Beyond Nicotine Lesson 1: Evaluating the Effects of Flavored Electronic Cigarettes on the Respiratory Immune System

This data interpretation activity enables students to analyze experimental data from studies assessing the impact of e-cigarette flavorings on the structure and function of the respiratory innate immune system and features the research of toxicologists at the University of North Carolina at Chapel Hill. E-cigarette liquids or "vaping" liquids are complex chemical mixtures; therefore, users are exposed to inhaled aerosols of varying chemical composition and these ingredients and mixtures are of interest to toxicologists. **In this activity, students learn about the experimental models and technologies being used to investigate the health effects of flavored e-cigarettes by interacting with published scientific data showing how flavored e-liquids and inhaled aerosols derived from these liquids influence biological responses (e.g., inflammation) of respiratory immune cells.**

Data from the following peer-reviewed article is featured in this activity:

Clapp P.W., Pawlak E.A., Lackey J.T., Keating, J.E., Reeber, S.L., Glish, G.L, & Jaspers. (2017). **Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function.** *American Journal of Physiology-Lung Cellular and Molecular Physiology,* 313(2), L278-L292. doi:10.1152/ajplung.00452.2016.

The featured research was made possible with NIH support to the Jaspers lab (T32 ES007126, P50 HL120100).

Learning Objectives

Upon completion of this lesson, students will be able to:

- Describe the components of vaping liquids and inhaled e-cigarette aerosols
- Describe the components of the innate immune system (respiratory epithelial cells + white blood cells) in the lungs and explain how some flavoring agents impair this system
- Explain how scientists are studying how flavoring agents in inhaled aerosols disrupts homeostasis through inflammation and/or immune suppression
- Interpret published scientific data (graphs and images) to assess how some flavorings impair immune cell functions, such as phagocytosis by macrophages

Curriculum Alignment

Advanced Placement Biology

This activity is aligned with all seven AP Biology Science Practices and the following Big Ideas:

Big Idea 2: Biological systems utilize free energy and molecular building blocks to grow, to reproduce, and to maintain dynamic homeostasis.

Enduring understanding 2.D: Growth and dynamic homeostasis of a biological system are influenced by changes in the system's environment.

Essential knowledge 2.D.1: All biological systems from cells to organisms to populations, communities and ecosystems are affected by complex biotic and abiotic interactions involving exchange of matter and free energy.

Essential knowledge 2.D.3: Biological systems are affected by disruptions to their dynamic homeostasis.
Essential knowledge 2.D.4: Plants and animals have a variety of chemical defenses against infections that affect dynamic homeostasis.

Enduring understanding 2.E: Many biological processes involved in growth, reproduction, and dynamic homeostasis include temporal regulation and coordination.

Essential knowledge 2.E.1: Timing and coordination of specific events are necessary for the normal development of an organism, and these events are regulated by a variety of mechanisms.
Essential knowledge 2.E.2: Timing and coordination of physiological events are regulated by multiple mechanisms.

Big Idea 3: Living systems store, retrieve, transmit, and respond to information essential to life processes.

Enduring understanding 3.B: Expression of genetic information involves cellular and molecular mechanisms.

Essential knowledge 3.B.1: Gene regulation results in differential gene expression, leading to cell specialization.

Big Idea 4: Biological systems interact, and these systems and their interactions possess complex properties.

Enduring understanding 4.C: Naturally occurring diversity among and between components within biological systems affects interactions with the environment.

Essential knowledge 4.C.2: Environmental factors influence the expression of the genotype in an organism.

Next Generation Science Standards

Disciplinary Core Ideas in Life Science
LS1: From Molecules to Organisms: Structures and Processes
LS3: Heredity: Inheritance and Variation of Traits

Scientific and Engineering Practices
Asking questions and defining problems
Analyzing and interpreting data
Developing and using models
Constructing explanations
Obtaining, evaluating, and communicating information

Crosscutting Concepts
Patterns
Cause and effect
Scale, proportion, and quantity
Systems and system models
Structure and function
Stability and change

