Cell Death Associated with ENDS Vapor Exposure

By

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Thesis

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Abstract of (Cell death associated with ENDS vapor exposure), by (Valeriia Syrovatska), ScM, Brown University, January 2023

The use of electronic nicotine delivery systems (ENDS) is continuously increasing both in the United States and the world as a whole. The lung epithelial cells and resident immune cells have an important role in maintaining the protective surfactant in the lungs, while macrophages play an important role in the immune response. Moreover, the balance of cell death, phagocytosis, and integrity of immune cells are crucial for the ability to properly respond to infectious challenges. Exposure to ENDS aerosol is associated with several adverse conditions like EVALI. However, there are still significant knowledge gaps pertaining to the mechanism of toxicity of ENDS and a full range of toxic effects. The purpose of our research study was to investigate the effect of ENDS on primary macrophages and epithelial cells to add to the scientific knowledge on the mechanism of toxicity of ENDS aerosol. To investigate the nature of cell death and measure the toxic effect associated with exposure to ENDS, we will perform a controlled exposure of cells to the ENDS vapor, while treating the other group of cells with HEPA-filtered air. To collect the data, we will perform flow cytometry. Finally, for the toxicological perspective on the vape automizer itself, which is a key component of the structural piece of the ENDS, we will perform a series of exposure experiments, followed by flow cytometry analysis. Results indicate that longer ENDS aerosol exposure induces greater pyroptotic activity and overall cell death in the sample of BMDMs. Moreover, longer incubation post-exposure gives macrophages time to respond to ENDS aerosol leading to stronger cell death and pyroptosis. Additionally, data indicate that the length of atomizer use is directly correlated to the degree of adverse response at a cellular level. These findings give us a new perspective on ENDS-associated toxic effects and, with further research, could play

a role in the development of public health campaigns and development of targeted therapeutics.

Chapter 1: Introduction

Electronic nicotine delivery systems (ENDS), also known as e-cigarettes or vape devices, are battery-operated devices that work by heating e-liquid, typically consisting of propylene glycol (PG), vegetable glycerin (VG), and nicotine. The use of ENDS has been increasing rapidly in recent years, particularly among young people. According to a report by the US Food and Drug Administration (FDA), more than 2.5 million middle and high school students are currently using e-cigarettes. Moreover, close to a third of vaping youth use ENDS on a daily basis. Despite the lack of significant scientific data supporting the claims made by tobacco companies, ENDS are often perceived as a safer alternative to conventional cigarettes. Example of the contrary would be e-cigarette-associated lung injury described by Salzman et al., 2019.

Due to the relative novelty of ENDS, the mechanisms of toxicity are still being established. Recent studies indicate the starting age for ENDS users has been decreasing every year, a cause for concern as the mechanisms of toxicity are still being established due to the relative novelty of ENDS (Besaratinia & Tommasi, 2020, Lyzwinski et al., 2022, Kaplan, 2019). It is crucial to increase the scale of research on the impact of ENDS aerosol because more and more people (particularly young adults) use electronic cigarettes with a false sense of security that aggressive marketing leads them to develop.

The toxicokinetics of vaping is a complex subject that bifurcates in to the ADME of nicotine by itself and vape aerosol as a whole (vegetable glycerin, propenyl glycol, and nicotine). Generally, absorption sites for nicotine include lung, oral/nasal mucosa, skin, and GI tract (Benowitz et al., 2009). However, in the case with electronic cigarettes, we are looking at inhalation, so skin would not be a major absorption site. Distribution of inhaled toxicant would include the crossing of the alveolar walls into the blood vessels, entering lymph and blood circulation. Metabolism of vape aerosol compounds is still under investigation, however, it can happen in the lung, GI tract, and liver, as it's an inhaled toxicant (Kaisar et al., 2016).

Recent research has raised concerns about the potential harmful effects of ENDS on human health. A paper by Serpa et al., 2020 shows that ENDS aerosol exposure leads to an increase in epithelial cell and macrophage death. Another observation made by Serpa et al. is the inhibition of most phagocytic and significant reduction in efferocytic activities due to the exposure to ENDS aerosol. Additionally, animal studies have demonstrated that exposure to ENDS vapor can enhance lung inflammation and impair immune responses to infectious challenge. A study done by Madison et al. reports that mice exposed to electronic cigarette vapor and infected with influenza demonstrate enhanced lung inflammation. These findings are in line with conclusions made by Wu et al., who claim that exposure to ENDS vapor is shown to increase general susceptibility to viral infections. This is an important development since viral susceptibility is directly regulated by the functionality of the immune system.

