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ORIGINAL ARTICLE

Electronic cigarette inhalation alters innate immunity and airway cytokines while increasing the virulence of colonizing bacteria

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Abstract
Electronic (e)-cigarette use is rapidly rising, with 20 % of Americans ages 25–44 now using these drug delivery devices. E-cigarette users expose their airways, cells of host defense, and colonizing bacteria to e-cigarette vapor (EV). Here, we report that exposure of human epithelial cells at the air–liquid interface to fresh EV (vaped from an e-cigarette device) resulted in dose-dependent cell death. After exposure to EV, cells of host defense—epithelial cells, alveolar macrophages, and neutrophils—had reduced antimicrobial activity against *Staphylococcus aureus* (*SA*). Mouse inhalation of EV for 1 h daily for 4 weeks led to alterations in inflammatory markers within the airways and elevation of an acute phase reactant in serum. Upon exposure to e-cigarette vapor extract (EVE), airway colonizer SA had increased biofilm formation, adherence and invasion of epithelial cells, resistance to human antimicrobial peptide LL-37, and up-regulation of virulence genes. EVE-exposed SA were more virulent in a mouse model of pneumonia. These data suggest that e-cigarettes may be toxic to airway cells, suppress host defenses, and promote inflammation over time, while also promoting virulence of colonizing bacteria.

Key message

- Acute exposure to e-cigarette vapor (EV) is cytotoxic to airway cells in vitro.
- Acute exposure to EV decreases macrophage and neutrophil antimicrobial function.
- Inhalation of EV alters immunomodulating cytokines in the airways of mice.
- Inhalation of EV leads to increased markers of inflammation in BAL and serum.
- *Staphylococcus aureus* become more virulent when exposed to EV.

Keywords E-cigarette vapor · Staphylococcal virulence · Cytotoxicity · Inflammatory lung disease · Antimicrobial peptide LL-37 · MRSA pneumonia

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Introduction

Electronic (e)-cigarette "vaping" is the inhalation of an electrically heated and aerosolized liquid containing nicotine. E-cigarettes were invented in 2003 and rapidly gained popularity through adolescent and adult smokers of all ages, sexes, and races [1]. E-cigarette use is tripling annually, with use increasing in both non-smoking and smoking children and adults [2–5]. Cigarette smokers who also use e-cigarettes do not have higher rates of intention of quit [6–8]. Annual sales of e-cigarettes now total $2 billion, and analysts predict they will outsell traditional cigarettes within 10 years. E-cigarette makers are flavoring and naming their wares to appeal to children and women, who prefer sweet flavors [9]. One in ten high schoolers have tried e-cigarettes in the past year, double that of the year prior [1]. Therefore, e-cigarette use is creating a new generation of nicotine addicts. Despite being advertised as a safe alternative to conventional combustible cigarettes, little is known about e-cigarettes' safety and their potentially deleterious effects on health.

E-cigarettes are composed of a cartridge of "e-liquid," which contains propylene glycol (PG) and/or vegetable glycerin (VG) plus nicotine. Many users mix their own e-liquid so that they can modify the nicotine content [10]. Nicotine absorption into the bloodstream is similar in the latest generation of e-cigarettes compared to conventional cigarettes [11]. E-liquid is introduced into a cartridge, which also contains an atomizer and heating vapor coil to produce the vapor. This cartridge is attached to a rechargeable battery.

The pathogenic bacteria *Staphylococcus aureus* (SA) persistently colonizes 20 % of humans and transiently colonizes 60 % [12]. We recently demonstrated that conventional cigarette smoke promotes SA pathogenicity via a general stress response and leads to increased virulence in vivo [13]. Suggesting that susceptibility of smokers to bacterial infections may be due in part to pro-virulent effects on colonizing bacteria. Because colonizing SA strains in the nasopharynx are exposed to all inhaled substance, including e-cigarette vapor (EV), we hypothesize that EV may affect SA pathogenicity.

The many toxic, inflammatory, and carcinogenic effects of conventional cigarette smoke have been well characterized over the past century [14]. However, it is unknown whether vaping e-cigarettes will also increase the incidence of inflammatory, infectious, or malignant lung diseases or systemic diseases. To evaluate the potential impact of e-cigarette use on susceptibility to bacterial infections and development of inflammatory responses, we undertook these studies of e-cigarette effects on both host and pathogen functions.

Methods

E-cigarettes

E-cigarettes were purchased from San Diego County gas stations and vaporiums, as well online from Xtreme Vaping. Flavored and unflavored e-liquids were selected, as well as with and without nicotine (Table 1). E-liquid was never added directly to media or cells; all exposures were done by creating aerosol ("vapor") with e-cigarette devices, via application of voltage to the heating coil within the e-liquid, and aerosolization. Unless otherwise noted, the e-cigarette liquid was mixed in the lab, using 50 % PG, 50 % VG, and 24 mg/mL (148 mM) nicotine (PG + VG + N; a common blend of e-liquid, based on content labels on multiple brands of e-cigarettes and by blogs of e-cigarette users who make their own e-liquid). Cartridges and batteries were bought from Xtreme Vaping, with each e-liquid brand having a dedicated cartridge, except the brands

Table 1 E-cigarette brands and composition

Brand	Materials	Type	Flavor
Free Masons Elixer	PG/VG with 14 mg/mL Nic	E-liquid	Treasury
Green Smart Living	PG/VG with 0.6 % (low) Nic PG/VG with 1.8 % (high) Nic	Vape pen with cartridges containing e-liquid	None
Grimm Creations	PG/VG with 18 mg/mL Nic	E-liquid	Highlander Grog
NJoy	PG	Vape pen containing e-liquid	None
Vape Addict Juices	PG/VG with 18 mg/mL Nic	E-liquid	California Blues
Vapure	PG/VG with Nic	E-liquid	None
Xtreme Vaping	e-liquids: PG, VG Nic: 50 mg/mL in PG Lithium batteries Cartridges/Tanks	Refillable vape pens for all brands of e-liquids and mixable liquids	None
Xtreme Vaping	80 % PG/20 % VG with 24 mg/mL Nic	E-liquid	Pure smoke

PG propylene glycol, *VG* vegetable glycerin, *Nic* nicotine

that came pre-loaded (NJoy and Green Smart Living (GSL)) (Table 1).

E-cigarette vapor extract

See Supplementary material.

