Macrophage Morphological Changes Due to iNOS Activation by LPS and Its Implication in Chronic Asthma

Amanda Smith, Ed.M.
Union City High School – Academy for Enrichment & Advancement
2210 Kerrigan Avenue
Union City, NJ 07087
asmith@union-city.k12.nj.us

Funded by the American Association of Immunologists – AAI Summer Research Program for Teachers

Mentored by
Debra Laskin, Ph.D.
Ernest Mario School of Pharmacy Rutgers University, Piscataway
160 Frelinghuysen Rd., Rm. 001, Piscataway, NJ 08854

Alba Rossi-George, Ph.D.
Ernest Mario School of Pharmacy Rutgers University, Piscataway
160 Frelinghuysen Rd., Rm. 001, Piscataway, NJ 08854

Table of Contents

Macrophage Morphological Changes Due to iNOS Activation by LPS and Its Implication in Chronic Asthma

Amanda Smith[1], Alba Rossi-George[2], and Debra Laskin[2]

[1]Union City High School, 2500 Kennedy Boulevard, Union City, NJ 07087
[2]Ernest Mario School of Pharmacy, Rutgers University, 160 Frelinghuysen Road, Piscataway, NJ 08854

Prolonged macrophage activation can lead to acute lung injury, including chronic diseases such as asthma, the most common chronic childhood disease. Research has shown that Latinos and children living in low socio-economic urban environments have a higher prevalence of having this disease due to factors such as increased exposure to pollution and other asthma allergens. Lipopolysaccharide (LPS) triggers an inflammatory immune response causing increased production of nitric oxide by macrophages via the inducible nitric oxide synthase (iNOS). Morphological changes induced within macrophages via LPS induction can be an important indicator of their contribution to the disease. In class, I plan to introduce a hands-on lab experience, where students will culture Raw 264.7 cells, a macrophage cell line, and treat them with LPS to induce a morphological change. The macrophage's phenotype will be analyzed and discussed in relation to the overall inflammatory immune response. Students will also be introduced to the immune system and its major cell types. They will analyze data from the Centers for Disease Control and Prevention, as well as literature reviews explaining how asthma is a chronic disease of the immune system. A final discussion on the government's role in citizens' health and the impact of environmental pollution will result in a letter writing assignment to the mayor of the city about this issue.

Teacher Guide

I. Overview

a. *Rationale:* The state of New Jersey recently revised its science standards to align more closely to national standards, which most states are moving towards adopting as well. There is one Cumulative Progress Indicator (CPI) that mentions anything about the topic of immunity in the life science curriculum. It indicates that students should be able to: "Describe how a disease is the result of a malfunctioning system, organ, and cell, and relate this to possible treatment interventions (e.g., diabetes, cystic fibrosis, lactose intolerance)." Keeping this standard in mind, as well as other state requirements such as having all biology students take the New Jersey Biology Competency Test, I thought that it was best to blend the topic of immunity into my current curriculum. The exposure of students to the topic of immunology leads to a more fluid introduction to this lesson. This lesson uses a real-world application problem of chronic asthma in urban populations and culminates in a hands-on laboratory exercise where students get to culture immune cells and observe their morphological changes when activated. The final activity of writing to the mayor about this health issue helps to generate student involvement in the community through science activism.

b. *Science concepts:*
- Specialized cells of the immune system
- Signaling responses to allergens / activation of immune cells
- Structure fits function: how morphology changes upon activation
- Analyzing data, linking cause and effect
- Relating environment to health and disease

c. *Recommended Placement*: The general course outline for Biology uses the following sequence: Nature of Science, Introduction to Evolution & Classification, **Cell Organelles & Types (Cell Specialization/Stem Cells)**, Cell Membrane Transport, Biochemistry, Photosynthesis & **Cellular Respiration**, Cell Reproduction, **DNA & Protein Synthesis, Genetics & Biotechnology**, Evolution incorporating genetics, and **Ecology**. Bolded topics indicate units where immunology topics can be infused through activities and discussion. The culminating lab exercise can occur during the Biotechnology unit or kept until Ecology when discussing human impacts on the environment.

d. *General goals of laboratory exercises:*
- Practice aseptic technique.
- Count cells using a Hemocytometer.
- Culture cells and treat with lipopolysaccharide.
    - Observe cells for changes in morphology.

e. *Technical Skills:*
- Use a pipette to transfer small volumes.
- Load a Hemocytometer and use to count cells under a microscope.

- Stain cells using a differential stain kit.
- Observe cells under the microscope.
- Follow a multi-day laboratory exercise of culturing and treating cells.

f. *Relevance:* Asthma rates have been on the rise in children, especially urban populations where they are constantly exposed to pollutants and allergens from older city buildings. This exercise is an easy way to link immunology to students' everyday lives while making them aware of health issues that can affect them personally. It also provides a way for them to positively impact see their own health and that of their community.

II. Science Standards

a. New Jersey Core Curriculum Content Standards

Standard 5.1B – Science Practices: Generate Scientific Evidence Through Active Investigations

- CPI 5.1.12.B.1: Design investigations, collect evidence, analyze data, and evaluate evidence to determine measures of central tendencies, causal/correlational relationships, and anomalous data.

Standard 5.1D - Science Practices: Participate Productively in Science

- CPI 5.1.12.D.2: Represent ideas using literal representations, such as graphs, tables, journals, concept maps, and diagrams.
- CPI 5.1.12.D.3: Demonstrate how to use scientific tools and instruments and knowledge of how to handle animals with respect for their safety and welfare.

Standard 5.3A – Life Science: Organization and Development

- CPI 5.3.12.A.2: Demonstrate the properties and functions of enzymes by designing and carrying out an experiment.
- CPI 5.3.12.A.3: Predict a cell's response in a given set of environmental conditions.
- CPI 5.3.12.A.6: Describe how a disease is the result of a malfunctioning system, organ, and cell, and relate this to possible treatment interventions (e.g., diabetes, cystic fibrosis, lactose intolerance).

b. National Science Education Standards

Content Standard A: Science as Inquiry

- Abilities Necessary to do Scientific Inquiry
- Understandings About Scientific Inquiry

Content Standard C: Life Science

- The Cell
- Matter, Energy, and Organization in Living Systems
- The Behavior of Organisms

Content Standard F: Science in Personal and Social Perspectives

- Personal and Community Health
- Environmental Quality

Content Standard G: History and Nature of Science

- Science as a Human Endeavor

III. Science Background

a. *Required Content Knowledge:* General biology knowledge in the areas bolded above will be needed to effectively infuse immunology concepts into these areas. Additional general immunology information needed includes knowing some of the different types of cells of the immune system and how they interact with antigens to cause chronic asthma.

