Immunology Overview:
From textbook to application

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I. Science Background

Overview

The purpose of this unit is to give students a general overview of the functioning of both the innate and adaptive immune systems, and to give students an opportunity to see how our knowledge of the immune system has been used to benefit human health. For this unit, the teacher should prepare by familiarizing themselves with the roles, functions, and dysfunctions of innate and adaptive immunity. A sufficient level of detail can be found in textbooks typically used in AP Biology classes, such as *Campbell Biology* by Urry et al., *Biology* by Raven et al., or *Principles of Life*, by Hillis et al. Alternatively, the Open Stax AP Biology textbook contains a good chapter covering essential information for this unit. The chapter can be accessed at: https://cnx.org/contents/bDIuMp-w@11.7:kx-jRce5@10/33-1-Innate-Immune-Response.

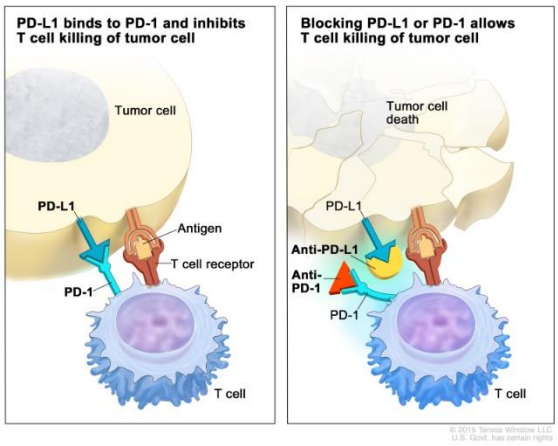
Immune Checkpoints and Checkpoint Therapy

The ELISA experiment at the end of the unit is centered around the recent development of so-called checkpoint inhibitors as effective treatments for certain forms of cancer. First discovered in the 1990's, checkpoint proteins are broadly grouped into two categories-those that act to stimulate the proliferation and/or activity of T cells, and those that act to inhibit the function of T cells. Proper balance of T cell activity is essential to immune homeostasis. For example, mice deficient in one inhibitory checkpoint protein, CTLA-4, die between 2-3 weeks of age due to a massive overproliferation of leukocytes and subsequent destruction of vital organs by hyperactive T cells. Similarly, mice deficient in another inhibitory checkpoint protein, PD1, develop symptoms consistent with an autoimmune disorder. Both CTLA-4 and PD1 are receptor proteins expressed on the surface of multiple immune cells, including T cells. When either protein binds one of its ligands (which include a protein called B7 for CTLA-4, and either PD-L1 or PD-L2 for PD1), the T cell is essentially told to "stand down". This is an important mechanism for limiting the intensity and/or duration of an immune response.

As cancer cells proliferate, they eventually begin to produce abnormal proteins known as tumor antigens, which can be recognized as abnormal by antigen-presenting cells such as dendritic cells. These cells can in turn stimulate the proliferation and activation of T cells, some of which can then infiltrate the tumor and destroy it.

However, some tumors, in the course of their progression, acquire mutations that cause them to express high levels of ligand for either CTLA-4 or PD1. When T cells enter into such a tumor, they receive the inhibitory signal from the tumor and become ineffective. Freed from attack by the immune system, the tumor can continue to grow.

Understanding this mechanism for how cancerous cells can evade destruction, researchers have developed treatments based on antibodies that bind specifically to the receptor (either CTLA-4 or PD1, most commonly). Binding of the antibody to the receptor prevents binding of the tumor-produced ligand, thereby preventing the inhibitory signal from being received by the T cell. This form of treatment has been termed **immune checkpoint blockade**.



Scientists James Allison and Tosuku Honju were awarded the 2018 Nobel Prize in Medicine or Physiology for their groundbreaking work in developing this approach.

References:

Khattri, R. et al. (1999). Lymphoproliferative Disorder in CTLA-4 Knockout Mice Is Characterized by CD28-Regulated Activation of Th2 Responses. J Immunol 162 (10) 5784-5791

Blank C. et al. (2003). Absence of programmed death receptor 1 alters thymic development and enhances generation of CD4/CD8 double-negative TCR-transgenic T cells. J Immunol. 171(9):4574-81.

II. Student Outcomes

a. Science Concepts

- Basics of the innate immune system
  - Functions of barriers, proteins, and cells in preventing establishment of infection by pathogens.
  - Signaling pathways that regulate inflammation and how NSAIDs regulate one of these pathways. Also, they will learn how organisms use negative feedback to control inflammation and how dysregulation can lead to chronic inflammation.
- Function of the adaptive immune system
  - Comparison of the function of B and T cells in primary vs. secondary immune responses; humoral vs. cellular immunity. Students will be able to identify and describe the functions of the major effector B and T cells, and explain how the development of memory cells is the basic for vaccines.
  - Students will learn how pathogens have evolved to evade/manipulate host defenses

- Students will learn how tumor cells avoid destruction by immune cells, and how current technology allows us to use a personalized medicine approach to 'reawaken' immune cells and target them to tumor cells.

b. Educational Standards:

Next Generation Science Standards (NGSS)

