

ORIGINAL RESEARCH

Phagocytosis and Inflammation: Exploring the effects of the components of E-cigarette vapor on macrophages

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E-cigarettes, macrophages, phagocytosis, cytokines, inflammation.

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Introduction

Electronic (E)-cigarettes are a recent innovation, reimagining the cigarette based on designs which have been proposed by inventors since the 60's and finally being commercialized in 2003 in China. They are advertised as a safe way to help people quit smoking tobacco cigarettes, on the basis that only water vapor is inhaled. In recent years, there has been a surge in the number of users, known as 'vapers', in many countries. Multiple studies identified most users as those attempting to cease smoking tobacco cigarettes or as an alternative where tobacco cigarette use is prohibited, as summarized in Table 1. However, a growing number of younger people are now using the devices, usually for social and novelty aspects (Schoenborn and Gindi 2015; Walsberger and Havill 2015; Singh et al. 2016; Wilson and Wang 2017). E-cigarettes are usually filled with an E-liquid which consists of

Abstract

E-cigarettes are perceived as harmless; however, evidence of their safety is lacking. New data suggests E-cigarettes discharge a range of compounds capable of physiological damage to users. We previously established that cigarette smoke caused defective alveolar macrophage phagocytosis. The present study compared the effect E-cigarette of components; E-liquid flavors, nicotine, vegetable glycerine, and propylene glycol on phagocytosis, proinflammatory cytokine secretion, and phagocytic recognition molecule expression using differentiated THP-1 macrophages. Similar to CSE, phagocytosis of NTHi bacteria was significantly decreased by E-liquid flavoring (11.65–15.75%) versus control (27.01%). Nicotine also decreased phagocytosis (15.26%). E-liquid, nicotine, and E-liquid+ nicotine reduced phagocytic recognition molecules; SR-A1 and TLR-2. IL-8 secretion increased with flavor and nicotine, while TNF α , IL-1 β , IL-6, MIP-1 α , MIP-1 β , and MCP-1 decreased after exposure to most flavors and nicotine. PG, VG, or PG:VG mix also induced a decrease in MIP-1 α and MIP-1 β . We conclude that E-cigarettes can cause macrophage phagocytic dysfunction, expression of phagocytic recognition receptors and cytokine secretion pathways. As such, E-cigarettes should be treated with caution by users, especially those who are nonsmokers.

a base made up of propylene glycol (PG), vegetable glycerine (VG), or a blend of the two, usually with a food safe flavor, and often with nicotine at concentrations varying up to 24 mg/mL. However, up to 250 different compounds have been identified in the resulting, inhaled, E-liquid vapor (Garcia-Gomez et al. 2016; Higham et al. 2016), with even more likely given the size cut-off in these studies. While there is definitive evidence of the dangers of cigarette smoking, the long-term health risks of E-cigarettes are not known. The laws governing E-cigarettes vary across the globe with Australian and Canadian laws being noteworthy by prohibiting the sale of E-liquid containing nicotine, identifying a need to separate out the effects of the flavoring compounds and nicotine.

Whilst E-cigarettes are pitched as being a perfectly harmless social pastime, data are emerging that even non-nicotine containing E-liquids may be damaging to the lungs and airways. E-liquids have been shown in vitro to:

Table 1. Adult* E-cigarette use in various countries.

Country	Year	Current smoker (%)	Ever users				Current users*			
			Total (%)	Nonsmoker (%)	Current smoker (%)	Ex smoker *	Total (%)	Nonsmoker (%)	Current smoker (%)	Ex smoker*
USA ¹	2014		12.6	3.2	47.6	55.4	3.7	0.4	15.9	22.2
Australia (NSW) ²	2015		8.4	3.2	36.5	N/A	1.3	0.7	4.9	N/A
Canada ³	2013		8.5	3.0	37.3	5.1	1.8	0.3	9.6	0.9
UK ⁴	2012	18.8	5.3	0.5	40.1	3.8	1.6	0.1	13	1.1
Korea ⁵	2013	24.1	6.6	0.7	21.8	4.8	1.1	0.2	0.3	4
New Zealand ⁶	2014	16.2	13.1	3.4	49.9	8.4	0.8	0.1	4.0	0.1
Japan ⁷	2015	20.5	6.6	3.51	18.3	7.7	1.29	0.6	3.2	1.75

*Definition of "adult", "current use", and "ex-smoker" differs between studies. Ever Use is define as having ever tried an E-cigarette even once¹⁻⁶. ¹Dockrell et al. 2013; ²Harrold et al. 2015; ³Reid et al. 2015; ⁴Schoenborn and Gindi 2015; ⁵Lee et al. 2016; ⁶Li et al., 2015; ⁷Tabuchi et al. 2016).

