Examining the Health Effects of Flavored Electronic Cigarettes

A data interpretation activity for students

This 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 will 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., inflammatory response and cellular respiration) of respiratory and immune cells.

This activity is divided into two parts:
Part I: Investigating the effects of e-liquids on respiratory immune cells
Part II: Investigating the effects of e-liquids and aerosols on cell metabolism

Data from the following peer-reviewed articles are featured in this activity:

Clapp P.W., Lavrich K.S., van Heusden C.A., Lazarowski E.R., Carson J.L., & Jaspers I. (2019). **Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function.** *American Journal of Physiology-Lung Cellular and Molecular Physiology.* 316(3), L470-L486. doi: 10.1152/ajplung.00304.2018.

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.

Hickman E., Herrera C.A., Jaspers, I. (2019. **Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst.** *Chem Res Toxicol*. 2019 Jun 17;32(6):982-985. doi:10.1021/acs.chemrestox.9b00171.

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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 [Part 1]
- Describe the mechanism (impaired mitochondrial function and reduced ATP formation) by which a flavoring chemical impairs cilia motility in respiratory epithelial cells [Part 2]

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

Scientific and Engineering Practices	Crosscutting Concepts
Asking questions and defining problems	Patterns
Analyzing and interpreting data	Cause and effect: mechanism and explanation
Developing and using models	Scale, proportion, and quantity
Constructing explanations	Systems and system models
Obtaining, evaluating, and communicating information	Structure and Function
	Stability and change

Disciplinary Core Ideas in Life Science

LS1: From Molecules to Organisms: Structures and Processes

LS3: Heredity: Inheritance and Variation of Traits

Background

Few consumer products have evolved as an environmental health concern as rapidly as electronic or e-cigarettes. These devices deliver nicotine, flavorings, and other additives to users through an inhaled aerosol, in a process knowing as vaping. Introduced in the United States just over a decade ago, the use of e-cigarettes has skyrocketed in recent years, especially among youth, with the advent of sleek, low-output devices like Juul (Cullen et al., 2018). The use of flavored e-liquids to deliver nicotine via discrete devices like Juul has been particularly appealing to teens and young adults. According to the 2018 National Youth Tobacco Survey, 1.5 million additional students reported using e-cigarettes in 2018 compared to 2017 (Cullen et al. 2018), and e-cigarettes are the most commonly used tobacco product among youth (Gentzke et al., 2019). Among youth, current e-cigarette users also reported using them more frequently than adults (Gentzke et al., 2019). Further, research has shown that young people who use e-cigarettes may be 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) such as natural killer cells and macrophages are modified in the respiratory mucosa of e-cigarette users. 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.

While the long-term health effects of 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 will 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 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 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.

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 when there is inflammation

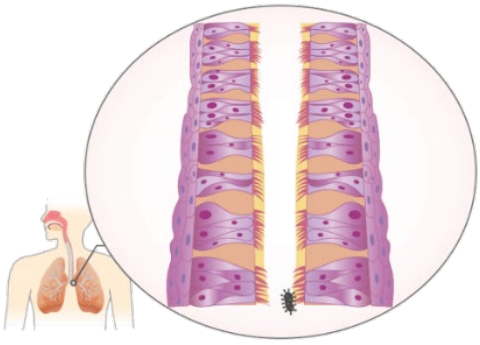

Image Credit: National Institutes of Health

in the lungs. These cells can kill pathogens and clear debris through a process called **phagocytosis.** They can also signal to 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.

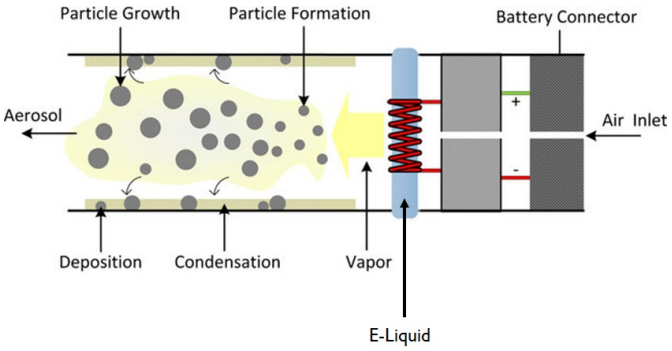

Image credit: Talih et al. Aerosol Science and Technology (Nov 2016)