Background

Few consumer products have evolved as an environmental health concern as rapidly as electronic or e-cigarettes. Officially known as “electronic nicotine delivery systems” or “ENDS”, these devices deliver nicotine, flavorings, and other additives to users through an inhaled aerosol, in a process known as vaping. Among youth, e-cigarettes have been the most commonly used tobacco product since 2014 and the use of these devices skyrocketed in 2017 with the advent of sleek and discreet ENDS devices like JUUL (Gentzke, et al., 2019; Cullen et al., 2018). JUUL's rise in popularity among youth led the US Food and Drug Administration and the US Surgeon General to declare youth e-cigarette use an epidemic in 2018 (US DHHS, 2018). According to the 2019 National Youth Tobacco Survey, over 5 million youth reported using e-cigarettes in 2019, up from 3.6 million in 2018, with 27.5% of high school students reporting current e-cigarette use (Cullen et al. 2019). Results also revealed that among high school students who exclusively used e-cigarettes, 72% used flavored e-cigarettes, with fruit, menthol or mint, and other sweet flavors being most commonly reported flavors (Cullen et al. 2019). With most flavored e-cigarettes also containing nicotine, and with nicotine exposure during adolescence known to be harmful to the developing brain, there is a concern not only about nicotine addiction among youth but also about research findings that indicate these youth are more likely to smoke combustible cigarettes in the future (NASEM, 2018). Thus, **e-cigarettes provide a timely and relevant context in which students can explore and refine their knowledge of fundamental biology concepts while communicating risk to a vulnerable population**.

This rise in e-cigarette use and in the frequency of use among youth is not only concerning because of exposure to nicotine but also because **e-liquids and aerosols derived from e-liquids contain flavoring agents and a variety of chemicals (such as formaldehyde and benzene) that can harm respiratory health**. For example, scientists have learned that the activity of leukocytes (white blood cells) commonly found in the respiratory mucosa, such as natural killer cells, neutrophils, and macrophages can be modified by exposure to e-liquids (Clapp et al. 2017). Furthermore, research is revealing that some flavoring agents, such as cinnamaldehyde, impair mitochondrial function in respiratory epithelial cells, which results in decreased ATP production, decreased cilia motility and reduced ability to keep the lungs clear of mucus (Clapp et al. 2019). Research in animals has also shown that e-cigarette exposure can impair bacterial and viral responses to common pathogens (Madison et al. 2019, Miyashita et al. 2018, Hwang et al. 2016). These research findings are especially concerning in light of the recent outbreak of SARS-CoV-2, which targets the respiratory system and causes the disease known as COVID-19.

Recognizing that flavored e-cigarettes were contributing to the teen vaping epidemic, the US FDA banned the sale of all flavored "cartridge-based ENDS", except for those containing menthol and tobacco flavoring agents effective January 2, 2020. While mango flavored JUUL pods are no longer available for sale, **a number of flavored tobacco products are exempt from this ban, including <u>disposable devices</u>** like Suorin and Smok, which in early 2019 were the most popular e-cigarette devices among high school students after JUUL (Cullen et al. 2019). **The ban also excludes approximately 15,000 <u>flavored e-liquids that can be used in refillable devices</u>**, also referred to as "tank systems" or "box mods." Given these loopholes, teens will still be able to gain access to flavored e-cigarettes, such as the disposable STIG and Puff Bar brands, some of which contain more nicotine than JUUL (Kaplan, 2020).

While the long-term health effects of flavored e-cigarettes are unknown due to the relatively recent entry of these devices into society, **research emerging from UNC-Chapel Hill and**

other institutions suggests that flavored e-liquids convey health risks beyond those associated with nicotine use.

In this activity, students explore how toxicologists are investigating the health effects of flavored e-cigarettes by interacting with published scientific data showing how inhaled aerosols from vaping liquids influence biological responses (e.g., inflammatory response) in respiratory immune cells.

Overview of the innate immune system in lungs

The innate immune system in the lungs is responsible for protecting the body from inhaled pathogens (e.g., bacteria and viruses) and chemical pollutants. The innate immune system in your lungs is made up of **two primary components**:

1) **The mucociliary escalator.** The epithelial cells that line your airways produce mucus and have cilia along the airway side of the cell. The cilia beat this mucus along with anything trapped in it up and out of the airway. This mucus is then cleared through either swallowing or coughing.

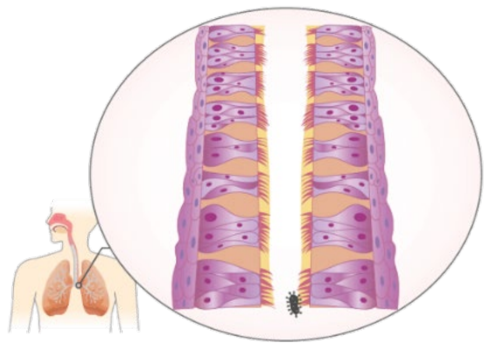

Image Credit: National Institutes of Health

2) **White blood cells (macrophages, neutrophils, natural killer cells).** Macrophages are found in the lungs, and neutrophils can be attracted to the lungs from the blood in response to inflammation. These cells can kill pathogens and clear debris through a process called **phagocytosis.** They can also signal other white blood cells to the site of infection or inflammation. In addition, neutrophils can release DNA and other molecules to the outside of the cell in order to bind and trap pathogens like bacteria. This release of extracellular DNA is known as a **neutrophil extracellular trap (NET)**. Natural killer (NK) cells are another type of white blood cell that patrol the body and identify and kill abnormal cells, including diseased cells.