Cells and culture methods

Two types of human epithelial cells were used for these studies—human keratinocytes (HaCaTs; CLS) and human lung alveolar type II epithelial cells (A549 cells; ATCC CCL-185) [15]. Both cell types express nicotinic receptors, which are believed to play a role in lung cancer pathogenesis [16, 17]. HaCaTs express nicotinic acid receptors GPR109A and B [18], while A549 cells express α5-nicotinic acetylcholine receptors (nAChRs) [19, 20]. HaCaTs and A549s were cultured in RPMI + 10 % fetal bovine serum (FBS). MH-S cells (ATCC CRL-2019) were cultured in RPMI + 10 % FBS + 0.05 mM beta-mercaptoethanol. MRSA USA300 was used for all mammalian cell infections and bacterial studies. Cultures of MRSA were started the day prior to assays and grown overnight in Todd Hewitt Broth (THB) at 37 °C with shaking. Control media and e-cigarette vapor extract (EVE) for mammalian cell studies were made using RPMI 1640 + 10 % FBS as the base media. EVE and control media for bacterial cell exposure were made with the addition of a small fraction of bacteriologic media: RPMI 1640 + 10 % FBS + 20 % THB as the base media. Both of these medias are accepted methods for CSE studies [21, 22]. Same-day MRSA subcultures were prepared in control media and 75 % EVE and grown to mid-log phase (OD_{600} 0.6–0.8) before centrifugation (1620×*g*, 10 min) and resuspension at 1×10^8 colony-forming units (CFU)/mL (OD_{600} 0.4) prior to dilution in assay media to the final CFU/mL. For macrophage assays, MRSA were pre-opsonized with mouse serum (Applied Biosystems) and incubated with 1×10^5 MH-S macrophages at a multiplicity of infection (MOI) of 0.1. For human neutrophil assays, MRSA were pre-opsonized with human serum for 30 min and then incubated for 2 h with neutrophils at an MOI of 0.1. For enumeration, MRSA was serially diluted and plated on THA.

Exposure of epithelial cells and macrophages to EVE and EV

See Supplementary material.

Neutrophil isolation from fresh human blood

See Supplementary material.

Bacterial killing by keratinocytes, macrophages, and neutrophils

See Supplementary material.

Mouse inhalation of e-cigarette vapor

Female CD-1 mice, 6–8 weeks old from Jackson Labs, were used for all studies. The SciReq inExpose system was used to limit EV effects to the respiratory tract. Mice were exposed to 9 s of EV per minute for 60 min. Experimental controls were also placed in mesh holders but breathed air alone (Air). Negative controls were kept in the mouse facility (Control). Mice were recovered in pre-warmed cages for 1 h. Mice were euthanized 24 h after their final exposure via inhaled CO_2. Blood was collected by intracardiac puncture followed by bronchoalveolar lavage (BAL) with 1 mL phosphate-buffered saline (PBS) three times. Right lung lobes were placed in RNA later. The left lobe was inflated with 4 % paraformaldehyde (PFA) for paraffin embedding and H&E and trichrome staining for blinded histologic analysis. BAL cells were counted via trypan blue exclusion, cytospun, and stained with Giemsa Wright for cell differential analysis. BAL cells and parenchymal lung cells were stained for multiple leukocyte markers and evaluated by flow cytometry. Blood was spun at 1620×*g* for 10 min to isolate serum. Serum and BAL supernatant were stored at −80 °C until use. All animal experiments were approved by both the VA and UCSD IACUC.

Proteome evaluation

Proteome profiler array (mouse cytokine array panel A, R&D Biosystems) was used according to manufacturer's instructions. Briefly, 1-mL samples were incubated with nitrocellulose membranes at 4 °C overnight. Membranes were washed, streptavidin-horseradish peroxidase applied, followed by chemiluminescent reagents. Membranes were exposed to film and processed through a developer.

Bacterial growth curves, biofilm formation, and hydrophobicity assays

See Supplementary material.

Adherence and invasion of human keratinocytes

See Supplementary material.

Antimicrobial peptide (LL-37) resistance and surface charge assays

See Supplementary material.

Murine MRSA pneumonia

Female CD-1 mice (6–10 weeks old, Charles River) were sedated with ketamine/xylazine and infected intranasally with 2×10^8 CFU MRSA in 75 μL. Mice were kept upright for 1 min and recovered with heads elevated at 30°. Mice were checked twice daily, with mortality and weight documented every 24 h. On day 4 post-infection, live mice were harvested via CO_2 asphyxiation. Left lungs were inflated with 10 % formalin for histology. Right lungs were weighed and homogenized using 1.0-mm zirconia/silica beads (Bio Spec Products) and MagNA Lyser (F. Hoffmann-La Roche). Of undiluted lung homogenate, 100 μL was spread on a THA plate, and the remainder was serially diluted and plated to enumerate surviving bacteria. Pneumonia experiments were repeated three times. All methods were approved by the VA IACUC, protocol no. 11-017. All efforts were made to minimize animal suffering.

qRT-PCR of MRSA

See Supplementary material.

Statistics

See Supplementary material.

Results

E-cigarette vapor extract reduces keratinocyte antimicrobial activity

Epithelial cells are the front-line defense against invasion and infection. To examine how EV affects host defense in the respiratory tract, we used e-liquid containing 50 % PG, 50 % VG, and nicotine, both 8 and 24 mg/mL (49 and 148 mM), which are common high and low nicotine concentrations used by humans. E-liquid was placed into the cartridge of an e-cigarette device and generated the aerosol (vapor). The vapor was bubbled through media to make EVE, and the EVE was applied to human keratinocytes (HaCaTs). HaCaTs are a well-established model for nasopharyngeal epithelium, one of the main sites of SA colonization and the gateway to the lungs, and give consistent results due to clonality [23, 24]. HaCaTs express nicotinic acid receptors GPR109A and B [18]. HaCaTs were exposed to EVE for 1 h at 37 °C with 5 % CO_2, followed by 2 h recovery in regular (e-cigarette vapor-free) media. Bacteria were not exposed to EVE.

Exposure of HaCaTs to EVE made from 24 mg/mL (148 mM) nicotine e-liquid (EVE High Nic) prior to bacterial infection led to 25 % greater methicillin-resistant *S. aureus* (MRSA) numbers compared to control ($P < 0.001$; Fig. 1a). Nicotine had a dose effect, with EVE made from 8 mg/mL (49 mM) nicotine e-liquid (EVE Low Nic) resulting in fewer bacteria compared to EVE High Nic but more bacteria compared to control ($P < 0.01$; Fig. 1a).