- Develop and use a model based on evidence to illustrate the relationships between systems or between components of a system (HS-LS-1-2)
- Plan and conduct an investigation individually and collaboratively to produce data to serve as the basis for evidence, and in the design: decide on types, how much, and accuracy of data needed to produce reliable measurements and consider limitations on the precision of the data (e.g., number of trials, cost, risk, time), and refine the design accordingly. (HS-LS1-3)
- Systems of specialized cells within organisms help them perform the essential functions of life. (HSLS1-1)
- Multicellular organisms have a hierarchical structural organization, in which any one system is made up of numerous parts and is itself a component of the next level. (HS-LS1-2)
- Construct an explanation based on evidence for how natural selection leads to adaptation of populations. (HS-LS4-4)
- Construct an explanation based on evidence that the process of evolution primarily results from four factors: (1) the potential for a species to increase in number, (2) the heritable genetic variation of individuals in a species due to mutation and sexual reproduction, (3) competition for limited resources, and (4) the proliferation of those organisms that are better able to survive and reproduce in the environment. (HS-LS4-2)
- Feedback mechanisms maintain a living system's internal conditions within certain limits and mediate behaviors, allowing it to remain alive and functional even as external conditions change within some range. Feedback mechanisms can encourage (through positive feedback) or discourage (negative feedback) what is going on inside the living system. (HS-LS1-3)

c. Recommended Placement

This unit will be taught in an AP Biology course following units on Evolution, Cell signaling and Molecular Biology. It could also fit in at a similar point in an Anatomy and Physiology class.

d. Technical Skills

- Students will perform ELISA assays on protein samples from fictional cancer patients, construct a standard curve, and use the results to recommend a course of treatment.
- Students will use the Limulus Amoebocyte Lysate assay to test for the presence of endotoxins. This will highlight both an important component of innate immunity as well as a widely used and extremely sensitive screen for bacterial contamination of pharmaceutical products.

e. Relevance The role of the immune system in preventing infection is of direct relevance to students. Understanding the functioning of the immune system will allow students to have informed opinions on topics such as vaccination that are controversial (at least in some circles), as well as a basic understanding of some of the emerging technologies that harness the power of the immune system to fight cancer.

III. Learning Objectives

Students will be able to:

- Describe the various ways in which the innate immune system protects organisms from infection
- List the main functions of each of the cells of the innate immune system
- Describe the general function of cytokines and chemokines in immunity
- Explain the function of complement
- Carry out Limulus Amoebocyte Lysate tests as a model of the complement cascade and the ability of the innate system to detect general pathogen-associated molecules
- Describe the process of inflammation and how it contributes to the defense against pathogens
- Diagram how cell signaling pathways regulate inflammation, and use this model to explain the function of NSAIDs in pain relief
- Describe generally how activation of the innate immune system leads to activation of the adaptive immune system
- Compare and contrast B and T cells at the levels of development, function, and roles in cellular vs. humoral immunity
- Use a paper model activity to visualize how antibodies are specific to particular antigens

- Describe how B and T cells that recognize self antigens are eliminated, and how defects in the process lead to many common autoimmune disorders
- Compare the primary and secondary immune responses, and explain how they relate to immunological memory and are the basis for vaccines
- Read a case study on host-pathogen interactions, and use evolutionary principles to explain how both pathogens and tumor cells can manipulate components of the immune system to facilitate their propagation.
- Read an article on how an emerging anti-cancer therapy involves disrupting interactions between tumor cells and T cells that inhibits T cell function.
- Carry out ELISA to assess PD-L1 expression in samples representing tumor cells from fictional patients. Students will compare their results to a standard curve and make a recommendation as to whether each patient is a good candidate for treatment with a monoclonal antibody that blocks the interaction between PD-L1 and PD-1.

V. Teacher Guide

A. Student Prior Knowledge and Skills

Students need to be familiar with different types of pathogens, in particular bacteria and viruses. They also need to know the principles of natural selection, and how the life cycles of common pathogens (such as large population size and short reproductive times) contribute to rapid evolution. Students will also need to understand cell signaling-including how signaling pathways can use cascades to amplify a response and negative feedback to tamp down a response.

A. Time Requirements

This unit is designed for 90 minute periods, meeting every other day. The unit will take 8 90 minute periods to complete.

Class Period	In class instruction/activities	Homework
1	<u>Notes: Innate immunity:</u> what is it in general, what types of organisms have it, what does it do and how? Students will work in small groups to read a case study outline a patient's response to a bacterial infection, and are asked to describe how the	Pre-reading assignment: Concept 35.1, *Campbell Biology In Focus* This section corresponds to an overview of the innate immune system.

	symptoms relate to each part of the response	
2	Review the roles of positive feedback/amplification and negative feedback in cell signaling. Discuss the utility of positive feedback, using complement activation as an example Watch video on septic shock to illustrate what can happen with an excessive innate immune response (https://www.youtube.com/watch?v=-bt-H5VQl5E) Discuss what might happen if bacteria were introduced to body intravenously (in IV fluid, for example). Give background information on Limulus Amoebocyte Lysate test. Students will carry out tests on unknown samples.	
3	Finish LAL lab. Students will generate a standard curve based on teacher-generated data. They will use this curve to estimate the concentration of their unknowns, complete analysis questions. Students will read a review article on cell signaling and inflammation. In groups, they will diagram a simplified picture of the role of COX-2 and prostaglandins in inflammation, and construct an explanation of how NSAIDs alleviate inflammation.	
4	Notes: Development and Function of B cells, T cells, and antibodies. Role of lymph organs and Antigen Presenting Cells Students will carry out paper modeling of antibody structure (Flinn Scientific) to visualize how antibodies are specific and how a	Pre-reading assignment: Concept 35.2 *Campbell Biology In Focus* This section corresponds to an overview of the adaptive immune system.

