Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function

Phillip W. Clapp,[1,2] Erica A. Pawlak,[2] Justin T. Lackey,[3] James E. Keating,[3] Steven L. Reeber,[3] Gary L. Glish,[3] and Ilona Jaspers[1,2]

[1]Curriculum in Toxicology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina; [2]Center for Environmental Medicine, Asthma, and Lung Biology, School of Medicine, University of North Carolina, Chapel Hill, North Carolina; and [3]Department of Chemistry, University of North Carolina, Chapel Hill, North Carolina

Clapp PW, Pawlak EA, Lackey JT, Keating JE, Reeber SL, Glish GL, Jaspers I. Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *Am J Physiol Lung Cell Mol Physiol* 313: L278–L292, 2017. First published May 11, 2017; doi:10.1152/ajplung.00452.2016.—Innate immune cells of the respiratory tract are the first line of defense against pathogenic and environmental insults. Failure of these cells to perform their immune functions leaves the host susceptible to infection and may contribute to impaired resolution of inflammation. While combustible tobacco cigarettes have been shown to suppress respiratory immune cell function, the effects of flavored electronic cigarette liquids (e-liquids) and individual flavoring agents on respiratory immune cell responses are unknown. We investigated the effects of seven flavored nicotine-free e-liquids on primary human alveolar macrophages, neutrophils, and natural killer (NK) cells. Cells were challenged with a range of e-liquid dilutions and assayed for their functional responses to pathogenic stimuli. End points included phagocytic capacity (neutrophils and macrophages), neutrophil extracellular trap formation, proinflammatory cytokine production, and cell-mediated cytotoxic response (NK cells). E-liquids were then analyzed via mass spectrometry to identify individual flavoring components. Three cinnamaldehyde-containing e-liquids exhibited dose-dependent broadly immunosuppressive effects. Quantitative mass spectrometry was used to determine concentrations of cinnamaldehyde in each of the three e-liquids, and cells were subsequently challenged with a range of cinnamaldehyde concentrations. Cinnamaldehyde alone recapitulated the impaired function observed with e-liquid exposures, and cinnamaldehyde-induced suppression of macrophage phagocytosis was reversed by addition of the small-molecule reducing agent 1,4-dithiothreitol. We conclude that cinnamaldehyde has the potential to impair respiratory immune cell function, illustrating an immediate need for further toxicological evaluation of chemical flavoring agents to inform regulation governing their use in e-liquid formulations.

e-cigarette; e-liquid; cinnamaldehyde; innate immune cell function

SMOKING CONTINUES TO BE the leading cause of preventable death, accounting for 12% of all mortalities among adults worldwide (69a). A link between cigarette smoke exposure, suppression of respiratory immune responses, and enhanced susceptibility to respiratory infection is well established (28–30, 32, 50, 51, 56). While tobacco use is steadily declining in the United States, electronic cigarettes (e-cigarettes) are rapidly gaining popularity, particularly among teens and young adults (4) who often perceive these products as less harmful than traditional cigarettes (2). This perception is in part the result of targeted marketing that identifies these products as a "safer alternative" to traditional cigarettes (55); 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 (64). Furthermore, lung macrophages from these exposed mice exhibited reduced phagocytic capacity. More recently, Hwang et al. exposed human epithelial cell lines, murine alveolar macrophages, and human neutrophils to "e-cigarette vapor extract" and observed dose-dependent cytotoxicity and reduced antimicrobial activity against *Staphylococcus aureus* (31). 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 (18, 38, 61).

E-cigarette devices aerosolize flavored e-liquids, which are typically composed of humectants [propylene glycol (PG) and/or vegetable glycerin (VG)], 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 (73). While many of these flavorings are classified as generally recognized as safe (GRAS) for oral consumption by the United States Food and Drug Administration (FDA), prolonged inhalation of some GRAS flavorings, such as diacetyl, 2,3-pentanedione, and acetoin, can cause irreversible lung disease (27b, 27c, 39). GRAS chemicals commonly used as e-liquid flavoring agents include aliphatic aldehydes (for fruity flavors) and aromatic aldehydes (for sweet and spicy flavors) (27a, 68a). The Flavor and Extract Manufacturers Association (FEMA) has identified over 1,000 GRAS flavorings that may pose a respiratory hazard due to possible volatility and irritant properties (18a); 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 (14, 34, 36, 37, 45, 47, 57, 72). However, most of these studies were conducted in nonhuman 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 natural killer (NK) cells from healthy nonsmoker 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 that 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.

MATERIALS AND METHODS

Subjects. Healthy individuals between the ages of 18 and 49 yr 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 criterial included pregnant or nursing females or individuals with a history of egg allergy, allergic rhinitis, aspirin therapy, asthma, immunodeficiency (human immunodeficiency virus or other), Guillain-Barre Syndrome, chronic obstructive pulmonary disease (COPD), cardiac disease, any chronic cardiorespiratory condition, or fever/respiratory illness within 3 wk before entry into study.

Subjects undergoing bronchoscopy received a physical examination, a routine blood panel with complete blood count and differential, serum electrolyte, glucose, and liver enzyme testing. Female subjects had to have a negative urine pregnancy test before bronchoscopy, and all volunteers were required to be free of chronic cardiovascular or respiratory illness and of acute respiratory illness within the proceeding 3 wk. All subjects undergoing bronchoscopy had forced expiratory volume in s (FEV1) and forced vital capacity (FVC) ≥80% predicted and FEV1/FVC ≥80% predicted normal for height and age, and were nonvapers and nonsmokers with no smoking history.

Subjects were matched for sex, age, and body mass index 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 (23). Briefly, a flexible fiber optic bronchoscope was inserted transnasally and wedged in a subsegment of the medial segment of the right middle lobe (5th or 6th generation). Saline was instilled and immediately aspirated to recover alveolar leukocytes. Recovered BAL fluid was centrifuged, and cells (>95% alveolar macrophages) were collected in RPMI-1640 medium (Thermo Fisher Scientific, Waltham, MA).

Neutrophil isolation. Venous blood was collected in Vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA) and allowed to cool to room temperature with gentle rocking before cell isolation. Neutrophils were isolated by density centrifugation of venous blood over Histopaque 1119 (Sigma-Aldrich, St. Louis, MO) and subsequently over a discontinuous Percoll (GE Healthcare Life Sciences, Marlborough, MA) gradient as previously described (10, 19). Isolated neutrophils were resuspended in RPMI-1640 medium containing 10 mM HEPES buffer and 0.5% fetal bovine serum (FBS). Cells were incubated at 37°C and 5% CO_2 for 30 min before challenges.

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

Flavored e-cigarette liquids. A panel of seven flavored nicotine-free e-liquids was purchased from a local vendor (The Vapor Girl, Chapel Hill, NC). 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 PG and VG at relative proportions of 55% and 45%, respectively. A mixture of 55% PG (Thermo Fisher Scientific) and 45% VG (Sigma-Aldrich) was used as a vehicle control for all e-liquid experiments.

Stimulation and quantification of neutrophil extracellular traps. Neutrophils were seeded at a density of 1×10^5 in 100 μl volume/well of a cell culture-treated 96-well plate and allowed to incubate at 37°C and 5% CO_2 for 30 min. Cells were then challenged with a final concentration of 1.0% e-liquid diluted in cell culture medium with or without 25 nM phorbol 12-myristate,13-acetate (PMA; Sigma-Aldrich), a protein kinase C (PKC) activator and potent neutrophil extracellular trap (NET) agonist. Wells were assayed for extracellular chromatin every hour during a 4-h challenge. Chromatin extruded by activated neutrophils was digested with 500 mU/ml micrococcal nuclease (Worthington Biochemical, Lakewood, NJ). Micrococcal nuclease activity was quenched with 5 mM EDTA following a 10-min digestion at 37°C. Chromatin released at each time point was quantified using the Picogreen double-stranded DNA kit (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol.