enlarge human lung fibroblast size, induce spindle formation and vacuolization, as well as decrease viability after exposure to individual or mixed components, to a similar degree as cigarette smoke (Lerner et al. 2015). E-cigarettes cause toxicity to NHBE48 and A549 airway and lung cells via oxidative stress (Cervellati et al. 2014; Scheffler et al. 2015), and both nicotine free and nicotine-containing E-cigarette vapor caused loss of lung endothelial barrier function (Schweitzer et al. 2015). In vivo studies in mice show E-cigarette exposure stunts growth (McGrath-Morrow et al. 2015) although this may be limited to nicotine-containing E-cigarettes (Larcombe et al. 2017). They have also been shown to induce an allergy-based asthma inflammatory response (Lim and Kim 2014) and increased methacholine response (Larcombe et al. 2017), and increasing susceptibility of mouse lung cells to viral infection (Wu et al. 2014; Sussan et al. 2015). Many of these mouse studies suffer from varied or nonphysiological delivery methods, as well as often lacking a solid basis in in vitro studies, thus this study presents a thorough in vitro study designed to be a basis for future in vivo studies.

We have long been interested in the effects of cigarette smoke on lung cell function. Our identification of defective alveolar macrophage phagocytic function in chronic obstructive disease (COPD) subjects and in response to cigarette smoke (especially with regard to the clearance of apoptotic material, termed efferocytosis), lead to the subsequent discovery of significantly increased apoptosis of epithelial cells in the airway of chronic obstructive pulmonary disease (COPD) subjects and cigarette smokers (Hodge et al. 2005; Hodge et al. 2007; Tran et al. 2016; Ween et al. 2016). These findings implicate defective efferocytosis with secondary necrosis and potentiation of the inflammatory milieu (Hodge et al. 2003, 2005).

In COPD, nontypeable *Haemophilus influenzae* (NTHi) is the most common cause of chronic bacterial airway colonization accounting for up to half of all isolates (King 2012). We found that airway macrophages from cigarette smokers and COPD subjects have a reduced ability to phagocytose bacteria, including NTHi (Hodge et al. 2006; Ween et al. 2016) which can potentially give rise to increased lung infections such as pneumonia. As up to 80% of COPD exacerbations can be attributed to NTHi, clearance by alveolar macrophages is important to reduce bacterial colonization, limit inflammation, and to prevent exacerbations (Sunakawa et al. 2011). Thus, we investigated how E-cigarettes affect the phagocytic ability of macrophages and explored mechanisms for this effect.

Materials and Methods

E-cigarette and E-liquids

For all experiments, an EVOD-2 was used. This device runs at 3.7 V and uses a dual coil with an internal wick and a resistance of 1.5 Ω . Three apple flavors were tested from two suppliers (Aussie Blue and Vape King) in a 70% PG:30% VG base (PG:VG), including one specifically tested to confirm the absence of diacetyl-acetyl propionyl (E-liquid 3). Nicotine (in PG from NicVape, SC) at 18 mg/mL in PG alone was also vaporized, also in combination with the three flavors, as well as PG alone (Vape King), VG alone (Vape King), and self mixed PG:VG. Based on the average users puff duration of 2.6 sec (Behar et al. 2015), 50 \times 3 sec puffs with 5 sec in between to allow the heating element to cool were bubbled through 10 mL of RPMI 1640 media supplemented with 2 mmol/L L-glutamine, 10% fetal calf serum (FCS), and Penicillin (12 μ g/mL) and Gentamycin (16 μ g/mL) (all from Life Technologies, Carlsbad, CA) (culture

medium). Control media was obtained, using the same device to pass air through media for the same duration as E-cigarette use. Cells were treated with 250 μ L for 48 well plates and 500 μ L for 24 well plates for 24 h.

Culture and preparation of THP-1 macrophage cell line

A THP-1 monocytic cell line (American Type Culture Collection, Manassas, VA) were maintained as per ATCC guidelines in culture medium and 0.05 mmol/L β -mercaptoethanol. was differentiated into macrophages by seeding at a density of 0.75×10^5 cells in 48 well plastic plates or 1.5×10^5 into 24 well plates, then stimulating with 45 μ mol/L phorbol 12-myristate 13-acetate (PMA) for 72 h as previously described (Ween *et al.* 2016). THP-1 monocytes were differentiated three days prior to treatment. Experiments were carried out within 10 passages and THP-1 cells were discarded at passage 30 to avoid contamination and genetic mutations from the original source.

Preparation of NTHi bacteria

A clinical isolate of NTHi was kindly provided by Dr Susan Pizzutto (Menziess School of Health Research, Northern Territory, Australia). NTHi was grown to log phase in Brain Heart Infusion (BHI) broth supplemented with hemin, β NAD and glycerol aliquoted and stored at -80°C . Viable quantification (colony-forming units per mL, cfu/mL) was determined by colony counts of serial dilutions plated onto chocolate agar as described (Ween *et al.* 2016). In our experience, viability is well maintained at -80°C . Bacteria were permeabilized for 30 min with 70% ethanol, centrifuged at 35,000g, washed and resuspended in 1xHBSS 10 mol/L HEPES pH 8.2 and stained with pHrodo Red (2 μ g/mL and 2×10^6 cells/mL, Life Technologies) at room temperature for 45 min on a shaker in the dark, washed twice in the HBSS/HEPES solution, resuspended in RPMI, and added at a 100:1 ratio of THP-1 cells in 500 μ L.