Electronic nicotine delivery systems have a complex and interconnected mechanism of action. The vapes themselves can range from tanks and mods that are large and refillable to disposable flavored "pens". The common element, however, in all

these devices is the compartment filled with liquid “vape juice” or PG/VG (50/50) mix with or without nicotine and a battery-powered heating element or, simply, an atomizer that turns the liquid component into vapor. The aerosol in turn is inhaled by the user when they draw on the device, as they would on a conventional tobacco cigarette. One way to interrogate the source of toxicity in ENDS is to look at the atomizer. In the tank vape, it consists of a coil, which serves as a heating element and some sort of wicking material that draws liquid onto the coil to be turned into vapor. Over time, the continuous heating of the coil and wicking fibers causes buildup, which is linked to progressive increase in oral cell cytotoxicity (Ureña et al., 2020). These findings introduce an often-overlooked variable of ENDS-associated adverse effects, which is atomizer age. A new study performed in 2022 by Goto et al. is interested in the in-vivo implications of aged coils in the vape on mice. Their findings suggest a significant risk of lung injury in mice associated with the use of aged coils. Another important finding was reported in an article by Jin et al., and who looked at formaldehyde and acetaldehyde emissions associated with aged coils and reported a strong correlation between prolonged use of same coils and high levels of aldehydes. Articles by Goto et al. and Jin et al. are based on in-vivo murine studies and introduce an important avenue in electronic cigarette research, however, the extent of human lung cell toxicity associated with atomizer age remains under investigation. Despite these findings, there are still significant knowledge gaps regarding the mechanisms of toxicity of ENDS and the full range of their toxic effects.

The purpose of this research project is to investigate the effects of ENDS on primary macrophages and epithelial cells to better understand the mechanisms of toxicity of ENDS aerosol. By doing so, we aim to provide new insights into the potential harmful effects of ENDS and contribute to the development of public health campaigns and

targeted therapeutics. We hypothesize that there is a direct relationship between the exposure to ENDS vapor and cellular survival disbalance, including increased levels of cell death, and more particularly, pyroptosis. Furthermore, the damaging effect of vaping on the human bronchia-epithelial cells is directly proportional to the duration of use of the same atomizer.

Chapter 2: Methods

Bone Marrow Derived Macrophages

Bone marrow derived macrophages (BMDM) were isolated from wild-type C57BL/6J mice by performing a hind leg dissection and full removal of the femur and tibia in mice euthanized with CO2. Bones were processed using a mortar and pestle with 20mL of RPMI 1640 media supplemented with 10% FBS, 12% L929 supernatant, and 1% penicillin/streptomycin to extract bone marrow. After filtration, cells were resuspended into fresh nutrient-dense media. To allow the macrophages to differentiate, they were split into non tissue culture treated flasks for 10 days.

Primary Human Bronchial Epithelial Cells (HBECs) and BEAS-2B Cell Line

BEAS-2B is a non-tumorigenic lung epithelial cell line derived from a human lung tissue. HBECs are primary human bronchial epithelial cells (PCS-300–010; ATCC). Cells were stored in liquid nitrogen in DMSO and grown in PneumaCult Medium system (Stemcell Technologies Inc.).

ENDS Aerosol Generation and Cell Exposure

Cells were plated in 12-well plates at 200 000 cells per well in 300ul of fresh media and incubated for 24 hours to settle into the non-tissue culture treated wells. The ENDS used for aerosol generation was Kangertech KBOW 160 with a Kangertech Protank 4. The dose dependence experiments were performed using the rebuildable atomizer deck with premade Kangertech nickel–chromium alloy RBA coils and Kangertech cotton wicks. The experiments were performed using the premade non-rebuildable atomizer to eliminate the possible atomizer variability. The E-liquid was composed of using the propenyl glycol and vegetable glycerin base (50/50) and nicotine at 18 mg/ml nicotine (N3876; Sigma). The exposure apparatus composed of airtight chamber with a peristaltic pump set to pull air at a rate of 1 L/min (Fig. 1A). The vaporizer was housed in a separate airtight chamber that had a (HEPA)-filter attached to the opening developed by Serpa et al., 2020. The aerosol exposure treatments included 6 second puff every 30 seconds. The negative control for these experiments consisted of cells treated with HEPA-filtered air. Sample size for all experiments was n=3.

