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Elevated Cellular Oxidative Stress in Circulating Immune Cells in Otherwise Healthy Young People Who Use Electronic Cigarettes in a Cross-sectional Single-Center Study: Implications for Future Cardiovascular Risk

Kelesidis: Immune Cell Oxidative Stress in E-Cigarette Vapers

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ABSTRACT

Background

Tobacco cigarettes (TC) increase oxidative stress and inflammation, both instigators of atherosclerotic cardiac disease. It is unknown if electronic-cigarettes (ECs) also increase immune cell oxidative stress. We hypothesized an ordered, “dose-response” relationship, with tobacco-product type as “dose”: lowest in non-smokers, intermediate in EC vapers, and highest in TC smokers, and the “response” being cellular oxidative stress in immune cell subtypes, in otherwise, healthy young people.

Methods and Results

Using flow cytometry and fluorescent probes, cellular oxidative stress was determined in in immune cell subtypes in 33 otherwise healthy young people: non-smokers (n=12), EC vapers (n=12), and TC smokers (n=9). Study groups had similar baseline characteristics, including age, sex, race and education level. A dose-response increase in pro-inflammatory monocytes and lymphocytes, and their cellular oxidative stress content amongst the three study groups was found: lowest in non-smokers, intermediate in EC vapers, and highest in TC smokers. These findings were most striking in $CD14_{dim}CD16^{+}$ and $CD14^{++}CD16^{+}$ pro-inflammatory monocytes and were reproduced with two independent fluorescent probes of cellular oxidative stress.

Conclusions

These findings portend the development of premature cardiovascular disease in otherwise healthy young people who chronically vape ECs. On the other hand, that the cellular oxidative stress is lower in EC-vapers compared to TC-smokers warrants additional investigation to determine if switching to ECs may form part of a harm-reduction strategy.
Registration Information: ClinicalTrials.gov (NCT03823885).

Key words: electronic cigarettes, tobacco cigarettes, nicotine, monocytes, reactive oxidative species

Non-Standard Abbreviations and Acronyms

COS = cellular oxidative stress

EC = electronic cigarette

NK = natural killer

ROS = reactive oxygen species

TC = tobacco cigarette

Clinical Perspective

What is new?

- Electronic cigarette (EC) vaping, which has grown to epidemic proportions among young people, is perceived as safer than tobacco cigarette (TC) smoking, but it remains unknown if otherwise healthy young EC vapers, like TC smokers, have increased oxidative stress and inflammation compared to non-smokers.
- A dose-response increase in pro-inflammatory monocytes and lymphocytes, and their cellular oxidative stress content was found: lowest in non-smokers, intermediate in EC vapers, and highest in TC smokers.

What are the clinical implications?

- These findings portend the development of premature cardiovascular disease in otherwise healthy young people who chronically vape ECs.
- On the other hand, that the cellular oxidative stress is lower in EC-vapers compared to TC-smokers warrants additional investigation to determine if switching to ECs may form part of a harm-reduction strategy.

Introduction

Oxidative stress and inflammation are implicated in the pathogenesis of most human diseases, including cardiovascular diseases[1]. Chronic exposure to excessive levels of reactive oxygen species (ROS) introduced through environmental exposures or through dysfunctional endogenous enzymatic systems overwhelm anti-oxidant defense systems, resulting in cellular damage and activation of circulating immune cells[1, 2]. Activated immune cells, in turn, generate additional ROS, driving oxidation of lipoproteins and further recruitment of monocytes and macrophages, which then enter the vascular wall. Thus, ongoing oxidative stress and inflammation contribute to the initiation and progression of atherosclerotic vascular disease that may present decades later.