	huge diversity of Abs can be made from a limited repertoire of genes.	
5	Compare and contrast humoral vs. cellular immunity, roles of specific effector cells. In groups: AP Biology POGIL-Immune System (Flinn Scientific)	Pre-reading assignment: Concept 35.3 *Campbell Biology In Focus* This section discusses humoral vs cellular responses as well as diseases associated with immune dysfunction.
6	Case study: Immune Evasion (From NCCSTS) Functions as excellent review for students to tie everything together, as well as to consider ways in which pathogens can evade the immune system	
7	Students read press release from the 2018 Nobel Prize in Physiology or Medicine, which describes the research behind immune checkpoint inhibition therapies, answer questions Notes: The immune system and cancer-what *should* happen, how tumor cells can evolve to avoid destruction, advent of immunotherapies.	Read Introduction to ELISA lab, answer pre-lab questions.
8	Notes: PD1 signaling as a potential drug target PD-L1 ELISA	Complete analysis questions for homework.

Teacher Instructions for ELISA Lab

This lab is based on using the Bio-Rad ELISA Immuno-Explorer Kit (Catalog #166-2400EDU, $138.00 for 12 student groups of 4 students each). This kit was chosen due to its relative economy, rapidity, and robustness of signal. Although I have not tested it yet with this kit, with other Bio-Rad kits I have had success in stretching reagents by reducing volumes/concentrations.

Materials required (aside from those provided in kit)

Per station:
Micropipettes capable of pipetting 50 µl
Box of disposable pipet tips
Waste container for used tips
Paper towels
Beaker or small bottle, 100-200 ml capacity
Sharpie
Paper towels

Teacher Preparation

Detailed instructions are found in the Instructor's Advance Laboratory Preparation Guide that comes with the kit.

2 days prior to lab (can be up to a week prior, as long as reconstituted reagents are kept refrigerated).

1. Make PBS, resuspend freeze-dried antigen, primary antibody, and secondary antibody to make 50x stock solutions. The provided antigen is chicken gamma-globulin, the primary antibody is rabbit anti-chicken polyclonal, and the secondary antibody is goat anti-rabbit, conjugated to HRP. (15 minutes).

2. Label student tubes according to kit instructions (30 minutes)

One day prior to lab

1. Dilute antigen, primary antibody, and secondary antibody to 1x stocks, aliquot into student tubes. Organize into racks (1 per group) in the refrigerator. (45 minutes)
2. Set out student workstations (pipets, tips, waste containers for used tips, disposable transfer pipets, beaker/bottle for wash buffer, Sharpie, stack of paper towels). (30 minutes).

Day of lab

1. Distribute tubes of reagents to each group (5 minutes)
3. Dispense wash buffer to each group's beaker. (5 minutes)
4. For patients 1 and 4, aliquot 0.25 ml 1x antigen to each group's tubes (10 minutes)
5. For patients 2 and 3, aliquot 0.25 ml PBS to each group's tubes (10 minutes)

Teacher's Instructions: LAL Endotoxin Lab

This lab is based on the ToxinSensor Chromogenic LAL Endotoxin Kit (GenScript, catalog # L00350, $180.00 for 32 reactions, enough for 9 student groups). A cheaper alternative is the ToxinSensor GelClot Endotoxin Assay Kit (GenScript, catalog #L00351, 103.00 for 40 reactions). The GelClot kit is non-quantitative, simply giving a +/- result of whether the toxin concentration exceeds 0.25 EU/ml. A positive result in this assay is simply coagulation of the sample-if you do not have access to a spectrophotometer, this would this would be a good alternative.

Materials required (aside from those provided in kit)

Water bath or heat block adjustable to 37°C
Vortexer (preferably 1 for every 2-3 student groups.
Spectrophotometer

Per student group
Micropipette capable of pipetting 100 µl
Micropipette capable of pipetting 500 µl
Box of disposable tips for each pipet
Waste container for used tips
Ice bucket

Teacher preparation:

Note: All kit reagents are best used within 1 week after reconstituting, but require refrigeration/freezing, as noted below.

3 days prior to lab (40 minutes)

1. Reconstitute LAL in 1.7 ml endotoxin-free water (provided in kit). Freeze until day of lab.
2. Resuspend Chromogenic substrate in 1.7 ml endotoxin-free water. Refrigerate until day of lab.

3. Reconstitute Color-stabilizer #1 in 10ml Buffer S. Refrigerate.
4. Reconstitute Color-stabilizer #2 and #3 by adding 10ml endotoxin-free water. Refrigerate until day of lab.
5. Dissolve lyophilized endotoxin standard by adding 2 ml endotoxin-free water. Mix thoroughly with repeated vortexing. Stable for 1 week in refrigerator (Do Not Freeze).
6. This endotoxin stock solution is 5EU/ml. Dilute to 1EU/ml by adding 200 µl of this stock to 800 µl water. Refrigerate.