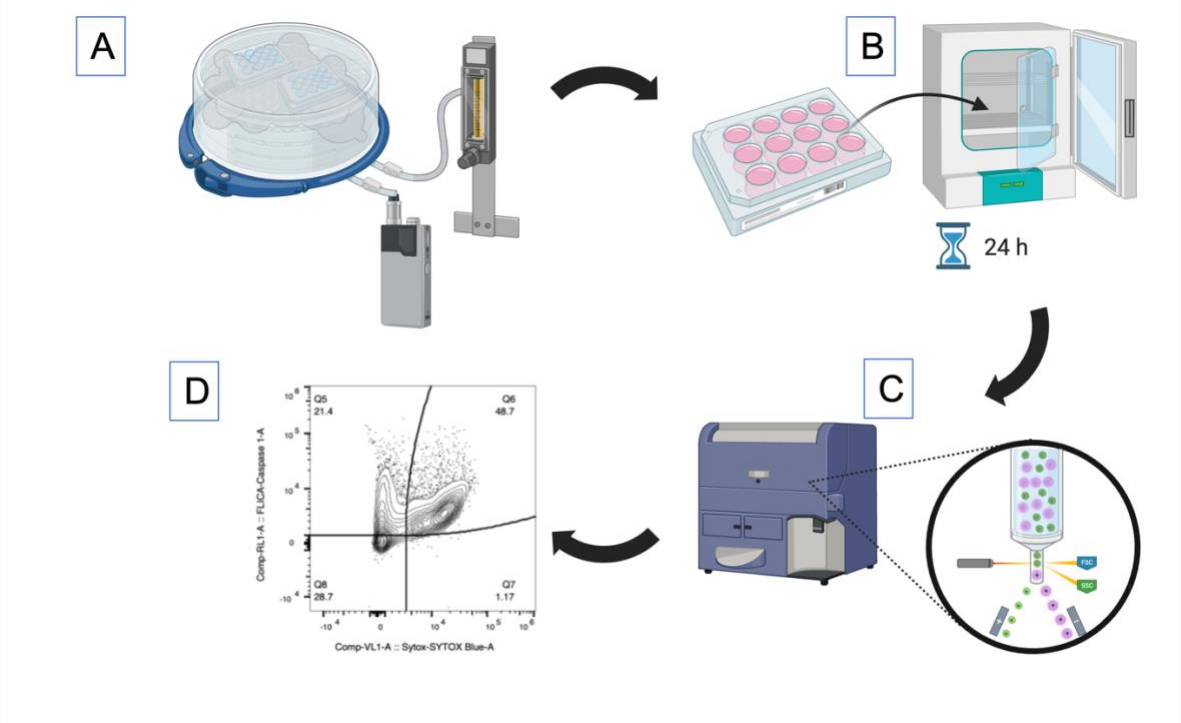

Figure 1. Model for investigation of vape-induced adverse effects on murine and human cell lines. Cells suspended in the media were exposed to ENDS aerosol with PG/VG and nicotine or HEPA-filtered air (A). Cell death was evaluated after 24 hours of incubation at 37C (B). Attune NxT Flow cytometer was used to identify cell death using sytox and pyroptosis using caspase 1 staining (C). Data was processed via the FlowJo software to produce representative scatter plots (D).

Staining and Flow Cytometry

After 24 hour incubation, the cell death investigation was conducted using flow cytometry assay (Fig. 1B-C). To investigate the nature of cell death, the samples were stained with SYTOX Blue, CellEvent Caspase-3/7 Green, and FLICA 660-YVAD-FMK Caspase-1 Assay Kit (ImmunoChemistry Technologies, LLC) as per manufacturer protocol. Single color stained and fully unstained samples served as positive and negative controls, respectively. Controls were used to establish laser compensation and to gate the flow cytometry plots. Raw data from the flow cytometry assay was processed with FlowJo software and further analyzed using GraphPad Prism (Fig. 1D). Statistical measures utilized for the data analysis included ANOVA and Tukey's multiple comparisons test.

Chapter 3: Results

To interrogate the adverse effect of ENDS aerosol on BMDMs, we performed a dose response experiment including 5- and 15-minute vaping sessions and an air exposure condition acting as a negative control (Fig. 2A). We have also compared samples analyzed after 4 or 24 hours to understand if the post-exposure incubation time changes the pattern of response we see. Data from these experiments shows that both 5- and 15-minute (HB+) exposure sessions leads to activation of caspase 1, which is a key component of pyroptosis (Fig. 2D). We also see a significant increase in overall cell death in HB+ conditions comparing to air, with 15-minute session resulting in more Sytox positive events (Fig. 2C). Longer incubation in these experiments lead to more significant overall cell death, however, pyroptosis markers remained at the same level (Fig. 2C-D). Cells were also stained to detect caspase 3/7 activation, which is a marker of apoptosis. The results indicate that BMDS also undergo significant apoptosis levels which are both dose dependent and post-exposure incubation length dependent (Fig. 2B).

To understand if our murine BMDM data is representative of the way human cells behave after exposure to ENDS aerosol, we have designed experiments that incorporated human bronchial epithelial cells (HBEC) and Beas2B cell lines. We have performed series exposure experiments using a new atomizer as a baseline to investigate the nature of toxic effect ENDS aerosol induces in cells. We exposed cells with 30 puffs of ENDS aerosol during the 15-minute sessions and analyzed cells after the first use of the new atomizer, second, and third, with air being a negative control. This experiment was also designed to investigate the dependence of adverse effect on the length of use of the atomizer. It is important because repeated prolonged use of vapes is not perceived as a threat or even point of caution by the everyday user.