Tobacco cigarette (TC) smoking is the most prevalent modifiable risk factor for numerous human diseases, including atherosclerosis, in which oxidative stress and inflammation are known to play a critical role[2, 3]. Over 90% of TC smokers begin smoking in their teens[4], but TC-related diseases are insidious, presenting only after decades of TC smoking. Each puff of TC smoke contains 10^{15} free radicals[5] and over 7000 different chemicals[6], several of which are known toxicants or even carcinogens. Major pro-oxidant constituents in TC smoke generate cellular production of ROS when they interact with cellular enzymatic systems[2]. Innate and adaptive immune cells such as myeloid cells (monocytes, macrophages, dendritic cells), NK cells and lymphocytes (B and T cells) are activated by TC smoking[7], and are also

major sources of systemic oxidative stress[8]. Cigarette smoke activates leukocytes to release reactive oxygen and nitrogen species and contributes to development and progression of atherosclerotic cardiovascular disease through several mechanisms such as secretion of pro-inflammatory cytokines and increased adherence of monocytes to the endothelium[2, 3]. Although cellular oxidative stress (COS) has been studied in the setting of tobacco smoking and atherosclerosis, there is limited evidence regarding COS among electronic-cigarette vapers.

Electronic-cigarettes (ECs) are the most rapidly rising tobacco product used in the US today. EC aerosol, generated from heating - without combustion - solvents, flavors, and usually nicotine, contains significantly lower levels of toxicants compared to TC smoke[9]. Due to the long lag time for disease presentation, the health risks of ECs relative to TCs are unknown, yet ECs have been promoted as a smoking cessation, harm reduction, strategy. Alarmingly, largely due to the perceptions that ECs are safe, EC vaping has reached epidemic levels in never-smoking middle and high school students, with 30% of high school seniors (typically 17-18 years old) reporting EC vaping in the previous month[10].

Although an urgent public health issue, the health risks associated with EC vaping, especially relative to TC smoking, remain unknown. The purpose of the current study was to pair sensitive flow cytometry with fluorescent probes to quantify the relative immune cell-type populations and their intra-cellular content of ROS in otherwise healthy young EC-vapers compared to

TC-smokers, and non-smokers. We hypothesized a continuum of oxidative stress and immune cell activation - essentially a “dose-response” relationship, with the “dose” defined as tobacco-product type: lowest in the non-smokers, intermediate in the chronic EC-vapers and highest in the chronic TC-smokers, and the “response” defined as measures of immune cell subtypes and their cellular oxidative stress.

MATERIALS AND METHODS

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request. HRM and TK had full access to all the data in the study and take responsibility for its integrity and the data analysis.

Materials

Flow cytometry reagents including flow cytometry staining buffers and antibodies were purchased from Biolegend. CellROX Green (catalog # C10444) and CellROX Deep Red (catalog # C10442) were obtained from Thermo Scientific.

Study Population

Healthy male and female volunteers between the ages of 21 and 45 years were eligible for enrollment if they were chronic ($\geq$ 1 year) 1) TC-smokers, or 2) EC-vapers (no dual users), or 3) non-smokers. Former TC-smokers were eligible if greater than 1 year had elapsed since quitting. End-tidal CO, elevated above 10 ppm in smokers, was measured in EC-vapers and non-smokers to confirm none were surreptitiously smoking TCs. All participants were required to meet the following criteria: (1) non-obese ($\leq$30 kg/m^2 body mass index); (2) no known health problems; (3) alcoholic intake $\leq$2 drinks per day and no regular illicit drug use, including marijuana, determined through screening questionnaire and urine toxicology testing; (4) no prescription medications (oral contraceptives allowed), (5) not exposed to

second hand smoke, or using licensed nicotine replacement therapies. The experimental protocol was approved by the Institutional Review Board at the University of California, Los Angeles and written, informed consent was obtained from each participant.

Experimental Protocol

After abstaining from caffeine, tobacco product use and exercise for at least 12 hours, fasting participants reported to the UCLA Clinical Translational Research Center at the same time of day, approximately 8AM. Blood was drawn by trained medical assistants and prepared for flow cytometry and measurement of cotinine levels.