Overview of e-liquids

Flavored e-liquids are chemical mixtures that, in addition to flavorings, include the base ingredients **propylene glycol (PG)** and/or **vegetable glycerin (VG)** and either nicotine or nicotine salts at concentrations ranging from 0% to 7% (which is very high).

Flavorings are chemicals that give flavored e-liquids their predominant taste and smell. Examples include cinnamaldehyde (spicy or cinnamon), benzaldehyde (cherry or almond), vanillin (vanilla), and ethyl vanillin (vanilla). However, many e-liquids include combinations of two or more flavors. PubChem (https://pubchem.ncbi.nlm.nih.gov/) is a great resource for looking up chemical formulas, molecular weights, structures, etc. for specific flavorings (or really any other chemical) you might be interested in having your class study. *Note: "Sini-cide" is a cinnamon flavored e-liquid referenced in this activity.*

E-liquids become aerosolized during vaping. The "vapor" generated by e-cigarettes is a complex mixture of particles and gases derived from aerosolizing e-liquid. An **aerosol** is a suspension of fine solid particles or liquid droplets in air or another gas. Due to their small size, these particles can be inhaled deep into lungs. Furthermore, when e-liquids are heated and aerosolized by e-cigarettes, the chemicals can be broken down or changed during the heating and aerosolizing process. How much the chemicals change depends on the type of e-cigarette and the device settings.

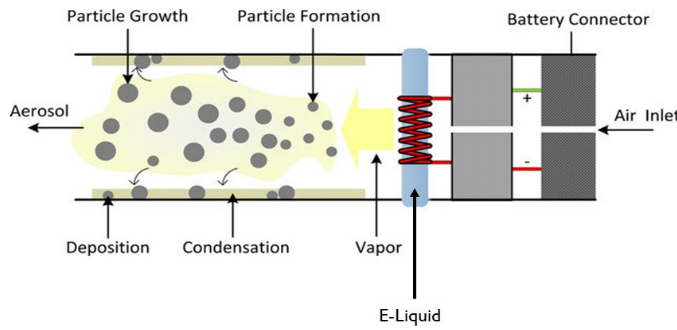

Image credit: Talih et al. November 2016 Aerosol Science and Technology

Experimental design and key toxicology concepts

Dose refers to how much of a chemical enters the body either through the nose, mouth or skin.

Dose response is the concept in toxicology that with increasing dose, or amount, of a chemical, you will see an increasing effect of that chemical on whatever function you are measuring.

Controls: In this activity, there are two control groups used:

1. **Media control** – this refers to cell cultures grown under normal growth conditions, with nothing extra added to the cells. Cells are taken from human subjects through blood draws or airway sampling and then placed in plastic culture dishes. In these dishes, they are covered by a liquid mixture called media that provides nutrients and a suitable growth environment for the cells. Treatments, like cinnamaldehyde, can also be added to the media. When a treatment is not added, that is called the media control.
2. **PG/VG vehicle control** - this refers to cell cultures being treated with PG/VG only to determine if there were any effects of the base liquid without the flavoring components and to determine whether effects were caused by the base liquid alone or just the flavoring components.

Statistical significance is indicated by asterisks (*) above specific bars on a bar graph. The number of asterisks indicates the extent of statistical significance. Statistical significance is important because it tells researchers that their results are likely real effects of a treatment or experiment versus being random effects due to variability in the experiment. P values indicate whether a comparison is statistically significantly different; a $P < 0.05$ is generally considered significant.

Activity Description

This activity offers **6 different guiding questions;** each question is accompanied by a set of figures from the featured articles and a series of question prompts to guide student data analysis and discussion.

Click here to access the Companion Slide Set

Teacher Preparation

- Read background information, review activity procedure and Companion Slide Set (PowerPoint), add any additional figures and/or slides if desired.
- Review the guiding questions posed by the activity and decide if your class will cover all of them or a select few depending on your instructional goals.
- Students should have a basic understanding of the structure and function of the human respiratory system, including the innate immune system, prior to introducing this activity.
- Determine how many guiding questions you will cover and the number of students per group.
- Make enough copies so that each group receives at least one set of figures for each guiding question to be covered. ***Note: Color copies are critical for question 4.***
- Make copies of the student worksheet, one per student (see end of lesson).

GUIDING QUESTIONS: Investigating the effects of e-liquids on respiratory immune cells

These guiding questions enable students to explore how respiratory immune cell structure and function is altered in response to e-liquids:

1. Do components of the e-liquid base (propylene glycol (PG) / vegetable glycerin (VG)) affect phagocytosis in macrophages and neutrophils?
2. Do flavored e-liquids affect phagocytosis in macrophages and neutrophils?
3. Do flavored e-liquids affect secretion of inflammatory signals like interleukin-8 (IL-8) by macrophages? by neutrophils?
4. Do flavored e-liquids affect neutrophil extracellular trap (NET) formation? [qualitative]
5. Do flavored e-liquids affect neutrophil extracellular trap (NET) formation? [quantitative]
6. Do flavored e-liquids affect natural killer (NK) cell function?