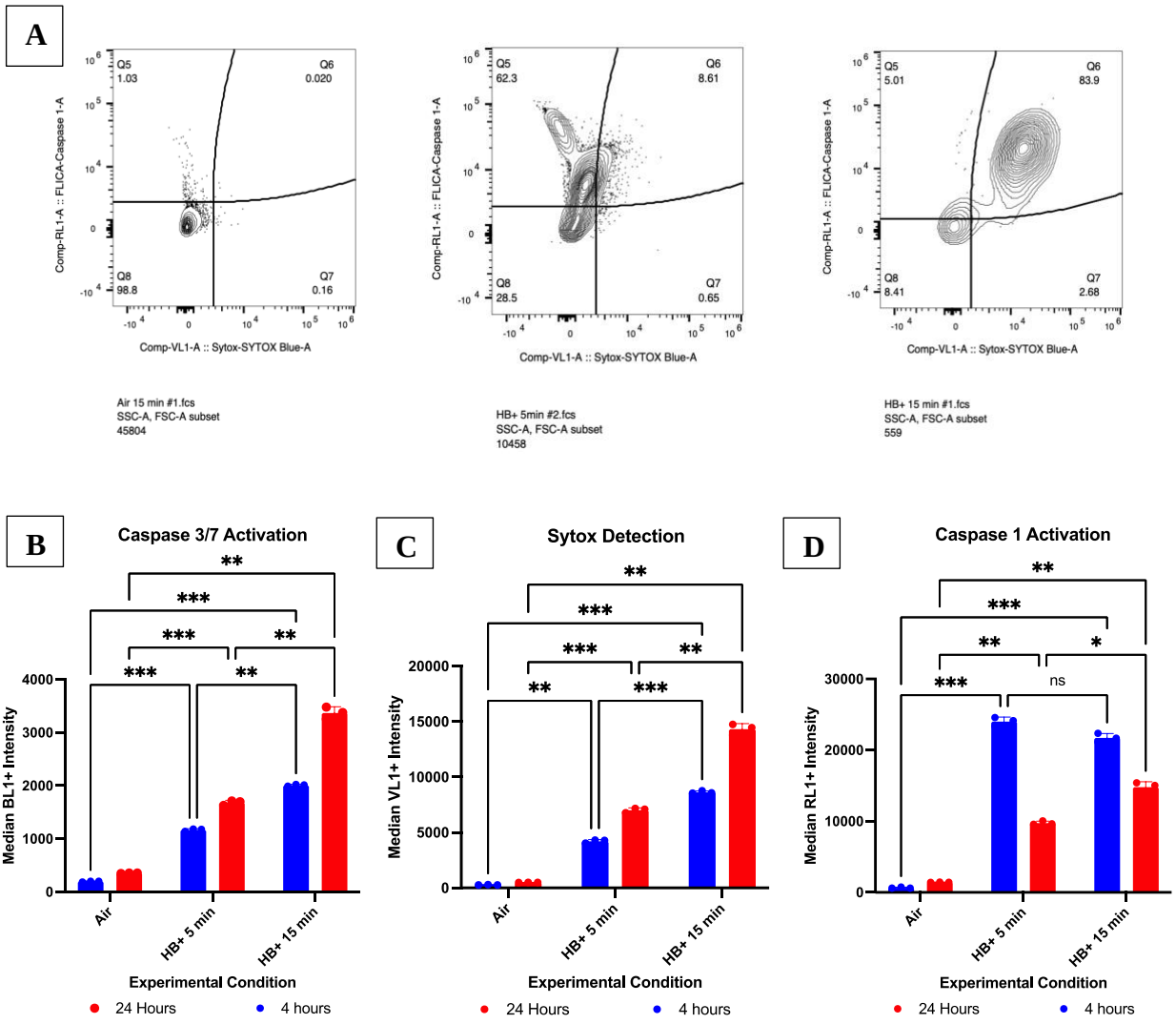

Figure 2. 24-hour post-exposure incubation is necessary to see the full pattern of response of BMDMs to ENDS aerosol. Bone marrow-derived macrophages were exposed to ENDS aerosol containing PG/VG and nicotine for a 5- or 15-minute session. Cells were further incubated in 37C for 4 or 24 hours, after which they were lifted and stained for flow cytometry with Sytox blue, Caspase 1, and Caspase 3/7. Experiment was performed with *n=3* per condition. Experimental conditions include HB+ 5 min and HB+ 15 min, which are ENDS aerosol exposure groups with 5- or 15-minute session respectively. Air exposure was used as a negative control. Graphs *A-C* show quantified median intensity of each marker used to interrogate the cell activation for each condition. Scatter plots in *A* identify the representative plots for each condition (from different experiments). Enclosed quadrants show stained populations of respective conditions. Graph *B* highlights caspase 3/7 activation breakdown. ***P<0.0005 in Air compared with HB+ 5 min at 4 and 24 hours. ***P<0.0005 in Air compared with HB+ 15 min at 4 hours. **P<0.005 in Air compared with HB+ 15 min at 24 hours. **P<0.005 in HB+ 5 min compared with HB+ 15 min at 4 and 24 hours. Graph C identifies median intensity of Sytox Blue detection. ***P<0.0005 in Air compared with HB+ 5 min at 24 hours. ***P<0.0005 in Air compared with HB+ 15 min at 4 hours. **P<0.005 in Air compared with HB+ 15 min at 24 hours. ***P<0.0005 in HB+ 5 min compared with HB+ 15 min at 4 hours. **P<0.005 in Air compared with HB+ 5 min at 4 hours. Graph *D* highlights caspase 1 activation breakdown. ***P<0.0005 in Air compared with HB+ 15 min at 4 hours. **P<0.005 in Air compared with HB+ 15 min at 24 hours. ***P<0.0005 in Air compared with HB+ 5 min at 4 hours. **P<0.005 in Air compared with HB+ 5 min at 24 hours. *P<0.05 in HB+ 5 min compared with HB+ 15 min at 24 hours. Flow cytometry plots were made using FlowJo software. Data were analyzed using ANOVA and Tukey's multiple comparisons test. Graphs were made using Graphing Prism.