Immunofluorescent analysis of NETs. Neutrophils were seeded on 13-mm round glass coverslips (0.15 mm thick) and challenged with 1.0% e-liquid diluted in cell culture medium with or without 25 nM PMA. Cells were fixed with 4% paraformaldehyde (PFA; Sigma-Aldrich) every hour during a 4-h challenge, and immunolabeling was conducted as described by Brinkmann et al. (10). Neutrophils were blocked [5% normal donkey serum (Jackson ImmunoResearch, West Grove, PA), 1% gelatin from cold water fish skin (Sigma-Aldrich), 1% bovine serum albumin (Sigma-Aldrich), and 0.05% Tween 20 (Sigma-Aldrich) in PBS (GIBCO/Thermo Fisher Scientific)] and incubated with a mouse anti-human neutrophil elastase primary antibody (Dako, Carpentaria, CA) that was detected with an Alexa Fluor 488-conjugated secondary antibody (Jackson ImmunoResearch). Negative controls were conducted using Isotype-matched (IgG1κ) labeling of NETs at each time point. Chromatin was labeled with Hoechst 33342 (Thermo Fisher Scientific). Coverslips were mounted on microscope slides with ProLong Gold Antifade Mountant (Thermo Fisher Scientific), and the edges were sealed with clear fingernail polish. Cells were imaged using a Nikon Digital Eclipse C-1 modular confocal microscope and Plan Apo VC 60 × /1.40 oil objective.

Neutrophil and alveolar macrophage phagocytosis. Neutrophils isolated from venous blood were seeded at a density of 1×10^5 cells/well of a black clear-bottom 96-well plate in RPMI-1640 media

with 10 mM HEPES buffer (Sigma-Aldrich) and 0.5% FBS (GIBCO/Thermo Fisher Scientific). Cells were allowed to incubate at 37°C and 5% CO_2 for 30 min. 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 min. Opsonized pHrodo Red *S. aureus* BioParticles (Thermo Fisher Scientific) 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 h to assess e-liquid-induced changes in phagocytic capacity.

Cells obtained from BAL fluid were seeded at a density of 5×10^4 cells/well of a black clear-bottom 96-well plate (Corning Life Sciences, Tewksbury, MA) in RPMI-1640 medium with 10% FBS. Cells were allowed to incubate at 37°C and 5% CO_2 for 2 h. Nonadherent 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 h. 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) 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%). 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 585 nm. 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 PG/VG vehicle at each dilution tested.

Dithiothreitol competition assay. Alveolar macrophages obtained from BAL fluid were seeded on black clear-bottom 96-well plates and allowed to incubate as described above. Nonadherent cells were removed, and adherent alveolar macrophages were challenged with cell culture medium alone, 500 μM cinnamaldehyde, 500 μM dithiothreitol (DTT; Sigma-Aldrich), or a combination of 500 μM cinnamaldehyde and 500 μM DTT for 30 min. Following challenge, opsonized pHrodo *S. aureus* BioParticles were added to the cells and incubated for an additional 4 h. Phagocytosis of bioparticles was quantified using the CLARIOstar fluorescent microplate reader as described above.

Immunofluorescent analysis of macrophage phagocytosis. Alveolar macrophages were seeded on 13-mm round glass coverslips (0.15 mm thick) at 2×10^5 cells/well of a cell culture-treated 24-well plate. Cells were allowed to incubate at 37°C and 5% CO_2 for 2 h and were challenged with 1, 0.5, or 0.25% dilutions of flavored e-liquid for 1 h as described above. Opsonized pHrodo Red *S. aureus* BioParticles were added to each well according to the manufacturer's protocol, and cells were fixed with 4% PFA after 4 h. Following 30 min fixation at 4°C, PFA was aspirated, and cells were blocked for 1 h 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) and Hoechst 33342 were diluted in block buffer to the manufacturer's recommended concentrations and applied to the cells for 1 h. Coverslips were washed with PBS and mounted on microscope slides with ProLong Gold Antifade Mountant, and the edges were sealed with clear fingernail polish. Cells were imaged using a Nikon Digital Eclipse C-1 modular confocal microscope and Plan Apo VC 60 × / 1.40 oil objective.

NK cell killing assay. NK cells (1×10^5) were added to duplicate polypropylene flow cytometry tubes with 0.25% e-liquids diluted in

cell culture medium for 1 h. During e-liquid incubation, K-562 human erythromyeloblastoid leukemia cells (ATCC, Manassas, VA) were labeled with carboxyfluorescein succinimidyl ester (Cayman Chemical, Ann Arbor, MI). Following e-liquid challenge, 3.2×10^4 carboxyfluorescein succinimidyl ester-labeled K-562 cells were added to each tube of NK cells and incubated for 4 h at 37°C and 5% CO_2. The entire cell suspension was then pelleted, stained with the LIVE/DEAD fixable dead cell stain kit (Thermo Fisher Scientific), and fixed with 0.5% PFA, and data were acquired on a BD LSRII Flow Cytometer as described in our previous studies (46).

Enzyme-linked immunosorbent assay. 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 h. Neutrophils were seeded at a density of 1×10^5 cells/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 h, followed by collection of supernatants. Medias collected postchallenge 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 enzyme-linked immunosorbent assay (ELISA) kits (BD Biosciences, San Jose, CA).

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. (52). Briefly, e-cigarette aerosol was produced by connecting a LAVABOX DNA 200 Box Mod (Volcano e-Cigs, Honolulu, HI) and a SMOK TFV4 Mini Tank with a sub-ohm (0.37Ω) TF-CLP2 Clapton Coil (SMOKtech, Shenzhen, China) to a 16-cm length of tubing (4.8 mm ID; Masterflex L/S 15, Vernon Hills, IL). A peristaltic pump (drive number 07522–20 and head number 77200–62; Cole-Parmer, Vernon Hills, IL) was used to pull aerosol from the e-cigarette device at a flow rate of 1 l/min. One puff of aerosol was generated every 30 s for 10 min (20 puffs total) using an output setting of 60 watts. Vaped aerosol was pumped through a series of alternating 250-μl pipette tips and Tygon tubing (1.5 mm ID, S3 E-3603; Saint-Gobain) to allow the aerosol to cool and condense. VEC for each e-liquid was collected in a sterile 1.5-ml microcentrifuge tube 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) using an Agilent DB-5MS capillary column (30 m, 0.25 mm ID, 0.25 μM film; Agilent Technologies, Santa Clara, CA). Samples were prepared by diluting 100 μl of e-liquid in 1 ml of methanol (optima grade) and vortexing for 30 s. Splitless injections (2 μl) were performed using a Bruker CP-8400 autosampler.

Cinnamaldehyde was quantified in e-liquid and VEC samples using the same GC-mass spectrometry (MS) system and operating conditions. Cinnamaldehyde standard solutions were prepared to concentrations of 7.22, 3.61, 1.44, 0.722, 0.361, and 0.181 mM 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 was used for quantitation of the Kola e-liquid, since it provided the limit of detection needed for the lower cinnamaldehyde concentration. Two reactions were used: mass-to-charge ratio (*m/z*) 131–77 and *m/z* 131–103. 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 1.5 mTorr, and the collision energy was 20 eV.