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 consists of two parts with each part being comprised of **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. **Depending on your instructional goals, you may want to complete either Part I or II or both.**

Click here to access the Companion Slide Set (For Parts I and II)

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. Depending on your instructional goals, you may want to complete either Part I or II or both.
- For Part I, 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.
- For part II, students should have a basic understanding of cellular respiration and more specifically, the role of glycolysis and aerobic respiration in generating ATP.
- 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 guiding questions 4 and 9.***
- Make copies of the student worksheet (Part I and/or Part II), one per student (see end of lesson).

Part I: 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?

Part II: Investigating the effects of e-liquids and aerosols on cell metabolism
These guiding questions enable students to explore how e-liquids and aerosols impact cellular respiration and a cellular process that requires energy (ciliary beating).

7. Do cinnamon-flavored e-liquids and aerosols impair ciliary beating in respiratory epithelial cells?
8. Does cinnamaldehyde impair ciliary beating in respiratory epithelial cells?
9. Does cinnamaldehyde change how energetic mitochondria are in the respiratory epithelial cells?
10. Does cinnamaldehyde impair mitochondrial function?
11. Does cinnamaldehyde impair glycolysis?
12. Does cinnamaldehyde impair neutrophil oxidative burst?

Activity Procedure
This activity utilizes the 5E instructional approach 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 Part I review the innate immune system, the function of phagocytosis, homeostasis, and inflammation.

Prior to conducting Part II review the innate immune system, cilia and epithelial cells, and the function of cellular respiration and the role of glycolysis and aerobic respiration in ATP production.

Prior to conducting this activity, introduce students to e-cigarettes, e-liquids, components of e-liquids and the basic concepts of toxicology covered in the background section.

1. **Engage | Part I:** 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.

Engage| Part II: Show the set of short videos provided in the Companion Slide Set (Slides 33-36) that provide visual evidence that Sini-cide (cinnamaldehyde) alters movement of cilia on respiratory epithelial cells. Use this to prompt student thinking about what might be causing this biological response (reduced cilia movement). Tells students they will be exploring experimental results to learn what might be responsible for this observation.

2. **Explore | Step I:** Depending on which set of guiding questions (Part I and/or Part II) you are using, describe experimental approach (source of cells and use of cell cultures) used by the Jaspers lab to prepare students for 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
   - 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

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Resources

Clapp P.W., Lavrich K.S., van Heusden C.A., Lazarowski E.R., Carson J.L., & Jaspers I. (2019). Cinnamaldehyde in flavored e-cigarette liquids temporarily suppresses bronchial epithelial cell ciliary motility by dysregulation of mitochondrial function. *American Journal of Physiology-Lung Cellular and Molecular Physiology. 316*(3), L470-L486. doi: 10.1152/ajplung.00304.2018.

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.

Cullen K.A., Ambrose B.K., Gentzke A.S., Apelberg B.J., Jamal A., & King B.A. (2018) Notes from the Field: Use of Electronic Cigarettes and Any Tobacco Product Among Middle and High School Students — United States, 2011–2018. *MMWR. Morbidity and Mortality Weekly Report,67*(45), 1276–1277. doi: http://dx.doi.org/10.15585/mmwr.mm6745a5

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

Hickman E., Herrera C.A., Jaspers, I. (2019. Common E-Cigarette Flavoring Chemicals Impair Neutrophil Phagocytosis and Oxidative Burst. *Chem Res Toxicol*. 2019 Jun 17;32(6):982-985. doi:10.1021/acs.chemrestox.9b00171.

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.

Other papers that might have interesting data for your class to consider:

Erythropel, H. C., Jabba, S. V, Dewinter, T. M., Mendizabal, M., Anastas, P. T., Jordt, S. E., and Zimmerman, J. B. (2018) Formation of flavorant-propylene Glycol Adducts With Novel Toxicological Properties in Chemically Unstable E-Cigarette Liquids Co-first authors. *Nicotine Tob. Res.* 1–11.