2 days prior to lab (90 minutes)

1. Make a dilution series using the 1 EU/ml stock to generate standards of 0.1, 0.05, 0.25, and 0.01 EU/ml. A flow chart is provided in the kit instructions. These will be used to generate a standard curve.
2. Prepare Sample 1 stock by mixing 100 µl of the 1 EU/ml stock with 1.4ml water. This will yield a concentration of 0.075 EU/ml.
3. Prepare Sample 2 stock by mixing 100 µl of the Sample 1 stock with 1.4 ml water. This will yield a concentration of 0.0075 EU/ml.
4. Carry out the LAL Chromogenic protocol to determine the absorbances for each standard stock that you prepared in Step 1

1 day prior to lab (45 minutes)

1. For each group, label 3 tubes “S1”, “S2”, and “B”, and add 100 µl of Sample 1 stock, Sample 2 stock, or water, respectively. Refrigerate.
2. For each group, label 4 tubes: "LAL", Stop #1, Stop #2, and Stop #3"
3. Add 350 µl LAL stock to the LAL tube, and 1.7ml of the appropriate stop solution to the other tubes.
4. Set out student work stations (pipets, tips, waste containers, etc.)
5. Set out vortexers
6. Set up water bath-turn on.

Day of lab

1. Set out student reagents in ice buckets.

Student Section

Case Study: Beauty and a (Bacterial) Beast

Jenny had never been happy with her nose, and she had saved for three years for the surgery. She consulted a plastic surgeon, and scheduled surgery for summer vacation. A few days after surgery Jenny was changing the dressings of gauze on her nose when she noticed increased swelling, redness, and pain at the incision site. By the next day she had a fever above 100°F. Jenny went back to the surgery clinic, where they took a swab of pus from the infected area for culture and prescribed an antibiotic. The next day the lab called to report that Jenny had a *Staphylococcus aureus* infection. The surgery and the nasal packing with gauze provided both an area for bacterial proliferation and an environment that contained air pockets giving the bacteria necessary amounts of oxygen for growth.

S. aureus is a bacterium found on the outside of the body, especially around the nose. About 30% of the population carries *S. aureus* at any particular time, and about 2/3 of people are at least occasional carriers. The bacteria can induce localized inflammation that causes capillary damage and gives the bacteria access to the bloodstream. *S. aureus* produces an antiphagocytic capsule, as well as secreting enzymes such as protease and lipase that destroy tissue, and coagulase that converts fibrinogen to a fibrin clot inside which the bacteria can grow. Many strains of *S. aureus* produce exotoxins and some strains are antibiotic resistant. Fortunately for Jenny, the *S. aureus* infecting her nose did not produce toxic shock syndrome toxin or exfoliative toxin, and her infection responded quickly to antibiotics.

QUESTIONS: Answer on your own paper

1. List as bullets with brief descriptions and in approximate chronological order the steps in the innate response to a bacterial infection. Include both the cells and the molecules that are involved in the response to the bacteria and what each does.

Example:

- Bacteria penetrate the skin and mucous membrane barriers through the surgical incision and enter the deeper layers of tissue.
- Bacteria begin to replicate at the site of infection.
- At the site of infection, . . .

2. What are the main types of phagocytic cells? How do they eliminate pathogens?

3. What is inflammation? What are the four signs/symptoms and what causes them? What is the purpose of inflammation? How are leukocytes (neutrophils and macrophages) signaled where to leave the circulation and enter the infection site?

4. What is the complement system? What activates this system, and what are its functions?

Case adapted and Modified from Dr. Janet Deckert at the University of Arizona. Her email is jdeckert@u.arizona.edu and her website is webMic419 Home

Why Do I Feel Better? How do Aspirin, Advil, and Aleve relieve pain?

Introduction: We've all been there-a bruise from soccer practice, aching arms the day after hitting the weight room, that random stress headache that hits after staying up late ~~playing video games~~ studying. Whatever the cause, you may have found yourself reaching for a pain reliever. Whether you reached for something old-school (aspirin), or a newer product, such as Aleve, you likely took something called a Non-Steroidal Anti-Inflammatory Drug (NSIAD). As implied by the name, these drugs affect inflammation in your body. But how? Your task is to find out…

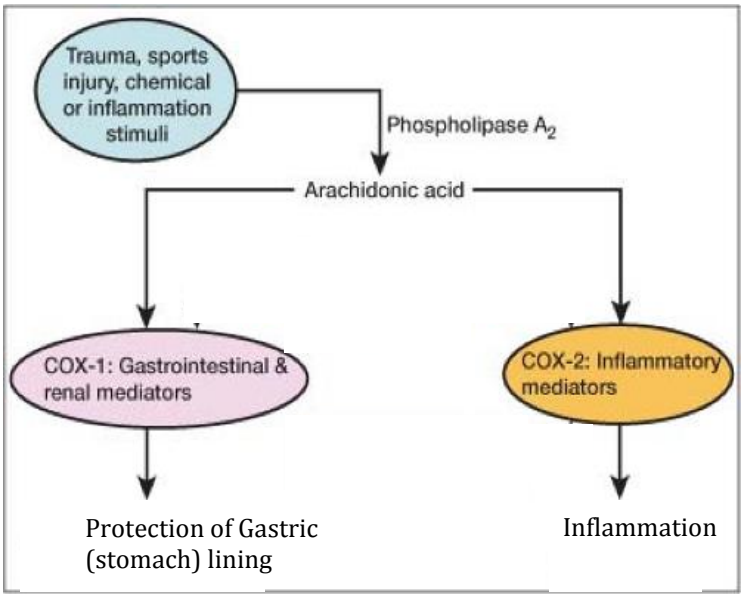
Assignment: Your job is to determine the mechanism of function for NSAIDs.

