Maternal e-cigarette exposure in mice alters DNA methylation and lung cytokine expression in offspring.

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Abstract

Introduction: E-cigarette usage is increasing, especially among the young, with both the general population and physicians perceiving them as a safe alternative to tobacco smoking. Worryingly, e-cigarettes are commonly used by pregnant women. As nicotine is known to adversely affect children *in-utero*, we hypothesised that nicotine delivered *via* e-cigarettes would negatively affect lung development. To test this we developed a mouse model of maternal e-vapour (nicotine and nicotine-free) exposure, and investigated the impact on the growth and lung inflammation in both offspring and mothers.

Method: Female Balb/c mice were exposed to e-fluid vapour containing nicotine (E-cig18mg/mL, equivalent to 2 cigarettes/treatment, twice daily,) or nicotine free (E-cig0mg/mL) from 6 weeks prior to mating until pups weaned. Male offspring were studied at postnatal day (P) 1, 20 and at 13 weeks. The mothers were studied when the pups weaned.

Results: In the mothers' lung, e-cigarette exposure with and without nicotine increased the pro-inflammatory cytokines IL-1β, IL-6 and TNF-α. In adult offspring, TNF-α protein levels were increased in both E-cig18 and E-cig0 groups, whilst IL-1β was suppressed. This was accompanied by global changes in DNA methylation.

Conclusion: In this study, we found that e-cigarette exposure during pregnancy adversely affected maternal and offspring lung health. As this occurred with both nicotine free and nicotine containing e-vapour the effects are likely due to by-products of vaporisation rather than nicotine.

Key words: e-cigarette, e-fluid, maternal programming, inflammatory cytokine

Introduction

Tobacco cigarette smoking during pregnancy is well known to be deleterious to respiratory health for both the mother and unborn child. Smoking is well known to increase the risk of both bacterial and viral infection (1) and while little evidence exists, at least similar effects would be expected in pregnancy. Similarly, maternal tobacco smoke exposure leads to increased risk of recurrent wheeze, development of asthma, bronchitis and hospitalisation for respiratory infections, and a lifelong decrease in pulmonary function for the offspring (reviewed in (2)). Recent studies have suggested that maternal smoking also predisposes to the development of chronic obstructive pulmonary disease (3). Nicotine delivered in cigarettes is well known to contribute to the detrimental effects of maternal tobacco smoking on offspring's lung development, leading to the suggestion that nicotine delivered by e-cigarette use would be similarly deleterious (4) . However, it is important to note that e-cigarettes deliver more than just nicotine.

Advertised as a safe alternative to tobacco cigarette, e-cigarettes have rapidly infiltrated the market since their first appearance in 2004. E-cigarette sales continue to increase with sales in traditional retail stores in the United States doubling between 2012 and 2013, equating to over $600 million in sales (5). Approximately 5% of adults in the United States are current users, with the highest prevalence in young adults (18-34 years old) (6). E-cigarette use among adolescents has also increased dramatically since 2011, with an estimated 3 million middle and high school student users in 2015 (7). E-cigarettes administer nicotine as a vapour, do not require tobacco combustion and therefore are assumed to remove the risks associated with tobacco cigarettes. However, numerous studies have reported the presence of toxic compounds in e-cigarette aerosol (8) and deleterious effects on a variety of cells *in vitro* (9-11). Despite the rising use of e-cigarettes and the potential risks, little is known as to the effect of e-cigarettes in utero.

Pregnant women may be particularly drawn to advertising campaigns that suggest e-cigarettes as a "safe" alternative to tobacco cigarettes. This opinion is echoed by the medical community, for example members of the American College of Obstetricians and Gynecologists consider e-cigarettes safer than tobacco cigarettes. Indeed, recent evidence also suggests that most pregnant women perceive e-cigarettes as a safer alternative to tobacco cigarettes (12, 13). Currently the use of e-cigarettes during pregnancy appears similar to tobacco cigarette smoking, with up to 15% of pregnant women using e-cigarettes (12). Taken together with data suggesting increased use amongst women of childbearing age (6), these studies reinforce the importance of determining the potential respiratory toxicity of e-cigarette use during pregnancy.

The development of asthma and COPD is associated with a variety of intrauterine exposures such as cigarette smoke. Such predisposition to lung disease is thought to be due to abnormal regulation of the inflammatory environment and/or physical changes to the lung structure. Both asthma and COPD are chronic inflammatory diseases of the airways and have traditionally been distinguished by the pattern, or phenotype, of airway inflammation. Early onset asthma is typically characterised by allergic eosinophilic inflammation (typified *via* increased IL-4, IL-5, and IL-13) whilst COPD is associated with non-allergic inflammation (for example increases in IL-6, IL-1β and TNF- α), although there exists overlap in inflammatory profiles between asthma and COPD (14). Numerous growth factors such as Scgb1a1 (Secretoglobin, or Clara cell secretory protein Ccsp) or any of the surfactant-associated protein genes (Sfpta, Sfptb, Sfptc) are important in determining the fate of airway and alveolar cells (15). Moreover, *in utero* changes in growth factors, such as Platelet Derived Growth Factor α (PDGFα) (16), or receptors, such as the Ephrin type-B receptor 2, effects alveolarization (17), and thus may contribute to the development and severity of future lung diseases.

Since E-cigarettes are potentially deleterious to the respiratory health of the developing lung *in vitro* the aim of the present study was to examine the potential effects of e-cigarettes in a mouse model of maternal exposure on respiratory immune status of both the mother and offspring.

Methods and materials

Animals

The animal experiments were approved by the Animal Care and Ethics Committee of the University of Technology Sydney (ACEC #ETH15-0025), and performed according to the Australian National Health & Medical Research Council Guide for the Care and Use of Laboratory Animals. Balb/C mice (Animal Resource Centre, Canning Vale, WA, Australia) had *ad libitum* access to standard laboratory chow and water while housed at 20±2 °C and maintained on a 12-h light, 12-h dark cycle (lights on at 06:00 h).

E-cigarette exposure and study design

After one week of acclimatisation, 7 weeks old female mice were randomised into three groups for exposure; room air (sham), e-cigarette liquid without nicotine (E-cig0) and e-cigarette liquid containing 18mg/mL nicotine (E-cig18). Both e-cigarette liquids contained 50% propylene glycol/50% vegetable glycerine and tobacco flavour additives (Vaper Empire, VIC, Australia). Only

female breeders were exposed with male breeders and offspring remaining in their home cage during exposure. For exposure, female mice were removed from their cages and placed in a custom designed 9L chamber filled with the e-cigarette fluid aerosols twice daily (2 x 15 minutes' exposure with a 5 minute washout interval, equivalent nicotine exposure to 2 tobacco cigarettes twice daily). At the end of the exposure protocol mice were returned to their cages. E-cigarette fluid aerosols were generated by an e-cigarette device (KangerTech NEBOX, KangerTech, Shenzen, China). Adult mice were exposed for 6 weeks prior to gestation, during gestation and lactation (up-to postnatal day 20), pubs were never directly exposed. The vapour was generated using 4 x 5s activations at 30 Watts for each exposure, with a 20s interval between each vaping to prevent overheating of the coil. The nicotine dose was adopted from our previous studies on the effect of maternal cigarette smoke exposure on offspring's health (18, 19). Sham exposed mice were treated exactly the same, but exposed to room air and not e-cigarette vape.

Female breeders were sacrificed when the pups weaned at postnatal day (P) 20. Male offspring were euthanized at day P1, P20, and 13 weeks. Liver, retroperitoneal fat, epididymal fat was harvested and weighed from the mothers and offspring. The lung was harvested from female breeders and offspring, snap frozen and stored at -80°C for mRNA and protein analyses. Blood from P20 offspring was collected via cardiac puncture after deep anaesthesia (4% isoflurane, 1% O_2) and cotinine measured in the plasma using an ELISA kit (Abnova, Taipei, Taiwan) as per manufacturer's instructions.

Real-time PCR

mRNA was analysed using pre-optimized and validated TaqMan® primers and probes Table 1, Thermo Fisher Scientific, MA, USA). See online supplement for full methods.