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.

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.

Part I | 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.

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.
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.

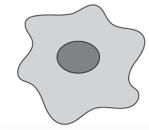
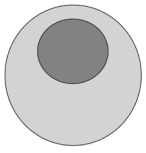
Data Analysis Questions:

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)	• Sinicide and Kola ↓ phagocytosis (Q2) • Sinicide ↓ IL-8 secretion (Q3)
Neutrophils 	↓ phagocytosis (Q1)	• Hot Cinnamon Candies, Banana Pudding, Menthol, Banana, and Sinicide ↓ phagocytosis (Q2) • Kola, Hot Cin Candies, Banana Pudding, Menthol, and Banana ↑ IL-8 secretion (Q3) • Kola and Sinicide ↑ NET formation (Q4 and Q5) • Hot Cin 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, have the ability to 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.

Part II | Activity Key

Guiding Question 7: Do cinnamon-flavored e-liquids and aerosols impair ciliary beating in respiratory epithelial cells?

Group 7 will evaluate the effects of cinnamon-flavored e-liquids and aerosols on ciliary function. Figure A shows the effects of 1% dilutions of flavored e-liquids (left side) on ciliary beat frequency and the percent of the imaged area that had cilia in motion (active area). Figure B shows the effects of vaped Sini-cide on ciliary beat frequency and the percent of the imaged area that had cilia in motion (active area).

Review Questions:

1. What is the mucociliary escalator?
   The cells that line your airways have cilia on the airway side of the cell. There is a layer of mucous on top of the cilia, and the cilia beat this mucous and anything trapped in it up and out of the airway. This is called the mucociliary escalator.
2. Why do you think cilia beat frequency and percent active area are endpoints the researchers were interested in studying?
   These endpoints are a good representation of how well the mucociliary escalator would be able to function since cilia beating is what moves the mucus up and out of the lungs.
3. Describe one consequence of impaired ciliary beat frequency in the lungs.
   Impaired ciliary beat frequency in the lung could cause mucus, pathogens, and or toxicants to get stuck in the lungs.

Data Analysis Questions:

1. Which e-liquid had the greatest effect on ciliary function? Why might this be?
   Sini-cide had the greatest effect on ciliary function. This could be because it has the highest concentration of the chemical(s) that are affecting the cilia.
2. Compare and contrast the effects of Sini-cide e-liquid and aerosol. Are they the same or different? Why might this be?
   Both e-liquid Sini-cide and vaped Sini-cide significantly reduced ciliary function. This could be because whatever chemical(s) in the e-liquid affect the cilia are also found in the aerosol.

Guiding Question 8: Does cinnamaldehyde impair ciliary beating in the respiratory epithelial cells?

Group 8 will evaluate the effects of cinnamaldehyde on ciliary function. These figures show the effects of different concentrations of cinnamaldehyde on ciliary beat frequency and the percent of the imaged area that had active cilia.

Review Questions:

1. What is the mucociliary escalator?
   The cells that line your airways have cilia on the airway side of the cell. There is a layer of mucous on top of the cilia, and the cilia beat this mucous and anything trapped in it up and out of the airway. This is called the mucociliary escalator.
2. Why do you think cilia beat frequency and percent active area are endpoints the researchers were interested in studying?
   These endpoints are a good representation of how well the mucociliary escalator would be able to function since cilia beating is what moves the mucous up and out of the lungs.
3. Describe one consequence of impaired ciliary beat frequency in the lungs.
   Impaired ciliary beat frequency in the lung could cause mucous, pathogens, and or toxicants to get stuck in the lungs.

Data Analysis Questions:

1. Which doses impaired ciliary function?
   10 mM and 15 mM cinnamaldehyde significantly impaired ciliary function.
2. What do you think would happen if cinnamaldehyde was inhaled as part of an e-cigarette aerosol? Why?
   If cinnamaldehyde was inhaled, the mucociliary escalator could be slowed or stopped because cinnamaldehyde can decrease ciliary beating.