Raw264.7 cells: These cells are from a murine (mouse) macrophage cell line, which is commonly used in immunological research. The cells are widely used for mouse models of severe respiratory disease studies and measure pro-inflammatory mediators released during infection. Raw264.7 cells are a type of monocyte that can respond to inflammatory signals. They are extremely sensitive to lipopolysaccharide (LPS) stimulation. When activated, their morphology changes from the more generic round cell shape to a specific dendritic morphology. This morphology change allows them to become antigen presenting cells for naïve T cells during a primary immune response. They also produce nitric oxide (NO), which is directly toxic to bacteria. [5, 9]

Lipopolysaccharide (LPS): LPS is a molecule that is found in the outer membrane of Gram-negative bacteria. It is an endotoxin and elicits a strong immune response in animals. LPS binds to the CD14 receptor complex and causes the secretion of pro-inflammatory cytokines (proteins secreted by cells that affect the behavior of nearby cells) in macrophages such as RAW264.7 cells. The Toll-like receptor 4 (TLR-4) has been found to recognize LPS as well. LPS has also been shown to have a role in activating transcription factors. [4, 9]

Chronic asthma and inflammation: The environment and genetic variation both play a role in the development of allergic diseases, including asthma. Increased rates of asthma in developed countries seems to be due to several factors such as lower exposure to infectious diseases in early childhood, an increase in environmental pollution and allergen levels, and dietary changes. LPS is associated with several bacterial diseases that promote inflammation which can cause lung tissue injury. Inflammation is an immune response that allows more cells of the immune system to respond to an infection while limiting the infection's spread. However, prolonged inflammation can be detrimental. Inflammatory signals can activate two types of macrophages, M1 and M2. Chronic lung disease is associated with a decrease in classically activated M1 macrophages, which are involved with host defense, and an increase in alternatively activated M2 macrophages that are involved in wound repair. Macrophages activated by LPS are known to release NO. Normally, NO helps to regulate airway vascular tone and pulmonary surface tension. An increase in NO production can lead to tissue injury. The general pathway of how this happens includes:
1. Small allergens enter the lungs and are able to diffuse into the mucosa.

2. LPS is recognized by macrophages that become activated (RAW264.7) and produce pro-inflammatory molecules such as cytokines and nitric oxide. NO is generated via enzyme-catalyzed reactions during cellular respiration. Large quantities are produced to help destroy invading pathogens at this point.

a. Phagocytosis of pathogens results in the production of antigenic fragments, which can then be presented to naïve T cells. Naïve T cells subsequently differentiate into helper T cells.

b. NO is produced specifically by the protein inducible nitric oxide synthase (iNOS). iNOS is formed in the lungs after lung injury occurs and inflammatory cytokines are released. When it is upregulated, there is an increase in NO production.

c. S-nitrosylation then occurs, where thiol groups on proteins are modified, forming S-nitrosothiol (SNO) groups. S-nitrosylation of surfactant protein-D (SP-D) breaks down the protein into smaller molecules that are pro-inflammatory, and activate NF-κB, a transcription factor that activates genes involved in defense against infection.

3. After exposure to molecules such as LPS, macrophages become hyper responsive. Over-expression of NO disrupts innate lung defenses that modulate lung inflammation. This leads to chronic inflammation and chronic asthma. [2, 3, 4, 6, 7]

b. *Laboratory Exercise Content Knowledge:* The teacher needs to be familiar with how to culture cells and aseptic technique to prevent contamination of the cells. This is to ensure that the cells stay alive and produce expected results for the students.

c. *Possible unfamiliar lab procedures/apparatus:* Procedures will be detailed on student handouts, and a list of possible unfamiliar apparatuses are listed below.

Incubator is used to regulate temperature and levels of carbon dioxide while cells grow.

Micropipette is a mechanized version of pipette that can measure volumes in microliters.

Cell Lifter is a small scraper used to remove cells that attached to bottom of Petri dishes during culturing.

Chamber Slides are a type of slide that allows cells to be cultured directly on it with a removable box chamber for media to be added/held. Chamber gets removed, slide is fixed, and it is ready to be viewed under the microscope.

IV. Student Outcomes & Learning Objectives

a. *Students will be able to:*

- Identify specific cell types of the immune system based on morphology and descriptions of their functions.
- Describe how immune cells respond to lipopolysaccharides in the body.
- Discuss how both genes and the environment influence a person's susceptibility to allergens.

- Culture Raw264.7 cells to determine their original morphology, and how they change upon activation by LPS.
- Analyze statistical data about asthma rates and causes.
- Relate the morphological changes seen in the Raw264.7 cells to the causes of chronic asthma.

b. *Learning Objectives:*
- Understand how chronic inflammation and asthma is caused by overactive immune cells.
- Describe how environmental factors contribute to chronic inflammation.
- Relate cell structure to function.
- Relate research in the lab to public health issues.

V. Time Requirements

a. *Content Teaching:* Infusing topics alongside currently taught curriculum items should add minimal time during the year. Teaching about the specific immune cells, asthma, the immune response, and review of previously learned material will take approximately 4 – 40 minute periods.

b. *Laboratory Exercise:* There will only be approximately 5 – 7 days (depending on having double lab periods or not) for practicing lab techniques and carrying out the lab exercise. There are about 2 additional days where only part of the time is required for the laboratory exercise, and the rest of the time will be used to start the analysis and discussion portion of the curriculum. In total it will take 9 – 11 days depending on the length of the class periods.