Western Blotting

The protein levels of Erk1/2, JNK, p38, p65, IL-1β, IL-6, and TNFα were measured by western blotting. See online supplement for full methods.

Global DNA methylation assay

A 5-methyl Cytosine kit (Zymo Research, CA USA) was used to assess global DNA methylation levels in the lung from the offspring at P1 (n=3). See online supplement for full methods.

Statistical analysis

Results are expressed as mean ± SEM and analysed by one-way ANOVA followed by Fisher's Least Significant (LSD) *post hoc* tests (Statistica 10, Statsoft Inc. OK, USA). P<0.05 was considered as the threshold for statistical significance.

Results

The Effect of E-cigarette Exposure on the Mothers

Anthropometry markers

Weight gain can be used as a global indication of health status in animal models. After the first 6 weeks of exposure, mothers exposed to room air (control) and mothers exposed to E-cig18 had similar weight gain (1.50±0.33g and 1.49±0.20g, respectively). Surprisingly, the mothers exposed to E-cig0 only had 1/3 weight gain of the Sham group (0.46±0.39g). When offspring were weaned, there was no difference in weight gain of the mothers between the three exposure groups (Sham: 6.43±0.57g, E-cig0: 6.95±0.50g, E-cig18: 5.66±0.79g,). Similarly, liver weight did not differ between the three groups (expressed as % body weight: control 5.93±0.22%, E-cig18 6.69±0.28%, E-cig0 6.74±0.37%). However, compared to the control group (expressed as % body weight 0.30±0.04%), retroperitoneal fat mass was significantly reduced in both the E-cig18 (0.18±0.02%) and E-cig0 (0.16±0.002%) mothers (P<0.05 for both).

Lung inflammatory markers

To determine the effect of e-cigarette exposure on the lung inflammatory environment, we measured pro-inflammatory cytokine protein levels in the lungs of mothers. IL-1β protein was increased in E-cig18 dams compared to both control and E-cig0 dams (p<0.01 and p< 0.05, respectively, Figure 1a). There was no difference in IL-1β protein between control and E-cig0 groups (p=0.11). IL-6 protein level was increased in the E-cig0 dams (p<0.05) but not the E-cig18 dams compared to control (Figure 1b). Compared to control, TNFα protein level was more than tripled in both E-cig18 and E-cig0 dams (both p<0.01, Figure 1c).

Increased cytokine production can occur by several mechanisms, but the most common is activation of transcription factors leading to transcription and translation. To investigate if e-cigarette exposure was activating pro-inflammatory signalling, we measured the expression of various pro-inflammatory signalling molecules known to be involved IL-1β and IL-6 production (Figure 2). Exposure to E-cig0 upregulated total Erk1/2 and total JNK, whilst E-cig18 upregulated only total

JNK. Activation of ERK1/2 and JNK was assessed by measuring phosphorylated protein. E-cig0 increased p-JNK but not p-Erk1/2. P38 and p65 NF-κB were not increased by either E-cig0 or E-cig18 in comparison to control animals.

The Effect of Maternal E-cigarette Exposure on Offspring

Anthropometry markers

At P1, there was no difference in body weight or organ weight among the offspring groups (sham, E-cig0 and E-cig18 (Table 2). At P20, E-cig0 offspring were heavier than both control and E-cig18 offspring ($p<0.05$ and $p<0.01$, respectively). E-cig18 offspring had reduced body weight compared to controls ($p<0.05$). In contrast, liver weight, as a percentage of body weight, was increased in E-cig18 offspring compared to control ($p<0.01$), but not in the E-cig0 offspring suggesting that the effect was mediated by nicotine. Both E-cig0 and E-cig18 offspring had bigger retroperitoneal fat mass compared to control offspring ($p<0.05$ for both), with E-cig0 having increased fat mass compared to E-cig18 ($P<0.05$). Epididymal fat mass was only increased in E-cig0 compared to both control and E-cig18 offspring ($p<0.01$ for both). At 13 weeks, there was no difference in body weight between the groups. However, liver weight in the E-cig0 offspring was reduced compared to both control and E-cig18 offspring ($p<0.05$). Retroperitoneal fat mass was increased in both E-cig18 and E-cig0 offspring compared to control ($p<0.05$), whereas there was no difference in epididymal fast mass.

As expected, blood cotinine levels were increased in E-cig18 offspring (9.12±1.17 ng/ml) at weaning compared to both control and E-cig0 offspring (2.83±0.63 and 3.31±0.57 ng/ml, respectively, n=9-10, $p<0.01$ for both *vs* E-cig18).

Lung developmental markers in early life

To determine the potential effect of *in utero* e-cigarette exposure on lung development we measured markers of alveoli development by qPCR. At P1 there was no difference in platelet-derived growth factor (*PDGF*) and ephrine B2 (*EPBP2*) mRNA expression among the three groups (Figure 3a,c). At P20, *PDGF* mRNA levels were significantly upregulated in the E-cig18 and E-cig0 offspring compared to the control group ($p<0.05$ for both, Figure 3b). mRNA levels of surfactant protein C (*Sftpc*) were similar among the groups at both P1 and P20 (Figure 3e,f).

Lung inflammatory markers

To investigate if the effects of *in utero* e-cigarette exposure on lung inflammatory homeostasis were different at adulthood we measured the levels of pro-inflammatory cytokines. At 13 weeks of age IL-1β protein levels were supressed in the lung of both E-cig18 and E-cig0 offspring compared to control (both $p<0.01$ *vs* Sham, Figure 4a). Although not significant, there was a trend for increased IL-6 in the E-cig0 group compared to control ($p=0.05$), whereas there was no change in E-cig18 offspring (Figure 4b). In contrast, TNFα protein levels were significantly increased in both E-cig18 and E-cig0 offspring compared to control ($p<0.01$ for both, Figure 4c).

We subsequently investigated the effect of maternal e-cigarette exposure on protein levels in lung homogenates of signalling pathways known to control inflammation (Figure 5). Although neither maternal e-cigarette exposure altered Erk1/2 levels compared with the controls, there was an increase in Erk1/2 protein in E-cig0 offspring compared to E-cig18 offspring. When adjusted for total Erk1/2, p-Erk1/2 was increased in E-cig18 mice compared to E-cig0 mice (Figure 5c). There were similarly disparate effects on JNK protein expression, with increased levels in E-cig18 mice ($p<0.05$ vs control, Figure 5d), but not E-cig0 mice, compared to controls. However, p-JNK protein and p-JNK adjusted for total protein were increased in mice exposed to E-cig0 compared to E-cig18 offspring ($p<0.05$ for both). Compared to control, p38 expression was increased in both E-cig0 and E-cig18 mice ($p<0.01$ vs control for both, Figure 5g), p-p38 was increased by only E-cig18, with similar ratios of total to phosphorylated p38 between the three groups (Figure 5i). There was no effect on total p65 of either maternal e-cigarette exposure (Figure 7j). However, the phosphorylated form was halved in E-cig18 offspring ($p<0.05$ *vs* control, Figure 5k) suggesting altered NFκB activation, but was not effected by E-cig0 exposure. The ratio between phosphorylated and total p65 was significantly reduced in the E-cig18 offspring ($p<0.01$ *vs* control, Figure 5l).

Activation of epigenetic remodelling events by global DNA methylation

DNA methylation is an important mechanism by which various genes can be controlled, with DNA hypermethylation (increased methylation) generally resulting in gene silencing. We assessed global DNA methylation in the lungs at P1, by measuring the amount of methylated cytosines (5 mC) in the sample relative to global cytidine (5 mC + dC). Compared to control offspring at P1, global methylation was more than tripled in E-cig0 offspring ($p<0.01$ *vs* both E-cig18 and control) and more than doubled in E-cig18 offspring ($p<0.05$ *vs* control Figure 6). To complement this data, we also measured the mRNA expression of a range of inflammatory cytokines known to be elevated in people with asthma and/or COPD. We found that maternal e-cigarette exposure increased the expression of inflammatory cytokines in P1 lungs. Interestingly, E-cig0, but not E-cig18, increased

IL-5, IL-13, and TNFα (Figure 6 B-D), whilst no changes were observed in IL-4, Il-1β or IL-6 in either e-cigarette exposure group (Figure 6 E-G).