Guiding Question 9: Does cinnamaldehyde change how energetic mitochondria are in respiratory epithelial cells?

Group 9 will evaluate the effects of cinnamaldehyde on the activity of the mitochondria. The set of images shows effects of cinnamaldehyde on the energetic state of the mitochondria (red = highly energetic, green = not energetic) with different doses of cinnamaldehyde. The graph quantifies the differences seen in the image. The y-axis is the red/green ratio.

Review Questions:

1. What is the primary way that epithelial cells lining our airway get energy to power the beating of cilia?
   ATP
2. What is mitochondrial membrane potential?
   Mitochondrial membrane potential is the proton gradient created by the electron transport chain during aerobic respiration. The movement of protons from high concentration in the intermembrane space to a low concentration in the mitochondrial matrix provides the energy needed for the cell to make ATP.

3. Describe one consequence of reduced mitochondrial function in airway cells.
   Reduced ATP levels, so reduced function for any process that requires ATP, such as ciliary beating.

Data Analysis Questions:

1. Describe what changes you see in the images as the dose of cinnamaldehyde increases.
   As the dose of cinnamaldehyde increases, the amount of red in the photos decreases and the amount of green increases which indicates that mitochondria are losing their membrane potential and therefore their ability to make ATP.
2. What does the decreased red/green ratio at higher doses of cinnamaldehyde mean for cellular function?
   It means that the mitochondria have been impaired and therefore ATP levels will be reduced in the cell. Lower levels of ATP will reduce the ability of the cell to perform any functions that require ATP, such as ciliary beating.

Guiding Question 10: Does cinnamaldehyde impair mitochondrial function?

Group 10 will evaluate the effects of cinnamaldehyde on mitochondrial function. These graphs show the effects of increasing doses of cinnamaldehyde on basal respiration (oxygen consumption) and ATP production.

Review Questions:

1. Why is mitochondrial function an important endpoint for this study?
   The mitochondria are the main cellular source of ATP, and ATP is what provides energy for ciliary beating.
2. Describe the relationship between basal respiration and ATP production.
   Basal respiration is the amount of oxygen that the mitochondria are consuming to move electrons through the electron transport chain and create the proton gradient necessary for ATP Production. However, this process isn't 100% efficient -- some of the energy (protons) is lost in the process. Measuring ATP production tells us how much of the oxygen and electrons were actually turned into ATP versus lost.
3. Describe one consequence of reduced mitochondrial function in airway cells.
   Reduced ATP levels, so reduced function for any process that requires ATP, such as ciliary beating.

Data Analysis Questions:

1. What was the effect of cinnamaldehyde on mitochondrial function?
   Cinnamaldehyde decreased mitochondrial function at 0.25, 0.5, and 5 mM.
2. What dose of cinnamaldehyde would you say is "non-toxic" and why?
   There was no effect of 0.05 mM cinnamaldehyde, suggesting that that dose is non-toxic.
3. Do these results agree with the results that group 9 discussed and why?
   Yes. Group 9's results showed that mitochondrial membrane potential is decreased, which correlates with decreased basal respiration and ATP production in this set of graphs.

Guiding Question 11: Does cinnamaldehyde impair glycolysis?

Group 11 will evaluate the effects of cinnamaldehyde on glycolysis. These graphs show the effects of increasing doses of cinnamaldehyde on glycolysis.

Review Questions:

1. Why is glycolysis an important endpoint for this study?
   Glycolysis is also a source of ATP in the cell and does not require oxygen. If mitochondria aren't functioning properly, the cell can acquire ATP from glycolysis.
2. Describe the relationship between basal glycolysis and glycolytic capacity.
   Basal glycolysis is glycolysis that is occurring normally in the cells. Glycolytic capacity is the maximum glycolysis that the cell can perform, typically to compensate for decreases in mitochondrial ATP production.
3. Describe one consequence of reduced glycolytic function of airway cells.
   Reduced ATP levels, so reduced function for any process that requires ATP, such as ciliary beating.

