Understanding potential mechanisms of harm

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REVIEW

Electronic Cigarettes: Not All Good News?

Understanding potential mechanisms of harm: the drivers of electronic cigarette-induced changes in alveolar macrophages, neutrophils, and lung epithelial cells

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Abstract

Electronic (e-) cigarettes are growing in popularity despite uncertainties regarding their long-term health implications. The link between cigarette smoking and initiation of chronic lung disease took decades to unpick so in vitro studies mimicking e-cigarette exposure aim to detect early indicators of harm. In response to e-cigarette exposure, alveolar macrophages adopt a proinflammatory phenotype of increased secretion of proinflammatory cytokines, reduction in phagocytosis, and efferocytosis and reactive oxygen species generation. These effects are largely driven by free radical exposure, changes in PI3K/Akt signaling pathways, nicotine-induced reduction in phagocytosis receptors, and impaired lipid homeostasis leading to a foam-like lipid-laden phenotype. Neutrophils exhibit disrupted chemotaxis and transmigration to chemokines, reduced phagocytosis and bacterial killing, and an increase in protease secretion without corresponding antiproteases in response to e-cigarette exposure. This is driven by an altered ability to respond and to polarize toward chemoattractants, an activation of the p38 MAPK signaling pathway and inability to assemble NADPH oxidase. E-cigarettes induce lung epithelial cells to display decreased ciliary beat frequency and ion channel conductance as well as changes in chemokine secretion and surface protein expression. Changes in gene expression, mitochondrial function, and signaling pathways have been demonstrated in lung epithelial cells to explain these changes. Many functional outputs of alveolar macrophages, neutrophils, and lung epithelial cells have not been fully explored in the context of e-cigarette exposure and the underlying driving mechanisms are poorly understood. This review discusses current evidence surrounding the effects of e-cigarettes on alveolar macrophages, neutrophils, and lung epithelial cells with particular focus on the cellular mechanisms of change.

alveolar macrophages; e-cigarettes; lung epithelial cells; mechanisms; neutrophils

INTRODUCTION

Electronic (e-) cigarettes, also known as electronic nicotine delivery systems (ENDS), are noncombustible nicotine delivery devices, which were developed in 2003 as an alternative to tobacco cigarettes. Despite origins as a smoking cessation tool, only 41% of e-cigarette users have quitting or harm reducing intentions (1). Furthermore, 80% of those who switch from cigarettes to e-cigarettes continue to use the device 1 yr after switching (2). Therefore, it is important that e-cigarettes are not considered only as short-term therapy. E-cigarettes serve to heat e-cigarette liquids (e-liquids), delivering a vapor to the lungs at subcombustion temperatures. E-liquids usually contain nicotine, a variety of flavoring compounds with over 7,700 currently described (3), humectants, propylene glycol (PG), and vegetable glycerin (VG). Production of sweet and fruity e-liquid flavors and exposure to advertising have increased the uptake of e-cigarette use by nonsmoker adolescents who are consequently 3.5 times more likely to initiate cigarette smoking, supporting the gateway to smoking hypothesis (4). The e-cigarette market is now worth an estimated $22.6 billion, supported by over 3.6 million e-cigarette users in the United Kingdom (1, 5–8) and over 40 million e-cigarette users worldwide (9).

The emergence of e-cigarette- or vaping-associated lung injury (EVALI) in the United States, acute severe respiratory distress with an absence of infection in otherwise healthy adults, has highlighted the potential dangers of e-cigarette use. Clinical presentation of EVALI includes dyspnea, fever, and tachycardia with bilateral ground glass opacities on computed tomography (CT). Of 81 patients with EVALI, 89% reported having used tetrahydrocannabinol (THC) and 9% used cannabidiol (CBD) e-liquids in the previous 90 days before symptom onset, raising further concerns around e-

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liquid components (10). Vitamin E acetate has been strongly linked with EVALI cases, particularly those where THC use has been reported (11). Vitamin E acetate is a supplement used to thicken some e-liquids and is considered safe for ingestion and skin application (12). However, upon inhalation, vitamin E acetate is delivered directly to the surfactant lining of the epithelium, interfering with the chemical properties and contributing to respiratory dysfunction in EVALI (11, 13, 14). The discovery of the dangers of vitamin E acetate raises further concern about e-liquid ingredients and the potential toxicity caused by vaporization. Since August 2019, over 2,800 cases of EVALI have been confirmed by the Centers for Disease Control and Prevention (CDC) with 68 confirmed deaths in the United States and at least 2 in the United Kingdom (15, 16).

Smoking cessation is a priority to reduce ill-health. Cigarette smoking is associated with initiating and worsening pathology in almost every chronic inflammatory lung disease (17) as well as causing a number of cancers, cardiovascular diseases, and strokes. Most notably, cigarette smoke is a causative factor in chronic obstructive pulmonary disease (COPD) (18). COPD is characterized by postbronchodilator forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) ratio of less than 0.70, symptoms including shortness of breath, chronic cough, sputum production, or wheezing and a history of smoking cigarettes or exposure to other noxious stimuli (19). COPD is associated with chronic lung inflammation resulting in progressively worsening and irreversible airflow obstruction driven by small airways destruction, emphysema, and chronic bronchitis (20). Risk of development of smoking-associated COPD is heightened by α-1 antitrypsin deficiency, the genetic condition resulting in the absence or dysfunction of the antiproteinase α-1 antitrypsin that normally protects the lungs from destruction by proteinases such as neutrophil elastase (NE) (21). Neutrophils are a central contributor to the chronic inflammation associated with COPD, as their recruitment, accumulation, and degranulation drives tissue damage, and their dysregulation in stable disease contributes to increased inflammation and impaired bacterial clearance (22). Pathophysiology is further driven by alveolar macrophages adopting a proinflammatory state, whereby secretion of inflammatory chemokines, interleukin (IL)-8 and monocyte chemoattractant protein-1 (MCP-1) recruits further immune cells to the small airways (23). Hyperplasia of airway epithelial cells contributes to tissue remodeling, which drives progression of airflow obstruction due to the stiffening of the airways and lung parenchyma (24). However, the link between these pathological drivers of COPD and smoking took decades to uncover, in part due to the slowly progressive nature of cigarette-associated lung damage, with often a lag of over three decades between smoking initiation and the development of characteristic airflow obstruction (25). This picture is further complicated by individual variance such as smoking behaviors, occupational and environment exposures, and genetic factors. This "slow-burning" disease is complex to model in cell, tissue, or animal-based models, where a short duration of smoke exposure is used. The same challenges are faced by researchers attempting to understand the signals of harm of e-cigarettes, as a similar disease progression may need to be considered.