Discussion

E-cigarettes are often considered a safe alternative to tobacco smoking but very little is known as to their potential toxicity, especially in susceptible populations such as pregnant mothers and their unborn children. Therefore, we developed a mouse model to investigate the effects of intrauterine e-cigarette exposure to determine the effect on respiratory health in both mothers and offspring. Long-term E-cigarette exposure induced significant inflammatory responses in the mothers, and this was independent of the effect of nicotine. *In utero* e-cigarette exposure had differential effects on the markers of lung development in early life. However, mice exposed to intrauterine e-cigarettes had altered homeostatic levels of several pro-inflammatory cytokines in adulthood, suggesting that e-cigarette exposure re-programmed immune homeostasis. Surprisingly, our results suggest that these effects appear partially independent of nicotine.

The adverse effect of tobacco smoking during pregnancy on offspring's birth weights is well known and contributes to increased risk of numerous chronic diseases (20). In the present study, *in utero* exposure to e-cigarette containing nicotine lowered birth weight (albeit not significantly), and led to reduced body weight at weaning. Surprisingly, offspring exposed to e-cigarette without nicotine *in utero* had increased fat mass at weaning compared to controls and *in utero* nicotine exposed mice. This may be due to prioritisation of maternal nutrients to suckling pups, as E-cig0 mothers had the least weight gain at weaning. At adulthood, there was no difference in fat mass between the e-cigarette offspring groups, but both groups had increased fat mass compared to control. Although the mechanisms for the increased fat mass are unknown, it may reflect abnormal metabolic function and have potential implications for the development of metabolic disorders.

It has been well accepted that tobacco smoke and nicotine exposure during prenatal and postnatal life can impair lung development (21). Therefore, we measured mRNA levels of three markers important in lung development; *PDGFα*, *EphB2* and *Sftpc*. E-cigarette exposure did not alter *EphB2* and *Sftpc*. In contrast, *PDGFα* was increased in both e-cigarette exposure groups i.e. with and without nicotine. Although the majority of the effect of *in utero* tobacco exposure on lung development is attributed to nicotine (22), the increase in *PDGFα* in the present study suggests that either the e-cigarette humectants or flavour additives increased *PDGFα* mRNA expression. PDGF

signalling is important to lung development and elevated PDGF signalling has been linked to lung fibrosis (23). Exposure to e-cigarette aerosol containing nicotine in the first 10 days of life has been shown to induce small increases in alveolar size in mice (24). In this study, we measured selected markers of lung development, which does limit the generalizability of the findings to all aspects of lung development. To fully investigate the abnormality in lung development, other molecular markers or anatomical changes using careful stereotactic techniques should be carried out.

The present findings suggest that e-cigarette exposure during pregnancy induces an abnormal inflammatory environment in the lung of both the mothers and offspring. Protein expression of IL-1β, IL-6 and TNFα were all increased in the lungs of mothers, with the effect on IL-6 and TNFα being independent of nicotine. A pro-inflammatory environment within the lung is consistent with previous findings reporting increased IL-6, MCP-1, IL-1α and β, IL-10 and IL-13 in bronchoalveolar lavage fluid of adult mice exposed to e-cigarette containing nicotine and tobacco flavourings (10). Similarly, exposure to e-cigarette vapour extract promotes pro-inflammatory responses from human neutrophils (25) and airway epithelial cells (10). Importantly, effects in airway epithelial cells were also independent of nicotine. As seen in the mothers, TNFα was also increased in the lungs of the offspring; however, in contrast to mothers, IL-1β was decreased in offspring exposed to e-cigarette. This effect was independent of nicotine. Our findings also suggest that inflammatory signalling pathways are differentially altered in mothers and offspring, with changes in ERK1/2 and JNK expression seen in mothers but changes in p38 and p65 in offspring. Taken together, these findings suggest that exposure to e-cigarettes induces immune dysregulation within the lung, and that the effect of *in utero* exposure can been seen in adulthood.

DNA methylation is a potential mechanism for the adverse impact of smoking during pregnancy on offspring health. Our findings suggest that *in utero* e-cigarette exposure leads to abnormalities in DNA methylation. Surprisingly, these effects appear to be independent of nicotine since global DNA methylation was increased in mice exposed to e-cigarette vapour with and without nicotine. In this study to identify potential epigenetic changes due to e-cigarette exposure we measured global DNA methylation. We report that maternal exposure to both e-cigarettes containing nicotine and without nicotine led to increased DNA methylation in the lungs of offspring. However, these changes in global DNA methylation do not provide information as to which parts of the genome are epigenetically reprogrammed. Therefore, future studies should to be carried out to identify site-specific methylation changes and their relationship to the potential consequences of e-cigarette use.

Recent evidence suggests that thousands of CpGs are differentially methylated in newborns and older children who were exposed to tobacco smoke *in utero* (26). These methylated sites corresponded to pathways involved in anatomical development, protein kinase activity and gene expression of pro-inflammatory cytokines. Taken together, these findings suggest that e-cigarette use during pregnancy is likely to induce epigenetic modifications in offspring that contribute to detrimental health outcomes.

Our mouse model adequately reflects important aspects of e-cigarette use in humans and thus provides important evidence for the effect of intrauterine e-cigarette exposure in humans. Firstly, we chose to use a flavoured e-cigarette as research suggests that almost all users under 30 and over half of adults use flavoured e-cigarettes (27). Tobacco flavour have been suggested to be particularly preferred by users (28), especially in current and former tobacco smokers (29). Secondly, serum cotinine levels in offspring of mothers exposed to E-cig18 in the present study (9.12±1.17 ng/ml) were similar to our findings in offspring of mothers exposed to tobacco cigarette smoke prior to gestation until weaning (19, 30). It is also consistent with the blood level in human infants of smoking mothers (31), suggesting that the model is sufficient to allow comparison with the effects of tobacco cigarette smoke. Although the effect of *in utero* exposure on offspring cotinine in humans is unknown, our model led to similar levels as seen in adult human users following a single session (32). Taken together, this suggests that our findings in the present mouse model likely reflect the potential for harm to human users and their unborn child after e-cigarette exposure.

The current study focused on the developmental and inflammatory aspects of maternal programming effects. Based on our previous studies in mouse models of maternal cigarette smoking, there is a significant gender difference. Male offspring are more susceptible to the effect of maternal smoking (delayed development and increased inflammatory responses in multiple organ systems). In future studies, we would like directly study gender differences in the offspring, as well as look at differences in maternal versus paternal smoking.

This study has limitations that need to be considered. In developing a model of *in utero* e-cigarette exposure we did not carry out mechanistic studies, have a tobacco exposure comparator group, use a range of e-cigarette exposure concentrations, or use several flavours of e-cigarettes. These are perhaps design flaws of our study. Of these limitations, we think that the lack of different flavours is perhaps the greatest limitation. Different flavours are likely to decompose to different toxic

compounds during vaporisation; therefore it may be possible that different effects are observed. However, we think that even though our study is limited by design and analysis the results are alarming and needs verification by other groups.

In conclusion, we report that in a mouse model of e-cigarette exposure during pregnancy, both nicotine and non-nicotine constituents induce inflammatory responses in the lung of both mothers and offspring. Our findings suggest that different signalling pathways may be altered in mother and offspring, and that epigenetic modifications are likely to contribute in offspring. Taken together, our study suggests that e-cigarette use during pregnancy should not be considered safe for future lung health of the child.

