Methods (supplemental)

E-cigarette vapour condensate was prepared using a novel method using 6 sterile tracheal suction traps (Unomedical, Denmark) connected in series and cooled in a dry ice/methanol bath (see Figure E1). All connections were sealed with parafilm to ensure even suction and connected to a vacuum source. As shown in Figure E1, 5 out of the 6 tubes were placed into a methanol dry ice bath. The 6^{th} tube was directly connected to the e-cigarette device to allow visual inspection of vapour flow through the rig. Tube 1, within the ice bath, was connected to a vacuum. Vaping was done under sterile conditions in a class 2 extraction hood to minimise risk of bacterial contamination.

Several papers have published data on puffing topography, based on which we calculated optimum puff duration (1-6). The mean puff duration was ~3 seconds. Our vaping model employed a puff time of 3 seconds every 30 seconds to allow time for the vapour to condense between each puff. This prevented overheating of the device.

A fully charged device was used for each acquisition. A fresh atomiser was used for each fresh preparation. Atomisers were pre-saturated with ECL/nfECL before use to ensure the unit did not dry-vape. 1.4ml of ECL/nfECL fluid was vaped from each device. Upon completion all 6 suction traps were normalised to room temperature and spun at 1500xg for 10 minutes to collect the condensate

E-cigarette devices

We used a second generation Kanger e-cigarette device which was the most popular in UK at the beginning of 2016 to produce condensate (Kanger LTD, Shenzhen, China- see Figure E1b).

4 devices were used in rotation. The device was fitted with a standard 650 mAh battery with a fresh 1.8 Ohm coil head (atomiser) was fitted for each preparation.

E-cigarette liquids

ECL with and without nicotine were obtained from American eliquids store (Milwaukee County Research Park, Wauwatosa) which uses pharmaceutical grade nicotine, adheres to FDA approved good manufacturing standards and has been used in previous animal exposure studies(7). Nicotine containing ECL was obtained at 36 mg/ml, nicotine free ECL (nfECL) was a 50:50 mixture of propylene glycol (PG) and vegetable glycerine (VG).

Gas chromatography-Flame ionisation detector (GC-FID)

GC-FID to determine nicotine concentration was performed by the University of Birmingham Chemistry department. GC-FID was performed using a GC system (Shimadzu GC-2010, Shimadzu, Japan), equipped with an FID. Separation of compounds was conducted with a Phenomenex ZB-Wax column (30m x 0.25mm (0.25um Film Thickness, Phenomenex Cheshire, UK), using Helium as the carrier gas. The inlet temperature was 250 °C (110.00 kPa) with a split ratio of 50:1 and the FID temperature was 250 °C. The oven temperature was programmed as follows: the column was ramped initially from 110 to 185 °C 0- 7.5 minutes, then increased from 185 to 210 °C for 2.5 minutes from 210 to 240 °C for a further 2 minutes and held at 240.00 °C for 3 minutes. Retention time for nicotine was 7.6 min internal standard quinoline was 8.2 min. L-Nicotine standard (Fisher Scientific, UK) was used as a reference standard.

AM isolation

Lung tissue was perfused with 0.15 M saline via pressure bag by inserting the needle (21-gauge) in bronchioles were possible. When saturated the tissue was gently massage to facilitate emptying lavage from the tissue ready for the next instillation. This process was repeated until the perfusate contains less than $1x10^4$ monocytes/ml. Cells were pelleted from the perfusate by centrifugation at 500xg, 5 mins. Mononuclear cells were then separated by gradient centrifugation using Lymphoprep, according to the manufacturer's instructions. Collected macrophages were washed, counted and assessed for purity by cytospin (8, 9). Average yields of alveolar macrophages were 75million cells per resection.

THP-1 macrophages

THP-1 human acute monocytic leukaemia cells (European Collection of Cell Cultures) were differentiated into macrophages by stimulation with 0.2 mM phorbol 12-myristate 13-acetate (PMA) for 24 hours. Adhered cells were washed with PBS and rested in RPMI (supplemented with 10% FBS, L-glutamine, penicillin and streptomycin) for 3 days before use(10).

Viability

Viability was assessed using CellTiter 96® AQueous One Solution using the manufacturer's instructions. Briefly, 25000 cells were seeded per well in a total volume of 100 µl RPMI supplemented medium. Cells were exposed to ECVC/ECL with and without nicotine for 6/24hour exposure. In some cases cell were treated with N-Acetyl-cysteine (NAC), Ly294002

(5-20nM) and PIK-75 (5-20nM), in the presence or absence of ECVC. After 24 hours, cells were washed twice with PBS and 100µl phenol red free RPMI added per well. 20µl of CellTiter 96® Aqueous One Solution Reagent was added into each well and incubated at 37°C for 4 hours in a humidified, 5% CO2 atmosphere. The reaction was stopped by addition 25 µl of 10% SDS to each well. The absorbance was read at 490nm using a Biotek Synergy2 plate reader.