Flow cytometry

Freshly isolated whole blood was immediately processed for flow cytometric determination of cellular ROS. Cellular oxidative stress was determined by the use of the CellROX® Green Reagent, a measure of total (cytoplasmic and nuclear) cellular ROS[11-13] and the use of the CellROX® Deep Red Reagent, a measure of cytoplasmic cellular ROS[14-16]. The efficiency of CellROX Green to determine COS has previously been validated in several cells including sperm, epithelial and melanoma cells, neurons, bacteria and immune cells such as macrophages[17]. The efficiency of CellROX Deep Red to assess COS has previously been validated in several cells including sperm, endothelial and epithelial cells, hepatocytes, neurons, cardiomyocytes and immune cells[15]. The CellROX deep Red has been previously used to detect the *ex vivo*

184 impact of cigarette smoke on cellular ROS by flow cytometry in
185 spermatocytes[16].
186 See Supplemental Materials for detailed methods.
187 **Determination of plasma cotinine levels**
188 The assay for plasma cotinine, using the methodology of
189 chromatography/mass spectrometry, was run by the commercial laboratory,
190 Quest Laboratories (Quest Diagnostics incorporated, Madison, NJ), with a
191 limit of quantitation of 2 ng/mL and a reference range in smokers of 16-145
192 ng/mL.
193 **Statistical analysis**
194 We hypothesized an ordered, dose-response relationship of oxidative stress
195 across the 3 study groups: lowest in non-smokers, intermediate in chronic
196 EC-vapers, and highest in chronic TC-smokers. We considered the “dose” to
197 be the type of tobacco product used, and the “response” to be the immune
198 cell subtype and its cellular oxidative stress. In order to test this hypothesis,
199 the *ordered trend (F) test* across the 3 ordered groups (non-smokers, EC-
200 vapers, TC-smokers) was computed under an analysis of variance (ANOVA)
201 model[18]. Means ± SEM are reported. If the overall trend p value or the overall
202 ANOVA p value was $\leq$0.05, then the pairwise post hoc *t* tests p values are
203 reported between 2 groups (Fisher LSD criterion). The ordered trend test was
204 considered statistically significant when $p \leq 0.05$. For continuous outcomes,
205 examination of normal quantile plots and the Shapiro-Wilks statistic
206 confirmed that the distributions followed the normal distribution. Overall and

pairwise p values for comparing categorical covariates (gender, race, education) across the 3 study groups were computed using the Fisher's exact test.

Sample size calculation

Our primary outcomes are COS in proinflammatory monocytes, given their role in cardiovascular disease[19]. Given absence of data regarding monocyte frequencies or COS in immune cells in EC-vapers, and based on data on frequencies of proinflammatory monocytes in otherwise healthy persons without clinical disease[20], a sample size of 9 participants per group (non-smokers, EC-vapers, and TC-smokers) was sufficient to permit detection of a delta of 2.9% with 80% power and two-sided alpha=0.05. Nine to twelve participants were included in each study group. This study, largely exploratory, is not powered to detect effect sizes with adjustments for multiple comparisons[21, 22]. It should be noted that this is an interim report of our study registered at ClinicalTrials.gov (NCT03823885), which is an acute exposure, crossover study.

RESULTS

Baseline Characteristics

A total of 33 participants, including 12 non-smokers (age 24.3±2.2 years, 5 female), 12 chronic EC-vapers (age 24.1±4.3 years, 4 female), and 9 chronic TC-smokers (age 24.9±4.1 years, 5 female) participated in the study. Baseline characteristics of the 3 groups are shown in Table 1. There were no differences among the groups in any variable, including age, sex, race, body mass index, or education level. All smokers and vapers used their tobacco product daily. Ten EC vapers reported using a “pod” device (e.g. JUUL), and one each used a “mod” or a “cigalike” device; all EC vapers used flavored, nicotine-containing liquid. Plasma cotinine levels were not significantly different in TC-smokers and EC-vapers (58 ng/ml vs 85 ng/ml respectively, p=0.34) consistent with similar, and relatively light, smoking burden.

Immune Cell Subtypes

To assess the impact of chronic smoking on immune cells, we first determined the frequency of immune cell subtypes among smoking groups (Figure 1A-J). Gating strategies for viability dye and antibody staining are shown in Supplemental Figure 1. Neutrophils, CD14dimCD16^{+} monocytes, natural killer (NK), T and B cells were found in the lowest proportion in the non-smokers, intermediate in the EC vapers, and in the greatest proportion in TC smokers and were lower in non-smokers compared to TC smokers (Figure 1 A-J).