References

1. Arcavi L, Benowitz NL. Cigarette smoking and infection. *Archives of internal medicine* 2004; 164: 2206-2216.
2. Zacharasiewicz A. Maternal smoking in pregnancy and its influence on childhood asthma. *ERJ open research* 2016; 2.
3. Perret JL, Walters H, Johns D, Gurrin L, Burgess J, Lowe A, Thompson B, Markos J, Morrison S, Thomas P, McDonald C, Wood-Baker R, Hopper J, Svanes C, Giles G, Abramson M, Matheson M, Dharmage S. Mother's smoking and complex lung function of offspring in middle age: A cohort study from childhood. *Respirology* 2016; 21: 911-919.
4. McEvoy CT, Spindel ER. Pulmonary Effects of Maternal Smoking on the Fetus and Child: Effects on Lung Development, Respiratory Morbidities, and Life Long Lung Health. *Paediatric respiratory reviews* 2017; 21: 27-33.
5. Giovenco DP, Hammond D, Corey CG, Ambrose BK, Delnevo CD. E-Cigarette Market Trends in Traditional U.S. Retail Channels, 2012-2013. *Nicotine & tobacco research : official journal of the Society for Research on Nicotine and Tobacco* 2015; 17: 1279-1283.
6. Kasza KA, Ambrose BK, Conway KP, Borek N, Taylor K, Goniewicz ML, Cummings KM, Sharma E, Pearson JL, Green VR, Kaufman AR, Bansal-Travers M, Travers MJ, Kwan J, Tworek C, Cheng YC, Yang L, Pharris-Ciurej N, van Bemmel DM, Backinger CL, Compton WM, Hyland AJ. Tobacco-Product Use by Adults and Youths in the United States in 2013 and 2014. *The New England journal of medicine* 2017; 376: 342-353.
7. Singh T, Arrazola RA, Corey CG, Husten CG, Neff LJ, Homa DM, King BA. Tobacco Use Among Middle and High School Students - United States, 2011-2015. Centers for Disease Control and Prevention Morbidity and Mortality Weekly Report. Washington, DC, USA: U.S. Government Printing Office; 2016. p. 361-363.
8. Cheng T. Chemical evaluation of electronic cigarettes. *Tobacco control* 2014; 23 Suppl 2: ii11-17.
9. Bahl V, Lin S, Xu N, Davis B, Wang YH, Talbot P. Comparison of electronic cigarette refill fluid cytotoxicity using embryonic and adult models. *Reproductive toxicology (Elmsford, NY)* 2012; 34: 529-537.
10. Lerner CA, Sundar IK, Yao H, Gerloff J, Ossip DJ, McIntosh S, Robinson R, Rahman I. Vapors produced by electronic cigarettes and e-juices with flavorings induce toxicity, oxidative stress, and inflammatory response in lung epithelial cells and in mouse lung. *PloS one* 2015; 10: e0116732.
11. Cervellati F, Muresan XM, Sticozzi C, Gambari R, Montagner G, Forman HJ, Torricelli C, Maioli E, Valacchi G. Comparative effects between electronic and cigarette smoke in human keratinocytes and epithelial lung cells. *Toxicology in vitro : an international journal published in association with BIBRA* 2014; 28: 999-1005.
12. Wagner NJ, Camerota M, Propper C. Prevalence and Perceptions of Electronic Cigarette Use during Pregnancy. *Matern Child Health J* 2017.
13. Mark KS, Farquhar B, Chisolm MS, Coleman-Cowger VH, Terplan M. Knowledge, Attitudes, and Practice of Electronic Cigarette Use Among Pregnant Women. *Journal of addiction medicine* 2015; 9: 266-272.
14. Barnes Peter J. Cellular and molecular mechanisms of asthma and COPD. *Clinical science* 2017; 131: 1541-1558.
15. Minoo P, Su G, Drum H, Bringas P, Kimura S. Defects in tracheoesophageal and lung morphogenesis in Nkx2.1(-/-) mouse embryos. *Developmental biology* 1999; 209: 60-71.
16. Bostrom H, Willetts K, Pekny M, Leveen P, Lindahl P, Hedstrand H, Pekna M, Hellstrom M, Gebre-Medhin S, Schalling M, Nilsson M, Kurland S, Tornell J, Heath JK, Betsholtz C. PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* 1996; 85: 863-873.
17. Wilkinson GA, Schittny JC, Reinhardt DP, Klein R. Role for ephrinB2 in postnatal lung alveolar development and elastic matrix integrity. *Dev Dyn* 2008; 237: 2220-2234.
18. Chan YL, Saad S, Pollock C, Oliver B, Al-Odat I, Zaky AA, Jones N, Chen H. Impact of maternal cigarette smoke exposure on brain inflammation and oxidative stress in male mice offspring. *Scientific Reports* 2016; 6: 25881.

19. Nguyen LT, Stangenberg S, Chen H, Al-Odat I, Chan YL, Gosnell ME, Anwer AG, Goldys EM, Pollock CA, Saad S. l-Carnitine reverses maternal cigarette smoke exposure-induced renal oxidative stress and mitochondrial dysfunction in mouse offspring. *Am J Physiol Renal Physiol* 2015; 308: F689-696.
20. Suter MA, Mastrobattista J, Sachs M, Aagaard K. Is there evidence for potential harm of electronic cigarette use in pregnancy? *Birth Defects Res A Clin Mol Teratol* 2015; 103: 186-195.
21. Gibbs K, Collaco JM, McGrath-Morrow SA. Impact of Tobacco Smoke and Nicotine Exposure on Lung Development. *Chest* 2016; 149: 552-561.
22. England LJ, Bunnell RE, Pechacek TF, Tong VT, McAfee TA. Nicotine and the Developing Human: A Neglected Element in the Electronic Cigarette Debate. *American journal of preventive medicine* 2015; 49: 286-293.
23. Noskovicova N, Petrek M, Eickelberg O, Heinzelmann K. Platelet-derived growth factor signaling in the lung. From lung development and disease to clinical studies. *American journal of respiratory cell and molecular biology* 2015; 52: 263-284.
24. McGrath-Morrow SA, Hayashi M, Aherrera A, Lopez A, Malinina A, Collaco JM, Neptune E, Klein JD, Winickoff JP, Breysse P, Lazarus P, Chen G. The effects of electronic cigarette emissions on systemic cotinine levels, weight and postnatal lung growth in neonatal mice. *PloS one* 2015; 10: e0118344.
25. Higham A, Rattray NJ, Dewhurst JA, Trivedi DK, Fowler SJ, Goodacre R, Singh D. Electronic cigarette exposure triggers neutrophil inflammatory responses. *Respiratory research* 2016; 17: 56.
26. Joubert BR, Felix JF, Yousefi P, Bakulski KM, Just AC, Breton C, Reese SE, Markunas CA, Richmond RC, Xu CJ, Kupers LK, Oh SS, Hoyo C, Gruzieva O, Soderhall C, Salas LA, Baiz N, Zhang H, Lepeule J, Ruiz C, Ligthart S, Wang T, Taylor JA, Duijts L, Sharp GC, Jankipersadsing SA, Nilsen RM, Vaez A, Fallin MD, Hu D, Litonjua AA, Fuemmeler BF, Huen K, Kere J, Kull I, Munthe-Kaas MC, Gehring U, Bustamante M, Saurel-Coubizolles MJ, Quraishi BM, Ren J, Tost J, Gonzalez JR, Peters MJ, Haberg SE, Xu Z, van Meurs JB, Gaunt TR, Kerkhof M, Corpeleijn E, Feinberg AP, Eng C, Baccarelli AA, Benjamin Neelon SE, Bradman A, Merid SK, Bergstrom A, Herceg Z, Hernandez-Vargas H, Brunekreef B, Pinart M, Heude B, Ewart S, Yao J, Lemonnier N, Franco OH, Wu MC, Hofman A, McArdle W, Van der Vlies P, Falahi F, Gillman MW, Barcellos LF, Kumar A, Wickman M, Guerra S, Charles MA, Holloway J, Auffray C, Tiemeier HW, Smith GD, Postma D, Hivert MF, Eskenazi B, Vrijheid M, Arshad H, Anto JM, Dehghan A, Karmaus W, Annesi-Maesano I, Sunyer J, Ghantous A, Pershagen G, Holland N, Murphy SK, DeMeo DL, Burchard EG, Ladd-Acosta C, Snieder H, Nystad W, Koppelman GH, Relton CL, Jaddoe VW, Wilcox A, Melen E, London SJ. DNA Methylation in Newborns and Maternal Smoking in Pregnancy: Genome-wide Consortium Meta-analysis. *American journal of human genetics* 2016; 98: 680-696.
27. Harrell MB, Weaver SR, Loukas A, Creamer M, Marti CN, Jackson CD, Heath JW, Nayak P, Perry CL, Pechacek TF, Eriksen MP. Flavored e-cigarette use: Characterizing youth, young adult, and adult users. *Preventive medicine reports* 2017; 5: 33-40.
28. Litt MD, Duffy V, Oncken C. Cigarette smoking and electronic cigarette vaping patterns as a function of e-cigarette flavourings. *Tobacco control* 2016; 25: ii67-ii72.
29. Berg CJ. Preferred flavors and reasons for e-cigarette use and discontinued use among never, current, and former smokers. *International journal of public health* 2016; 61: 225-236.
30. Vivekanandarajah A, Chan YL, Chen H, Machaalani R. Prenatal cigarette smoke exposure effects on apoptotic and nicotinic acetylcholine receptor expression in the infant mouse brainstem. *Neurotoxicology* 2016; 53: 53-63.
31. Luck W, Nau H. Nicotine and cotinine concentrations in serum and urine of infants exposed via passive smoking or milk from smoking mothers. *The Journal of pediatrics* 1985; 107: 816-820.
32. St Helen G, Havel C, Dempsey DA, Jacob P, 3rd, Benowitz NL. Nicotine delivery, retention and pharmacokinetics from various electronic cigarettes. *Addiction (Abingdon, England)* 2016; 111: 535-544.