The passage at the following link will be helpful:

https://www.intechopen.com/books/nonsteroidal-anti-inflammatory-drugs/mechanism-of-action-of-nonsteroidal-anti-inflammatory-drugs.

Answer the following questions (you are not limited to the above site in finding this information).

1. What was the first NSAID used by people?
2. In general, what are the most common symptoms alleviated by taking an NSAID?
3. Briefly describe the functions of:
    - **a.** COX-1
    - **b.** COX-2
4. At a molecular level, how do NSAIDs inhibit the function of these enzymes?
5. Briefly describe the functions of prostaglandins (**focus on their roles in inflammation**, <u>including</u> what cells they influence).
6. Examine the diagram below. Add in labels where NSAIDs are predicted to act.
7. Newer-generation NSAIDs like Celebrex specifically inhibit COX-2. Based on the information in the diagram, why would taking a COX-2 specific NSAID have fewer side effects than taking something like ibuprofen, which inhibits both COX-1 and COX-2?



Immune Checkpoint Therapy

Last year, two scientists, James Allison and Tasuku Honjo, won the Nobel Prize in Physiology or Medicine. Their research into the functioning of the human immune system has led to the development of exciting new cancer treatments where the patient's own immune system is activated to attack and destroy tumors. For some types of cancer, these therapies have resulted in dramatic, long-term remission of cancer. To understand how this works, read the article at the site below, and answer the questions below the link.

Link: https://www.nobelprize.org/prizes/medicine/2018/press-release/

Analysis Questions

1. Why is it essential that the function of immune cells like T cells is subject to both positive and negative regulation?

2. What can result if the immune system is excessively active?

3. What are the normal functions of the CTLA-4 and PD1 proteins?

4. What was Dr. Allison's hypothesis for the experiment in which he tested the effect of CTLA-4 blockade on mice with tumors? What did he use to block the function of CTLA-4?

4. Based on what you know about antibodies, would an antibody against CTLA-4 only bind to CTLA-4, or would it bind to other proteins (such as PD1) as well? Explain.

5. What side effects can result from this type of therapy?

PD-L1 ELISA Lab

Background

As you have learned, immune checkpoint therapy has been shown to cause dramatic, long-lasting improvements for patients suffering from a number of different types of cancer. However, such results are not at all uniform-for some types of cancer, up to 50% of patients may experience long-term remission, while for others, only 20% of patients may see such improvement. Also, there are many cancers against which this therapy is ineffective. Why is this? One reason is that not all tumors are alike-even among tumors that actively inhibit the activity of immune cells that would otherwise destroy them, not all of them do so by using CTLA-4 or PD-1, the two most common targets for checkpoint therapy.

Recently, doctors have developed tests that help them determine if a patient is likely to benefit from checkpoint therapy. One of these involves testing tumor cells for high levels of expression for a protein called PD-L1, which is a protein that cancer cells can use to "shut off" T cells by binding to the PD1 protein on the surface of the T cell. Tumors that express high levels of PD-L1 are good candidates for inhibitors that block the PD1 pathway. In contrast, tumors that don't express PD-L1 are likely using some other mechanism to avoid destruction by the immune system, and so these patients would be unlikely to benefit from PD1 blockade. In this lab, you will be using a common lab technique called ELISA to test (fictional) patient samples to assess their levels of PD-L1 expression.

Prelab

Read the *Introduction* section of the student manual, and answer the Prelab questions below.

Prelab Questions

1. What does ELISA stand for?

2. What is an example of a use for the ELISA assay?

3. What is an antibody-based kit that you could buy at the pharmacy?

4. Define: Primary antibody, secondary antibody

5. What is the role of HRP (Horseradish Peroxidase) in this assay?

6. Draw labeled diagram showing an interaction between an antigen, a primary antibody, and a secondary antibody

PD-L1 ELISA Lab-Data and Analysis

Addendum to Laboratory Procedure:

Each table group will be testing 4 patient samples-each sample is in a numbered yellow tube in your ice bucket. Instead of recording your initials on the tubes in your 12-well strip, use the numbers from the patient tubes as follows:

Strip 1: Tubes 7,8,9: Patient 1
Tubes 10,11,12: Patient 2

Strip 2: Tubes 7,8,9: Patient 3
Tubes 10,11,12: Patient 4

Carry out the remaining steps in the protocol as described

Following Step 14, you should observe a color change in some of the tubes. Record your results in the table below. Record a "-" for no color change, and a "+" for a blue color change. If you feel any of the lanes are a darker blue than others, you can use ++" for a more intense blue color change.

Table 1: Results of PD-L1 ELISA

Strip	1	2	3	4	5	6	7	8	9	10	11	12
1												
2												

Analysis

1. Tubes 1-3 in each strip were positive controls-tests that should be positive, if all of the reagents in the kit are all functioning properly. What antigen do they all contain?