Activity Procedure

This activity utilizes the 5E instructional model and can be conducted as a jigsaw activity, with a small group of students evaluating one question, or it can be adapted into worksheets, lecture-based discussion, or stations.

Prior to conducting this activity:

1) Introduce or review the innate immune system, the function of phagocytosis, homeostasis, and inflammation
2) Introduce students to e-cigarettes, e-liquids, components of e-liquids and the basic concepts of toxicology covered in the background section

1. **Engage:** Ask students how a toxicologist might go about studying the effects of flavorings on the innate immune system. Listen and record student responses on the board without indicating whether their responses are correct.

2. **Explore | Step I:** Describe experimental approach (source of cells and use of cell cultures) used by the Jaspers lab to prepare students for the data analysis activity. *See Companion PowerPoint for accompanying visuals.*

3. Explore | Step 2: Begin jigsaw activity by distributing the set of figures for each guiding question to be covered. Ask students to read their assigned question prompts and examine the accompanying figures in order to formulate their answers. Students should practice using available evidence to answer the questions on their worksheet. Students could also make a poster or google slide presentation to summarize what they learn prior to sharing with the class.

4. Explain: One at a time, ask groups to report their findings to the class, summarizing the answer to their guiding question. A key is provided at the end of this activity. To keep the class on task during this part, ask students to complete the summary table on their worksheet while they hear from other groups.

5. Elaborate: Once all groups have reported out, summarize the activity as a class and ask one or more of these culminating discussion questions:

- What broad conclusions can you make based on these data?
    - Flavored e-liquids can impair function of our innate immune cells (macrophages, neutrophils and natural killer cells).
- Why did the authors use nicotine-free e-liquids in these experiments?
    - The authors were interested in the effects of the flavorings specifically.
    - The presence of nicotine could impact the data.
    - Nicotine concentration is not always accurately reported in e-liquids, so if nicotine-containing e-liquids were used, it could still be hard to control for the concentration of nicotine.
- Based on these data, which e-liquid flavors would you consider less safe to inhale?
    - Hot Cinnamon Candies, Kola, Sini-cide
- What ideas do you have for follow-up experiments to expand on the data provided here?
    - Study what chemicals are in the e-liquids.
    - Study other flavors of e-liquids.
    - Figure out what the most accurate dose is to use on these cells.
    - Study other cell types or other cell functions.
    - Study why these flavored e-liquids affect immune cell function (mechanism).
- Why do you think different cell types (e.g., macrophages and neutrophils) responded differently to certain flavorings such as “Hot Cinnamon Candies” (Guiding Question 2)?
    - Scientists don’t exactly know why macrophages and neutrophils might respond differently to the same chemical/pathogen, but here are some reasonable hypotheses supported by scientific research:
    - Neutrophils generally are more reactive than macrophages; they are recruited to sites of inflammation to respond to pathogens whereas macrophages reside in tissues and patrol for pathogens.
    - Neutrophils have less of an ability to defend themselves against toxicants via antioxidant and metabolizing enzymes.
    - Neutrophils are short lived and are quickly regenerated in the body, so they aren’t programmed for survival like tissue-resident macrophages.

6. **Evaluate**: Assess student learning by doing one or more of the following:

- Collect completed worksheets
- Ask students to summarize their responses to their assigned guiding question in writing or by creating a short presentation
- Ask students to identify a new research question based on what they have learned, develop a hypothesis and, on paper, design an experiment to test their hypothesis. Students can submit their ideas to the Jaspers lab by emailing their ideas to jasperslabunc@gmail.com

7. **Extend:** There are opportunities to **extend student learning by inviting them to read, evaluate, and communicate information** about this topic in the context of the E-cigarette or Vaping Use-Associated Lung Injury (EVALI) outbreak 2019 and of emerging evidence that vaping could impair the respiratory immune response to influenza or SARS-CoV-2 (Novel Coronavirus):

E-cigarette or Vaping Use-Associated Lung Injury (EVALI)

According to the CDC, from August 2019 to February 2020, over 2,800 people were hospitalized and 68 died as a result of e-cigarette or vaping use-associated lung Injury (EVALI). While scientists don't know the cause of EVALI, there is evidence that vitamin E acetate used to dilute and/or thicken marijuana containing vaping liquid may have contributed to the outbreak (CDC). While this additive is safe for use in skin creams and nutritional supplements it is not safe to inhale but scientists do not know the mechanism by which it could have caused EVALI. This finding reinforces one of the key messages of this lesson, that chemicals (such as flavorings, or vitamin E acetate) may be safe for ingestion but not safe for inhalation.