Cellular Oxidative Stress in CD45$^+$ Immune Cells

Given the lack of data on the impact of EC vaping on cellular oxidative stress **(**COS), we then determined the relative impact of chronic TC smoking or EC vaping on COS as measured by flow cytometry using the fluorescent probes CellROX Green, a measure of total (cytoplasmic and nuclear) cellular ROS, and CellROX Deep Red, a measure of cytoplasmic cellular ROS. There was a dose-response relationship among the three study groups for the percentage of CD45$^+$ immune cells that were positive for total (Figure 2A, B) and cytoplasmic (Figure 2C, D) ROS (lowest in non-smokers, intermediate in EC-vapers, and greatest in TC-smokers). Additionally, the mean fluorescence intensity (MFI) of total (Figure 2E, F) and cytoplasmic (Figure 2 G, H) ROS in CD45$^+$ immune cells also demonstrated this same, consistent dose-response relationship. Between group comparisons consistently showed significantly greater COS in TC-smokers compared to non-smokers (Figure 2A-H). Cytoplasmic ROS was greater in TC-smokers compared to EC-vapers as well (Figure 2C, D).

Cellular Oxidative Stress in Specific Immune Cell Types

We then determined the impact of smoking exposures on COS among immune cell types (Figures 3, 4, 5). Group comparisons between TC smokers and EC vapers showed that there were no differences in ROS in neutrophils (Figure 3 A-D). The proportion of B cells that had detectable total ROS

(Figure 3I) and the proportion of NK (Figure 3G), B (Figure 3K) and total $CD3^+$, $CD4^+$ and $CD8^+$ T cells (Figure 4C, G, K) that had detectable cytoplasmic ROS was greater in TC smokers compared to EC vapers. Similar data were seen for the mean content for cytoplasmic ROS in NK cells (Figure 3H) and for the mean content for total (Figure 4J, Figure 5J) and cytoplasmic (Figure 4L, Figure 5H, L, P) ROS in $CD8^+$ T cells (Figure 4 J, L) and proinflammatory monocytes (Figure 5 H, J, L, P). There were no differences in total ROS (Figure 3E, F), the mean content for total (Figure 3J; 4B, F) and cytoplasmic (Figure 3L) ROS in NK (Figure 3E, F) and B cells (Figure 3L) in TC smokers compared to EC vapers.

Group comparisons between TC smokers and non-smokers showed that the proportion of B cells (Figure 3I, K) and proinflammatory monocytes (Figure 5 C, E, G, K, L, M, O) that had detectable cellular total (Figures 3I, 5E, L, M) and cytoplasmic (Figures 3K, 5 C, G, K, O) ROS was greater in TC smokers compared to non-smokers. Similar results were seen for cytoplasmic ROS in NK (Figure 3G), B (Figure 3K), T cells (Figure 4C), T cell (Figure 4 G, K) and monocyte (Figure 5 C, G, K, O) subsets. The mean cellular content for total (Figure 4 J, Figure 5 F, J) and cytoplasmic (Figure 4 L, Figure 5H, L, P) ROS was higher in $CD8^+$ T cells (Figure 4 J, L) and proinflammatory monocytes (Figure 5 F, H, J, L, P) in TC smokers compared to non-smokers. Similar trends ($0.05<p<0.10$) were observed in neutrophils (Figure 3D), NK (Figure 3F), T cells (Figure 4D) and monocyte subsets (Figure 5D, N) but were not

consistent among independent readouts of COS. There were no other consistent differences in measures of COS in immune cell types between TC smokers and non-smokers (Figures 3A-F, H, J, L; 4 A, B, D, F, I; 5 A, B, D, N).

Group comparisons between EC vapers and non-smokers showed that EC vapers had higher proportion of monocyte subsets (Figure 5C, G, K, O) that had detectable total (Figure 5 E, I, M) and cytoplasmic (Figure 5C, G, K, O) ROS compared to non-smokers. Similar results were seen for cytoplasmic ROS in NK (Figure 3G) and $CD4^+$ T cells (Figure 4G) and the mean cellular content for total (Figure 5J) and cytoplasmic (Figure 5H, L) ROS in proinflammatory monocytes. There were no differences in other measures of COS in other immune cell types between compared groups (Figures 3A-F, H-L; 4, 5A, B, D, F).