Apoptosis

Apoptosis was assessed by flow cytometry using an Annexin V assay (BD Biosciences, UK) in combination with the vital dye propidium iodide (PI) (Sigma-Aldrich, UK). Briefly, 0.5M macrophages were seeded per well and cultured for 24 hours prior to challenge. Macrophages were challenged with ECVC/nfECVC for 24 hours. In some cases cell were treated with NAC in the presence or absence of ECVC. Cells were trypsinised and washed 1x in PBS/BSA/EDTA and 2x in Annexin V binding buffer (BD). Cells were stained with Anti-Annexin V-FITC conjugate (BD) for 15 minutes on ice and washed a further 2x with Annexin V binding buffer. Propidium Iodide (PI, Sigma) was added as appropriate to stain necrotic cells. Macrophages remaining unlabelled ($Annexin^-$/ Pi^-) are live cells. Cells positive for Annexin V only ($Annexin^+$/ Pi^-) are undergoing early apoptosis, whilst cells positive for both Annexin V and PI ($Annexin^+$/ Pi^+) are late apoptotic cells. Macrophages staining PI only ($Annexin^-$/ Pi^+) have undergone necrosis. Detection of labelled cells was done using a BD Accuri C6.

Cellular Reactive Oxygen Species Assay

Reactive Oxygen Species (ROS) was measured using DCFDA assay (Abcam ab113851) according to manufacturer's instructions (Abcam, Cambridge, UK). Cells were seeded at

25000 cells per well in 100 µL and allowed to adhere overnight. 100 µL/well of the diluted 2',7' –dichlorofluorescin diacetate (DCFDA) solution was added per well and incubated for 45 minutes at 37°C in the dark. Macrophages were then treated with 0.5% ECVC/nfECVC for 4 hours. Plates were measured Using a Biotech Synergy plate reader at Ex/Em= 485/535nm in end point.

Phagocytosis assay

Phagocytosis assay was carried out using pHrodo® red *E. coli* or *S.aureus* BioParticles® (Invitrogen, UK) according to the manufacturer's instructions, in 96 well plate format. Phrodo beads were prepared according to manufacturer's instructions to a final concentration of 1 mg/ml. Macrophages were seeded at 50000 cells per well in black well, clear bottomed 96 well plates (VWR, Sussex, UK) and allowed to adhere overnight. Macrophages were then treated with 0.5% ECVC/nfECVC. 50 µL of Phrodo bead suspension was added per well and incubated for 6 hours at 37°C. After 6 hours, cells were washed 3x with PBS before adding of 100 µl fresh PBS for reading. Fluorescence was then measured Using a Biotech Synergy plate reader at Ex/Em= 509/533 nm in end point.

Viability assessment after PI3K inhibition

Viability was assessed using CellTiter 96® AQueous One Solution using the manufacturer's instructions. Briefly, 25000 cells were seeded per well in a total volume of 100 µl RPMI supplemented medium. Cells were exposed to ECVC/nfECVC 24hour exposure in the presence or absence of PI3K inhibitor PIK-75 (Figure 8.) or Ly292004 (Figure E5.). After 24 hours, cells were washed twice with PBS and 100µl phenol red free RPMI added per well. 20µl of CellTiter

96® AQueous One Solution Reagent was added into each well and incubated at 37°C for 4 hours in a humidified, 5% CO2 atmosphere. The reaction was stopped by addition 25 µl of 10% SDS to each well. The absorbance was read at 490nm using a Biotek Synergy2 plate reader.

Statistical Analyses

All statistical analyses were performed using GraphPad PRISM 6.0 software package (San Diego, CA). Parametric results are expressed as the *mean ±* SEM and unless specified otherwise all results are representative of 6-8 independent experiments performed in duplicate. Differences between multiple treatments were compared by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-test. Non-parametric results are expressed as the median with interquartile range and unless specified otherwise all results are representative of at 6-8 independent experiments performed in duplicate. Differences between multiple treatments were compared by Kruskal-Walis test followed by Dunn's multiple comparison post-test. A p value of ≤0.05 was considered to represent a statistically significant difference.

Results (Supplemental)

Validation of our model system of condensing vaped ECL .