Exposure to e-cigarette vapor at the air–liquid interface induces cell death in epithelial cells

To determine whether the decreased ability to control the Staphylococcal infection was due to cell death induced by EVE, HaCaTs were exposed to EVE, allowed to recover for 2 h, harvested, and enumerated by trypan blue exclusion. For increased physiologic relevance, human epithelial cells (HaCaTs and A549s) were grown on transwells with the apical surface exposed to air or EV. Both direct exposure of the apical cell surface to EV and incubation in EVE led to significant cell death, ranging from 50 to 70 % for vapor made from PG and PG + N and 99 % for vapor made from VG and VG + N (air-interface data shown in Fig. 1b). Nicotine at 2 mg/mL in media applied to the apical surface of cells for 15 min killed 32 % of cells. Addition of nicotine to the e-liquid base PG increased cytotoxicity by 20 %. No difference was seen when nicotine was added to VG (VG + N); however, this may have been due to maximal cytotoxicity seen by VG alone. Exposure of A549 cells to EVE induced cell death in a dose-dependent manner (Fig. 1c).

Multiple e-cigarette brands were tested to determine whether the cytotoxic effect was generalizable. Cytotoxicity was seen with four of the six additional brands. Direct exposure on air–liquid interfaces led to 96, 99, 97, and 41 % cell death with Pure Smoke, Vapure, Treasury, and Highlander Grog, respectively (Fig. 1d). Cell death was no different at 2 and 18 h post-exposure. Addition of flavors to e-liquids did not influence cytotoxicity. Exposure to NJoy, a pre-loaded cartridge type e-cigarette, and Cali Blues, an e-liquid added to a conventional e-cigarette for vaping (Table 1), did not induce cytotoxicity.

Cell death caused by e-cigarette vapor exposure occurs through necrosis

To characterize the method of cytotoxicity induced by e-cigarette vapor exposure, lactate dehydrogenase (LDH) levels were measured in A549 cells after exposure to vaped e-liquid components. Compared to LDH release from air-exposed A549 control cells, exposure to each vaped e-liquid component led to significantly higher levels of LDH released into the supernatant (Fig. 1e). The high levels of LDH found within 2 h of EV exposure are indicative of necrotic cell death. Cells also stained positively for EthD-IIIi and surface Annexin-V 2 h after exposure (Supplemental Fig. S1).

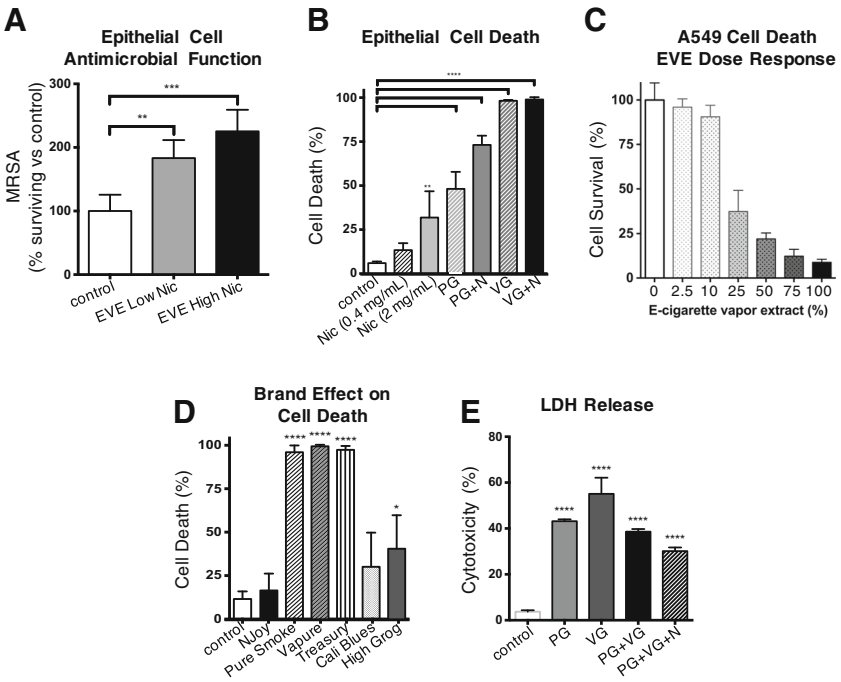

Fig. 1 E-cigarette vapor exposure decreases the ability of keratinocytes to fight bacterial infection via induction of cell death. **a** Keratinocytes exposed to EVE containing low nicotine (8 mg/mL, 49 mM, prior to vaping; *EVE Low Nic*) and high nicotine (24 mg/mL, 148 mM, prior to vaping; *EVE High Nic*) or control (*no EVE*) for 1 h, followed by 2 h recovery in regular media, were unable to control MRSA growth during infection. **b** Exposure of the apical cell surface to the three most common e-cigarette vapor components (each was aerosolized via e-cigarette devices) or control (air only, no EV) for 15 min, followed by 2 h recovery, led to significant keratinocyte cell death, as measured by trypan blue exclusion. **c** Exposure of the human airway epithelial cell line A549 to EVE also led to cytotoxicity, in a dose-dependent manner. **d** Multiple different brands of e-cigarette vapors caused cell death. **e** Cytotoxicity was confirmed via LDH release assays. All conditions were run in triplicate and experiments were run ≥3 times. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$

E-cigarette vapor extract reduces macrophage antimicrobial activity

Alveolar macrophages (AMs) are pivotal regulators of inflammatory signals that orchestrate innate immune responses against infectious pathogens, and they are responsible for clearance of inhaled/aspirated bacteria. Exposure of MH-S cells to EVE led to a reduction in antimicrobial activity. Pre-exposure of AMs to EVE High Nic led to 535 % higher MRSA numbers compared to MRSA recovered from control macrophages ($P<0.0001$; Fig. 2a). Exposure of MH-S cells to EVE Low Nic resulted in 395 % greater MRSA numbers ($P<0.001$). The overgrowth of MRSA in the presence of macrophages exposed to EVE High Nic, where MRSA were able to completely resist killing and grow, may be due to impairment of macrophage antibacterial functions by EVE.

