma camera (gamma scintigraphy) to determine their rate of egress (61). Immediately following controlled inhalation of Tc99m-SC particles, an initial deposition scan is recorded followed by imaging with gamma camera at regular intervals to monitor the egress of Tc99m-SC particles from the lung. The temporal analysis of particle egress allows for the calculation of particle

retention as a percent or fraction of the initial deposition. Using this approach, healthy e-cigarette users could vape cinnamaldehyde-containing e-liquids prior to inhalation of Tc99m-SC particles, and the effects of cinnamaldehyde on mucociliary clearance could be quantified. This translational approach would allow a direct comparison between our *in vitro* data and real-word exposures in healthy e-cigarette users.

5.2.3 Spontaneous recovery of cilia motility and the role of ALDH

An interesting phenomenon described in Chapter 3 is the spontaneous recovery of cilia motility without the removal of cinnamaldehyde. We are investigating underlying mechanisms of this recovery; however, it is currently unclear what, precisely, is driving this response. Previous work on the dermatologic and toxicologic assessment of cinnamaldehyde indicates that cinnamaldehyde is rapidly metabolized to cinnamic acid (62). Studies in rodents show that cinnamic acid undergoes β-oxidation to form benzoic acid, which then undergoes glucuronidation reactions to form glycine or glucuronic acid conjugates, which are eliminated in the urine (62). Very little has been reported about the enzymatic oxidation of cinnamaldehyde. Wang and colleagues report that cytosolic aldehyde dehydrogenase 1 (ALDH1) and mitochondrial aldehyde dehydrogenase 2 (ALDH2) purified from human liver effectively detoxify cinnamaldehyde *in vitro* (63). More recent work from Solobodowska identified ALDH1A1 and ALDH3A1 as effective enzymes for eliminating cinnamaldehyde; however, ALDH1A1 was significantly more effective (a K_M value 35 times lower than ALDH3A1). While we did not quantify the conversion of cinnamaldehyde to cinnamic acid or other metabolites, it is plausible that the recovery of mitochondrial function and cilia motility rely on the metabolism of cinnamaldehyde. Additionally, our finding reported in Chapter 4 indicate that cinnamaldehyde reacts with GSH and other studies have suggested

that other cysteine-rich proteins may act as "electrophile sinks" (64). Hence, the rapid metabolism or sequestration of cinnamaldehyde could be among the underlying mechanisms of the rapid reversal of the ciliary stasis and decreases in ATP synthesis.

Based on these data, future studies investigating the mechanisms of cinnamaldehyde metabolism in hBE cells and the role of ALDH enzymes would be useful. Furthermore, if future studies indicate that ALDH enzymes play an important role in hBE cell metabolism and the protection/restoration of ciliary function, it would be extremely useful to investigate whether populations with frequently-occurring ALDH inactivation mutations (e.g., Asians and Native Americans) have enhanced susceptibility to the effects of cinnamaldehyde and other aldehyde e-cigarette flavoring agents (65, 66).

5.2.4 The inclusion of aldehyde flavorings in e-cigarette carbonyl quantification

Many investigators have attempted to make head-to-head comparisons of the quantities of toxic aldehydes in cigarette smoke versus aerosols generated when vaping. In a 2014 study by Goniewics and colleagues, aerosols generated from 12 "cig-a-like" type e-cigarettes were evaluated for the presence of toxic compounds found in cigarette smoke (67). Formaldehyde, acetaldehyde, acrolein, and o-methylbenzaldehyde were present in the e-cigarette aerosol. However, concentrations of these carbonyls ranged between 9 and 450 times lower than the concentrations in an equivalent volume of cigarette smoke. Similarly, work by Uchiyama et al. reported the carbonyl content of aerosols generated from 13 Japanese "cig-a-like" type e-cigarettes (68). Formaldehyde, acetaldehyde, acrolein, propanal, glyoxal, and methylglyoxal were generated by 9 of the e-cigarettes tested at concentrations of 140, 120, 40, 46, 23, and 21μg/10 puffs, respectively. In a recent letter to the New England

Journal of Medicine, Jensen and colleagues reported that a 2nd generation "tank-style" e-cigarette with a 5.0V output setting produced 380±90µg of formaldehyde in 10 puffs; concentrations much higher than previous studies and exceeding formaldehyde levels of traditional cigarettes (69). However, this study was criticized for using power levels that likely resulted in "dry-puff" conditions, which confound the finding. More recently, a study by Sleiman et al. reported extremely high aldehyde emissions from a 2nd generation e-cigarette device equipped with a dual-coil atomizer (70). The authors quantified formaldehyde, acetaldehyde, and acrolein at 48200µg, 19080µg, and 10060µg per gram of vaped e-liquid, respectively. These levels far exceed carbonyl levels found in cigarette smoke. The average formaldehyde content of a single cigarette under intense smoking conditions is reported to be 74µg (71). Based on this value, the concentration of formaldehyde measured in one gram of vaped e-liquid in the Sleiman study would equate to that of approximately 650 cigarettes. Interestingly, a recent replication study of Sleiman's work by Farsalinos and colleagues reported significantly lower quantities of formaldehyde, acetaldehyde, and acrolein using the same e-liquid and device used by Sleiman. Farsalinos quantified formaldehyde, acetaldehyde, and acrolein at 4259.6µg, 2156.2µg, and 623.6µg per gram of vaped e-liquid, respectively. The reductions in carbonyl content suggest that the prior measurements by Sleiman may have been due to "dry-puff" conditions. While the concentrations reported by Farsalinos are significantly lower than those reported by Sleiman, 4259.6µg of formaldehyde in 1 gram of vaped e-liquid equates to the amount of formaldehyde generated by approximately 57 cigarettes; an exposure concentration that is still cause for concern.