Figure Legends

Figure 1. Protein levels of IL-1β (a), IL-6 (b), and TNFα (c) in the mothers' lungs. Results are expressed as mean ± SEM. In the representative gel images, all samples were run on the same gel but rearranged to correspond to the graph layout. * P<0.05, ** P<0.01 vs Sham. N=6

Figure 2. Protein levels of factors involved in inflammatory signaling pathways in the mothers' lungs. Results are expressed as mean ± SEM. In the representative gel images, all samples were run on the same gel but rearranged to correspond to the graph layout. *P<0.05, **P<0.01 vs Sham, † P<0.05 vs E-cig18, n=6.

Figure 3. mRNA expression of alveoli developmental markers platelet derived growth factor (PDGF, a,b), Ephrine B2 (EphB2, c,d) and surfactant protein c (Sftpc, e,f) in offspring's lung at postnatal day (P) 1 and P20. Results are expressed as mean ± SEM. ** P<0.01 vs Sham, n=6-8.

Figure 4. Protein levels of IL-1β (a), IL-6 (b), and TNFα (c) in the offspring lungs at 13 weeks. Results are expressed as mean ± SEM. In the representative gel images, all samples were run on the same gel but rearranged to correspond to the graph layout. * P<0.05, ** P<0.01 vs Sham. N=6

Figure 5. Protein levels of factors involved in inflammatory signaling pathways in offspring lungs at 13 weeks. Results are expressed as mean ± SEM. In the representative gel images, all samples were run on the same gel but rearranged to correspond to the graph layout. *P<0.05, **P<0.01 vs Sham; †P<0.05, ††P<0.01 vs E-cig18, n=7-8.

Figure 6. Global DNA methylation, and inflammatory cytokine expression in offspring's lung at P1. Results are expressed as mean ± SEM. a) Global DNA methylation as assessed by % methylated cytosines (5 mC) in the DNA, n=3. b to g, mRNA expression of inflammatory cytokines., n=6-8. *P<0.05, **P<0.01 vs Sham, † P<0.01 vs E-cig18

Table 1. TaqMan probe sequence (Thermo Fisher Scientific, MA, USA).

Gene	NCBI references	Probe Sequence	Assay ID
Pdgfa	NM_008808.3	GAGGAGGAGACAGATGTGAGGTGA	Mm01205760_m1
Ephb2	NM_001290753.1	AACCATGACAGAAGCCGAGTACCAG	Mm01181021_m1
Sftpc	NM_011359.2	GGAGAGTCCACCGGATTACTCGGCA	Mm00488144_m1

Table 2 Anthropometry of male offspring at P1, P20 and 13 weeks.

P1	**Sham**	**E-cig18**	**E-cig0**	**ANOVA**
Body weight (g)	1.62±0.08	1.50±0.06	1.58±0.05	P=0.41
Liver (g)	0.069±0.003	0.065±0.002	0.065±0.002	P=0.48
Liver (%)	4.30±0.11	4.35±0.10	4.15±0.10	P=0.34
P20				
Body weight (g)	10.36±0.25	9.74±0.12*	10.99±0.21*††	P<0.01
Liver (g)	0.42±0.01	0.44±0.01	0.48±0.01**†	P<0.01
Liver (%)	4.12±0.11	4.54±0.10**	4.39±0.08	P=0.01
Retroperitoneal fat (g)	0.015±0.001	0.019±0.001	0.041±0.0058**††	P<0.01
Retroperitoneal fat (%)	0.15±0.01	0.20±0.01*	0.37±0.048**†	P<0.01
Epididymal fat (g)	0.065±0.005	0.070±0.004	0.109±0.009**††	P<0.01
Epididymal fat (%)	0.64±0.04	0.66±0.05	0.99±0.07**††	P<0.01
13 weeks				
Body weight (g)	26.49±0.52	26.01±0.32	26.02±0.33	P=0.63
Liver (g)	1.33±0.05	1.31±0.03	1.18±0.02*†	P=0.02
Liver (%)	4.99±0.12	5.02±0.09	4.54±0.09**††	P<0.01
Retroperitoneal fat (g)	0.17±0.02	0.21±0.02	0.21±0.01	P=0.14
Retroperitoneal fat (%)	0.63±0.05	0.80±0.07*	0.80±0.05*	P=0.045
Epididymal fat (g)	0.62±0.05	0.58±0.02	0.61±0.02	P=0.69
Epididymal fat (%)	2.32±0.16	2.23±0.07	2.34±0.08	P=0.77

Data are expressed as mean ± S.E.M (n=14-20). *P<0.05, **P<0.01 vs Sham. †P<0.05, ††P<0.01 vs E-cig18.

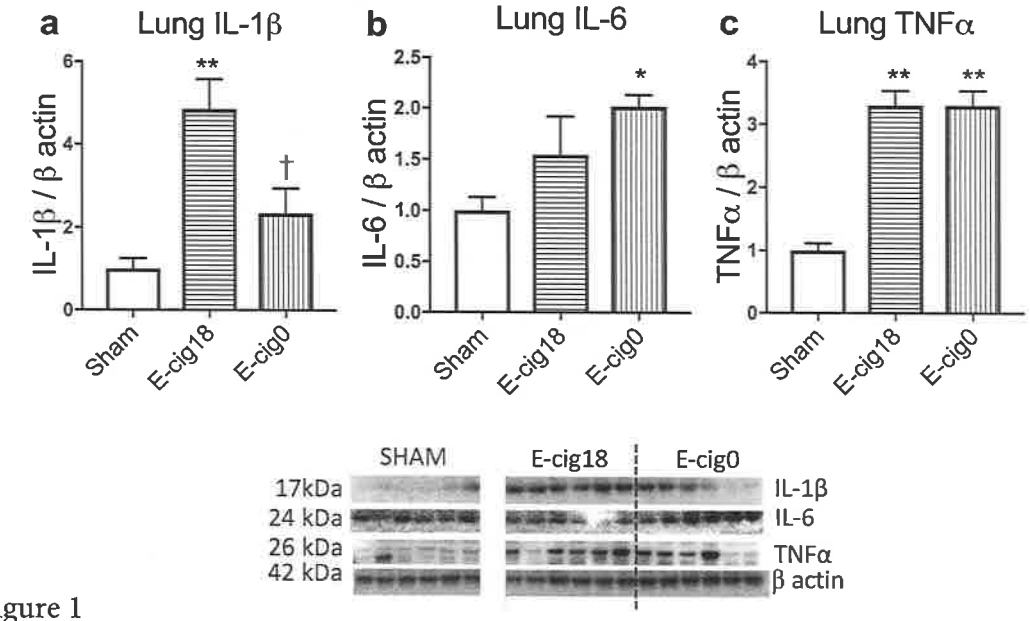
a
Lung IL-1β
IL-1β / β actin
6
4
2
0
**
†
Sham
E-cig18
E-cig0
b
Lung IL-6
IL-6 / β actin
2.5
2.0
1.5
1.0
0.5
0.0
*
Sham
E-cig18
E-cig0
c
Lung TNFα
TNFα / β actin
4
3
2
1
0
**
**
Sham
E-cig18
E-cig0
SHAM
E-cig18
E-cig0
17kDa
24 kDa
26 kDa
42 kDa
IL-1β
IL-6
TNFα
β actin