To determine whether and what the long-term harm of e-cigarettes might be, models to mimic the exposure conditions in vitro and in vivo have been developed by researchers yet lack of standardization in experimental methods has resulted in differing experimental methods (26). E-cigarette vapor extract systems rely on vapor constituents quickly dissolving into media with high degrees of dilution and loss of some insoluble elements. E-cigarette vapor extract is best for direct comparison with cigarette smoke extract, which has been used in cigarette studies. E-cigarette vapor condensate is generated by vaporizing and condensing e-liquids, which is then diluted with media and used to treat cells. This represents the physiological behavior of e-cigarette vapor, vaporizing in the e-cigarette and condensing in the airway, more accurately than e-cigarette vapor extract treatment (27). Both e-cigarette vapor extract and condensate exposure models account for chemical changes induced by vaporization, preferable compared with unvaporized liquid treatment. The models mentioned thus far do not simulate direct vapor exposure; however, in the air-liquid interface model, cells are cultured on membranes and e-cigarette vapor is drawn over the apical surface of the cells. Each exposure model has advantages and limitations, and various methods have been used to quantify level of exposure including measurements of particulate deposition by optical density and nicotine quantification by mass spectrometry. In addition to variations in exposure model is the further complication of variations in devices, device settings, and e-liquids. The first devices to be invented, first-generation or cig-a-like devices, were disposable, non-refillable, and looked like traditional cigarettes. Later came the more advanced pen-like second-generation devices, with refillable tank and rechargeable battery. Second-generation devices rapidly overtook first generation due to the cost-effectiveness of a refillable and rechargeable device. However, development of larger volume tanks and high-power batteries formed the basis of third-generation devices. Most recently, the fourth generation of e-cigarettes allows user modification of temperature, power output, and airflow settings. In addition, these devices have larger tanks, longer battery life, and more customizable features than ever. Studies have shown that higher battery outputs lead to increased carbonyl levels in vapor (28, 29). The ability to customize settings, along with the huge variety of e-liquids—from flavors to nicotine concentrations)—allows the user to adapt their device to suit their preferred vaping habits. However, this poses its own challenges in assessing effects of vaping on health as the combination of device, settings, and e-liquid differ from user to user.

Given the primary cells implicated in chronic smoking-related lung disease are lung epithelial cells, alveolar macrophages, and neutrophils, this review will assess evidence of cellular dysfunction for these cell types in response to e-cigarette exposure, summarized in Fig. 1.

ALVEOLAR MACROPHAGES

Alveolar macrophages are specialized resident innate immune cells residing in the lung alveoli and proximal airways. Existing in alveolar spaces, alveolar macrophages constantly encounter and clear an array of inhaled toxins,