VI. Advance Preparation

a. *Content Teaching:* Edits and/or additions to lectures and activities throughout curriculum will need to be made to incorporate immunology topics, including:
- Discuss specific cell types of the immune system.
- Understand organelles' (ribosomes, endoplasmic reticulum, lysosomes) roles and functions within the immune system.
- Understand that malfunctions in cellular respiration lead to excess reactive oxygen, NO production, inflammation.
- Complete a virtual stem cell lab to see how hematopoietic stem cells differentiate into different cells of the immune system , and discuss adult stem cells and their relation to the immune system.

b. *Equipment / Materials needed for lab exercise:*
- Equipment
  - Hemacytometer(s) $147.50/ pack 50
  - Tissue culture treated Petri dishes $38.40

- Cell lifters $21.74
- 10 µl pipette $43.75
- 37° Incubator
- Chamber slides $135.38

– Materials
  - Raw264.7 cells or a similar line kept frozen in a deep freezer until ready to use (or get right before culturing starts)
  - Trypan blue, 100ml $14.09
  - Dubelco's Modified Eagle Medium (DMEM) high glucose with phenol, 1,000 ml $32.64
  - L-Glutamine, 100ml* $22.17
  - Fetal bovine serum (FBS), 50 ml* $30.07
  - Penicillin-streptomycin (Pen-Strep), 100 ml* $17.94
  - Lipopolysaccharide (LPS), 1,000 ng/ml
  - Poly-L lysine hydrobromide, 10 mg* $28.25
  - Differential stain kit $26.50
  - Acetone
  - Clear nail polish

Total cost is $601.71. Materials can be ordered from Fisher Scientific and Life Technologies (Invitrogen). The cells and LPS were donated by the research lab. Those items marked with an asterisk (*) need to be kept frozen. All other materials require refrigeration.

c. *Approximate preparation time:* The complete growth medium is the only solution that needs to be prepared ahead of time, and can be kept in a refrigerator until ready to be used. Bring to room temperature in a hot water bath before use.

– 500 ml of complete growth medium
  - DMEM 440 ml
  - FBS 50 ml
  - Pen-Strep 5 ml
  - L-Glutamine 5 ml

VII. Materials and Equipment

a. Designed for a class of 24
12 computers with access to the internet for:
  1. Stem cell online lab
  2. Hemacytometer simulation website
  3. Pipetting skills simulation

Materials listed in order of use in section VI and detailed in student handouts.

b. *Possible substitutes:* It is possible to culture the cells in the petri dishes, and then prepare slides of the cells without using the chamber slides, one of the highest cost materials in this activity. Hemacytometers are also expensive, a

single one may be less than the disposable pack listed here or the school may already own some. Those two items if not purchased can take almost $300 off of the cost of this activity.

c. *Precautions and safety:* Always practice aseptic technique to keep from contaminating experiment. Materials should not be stored in a refrigerator with food items so they are not confused with food or contaminated. Petri dishes and chamber slides should be disposed of as biomedical waste as they contain cells.

VIII. Student Prior Knowledge and Skills

a. *Content Knowledge:* Students will be learning about the immune system throughout the year within their normal biology curriculum. However, they should understand that cells send, receive, and respond to signals, which in turn can cause them to produce specific proteins. The processes are involved in each. They should also know how structure relates to function, and that a change in structure will cause a change in function (or for this activity, an activation).

b. *Technical Skills:* Students should understand basic lab safety procedures, aseptic technique, measuring skills, and how color changes can indicate test results.

IX. What is Expected from Students

a. Pipetting practice worksheet

b. Complete Hemacytometer simulation

c. Concept map showing the steps for a macrophage to become activated by LPS

d. Lab report

e. Letter to Mayor about asthma in the city

X. Anticipated Results

a. *Data Results:* Accurate counting of cells with Hemacytometer and proper dilution of cells into chamber slides. Visual confirmation of macrophage activation by LPS.

b. *Possible sources of error:* Inaccurate measuring of samples or chemicals, inaccurate counting of cells, improper calculation for diluting cells, improper incubating of cells, cells not fixed to chamber slides properly.

XI. Classroom Discussion

a. Question students' understanding about structure and function; use other examples in biology and life. Examples: protein structure and denaturing, structure and function of a kitchen chair vs. folding chair.

b. Discussions on aseptic technique include relating to what hospitals do to prevent infection and experimental design and ensuring the scientist knows what is causing the change.

c. Discussion on the need for counting cells and knowing how many to put in one chamber slide relates to cell population and amount of available nutrients and room to grow.

d. Discussion of process occurring in petri dishes includes macrophage's activation.

XII. Assessment

a. Accurate counting of cells

b. Confirmed activation of macrophages by pictures and diagrams

c. Test on immunology concepts related to macrophage activation and lab techniques

Lesson Plans

I. Lesson 1: Introduction to the Immune System

Introduction – There are two major parts of the immune system, the innate immune system and the adaptive immune system. They work together to keep an organism alive and healthy. Within each part of the system, there are a variety of immune cells that each has their own role. Many work in concert and respond to infections and foreign materials by releasing molecules and activating other cells in a cascade effect. Some of these cells, their morphology, and function in the body have been discussed in previous units. These two days provide for a review and more in-depth look into several cell types: neutrophils, monocytes, including macrophages and dendritic cells, naïve T cells, T helper cells, B cells, and natural killer (NK) cells. How they arise from hematopoietic stem cells, the flow of activation of each different cell, molecules and organelles involved is discussed. Understanding pathways helps to show the complementary and redundant functions of the cells, as well as how distinguishing the cause to then find a treatment for diseases like chronic asthma is not an easy task.

Class Time - Two 40-minute periods, lecture

Materials – Lecture slides with student response questions, graphic organizer worksheet (if desired for guidance in organization or have students create own)

Procedure

1. Do Now – What types of immune cells do you know about? How many can you name and what are their functions?

2. Lecture on types of cells. Intersperse student response questions throughout slides to ensure understanding and to clear up any misconceptions about cell types.
3. Have students create a graphic organizer detailing the types of immune cells gone over in lecture.

Data – graphic organizer of cell types

Analysis / Discussion

1. Discuss Do Now question to introduce topic.
2. What are the two major lineages that arise from hematopoietic stem cells?
   A: Myeloid lineage and lymphoid lineage
3. What types of cells can naïve T cells differentiate into?
   A: CD8 (cytotoxic T cells), and CD4 (Helper T 1 cells and Helper T 2 cells)
4. How are dendritic cells related to naïve T cells?
   A: Dendritic cells present the antigens that help differentiate T cells.
5. How are T cells related to B cells?
   A: T Helper 1 and T Helper 2 cells activate B cells
6. What is the difference between an antigen and an antibody?
   A: An antigen is a piece of a pathogen or chemical that stimulates antibody generation, an antibody is a protein that binds specifically to toxins and neutralizes their activity.