The GC oven was programmed to hold the temperature at 50°C for 2 min, ramp to 300°C at a rate of 15°C/min, and hold at 300°C for 6.33 min for a total run time of 25 min. The transfer line and electron ionization source were held at 250°C throughout the analysis. Compound identification and peak integration were 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 the legends for Figs. 1–8. 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 least-significant difference 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.

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 *S. aureus* BioParticles. The BioParticles used for this study emit a minimal fluorescent signal at physiological pH

(~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 PG/VG vehicle alone produced a slight but significant reduction in BioParticle phagocytosis (Fig. 1*A*). Of the seven e-liquids tested, only Kola (1%) and Sini-cide (1, 0.5, and 0.25%) significantly suppressed phagocytosis compared with the PG/VG vehicle control (Fig. 1*B*). 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 (Fig. 1*C*). 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 (Fig. 1*D*). 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 (Fig. 1*E*).

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, whereas 0.5% Sini-cide challenge significantly suppressed IL-6 secre-
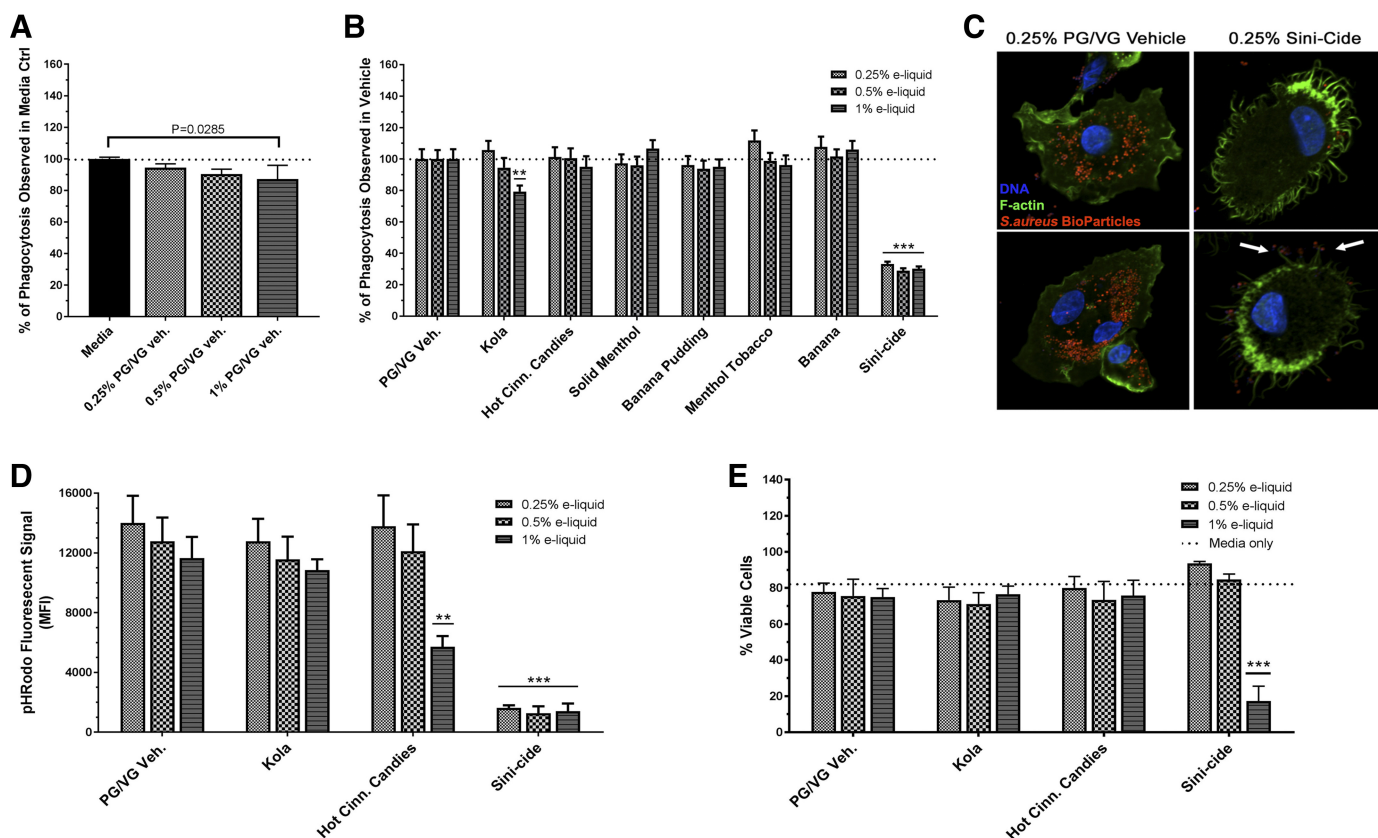

Fig. 1. Effects of flavored e-liquids on alveolar macrophage phagocytosis. Alveolar macrophages were challenged with 1, 0.5, and 0.25% dilutions of propylene glycol/vegetable glycerin (PG/VG) vehicle (*A*) or electronic cigarette liquids (e-liquids, *B*) for 1 h before the addition of *Staphylococcus aureus* BioParticles. Phagocytosis was assessed 4 h 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, nonphagocytosed 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.
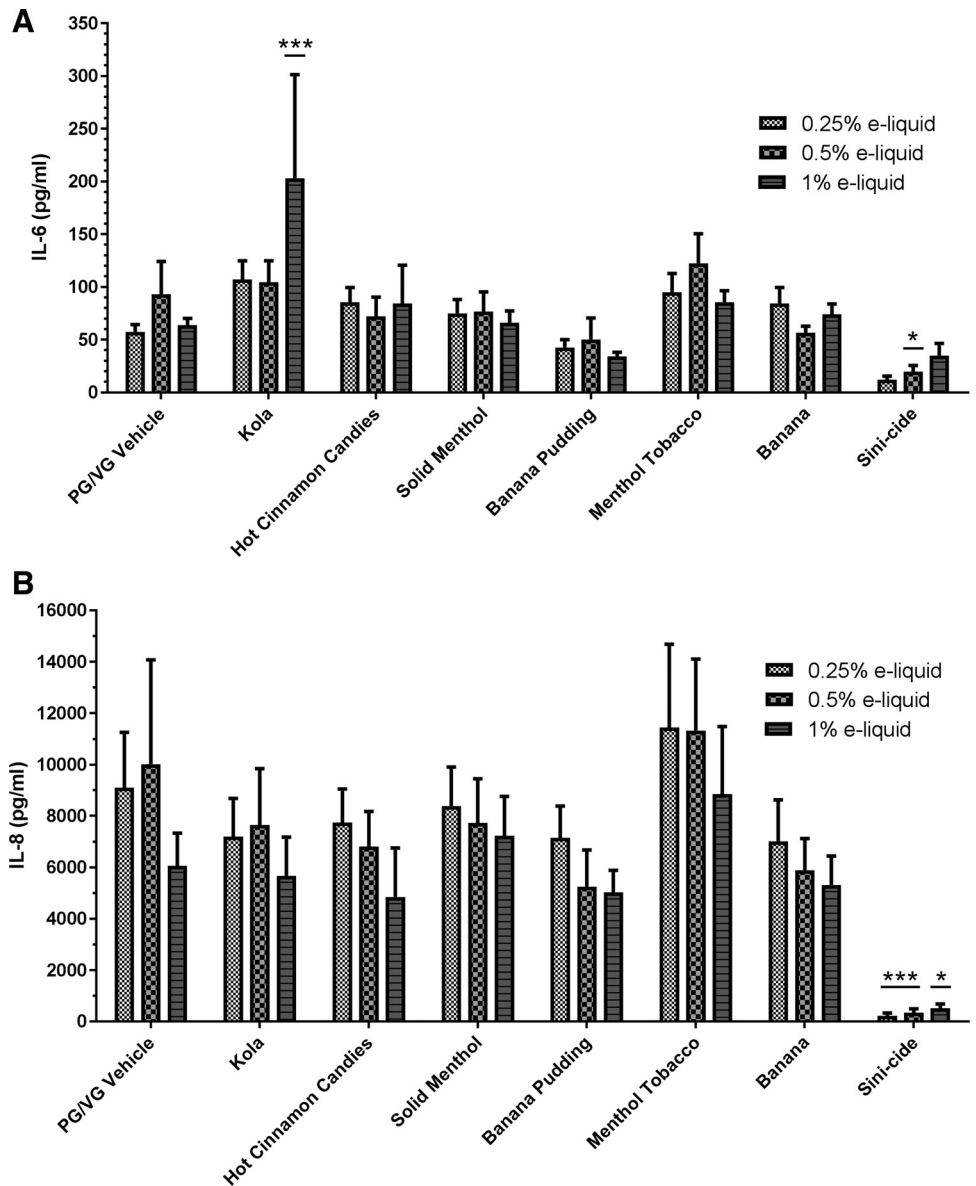
Fig. 2. Effects of flavored e-liquids on alveolar macrophage cytokine secretion. Alveolar macrophages were challenged with 1, 0.5, and 0.25% e-liquid dilutions for 24 h and evaluated for changes in interleukin (IL)-6 (*A*) and IL-8 (*B*) protein secretion (*n* = 5). Data are presented as means ± SE. Significance: *P < 0.05 and ***P < 0.001 compared with vehicle control.