Cells were exposed to e-cigarette aerosol with propylene glycol/vegetable glycerin and nicotine for one 15-minute vaping session starting with a fresh new prebuilt atomizer and using it for each run. Cell death was further evaluated after 24 hours of incubation at 37°C. Flow cytometry was used to identify the type of cell death using Sytox blue, caspase 3/7, and Caspase 1 staining. HEPA-filtered air exposure served as a control in this experiment. The experimental conditions included samples exposed to a new atomizer, after 30 puffs, and after 60 puffs. All samples underwent exposure once, with n=3 per each condition. The resulting data suggests there is a direct correlation between the length of atomizer use and cell death Beas2Bs. The flow cytometry plots in Fig. 3A show a progression in pyroptotic activation and cell death, which we can qualitatively observe via upper-right quadrant cell accumulation. The identified progression is directly correlated to the length of atomizer use. Results were quantified by using the percentage of events in each quadrant. ANOVA and Tukey's multiple comparisons test identified there was significantly more pyroptotic activity in Beas2bs in the "old" atomizer (used for 60 puffs prior to this session) group than in both the new atomizer condition and the air control (Fig. 3C). Additionally, data indicates progressively less unaffected cells with each consecutive use of the structural vape components. With the third run of the atomizer, we see a significant decrease in unstained cells comparing to all other conditions (Fig. 3B). These data suggest that the percentage of cells that remains uninflamed and alive after one vapor exposure session decreases with each use of the same atomizer (Fig. 3B). Ultimately, we looked at the total percent of sytox stained cells. The results show a significant increase in overall BEAS 2Bs cell death in the "60 puffs" condition, which is the third run of the atomizer after replacement.