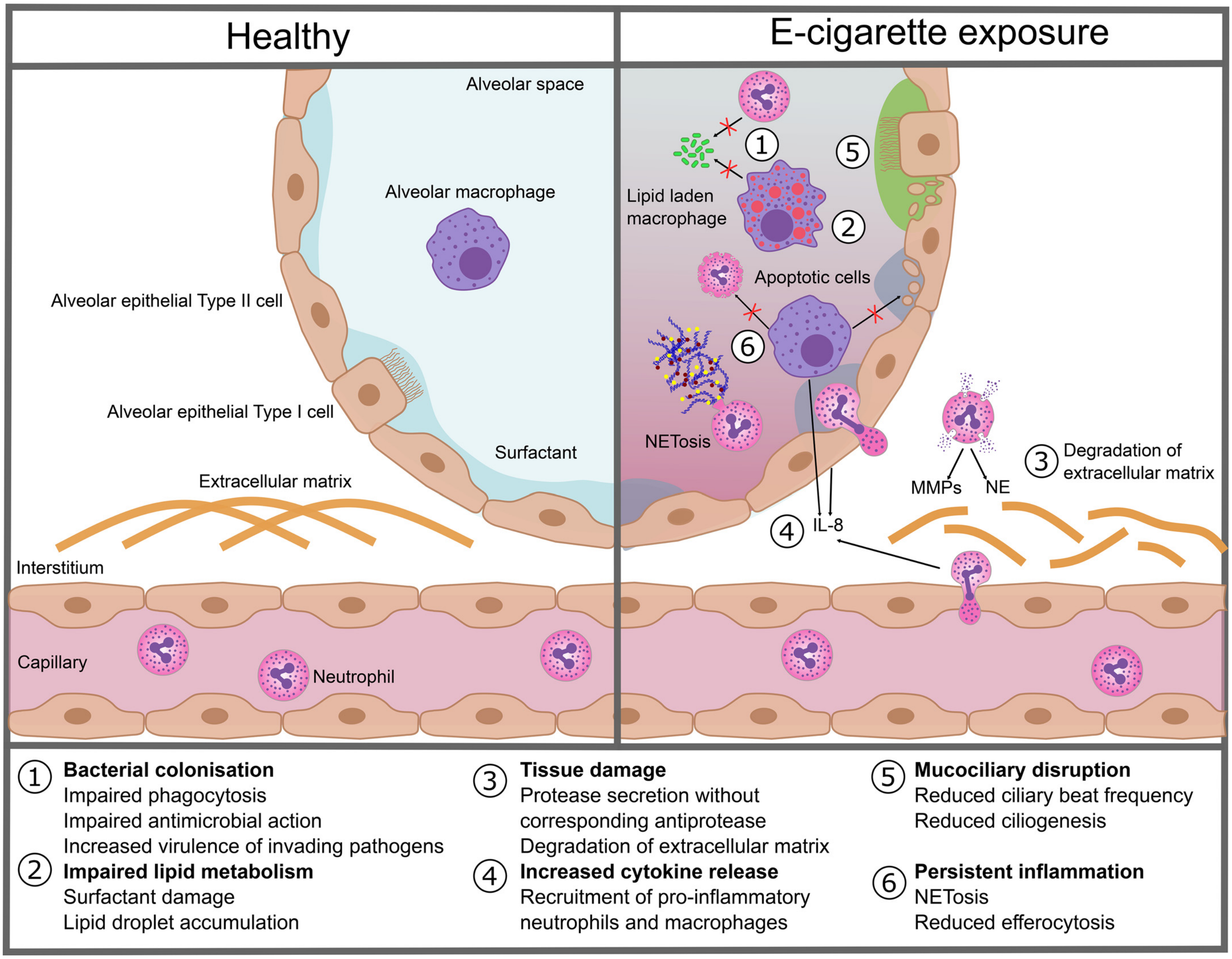

Figure 1. A summary of the effects of e-cigarette vapor exposure on lung homeostasis driven by alterations in important lung cells. In response to e-cigarette exposure, functional changes in a range of cell type and subsequent dysfunctional interactions lead to changes characteristic of oxidative stress: *1*) bacterial colonization is driven by impaired phagocytosis and bacterial killing by alveolar macrophages and neutrophils, exacerbated by evidence of increased virulence by invading pathogens; *2*) impaired lipid metabolism by alveolar macrophages and alveolar type II epithelial cells leads to damaged surfactant and the formation of lipid-laden macrophages that exhibit abnormal processing of both endogenous and exogenous lipids; *3*) tissue damage to the interstitium and epithelial cell layer is caused by recruitment of neutrophils and their subsequent degranulation of proteases such as matrix metalloproteases (MMPs) and neutrophil elastase (NE) without a corresponding increase in antiproteases such as α 1 antitrypsin, leading to degradation of the extracellular matrix; *4*) increased cytokine release by alveolar macrophages and epithelial cells recruits proinflammatory neutrophils and monocytes to the airspaces, causing tissue destruction as they transmigrate; *5*) mucociliary disruption causes accumulation of mucus due to reduced ciliogenesis and ciliary beat frequency; *6*) persistent inflammation driven by reduced efferocytosis by alveolar macrophages, subsequent accumulation of apoptotic cells and neutrophil NETosis. These substantial changes are driven by functional changes in neutrophils, alveolar macrophages, and epithelial cells as a result of e-cigarette exposure. As a result, the alveolar spaces become inflamed and changes, some irreversible, occur, which can lead to the development of a range of respiratory diseases including bronchiectasis, chronic obstructive pulmonary disease, and emphysema. IL-8, interleukin 8; NET, neutrophil extracellular trap.

microbes, and antigens, assessing necessity for inflammatory responses and maintaining tissue homeostasis (30). Disruptions in the function of alveolar macrophages can contribute to the pathogenesis of lung disease such as COPD and enhance susceptibility to infection such as pneumonia (31, 32). Cigarette smoke exposure causes a proinflammatory alveolar macrophage phenotype as well as aberrant autophagy, impaired phagocytosis, and efferocytosis-driving inflammation in the lungs and reducing the clearance of infections and cell debris (33–35).

Alveolar Macrophages and E-cigarettes

Table 1 details the experimental methods and downstream effects of e-cigarette exposure to primary alveolar macrophages or cell line-derived macrophages. In an e-cigarette vapor condensate model, significant cell toxicities have been shown, which are independent of, but worsened by, the presence of nicotine; yet in an e-cigarette vapor extract model there was no significant cytotoxicity (36, 37). This may be because e-cigarette vapor condensate captures more of the

Table 1. *A summary of the effects of e-cigarette vapor exposure on alveolar macrophage effector functions*

Effector Function	E-Cigarette Model	ECL Nicotine Concentration	Exposure Model	Exposure Duration	Cell Type	Results	Reference
Viability	Second generation (Kanger Ltd)	36 mg/mL and 0 mg/mL	ECVC (0.8% concentration)	24 h	Human alveolar macrophages	36 mg/mL nicotine: 40.87%, 0 mg/mL nicotine: 77.87% live cells vs. 92.5% live cells in UTC	(36)
	QHIT e-cigarette (Puff, Moncalleri, Italy) 3.7 V	8 mg/g and 0 mg/g	100% ECVE (40 × 60 mL puff, 3 s, 30 s break)	0, 3, 6 days	THP-1 cell line	No change in toxicity with or without nicotine	(37)
Reactive oxygen species	Second generation (Kanger Ltd)	36 mg/mL	ECVC (0.5% concentration)	4 h	Human alveolar macrophages	50-fold increase in ROS production vs. UTC	(36)
Phagocytosis/antimicrobial activity	Vape pen (Green smart living/ Njoy/Xtreme Vaping)	24 mg/mL and 8 mg/mL	ECVE [0.2–0.26 mg/mL nicotine (1.2–1.6 mM)]	1 h	Human alveolar macrophages	24 mg/mL nicotine: 535%, 8 mg/mL nicotine: 395% increase in MRSA population size vs. UTC	(38)
	LAVABOX DNA 200 Box Mod (Volcano e-cigs)	0	ECVC (55% PG/45% VG) (1% concentration)	1 h	Human alveolar macrophages	Significant reduction in *S. aureus* phagocytosis vs. UTC	(39)
	Second generation (Kanger Ltd)	36 mg/mL and 0 mg/mL	ECVC (0.5% concentration)	6 h	Human alveolar macrophages	36 mg/mL nicotine: 30% reduction, 0 mg/mL nicotine: 50% reduction in *E. coli* phagocytosis vs. UTC	(36)
	QHIT e-cigarette (Puff, Moncalleri, Italy) 3.7 V	0	100% ECVE (40 × 60 mL puff, 3 s, 30 s break)	0, 3, 6 days	THP-1 cell line	Reduced phagocytosis of *M. tuberculosis* at all-time points	(37)
Efferocytosis	EVOD-2 3.7 V, 1.5 Ω	18 mg/mL and 0 mg/mL	100% ECVE (50 × 3 s puffs, 5 s break)	24 h	THP-1 cell line	18mg/mL nicotine: Significant reduction in efferocytosis compared with controls 0 mg/mL nicotine: No effect on efferocytosis	(40)
Cytokine/protease release	Second generation (Kanger Ltd)	26 mg/mL	ECVC (0.5% concentration)	24 h	Human alveolar macrophages	Increased secretion of IL-6, TNF-α, IL-8, MCP-1, and MMP-9 vs. UTC	(36)
	EVOD-2 3.7 V, 1.5 Ω	18 mg/mL	ECVE (50 × 3 s puffs, 5 s break)	24 h	THP-1 cell line	No change in IL-8, IL-1β, MIP-1β, or MCP-1 secretion but a reduction production of IL-6, MIP-1α, and TNFα vs. UTC	(41)
	None	1.8% or 0	1% PG/VG (55:45 v/v) media	Overnight	THP-1 cell line	1.8% nicotine: increased secretion and activity of MMP-2 and MMP-9 vs. UTC 0 nicotine: No change in MMP-2 or MMP-9 vs. UTC	(42)
	QHIT e-cigarette (Puff, Moncalleri, Italy) 3.7 V	8 mg/g and 0 mg/g	100% ECVE (40 × 60 mL puff, 3 s, 30 s break)	3 days	THP-1 cell line	IL-8 was the highest produced cytokine and IL-1β was highly produced	(37)