Phagocytosis assay

PMA-differentiated THP-1 cells were treated with 250 μ L of E-cigarette media for 24 h. Supernatant was collected, and debris removed by centrifugation at 500g. Supernatants were stored at -80°C without protease inhibitor for a max of 6 weeks for LDH assays described below. NTHi were incubated with THP-1 macrophages for 90 min at 37°C 5% CO_2 . Nonphagocytosed NTHi were removed, the wells rinsed, and the THP-1 cells incubated with ice cold 1 x HBSS 10 mmol/L HEPES pH 8.2 for

15 min before lifting with a bulb pipette. Macrophages were centrifuged at 1100g, vortexed, washed with HBSS/HEPES solution before reading 10,000 events on the FACSCanto II flow cytometer (BD Biosciences, San Jose, CA). As pHrodo is only brightly fluorescent at low pH, found intracellularly, no quenching was required. Macrophages not exposed to NTHi were used as a gating control and phagocytosis was assessed, using FACS DIVA 7.0 (BD Biosciences) and data expressed as the percentage of positive cells. Gating strategies are supplied (Fig. S1).

Flow cytometry of cell surface markers

Thereafter, 2×10^5 THP-1 cells were seeded in 24 well plates and differentiated as described above. Cells were treated with E-cigarette media for 24 h. Supernatant was collected, spun at 500g for 10 min to remove debris, and stored at -80°C in the presence of protease inhibitors for cytokine bead array (CBA) analysis as described below. Cells were incubated in ice cold PBS for 15 min before lifting with a bulb pipette. Cells were washed with 0.5% BSA in isoflow (BD Biosciences) and pelleted. Cells were incubated with 2 μ L conjugated antibodies (SR-A1 APC #FAB2708A R& Systems, MN, TLR-2 PE #FAB6248P, R&D systems, TLR-4 APC #17-9917-41 EBiosciences, CA) for 10 min in the dark, and washed. Unstained-treated THP-1 cells were used for gating controls. Fifty thousand events were collected and cell surface markers analyzed, using FACS DIVA 7.0 and expressed as % positive, and also MFI of cells constitutively expressing the marker. Gating strategies are supplied (Fig. S2).

LDH assay

The Lactate dehydrogenase (LDH) assay was carried out following the manufacturer's instructions (Roche, IN). In a separate well: Tween-20 was added at 2% v/v, mixed and incubated for 5 min to lyse cells for as a 100% control for total potential max LDH release. Data are presented as a percentage of the lysed cell control for each replicate experiment.

CSE preparation

Smoke from 2x reference cigarettes (1R5F, University of Kentucky, KY) was bubbled through 10 mL RPMI 1640 media (Life Technologies), supplemented with 12 μ g/mL penicillin 16 μ g/mL gentamycin (Life Technologies), and L-glutamine (2 mmol/L, Life Technologies), using a vacuum for 5 min per cigarette. Cigarette smoke extract (CSE) was prepared and used at a concentration of 10%. Cells were treated with 10% CSE or E-cigarette media for 24 h in parallel experiments.

Analysis of secreted inflammatory markers via CBA

Cytometric bead arrays (CBA)(BD Biosciences) were performed as per the manufacturer guidelines for cell culture supernatants. CBA were analyzed on a FACSCantoII with FCAP array software. Human soluble protein Flex sets were used, cytokines targeted were TNF α , INF γ , MIP1 α , MIP1 β , IP-10, IL-1 β , IL-6, IL-8, IL-10, IL-12p70, and MCP-1.

Statistical analyses

The Wilcoxon signed rank test for paired data, Kruskal–Wallis nonparametric ANOVA with Mann–Whitney *U* test were employed for statistical analysis. SPSS v23 software was utilized to perform all statistical analysis and differences between groups of *P* < 0.05 considered significant.

Results

E-cigarette components toxicity on THP-1 macrophages

In this study, we assessed the effects of E-cigarette vapor on PMA-differentiated THP-1 macrophages. Firstly we assessed toxicity of E-cigarette vapor on THP-1 macrophages via release of lactate dehydrogenase, and found that none of the three apple flavors, base alone, or nicotine alone induced any significant toxicity at the exposure tested when compared with a total LDH release lysis control (all <6% of lysis max, Fig. 1).