Vaping and respiratory immune response to influenza or SARS-CoV-2 (Novel Coronavirus)

Scientists do know that e-cigarette exposure can impair the body's immune response to viruses; in one recent experiment mice exposed to unflavored e-cigarette aerosol with or without nicotine for 4 months were not able to clear flu infection as well (Madison et al., 2019). In another study from the Jaspers lab, e-cigarette users, smokers, and nonsmokers were administered the nasal spray flu vaccine (a safe way to study viral responses) and their bodies' responses to the flu vaccine were monitored to determine if there were differences in antiviral response between groups. Data revealed that e-cigarette users had significantly lower levels of a flu-specific antibody (proteins that help the body remember a past viral exposure) than nonsmokers or e-cigarette users (Rebuli et al. under review). Some scientists and doctors have speculated that e-cigarette use may make people more susceptible to COVID-19, the infection caused by SARS-CoV-2 (Novel Coronavirus), though it is too soon to know if this connection exists (Lewis, 2020).

Authors

Dana Brown Haine, MS, K-12 Science Education Manager, Center for Public Engagement with Science, UNC-Chapel Hill Institute for the Environment
Elise Hickman, PhD candidate in the research lab of Ilona Jaspers, PhD at UNC-Chapel Hill

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Centers for Disease Control and Prevention. Outbreak of Lung Injury Associated with the Use of E-Cigarette, or Vaping, Products. https://www.cdc.gov/tobacco/basic_information/e-cigarettes/severe-lung-disease.html Accessed May 6, 2020.

Clapp P.W., Pawlak E.A., Lackey J.T., Keating, J.E., Reeber, S.L., Glish, G.L, & Jaspers. (2017). Flavored e-cigarette liquids and cinnamaldehyde impair respiratory innate immune cell function. *American Journal of Physiology-Lung Cellular and Molecular Physiology, 313(2)*, L278-L292. doi:10.1152/ajplung.00452.2016. *For pdf version contact: jasperslabunc@gmail.com

Cullen, K.A., Gentzke, A.S., Sawdey, M.D., et al. (2019) E-cigarette Use Among Youth in the United States, 2019. *JAMA*;322(21):2095-2103. doi:10.1001/jama.2019.18387

Gentzke, A. S., Creamer, M., Cullen, K. A., Ambrose, B. K., Willis, G., Jamal, A., & King, B. A. (2019). Vital Signs: Tobacco Product Use Among Middle and High School Students — United States, 2011–2018. *MMWR. Morbidity and Mortality Weekly Report, 68*(6), 157-164. doi:10.15585/mmwr.mm6806e1.

Hwang, J.H., et al (2016) Electronic cigarette inhalation alters innate immunity and airway cytokines while increasing the virulence of colonizing bacteria. *J Mol Med.* 94(6):667-79

Kaplan, S. (2020). Teens Find a Big Loophole in the New Flavored Vaping Ban. *New York Times.* Accessed May 6, 2020.

Lewis, T. (2020). Smoking or Vaping May Increase the Risk of a Severe Coronavirus. *Scientific American* Infection
https://www.scientificamerican.com/article/smoking-or-vaping-may-increase-the-risk-of-a-severe-coronavirus-infection1/
Accessed May 6, 2020.

Madison, M.C., et al (2019) Electronic cigarettes disrupt lung lipid homeostasis and innate immunity independent of nicotine. *J Clin Invest.* 129(10):4290-4304.

Miyashita, L., et al. (2018) E-cigarette vapour enhances pneumococcal adherence to airway epithelial cells. *Eur Respir J.* 7;51(2). pii: 1701592.

National Academies of Sciences, Engineering, and Medicine. (2018). *Public Health Consequences of E-Cigarettes.* Washington, DC: The National Academies Press. https://doi.org/10.17226/24952.

US Department of Health and Human Services. (2018) *Surgeon General's advisory on e-cigarette use among youth.* https://e-cigarettes.surgeongeneral.gov/documents/surgeon-generals-advisory-on-e-cigarette-use-among-youth-2018.pdf.

Resources and other papers that might have interesting data for your class to consider:

Other Recent Jaspers Lab Research:

Clapp, P.W., Jaspers, I. (2017) Electronic Cigarettes: Their Constituents and Potential Links to Asthma. *Curr Allergy Asthma Rep.* Oct 5;17(11):79

Escobar, Y.H., Nipp, G., Cui, T., Peters, S.S., Surratt, J.D., Jaspers, I. (2020) In Vitro Toxicity and Chemical Characterization of Aerosol Derived from Electronic Cigarette Humectants Using a Newly Developed Exposure System. *Chem Res Toxicol.*

Chemical Characterization of E-Cigarettes:

Behar, R. Z., Luo, W., McWhirter, K. J., Pankow, J. F., and Talbot, P. (2018) Analytical and toxicological evaluation of flavor chemicals in electronic cigarette refill fluids. *Sci. Rep. 8*, 8288.