Studies investigating the effect of e-cigarettes on alveolar macrophage viability, reactive oxygen species generation, phagocytosis, efferocytosis, and cytokine release are included. The exposure design and cell type have been included for comparison between findings. Only experiments using the humectant bases with or without nicotine in the absence of any flavorings have been included to aid understanding of the fundamental effects of e-cigarette exposure on alveolar macrophages. *E. coli*, Escherichia coli; ECVC, e-cigarette vapor condensate; ECVE, e-cigarette vapor extract; IL, interleukin; MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; MMP, matrix metalloproteinase; MRSA, methicillin-resistant *Staphylococcus aureus*, *M. tuberculosis*, *Mycobacterium tuberculosis*; PG, propylene glycol; ROS, reactive oxygen species; *S. aureus*, *Staphylococcus aureus*, TNF, tumor necrosis factor; UTC, untreated control; VG, vegetable glycerin.

vapor constituents, as it does not rely upon dissolving. In a range of studies examining cytokine secretion from both THP-1 cell lines and bronchoalveolar lavage (BAL)-derived macrophages, proinflammatory cytokines, most notably the robust recruiter of neutrophils IL-8, are upregulated in response to e-cigarette vapor condensate, extract, and supplemented media treatments (36, 37, 42). However, contradictory findings have been reported for other cytokines, such as one study showing an upregulation of tumor necrosis factor (TNF)-α after alveolar macrophage exposure to 50% PG: 50% VG condensate whereas another study showed a downregulation after 70% PG: 30% VG extract exposure in THP-1 cells (36, 41). This form of comparison makes it difficult to deduce whether varying results are due to different exposure models, cell types, or base humectants.

Conversely, effects on antimicrobial activity appear to be independent of nicotine content. Alveolar macrophages exposed to e-cigarettes exhibited reduced antimicrobial activity to gram-positive and gram-negative bacteria regardless of nicotine content and this is exacerbated by the increased virulence and growth rates of infecting pathogens (36–39). E-liquids have been shown to upregulate Ca^{2+} signaling in THP-1 cells and have been implicated in many cellular functions, including phagocytosis, so may represent a driving mechanism of impaired phagocytosis (43, 44). Furthermore, alveolar macrophages exposed to nicotine-containing e-cigarette vapor extract exhibited significant reduction in efferocytosis of apoptotic epithelial cells (40). Inability to clear cellular debris and dying cells causes persistent inflammation of the airway.

In vitro models and even cohort studies carried out to date model the short-term effects of vaping. Murine studies have been used to further examine the chronic effects. Using a chronic vapor exposure model, Maddison et al. (45) recapitulated the lipid-laden phenotype of alveolar macrophages seen in EVALI (46).

Mechanisms

Despite several decades of research, the mechanism(s) of action of cigarette smoke-mediated harm related to macrophage function are still not completely understood. Among the 5,000 different chemicals present in cigarette smoke (47, 48), the presence of many of the same harmful constituents that drive respiratory disease mechanisms have been found in e-cigarette vapor including carbonyl compounds (such as acrolein and formaldehyde), volatile organic compounds, and tobacco-specific nitrosamines (49). Recent exploratory evidence examining the effects of e-cigarettes on alveolar macrophages can broadly define potential mechanisms as *1*) nicotine independent and driven by pathways activated by free radicals, aldehydes, and the PI3K pathway; *2*) nicotine dependent and driven by cellular changes as a result of nicotine binding cell surface receptors; and *3*) lipid metabolism driven by the altered ability to process lipids.

Nicotine independent.

Although most vapers are ex-smokers, an emerging cohort of previously never-smoker vapers appears to have much greater prevalence of vaping without nicotine. Although flavorings differ, the humectant base components PG and VG are common to all e-liquids. For this reason, it is important to assess the nicotine-independent effects of e-liquids. Phagocytosis is dysregulated by e-cigarette vapor condensate treatment independent of nicotine, and partially restored by antioxidant/antialdehyde, *N*-acetyl cysteine (NAC) cotreatment, suggesting a free radical/aldehyde driven mechanism (36). THP-1 macrophages exhibit upregulated reactive oxygen species (ROS) production in response to nicotine-free e-cigarette vapor condensate exposure (36). The PI3K/Akt pathway is known to mediate ROS production (50), and work has demonstrated pan-PI3K inhibition and specific PI3Kα inhibition improved viability and partially restored phagocytosis after nicotine-free e-cigarette vapor condensate challenge (36, 51). This work demonstrates nicotine is not the sole active component of e-cigarette vapor, yet needs further exploration.

