

MEDICAL IMMUNOLOGY LAB
(MB417/517)

Malcolm Lowry, Ph.D.
Department of Microbiology
Oregon State University

Immunology Lab

MB 417/517

Credits: 2

The course meets 4 hours per week in laboratory. Nash Hall 3rd floor teaching lab.

Pre-requisites: MB 416 (may be taken as co-req), MB 303

Learning resources (required): MB 417/517 lab manual (available in the OSU bookstore)

Instructor: Malcolm Lowry, Ph.D.
Department of Microbiology
Office: 453 Linus Pauling Science Center
Email: malcolm.lowry@oregonstate.edu
Phone: 737-8309

This laboratory will provide exposure to up to date techniques in immunology using several newer technologies as well as more basic experimental approaches.

Student Learning Outcomes for MB417

Upon completion of this class students should know how to:

1. Collect experimental data aimed at understanding the function of the immune system.
2. Analyze experimental data and construct valid conclusions regarding the meaning of the experiment.
3. Formulate and present a well-organized and concise report of the experimental findings.
4. Communicate recent research progress in the field from scientific journal articles and critically evaluate the methods and conclusions.

Evaluation of student performance for MB417

Lab reports and lab participation = 50%

Midterm exam = 20%

Presentation = 10%

Final exam = 20%

Student Learning Outcomes for MB517

Upon completion of this class students should know how to:

1. Collect experimental data aimed at understanding the function of the immune system.
2. Analyze experimental data and construct valid conclusions regarding the meaning of the experiment.
3. Formulate and present a well-organized and concise report of the experimental findings.
4. Communicate recent research progress in the field from scientific journal articles and critically evaluate the methods and conclusions.
5. Write a critical review of a current research paper from a major journal in the field and extend the topic into what could be done next in further research.

Evaluation of student performance for MB517

Lab reports and lab participation = 50%

Midterm exam = 20%

Presentation = 5%

Written paper critique = 5%

Final exam = 20%

Student conduct (<http://studentlife.oregonstate.edu/studentconduct/offenses-0>)

The following information is summarized from the OSU Student Conduct Regulations

Students are expected to be honest and ethical in their academic work. Academic dishonesty is defined as an intentional act of deception in one of the following areas:

- *cheating- use/attempted use of unauthorized materials, information or study aids
- *fabrication- falsification or invention of any information
- *assisting- helping another commit an act of academic dishonesty
- *tampering- altering or interfering with evaluation instruments and documents
- *plagiarism- representing the words or ideas of another person as one's own

When evidence of academic dishonesty comes to the instructor's attention, the instructor will document the incident, permit the accused student to provide an explanation, advise the student of possible penalties, and take action. The instructor may impose any academic penalty up to and including an "F" grade in the course after consulting with his/her department chair and informing the student of the action taken.

The goal of Oregon State University is to provide students with the knowledge, skill and wisdom they need to contribute to society. Our rules are formulated to guarantee each student's freedom to learn and to protect the fundamental rights of others. People must treat each other with dignity and respect in order for scholarship to thrive. Behaviors that are disruptive to teaching and learning will not be tolerated, and will be referred to the Student Conduct Program for disciplinary action. Behaviors that create a hostile, offensive or intimidating environment based on gender, race, ethnicity, color, religion, age, disability, marital status or sexual orientation will be referred to the Affirmative Action Office.

Student Conduct Expectations link:

<http://studentlife.oregonstate.edu/code>

Statement Regarding Students with Disabilities: Accommodations for students with disabilities are determined and approved by Disability Access Services (DAS). If you, as a student, believe you are eligible for accommodations but have not obtained approval please contact DAS immediately at 541-737-4098 or at <http://ds.oregonstate.edu>. DAS notifies students and faculty members of approved academic accommodations and coordinates implementation of those accommodations. While not required, students and faculty members are encouraged to discuss details of the implementation of individual accommodations.

Reach Out for Success: University students encounter setbacks from time to time. If you encounter difficulties and need assistance, it's important to reach out. Consider discussing the situation with an instructor or academic advisor. Learn about resources that assist with wellness and academic success at oregonstate.edu/ReachOut. If you are in immediate crisis, please contact the Crisis Text Line by texting OREGON to 741-741 or call the National Suicide Prevention Lifeline at 1-800-273-TALK (8255)

Schedule of MB 417/517 lab

1. September 25: Lab 1: Histology of the immune system. Differential blood count
2. October 2: Lab 2: Phagocytosis of pathogens by macrophages
3. October 9:
October 11: Lab 3: Nitric oxide production in response to microbial stimuli. Part 1.
Friday- 1pm Part 2 of Lab 3 Nitric oxide lab.
4. October 16: Lab 4: Macrophage intracellular killing of bacteria
Lab report from lab#1 and #2 due
5. October 23: Lab 4 part 2: CFU counting from Lab 4
Lab 5 part 1: SDS PAGE and western blotting 1
Midterm exam
6. October 30: Lab 5 part 2: SDS PAGE and Western blotting part 2
Lab report #3 due
Immuno-olympics topics and teams will be assigned
7. November 6: Lab 6: Analysis of B and T lymphocyte activation by Flow cytometry
8. November 13: Lab 7: Lab 7 ELISA part 1, Immuno-olympics discussion
Lab report #4 due
9. November 20: Lab 7: ELISA Part 2.
Lab final exam: taken during incubation time for ELISA
Lab report #5 due
10. November 27: Discussion of flow cytometry Lab 6 data
11. December 4: Immuno-Olympics team challenge.
Lab report #6 and #7 due

Note: Please read the lab procedure before coming to class. Some of these labs are very challenging, and you will make mistakes if you don't have a good idea of what you are going to do that day.