tion relative to the PG/VG vehicle control (Fig. 2*A*). Sini-cide e-liquid significantly suppressed alveolar macrophage IL-8 secretion across all dilutions tested (Fig. 2*B*).

Effects of flavored e-liquids on neutrophils. We next 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 (Fig. 3*A*). 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 (Fig. 3*B*). Although toxicity was observed at higher concentrations for Kola (1%) and Sini-cide (1 and 0.5%) flavors, none of the flavors showed significant toxicity at 0.25%, suggesting that suppression of phagocytosis was not the result of e-liquid-induced cytotoxicity (lactate dehydrogenase 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 because of 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 (Fig. 3*C*).

We next 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 (Fig. 3*D*). Specifically, Kola (1 and 0.5%), Hot Cinnamon Candies (0.5%), Banana Pudding (1, 0.5, and 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 because
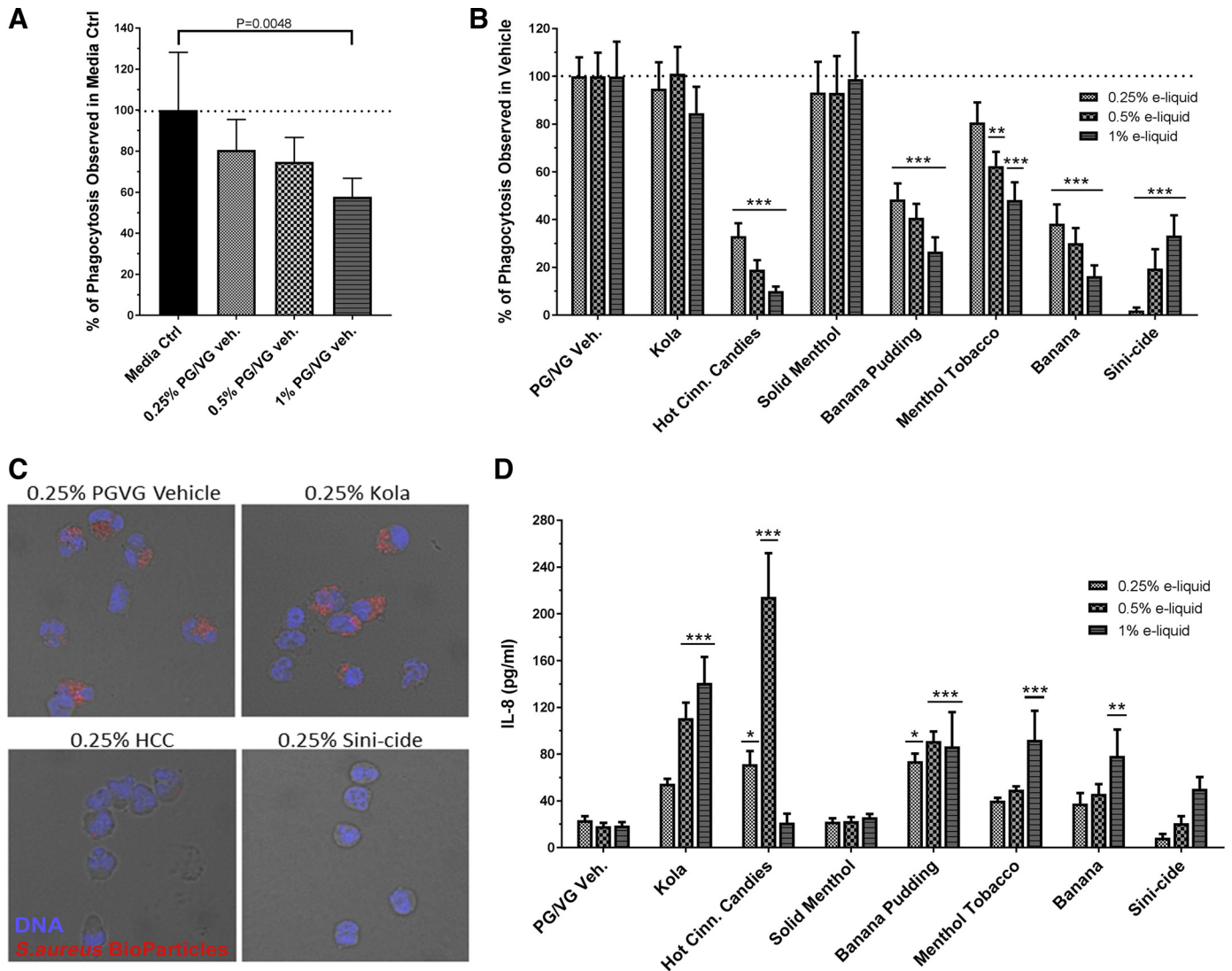
Fig. 3. Effects of flavored e-liquids on neutrophil phagocytosis. Neutrophils were challenged with 1, 0.5, and 0.25% dilutions of PG/VG vehicle (*A*) or e-liquids (*B*) for 30 min before addition of *S. aureus* BioParticles. Phagocytosis was assessed 4 h after the addition of Bioparticles ($n = 5$). *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 4 h 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.

of the significant cytotoxicity observed with the 1 and 0.5% challenges.