E-cigarette vapor extract from multiple brands reduces neutrophil antimicrobial activity in a nicotine concentration-dependent manner

Human polymorphonuclear leukocytes (hPMNs) play a vital role in host defense of the lungs against bacterial pathogens and are found in abundance in the airways of conventional cigarette smokers—the largest population of current e-cigarette users. hPMNs isolated from blood were used for these studies. Once primed and exposed to activating stimuli, such as bacteria, blood hPMNs have function similar to mature neutrophils within sites of inflammation and infection [25]. EVE exposure of hPMNs reduced antimicrobial function, leading to 46 % greater MRSA numbers after EVE High Nic exposure ($P<0.05$; Fig. 2b). Bacteria were not exposed to EVE.

Each of the innate immune cell types tested, epithelial cells, AMs, and hPMNs, had decreased abilities to kill and control MRSA growth at higher nicotine concentrations, suggesting that nicotine suppresses antimicrobial function in multiple cell types in a dose-dependent manner. To evaluate the effect of nicotine, separate from that of EV, we ran infection assays with nicotine added directly to media. Because neutrophils are one of the most relevant airway cells to the main e-cigarette using population, we utilized them for the remaining in vitro studies. hPMNs were incubated with incremental concentrations of nicotine (0.4, 1.0, and 2.0 mg/mL–2.5, 6, and 12 mM; similar to serum nicotine levels in cigarette smokers and e-cigarette users—therefore less than levels in airways exposed to inhalants).

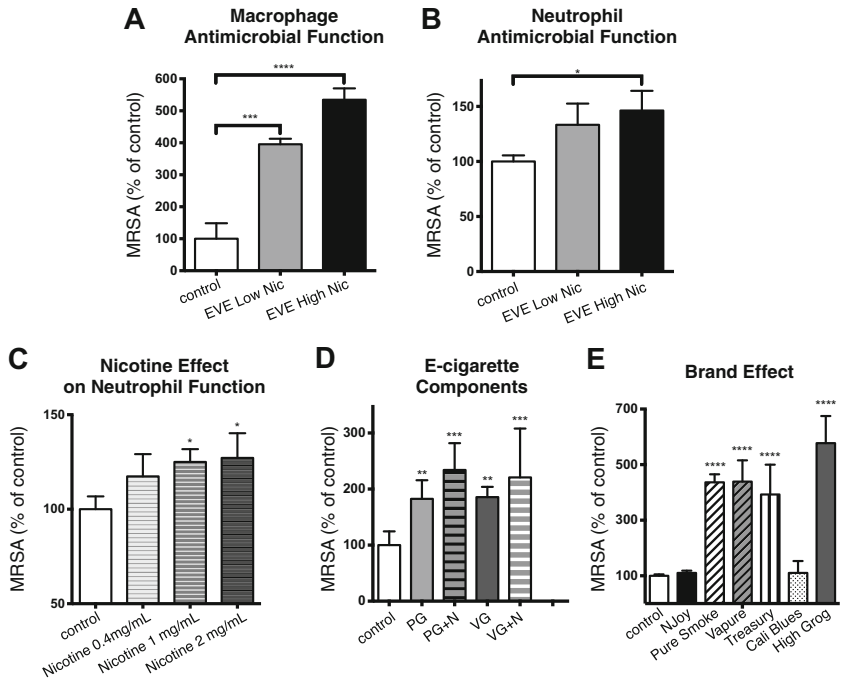

Fig. 2 E-cigarette vapor alters macrophage and neutrophil antimicrobial function. **a** Alveolar macrophages exposed to EVE Low Nic and EVE High Nic for 1 h followed by a 2-h recovery period in control media containing no EV components, and enumeration for live cells, had decreased antimicrobial activity against MRSA. **b** Human neutrophils from blood had decreased antimicrobial activity after 1 h EVE exposure, followed by a 2-h recovery period in control media and enumeration for live cells, as demonstrated as higher MRSA survival during infection. **c** Nicotine alone suppressed neutrophil antimicrobial activity. **d** The main components of e-cigarette liquid, PG, and VG, contributed to decreased antimicrobial function. When combined with nicotine (PG + N and VG + N), greater effects were found. **e** Multiple, but not all, brands caused similar decrements in neutrophil antimicrobial function. All conditions were run in triplicate and experiments were run ≥3 times. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$

Moderate suppression of hPMN antimicrobial function was found, with increased MRSA survival by 17 % (2.5 mM), 25 % (6 mM), and 27 % (12 mM) were found, respectively (Fig. 2c).

The modest effect of nicotine on antimicrobial function suggests that other elements of EV may be playing a role. We vaped PG and VG on their own and vaped PG and VG with nicotine (+N). Each vaped component led to increased bacterial survival when hPMNs were infected post-exposure (Fig. 2d). The addition of nicotine into the e-liquid bases (PG + N and VG + N) further reduced killing capacity of neutrophils, and increased the corresponding numbers of MRSA (Fig. 2d), demonstrating that nicotine also causes hPMN dysfunction.

Lastly, we tested EVE from six popular brands. Neutrophil exposure to four of the brands increased MRSA survival and growth relative to control (Fig. 2e). E-liquid without flavor (Vapure in Fig. 2e and Xtreme Vaping in Fig. 2d) resulted in similar bacterial survival as those with flavors (Pure Smoke, Treasury, and Highlander Grog). No difference in bacterial counts was observed when neutrophils were exposed to EVE made from NJoy, a pre-loaded cartridge type e-cigarette system, or California Blues flavored e-liquid.

Mouse inhalation of e-cigarette vapor leads to increased levels of inflammatory cytokines in bronchoalveolar lavage

Mice inhaled EV for 60 min, once daily, for 4 weeks. Total cell counts in the BAL fluid were no different in e-cigarette mice compared to experimental air-exposed controls ($n=6$ mice per group; Fig. 3a). There were no alterations in the types of cells patrolling the airways, with >99 % macrophages and <1 % neutrophils, eosinophils, and lymphocytes in BAL of all groups (Fig. 3b, c). By flow cytometry, there was no difference in neutrophil, eosinophil, and lymphocyte markers ($P=$n.s.; data not shown). There was no evidence of emphysematous changes, fibrosis, or overt inflammation by blinded histologic grading of H&E- and trichrome-stained lungs (Fig. 3d).