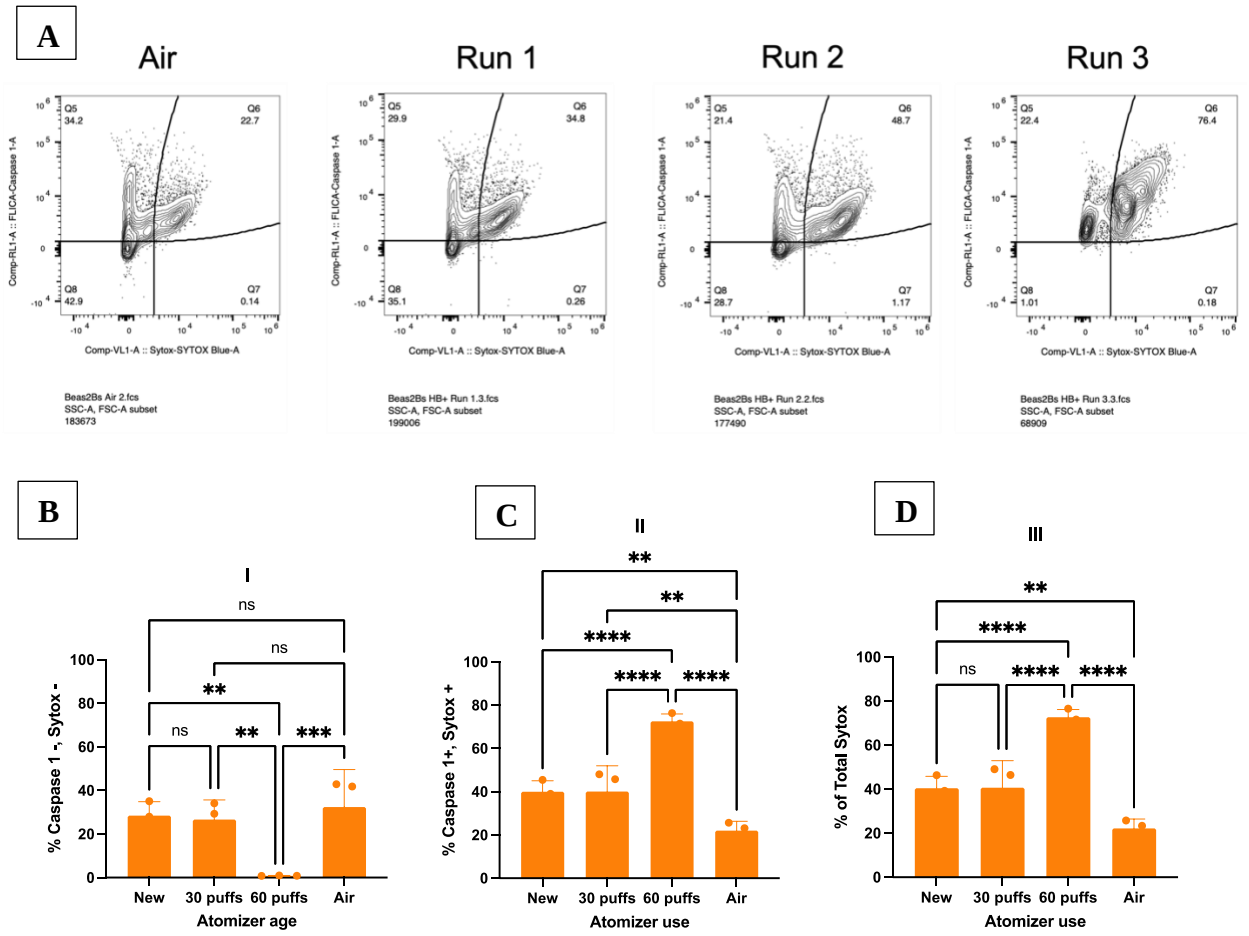

Figure 3. There is a direct correlation between the length of atomizer use and cell death in human Beas2Bs. Human non-tumorigenic lung epithelial cell (Beas2Bs) were exposed to ENDS aerosol with PG/VG and nicotine for one, two, or three 15-minute vaping sessions, each containing 30 puffs or HEPA-filtered air (Air) control. Cell death was evaluated after 24 hours of incubation at 37C. Experiment was performed with *n=3* per condition. Flow cytometry was used to identify cell death type using Sytox blue and Caspase 1 staining. Plots in part *A* are showing cell distribution in the frame of quadrants representative of stained populations. *B-D* are the quantification of quadrant analysis. The plot that highlights cells negative for Caspase 1 and Sytox is shown in *B*. ***P*<0.005 in Run 1 (new atomizer) compared with Run 3 (after 60 puffs). ***P*<0.005 in Run 2 (after 30 puffs) compared with Run 3 (after 60 puffs). ****P*<0.0005 in Run 3 (60 puffs) compared with Air. Graph plotting the cells positive for both caspase 1 and Sytox is shown in *C*. ***P*<0.005 in Run 1 (new atomizer) compared with Air. ***P*<0.005 in Run 2 (after 30 puffs) compared with Air. *****P*<0.0001 in Run 3 compared with Air in Beas2Bs. **** *P*<0.0001 in Run 3 compared with new atomizer. The plot that highlights cells that are dead and stained with Sytox is shown in *D*. ***P*<0.005 in Run 1 (new atomizer) compared with air. *****P*<0.0001 in Run 3 compared with Air, Run 1 and Run 2. Flow cytometry plots were made using FlowJo software. Data were analyzed using ANOVA and Tukey's multiple comparisons test. Graphs were made using Graphing Prism.

The described experiment was repeated using human broncho-epithelial cells (HBECs) to interrogate the possible difference in adverse effect on another human cell line. Although the overall conclusions of this experiment also indicated progressive pyroptotic activation and cell death related to atomizer age, there were some differences. The qualitative flow cytometry plots showing HBECs in different conditions show a clear increase in double positive (sytox and caspase 1), which indicates increase in pyroptotic cell death (Fig. 4A). The quantitative view of the double positive quadrant is characterized by the significant difference between the third run of the atomizer (after 60 puffs) and the air condition (Fig. 4C). The data also indicated a difference between new atomizer and air, which suggests pyroptotic activity is induced by aerosol from the new atomizer as well, although it is certainly greater in the older atomizer condition (Fig. 4C). The unstained population analysis did not show significant results, however, the low percentage of unaffected cells may contribute to this statistical outcome (Fig. 4B). Additionally, we recorded a statistically significant increase in overall cell death labeled with the sytox blue stain (Fig. 4D).

These findings have important implications for the safety of ENDS use. They suggest that the long-term effects of ENDS on human health may not only depend on the amount of ENDS aerosol exposure, but also on the condition of the ENDS device itself. Further research is needed to fully understand the mechanisms by which ENDS may induce pyroptosis and other adverse effects on human health. This information can be used to develop safer ENDS devices and to inform regulatory decisions regarding the use of ENDS.