Nicotine dependent.

Nicotine concentrations in e-liquids differ significantly between vapers, with users titrating nicotine intake to meet their addiction needs (52). Reduced efferocytosis of apoptotic epithelial cells has been reported (40), driven by a reduction in apoptotic cell receptor (CD36 and CD44) expression on the surface of alveolar macrophages following exposure to humectant bases PG and VG as well as nicotine. However, this treatment alone did not translate into functional reduction in efferocytosis in the absence of nicotine (40). Similarly, alveolar macrophages exhibit reduced expression of phagocytic receptors SR-A1 and TLR2 in response to nicotine-containing e-cigarette vapor extract, contributing to the reduction in phagocytosis (41). Release of proteinases is driven by an increase in intracellular Ca^{2+} levels in response to the opening of the ligand-gated nicotine receptors [nicotinic acetylcholine receptor (nAChRs)]. Macrophages express four types of nAChRs: CHRNB1, CHRNA5, CHRNA6, and CHRNA7 and their activation by nicotine increases intracellular Ca^{2+} levels and NF-κB signaling (42, 53).

Lipid metabolism.

Macrophages contribute to lipid metabolism and homeostasis in a range of tissues including the liver, spleen, and lungs, as they clear lipids from cell debris and excess cholesterol (54). In the lungs, alveolar macrophages catabolize surfactant and along with alveolar type II cells regulate pulmonary surfactant reducing surface tension and contributing to immune host defense against invading pathogens (55). In patients with EVALI, particularly those who use THC products, lipid-laden macrophages that stain heavily with oil red O stain have been reported in histological analyses of bronchoalveolar lavage (BAL) samples (10, 56). The derivation of these unique foam-like cells in EVALI is uncertain due to the presence in young, otherwise healthy individuals with an absence of hyperlipidemia (57). In addition, exogenous lipid exposure from oil-based e-cigarette vapor and persistent inflammation driving formation of lipid-laden macrophages, further evidence has shown that disruption of lipid metabolism causes lipid accumulation from endogenous sources in a nicotine-independent mechanism (45, 58). Although lipid-laden macrophages are not unique to patients with EVALI, and have been shown to be present in nonvapers and smokers (59–61), they contribute to the pathogenesis of exogenous lipoid pneumonia, as in the presentation of EVALI (62). These lipid-laden macrophages have impaired function, particularly phagocytosis (54, 63), so may explain some of the functional implications of e-cigarette exposure reported. Finally, in vivo, lamellar bodies that regulate surfactant catabolism appeared poorly organized and irregular, suggesting changes in surfactant processing had occurred (45). Impaired surfactant homeostasis by macrophages causes excess lipid deposition in the airway and accumulation of intracellular lipids (64).

NEUTROPHILS

Neutrophils are essential innate immune cells, constituting 70% of circulating leukocytes and are rapidly recruited to sites of infection or injury in tissues (65). Neutrophils play an important role in tissue homeostasis including inflammation initiation and resolution. Neutrophils have an arsenal of killing mechanisms, which can be deployed in response to infection, phagocytosis, degranulation and neutrophil extracellular traps (NET)osis (66).

Neutrophil dysfunction has been reported in a range of disease states including COPD, sepsis, and inflammaging (67–70). Cigarette smoking upregulates neutrophil numbers in BAL, hinders normal neutrophil function, and promotes a proinflammatory phenotype (71–73), leading to persistent inflammation and increased tissue damage, which can cause early changes in the lung associated with progression to COPD. In health, there is an obligate area of tissue damage following neutrophil degranulation; however, disturbances of the proteinase-antiproteinase balance lead to uncontrolled promotion of tissue destructive proteinases including NE without the corresponding inhibition by antiproteinases such as α-1 antitrypsin (74, 75).

Neutrophils and E-cigarettes

Murine models initially showed unchanged neutrophil numbers in BAL after e-cigarette exposure (76); however, further delineation of humectants, nicotine, and flavorants found significant infiltration of neutrophils into the lung after exposure to 12 mg/mL nicotine flavorless e-liquid (50/50 PG/VG) (77). These studies used whole body exposure methods, which may have diluted out the impact on lung tissue. This finding supports the recently reported EVALI cases, cytopathological findings revealed neutrophil infiltration into the airways (58% of BAL cells were neutrophils) (10). However, data from cohort studies have reported no difference in neutrophil numbers in either BAL or induced sputum samples of e-cigarette users compared with nonsmokers and smokers (42, 78). Despite mixed findings on lung infiltration of neutrophils as a result of e-cigarette use, neutrophilic granule enzymes including NE and matrix metalloproteases (MMPs) are consistently elevated (42, 78). Importantly, a lack of corresponding increase in antiproteinases (42, 78, 79) allows unopposed tissue destruction by proteinases and increases the potential for bystander tissue damage upon neutrophil recruitment to the airways.

E-cigarette vapor extract has not been reported to be cytotoxic to neutrophils; however, markers of neutrophil activation are elevated after exposure, including morphological shape change and activation marker expression (CD11b and CD66b) (79). Much of the literature on neutrophil function in response to e-cigarette vapor exposure is difficult to interpret, as authors neglect to report concomitant effects on neutrophil viability in these experiments.

Previous work has highlighted the ease in which primed neutrophils degranulate and subsequently easily get caught in the lung (80, 81). Neutrophils exhibit a diminished ability to produce ROS, which coincides with the reduced ability to migrate through a membrane toward a chemoattractant after exposure to e-cigarettes (82). Phagocytosis of *Escherichia coli* and *Staphylococcus aureus* by circulating neutrophils is significantly reduced by e-cigarette exposure independently of nicotine; indicating that e-cigarette use may limit the control and elimination of bacterial infections, even within the blood (39, 82). The effect of e-cigarette exposure on NETosis varies depending on the exposure model and NET inducer investigated, with mixed reports of increasing suppression and sensitivity to NET production. In a report examining neutrophils from vaper's BAL, neutrophils were more sensitive to NET-stimulating Phorbol 12-myristate 13-acetate (PMA) than controls (78). Table 2 summarizes the key studies investigating neutrophil responses to e-cigarette exposure, including the device type, exposure model, and functional findings.