2. Tubes 4-6 are negative controls--tests that should be negative, due to the lack of any added antigen. What could be a possible reason that you could get a positive signal in one of these tubes?

3. Suppose that your patient samples all came up negative, but so did your positive controls. What could you conclude about whether your patient samples express the antigen?

4. Suppose that you had one or more patient samples that were positive for antigen (changed color), but 1 or more of your negative control tubes also changed color. What could you conclude about whether your patient samples express the antigen?

5. Based on your results, which patients would be the best candidates for anti-PD1 checkpoint therapy?

Blue-Bloods: How Horseshoe Crabs Save Lives

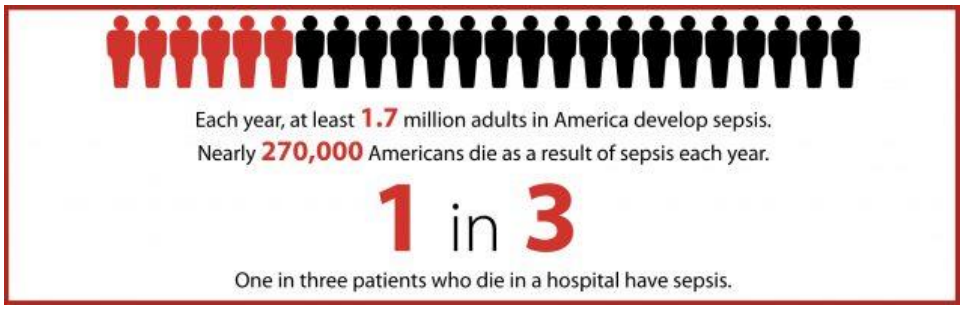
Part 1: An outpatient procedure turns into a life or death struggle

Diana Kane, MD, knew something was very wrong. It had been a week since she had undergone an outpatient procedure at a local surgical center and for the past five days she had been experiencing symptoms of an infection, including fever and fatigue. Embodying the old adage that doctors make the worst patients, she urged herself to press on through her workweek. But, soon enough, Kane was overwhelmed by the reality of her situation.

On the morning of day six, as she watched her husband, a paramedic, get ready for a 16-hour shift, she told him that if he went to work, she would be dead by morning. Within the hour, they were in the Emergency Department at Chester County Hospital. The nurses and physicians — all people she knew well — tended to her with incredible urgency. Kane had come around and admitted she was sick, but she was having trouble reconciling their austere demeanors and looks of concern with how she was feeling. Was she really dying? She couldn't tell. She was living in a fog.

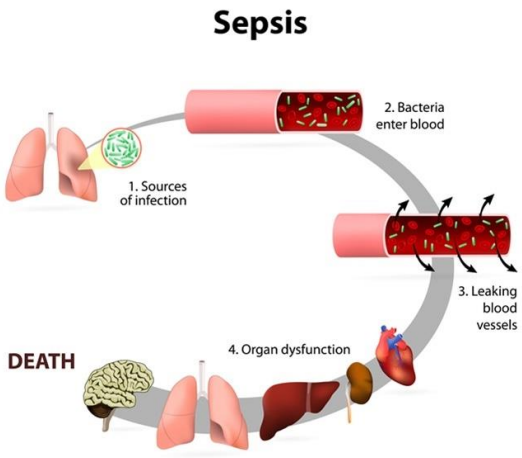
She was given blood, fluids, and antibiotics. She ended up spending seven days in the hospital, and received antibiotics and fluids via an intravenous (IV) line for her first month at home. All told, it was nine weeks before she began to feel normal and ready to return to work. "That first week home, I remember walking to the bottom of my driveway to get the mail and I couldn't get back to the house. It was too draining," she says.

It turns out Kane had contracted an infection in one of her legs from an instrument that was used during her outpatient procedure and subsequently joined the nearly 2 million Americans who each year develop a life-threatening condition called **sepsis.***



Graphic downloaded from https://www.cdc.gov/sepsis/datareports/index.html

*Adapted from https://www.pennmedicine.org/news/news-blog/2018/october/surviving-sepsis-how-one-doctors-personal-experience-is-helping-improve-care-for-patients-and-staff



What is sepsis?

Each year, more than 200,000 people in the United States die from sepsis, a condition caused by an overwhelming immune

response that can quickly lead to organ failure. In response to infection, particularly when the pathogen enters the bloodstream, the body releases pro-inflammatory molecules into the blood to combat the infection. Those chemicals trigger widespread inflammation, which leads to blood clots and leaky blood vessels. As a result, blood flow is impaired, and that deprives organs of nutrients and oxygen and leads to organ damage. In severe cases, one or more organs fail. In the worst cases, blood pressure drops, the heart weakens, and the patient spirals toward septic shock. Once this happens, multiple organs—lungs, kidneys, and liver—may quickly fail, and the patient can die. The progression from mild symptoms of infection to total collapse of multiple organ systems and death can be astonishingly fast. For example, Jim Henson, creator of the Muppets, began feeling flu-like symptoms on a Saturday, was admitted to a major New York Hospital on Tuesday morning, and died less than 20 hours later.