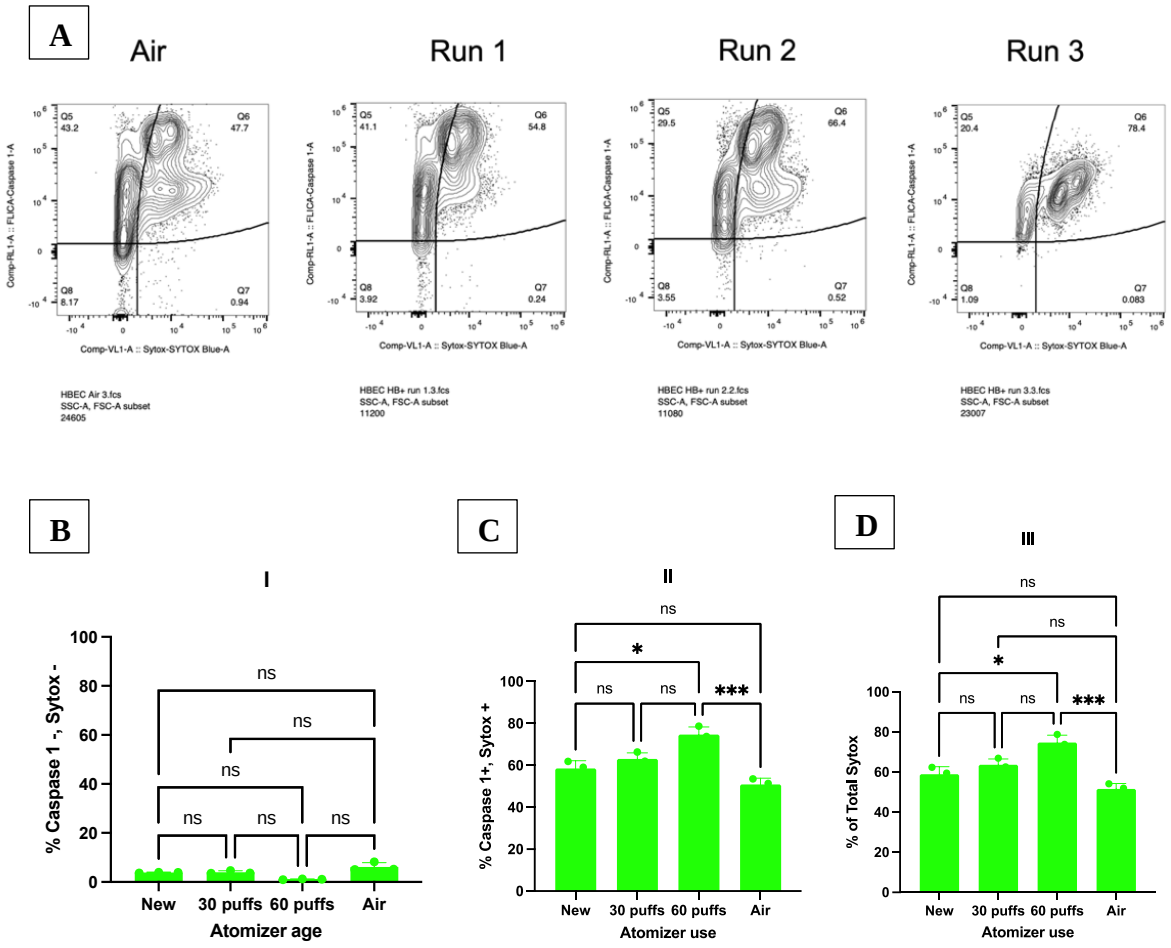

Figure 4. There is a direct correlation between the length of atomizer use and cell death in HBECs. Human broncho-epithelial cells (HBECs) were exposed to ENDS aerosol with PG/VG and nicotine for one, two, or three 15-minute vaping sessions, each containing 30 puffs or HEPA-filtered air (Air) control. Cell death was evaluated after 24 hours of incubation at 37C. Experiment was performed with *n=3* per condition. Flow cytometry was used to identify cell death type using Sytox blue and Caspase 1 staining. Plots in part *A* are showing cell distribution in the frame of quadrants representative of stained populations. *B-D* are the quantification of quadrant analysis. The plot that highlights cells negative for Caspase 1 and Sytox is shown in *B.* Graph plotting the cells positive for both caspase 1 and Sytox is shown in *C*. ***P<0.0005 in Run 3 (60 puffs) compared with Air. *P<0.05 in Run 1 (new atomizer) compared with Run 3 (after 60 puffs). The plot that highlights cells that are dead and stained with Sytox is shown in *D*. *P<0.05 in Run 3 (after 60 puffs) compared with new atomizer. ***P<0.0005 in Run 3 (60 puffs) compared with Air. Flow cytometry plots were made using FlowJo software. Data were analyzed using ANOVA and Tukey's multiple comparisons test. Graphs were made using Graphing Prism.