Mechanisms

Few studies have investigated the mechanisms of e-cigarette effects on neutrophils and those that have are largely driven by speculative hypotheses. The mechanism for dysregulated chemotaxis remains unclear. One explanation is that chemotaxis is dysregulated by e-cigarette-mediated changes in membrane fluidity and inability to polarize f-actin polymerization in response to a chemotactic agent [*N*-formylmethionine-leucyl-phenylalanine (fMLP)] (82), yet the cause of this remains unclear. NETosis relies upon the activation of protein kinase C (PKC) activation (83); however, the impact of e-cigarette exposure on PKC activation in neutrophils or an alternative pathway to NETosis has not been investigated. Evidence from cigarette smoke extract and nicotine treatments on neutrophils shows upregulation of p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK) and nuclear factor (NF)-κB pathways (84). In response to high-dose e-cigarette vapor extract treatment, neutrophils exhibit p38 MAPK activation but no change in ERK or NF-κB pathway activation (79). Further investigation revealed secretion of MMP-9 by e-cigarette-exposed neutrophils is driven by p38 MAPK activation and can be reduced by chemical inhibition of this pathway (79). This indicates that there are both differential and overlapping mechanisms of e-cigarette damage compared with cigarette damage, which need further investigating to understand the possible impacts of chronic vaping. The mechanism of e-cigarette driven impaired neutrophil phagocytosis remains unknown. There may be receptor sensitivity/cleavage that prevent pathogen recognition, cytoskeletal rearrangement issues that prevent the internalization of the microbe, issues with assembly of NADPH oxidase preventing bacterial killing or another cause of this dysfunction, requiring more robust studies to confirm the driving mechanism of these effects. However, baseline ROS generation from neutrophils treated with e-

Table 2. *A summary of the effects of e-cigarette vapor exposure on neutrophil effector functions*

Effector Function	E-Cigarette Model	ECL Nicotine Concentration	Exposure Model	Exposure Duration	Cell Type	Results	Reference
Viability	VIP 1100 mAh battery with V5/CE5 Clearomizer	24 mg	ECVE (optical density values: 0.001, 0.003, 0.01, 0.03, 0.1)	6 h	Isolated peripheral blood neutrophils of never smokers	No effect on neutrophil apoptosis or necrosis at any concentrations but significant morphological changes and increased cell activation markers (CD11b and CD66b)	(79)
Reactive oxygen species	Kanger Miniprotank glassomizers attached to Kanger eVOD variable voltage 1,000 mAh battery	24 mg/mL	ECVE (100%, 75%, 50%, 25% concentrations)	20 min	Isolated peripheral blood neutrophils	Baseline ROS production significantly reduced by 100%, 75%, and 50% ECVE vs. UTC PMA-induced ROS unaffected	(82)
Phagocytosis/antimicrobial activity	LAVABOX DNA 200 Box Mod (Volcano e-cigs) and SMOK TFV4 mini tank with sub-ohm coil	0	ECL (PG/VG vehicle control) (1%, 0.5%, or 0.25% concentration)	30 min	Isolated peripheral blood neutrophils	Significantly reduced phagocytosis of *S. aureus* vs. UTC	(39)
	Kanger Miniprotank glassomizers attached to Kanger eVOD variable voltage 1,000 mAh battery	24 mg/mL	ECVE (100%, 75%, 50%, 25% concentrations)	30 min	Isolated peripheral blood neutrophils	40% reduction in phagocytosis of *E. coli* and *S. aureus* when treated with 100% ECVE vs. UTC	(82)
Cytokine/proteinase release	VIP 1100 mAh battery with V5/CE5 clearomizer	24 mg	ECVE (optical density values: 0.001, 0.003, 0.01, 0.03, 1)	6 h	Isolated peripheral blood neutrophils of never smokers	Significantly upregulated MMP-9 secretion and activity at 0.003 and 0.01 OD concentrations vs. UTC. Increase in NE secretion at 0.003 OD but decreased secretion at 0.03 and 0.1 OD vs. UTC	(79)
	N/A	N/A	N/A	N/A	BAL proteinase levels	NE, MMP-2, and MMP-9 secretion and activity were significantly elevated in BAL of vapers compared with nonsmokers Antiproteinases (A1AT, CLPI, and TIMP-1/2) unchanged in BAL of vapers compared with nonsmokers	(42)
	None	18 mg/mL	PG/VG (55:45 v/v)	4 h	Isolated peripheral blood neutrophils	Significantly increased NE secretion vs. UTC	(42)
Migration	Kanger Miniprotank glassomizers attached to Kanger eVOD variable voltage 1,000 mAh battery	24 mg/mL	ECVE (100%, 75%, 50%, 25% concentrations)	20 min	Isolated peripheral blood neutrophils	ECVE (50%, 75%, and 100%) significantly reduced migration of neutrophils to fMLP vs. UTC	(82)
NETosis	N/A	N/A	N/A	N/A	Isolated peripheral blood neutrophils from smokers, e-cigarette users and healthy individuals	Significantly more sensitive to NET stimuli than smokers or healthy individuals	(78)
	Kanger Miniprotank glassomizers attached to Kanger eVOD variable voltage 1,000 mAh battery	24 mg/mL and 0 mg/mL	ECVE (100%, 75%, 50%, 25% concentrations)	20 min	Isolated peripheral blood neutrophils	24 mg/mL: No change in spontaneous or nigericin-induced NET production vs. UTC, but increasing suppression of PMA-induced NETS with increasing ECVE concentration0 mg/mL: Significantly suppressed PMA-induced NET production vs. UTC	(82)