E-cigarettes cause decreased macrophage phagocytic capacity

Given that our previous studies had shown decreased alveolar macrophage phagocytic function in COPD subjects and smokers, and in response to cigarette smoke extract in vitro (Hodge et al. 2007), we investigated phagocytosis, using differentiated THP-1 macrophages exposed to cigarette smoke, and E-liquid components. Exposure to all three apple flavors caused a significantly decreased ability of macrophages to phagocytose NTHi (11.65–15.79%) versus control (27.01%), as did nicotine at 18 mg/mL (15.26%). There was no statistical difference between E-liquids with and without nicotine. PG, VG, and PG:VG had no significant effects on phagocytosis (24.5–27.66% vs. 27.01% for control). The reduction in phagocytosis in the presence of 10% CSE (8.2%) was consistent with our previous findings (Tran et al. 2016; Ween et al. 2016) (Fig. 2). The negative control, cytochalasin D, confirmed the assay and gating optimization (Fig. 2).

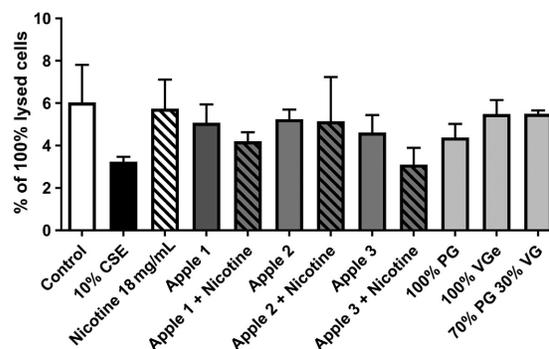


Figure 1. Lactate dehydrogenase (LDH) levels indicating toxicity of E-cigarette treated cells. THP-1 PMA-differentiated macrophages were treated with 10% CSE and media infused with 50 puffs of components of E-liquids, including three apple flavors \pm Nicotine (in 70:30 PG:VG), PG, VG, or 70:30 PG:VG for 24 h. Supernatant was collected, debris removed, and lactate dehydrogenase levels measured and expressed as a percentage of 100% lysis with Tween-20. *n* = 4 separate experiments. Data represents mean \pm SEM.

E-cigarettes reduce surface macrophage phagocytosis receptor expression

Macrophages express a range of cell surface receptors in order to recognize phagocytic targets including bacteria. Consistent with our previous studies using cigarette smoke, we show that exposure to E-cigarette vapor also reduces expression of the phagocytosis receptor, scavenger receptor (SR)-A1 (Fig. 3A). This was also non-nicotine dependent with all three apple flavors showing a significant decrease in SR-A1 (9.05–10.75%) versus control treatment (19.04%). Nicotine alone also induced a significant reduction in SR-A1 (8.18%), whilst PG, VG, and PG:VG showed no significant difference from control treatment. Given the role of toll like receptors (TLR) in the inflammatory response to bacteria, and their known ability to recognize prokaryotic targets, we also investigated the expression of TLR-2 and TLR-4. Our data showed that our THP-1 cells expressed very high levels of TLR-2 (>90% positive). We found a significant decrease in the percentage of TLR-2 positive cells with 24 h treatment with 10% CSE (34.77%, Fig. 3B). However, no change in the number of TLR-2 positive cells when treated with any component of the E-liquid. Interestingly, when we looked at mean fluorescent intensity (MFI), we found significant decreases when treated with all three flavors (360–440 MFI) as well as nicotine alone (299 MFI) when compared with control (635 MFI), but no significant change with PG, VG, and PG:VG treatment versus control (511–576 MFI, Fig. 3C), indicating a decrease in the number of receptors on each cell. We also observed that

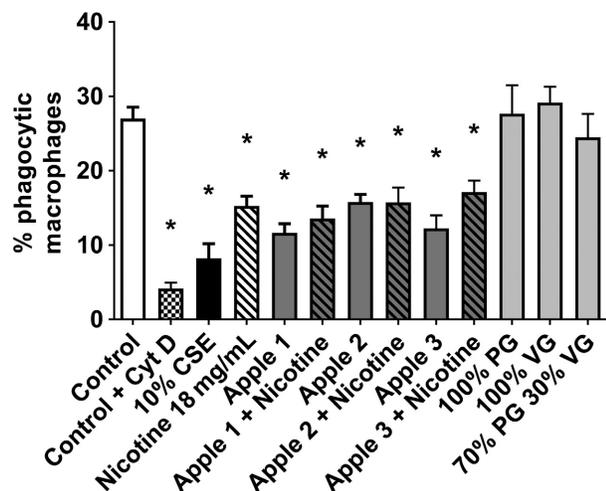


Figure 2. E-cigarette exposure decreases phagocytic ability of macrophages. THP-1 PMA-differentiated macrophages were treated with 10% CSE and media infused with 50 puffs of components of E-liquids, including three apple flavors \pm Nicotine (in 70:30 PG:VG), PG, VG, or 70:30 PG:VG for 24 h. Cells were incubated with pHrodo Red labeled NTHi for 1.5 h, and nonphagocytosed bacteria removed. Due to pH reactivity of pHrodo Red, only THP-1 with intracellular NTHi were counted as positive in the red channel on a flow cytometer. Expressed as mean percentage positive macrophages \pm SEM; $n = 5$ separate experiments performed in duplicate. Significance from control, $P < 0.05$, Mann-Whitney U test.