Evaluation of BAL by proteome array determined that three cytokines, KC, IL-1ra, and TREM-1, were all elevated after 4-week-long exposures. These cytokines had >10 % higher protein levels in e-cigarette-exposed mice compared to air-exposed experimental controls. KC and TREM-1 had >10 % higher levels after both 2- and 4-week exposures (Fig. 3e and Supplemental Fig. S2). Protein levels of several cytokines in the BAL decreased by >50 % compared to

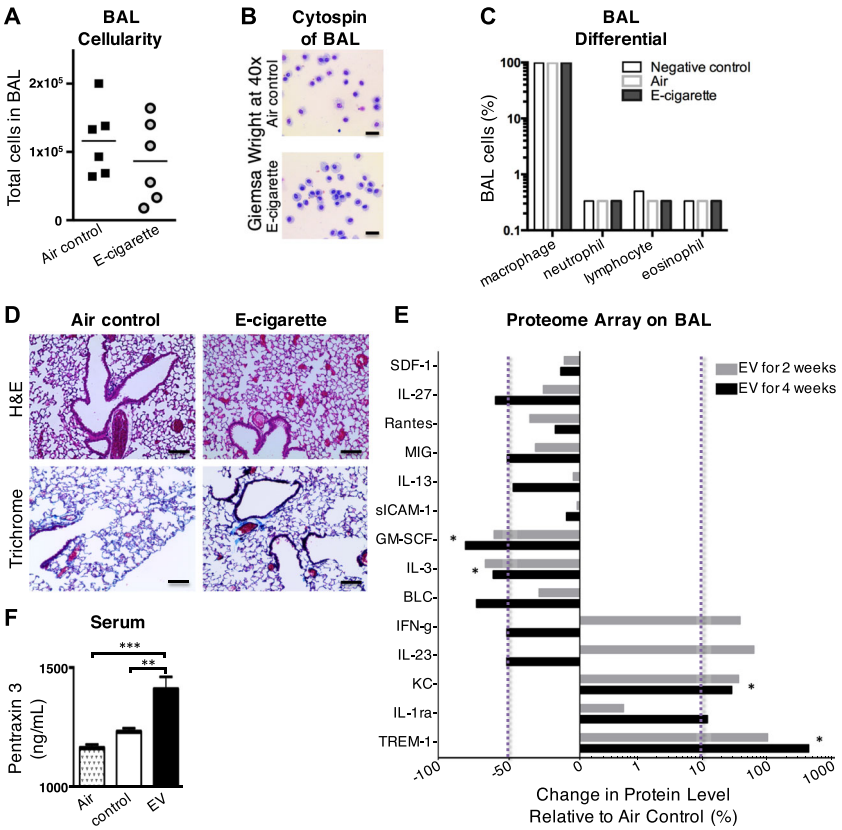

Fig. 3 Mice which inhaled e-cigarette vapor for 4 weeks had increased inflammatory markers in their BAL and serum, compared to control mice. **a** Inhalation of e-cigarette vapor for 1 h per day for 4 weeks did not alter the quantity of cells in the airways, compared to air-exposed control mice. **b**, **c** There was no difference in the types of cells within the BAL of e-cigarette vaping mice, with >99 % macrophages in both e-cigarette and control groups and <1 % neutrophils. *Scale bars*, 20 μm. **d** No changes of emphysema, fibrosis, or airway remodeling were found by histologic grading. *Scale bars*, 200 μm. **e** Two inflammatory markers were elevated in the BAL of e-cigarette mice, with levels >10 % higher compared to air controls (*asterisk*), and two immunomodulatory cytokines had levels <50 % that of controls. **f** Acute phase reactant Pentraxin 3 was elevated in the serum of EV-exposed mice compared to negative and air controls. $n = 6$ for all groups. $^{**}P < 0.01$, $^{***}P < 0.001$

controls, with two cytokines being >50 % decreased at both 2 and 4 weeks: GM-CSF and IL-3.

Mouse inhalation of e-cigarette vapor is associated with elevations in acute phase reactant Pentraxin 3 in serum

Cigarette smoke inhalation is known to cause systemic inflammation, measured by rises in acute phase reactants in the serum [26, 27]. To evaluate whether EV inhalation also affects systemic inflammation, we assessed serum levels of Pentraxin 3, an acute phase reactant in mice. E-cigarette mice had higher levels of Pentraxin 3 in their serum compared to controls (Fig. 3f).

E-cigarette vapor suppresses MRSA growth with moderate bactericidal activity

We previously demonstrated that conventional cigarette smoke increases the virulence of human pathogen SA by inducing a general stress response [13]. E-cigarettes are a young invention and as such are frequently compared to their cousin, the tobacco cigarette. One of the questions most frequently asked by e-cigarette users and combustible tobacco smokers alike is: Do e-cigarettes have the same adverse affects as conventional tobacco and to the same degree? Therefore, we designed these studies to determine if e-cigarette vapor promotes SA virulence to the same degree as cigarette smoke. We chose SA for these studies because of its high prevalence (60 % of the population are colonized intermittently) and its presence in the nasopharynx where it is exposed to inhalants.

We first examined the growth kinetics during exposure to EVE. MRSA failed to show logarithmic growth in the presence of EVE, nicotine, and individual e-cigarette components PG and VG +/− nicotine (Fig. 4a). We next established that e-cigarette components were primarily bactericidal in their inhibitive function of growth by plating MRSA every hour, where plated bacteria showed reductions in CFU in PG and VG +/− nicotine over the first 2 h of exposure (data not shown). We exposed MRSA to multiple brands of e-cigarettes and found growth suppression by four of five brands, suggesting that a common ingredient or mechanism,