Talih S., Salman R., El-Hage R., Karam E., Karaoghlanian N., El-Hellani A., Saliba N., Shihadeh A. (2019) Characteristics and toxicant emissions of JUUL electronic cigarettes. *Tob Control.* Nov;28(6):678-680.

Tierney, P. A., Karpinski, C. D., Brown, J. E., Luo, W., and Pankow, J. F. (2016) Flavour chemicals in electronic cigarette fluids. *Tob. Control 25*, e10.

Review of Respiratory Effects of E-Cigarettes:

Gotts J.E., Jordt S.E., McConnell R., Tarran R. (2019) What are the respiratory effects of e-cigarettes? *BMJ* 366: l5275

Activity Key

Guiding Question 1: Do components of the e-liquid base (propylene glycol (PG) / vegetable glycerin (VG)) affect phagocytosis in macrophages and neutrophils?

Group 1 will evaluate the effects of the e-liquid base (PG/VG only) on phagocytosis in macrophages and neutrophils. This set of figures shows the effects of different dilutions of PG/VG (x-axis) on either macrophage or neutrophil phagocytosis (y-axis).

Review Questions:
1. What is phagocytosis? What cell features enable phagocytosis?
Phagocytosis is the engulfment of a solid particle or particles, such as debris or bacteria. Cell features involved in phagocytosis include receptor binding and signaling, actin-myosin contraction, lysosomes, phagosomes, and reactive oxygen species.

2. Why do you think phagocytosis an endpoint the researchers were interested in studying?
Phagocytosis is an important cellular process performed by these cells to protect the body from pathogens.

3. Describe one consequence of impaired phagocytosis by white blood cells in the lung.
White blood cells would not be able to fight invading pathogens, so the body would not be as well protected from disease.

Data Analysis Questions:
1. Why did the authors use 0.25-1% dilutions of PG/VG?
When the e-liquid is vaporized, the dose that your cells are exposed to becomes much lower than the e-liquid that was loaded into the device. While scientists don't know exactly what dose of PG/VG the cells in your body are exposed to, by diluting the e-liquids in this experiment, they hoped to get closer to a biologically relevant dose.

2. Compare and contrast the effects of PG/VG on phagocytosis by lung neutrophils and macrophages.

a) Does PG/VG affect phagocytosis in either cell type? I
PG/VG at a 1% dilution significantly decreased phagocytosis by both cell types.

b) Was one cell type more sensitive to the effects of PG/VG than the other?
Neutrophils appear to be more sensitive to the effects of PG/VG than macrophages (neutrophils are generally more reactive than macrophages - see the *Elaborate* section above for more information).

Guiding Question 2: Do flavored e-liquids affect phagocytosis in macrophages and neutrophils?

Group 2 will evaluate the effects of flavored e-liquids on phagocytosis in macrophages and neutrophils. This set of figures shows the effects of different dilutions of flavored e-liquids (x-axis) on either macrophage or neutrophil phagocytosis (y-axis).

Review Questions:

1. What is phagocytosis? What cell features enable phagocytosis?
Phagocytosis is the engulfment of a solid particle or particles, such as debris or bacteria. Cell features involved in phagocytosis include receptor binding and signaling, actin-myosin contraction, lysosomes, phagosomes, and reactive oxygen species.

2. Why is phagocytosis an endpoint the researchers were interested in studying?
Phagocytosis is an important cellular process performed by these cells to protect the body from pathogens.

3. Describe one consequence of impaired phagocytosis by white blood cells in the lung.
White blood cells would not be able to fight invading pathogens, so the body would not be as well protected from disease.

Data Analysis Questions:

1. Why did the authors use 0.25-1% dilutions of PG/VG?
When the e-liquid is vaporized, the dose that your cells are exposed to becomes much lower than the e-liquid that was loaded into the device. While scientists don't know exactly what dose of PG/VG the cells in your body are exposed to, by diluting the e-liquids in this research, they hoped to get closer to a biologically relevant dose.