In addition to phagocytosis, neutrophils exert their antibacterial function via the formation of 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 25 nM PMA, a PKC agonist and potent activator of NET formation. Challenge of neutrophils with a 1% dilution of PG/VG with and without 25 nM PMA did not induce or alter NET formation compared with NET formation generated with media alone and media with 25 nM PMA (Fig. 4*A*). Similar to PG/VG, e-liquid challenges without the addition of PMA did not stimulate normal NET formation; however, 1% Sini-cide induced chromatin release at all time points (Fig. 4*B*). The pattern of chromatin extrusion observed with the Sini-cide challenge did not follow the oxidant-driven temporal progression of NET

formation (13, 19) or the rapid oxidant-independent generation of NETs (54), 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 25 nM PMA, and NET extrusion was quantified over 4 h. Stimulation of neutrophils with 1% Kola significantly enhanced chromatin release at 3 h, whereas challenge with 1% Hot Cinnamon Candies suppressed PMA-induced NET formation at 4 h (Fig. 4*C*). 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 (12) using confocal microscopy. Figure
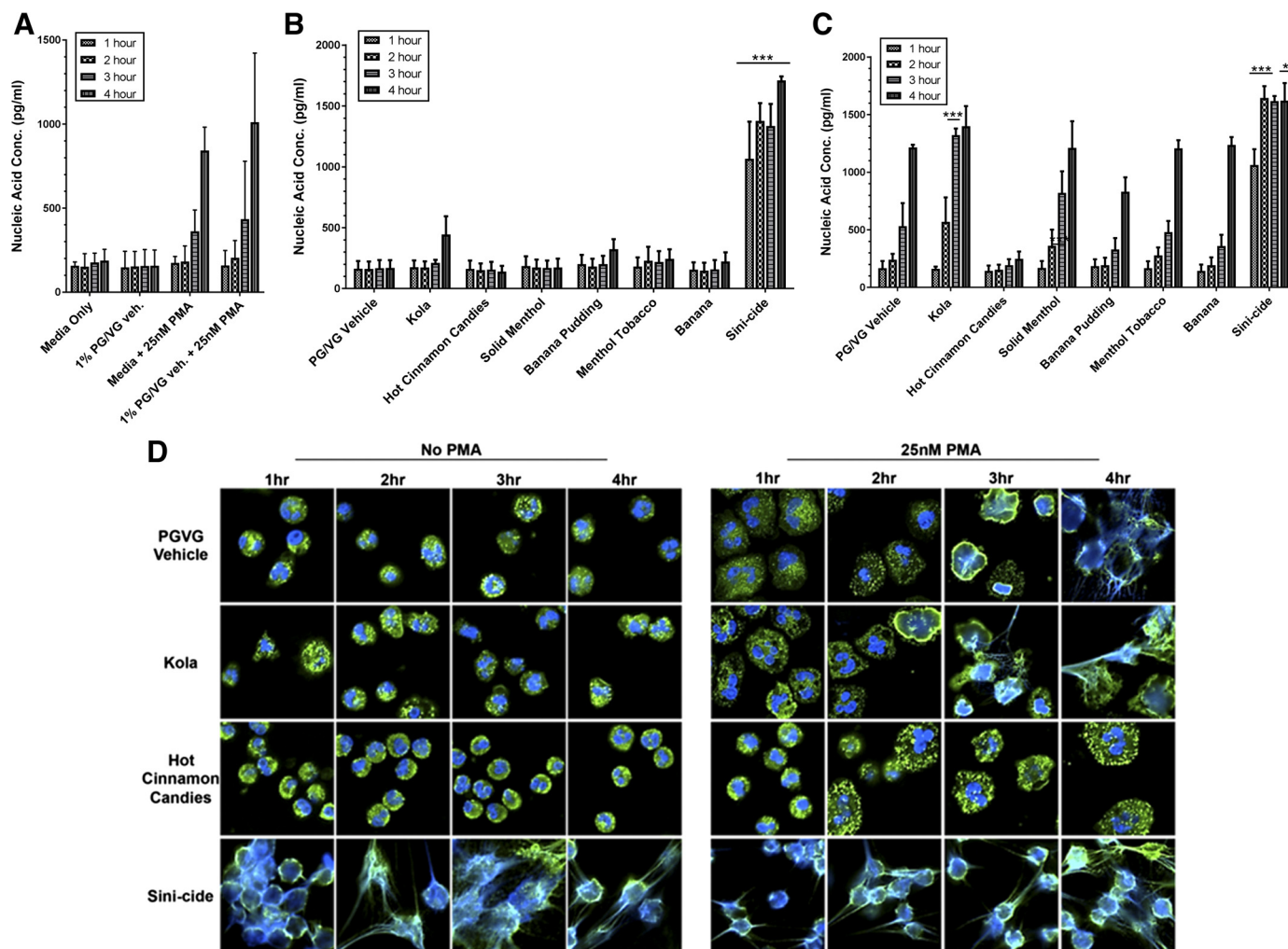
Fig. 4. Effects of flavored e-liquids on neutrophil extracellular trap (NET) formation. Quantitation of nucleic acid released during challenge with a 1% dilution of PG/VG vehicle (*A*) or e-liquid challenges with 25 nM phorbol 12-myristate,13-acetate (PMA, *B*) and without 25 nM PMA (*C*) (*n* = 3). *D*: confocal immunofluorescence microscopy of neutrophils challenged with 1% flavored e-liquids, with and without 25 nM PMA. Data are presented as means ± SE. Significance: *$P < 0.05$ and ***$P < 0.001$ compared with vehicle control.

4*D* indicates that Sini-cide was cytotoxic, resulting in chromatin release by cell lysis within the 1st h of exposure, confirming the nonspecific release of DNA seen in Fig. 4, *B* and *C*. 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 h, rather than 4 h. 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 has differential effects on NET formation. Furthermore, the visual illustration in Fig. 4*D* largely reflects the quantitative assessment of chromatin release reported in Fig. 4, *B* and *C*.

Numerous studies have reported that nicotine elicits a variety of immune suppressive responses (31, 58, 59) and prolongs neutrophil survival (3, 43, 70). To investigate whether nicotine could alter the observed e-liquid-induced suppression of neutrophil phagocytosis, we added nicotine to the PG/VG 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 (Fig. 5*A*). In addition, emerging evidence indicates that thermal decomposition of e-liquids during the vaping process can modify the chemical composition of the aerosol mixtures (35). To examine whether and how aerosolization of e-liquids affects the suppressive effects on neutrophil phagocytosis, we prepared VEC, similar to previously published methods (52). Figure 5*B* shows that stimulation of neutrophils with PG/VG vehicle, Kola, Hot Cinnamon Candies, or Sini-cide VEC did not significantly alter neutrophil phagocytosis compared with the unheated e-liquid challenges. These data suggest that vaping and aerosolization of these e-liquids do not significantly modify the ability to suppress neutrophil phagocytosis.