II. Lesson 2: Nitric Oxide, Inflammation and the Immune Response

Introduction – To understand how a process works, it is good to study what happens when things go wrong. As the immune system combats an infection, inflammatory molecules are produced. Inflammation occurs to allow for more innate immune response cells to enter the tissue and destroy pathogens, as well as increasing the flow of lymph to nearby tissues to help initiate the adaptive immune response. Nitric oxide is a very reactive molecule. It is produced by macrophages to help with the inflammatory response and can react to form hydrogen peroxide which can be used to destroy pathogens as well. Lipopolysaccharide is a molecule found on the surface of Gram-negative bacteria and triggers an immune response that produces nitric oxide. Sometimes, the macrophages that produce it become hyper-responsive and correlations have been seen between them and chronic diseases such as asthma. [4, 6, 7]

Class Time – Two 40-minute periods, lecture and related assignment

Materials – Lecture slides, flow chart worksheet, literature article

Procedure

1. Do Now: What do you think are some possible effects of the immune system responding too much? Would they be positive, negative, or neutral?
2. Lecture on inflammation as part of the immune response and introduction to nitric oxide production.
3. Read article “The Inflammatory Response in the Pathogenesis of Asthma”.

Provide definitions for new words. Students write a summary of how asthma occurs.
4. Create flow chart of the steps that lead to chronic inflammation based on lecture notes and article.

Data – flow chart of inflammatory response

Analysis / Discussion
1. Discuss Do Now question to assess students' current understanding of how the immune system works and possibly uncover any misconceptions.
2. Question students during lecture about the inflammatory response.
3. Analysis occurs in written summary of article and in creation of concept map.

III. Lesson 3: Introduction to Laboratory Procedures and Aseptic Technique

Introduction – Cell culture models are used to study the immune response, the types of cells that are involved, and their respective signaling pathways. To ensure that an experiment is carried out properly, aseptic technique needs to be strictly followed. Using aseptic technique keeps cultures from being contaminated by foreign microorganisms in the environment of the lab room. This can include airborne microbes or those found on surfaces and unsterilized equipment. Such contamination can ruin or skew the results of an experiment and make results invalid. Along the same lines, making accurate measurements also ensures accurate results of an experiment. Using equipment both correctly and safely are two of the biggest keys to ensuring a well carried out experiment. Micropipettes allow for accurate measurements of volumes in the microliter range when used properly, and a Hemacytometer allows for accurate counting of cells. As cells themselves are microscopic, it only takes a few microliters of a chemical to induce a change in them. Too much can end up killing the culture, resulting in loss of results, time, and money. Knowing how many cells you have in a solution, allows for accurate growth time when culturing cells, providing enough medium and nutrients for them to grow, and knowing how many to seed with when sub-culturing.

Class Time – One 40-minute period, one 80-minute lab period

Materials – Lecture slides on aseptic technique and measuring with a micropipette
Per Group:
Pipette & Hemacytometer practice worksheet to accompany virtual labs
Computer with access to the internet and Flash installed
Squeeze bottle of disinfectant
Papertowels
10 µl micropipettor with disposable tips
Bottles with colored water (to practice measuring)
3 test tubes
Container to dispose of used pipette tips in
Lab worksheets for Raw264.7 cells

Procedure

1. Do Now – What does it mean when somebody goes into septic shock? Then what does aseptic mean? How can you practice being aseptic in a lab setting?
2. Go over aseptic technique lecture slides and measuring with micropipette lecture slides.
3. Watch how to make a serial dilution: http://ats.udel.edu/showcase/micropipette.php Complete questions on worksheet while viewing. Then, complete lab simulation at: http://www.udel.edu/present/Becky/lehman/ Read the Help section to learn how to control a micropipette. Instructor visually confirms accurate completion of the lab and by seeing score on simulation.
4. Complete virtual Hemacytometer lab simulation at: http://amrita.vlab.co.in/?sub=3&brch=188&sim=336&cnt=1. Answer questions on worksheet.
5. Practice aseptic technique in actual lab. Include opening containers without putting lid on lab bench, changing pipette tips, removing a sample with a pipette without touching the sides of the bottle, sterilizing lab bench and equipment before and after use.
6. Pass out lab sheets for Culturing Raw264.7 cells. Go over what will be happening in the activity. Assign pre-lab questions for homework.

Data – Pipette virtual laboratory simulation results, results of aseptic technique quiz

Analysis / Discussion

1. Discuss aseptic technique and its usefulness in a lab setting as part of Do Now and aseptic lecture.
2. Discuss virtual pipette lab and Hemacytometer lab.
3. Analyze and discuss individual student aseptic technique during practice.

IV. Lesson 4: Culturing and Treating Raw 264.7 Cells

Introduction – Cell culture techniques are widely used in basic science research. Thus, knowledge of how to culture and treat cells is paramount. Raw264.7 cells are the most commonly used murine macrophage cell line and are commonly used when studying respiratory diseases. They are highly sensitive to lipopolysaccharides (LPS), and show a morphological change when they are exposed to the endotoxin. Prior to activation circulating monocytes appear to be spherical under the microscope. They differentiate into mature macrophages on entering specific tissues. Raw264.7 cells also demonstrate this spherical morphology in culture. Exposure to antigens such as LPS induces a dendritic morphology where the cells grow branched projections that look similar to the dendrites of neuron cells. At this stage, they are able to become antigen-presenting cells and interact with T cells by presenting them with peptide antigens on the surface of MHC molecules.

Class Time – 4 days, requiring time in the lab room all four days. Possible sequence:
Day 1: One 80-minute lab period
Day 2: One 40-minute period in lab room

Day 3: One 80-minute lab period
Day 4: One 80-minute period in lab room (possible to split into two days)

Materials – see lab sheets

Procedure – Complete lab activity following procedure on lab sheets. If there is downtime, start Lesson 5 concurrently.

Data – See lab sheets. It is expected to see the Raw264.7 cells to have the dendritic morphology as discussed in lecture.

Analysis / Discussion

1. Discuss prelab questions and procedure for culturing Raw264.7 cells.
2. Compare results between lab groups. Determine any unexpected results that are not due to contamination/improper aseptic technique.
3. Discuss lab results and procedures that were followed.
4. Answer additional questions on lab sheets.