2. Compare and contrast the effects of flavorings on phagocytosis by lung neutrophils and macrophages.
In macrophages, Kola (Cola flavored) decreased phagocytosis at the highest dose, and Sini-cide (cinnamon flavored) decreased phagocytosis at all doses. In neutrophils, a dose response was seen for hot cinnamon candies, banana pudding, menthol tobacco, and banana flavored e-liquids. Sini-cide also decreased neutrophil phagocytosis but in a reverse pattern, which was likely caused by toxicity and cell death during the experiment. *Note: The phagocytosis assay used in this experiment is dependent upon particles fluorescing when they are phagocytosed because the phagosome (pocket they are taken into in the cell) is very acidic. When Sini-cide caused cell death, the media they were growing in became very acidic and gave a false positive reading for increased phagocytosis.)*

3. Did any of the flavors have the same effect on both cell types? Was one cell type more sensitive?
Sini-cide decreased phagocytosis in both macrophages and neutrophils when compared to the PG/VG control. Neutrophils were overall more sensitive to the flavorings.

Guiding Question 3: Do flavored e-liquids affect secretion of inflammatory signals like interleukin-8 (IL-8) by macrophages? by neutrophils?

Group 3 will evaluate the effects of flavored e-liquids on secretion of IL-8 by macrophages and neutrophils. This set of figures shows the effects of different dilutions of flavored e-liquids (x-axis) on macrophage and neutrophil IL-8 secretion (y-axis). IL-8 is an example of a cytokine, which are protein signals released by one cell to attract other cells to the area or influence the activity of other cells that are already present. *Note: Neutrophils and macrophages have different magnitudes (y-axis values) of IL-8 secretion in the vehicle control group,* ***so it is most important to pay attention to how the e-liquids changed their IL-8 secretion from the PG/VG group within each cell type.***

Review Questions:
1. What is the function of interleukin-8 (IL-8)?
IL-8 is secreted by one cell in order to attract other immune cells to the site of its release thereby promoting inflammation.

2. Would an increase in IL-8 secretion cause increased or decreased inflammation?
Increased inflammation.

3. What could be some potential consequences of altered IL-8 secretion by these cells?
If macrophages can't secrete IL-8, they can't recruit neutrophils to the lungs to help fight infections. If neutrophils secrete excess IL-8, that can trigger more inflammation.

Data Analysis Questions:
1. Compare and contrast the effects of the flavorings on IL-8 secretion by neutrophils and macrophages.
In macrophages, Sini-cide resulted in significantly decreased IL-8 production while in neutrophils, IL-8 production increased (in comparison to the PG/VG control) in a mostly dose-dependent fashion for all flavors except solid menthol. Hot cinnamon candies at 1% dilution did not result in increased IL-8 production as expected perhaps because of cell death (cytotoxicity).

2. Did any of the flavors have the same effect on both cell types? Was one cell type more sensitive than the other?
The effects were not that similar between the two cell types. Neutrophils appear to be more sensitive; there was a dose-response increase in IL-8 production from neutrophils treated with the flavored e-liquids. See the *Elaborate* section for discussion sensitivity.

Guiding Question 4: Do flavored e-liquids affect neutrophil extracellular trap (NET) formation?

Group 4 will evaluate **qualitative data** to assess the effects of flavored e-liquids on neutrophil extracellular trap formation. This figure shows the effects of 1% dilutions of flavored e-liquids (left side) on neutrophil extracellular trap (NET) formation.

Group 4, continued

Review Questions:
1. What are neutrophil extracellular traps (NETs)?
NETs are the release of DNA from a neutrophil to bind and trap pathogens, such as bacteria.

2. Why do you think NETs are physiologically important?
NETs are one way that neutrophils can protect the body from invading pathogens and therefore prevent disease.

3. Describe one consequence if neutrophil extracellular traps cannot form properly.
Neutrophils are impaired in their ability to fight invading pathogens, so the body is not as well protected from disease.

Data Analysis Questions:
1. Describe what you see in the figures, including any patterns you see. What conclusions can you draw from these images?
When the neutrophils were stimulated to form extracellular traps, PG/VG vehicle and Kola formed extracellular traps by 4 hours, but Hot Cinnamon Candies did not. Sini-cide formed extracellular traps at all time points.

2. Why might neutrophils appear to be forming extracellular traps when exposed to Sini-cide after only one hour of stimulation?
Sini-cide is toxic to neutrophils at the dose they received. It caused cell death and DNA release (NET formation).

Guiding Question 5: Do flavored e-liquids affect neutrophil extracellular trap (NET) formation?

Group 5 will assess **quantitative data** to evaluate the effects of flavored e-liquids on neutrophil extracellular trap (NET) formation*.* This figure shows the effects of different dilutions of flavored e-liquids (x-axis) on neutrophil extracellular trap (NET) formation (y-axis). Note: at the beginning of the experiment, the neutrophils were stimulated with phorbol 12-myristate 13-acetate (PMA), which causes them to make extracellular traps. The DNA release was then measured over 4 hours.

Review Questions:
1. What are neutrophil extracellular traps (NETs)?
NETs are the release of DNA from a neutrophil to bind and trap pathogens, such as bacteria.