Exactly why the body's immune response spirals out of control in these cases is uncertain, but one thing is clear-once started, septic shock can be very difficult to stop, even if the original infection is cleared with antibiotics. Physicians therefore place heavy emphasis on early detection of sepsis-studies show that for every hour of delay in starting treatment, the chance of death can increase be as much as 8%.*

*http://www.ncbi.nlm.nih.gov/pubmed/16625125

Video: We will watch the following Khan Academy video on the physiology and symptoms of septic shock (https://www.youtube.com/watch?v=-bt-H5VQl5E). Following the video, answer the following questions:

1. White blood cells release molecules such as nitrous oxide in response to encountering a pathogen. What are the two effects on blood vessels of molecules like nitrous oxide?

2. Under normal conditions (an infection in the peripheral tissues, not in the blood), what is the purpose of nitrous oxide release?

3. How do these effects (from question 1) contribute to low blood pressure?

4. Describe how cytotoxic molecules released by white blood cells contribute to septic shock.

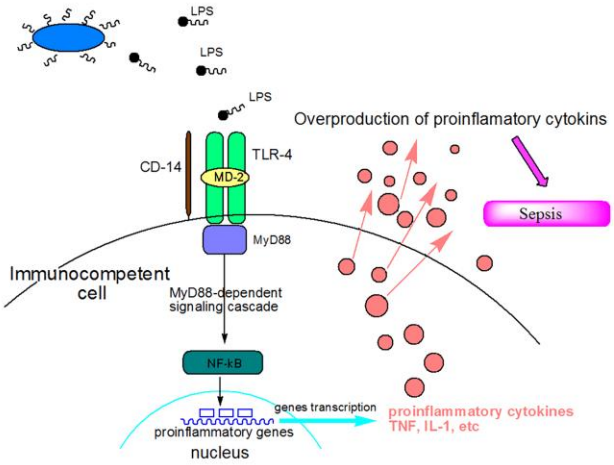
5. What is the cause of ARDS (Acute Respiratory Distress Syndrome)?

6. People experiencing septic shock are typically given large volumes of IV fluids. What is the purpose of this?

Sepsis is a leading cause of death in hospitals

Sepsis is a major challenge in hospitals, where it's one of the leading causes of death. It is also a main reason why people are readmitted to the hospital. There are many contributing factors to this. First, many patients are already immunocompromised in some way-they many already have an infection, or their immune systems may be weakened by coping with injuries and/or other illnesses. Also, invasive medical procedures such as insertion of IV's, breathing tubes, catheters, and other medical equipment can introduce bacteria into the bloodstream and bring on the condition. In addition to the possibility of bacteria being introduced into the body on equipment, hospitals must also be diligent about possible bacterial contamination of any fluids (such as saline, medicinal drugs, chemotherapy agents, etc.) that enter the patient's blood.

Even worse, we must be concerned not only about contamination with live bacteria, but also with certain bacterial compounds called **endotoxins** that can be released by some types of bacteria. The most common endotoxin is called lipopolysaccharide (LPS), a carbohydrate component of the cell walls of some bacteria. LPS is recognized by receptor proteins on many immune cells (see diagram to right) Humans are extremely sensitive to LPS-a dose of 50 micrograms (a microgram is 1 millionth of a gram) can induce sepsis by itself in the absence of any infection. How do we ensure that our medicines and medical devices are free of both pathogens and endotoxins?



https://www.creative-biolabs.com/drug-discovery/therapeutics/lps-induced-rodent-sepsis-model.htm

Enter *Limulus*

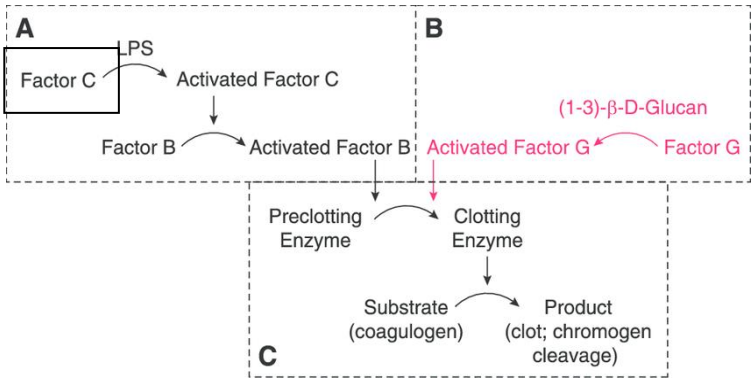
The humble horseshoe crab, *Limulus polyphemus*, would seem an unlikely source of help in our fight against bacteria. A marine relative of spiders and scorpions, horseshoe crabs lack an adaptive immune system altogether. Not only that, but they utilize a compound called hemocyanin to carry oxygen, which gives horseshoe crab blood a striking blue color. *Limulus* does have a powerful innate immune response, though, one that is fantastically sensitive to LPS. Cells called amoebocytes circulate in the blood, and upon detection of LPS, release a chemical called **coagulogen**, inducing the formation of a gel-like clot, trapping any bacteria present.