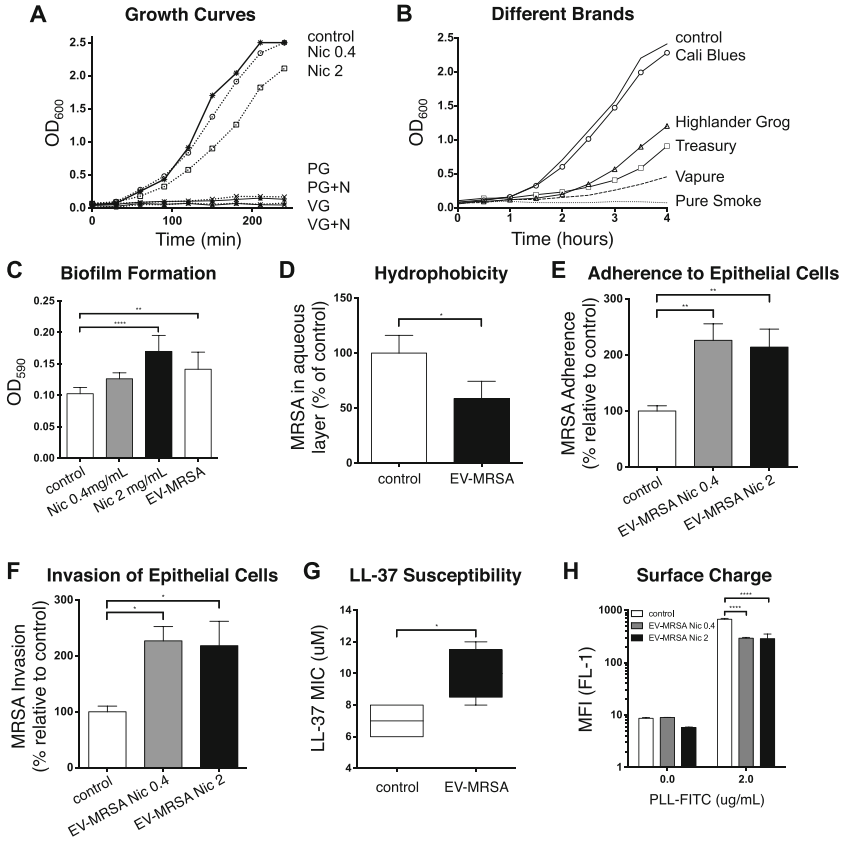

Fig. 4 E-cigarette vapor suppresses MRSA growth and induces cell surface changes. Control bacteria were grown in control media without EV components. **a** Nicotine alters MRSA growth in a dose-dependent fashion, while vaped PG, VG and PG, or VG with nicotine completely suppress MRSA growth. **b** Four brands of e-cigarettes suppressed MRSA growth, while one had no effect. For the remaining studies, bacteria were growth in e-cigarette vapor (*EV*) exposed or control media to mid-log phase, spun, and rinsed 2× in PBS to remove EV. **c** Nicotine induces higher biofilm formation by MRSA, as well as EV containing nicotine. **d** MRSA became more hydrophobic—fewer bacteria found in the aqueous layer—with EV exposure. **e** EV-exposed MRSA were better able to adhere to human epithelial cells. **f** EV increased the ability of MRSA to invade epithelial cells. Nicotine concentration did not affect the ability of MRSA to adhere to or invade cells. **g** Exposure to EV increases MRSA resistance to killing by the human antimicrobial peptide LL-37. **h** EV induces changes in MRSA surface charge, leading to bacterial cell surfaces becoming less negative/more positive, and thus resistance to binding by cationic antimicrobial molecules. All conditions were run in triplicate and experiments were run ≥3 times. $*P<0.05$, $**P<0.01$, $***P<0.001$, $****P<0.0001$

such as heating temperature or voltage applied to the e-liquid, as the root cause (Fig. 4b).

E-cigarette vapor and nicotine induce biofilm formation in MRSA

Biofilm is a defense mechanism by which bacteria increase the distance to their cell wall, decreasing the ability of antibiotics and other antimicrobials to kill these pathogens. MRSA exposed to EV (EV-MRSA) had increased biofilm formation in a dose-dependent manner with respect to nicotine (Fig. 4c). Interestingly, biofilm formation is induced in response to conventional cigarette smoke as well, which we previously determined to be induced by oxidative stress [13]. In these studies, incubation of MRSA with nicotine alone showed higher biofilm-associated biomass than control, suggesting a novel pathway in inducing biofilm production that is at play in both e-cigarette vapor and cigarette smoke exposures.

E-cigarette vapor increases MRSA hydrophobicity and adherence and invasion of keratinocytes

Hydrophobicity is an important factor in the ability of bacteria to adhere to the epithelial cells [28, 29]. Bacterial adherence to the host epithelium is the first step in invasive staphylococcal infections while promoting colonization of keratinocytes in the nasopharynx, axilla, and inguinal regions [30]. Here, we revisit the human keratinocyte HaCaT cell line but evaluate the effects of e-cigarette vapor on MRSA function, specifically the ability to adhere to and invade HaCaT cells (which have not been exposed to e-cigarette vapor). Exposure to EV resulted in increased hydrophobicity of MRSA, where 31 % fewer bacteria were found in the aqueous layer (Fig. 4d). Infection of

HaCaT cells showed increased adherence by EV-MRSA when compared to control (Fig. 4e). Extending the infection time and quantifying the bacteria solely inside of HaCaT cells showed that EV exposure increased MRSA's ability to invade and persist within human keratinocytes (Fig. 4f).

E-cigarette vapor increases MRSA resistance to the human antimicrobial peptide LL-37

Increased survival of internalized MRSA suggests that e-cigarette exposure may induce resistance to killing by epithelial cells. Human keratinocytes both secrete and have the antimicrobial peptide LL-37 within their walls. Minimum inhibitory concentrations (MICs) of LL-37 for control MRSA and EV-MRSA were determined. Duplicate samples from four separate experiments yielded MIC of 7 μM for control MRSA and 10 μM for EV-MRSA ($P = 0.014$ and Fig. 4g).

An established mechanism by which bacteria become more virulent is alterations in surface charge, to make the cells more cationic. Most bacteria have predominantly anionic surfaces, which are targeted by the human innate immune system [31, 32]. LL-37's mode-of-action utilizes charge interactions where its cationic charge drives the preferential targeting of negatively charged bacterial lipids [31–33]. The moderate increase in resistance to LL-37, therefore, may be due to modulation of MRSA surface charge upon EV exposure. Using a cationic molecule poly-L-Lysine (PLL) to model LL-37 charge interaction, we found reductions in PLL-FITC binding to MRSA cultured in EVE with low and high nicotine concentrations (Fig. 4h). Nicotine level did not impact the binding of PLL-FITC.