Studies investigating the effect of e-cigarettes on neutrophil viability, reactive oxygen species generation, phagocytosis, cytokine release, migration, and NETosis are included. The exposure design and cell type has been included for comparison of findings. Only experiments using the humectant bases with or without nicotine in the absence of any flavorings have been included to aid understanding of the fundamental effects of e-cigarette exposure on neutrophils. A1AT, alpha 1 antitrypsin; CD, cluster of differentiation; *E. coli*, Escherichia coli; ECVC, e-cigarette vapor condensate; ECVE, e-cigarette vapor extract; IL, interleukin; MMP, matrix metalloproteinase; NET, neutrophil extracellular trap; OD, optical density; PG, propylene glycol; PMA, phorbol myristate acetate; ROS, reactive oxygen species; *S. aureus*, *Staphylococcus aureus*; SLPI, secretory leukocyte protease inhibitor, TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor; UTC, untreated control; VG, vegetable glycerin.

cigarette vapor extract may signify an inability to rapidly assemble NADPH oxidase, which has implications for bacterial killing (85). Neutrophils express nicotine receptor variants CHRNB1, CHRNA5, and CHRNA6 (42, 86) and exhibit an increase in intracellular Ca^{2+} concentration in response to e-cigarette exposure without a corresponding upregulation of NF-κB activity, as is seen with direct nicotine-only treatment (42, 79, 87). Therefore, the downstream pathways of this receptor and its consequences for neutrophil functions require further investigations. Many questions remain about the causes of neutrophil dysfunction in response to e-cigarette exposure, meaning robust in vitro studies are required.

EPITHELIAL CELLS

Once considered to function purely as a static barrier, the airway epithelium is now recognized for its diverse range of functions and its importance in orchestrating inflammation and tissue remodeling (88). Consisting of a range of cell types including secretory, ciliated, and type I/II alveolar cells, the airway epithelium is a dynamic tissue required to protect the lung against invading pathogens (89). Airway epithelial cells are central to lung health and their dysfunction contributes to lung diseases including asthma, COPD, and cystic fibrosis (88–90). Cigarette smoke exposure increases epithelial layer permeability and reduces cell-cell contacts (91, 92). In addition, impairing the mucociliary escalator drives mucus build up in the airways by impairing differentiation and function of ciliated epithelial cells that contribute to the development of chronic bronchitis, a feature of COPD (93, 94).

Epithelial Cells and E-cigarettes

Experimental models have attempted to recapitulate lung conditions to investigate the functional impact of e-cigarettes on epithelial cells. Table 3 summarizes the key findings regarding epithelial cell viability, ciliary parameters,

Table 3. *A summary of the effects of e-cigarette vapor exposure on epithelial cell effector functions*

Effector Function	E-Cigarette Model	ECL Nicotine Concentration	Exposure Model	Exposure Duration	Cell Type	Results	Reference
Viability	Third-generation device	1.2%	ECVE	72 h	BEAS-2B	Significant toxicities at 5% and 10% concentrations vs. UTC	(95)
	EVOD-2 3.7 V, 1.5 Ω	18 mg/mL and 0 mg/mL	100% ECVE (50 × 3 s puffs, 5 s break)	24 h	HBEC	No increase in apoptosis or necrosis	(96)
	VC-1 exposure system	36 mg/mL, 0 mg/mL, and 100 μM (aerosolized, calculated final exposure: 100 nM)	Air-liquid interface	36 puffs (1 every 30 s)	NHBE from non-smoker donors	Viability was unaffected vs. UTC	(76)
	Voltage-variable ENDS device from Vision Spinner II 1,600 mAh	1.1%	Air-liquid interface	0–60 min	HBEC	No effect on cellular viability vs. UTC (except at 60-min exposure)	(97)
Ciliary parameters	VC-1 exposure system	36 mg/mL	Air-liquid interface	8 h	NHBE from non-smoker donors	Reduced ciliary beat frequency but no change in percentage of ciliated cells vs. UTC	(76)
	Information not available	2.4%	Air-liquid interface	20 puffs	NHBE from non-smoker donors	Reduced ciliary beat frequency	(98)
Ion channel function	Voltage-variable ENDS device from Vision Spinner II 1,600 mAh	1.1%	Air-liquid interface	0–60 min	HBEC	Dose-dependent inhibition of chloride ion transfer vs. UTC	(97)
Cytokine release	Third-generation device	1.2%	ECVE	72 h	BEAS-2B	Significantly increased CXCL8, collagen I, and fibronectin secretion vs. UTC	(95)
	N/A	Nicotine-supplemented media (0, 1 or 10 μM)	Air-liquid interface	5 days	NHBE from non-smoker donors	Elevated secretion of IL-6, IL-8, and a trend toward an increase in MCP-1 secretion vs. UTC	(76)
	Steamo Nova2 e-cigarette. 2.2 Ω, 2.6 V	18 mg/mL (calculated final exposure: 2.4 mg)	Air-liquid interface	25 min (3 s puff every 29 s)	Calu-3 cell line	Significant increase in IL-8 secretion 24 h after exposure	(99)
	EVOD-2 3.7 V, 1.5 Ω	18 mg/mL	100% ECVE (50 x 3 s puffs, 5 s break)	24 h	HBEC	Significant reduction in TNF-α, MIP-1β, and IP-10 secretion; however, no change in IL-6, IL-8, or MIP-1α	(96)
Protein expression	SIGELEI 150 W with TFV4 top refill tank	0 (PG/VG 55/45)	Air-liquid interface	36 puffs (measured 24 h later)	Primary HBEC	3-4 fold increase in MUC5AC levels and significant increase in CYP1B1	(100)

Studies investigating the effect of e-cigarettes on epithelial cell viability, ciliary parameters, ion channel function, cytokine release, and protein expression are included. The exposure model and cell type have been included for comparison of findings. Only experiments using the humectant bases with or without nicotine in the absence of any flavorings have been included for simplicity and to aid understanding of the fundamental effects of e-cigarette exposure on this important cell type. CYP1B1, cytochrome P450 family 1 subfamily B member 1; ECVC, e-cigarette vapor condensate; ECVE, e-cigarette vapor extract; HBEC, human bronchial epithelial cultures, IL, interleukin, MCP, methyl-accepting chemotaxis protein; MUC5A, mucin 5AC; NHBE, normal human bronchial epithelial; PG, propylene glycol; TNF, tumor necrosis factor; UTC, untreated control; VG, vegetable glycerin.

cytokine release, ion channel function, and protein expression after e-cigarette exposure.