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 before 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
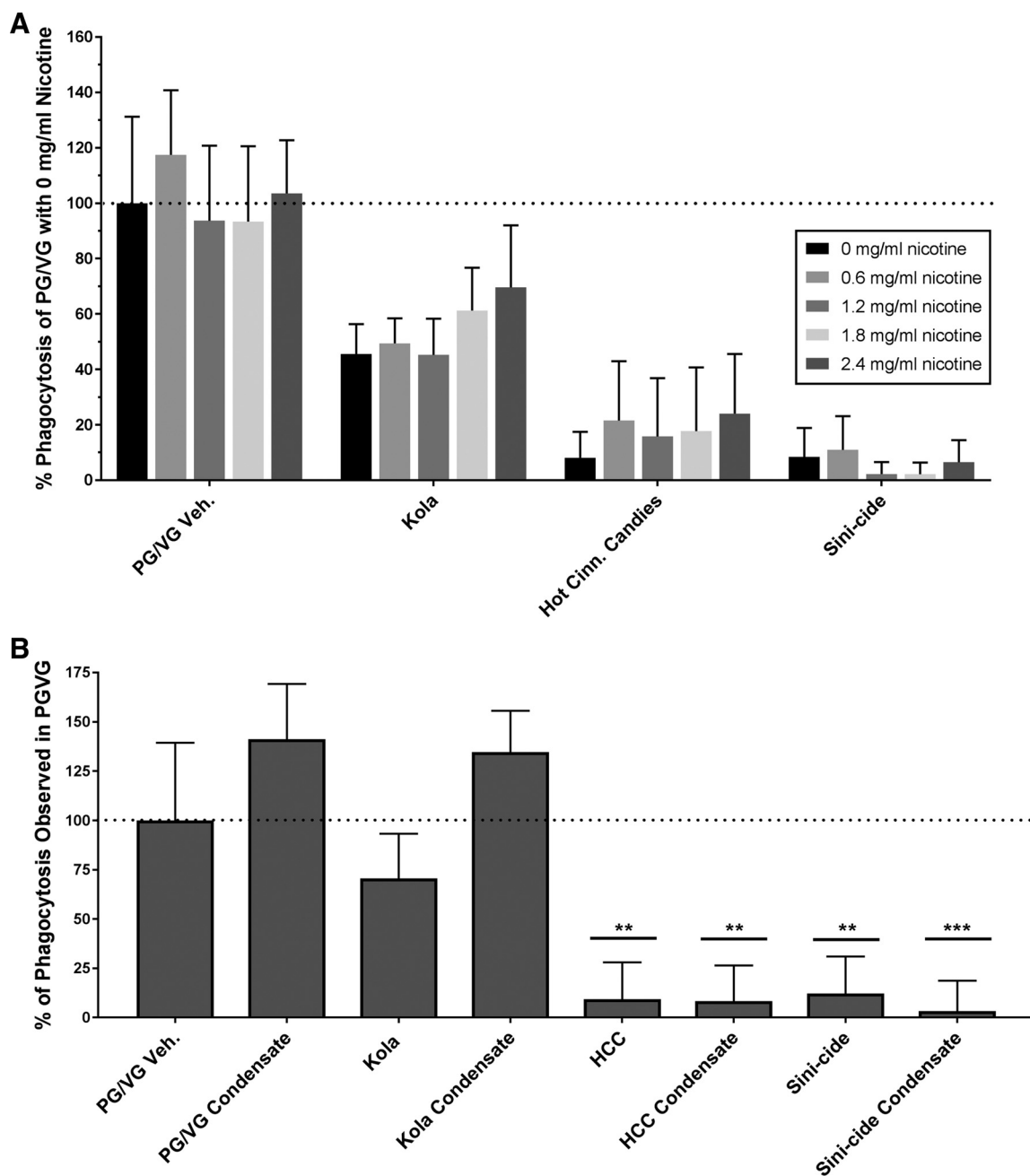
Fig. 5. Effects of nicotine and vaping on e-liquid-induced changes in neutrophil phagocytosis. Nicotine was added to the Kola, Hot Cinnamon Candies, Sini-cide, and PG/VG vehicle e-liquids at concentrations commonly stocked in vape shops (2.4, 1.8, 1.2, and 0.6%). *A*: nicotine-containing e-liquids were 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 4 h 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 30 min before the addition of *S. aureus* BioParticles. Phagocytosis was assessed 4 h 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.

dilution. Following 4 h incubation, the ability of the NK cells to eliminate target cells was determined using flow cytometry (Fig. 6*A*). 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 compared with the PG/VG vehicle control (Fig. 6, *A* and *B*). Reductions in NK cell killing capacity were not the result of a loss of NK cell viability (data not shown).

Chemical composition of flavored e-liquids. GC-coupled MS was used to determine the qualitative chemical composition of the seven 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 S1 (Supplemental data for this article is available on the Journal website.)]. Quantitative MS analysis for cinnamaldehyde con-
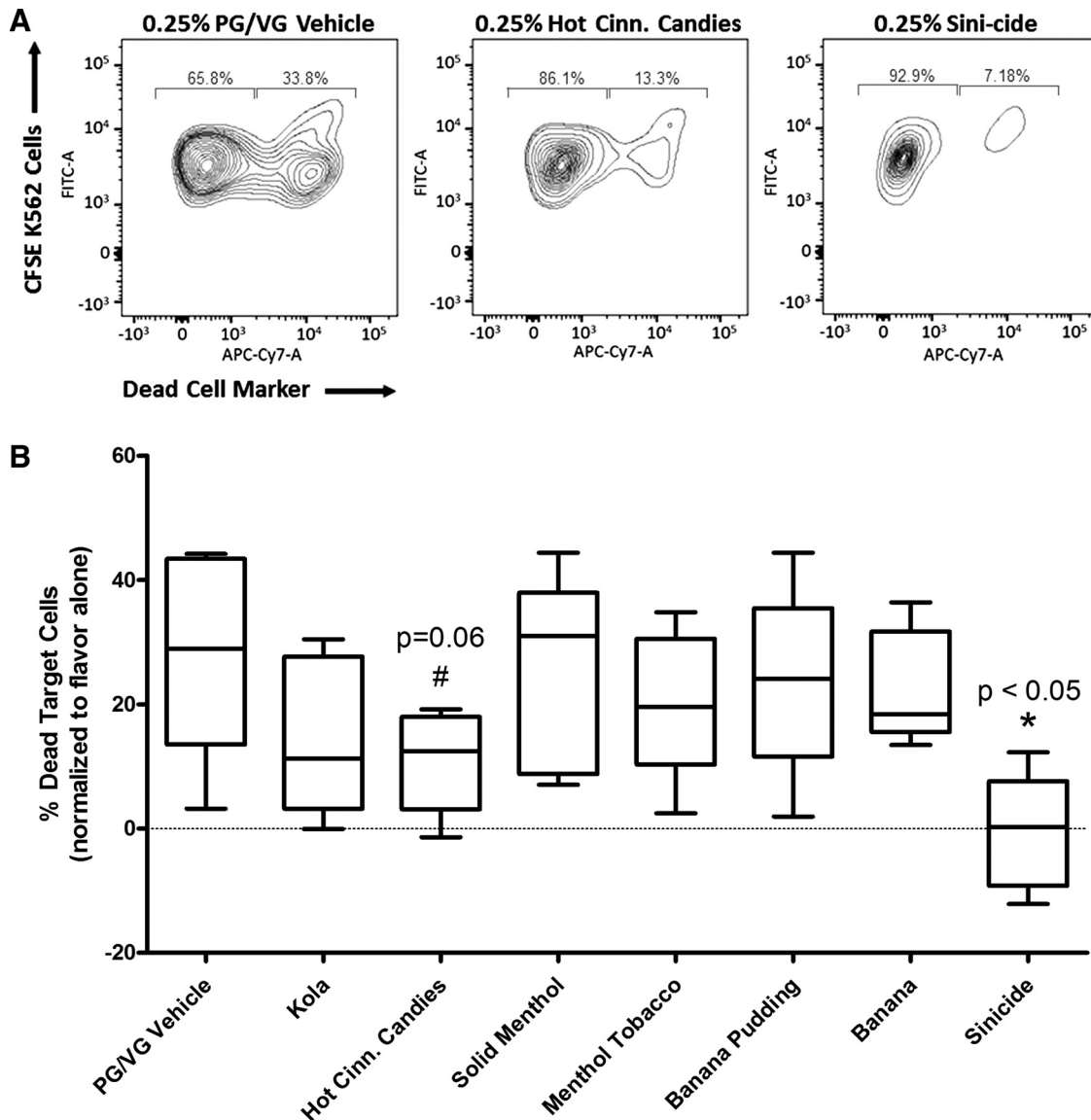
Fig. 6. Effects of flavored e-liquids on natural killer (NK) cell-mediated cytotoxicity. NK cells were incubated for 1 h with 0.25% e-liquids and then combined with carboxyfluorescein succinimidyl ester (CFSE)-labeled K562 target cells for 4 h. *A*: flow cytometry analysis of CFSE bright target cells 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.

tent in these flavored e-liquids revealed that the Kola, Hot Cinnamon Candies, and Sini-cide contained 0.896 ± 0.360, 39.328 ± 3.414, and $1,131.0 \pm 19.9$ mM, respectively (Table 1). Based on these measured concentration 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 reflecting wall loss caused by the system generating the condensate.