Figure 1

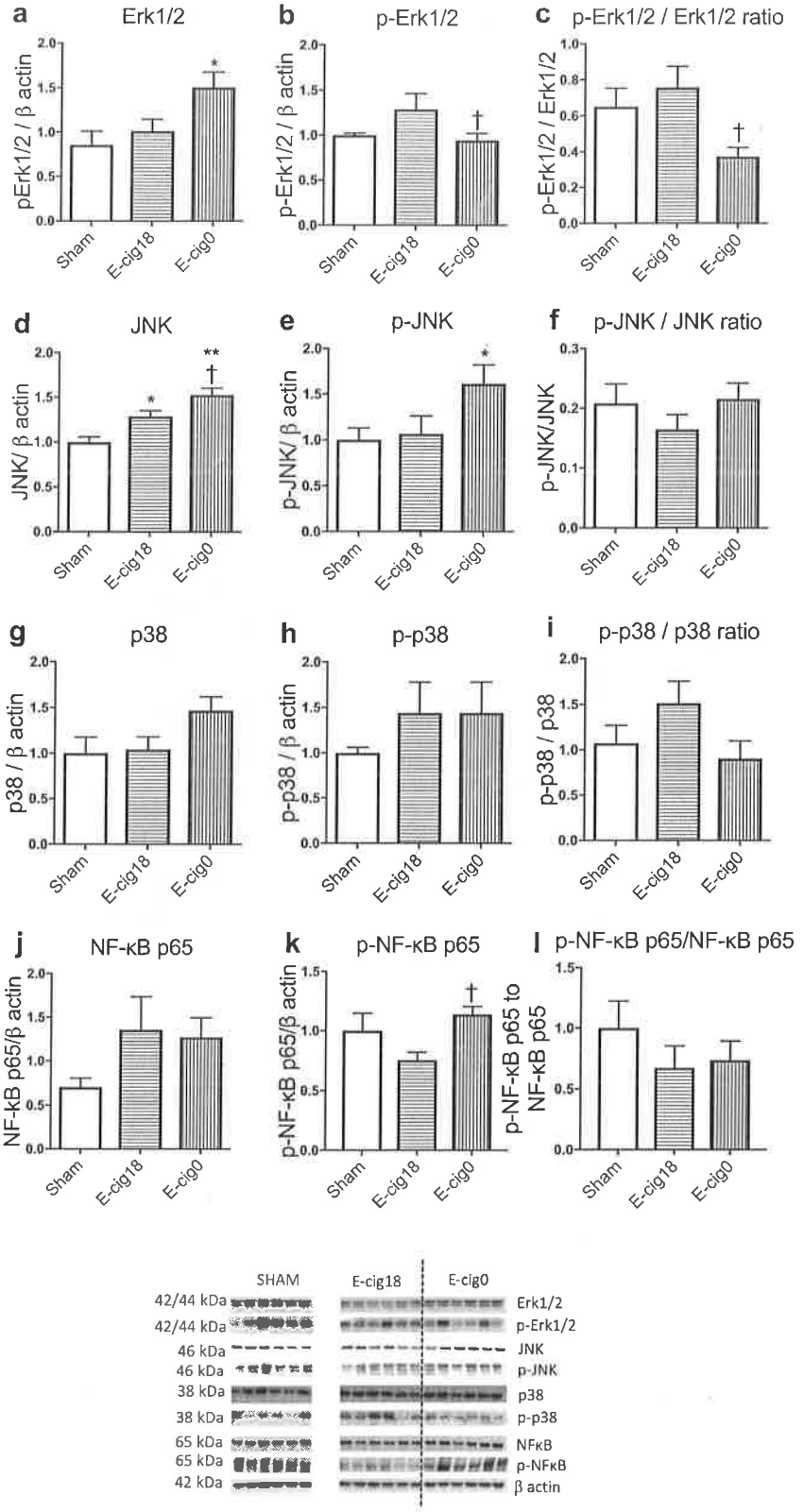
a
Erk1/2
pErk1/2 / β actin
b
p-Erk1/2
p-Erk1/2 / β actin
c
p-Erk1/2 / Erk1/2 ratio
p-Erk1/2 / Erk1/2
d
JNK
JNK/ β actin
e
p-JNK
p-JNK/ β actin
f
p-JNK / JNK ratio
p-JNK/JNK
g
p38
p38 / β actin
h
p-p38
p-p38 / β actin
i
p-p38 / p38 ratio
p-p38 / p38
j
NF-κB p65
NF-kB p65/β actin
k
p-NF-κB p65
p-NF-κB p65/β actin
l
p-NF-κB p65/NF-κB p65
p-NF-κB p65 to NF-κB p65
Sham
E-cig18
E-cig0
SHAM
E-cig18
E-cig0
42/44 kDa
Erk1/2
42/44 kDa
p-Erk1/2
46 kDa
JNK
46 kDa
p-JNK
38 kDa
p38
38 kDa
p-p38
65 kDa
NFκB
65 kDa
p-NFκB
42 kDa
β actin

Figure 2

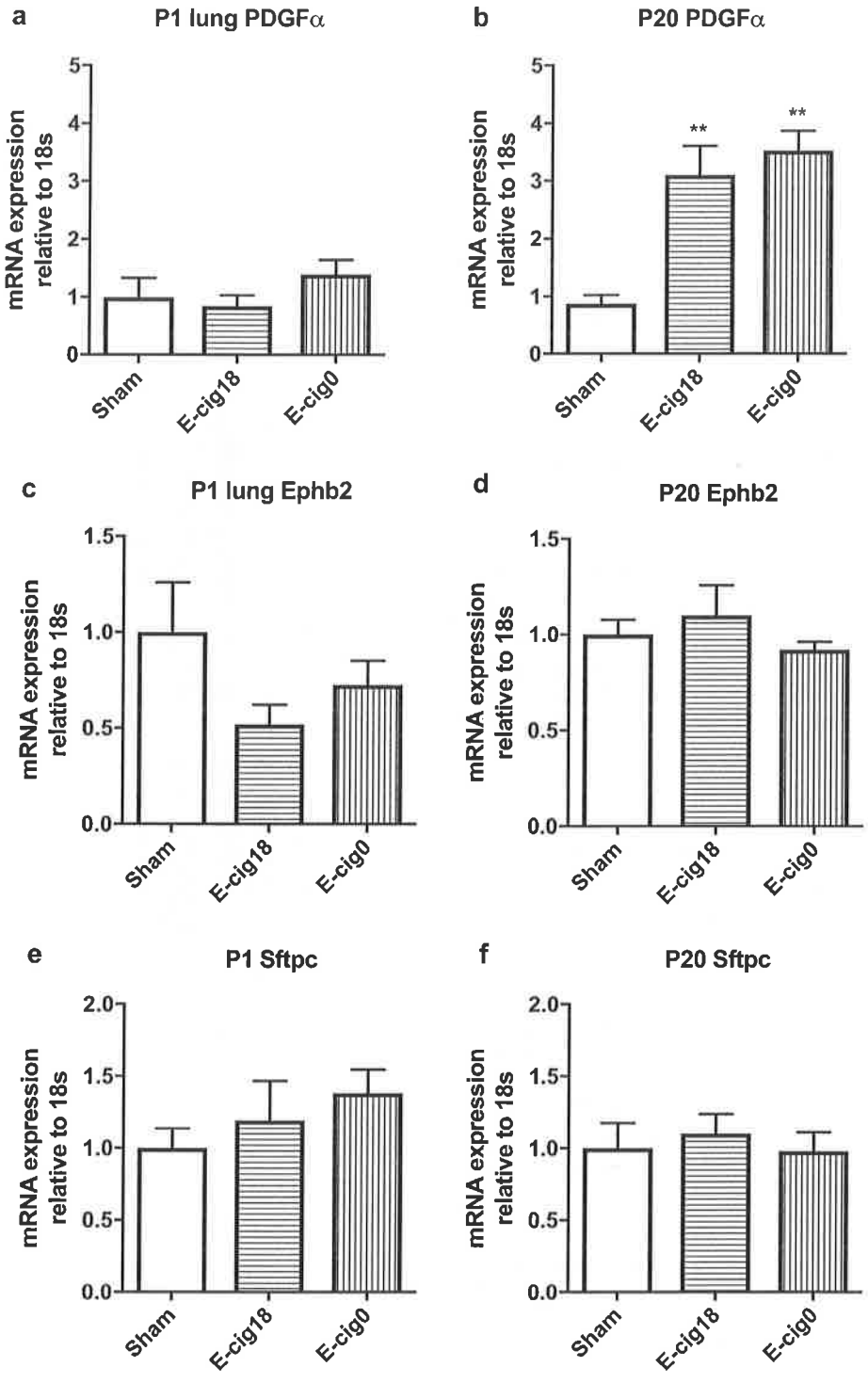
a
P1 lung PDGFα
mRNA expression relative to 18s
5
4
3
2
1
0
Sham
E-cig18
E-cig0
b
P20 PDGFα
mRNA expression relative to 18s
**
**
Sham
E-cig18
E-cig0
c
P1 lung Ephb2
mRNA expression relative to 18s
1.5
1.0
0.5
0.0
Sham
E-cig18
E-cig0
d
P20 Ephb2
mRNA expression relative to 18s
Sham
E-cig18
E-cig0
e
P1 Sftpc
mRNA expression relative to 18s
2.0
1.5
1.0
0.5
0.0
Sham
E-cig18
E-cig0
f
P20 Sftpc
mRNA expression relative to 18s
Sham
E-cig18
E-cig0