E-cigarette vapor increases MRSA virulence in a mouse model of pneumonia

To assess the effect of EV exposure on MRSA virulence in a physiologic setting, we infected mice with MRSA grown in control media versus EVE (GSL High Nic). All 16 mice infected with control MRSA survived while 25 % of mice infected with EV-MRSA died ($P = 0.035$; Fig. 5a). Bacterial loads from surviving mice at day 4 demonstrated tenfold higher bacterial burdens in EV-MRSA-infected lungs ($P < 0.01$; Fig. 5b). None of the seven control mice harvested had more than four colonies grow from their lungs (Supplemental Fig. S3). Within this physiologic model, EV exposure enhanced MRSA virulence.

E-cigarette vapor induces virulence gene expression changes in MRSA

To determine the mechanism through which EVE-exposed MRSA becomes more virulent, we evaluated the expression of several well-known virulence factors. Relative quantification (RQ) values of coagulase *coa*, intracellular adhesion *icaA*, quorum sensing *agrA*, staphylococcal protein A *spa*, α-hemolysin *hla*, α-phenol soluble modulin *psm-α*, and

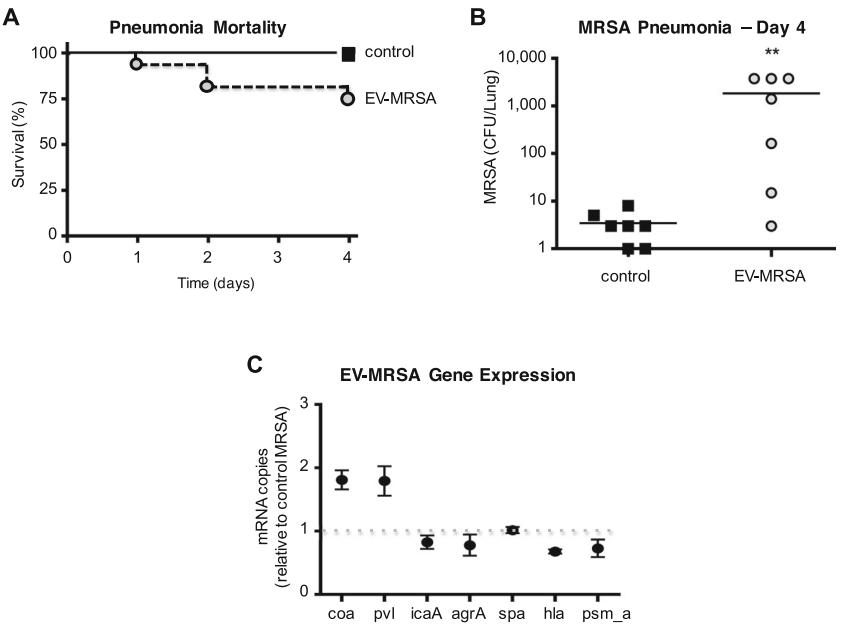

Fig. 5 One 2-h exposure to e-cigarette vapor increases MRSA virulence, leading to higher bacterial counts in infected mice, and higher mortality. **a** Mice infected intranasally with 3×10^8 CFU MRSA in 75 μL EV-MRSA led to 25 % mortality, while none of the mice infected with control MRSA died ($P = 0.035$; $n = 16$ mice per group). **b** The majority of mice harvested at 4 days post-EV-MRSA intranasal infection had bacteria remaining in their lungs, while control MRSA-infected mice primarily had cleared the bacteria from their lungs ($P < 0.01$; $n = 7$ per group). **c** MRSA exposed to EV had up-regulation of *coa* and *pvl* genes and down-regulation of *icaA*, *agrA*, *hla*, and *psm_a*. All conditions were run in triplicate, and experiments were run ≥3 times

Panton-Valentine leukocidin *pvl* were obtained with 16s rRNA *16s* as the housekeeping gene. Expression of coagulase (*coa*) and Panton-Valentine leukocidin (*pvl*) increased by 1.68- and 1.56-fold, respectively, after EVE exposure (Fig. 5d). Expression of *spa* did not change with EVE exposure while *icaA*, *agrA*, *hla*, and psm decreased.

Discussion

E-cigarettes are often advertised as a safer alternative to traditional cigarettes, whose toxic effects of which have been studied for decades. The data presented here demonstrate the harmful effects of EV on human epithelial and innate immune cells in vitro and their impact on the virulence of a common human pathogen that colonizes the upper airways (Fig. 6). Direct exposure of human keratinocytes to EV for 15 min induced cytotoxicity, suggesting that EV contains substances toxic to mammalian cells. Rapid cell staining with Annexin-V and EthD-IIIi after e-cigarette vapor exposure suggests that cells are undergoing necrotic cell death; however, studies blocking individual pathways would provide further insight. To evaluate which component(s) may be contributing to cytotoxicity, separate components of e-liquid were vaped: PG, VG, and nicotine. Vapor produced from these separate components also caused cell death. Testing vapor created from e-liquids from seven different companies led to similar results on mammalian and bacterial cells, demonstrating that our findings are not limited to one formula or brand. A limitation of our studies is the use of cell lines instead of primary cells from human airways. HaCaTs are commonly used for in vitro toxicology studies [34–36], but in the future, it will be important to evaluate EV effects on primary human airway cells for a more comprehensive understanding of potential toxicities.

In vivo, lung epithelium has other features to protect it from inhaled toxins, such as a mucous layer. Therefore, our in vitro findings may not be replicated in human airways. However, since it will be decades until we have epidemiologic data on e-cigarette effects, in vitro models may help by giving some signal as to how the inhalation of these chemicals may affect human cells and organ systems. It is well known that cigarette smoke is highly toxic to human cells, even at very low concentrations (10 % cigarette smoke extract kills 100 % of epithelial cells in vitro). Therefore, the cytotoxicity of EV (25 % EVE kills 40–50 % of cells, and 10 % EVE kills <5 % of cells) is less than that of cigarette smoke. These results are in agreement with those of Farsalinos et al. [37], who demonstrated that e-cigarette vapor induced cytotoxicity in cardiomyocytes but required higher concentrations as compared to conventional cigarette smoke, as well as Schweitzer et al. [38], who demonstrated that e-cigarette vapor disrupts endothelial barriers and promotes inflammation.