V. Lesson 5: Asthma Rates and You

Introduction – Research has no relevance without a real-world application. Understanding what triggers the immune response in the lungs and what causes lung tissue injury leading to asthma should also point to a way to prevent it. The environment plays a role in developing allergic diseases. Additional research has shown that specific populations (Latinos, African Americans, and those living in urban areas) have a higher rate of asthma even though the air is cleaner now than in the past. This can be attributed to several factors including lifestyle choices such as smoking, rates of obesity, access and quality of treatment, and environmental factors such as pollution and allergens. Older buildings that are often found in urban areas are incubators for mold, cockroach droppings, and dust mites, all key triggers of asthma. Early exposure may also cause the hyper-response seen in chronic asthmatics. This chronic condition can become a public health issue for cities. The local government can therefore try to take some steps to curb rates of asthma within their own cities.

Class Time - two 40-minute days

Materials – articles explaining chronic asthma disparities
CDC statistics about asthma
Letter to Mayor activity sheet

Procedure

1. Do Now: How many people do you know that have asthma? What can trigger an asthmatic episode?
2. Discuss asthma rates in America as posted by the CDC.
3. Read over articles describing the disparities between certain populations and why Latinos have higher rates of asthma.
4. Use information from articles and knowledge of the immune response to

asthma triggers to write a letter to the mayor about what should be done for his citizens.

Data - N/A

Analysis / Discussion

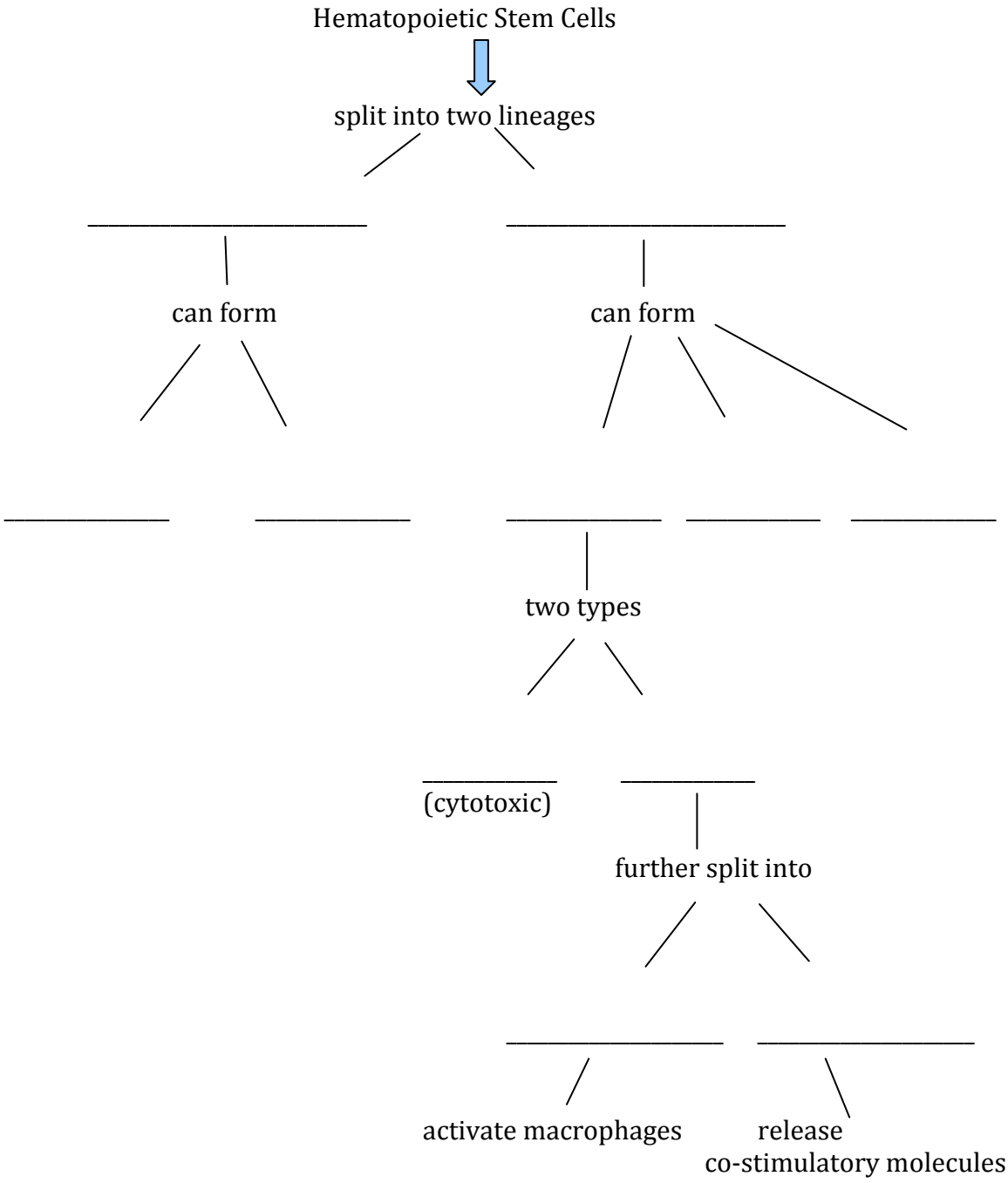
1. Analyze data tables from CDC, found here: http://www.cdc.gov/nchs/data/series/sr_10/sr10_254.pdf Asthma Prevalence by Age: http://www.cdc.gov/asthma/nhis/2011/table2-1.htm Percentages of those with asthma that do and do not have insurance coverage: http://www.cdc.gov/asthma/asthma_stats/Insurance_Asthmasta.pdf
2. Discuss any noticeable correlations from data tables.
3. Compare information from CDC data tables to what was read in articles about populations that have high rates of asthma. Discuss personal opinions on why these rates are seen.
4. Discuss what can be done to help reduce these rates.