There was a dose-response relationship among the three study groups for the mean percent of NK (Figure 3G), B (Figure 3K), T cells (Figure 4) and monocyte (Figure 5 C, G, K, O) subtypes with cytoplasmic ROS; lowest in the non-smokers, intermediate in EC vapers, and greatest in TC-smokers. The mean percentage of proinflammatory monocytes positive for total ROS (Figure 5E, I, M), the mean cellular content for total (Figure 5 F, J, N) and cytoplasmic ROS in proinflammatory monocytes (Figure 5 H, L, P) and T cell subtypes (Figure 4 G, K) also followed this same pattern. The COS findings in

different immune cell subpopulations and whether or not the dose-response relationship was observed are summarized in Figure 6.

DISCUSSION

To our knowledge, this is the first study to report alterations in the proportion of circulating innate and adaptive immune cells, as well as their cellular oxidative stress (COS) content, in otherwise healthy young people who are chronic EC-vapers or TC-smokers compared to non-smokers. Overall, we found a marked and consistent dose-response increase in pro-inflammatory monocytes and lymphocytes, and their total cellular and cytoplasmic ROS content amongst the three study groups: lowest in the non-smokers, intermediate in EC-vapers, and highest in TC-smokers. These findings were most striking in $CD14_{dim}CD16^{+}$ and intermediate $CD14^{++}CD16^{+}$ pro-inflammatory monocytes and were reproduced with 2 independent fluorescent probes that determine total (CellROX Green) and cytoplasmic (CellROX Deep Red) cellular ROS.

Oxidative stress plays a major role in inflammation and cellular activation and is a major contributor to atherosclerotic cardiovascular disease[1-3]. The presence of excessive ROS has been termed the “convergent signaling hub” that underlies inflammatory diseases- including smoking-related atherosclerotic disease[23]. These findings of increased COS in key innate and adaptive immune cell sub-types portend the future development of premature atherosclerosis in otherwise healthy young people who chronically vape ECs.

TC smoking is a significant independent risk factor for many chronic and lethal diseases in humans[1, 2]. Given the powerfully addictive nature of

nicotine and the low rate of successful smoking cessation, ECs have been proposed as a potential harm-reduction strategy, with the ultimate goal of reducing morbidity and mortality while satisfying nicotine addiction[24]. ECs may emit fewer toxicants and carcinogens compared to TCs, but our findings confirm that their chronic use is associated with increased innate and adaptive immunity with increased COS. Although the proportion of immune cells subtypes, and their burden of COS, may be less in chronic EC-vapers compared to TC-smokers, it remains unproven and unknown if there is a "safe" level of chronic oxidative stress and inflammation.

Previous attempts to predict the adverse future health effects of ECs have been hampered by methodological limitations, such as relying on *in-vitro* model systems or focusing on acute, not chronic, EC exposure; additionally, most studies have been significantly underpowered[25-29]. In one of the few studies of health effects in chronic EC-vapers, we reported an increased susceptibility to, but not actual presence of, chronic oxidative stress, estimated by LDL oxidizability, compared to healthy non-smoking controls[30]. Traditional, clinical biomarkers of inflammation, including fibrinogen and C-reactive protein, were not elevated[30]. Admittedly, measurements of biomarkers in plasma lack sensitivity to elucidate the effects of ECs on oxidative stress and immune cell activation.

We found that COS was consistently elevated in $CD14_{dim}CD16^{+}$ and intermediate $CD14^{++}CD16^{+}$ pro-inflammatory monocytes of TC smokers and EC vapers compared to non-smokers. $CD14^{+}CD16^{+}$ monocytes are known

contributors to atherosclerotic cardiovascular disease[31-33], have increased chemotactic properties and are potent secretors of IL-1, IL-6 and TNF-α[34]. However, their specific roles in atherosclerosis progression, lesion stability and clinical events are uncertain. This monocyte subpopulation was also associated with increased vascular superoxide production in vascular dysfunction[35]. Consistent with our data, it has been shown that $CD14^+CD16^+$ monocytes have lower levels of anti-oxidant genes and increased aerobic respiration and ROS production capacities[36]. Given that oxidative stress is a known instigator of atherosclerosis[2, 3], it remains to be shown whether increased prooxidant capacity of $CD14^+CD16^+$ monocytes in the setting of EC vaping during lung chemotaxis may contribute to subsequent oxidative stress in arteries, portending the development of premature cardiovascular disease in otherwise healthy young people who chronically vape ECs.