Effects of cinnamaldehyde on alveolar macrophage, neutrophil, and NK cell function. MS 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 end points. Alveolar macrophages were incubated with cinnamaldehyde for 1 h before the addition of *S. aureus* BioParticles. Changes in phagocytosis were assessed after 4 h. A significant reduction in phagocytosis was observed with cinnamaldehyde concentrations ≥ 312.5 μM, whereas cinnamaldehyde-induced cell death was only apparent at the 10 mM concentration (Fig. 7*A*). Similarly, neutrophils were challenged for 30 min with a broad range of cinnamaldehyde concentrations, and phagocytosis of BioParticles was assessed. Neutrophil phagocytosis was significantly inhibited at cinna-

Table 1. *Quantitation of cinnamaldehyde in e-liquids and vaped e-liquid condensates*

	Kola	Hot Cinnamon Candies	Sini-cide
Neat e-liquid, mM	0.896 ± 0.360	39.32 ± 3.41	1,131 ± 19.9
Dilution, mM			
1%†	0.00896	0.393	11.31*
0.5%†	0.00448	0.197	5.66*
0.25%†	0.00224	0.0983	2.83*
Vaped e-liquid condensate, mM	0.742 ± 0.035	9.807 ± 0.743	183.9 ± 4.089

Values are means ± SD. *Units are mM. †Cinnamaldehyde in e-liquid dilutions is extrapolated from neat e-liquid measurements.

maldehyde concentrations of 10 μM and greater. Viability was significantly reduced with 5 and 10 mM cinnamaldehyde exposures (Fig. 7*B*). 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 1 h before incubation with fluorescently labeled K562 target cells. Flow cytometry analysis revealed that NK cell killing efficiency was significantly reduced with cinnamaldehyde challenges between 78 μM and 1 mM. Exposure of target cells to cinnamaldehyde concentrations >1.25 mM resulted in significant cell death independent of NK cell activity. Cinnamaldehyde significantly reduced NK cell viability at concentrations of 625 μM and greater (Fig. 7*C*). The inhibitory (IC_{50}) and cytotoxic (LC_{50}) concentrations for cinnamaldehyde on each end point in the respective cell type tested are reported in Table 2.

Cinnamaldehyde is a potent electrophile that likely reacts with free sulfhydryl groups on cysteine residues on proteins (41). 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 DTT. Cinnamaldehyde-mediated suppression of alveolar macrophage phagocytosis was reversed by coexposure with cinnamaldehyde and DTT (Fig. 8), suggesting protein thiol modification as a potential mechanism for cinnamaldehyde-induced immune cell dysfunction.

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 (16, 24, 44, 66). Recent publications have highlighted the potential risk for exposure to reactive chemicals (1, 6, 9, 22, 60, 63) and toxicants (21, 35, 69), 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 (42). 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 end points across all three human primary immune cell types, our data demonstrate that cinnamaldehyde-containing e-liquids had the most immunosuppressive effects across the different end points 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 of 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-cigarette 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 (8), 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 (8). 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 (40). 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). 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 consist predominately of alveolar and airway macrophages, neutrophils in the perialveolar vasculature, and NK cells in the nasal passages and sinuses (30), 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 increase potential susceptibility to respiratory infections. Hwang et al. recently reported that e-cigarette vapor extract reduced both macrophage and neutrophil antimicrobial activity against *S. aureus* (31). However, the effect was attributed to nicotine, PG, 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 *S. aureus*; however, the impaired clearance we observed was flavor specific, with phagocytosis being suppressed more broadly in neutrophils (Figs. 1 and 3). Moreover, the addition of nicotine to our e-liquid challenges did not significantly alter the suppression of phagocytosis
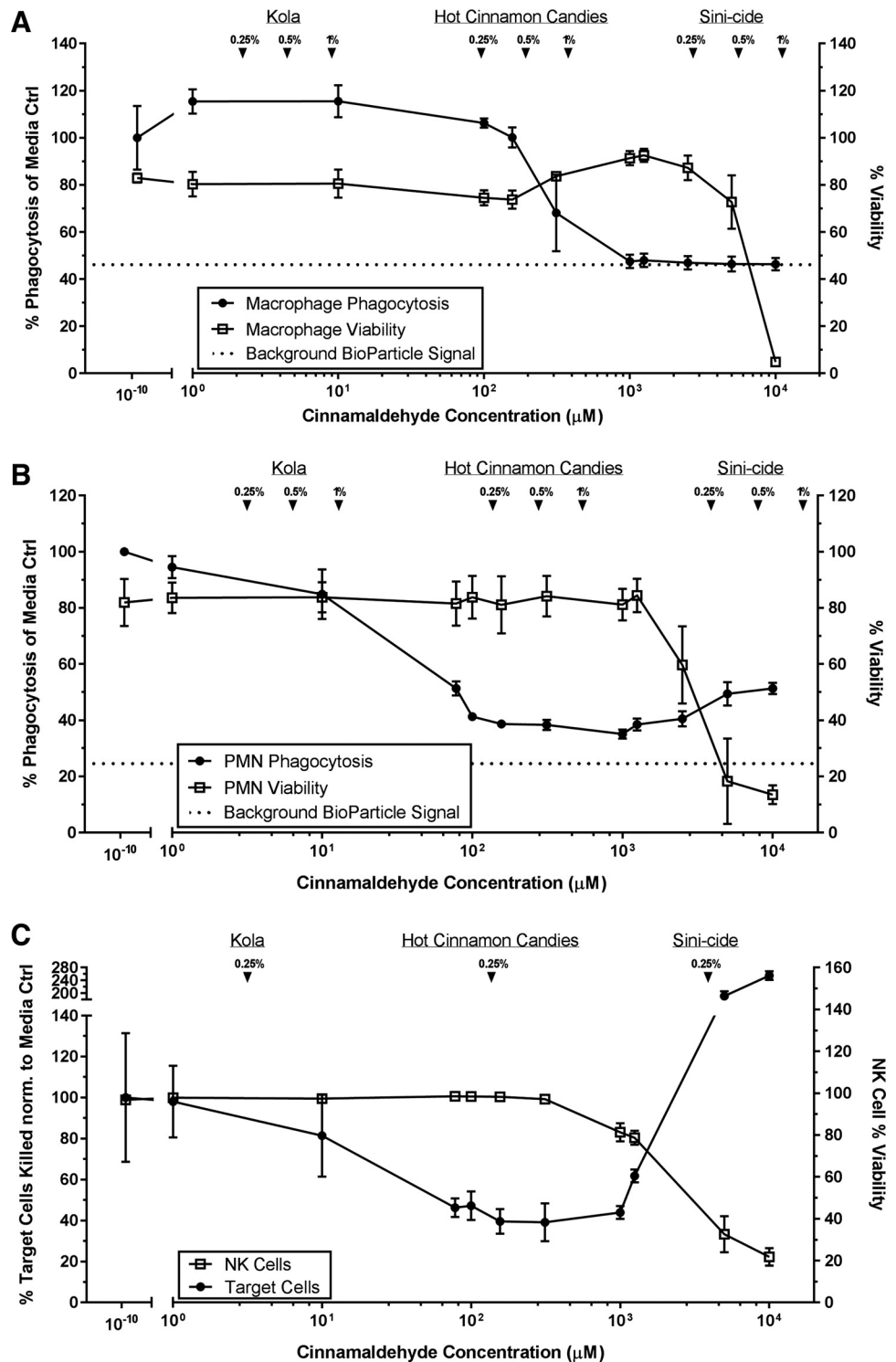
Fig. 7. Effects of cinnamaldehyde on alveolar macrophage, neutrophil, and NK cell function. Alveolar macrophages (*A*) (*n* = 3) and neutrophils (*B*) (*n* = 3) were challenged with a broad range of cinnamaldehyde concentrations. Cinnamaldehyde-induced changes in BioParticle phagocytosis and cell viability 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.