Student Worksheets

Name: _____ Date: _____

Cell Types of the Immune System

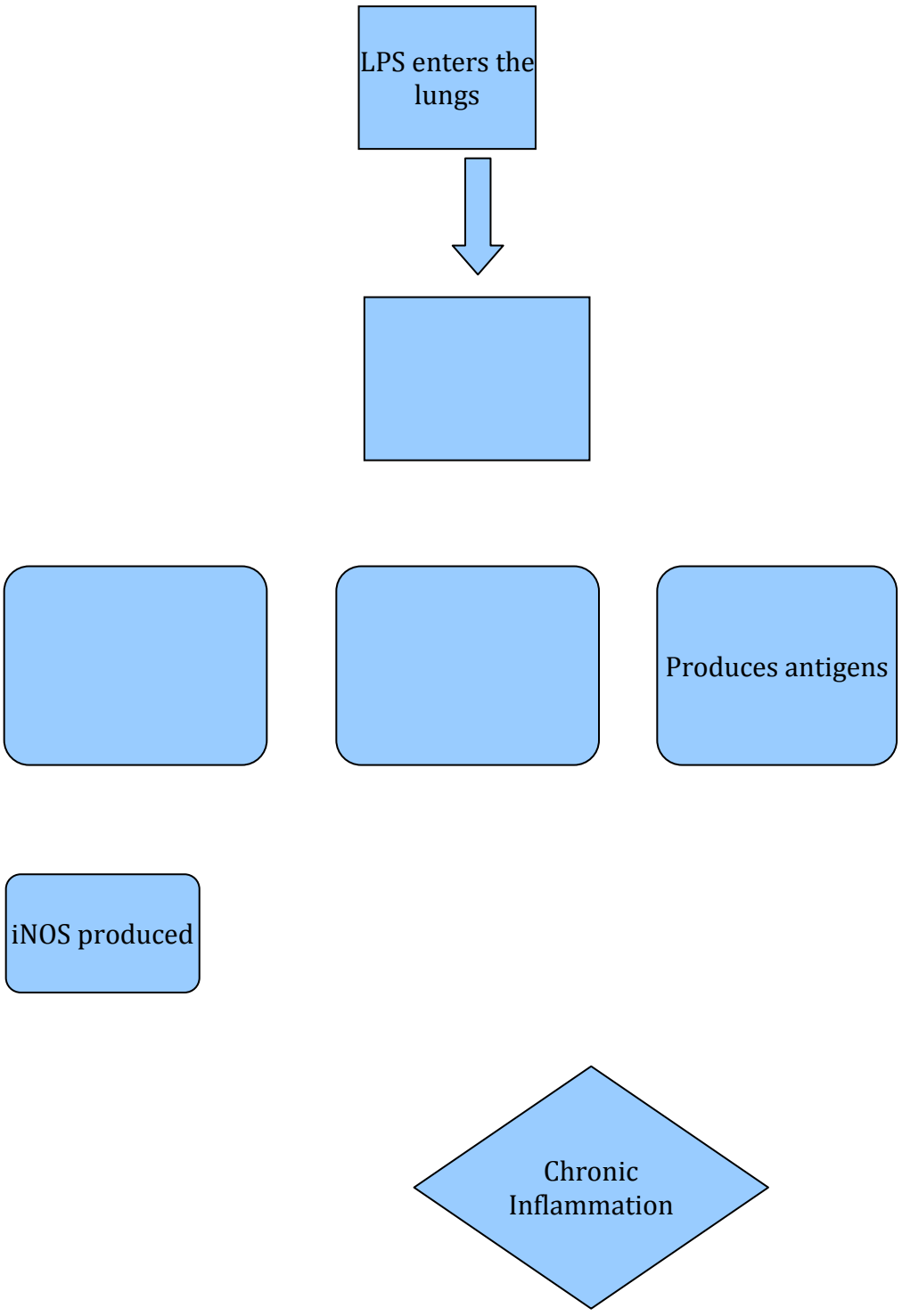
Directions: Fill in the blanks with the correct cell types. Then, draw arrows showing how some of the cells work with each other.



Name: _____ Date: _____

Inflammatory Response Flow Chart

Directions: Complete the following flow chart that shows the signaling pathway and cells involved in the immune response after the introduction of the allergen lipopolysaccharide. Include arrows to show the flow of the process, and add any additional boxes that may be needed.



Name: _____ Date: _____

The Inflammatory Response in the Pathogenesis of Asthma Article Review

Directions: Read the following article. It can be found for free at: http://www.jaoa.org/content/111/11_suppl_7/S11.full.pdf. Then answer the following questions and analysis on a separate sheet of paper.

Words to Know

- pathogenesis – the development of a disease
- bronchoconstriction – narrowing of the airways in the lungs due to the tightening of surrounding smooth muscle, causing breathing difficulty
- syndrome – a group of symptoms that occur together and characterize a particular disorder, disease, or the like
- endotype – a subtype of a condition or disease
- epithelium – tissue that lines the cavities and surfaces of structures throughout the body
- cytokines – small signaling molecules that are secreted by specific cells of the immune system, carrying signals locally and having an effect on other cells

Article Questions

1. What triggers induce bronchoconstriction?
2. How many asthma endotypes are there, and what did they use to classify them?
3. Ultimately, what are the effectors of chronic inflammation?
4. Which cytokine induces key changes in dendritic cells?
5. What important role do chemokines play?
6. What structural changes occur due to chronic inflammation?

Analysis Response

Write a summary of the allergic response and T-Helper 2 Lymphocytes. Include what cells and chemicals are involved.

Name: _____ Date: _____

Laboratory Skills Practice
Using the Micropipette and Hemacytometer

Directions for the Micropipette

1. Go to this website and view the following video:
   http://ats.udel.edu/showcase/micropipette.php

2. Answer the following questions:
   1. Where is the decimal located?

   2. How much water is added to each tube?

   3. How much dye is transferred to the first tube?

   4. How much of the solution from the first tube is then transferred to the next tube?

   5. Why do you discard 1 ml of liquid from the last tube?

   6. How should the serial dilution look when you are done?

3. Using the procedure seen in the video, complete your own serial dilution at this website: http://www.udel.edu/present/Becky/lehman/ Click on the help button to learn how to use the simulation.

4. Answer the following questions:
   1. How do you change the volume on the pipette?

   2. What button allows you to depress the plunger?

   3. How many times will you need to change the pipette tip?

5. You must show the instructor your simulation screen when you are done with the serial dilution. A score of 90 or better is required to pass the simulation.