Figure 3

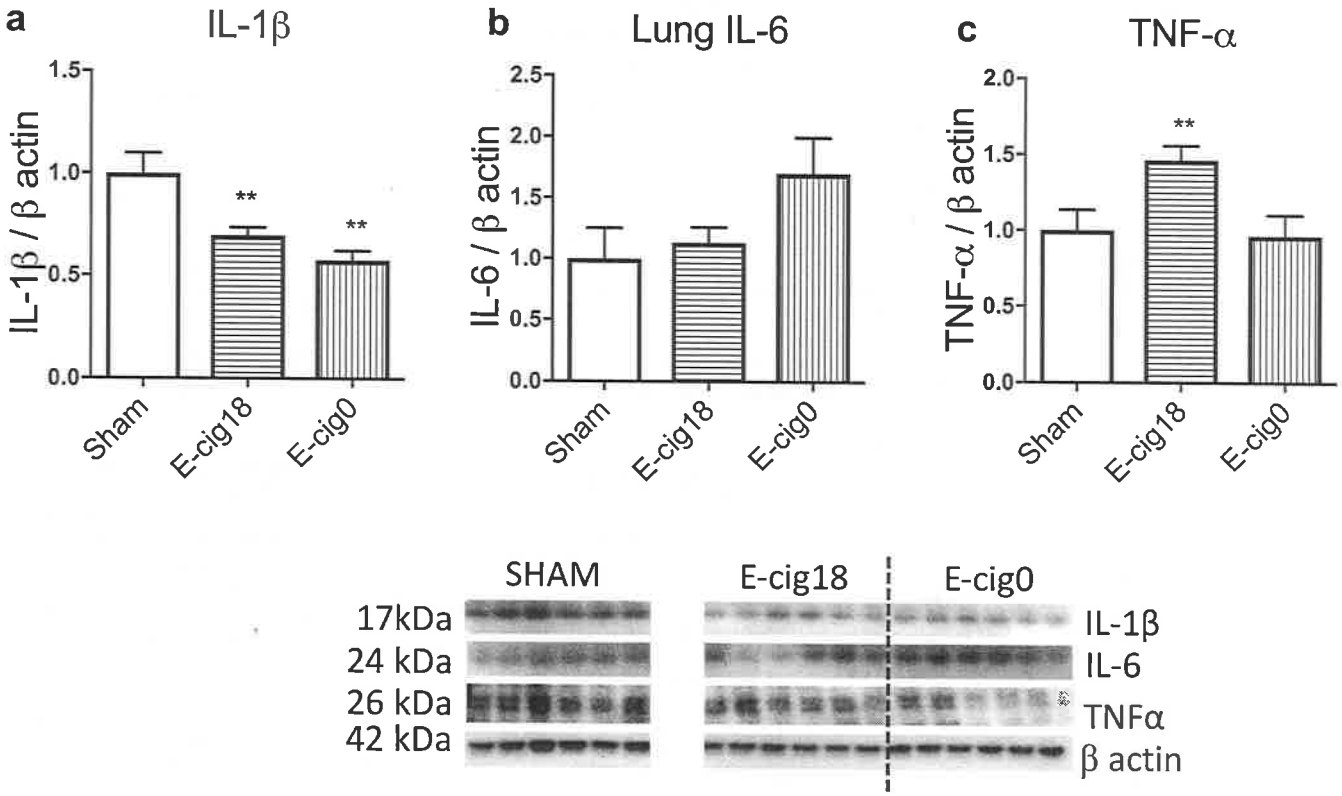
a
IL-1β
IL-1β / β actin
1.5
1.0
0.5
0.0
**
**
Sham
E-cig18
E-cig0
b
Lung IL-6
IL-6 / β actin
2.5
2.0
1.5
1.0
0.5
0.0
Sham
E-cig18
E-cig0
c
TNF-α
TNF-α / β actin
2.0
1.5
1.0
0.5
0.0
**
Sham
E-cig18
E-cig0
SHAM
E-cig18
E-cig0
17kDa
24 kDa
26 kDa
42 kDa
IL-1β
IL-6
TNFα
β actin