The direct quantification of ROS is a valuable and promising biomarker that can reflect the disease process. However, given the short half-life of these species, their measurement in biological systems is complex. Determination of ROS has several methodological concerns and global ROS measurements need to be avoided[37]. Identifying individual molecular targets of redox regulation is needed and the complexity of COS can be studied only at the single cell level[12]. Approaches, such as mass spectrometry, spectrophotometric or luminescence methods, have major methodological limitations[38]. Although there is no single method that detects ROS that does not have limitations, the relative differences among different

393 samples may be assessed reasonably and the bias of each method to detect
394 ROS could be overcome by the evaluation of oxidative stress by using more
395 than one criterion[12]. Flow cytometry is one of the most powerful tools for
396 single-cell analysis of the immune system. Many fluorescent probes for the
397 detection of reactive species have been developed in the last years, with a
398 different degree of specificity and sensitivity[12].
399 The CellROX Deep Red has been previously used to detect the ex vivo
400 impact of TC smoke on cellular ROS by flow cytometry in spermatocytes[16].
401 The use of these fluorochromes for determination of COS in immune cells has
402 previously been validated both *in vitro*[17] and *in vivo*[39]. The CellROX ROS
403 detection reagents are bright and stable ROS sensors that offer significant
404 advantages over existing ROS sensors because they are compatible with
405 labeling in different media and can be used with fixatives[40]. This combined
406 use has previously been described in non-immune cells[41]. To the best of our
407 knowledge, this study is pioneering in evaluating the efficiency of these
408 probes in detecting ROS production among unique immune cell subsets.
409 Our study has limitations. Unlike animal studies, participants in human
410 studies are heterogeneous. It is possible, but unlikely, that unmeasured,
411 confounding differences exist among the three study groups, besides the
412 obviously different smoking habits, to explain the marked and consistent
413 differences in the proportion of immune cell subtypes and their oxidative
414 stress. However, by any major demographic measure including age, sex,
415 race, and education level, the three study groups were markedly similar

(Table 1). EC vaping is difficult to quantify objectively and then compare to commonly used measures of TC smoking (e.g. number of cigarettes per day). Since all of our vapers used ECs with nicotine, plasma cotinine levels were used as an objective, quantifiable measure, common to both EC and TC users, that could be compared between groups to estimate relative tobacco product burden. Our study is a small single-center study, and not powered to detect effect sizes with adjustment for multiple comparisons. Rather, consistency, direction, and magnitude of the effect in conjunction with the nominal p values were considered in order to help distinguish true and false-positive findings[21, 22]. Accordingly, by leveraging the powerful technique of flow cytometry coupled to two different sensitive fluorescent probes, we were able to find a consistent dose-response relationship in COS among the three study groups that was repeated in both innate and adaptive immune cells. We acknowledge, however, that confirmation of these findings in additional participants is warranted.

In conclusion, our study is the first to report an increased proportion of pro-inflammatory monocytes/macrophages, natural killer, and T and B lymphocytes, in otherwise healthy young people who are chronic EC-vapers compared to non-smokers. This increased proportion of innate and adaptive immune cell subtypes is coupled with the finding that chronic EC-vapers have elevated cellular oxidative stress as well. Since low-grade oxidative stress and inflammation have been identified as the underlying mechanism that instigates and perpetuates atherosclerotic vascular disease that may

manifest only decades later, these findings have important future health implications for young people who vape. On the other hand, that the COS is lower in chronic EC-vapers compared to TC-smokers is intriguing and warrants additional investigation to determine if switching to ECs may indeed avoid activation of downstream detrimental cellular pathways, supporting their role as part of a harm-reduction strategy for cardiovascular disease. Future studies delineating the specific cellular pathways impacted in humans who chronically use ECs compared to TCs may provide further insights into their relative health risks, and whether switching to ECs will result in harm reduction.