6. Teacher's approval signature: _____

Directions for the Hemacytometer

1. Go to the following website: http://amrita.vlab.co.in/?sub=3&brch=188&sim=336&cnt=1 and click through the tabs to find answers to the following questions.
    1. What is the purpose of the Hemacytometer?
    2. What measurement is used for the Hemacytometer?
    3. What volume does the Hemacytometer hold?
    4. Where is the cell suspension loaded?
    5. What property allows the suspension to be drawn into the space?
    6. How are viable and non-viable cells differentiated?
    7. What other piece of lab equipment is required to count the cells? What magnification should you use?
    8. What are possible sources of error when using a Hemacytometer?
2. Complete the self-evaluation.
3. Click on the Simulator tab. Increase the magnification to 100X. Count how many viable cells are present.
4. How to count cells when looking at the Hemacytometer: Include cells touching the middle line of the outside grid border on top and left, but exclude cells touching on bottom and right (see sample grid in Procedure tab).
5. Calculation: Count the 4 corner squares. Input the number of viable and dead cells into the table in the simulator and on the sheet here.

Data Table 1

	Viable Cell Count	Dead Cell Count
Square 1		
Square 2		
Square 3		
Square 4		
Total		

Data Analysis

1. What is the percentage cell viability?

2. What is the amount of viable cells per milliliter?

3. If you want to seed a dish with 2,000 cells, what volume of media should be transferred?

4. If you want to seed a dish with 10,000 cells, what volume of media should be transferred?

Name: _____ Date: _____

Laboratory Skills Practice
Aseptic Technique

Directions: Answer the following questions, and then, practice aseptic technique according to the procedure.

1. What does aseptic mean?

2. What is the purpose behind aseptic technique?

3. When removing the lid of a bottle, where should you put it?

4. How do you prevent cross-contamination when pipetting liquids?

5. When should you wash your hands?

6. What should you do to your lab bench?

Procedure

1. Prepare yourself and your lab bench for work.
2. Obtain a micropipette, a bottle of red colored water, a bottle of blue colored water, and a test tube rack with three test tubes.
3. Keep discarded pipette tips on a separate paper towel on the lab bench.
4. Pipette 2 ml of red colored water into the first and second test tubes, following aseptic technique.
5. Pipette 2 ml of blue colored water into the second and third test tubes following aseptic technique.
6. After answering the analysis questions, clean up the lab bench. Pour the colored water down the drain and return the bench to how it looked when you started.

Analysis

1. What did you do to prepare for the activity?

2. What color is the water in the three test tubes?

3. How many pipette tips did you use?

4. Is there any color left on the pipette tips? What is it?

5. What is the last thing that you did at the end of this lab exercise?

6. Based on your answers here, did you follow aseptic technique? How can you tell?

Name: _____ Date: _____

Lab: Culturing and Treating Raw264.7 Cells

Introduction: Raw264.7 cells are a murine macrophage cell line that is commonly used in studies of lung disease. They originally came from mice, and are sensitive to lipopolysaccharide (LPS) stimulation. Culturing refers to growing and preparing cells in a specific way. This lab activity is designed to teach the basics of cell culturing and performing the steps aseptically. For the cells to live and grow, they require nutrients and the right environment. The cells will be allowed to grow after being unfrozen, and then, they will be treated with LPS. The macrophages will undergo a morphological change as they recognize the LPS and prepare to become antigen-presenting cells for naïve T cells. As the function of the cells is directed by the LPS, their structure must change.

Purpose: _____
_____

Prediction: Based on prior knowledge and the introduction, predict what will happen to the cells after treatment with LPS. _____
_____

Prelab Questions: Answer on a separate sheet of paper in complete sentences.

1. What type of cells are Raw264.7 cells?
2. How long are the cells cultured?
3. How early should the chamber slides be prepared?
4. How many cells are to be seeded in each chamber slide?
5. What is the purpose of the Dif-Quick Stain Kit?

Materials:

- Raw264.7 cells
- Tissue culture treated Petri dish
- Complete medium
- Micropipette with disposable tips
- Trypan blue solution
- Hemacytometer
- Lipopolysaccharide
- Cell lifters
- Chamber slides
- Poly-l lysine hydrobromide
- Acetone
- Dif-Quick stain kit
- Clear nail polish
- Microscope
- Incubator

Procedures:

Day 1 - 2

1. Cells have already been defrosted and placed into tissue culture treated culture dishes with 500mL complete growth medium.
2. Place dishes in incubator and incubate at 37°C until cells reach ~60% confluence (1-2 days).

3. Take dishes out of incubator. Detach cells from the bottom of the culture dishes by using cell lifters. Gently scrape the bottom of the dishes using the float end of the lifter like a spatula.
4. Prepare a 1:1 suspension of the cell solution in trypan blue. (10µl:10µl) **MAKE SURE TO USE GOGGLES AND GLOVES. TRYPAN BLUE IS A MUTAGEN.**
5. Using a pipette, load the hemocytometer. Add enough of the suspension to fill the chamber without overflowing it. Place the Hemocytometer under the microscope and get in focus under high power (100X). Count the viable and nonviable cells to determine the solution's concentration.
6. *Prepare chamber slides 1-2 days before being needed, using poly-l lysine hydrobromide to coat chambers.*
7. Dilute cells to 200,000 cells per milliliter. Determine the volume required for this concentration using the calculations listed under Data Table 1. Add 1 milliliter of the cell suspension and 1 ml complete growth medium to each chamber slide.
8. Incubate the chamber slides at 37°C for 24 hours.