Chapter 4: Discussion and Conclusions

The findings of this study provide valuable insights into the mechanism of toxicity of ENDS aerosol and its effects on primary macrophages and epithelial cells. The results show that longer exposure to ENDS aerosol leads to greater pyroptotic activity and overall cell death in BMDMs, and that longer incubation post-exposure allows for stronger expression of the response to ENDS aerosol. This suggests that the toxic effects of ENDS on immune cells may be dependent on both duration of exposure and time allowed for cells to respond. Furthermore, the study's observations regarding the dependence of vaping-associated adverse responses on atomizer use provide new insights into the potential toxic effects of ENDS. The finding that adverse responses are significantly stronger after the second use of the atomizer suggests that repeated use of ENDS may have a cumulative effect on the body's response to the aerosol. This is particularly concerning given the high rates of daily use among young ENDS users. In their study on endothelial progenitor cells (EPC), Antoniewicz et al. identified that only 10 puffs of e-cigarette aerosol exposure have caused an increase in EPCs, suggesting the possibility of acute endothelial injury. These findings put the short-term exposure of cells to vape aerosol into the possible perspective of human relevance, highlighting the importance of continued research on the effects of ENDS on human health. Furthermore, it is important to consider the possible reasons for the identified correlation between the use of the atomizer and the adverse effect on cells.

There are several potential reasons why the atomizer, or heating element, in a vape device could be toxic, including the design or materials used in the atomizer, overheating of e-cigarette liquid, and contamination. Some atomizers are made with low-

quality materials that can release toxic chemicals when heated. For example, some atomizers use a wire made of cheap, low-grade stainless steel that can release toxic metals when heated. A study done by Haworth-Duff et al. identified varying levels of metals both between different atomizer manufacturers and coil batches (Haworth-Duff et al., 2022). Literature review on the topic revealed conflicting results on the toxic particle release over time. Previous research has shown that devices that are modified to operate at higher temperatures or used incorrectly can contribute to increased toxic effect. Some examples of user module modification associated with higher puff volume is “sub-ohming” and opening/closing of the vents (Cancelada et al., 2021). The study suggested that the heating of e-cigarette liquids can produce formaldehyde and other toxic compounds. These compounds can be inhaled by the user and may be harmful to the respiratory system. Research has also been done to investigate the possibility and extent of toxic metal particulate release into the e-liquid over time. That parameter was assessed via evaluating mass concentrations of metals. The findings indicate trace amounts of toxic metals, including chromium, nickel, lead, manganese, and zinc (Olmedo et al., 2018). These observed toxic effects of ENDS on immune cells suggest that long-term use of these devices may have negative impacts on respiratory health.

Ultimately, our observations regarding the dependence of vaping-associated adverse responses on atomizer use suggest that repeated use of ENDS without frequent atomizer change may have an effect on the cells’ response to the e-cigarette aerosol. These findings highlight the need for continued research on the effects of ENDS on human health and the importance of public health efforts to regulate these devices. This information can be used to develop safer ENDS and to inform regulatory decisions.

Some limitations of this study could include sample size, external validity, and limited duration of exposure. The sample size of the study may be too small to accurately represent the entirety of the effect ENDS have on the cell types interrogated. The study may also not be representative of the effects of ENDS on the general human population, as it was conducted on murine primary macrophages and human epithelial cells in a controlled laboratory setting. The use of a control group of cells exposed to HEPA-filtered air allows for comparison, but the results may not accurately reflect the effects of ENDS in humans, as the cells were not exposed to other potential factors in the body that could affect their response. Another limitation could be time, the duration of exposure to ENDS aerosol in the study may not accurately reflect the long-term effects of ENDS use in humans. Finally, there may be other variables that could affect the results of the study, such as the specific types and concentrations of e-liquids used, the characteristics of the ENDS devices, and the individual characteristics of the cells used in the study. It is important to consider these and other potential limitations when interpreting the results of this study and when drawing conclusions about the effects of ENDS on human health.

There are several potential next steps that could be taken following this study on the effects of ENDS on primary macrophages and epithelial cells. These could include expanding the sample size, examining the effects of long-term exposure, testing the effects of different flavors, and conducting in-vivo studies. Conducting the controlled exposure experiments on a larger sample of cells to confirm the findings of the current study and to determine if the results are consistent across a wider range of subjects would be beneficial. Additionally, to understand the full range of toxic effects of ENDS aerosol, it would be valuable to study the effects of long-term exposure to ENDS vapor. This could involve exposing cells to ENDS aerosol for extended periods of time and measuring the

resulting toxic effects. Another avenue for further investigation could be vape specifications. ENDS products are available in a variety of flavors, and it is possible that different flavors may have different toxic effects. To investigate this possibility, it would be useful to expose cells to ENDS vapor in different flavors and compare the results. Finally, while in-vitro studies, such as the controlled exposure experiments described in this study, are important for understanding the mechanisms of toxicity, it is also important to conduct in-vivo studies to examine the effects of ENDS on living organisms. This could involve exposing animal models to ENDS vapor and investigating the resulting effects by recording long term symptoms, changes in behavior, ability to fight off infections, and post-mortem tissue examinations.

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