A detailed validation of our model of condensing vaped ECL is available online. The model has good reproducibility in terms of volume of recovery (60.8%), nicotine content (87%), as well as particulates (OD at 370nm). Further we confirm that there was no difference in the effects of a standardised dose of ECVC on AMs and that ECVC loses its effects over time with storage (Figure E3, $p<0.0001$).

We assessed the reproducibility of the technique in terms of recovery volume, nicotine content and OD at 370nm to assess generation of particulates (see Table 1b). Using 6 fresh preparations of ECVC mean nicotine content was 26.52mg/ml, a recovery rate of 87% compared to input. Mean recovery by volume was 851.7 µl, CV 3.36%. Generation of particulates as measured by O.D found an average increase of 2.44 fold, CV 0.2% (Table 1 a+b).

The effect of the 6 different batches of (0.8%) ECVC on AM viability was assessed however no significant difference was observed between the fresh preparations (ECVC $p=0.1633$, nfECVC $p= 0.1401$) (see Figure E2).

ECVC loses potency over time

CSE used in traditional smoking studies has been shown to lose potency over time therefore we assessed the effect of ECVC/nECVC on macrophages on the day of preparation (day1) and at day 5, 7, 11 and 16 post preparation. Cells were exposed to 0.8% ECVC/nfECVC for 24h.

There was a significant loss of cytotoxicity after exposure to both ECVC and nfECVC, 7 days post preparation (Figure E3, $p<0.0001$). All subsequent experiments were therefore carried out within 48h of preparation to ensure consistency between experiments.

Pro-inflammatory cytokine, chemokines and proteases are induced by 24 hours exposure to 0.5% ECVC

Induction of pro-inflammatory cytokines, chemokines and metalloprotease is summarised in Table 2. 0.5% ECVC significantly induced production of pro-inflammatory cytokines; IL-6 ($p<000.1$) and TNF-α ($p<0.001$), pro-inflammatory chemokines; CXCL8 ($p<0.0001$) and MCP-1 ($p<0.01$) and matrix metalloprotease 9 (MMP-9) ($p<0.0001$) compared to UTC and nfECVC. 0.5% nfECVC significantly induced production of IL-6 ($p<0.001$), CXCL-8 ($p<0.0001$) and MMP-9 ($p<0.0001$) compared to UTC.

ECVC significantly inhibits phagocytosis by AM and THP-1 macrophages

Incubation of THP-1 macrophages with ECVC and nfECVC reduced pHrodo *S. aureus* BioParticles® phagocytosis by 60.9% ($p<0.0001$) and 62.9% respectively ($p<0.0001$, Figure E4).

Effect of N-acetyl cysteine treatment following ECVC challenge upon THP-1 derived macrophages

ROS production in response to cigarette smoking (or smoke extract) has been implicated as a mediator of adverse effects (11) therefore we examined the possible utility of N-acetyl

cysteine **(**NAC) treatment in reducing the harmful effects of ECVC in THP-1 macrophages. Both AM and THP-1 macrophages were used for these experiments due to the large number of experimental conditions and numbers of cells required.

NAC can restore phagocytic function of ECVC treated macrophages

THP-1 macrophages were also used to assess phagocytosis of *S. aureus* pHrodo bioparticles® in the presence of ECVC + NAC (See Figure E4). Condensate treatment reduced phagocytosis: ECVC 60.9% reduction ($p<0.0001$), nfECVC 62.9% reduction ($p<0.0001$). In keeping with the *E. coli* phagocytosis data, simultaneous treatment with 5mM NAC significantly restored phagocytic function; ECVC 22% restoration of activity ($p<0.0001$), nfECVC 24.8% restoration of activity ($p<0.0001$) (Figure E4).

ECVC effects upon THP-1 macrophage viability and apoptosis are attenuated by inhibitors of Phopshopinositol3 kinase (PI3K)

ROS induced lung inflammation in COPD has been reported to be associated with PI3kinase activation(12). To explore a role for PI3 kinase in ECVC induced responses we used the pan-inhibitor LY294002 (5nM) as well as an isoform selective inhibitor (PIK75 10nM).

Both general PI3K inhibition (Figure E5a) and PI3K alpha isoform inhibitor (PIK-75, Figure 7) attenuated the effects of ECVC (Ly294002; 37.4%, PIK75; 35% more viable than with ECVC alone, $p<0.0001$). This protective effect was also evident when cells were challenged with nfECVC (Ly294002 25 %, PIK75 29.2% more viable than with nfECVC alone $p<0.0001$).

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