High concentrations of e-cigarette vapor extract trigger epithelial cell toxicities comparable to cigarette smoke extract exposure but how these doses compare with user exposure is unclear, as no data regarding e-cigarette vapor extract characteristics were provided (95). Other findings have indicated no induction of apoptosis or necrosis by unflavored e-cigarette exposure (40, 76, 97). Dysfunctional cilia beat frequency and motility have been reported in human nasal epithelial cells (HNEC) and normal human bronchial epithelial (NHBE) cells exposed to e-cigarettes in a manner similar to that seen with cigarette smoke extract (76, 98). Although the authors describe a return to baseline function over time, they do not rule out the potential of lasting damage with chronic exposure (98). Beas-2B, NHBE and Calu-3 cells exposed to e-cigarette vapor exhibit an increase in IL8 secretion (76, 78, 95). Extracellular matrix (ECM) proteins, collagen 1 and fibronectin, are secreted in a concentration-dependent manner in Beas-2B and airway smooth muscle cells exposed to e-cigarette vapor extract, leading to formation of mesh-like fibers observed in e-cigarette vapor-exposed HNECs and contributes to tissue remodeling (95, 98).

Mechanisms

Signaling molecules have been implicated as contributors to the functional changes observed by e-cigarette exposure to epithelial cells. α7nAChRs on the surface of epithelial cells upregulate the PKC and ERK signaling pathways after stimulation with nicotine (101, 102). These pathways are involved with inflammatory responses and controlled cell death in epithelial cells but focused investigations on these molecules and their contribution to dysfunction after e-cigarette exposure are lacking (103). The regulator of ciliogenesis, FOXJ1, is downregulated in nicotine-containing e-cigarette-exposed NHBE cells, suggesting a possible mechanism for the reduction in ciliary beat function (76). Cigarette smoke inhibits cystic fibrosis transmembrane regulation (CFTR) in a Ca^{2+}-dependent manner, which can be reprised by direct elevations in Ca^{2+} (104, 105). Independent of changes in gene expression, conductance of the CFTR channel is impaired by e-liquid in a similar manner to that caused by cigarette smoke extract (76, 97). This is an effect of vaporized but not unvaporized e-liquid and drives airway dehydration (97) indicating the change in chemical composition, which occurs during vaping is a key factor in this activity. Reactive carbonyl species generated during vaping (28, 106) must therefore be the key active agent in mediating this effect. In addition, ion channel conductance of the big potassium (BK) channel is reduced by e-cigarette vapor in airway epithelial cells due to a reduced expression in α subunit gene (KCNMA1) (76), which leads to impairment of fluid homeostasis and air surface liquid (107). E-cigarette-exposed Beas-2B cells exhibit mitochondrial uncoupling after exposure to e-cigarette vapor extract as well as increased glycolysis at high e-cigarette vapor extract concentrations, suggesting a pivotal role of dysfunctional mitochondria in the aberrant function of airway epithelial cells that lead to airway remodeling and scarring after persistent insult or injury such as that caused by e-cigarette usage (95, 108). The mechanism driving cytokine release not been investigated; however, IL8 was not upregulated by nicotine-free vapor exposure in NHBE cells, indicating this may be a nicotine-dependent effect (76). These molecules identified as potential mechanisms of the effects seen on epithelial cells are promising but lack in-depth investigations to conclusively report the mechanism of damage.

SUMMARY

The current evidence surrounding the potential impact of e-cigarette exposure on alveolar macrophages, neutrophils, and lung epithelial cells is limited but building. To date, studies have been small and exposure durations short. There have been many variations in exposure model, e-cigarette device and e-liquid used, and there are still important gaps to be filled. However, there are consistent signals of short-term harm in alveolar macrophage, neutrophil, and epithelial dysfunction. These short-term effects are likely to have long-term implications with repeated exposure. For example, e-cigarette-driven decline in efferocytosis by alveolar macrophages leads to reduced clearance of apoptotic neutrophils, driving neutrophil-induced inflammation and secondary necrosis, which has been shown to contribute to the pathophysiology of COPD (109, 110). In addition, impaired neutrophil chemotaxis and susceptibility to NET formation, as caused by e-cigarette exposure, has also been demonstrated in neutrophils COPD patients (22, 111). Finally, increased IL-8 secretion from lung epithelial cells has been associated with worse obstruction in COPD, with a multifactorial impact on disease pathophysiology (112). Demonstrating the relationship between cellular dysfunction reported in e-cigarette studies and those driving pathophysiology in just one chronic inflammatory lung disease with significant quality of life burdens highlights potential for chronic disease development with persistent e-cigarette exposure. Extensive studies investigating the contribution of nicotine receptors, signaling pathways, transcriptional/epigenetic alterations, and metabolic changes across all cell types are needed. Although large longitudinal studies of vapers will be required to fully determine the effects of chronic vaping, these studies will take years, by which time e-cigarettes may be embedded within society. Better in vitro studies may give us crucial insight into how e-cigarettes affect key cell types and importantly how these mechanisms differ from that of cigarette smoking. Until these processes are understood, we cannot reliably inform policy makers, healthcare professionals, or the public on the safety of e-cigarettes.

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AUTHOR CONTRIBUTIONS

A.E.J. prepared figures; A.E.J. and A.S. drafted manuscript; A.E.J., E.S., and A.S. edited and revised manuscript; A.E.J., E.S., D.R.T., and A.S. approved final version of manuscript.

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