Data Analysis Questions:

1. What was the effect of cinnamaldehyde on glycolysis?
   Cinnamaldehyde decreased basal glycolysis at 0.25, 0.5, and 5 mM and decreased glycolytic capacity at 0.5 and 5 mM.
2. What dose of cinnamaldehyde would you say is "non-toxic" and why?
   There was no effect of 0.05 mM cinnamaldehyde, suggesting that that dose is non-toxic.
3. What is the significance of these results in the context of the results from groups 9 and 10?
   These results show that glycolysis is also affected by cinnamaldehyde, meaning that glycolysis will not be able to compensate for decreases in mitochondrial function. This partially explains why cilia function was decreased.

Guiding Question 12: Does cinnamaldehyde impair neutrophil oxidative burst?

Group 12 will evaluate the effects of cinnamaldehyde on neutrophil oxidative burst. One way that neutrophils kill bacteria is through turning oxygen into a more reactive form of oxygen that can act as an antimicrobial agent. This is called an oxidative burst. Neutrophils rely on glycolysis to generate the energy (ATP) needed for oxidative burst. These graphs show the effects of increasing doses of cinnamaldehyde on neutrophil oxidative burst and glycolysis (which is the source of ATP for oxidative burst).

Review Questions:

1. Why are neutrophils an important cell type to study in the lung?
   Neutrophils are part of the innate immune system that protects your body from inhaled pathogens.
2. What is oxidative burst? Where do neutrophils get the energy for oxidative burst?
   Oxidative burst is when neutrophils turn oxygen into more reactive/toxic types of oxygen that can then kill bacteria (acting as an antimicrobial agent). Neutrophils get the energy (ATP) to do this from glycolysis.

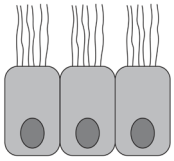
3. Describe one consequence of decreased oxidative burst.
   Neutrophils would not be able to fight invading pathogens as well, so the body would not be as well protected from disease.

Data Analysis Questions:

1. What was the effect of cinnamaldehyde on oxidative burst (antimicrobial activity)?
   Cinnamaldehyde decreased oxidative burst at 0.25-2 mM.
2. What was the effect of cinnamaldehyde on glycolysis?
   Cinnamaldehyde decreased glycolysis at 0.25-2 mM.

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 Function	Summary of Findings	
	Impact of Liquids	Impact of aerosol
Ciliary beating 	• Sinicide ↓ cilia beating (Q7) • Cinnamaldehyde ↓ cilia beating (Q8)	• Sinicide ↓ cilia beating (Q7)
Glycolysis	• Cinnamaldehyde ↓ basal glycolysis and glycolytic capacity (Q11)	
Mitochondria	• Cinnamaldehyde ↓ mitochondrial energy (membrane potential) (Q9) • Cinnamaldehyde ↓ basal respiration and ATP production (Q10)	
Oxidative Burst (Neutrophils) 	• Cinnamaldehyde ↓ neutrophil antimicrobial function and glycolysis (Q12)	

In one or two sentences, summarize the major conclusion(s) from these studies.
Cinnamaldehyde-containing e-liquids and cinnamaldehyde have the ability to impair cilia beating and bioenergetic functions such as glycolysis and respiration in airway cells. Cinnamaldehyde can also impair bioenergetics in neutrophils, decreasing their antimicrobial function. Together these data

suggest that vaping cinnamaldehyde-containing e-liquids could cause 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 options for this, including doing bioenergetics in different cell types, figuring out exactly which enzymes are affected, and performing similar experiments with different flavors/flavoring chemicals.

Examining the Health Effects of E-Cigarettes: Part I

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.

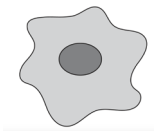
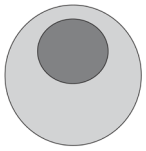
2.

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?

Examining the Health Effects of E-Cigarettes: Part II

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.

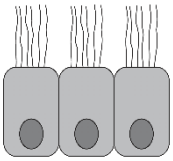
2.

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 Function	Summary of Findings	
	Impact of e-liquids	Impact of aerosols
Ciliary beating 		
Glycolysis		
Mitochondria		
Oxidative Burst (Neutrophils) 		

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?