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Conflict of Interest Disclosures

None.

SUPPLEMENTAL MATERIAL

Supplemental Methods

Supplemental Figure 1 and Supplemental Figure 1 Legend

Supplemental References 42-70

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Table 1

Baseline Characteristics

	Non-Smokers	EC-Vapers	TC-Smokers	p value
	N=12	n=12	n=9	
Age (years)	24.3±2.15	24.1±4.34	24.9±4.08	0.54
Sex (M/F)	7/5	8/4	4/5	0.61
Race				0.65
White	4	6	2	
Asian	4	5	3	
Black	2	0	1	
Hispanic	2	1	1	
Unknown	0	0	2	
BMI (kg/m^2)	24±3.66	22.6±2.89	23.0±3.47	0.37
Plasma cotinine (ng/ml)	0	85.0±126.2	58.0±39.5*	
Highest Level Education				1.0
$\leq$ High school	0	0	0	
$\geq$ College	12	12	9	

Values ± SD

*p=0.34, EC-vapers vs TC-smokers

BMI= body mass index, EC = electronic cigarette, TC = tobacco cigarette

FIGURE LEGENDS

Figure 1. Frequency of immune cell types among smoker groups. Flow cytometry was used to determine the percent of different immune cell types in $CD45^+$ immune cells (**A–J**). The compared groups were nonsmokers (NS, white), electronic cigarette vapers (EC vapers, light grey) and tobacco cigarette smokers (TC smokers, dark grey). Summary of data (% cellular marker$^+$ of parent population) are shown for $CD45^+CD15^+CD16^+CD14^-$hi-SSC neutrophils (**A**), $CD45^+CD14^{++}CD16^-$ classical monocytes (**B**), $CD45^+CD14^{++}CD16^+$ intermediate monocytes (**C**), $CD45^+CD14dimCD16^+$ non-classical (patrolling or $CD14^+CD16^{++}$) monocytes (**D**), $CD45^+CD14^+CD16^+$ total proinflammatory monocytes (intermediate and non-classical)(**E**), $CD45^+CD3^+$ T cells (**F**), $CD45^+CD3^+CD4^+$ T cells (**G**), $CD45^+CD3^+CD8^+$ T cells (**H**), $CD45^+CD3^-CD56^+CD16^+$ NK cells (**I**), $CD45^+CD19^+$B cells (**J**). Data represent box and whisker boxes that display the minimum, mean and maximum (n = 9-12 participants per group). The Analysis of Variance (ANOVA) statistical test was used to compare 3 groups and the t- test was used to compare 2 groups. The trend p analysis tested the continuum of the difference in measures among groups in an ordered direction (NS→ EC vapers→ TC smokers) (*P < 0.05, **P < 0.01, ***P < 0.001).