Discovery of this reaction led to the development of the **Limulus Amoebocyte Lysate (LAL)** assay, where medical products can be tested in a rapid and reliable fashion. The test is simple-amoebocytes are broken open (lysed) to release coagulogen,

and this extract is mixed with the fluid to be tested. Formation of a thick gel indicates the presence of hazardous bacterial toxins. Approximately half a million horseshoe crabs are captured, bled, and released each year to support this testing. Not surprisingly, being removed from the ocean for several hours and having a significant proportion of your blood removed (20-40%) is stressful to these animals-leading to a mortality rate of at least 20%. Fortunately, new techniques involving the *in vitro* production and purification of the relevant horseshoe crab proteins are beginning to enter the market, but for now, the traditional LAL test is still the gold standard for ensuring the safety of medical and pharmaceutical products.

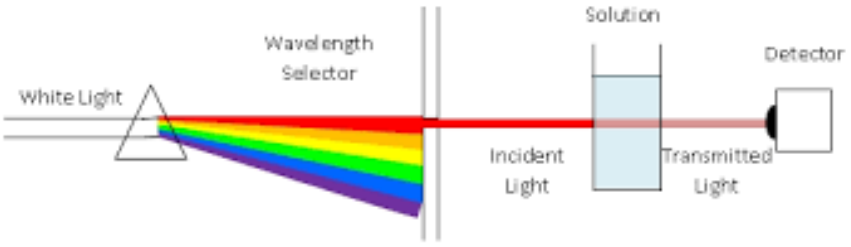
The Chromogenic LAL Test

Although formation of a gel is an accurate test of whether endotoxin is present, it does not tell us *how much* is present. You will be carrying out a variation on the traditional LAL test. Rather than simply testing for the presence/absence of endotoxin, you will be testing for the activity of an enzyme called Clotting Factor C, which is activated by binding to LPS. This initiates a cascade of effects that culminates in the processing of coagulogen into an active, clot-forming structure. (see diagram below).



Munford, Robert. (2016). Endotoxemia--menace, marker, or mistake?. Journal of Leukocyte Biology. 100. 10.1189/jlb.3RU0316-151R.

The amount of Clotting Factor C activity is proportional to the amount of LPS that is present in the solution being tested. By adding a **chromogenic (color-generating)** substrate to the reaction, we can monitor the reaction simply by observing a color change. The amount of activity can be quantified by using a spectrophotometer to measure the amount of colored product produced (see below).



The difference between the incident and transmitted light indicates the absorbance

https://di.uq.edu.au/community-and-alumni/sparq-ed/sparq-ed-services/spectrophotometry

Procedure

a. Wear nitrile gloves whenever handling the vials or pipettes.
b. Use a fresh pipet tip each time

1) You will be testing 2 samples, plus a “blank” (water known to be LPS-free). Label your 3 vials “S1”, “S2”, and “B”

   Use a micropipette to add 100 µl of each test sample into a separate endotoxin-free vial. Add 100 µl of LPS-free water to a third vial.

2) Vortex each vial for 30 seconds.

3) Add 100 µl of reconstituted LAL to each vial. Cap the vials and mix well by swirling gently.

4) Place all vials in the 37°C water bath. Incubate **10 minutes.**

5) After incubation, add 100 µl of reconstituted chromogenic substrate solution to each vial. Cap the vials and swirl gently to mix well. **Do not shake or vortex to avoid foaming.** Incubate at 37°C for **6 minutes**.

6) Add 500 µl of reconstituted Color-stabilizer #1 (Stop Solution) to each vial and swirl gently to mix well. Do not shake or vortex to avoid foaming. Add 500 µl of reconstituted Color-stabilizer #2 to each vial and mix well. Finally, add 500 µl of reconstituted Color-stabilizer #3 to each vial. Gently swirl each vial to mix well.

7) Load a cuvette with distilled water, place into the spectrophotometer, and press “blank”. This adjusts the spectrophotometer to zero absorbance.

8) Read the absorbance of each reaction vial at 545 nm Record your data in Table 1

Table 1: Your data:

Vial No.	Sample	Absorbance at 545nm	∆ Absorbance*	Endotoxin concentration (EU/ml)**
1	Blank (LPS-free water)		—	—
2				
3				

*(Absorbance of sample-absorbance of blank)

** The amount of LPS in a sample is reported in Endotoxin Units (EU). 1 EU=1 picogram (pg) of endotoxin, which is approximately the amount of endotoxin in 105 bacteria.

Your teacher will provide you with data showing the absorbance of known amounts of LPS in this assay. Fill in Table 2 below with this information.

Table 2: Standards

Sample	**Absorbance at 545nm**	**Δ Absorbance**
Blank (LPS-free water)		—
0.01 EU/ml Standard		
0.025 EU/ml Standard		
0.05 EU/ml Standard		
0.1 EU/ml Standard		

Calculation of Endotoxin Concentration

Under standard conditions, the absorbance at 545 nm shows a linear relationship with the concentration in the range of 0.01 to 1 EU/ml. On a piece of graph paper, plot the absorbance for the four standards from Table 2 on the x-axis and the corresponding endotoxin concentration in EU/ml on the y-axis. Draw a best-fit straight line between these points. You can then use the absorbances you measured for your samples to estimate the endotoxin concentrations-add these values to Table 1.

Analysis

1. For some applications, the maximum allowable endotoxin concentration is 0.2EU/kg body weight. If you were to introduce 50 ml of the solutions you tested into a 50kg patient, would either solution exceed the maximum endotoxin exposure? Show your work for each solution below.