While the studies described here make efforts to compare and contrast the reactive aldehydes in traditional cigarettes and e-cigarettes, the contribution of flavoring agents to the total aldehyde is not considered. The Sinicide e-liquid in our studies contained approximately 1.14M cinnamaldehyde (Chapter 2). Based on this concentration, vaping 1ml of this e-liquid (5 to 20 5-second puffs depending on the device settings) would expose the user to 150.66mg of cinnamaldehyde. A study by Fujioka et al. found that the total carbonyl content recovered from regular size cigarettes ranged from 1.92mg per cigarette to 3.14mg per cigarette (72). This would indicate that the cinnamaldehyde content alone in 1ml of Sinicide e-liquid is 78.5% to 48.0% of the total content of a typical cigarette. Depending on the other carbonyls present in Sinicide aerosols (i.e. other flavorings, thermal degradation products of PG and VG, etc.), the total carbonyl content of 1ml of this e-liquid could be comparable to or greatly exceed the carbonyl content of a traditional cigarette. Based on our studies, cinnamaldehyde can directly affect cellular functions, suppress antioxidant responses, and deplete GSH levels. These data provide a clear indication that cinnamaldehyde (and likely other carbonyl flavoring agents) should be included in future studies assessing the carbonyl content of e-cigarette aerosols and e-liquids.

5.2.5 Quantifying cinnamaldehyde-protein adducts in cells and e-cigarette users

The data here indicate that cinnamaldehyde has concentration-dependent effects on various cell types and critical cell processes. However, we did not investigate specific molecular drivers of these responses. The predominant mechanism by which α,β-unsaturated aldehydes disrupt cellular functions is via protein modification. Nucleophilic additions can occur at either the carbonyl group (1,2-addition) or β-carbon (1,4-addition). Addition at the β-carbon (i.e., Michael addition) results in the formation of a stable, covalent modification.

Weak bases, including alcohols, thiols, primary amines, and secondary amines can readily form Michael adducts with α,β-unsaturated aldehydes (73, 74). Nucleophilic addition at the carbonyl group (Schiff base formation) are less stable than Michael additions and believed to occur less frequently during aldehyde-protein interactions (75). A logical next step for our work is to investigate cinnamaldehyde-protein adducts in order to identify underlying molecular drivers of cellular dysregulation. To this end, hBE cells or respiratory immune cells could be exposed to cinnamaldehyde and cell lysates collected for adduct proteomic analysis by high-performance liquid chromatography (HPLC)-tandem mass spectrometry (MS/MS). This would provide an effective approach for, 1) identifying adducted proteins, and 2) determining which amino acid is adducted (i.e. whether the adduction is a Michael addition or a Schiff base). To expand this work further, nasal-scape biopsies of the inferior nasal turbinate epithelium could be collected from non-smokers and healthy e-cigarette users and analyzed for aldehyde-protein adducts using this HPLC-MS/MS approach.

5.3 Concluding remarks

In summary, the data presented in this dissertation significantly add to our knowledge of how common food-safe flavoring agents, in the context of e-cigarette use, can affect respiratory innate defense responses. Specifically, we demonstrate that the α,β-unsaturated aldehyde flavoring agent cinnamaldehyde, at sufficient exposure doses, can impair respiratory innate immune cell functions, suppress airway cilia motility by dysregulation of mitochondrial respiration and glycolytic function, and modify airway epithelial cell redox homeostasis by depletion of glutathione (GSH) and dose-dependent modulation of Nrf2 antioxidant pathway activation. These findings are cause for concern as electrophilic flavoring agents, such as cinnamaldehyde, are commonly used to flavor e-cigarettes and there

is currently no regulation governing the quantities used in these products. E-cigarettes may have the potential to reduce cigarette consumption and improve smoking-related morbidity and mortality rates. However, as we stated in Chapter 1, the net effect of e-cigarettes on public health, whether harm or benefit, depends on: 1) their effect on youth transition to tobacco smoking, 2) their effect on adult smoking cessation, and 3) their intrinsic toxicity. Our work, as well as the work of others, clearly indicates that flavoring additives, at concentrations observed in e-liquids, can significantly disrupt normal cell physiology in ways that may have implications for the development and exacerbation of respiratory disease. As the high concentrations of flavorings in e-liquids likely contribute to the intrinsic toxicity of e-cigarettes, the quantities of flavoring agents used in these products should be regulated. To this end, the information provided here may be particularly useful in the development of health-protective policies aimed at reducing or eliminating harmful and potentially harmful chemicals from e-cigarettes.

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