THP-1-differentiated macrophages had extremely low levels of TLR-4 expression ($<4\%$, Fig. 3D).

E-cigarettes alter cytokine secretion

Cigarette smoking causes significant changes to the inflammatory environment in the lung and alveolar macrophages are a key source of the cytokines involved in the inflammatory process. Thus, we investigated whether E-cigarette exposure altered the cytokine secretion profile of THP-1-differentiated macrophages, using a cytokine bead array that provides very high sensitivity to 5 pg/mL in cell supernatant. We showed that secretion of the neutrophil chemoattractant IL-8 significantly increased in response to 10% CSE (282526 pg/mL), nicotine (191779 pg/mL), and all three apple flavors (158743–173995 pg/mL), but not to PG, VG, or PG:VG compared with control treatment (81786 pg/mL, Fig. 4A). In contrast, IL-6, IL-1 β , MIP-1 α , MIP-1 β , MCP-1, and TNF α all significantly decreased in response to 10% CSE as well as E-liquid 2 and 3, and nicotine. IL-6 and MCP-1 also significantly decreased in macrophages treated with E-liquid 1. Interestingly, MIP-1 α , IL-6, and TNF α also showed significant decreases with PG, but not VG or PG:VG. IP-10

only showed a significant decrease versus control with 10% CSE treatment (Fig. 3B–H). IFN γ , IL-10, and IL-12p70 were below detection levels.

Discussion

It has been well documented that tobacco cigarettes are toxic to airway cells (Wu and Lee 1985; Matsumoto et al. 1998; Wright et al. 1999); however, we have described that at lower concentrations, they do not provide a toxic effect to macrophages, but rather affects their function. Whilst E-cigarettes are a relatively new product on the market, many papers now show that they too have toxic effects on a range of cells (Bahl et al. 2012; McGrath-Morrow et al. 2015; El Golli et al. 2016; Sancilio et al. 2016; Sherwood and Boitano 2016; Welz et al. 2016) including airway epithelial cells (Scheffler et al. 2015; Schweitzer et al. 2015). This effect is non-nicotine dependent, and there has been particular interest in certain flavors including tobacco, cherry, and cinnamon, that have been shown to be more toxic than others (Bahl et al. 2012; Behar et al. 2014; Kosmider et al. 2016). In this study, we tested three apple E-liquids \pm added nicotine (18 mg/mL) from two different retailers including one specifically tested to be diacetyl and acetyl propionyl (DA-AP) free, which has been associated with popcorn lung (Modi et al. 2008; Halldin et al. 2013). We found that there was no cell death associated with the treatment of THP-1-differentiated macrophages with these flavors with or without added nicotine (Fig. 1). Our CSE data supported our previous findings (Hodge et al. 2011; Ween et al. 2016) as well as other studies showing cigarette smoke exposure can inhibit bacterial phagocytosis (Shang et al. 2011). To date, there has been no studies on toxicity of E-cigarettes on macrophages. It is possible that further investigation of a broader range of flavors may identify compounds with toxic effects on macrophages.

NTHi bacteria is the most common lung bacteria in COPD patient exacerbations (King 2012), and it is also common in cystic fibrosis (Bilton et al. 1995) and chronic bronchitis patients (Bandi et al. 2001). We and others have shown that smokers and COPD patient alveolar macrophages (Hodge et al. 2007; Taylor et al. 2010; Berenson et al. 2013) as well as THP-1-differentiated macrophages and healthy control alveolar macrophages treated with CSE have decreased ability to phagocytose this bacteria (Bozinovski et al. 2011; Hodge et al. 2011; Thimmulappa et al. 2012; Bain et al. 2016; Ween et al. 2016). Thus, we investigated whether E-cigarettes caused a similar phagocytic dysfunction utilizing our established THP-1 model. In this study, we opted to utilize the relatively new dye, pHrodo Red which has minimal fluorescence at neutral to high pH but becomes brightly fluorescent in the low pH