Figure 2: Cellular oxidative stress in CD45$^+$ immune cells among smoker groups. Flow cytometry was used to determine total (nuclear and cytoplasmic) and cytoplasmic ROS. The compared groups were nonsmokers (NS, white), electronic cigarette vapers (EC vapers, light grey) and tobacco cigarette smokers (TC smokers, dark grey). Representative data of percentage of immune (CD45$^+$) cells that had positive staining for CELLROX Green among compared groups are shown in **A.** Summary of data for **A** is shown in **B**. Representative data of percentage of CD45$^+$ cells that had positive staining for CELLROX Deep Red among compared groups are shown in **C.** Summary of data for C is shown in **D**. Representative data of CellROX Green ΔMFI in CD45$^+$ cells are shown in **E**. Fluorescence intensity of a positive cell population was compared to a negative cell population (fluorescence minus one negative control for staining) (ΔMFI). Summary of data for **E** is shown in **F.** Representative data of CellROX Deep Red ΔMFI in CD45$^+$ cells is shown in **G**. Summary of data for **E** is shown in **H.** Data represent box and whisker boxes that display the minimum, mean and maximum (n = 9-12 participants per group). The Analysis of Variance (ANOVA) statistical test was used to compare 3 groups and the t- test was used to compare 2 groups. The trend p analysis tested the continuum of the difference in measures among groups in an ordered direction (NS→ EC vapers→ TC smokers) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 3: Cellular oxidative stress in neutrophils, NK cells and B cells among smoker groups. Flow cytometry was used to determine total (nuclear and cytoplasmic) and cytoplasmic ROS. The compared groups were nonsmokers (NS, white), electronic cigarette vapers (EC vapers, light grey) and tobacco cigarette smokers (TC smokers, dark grey). Summary data of percentage of immune cells that had positive staining for CELLROX Green **(A, E, I, M)** and CELLROX Deep Red **(C, G, K, O)** and for ΔMFI CellROX Green **(B, F, J, N)** and ΔMFI CellROX Deep Red in cells **(D, H, L, P)** among compared groups are shown for $CD45^{+}CD15^{+}CD16^{+}CD14^{-}$hi-SSC neutrophils (**A-D**), $CD45^{+}CD3^{-}CD56^{+}CD16^{+}$ NK cells **(E-H**), $CD45^{+}CD19^{+}$B cells **(I-L)** and $CD45^{+}CD3^{+}$ T cells **(M-P**). Data represent box and whisker boxes that display the minimum, mean and maximum (n = 9-12 participants per group). The Analysis of Variance (ANOVA) statistical test was used to compare 3 groups and the t- test was used to compare 2 groups. The trend p analysis tested the continuum of the difference in measures among groups in an ordered direction (NS→ EC vapers→ TC smokers) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 4: Cellular oxidative stress in T cell subsets among smoker groups. Flow cytometry was used to determine total (nuclear and cytoplasmic) and cytoplasmic ROS. The compared groups were nonsmokers (NS, white), electronic cigarette vapers (EC vapers, light grey) and tobacco cigarette smokers (TC smokers, dark grey). Summary data of percentage of

immune cells that had positive staining for CELLROX Green **(A, E, I)** and CELLROX Deep Red **(C, G, K)** and for ΔMFI CellROX Green **(B, F, J)** and ΔMFI CellROX Deep Red in cells **(D, H, L)** among compared groups are shown for $CD45^{+}CD3^{+}$ T cells (**A-D**), $CD45^{+}CD3^{+}CD4^{+}$ T cells (**E-H**), and $CD45^{+}CD3^{+}CD8^{+}$ T cells (**I-L**). Data represent box and whisker boxes that display the minimum, mean and maximum (n = 9-12 participants per group). The Analysis of Variance (ANOVA)statistical test was used to compare 3 groups and the t- test was used to compare 2 groups. The trend p analysis tested the continuum of the difference in measures among groups in an ordered direction (NS→ EC vapers→ TC smokers) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 5: Cellular oxidative stress in monocyte subsets among smoker groups. Flow cytometry was used to determine total (nuclear and cytoplasmic) and cytoplasmic ROS. The compared groups were nonsmokers (NS, white), electronic cigarette vapers (EC vapers, light grey) and tobacco cigarette smokers (TC smokers, dark grey). Summary data of percentage of immune cells that had positive staining for CELLROX Green **(A, E, I, M)** and CELLROX Deep Red **(C, G, K, O)** and for ΔMFI CellROX Green **(B, F, J, N)** and ΔMFI CellROX Deep Red in cells **(D, H, L, P)** among compared groups are shown for $CD45^{+}CD15^{+}CD16^{+}CD14^{-}$hi-SSC neutrophils (**A-D**), $CD45^{+}CD3^{-}CD56^{+}CD16^{+}$ NK cells **(E-H**), $CD45^{+}CD19^{+}$B cells **(I-L)** and $CD45^{+}CD3^{+}$ T cells **(M-P**). Data represent box and whisker boxes that

display the minimum, mean and maximum (n = 9-12 participants per group). The Analysis of Variance (ANOVA) statistical test was used to compare 3 groups and the t- test was used to compare 2 groups. The trend p analysis tested the continuum of the difference in measures among groups in an ordered direction (NS→ EC vapers→ TC smokers) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Figure 6: Ordered, “dose-response” relationship in cellular oxidative stress among immune cell types and smoker groups, with tobacco-product type as “dose”.