observed with exposure to nicotine-free e-liquids (Fig. 5). Although nicotine, at sufficient concentrations, may independently suppress innate immune cell activation and impair the clearance of *S. 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 (36). 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 DTT. The observed cinnamaldehyde-mediated suppression of alveolar macrophage

Table 2. *The inhibitory and cytotoxic potential of cinnamaldehyde on human primary alveolar macrophages, neutrophils, and NK cells*

	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

Values are means $\pm$ SD. NK, natural killer; IC_{50}, half-maximal inhibitory concentration; LC_{50}, half-maximal cytotoxic concentration.

phagocytosis was reversed by coexposure with DTT, suggesting that the sulfhydryl-modifying activity of cinnamaldehyde correlates with cinnamaldehyde-induced reductions in phagocytic capacity (Fig. 8). Although 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* (20), *Klebsiella pneumoniae* (33), *Legionella pneumophila* (65), and *S. aureus* (48). 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 antibacterial function of neutrophils (49). Furthermore, recent studies of neutrophil function (including cell migration, ROS generation, degranulation, phagocytosis, and NET generation) in COPD patients indicate impaired responses that predispose toward reduced bacterial clearance and increased inflammation (62). 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 (49). Although levels of acrolein in e-liquids and e-cigarette aerosols are reported to be substantially lower than those present in cigarette smoke (24), 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 *S. aureus* in a dose-dependent manner (Fig. 3, *A* and *B*), and subsequent challenge with cinnamaldehyde alone recapitulated the effect (Fig. 7*B*). Moreover, cinnamaldehyde-induced inhibition of phagocytosis was observed at low micromolar concentrations ($IC_{50} = 32.4 \pm 12.9$ μM), more than two orders of magnitude lower than those eliciting cytotoxicity ($LC_{50} = 3.35 \pm 0.84$ mM) (Table 2).

Neutrophils also eliminate bacterial pathogens by expelling extracellular traps composed of chromatin and antimicrobial peptides originating from intracellular vesicles (11, 19, 62). NET formation is initiated by stimulation of surface receptors, including Toll-like receptors, cytokine receptors, and fragment crystallizable receptors, resulting in activation of PKC and the NADPH oxidase complex (26, 62). Whereas the exact role of NETs in respiratory disease remains unclear, accumulation of NETs has been implicated in the pathogenesis of numerous noninfectious diseases, inflammatory disorders, and autoim-

mune disorders, including cystic fibrosis, asthma, and COPD (27). 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 (71). Our finding that 1% Kola (8.96 μM cinnamaldehyde) significantly increased the rate of PMA-induced NET formation, whereas 1% Hot Cinnamon Candies (393.28 μM cinnamaldehyde) significantly decreased the rate of PMA-induced NET formation, suggests that cinnamaldehyde may elicit a concentration-dependent bimodal response (Fig. 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 use activating and inhibiting receptors to interrogate host cells for molecular changes indicative of damage or infection (68). 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 (67), and cigarette smoke reduces NK cell-mediated killing of cancer cells in vitro (17). 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 (30). Here, we expanded on the current literature by evaluating the effects of flavored e-liquids and cinnamaldehyde flavoring on NK cell function. Our data indic-
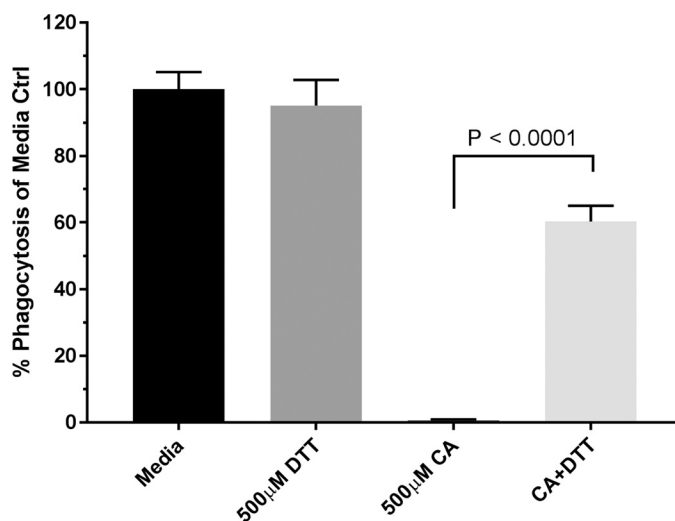

Fig. 8. Coexposure of alveolar macrophages with cinnamaldehyde and DTT reverses cinnamaldehyde-induced suppression of phagocytosis. Alveolar macrophages were challenged with cell culture medium, 500 μM DTT, 500 μM cinnamaldehyde, or 500 μM DTT and 500 μM cinnamaldehyde, and changes in BioParticle phagocytosis were evaluated ($n = 3$). Data are presented as means $\pm$ SE.

ate 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 (Fig. 5). Exposure to cinnamaldehyde alone recapitulated this effect, inhibiting NK cell killing at low micromolar concentrations (IC$_{50}$ = 20.5 ± 5.4 μM) (Fig. 7 and Table 2). 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 FDA in 2009, are broadly used in the manufacture of e-cigarettes and refill e-liquids (73) and contribute to the surging popularity of these products (15). 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 (39). A recent study published by Allen et al. screened 51 unique flavored e-liquids for the presence of diacetyl, acetoin, and 2,3-pentanedione (1) 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 the result of chemical flavorings (7, 25). 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 (5). 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 (22). 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 broadly contain potentially hazardous chemicals and illustrate the need to investigate the respiratory effects of common flavorings used in the manufacture of e-liquids.

DISCLAIMERS

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health or the Food and Drug Administration.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

P.W.C., E.A.P., S.L.R., G.L.G., and I.J. conceived and designed research; P.W.C., E.A.P., J.T.L., J.E.K., and S.L.R. performed experiments; P.W.C., E.A.P., S.L.R., G.L.G., and I.J. analyzed data; P.W.C., E.A.P., and I.J. interpreted results of experiments; P.W.C. and E.A.P. prepared figures; P.W.C. and I.J. drafted manuscript; P.W.C., E.A.P., J.T.L., J.E.K., S.L.R., G.L.G., and I.J. edited and revised manuscript; P.W.C., E.A.P., J.T.L., J.E.K., S.L.R., G.L.G., and I.J. approved final version of manuscript.

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