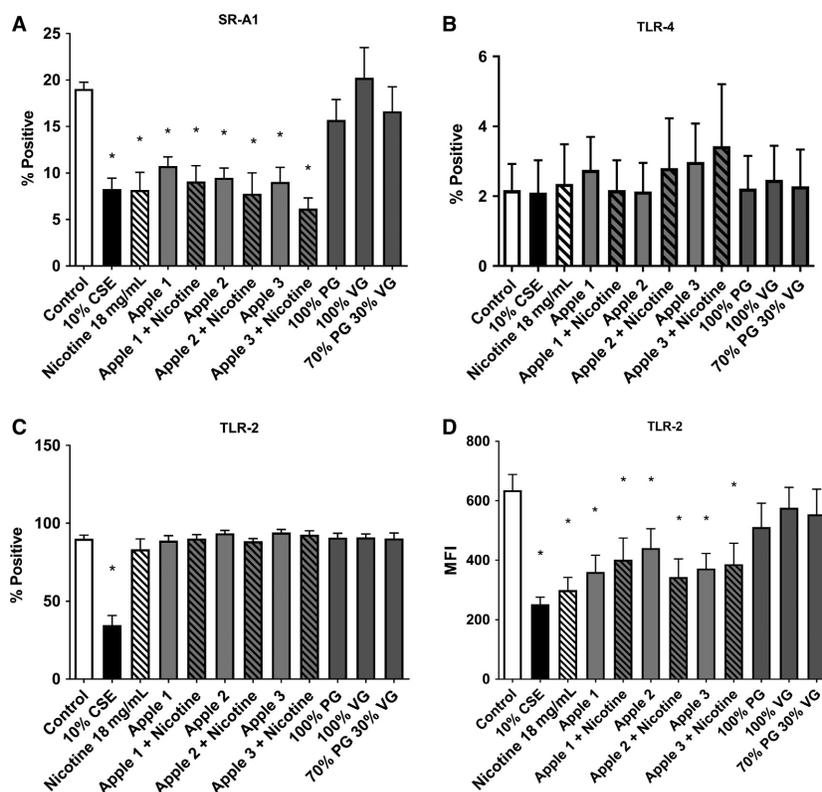


Figure 3. E-cigarette exposure inhibits phagocytic surface receptor expression. THP-1 PMA-differentiated macrophages were treated with 10% CSE and media infused with 50 puffs of the components of E-liquids, including three apple flavors \pm Nicotine (in 70:30 PG:VG), PG, VG, or 70:30 PG:VG for 24 h. Cells were lifted, stained with conjugated antibodies for (A) SR-A1 (B, C) TLR-2, and (D) TLR-4 and percentage positive of MFI measured \pm SEM using flow cytometry. $n = 4$ separate experiments performed in duplicate. Significance from control, $P < 0.05$, Mann–Whitney U test.

environment present in endosomes where phagocytosed bacteria are broken down. This newer stain does not require the quenching of any NTHi bound to the surface of the macrophages that previously used stains required. It also allowed us to differentiate between NTHi bound to the surface of macrophages and those phagocytosed, by placing the cells in pH 3 for 5 min after reading the phagocytosis number. By reading the cells again and comparing the difference in pHrodo red positive cells, we found that very few bacteria were bound to the surface of the macrophages (data not shown). Our data are consistent with other early E-cigarette studies which show greater survival of MRSA (Hwang et al. 2016) and mouse colonization with *Streptococcus pneumoniae* and influenza viral load (Sussan et al. 2015).

Our laboratory and others had previously shown that one of the reasons why cigarette smoke exposed macrophages had reduced phagocytic ability was a reduced level of expression of a range of bacterial recognition receptors SR-A1 (Heguy et al. 2006), TLR-2 (Droemann et al. 2005; Heguy et al. 2006; Kent et al. 2008), and TLR-4

(Droemann et al. 2005; Heguy et al. 2006; Kent et al. 2008). Thus, we assessed whether exposure to E-cigarette vapor-induced reduction of phagocytic ability was similarly linked to bacterial recognition receptors. We observed reduced scavenger receptor SR-A1 as well as reduced MFI of TLR-2 on the surface of THP-1 cells exposed to E-liquid \pm nicotine, and nicotine alone, but not the glycol bases, PG and VG. Some studies have also shown that SR-A1 deficient mice were more susceptible to pneumococcus and staphylococcus aureus infections (Thomas et al. 2000; Arredouani et al. 2006). This is the first data that shows that E-cigarettes may alter expression of bacterial recognition receptors on the surface of macrophages resulting in reduced phagocytic ability. We saw very low expression of TLR-4 on THP-1 cells, which shows that they are not a 100% ideal model for all bacterial recognition receptors, and further experiments will need to be done with primary alveolar macrophages to investigate the full range of receptors.

We furthermore analyzed the baseline levels of cytokines secreted by THP-1-differentiated macrophages