Impaired bacterial killing by neutrophils, macrophages, and epithelial cells by any method is detrimental to the normal function of host defenses and may increase susceptibility to bacterial infections. In our model, it appears that one

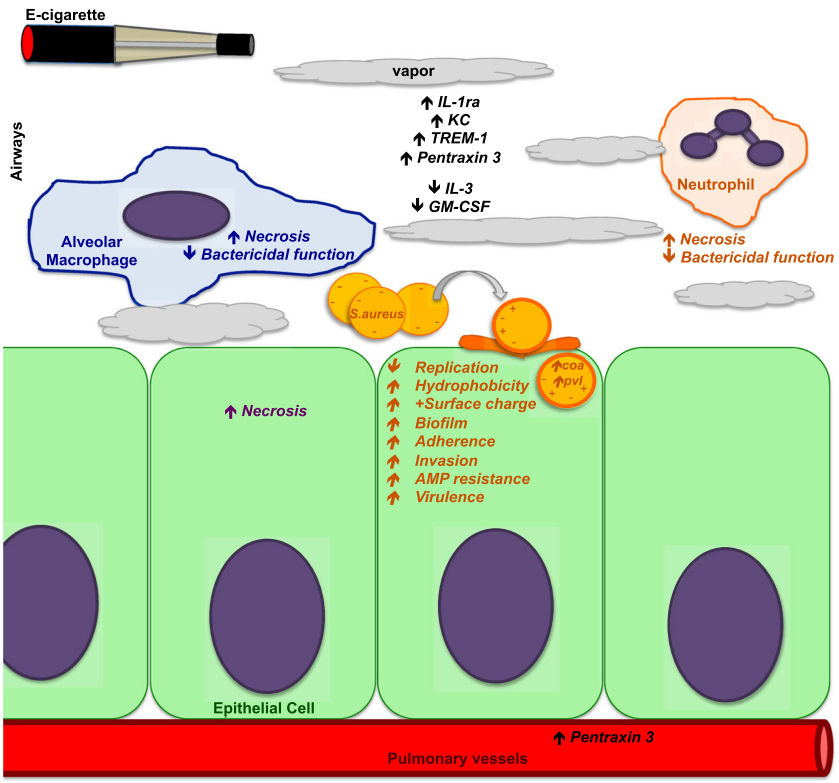

Fig. 6 Model of potential e-cigarette vapor effects on lungs

mechanism of suppression of innate immunity is via cytotoxicity by EV, which is suggestive of diffuse cellular damage as a possible effect of EV inhalation. Inefficient removal of dying cells may lead to autoimmune-related inflammatory signaling [39]. And necrotic cell death, which is one of the pathways induced by EV in our studies, can lead to strong inflammatory responses [40]. The pro-inflammatory cytokine TREM-1, elevated in the BAL of mice exposed to e-cigarette vapor daily (Fig. 3e), is a mediator of necrotic cell signaling and particularly augments the pro-inflammatory response of monocytes and macrophages [41]. TREM-1 is also associated with multiple chronic inflammatory conditions, including COPD, interstitial lung disease (cryptogenic organizing pneumonia), and rheumatoid arthritis [42–44]. The decreases found in IL-3 and GM-CSF in the BAL of e-cigarette exposed mice are concerning for increased susceptibility to bacterial and fungal infections, as both of these cytokines are important for rapid activation of host defenses during early stages of infection. Other groups have demonstrated increased susceptibility to infection after EV exposure [45, 46], and our findings provide further evidence that the innate immune system is altered by e-cigarette exposure.

The absence of changes in BAL cellularity and differential after inhalation of EV for an hour a day, 5 days per week for 4 weeks, was reassuring in that EV exposure did not induce neutrophil, eosinophil, or lymphocyte influx. However, the pattern of elevated cytokines found in the airways of mice that inhaled EV suggests that airway macrophages and epithelial cells may be affected by chronic EV inhalation and modulate the release of immune activation and pro-inflammatory cytokines in response (Fig. 6). Inhalation of cigarette smoke is known to cause both local and systemic inflammation, leading to local and systemic disease. Some elevations of pro-inflammatory cytokines after e-cigarette inhalation are similar to what is seen with chronic tobacco cigarette inhalation [14]. Also, elevation of Pentraxin 3 in the serum of mice that inhaled EV for 4 weeks indicates that inhalation of e-cigarettes may lead to systemic inflammation and disease, such as coronary artery disease and stroke.

Evaluating EV effects on bacterial airway cells found that human colonizer and pathogen *S. aureus* became more virulent upon exposure to EV in a murine model of pneumonia. qRT-PCR demonstrated up-regulation of virulence factors *coa* and *pvl* with EV exposure. Clotting factors such as Coa are essential for abscess formation in *Staphylococcus* [47], and therefore the elevated expression of *coa* is consistent with the abundance of abscesses that were seen only in lungs of mice infected with EVE-MRSA. PVL is postulated to be a pore-forming cytotoxin involved in leukocyte destruction and necrosis, and its increased expression likely contributes to MRSA pathogenesis [48]. Both of these factors are associated with increased virulence, but the mechanism of increased expression is unknown as yet [49, 50]. In vitro assays showed increased biofilm formation, shifts in cell surface towards a more positive charge, and resistance to human antimicrobial peptide LL-37. Biofilm and cationic surface charge both enhance *S. aureus* survival by making the bacteria unrecognizable and disrupting charge interaction that drives LL-37 function. Decreased susceptibility of *S. aureus* to human antimicrobial peptides is an alarming concern for the current state of antibacterial therapy where the antibiotic development pipeline is running dry. Overall, these data suggest that *Staphylococcal* infections in vapers may be more deadly and difficult to treat. Subsequent experiments with PG and VG +/− nicotine exposure may help distinguish which specific components are up-regulating the virulence factors and how other gene expressions may be modulated by EV exposure.

In this paper, we demonstrate that e-cigarette vapor increases the virulence potential of *S. aureus* while impairing innate immune function and inducing cytotoxicity in human cells with direct exposure (Fig. 6). Our data suggest that vaping e-liquid has the potential to decrease host ability to fight infection, promote virulence in bacterial colonizers of the airways, and promote inflammation, overall having the potential to cause significant inflammatory lung and systemic diseases.

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Author contributions JHH, EM, WMO, SB, SD, and LCA designed the research. JHH, ML, KS, SE, SD, EM, DM, AM, SD, SB, WMO, and LCA performed the research and analyzed the data. JHH, SE, SB, ML, DTP, WMO, and LCA performed statistical analyses and edited the paper. JHH, SD, SB, and LCA wrote the paper.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no competing interests.

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