Figure 4

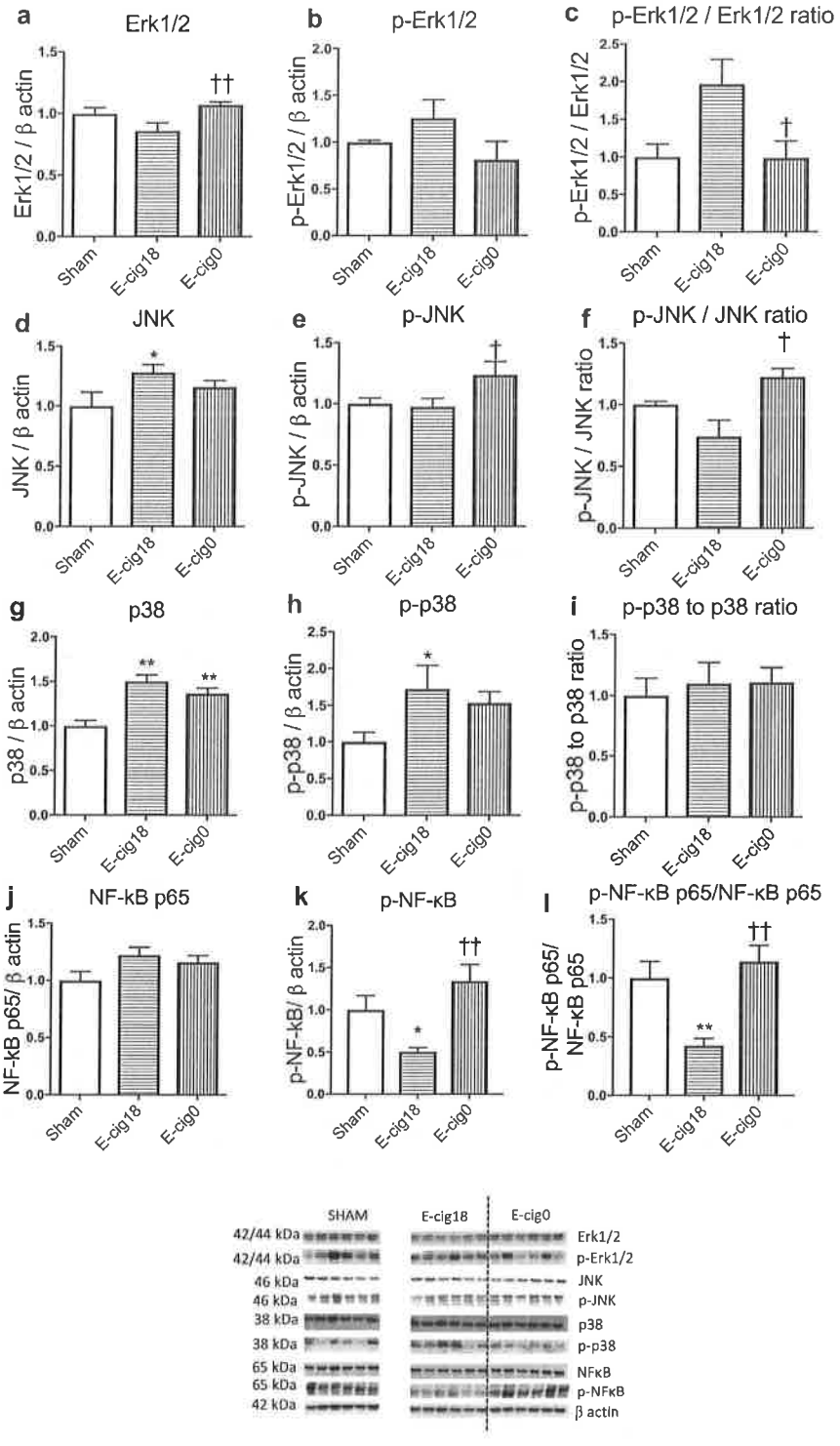
a
Erk1/2
Erk1/2 / β actin
b
p-Erk1/2
p-Erk1/2 / β actin
c
p-Erk1/2 / Erk1/2 ratio
p-Erk1/2 / Erk1/2
d
JNK
JNK / β actin
e
p-JNK
p-JNK / β actin
f
p-JNK / JNK ratio
p-JNK / JNK ratio
g
p38
p38 / β actin
h
p-p38
p-p38 / β actin
i
p-p38 to p38 ratio
p-p38 to p38 ratio
j
NF-kB p65
NF-kB p65/ β actin
k
p-NF-κB
p-NF-kB/ β actin
l
p-NF-κB p65/NF-κB p65
p-NF-κB p65/ NF-κB p65
Sham
E-cig18
E-cig0
SHAM
E-cig18
E-cig0
42/44 kDa
42/44 kDa
46 kDa
46 kDa
38 kDa
38 kDa
65 kDa
65 kDa
42 kDa
Erk1/2
p-Erk1/2
JNK
p-JNK
p38
p-p38
NFκB
p-NFκB
β actin

Figure 5

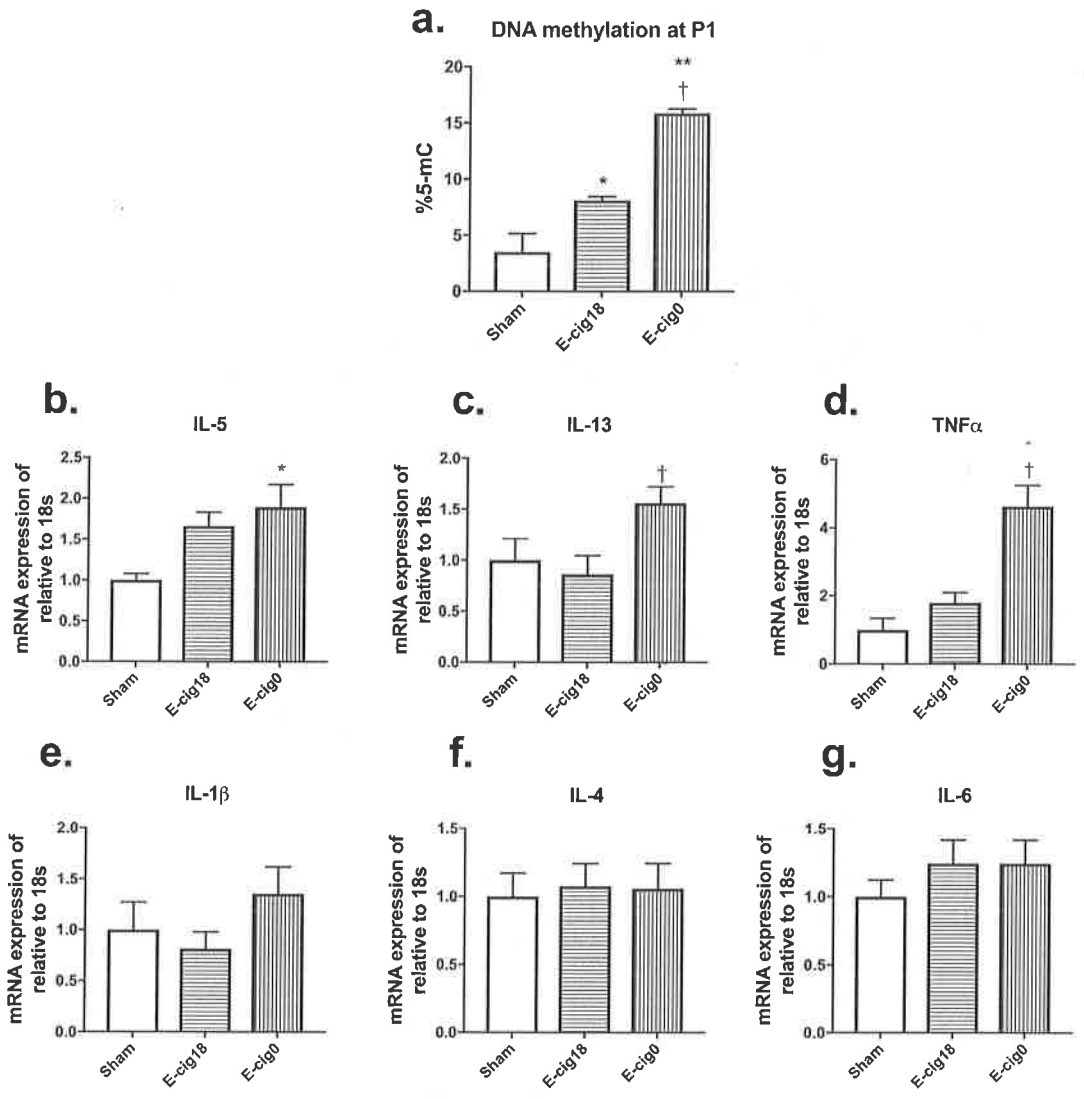
a.
DNA methylation at P1
%5-mC
20
15
10
5
0
**
†
*
Sham
E-cig18
E-cig0
b.
IL-5
mRNA expression of relative to 18s
2.5
2.0
1.5
1.0
0.5
0.0
*
Sham
E-cig18
E-cig0
c.
IL-13
mRNA expression of relative to 18s
2.0
1.5
1.0
0.5
0.0
†
Sham
E-cig18
E-cig0
d.
TNFα
mRNA expression of relative to 18s
6
4
2
0
†
Sham
E-cig18
E-cig0
e.
IL-1β
mRNA expression of relative to 18s
2.0
1.5
1.0
0.5
0.0
Sham
E-cig18
E-cig0
f.
IL-4
mRNA expression of relative to 18s
1.5
1.0
0.5
0.0
Sham
E-cig18
E-cig0
g.
IL-6
mRNA expression of relative to 18s
1.5
1.0
0.5
0.0
Sham
E-cig18
E-cig0

Figure 6