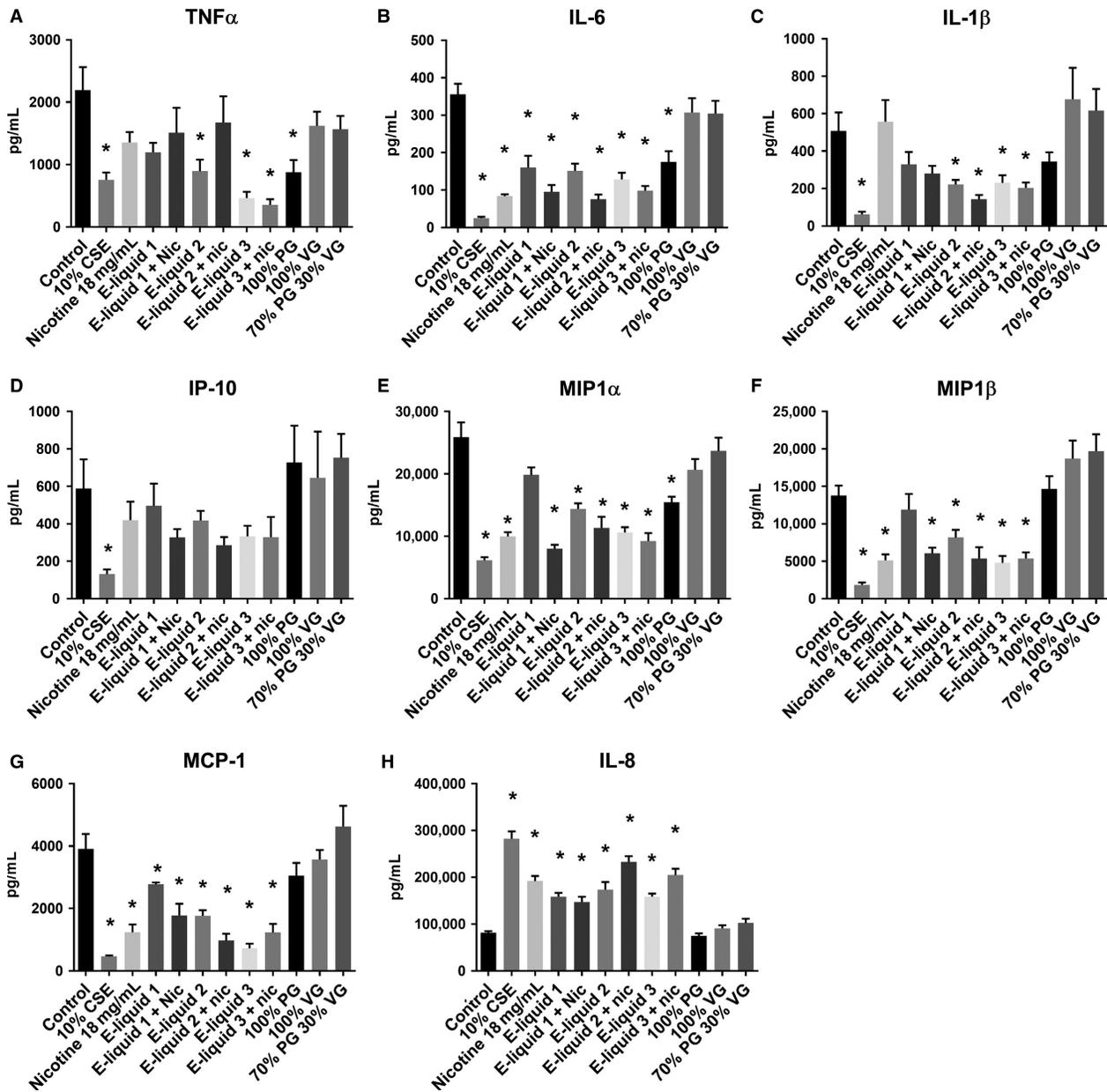


Figure 4. Cytokine profile of E-cigarette exposed cells. THP-1 PMA-differentiated macrophages were treated with 10% CSE and media infused with 50 puffs of the components of E-liquids, including three apple flavors \pm Nicotine (in 70:30 PG:VG), PG, VG, or 70:30 PG:VG for 24 h. Supernatant was collected, debris removed, and (A) IL-8, (B) TNF α , (C) IL-6, (D) IL-1 β , (E) IP-10, (F) MIP-1 α , (G) MIP-1 β , and (H) MCP-1 measured using a cytokine bead array. $n = 5$ experiments assessed in duplicate. Significance from control, $P < 0.05$, Mann-Whitney U test. Data represent mean \pm SEM.

exposed to E-cigarette vapor to determine whether E-cigarettes could have an effect on inflammatory responses in the lung. We observed an increase in baseline of the neutrophil chemoattractant IL-8 secretion with exposure to cigarette smoke extract and E-liquid \pm nicotine vapor. IL-8 secretion has previously been shown to be increased in macrophages exposed to cigarette smoke exposure

(Nordskog et al. 2005; Walters et al. 2005; Yang et al. 2007; Birrell et al. 2008; Kent et al. 2008, 2010; Mortaz et al. 2009; Paul-Clark et al. 2009; Metcalfe et al. 2014) as well as in BALF of smokers (McCrea et al. 1994; Weselius et al. 1997) versus nonsmokers. However, we saw a decrease in the secretion of a range of other cytokines including TNF α , IL-6, MIP-1 α , MIP-1 β , MCP-1, IL-1 β ,

and IP-10 when treated with E-liquid \pm nicotine, nicotine, or cigarette smoke extract. IL-12, IFN γ , IL10 were undetected in the cells supernatant (Data not shown).