2. Why do you think NETs are physiologically important?
NETs are one way that neutrophils can protect the body from invading pathogens and therefore prevent disease.

Group 5, continued

3. Describe one consequence if neutrophil extracellular traps cannot form properly.
Neutrophils are impaired in their ability to fight invading pathogens, so the body is not as well protected from disease.

Data Analysis Questions:

1. Why did the researchers quantify DNA to measure NET formation?
Neutrophils release DNA during NET formation, so the amount of DNA released correlated with their ability to form NETs.

2. What was the effect of the flavorings on neutrophil extracellular trap formation?
Kola significantly increased NET formation at 3 hours, and hot cinnamon candies impaired NET formation. Sini-cide (cinnamaldehyde) increased NET formation at all time points, but this was likely because it was toxic to the cells.

Guiding Question 6: Do flavored e-liquids affect natural killer cell function?

Group 6 will evaluate the effects of flavored e-liquids on natural killer cell function. This box plot shows the effects of 0.25% dilutions of flavored e-liquids (left side) on neutrophil extracellular trap (NET) formation. While the plot can look intimidating to students tell them the horizontal line in the middle of each box represents the median value and students can compare results from each flavoring to that of the control (PG/VG vehicle). The height of the box conveys the range of values for a particular treatment.

Review Questions:

1. What function do natural killer cells perform in the body?
They patrol the body and identify and kill abnormal cells, including diseased cells like cancer cells.

2. Describe the consequences if these cells are not able to function properly.
Diseased and abnormal cells will not be killed and will remain in the body, potentially causing or exacerbating disease.

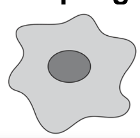
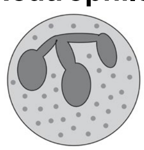
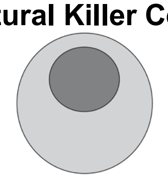
Data Analysis Questions:

1. Briefly describe your observations of the data.
Hot Cinnamon Candies and Sini-cide decreased the number of target cells the natural killer cells were able to kill.

2. Were any flavors more likely to impair natural killer cell function than others?
See answer to question 3 above. These questions could be grouped together.

Summary of Data: ***Complete this section as your classmates share their findings.***
Use the following symbols to summarize findings from each group:

X = no effect ↑ = stimulation ↓ = suppression

Cell Type	Summary of Findings	
	Impact of PG/VG	Impact of Flavored E-Liquids
Macrophages 	↓ phagocytosis (Q1)	• Sini-cide and Kola ↓ phagocytosis (Q2) • Sini-cide ↓ IL-8 secretion (Q3)
Neutrophils 	↓ phagocytosis (Q1)	• Hot Cinnamon Candies, Banana Pudding, Menthol, Banana, and Sini-cide ↓ phagocytosis (Q2) • Kola, Hot Cinn Candies, Banana Pudding, Menthol, and Banana ↑ IL-8 secretion (Q3) • Kola and Sini-cide ↑ NET formation (Q4 and Q5) • Hot Cinn Candies ↓ NET formation (Q5)
Natural Killer Cells 		• Hot Cinn Candies and Sinicide ↓ NK cell ability to kill target cells (Q6)

In one or two sentences, summarize the major conclusion(s) from these studies.
Flavored e-liquids, particularly those that are cinnamon-flavored, can impair immune cell function. Impaired immune cell function could result in increased chance of respiratory infection.

What is a follow up question or experiment the researchers could use to find out more about the effects of e-cigarettes on the respiratory immune system?
Many possible answers, including experiments with epithelial cells, different flavors, using an aerosol instead of an e-liquid, using specific chemicals instead of mixtures, etc.

Beyond Nicotine: Evaluating the Effects of Flavored Electronic Cigarettes on the Respiratory Immune System

Name ________________________________ Group Number: ____

Write down your assigned guiding question:

Data Review Questions: ***Record your answers to your group's questions below.***

1.

2.

3.

Data Analysis Questions:

1.

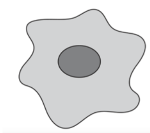
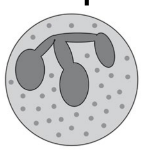
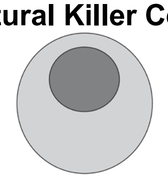
2.

3.

What additional questions do you have?

Summary of Data: ***Complete this section as your classmates share their findings.***
Use the following symbols to summarize findings from each group:

X = no effect ↑ = stimulation ↓ = suppression

Cell Type	Summary of Findings	
	Impact of PG/VG (e-liquid base)	Impact of flavoring
Macrophages 		
Neutrophils 		
Natural Killer Cells 		

In one or two sentences, summarize the major conclusion(s) from these studies.

What is a follow up question or experiment researchers could use to find out more about the effects of e-cigarettes on the respiratory immune system?