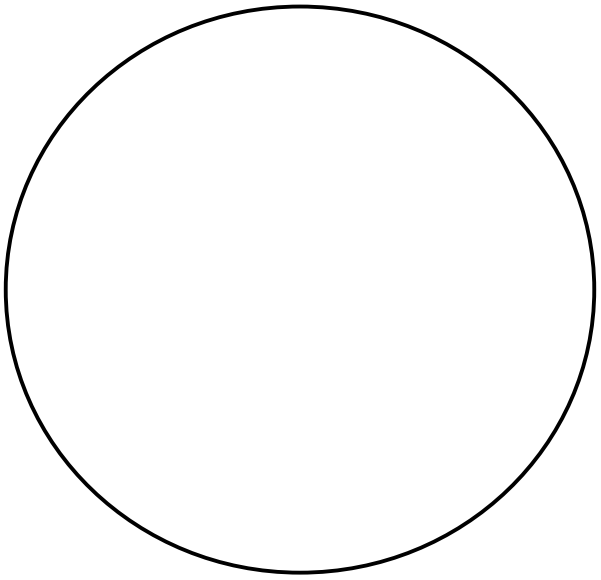
Day 3

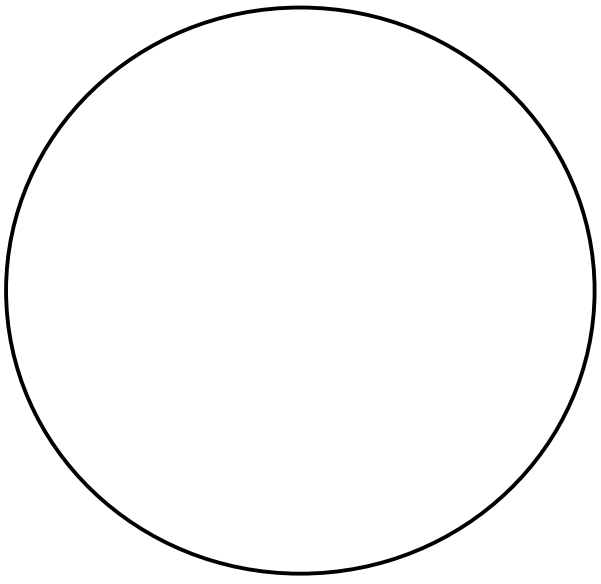
9. Remove medium using pipette, and add 1 mL fresh medium.
10. Add 1,000 ng/mL LPS to chamber slides.
11. Incubate the chamber slides at 37°C for 24 hours.

Day 4

12. Aspirate medium off of cells using a pipette. Try to remove as much of the medium without actually touching the bottom of the slide where the cells are attached.
13. Fix slides by immersing in acetone for 2 – 5 minutes.
14. Stain using Dif-Quick Stain Kit directions.
15. Remove chamber walls by pulling straight up. Affix coverslip to slide using clear nail polish.
16. View under microscope. Record observations. Cells should appear purplish in color from the stain.

Data: Record your observations before LPS treatment, and after.





_____ _____

Hemacytometer Data:

Data Table 1

	Viable Cell Count	Dead Cell Count
Square 1		
Square 2		
Square 3		
Square 4		
Total		

The total number of cells per ml will be determined using the following calculations:

- %Cell Viability = [Total Viable cells (Unstained) / Total cells (Viable +Dead)] X 100.
- Viable Cells/ml = Average viable cell count per square x Dilution Factor x 10^4/
- Average viable cell count per square = Total number of viable cells in 4 squares / 4.
- Dilution Factor = Total Volume (Volume of sample + Volume of diluting liquid) / Volume of sample.
- Total viable cells/Sample = Viable Cells/ml x The original volume of fluid from which the cell sample was removed.
- Volume of media needed = (Number of cells needed/Total number of viable cells) x 1000.

Analysis: Answer the following questions in complete sentences on a separate sheet of paper.

1. Show calculations for determining the volume needed for the dilution of 200,000 cells per milliliter.
2. How accurate do you feel your counting was using the Hemocytometer? Was it as easy as the simulation? Explain.
3. Compare the cells before and after LPS treatment. How do they look? What has happened to them?
4. Could anything besides the LPS have caused this change? Explain.
5. Do you see anything else under the microscope besides the Raw264.7 cells? What could it be? Should it be there?

Conclusion: Answer the following questions in complete sentences on a separate sheet of paper.

1. Were your predictions correct? Why or why not?
2. Why do the cells change their morphology in the presence of LPS?
3. What does this lab activity demonstrate about structure and function?

Name: _____ Date: _____

Asthma Rates and You

Directions: You have read about what triggers an allergic, asthmatic response, as well as the disparities between populations and asthma rates. You also have your own personal experiences involving asthma and the effect it has on people who manage asthma every day. Use this information to develop a public health plan and help the residents of Union City. This plan will be sent to the mayor for his consideration. This plan needs to be plausible, able to achieve the set goal, and cost-effective for the mayor to implement.

Requirements:

- Letter should be typed, double spaced with 1 inch margins.
- Addressed to the Mayor.
- Provides outline of a public health plan to reduce asthma rates in Union City.
- Includes data and scientific background to support the plan.
- Minimum length is two pages.

Use the space below for planning.

References

1. Amrita Labs – Hemacytometer simulation. Retrieved from: http://amrita.vlab.co.in/?sub=3&brch=188&sim=336&cnt=1

2. Bryant-Stephens, T. (2009). Asthma disparities in urban environments. *Journal of Allergy and Clinical Immunology, 123*, 1199-1206. doi:10.1016/j.jaci.2009.04.030

3. Canino, A., Koinis-Mitchell, D., Ortega, A.N., McQuaid, E.L., Fritz, G.K., Alegria, M. (2006). Asthma disparities in the prevalence, morbidity, and treatment of Latino children. *Social Science & Medicine, 63,* 2926-2937. doi: 10.1016/j.socscimed.2006.07.017

4. Guo, C-J, Atochina-Vasserman, E.N., Abramova, E., Foley, J.P., Zaman, A., et al. (2008). S-nitrosylation of surfactant protein-D controls inflammatory function. *PloS Biology 6*(11), 2414-2423. doi: 10.1371/journal.pbio.0060266

5. Hartley, J.W., Evans, L.H., Green, K.Y., Naghashfar, Z., Macias, A.R., Zerfas, P.M., Ward, J.M. (2008). Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology, 5*(1). doi: 10.1186/1742-4690-5- http://www.retrovirology.com/content/5/1/1

6. Ishmael, F.T., (2011). The Inflammatory Response in the Pathogenesis of Asthma. *The Journal of the American Osteopathic Association, 111*(11). Supplement 7, S11-S17. Free via open access/creative commons: http://www.jaoa.org/content/111/11_suppl_7/S11.full.pdf

7. Laskin, D.L., Sunil, V.R., Gardner, C.R., Laskin, J.D. (2010). Macrophages and Tissue Injury: Agents of Defense or Destruction? *The Annual Review of Pharmacological and Toxicology, 51*, 267-288. doi: 10.1146/annurev.pharmtox.010909.105812

8. Murphy, K., Travers, P., & Walport, M. (2008). *Janeway's Immunobiology, 7th Ed*. New York, NY: Garland Science.

9. Saxena, R.K., Vallyathan, V., Lewis, D.M. (2003). Evidence for lipopolysaccharde-induced differentiation of RAW264.7 murine macrophage cell line into dendritic like cells. *Journal of Biosciences, 28*(1), 129-134. http://www.ias.ac.in/jbiosci/feb2003/129.pdf