This cigarette smoke result was somewhat surprising at first given previous studies with bronchoalveolar fluid (BALF) and or sputum from smokers and COPD patients had shown increases in some of these cytokines, especially IL-1 β (Keatings et al. 1996; Traves et al. 2002; Grumelli et al. 2004; Yang et al. 2007; Gualano et al. 2008; Brozyna et al. 2009; Li et al. 2014). However, further scrutiny of the literature found that many studies had reported similar findings. IL-6 was decreased in BALF of smokers versus nonsmokers (McCrea et al. 1994) with reduced secretion by cultured alveolar macrophages from smokers compared with nonsmokers (Soliman and Twigg 1992; McCrea et al. 1994; Sauty et al. 1994; Mikuniya et al. 1999; Bardelli et al. 2005; Chen et al. 2007) and those exposed to cigarette smoke (Nordskog et al. 2005; Birrell et al. 2008; Bozinovski et al. 2011). IL-1 β secretion also decreased in macrophages exposed to cigarette smoke (Ouyang et al. 2000; Nordskog et al. 2005; Birrell et al. 2008) as well as in cultured alveolar macrophages from smokers compared with nonsmokers (Brown et al. 1989; Soliman and Twigg 1992; Sauty et al. 1994; Birrell et al. 2008). TNF α secretion likewise decreased in macrophages exposed to cigarette smoke (Ouyang et al. 2000; Birrell et al. 2008; Bozinovski et al. 2011) and in cultured alveolar macrophages from smokers compared with nonsmokers (Yamaguchi et al. 1993; Sauty et al. 1994; Bardelli et al. 2005; Chen et al. 2007). Similarly, MIP-1 α (Birrell et al. 2008), MIP-1b (Nordskog et al. 2005), IP-10 (Bozinovski et al. 2011) and MCP-1 (Nordskog et al. 2005) secretion decreased in macrophages exposed to cigarette smoke.

Limited reports in the literature of the effects of E-cigarettes and their components on cytokine levels support our findings that E-cigarettes can induce a cytokine response in cells. IL-8 was shown to increase in neutrophils exposed to E-cigarettes (Higham et al. 2016). Decreased IL-1 β and TNF- α was observed in PBMCs exposed to nicotine (Ouyang et al. 2000) and further studies showed that cytokines including IL-6 and MCP-1 were decreased in the BALF of E-cigarette exposed mice (Lerner et al. 2015; Sussan et al. 2015). There have been no reports in the literature showing any changes for PG alone (as found in the present study), which we found very interesting given there is great discussion in vaping forums regarding the pros and cons of PG versus VG for taste and vapor production in various E-cigarette generations, and the awareness that PG has already been shown to be a skin (Lessmann et al. 2005) and lung irritant (Wieslander et al. 2001; Varughese et al. 2005).

The reasons for the decreased cytokine secretion in response to E-cigarettes and cigarette smoke are unclear; however, the low expression of TLR-4 on our THP-1-differentiated macrophages may have had an effect. IL-8 is known to be released via a TLR-2 mediated pathway but not TLR-4 (Paul-Clark et al. 2009), while many of the other cytokines are known to be released via a TLR-4-mediated pathway (ref). One study showed that MIP-1 α and MIP-1 β could trigger of TNF- α and IL-6 secretion by macrophages, so there could also be negative feedback loops in play (O'Grady et al. 1999). Furthermore, TLR-4-deficient mice have been shown to have a reduced neutrophil recruitment in response to cigarette smoke as well as a reduced IL-1 β and IL-6 response (Doz et al. 2008). Additionally, this is an in vitro culture system, and alveolar macrophages do not exist in isolation, so it is also likely that cytokine release into BALF is a more complicated procedure involving cross talk between the various cell types present in the airway as well as secretion by more than one cell type. Regardless, we show that E-cigarettes have an ability to alter cytokine responses from cells, and future studies should look at the BALF of healthy controls compared with vapers to assess physiological cytokine changes in the airways from E-cigarette use.

We conclude that E-cigarettes can cause phagocytic dysfunction of macrophages via alteration of bacterial recognition receptors and can alter cytokine secretion pathways. As such, E-cigarettes should be treated with caution by users, especially those who are nonsmokers, as this data adds to the growing literature showing E-cigarettes can potentially cause harm to a variety of cells, especially those in the airway where exposure is most direct. Future studies should also focus on whether E-cigarettes reduce the physiological changes seen in the airways of smokers and any long-term usage risks.

Conflict of Interests

The Authors have no competing interests or conflicts of interest to declare. No funding for this study or the authors salaries was received from any tobacco, E-cigarette, or quitting aid company.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1: Gating strategy for phrodo phagocytosis assays.

Figure S2: Example of gating strategy for marker expression on THP-1 macrophages.