LAB NOTEBOOKS

The lab notebook is an important part of your grade. It will be graded as indicated on the lab schedule. You may use separate folders to hand in lab reports that are due. This will allow you to remove pages/reports for grading, or add in computer-typed introduction/discussion/results sections. Unless otherwise specified by the instructor, each individual lab report should have the following components:

Heading: Exercise # and Title of the lab
Your name
Your partners' name (if applicable)

Purpose (2 pts): A short paragraph describing the objectives of the lab. Do not just copy what is written in the lab instructions! (you will get a zero if you plagiarize from the lab manual or any other source).you should address: Why did we conduct exercise? What information were we trying to obtain? What concepts were being demonstrated? What protocol or method or technique did we use to obtain the information? The purpose is typically just 1-2 paragraphs, but it should be complete.

Procedure (1 pt): Brief outline. You may reference the lab manual, but you **must** include any changes from printed protocol.

Results (3 pts): Detailed, well-organized, clearly labeled.

Must attach pages of raw data to get full credit
Results should be clearly organized, preferably as table or chart
Drawings
Graphs – should have titles and clearly labeled axes and scales.
Calculations

When presenting data, especially tables, assume that the reader is not intimately familiar with the lab procedure, and make sure that every entry is properly labeled and that all information necessary for understanding the results is included.

Conclusions/

Discussion (4 pts): Explain results. Summarize conclusions you can draw from the results.

What do the results indicate? This is the most important section, explain the reasoning for what the results mean.
Were the objectives listed in the purpose section accomplished? Why or how?
What problems were encountered?
Were there any unexpected results? What are possible explanations?
Indicate things that might be done differently if the lab were repeated.

Note: If you use any outside sources, you MUST provide a complete reference.

LABORATORY RULES

Carefully read the following items relating to laboratory safety:

- 1) **DO NOT:** eat, drink, chew gum or tobacco. Open beverage containers must be left on the hallway shelf outside lab. Keep your hands out of your mouth and eyes.
- 2) You are required to provide and wear a lab coat at all times in lab. Wear shoes that would protect feet from broken glass (preferably autoclavable). Only the necessary supplies should be kept on your bench top; everything else can be placed in cupboards (keep the aisles clear!). Backpacks/coats should not be left on bench tops. **WARNING: CONTAMINATED PERSONAL OBJECTS WILL BE AUTOCLAVED.**
- 3) Protective eye goggles are required for all experiments using corrosive or toxic chemicals or potentially pathogenic organisms.
- 4) No unauthorized visitors in lab.
- 5) **CLEAN** desktop with LpH **DISINFECTANT** at the beginning and end of class. Wash hands with soap before leaving the lab.
- 6) **LOCATE** the safety equipment in the lab - fire extinguisher, fire blanket and eye shower. The full body shower is located at the entrance to the media room (Nash 324) for 3rd floor labs or lab entrance (Nash 554) for 5th floor labs.
- 7) **BUNSEN BURNERS** in the lab have almost invisible flames – be careful of loose clothing and tie back long hair during lab to avoid contact with flame. Turn burners completely off when finished. If at any time you smell gas in the lab, check to make sure your burner is turned all the way off. If it is not your burner, notify the lab TA immediately.
- 8) **BROKEN GLASSWARE** - Call the instructor or TA to assist you. **Do not dispose of any glassware in the regular garbage cans.**
 - a. **Contaminated glass** is placed in large can on discard table for autoclaving and subsequent disposal in broken glass box.
 - b. **Non-contaminated glass** is placed in the large cardboard broken glass box.
- 9) **INJURY** - If you cut yourself in the lab, inform the instructor or TA so that the wound can be properly disinfected. If the injury needs professional assistance, you will be escorted to the Health Center or proper facility.
- 10) **SPILLAGE** – If you spill anything in lab, inform the instructor or TA so that they can assist you in proper clean-up. If culture is spilled on your clothing or belongings, they may require decontamination to assure your safety.
- 11) **PIPETTING** - Do not pipette by mouth. When using rubber bulbs, insert the pipette gently into the bulb to avoid breaking the pipette and potentially cutting yourself.
- 12) **TO AVOID CONTAMINATION** - Microbes are found everywhere, use common sense to avoid contamination.

- a. You must not allow any part of yourself or any other nonsterile object to touch growth media.
- b. Do not remove the lid from your Petri dish completely, instead lift it and hold it above the dish to protect the media from dust.
- c. Flame tube mouths after you open them and before you close them to kill dust borne contaminants.
- d. Do not put test tube caps or Petri dish lids down on the counter.
- e. Hold tubes and flasks so that the mouth is as horizontal as possible to minimize exposure to dust.
- f. Sterilize your loop or inoculating needle by heating it until it glows. Start with the wire above the loop in the "cool" yellow part of the flame and end with the loop itself in the "hot" blue cone. Do this before and after you transfer each culture.

13) **INCUBATING** - Carefully label all materials to be incubated with your name and organism identification.

- a. Label **culture plates** on **agar side** with your name and organism identification. Incubate plates **agar side up**.
- b. Label **culture tubes** on the **glass (not plastic caps)** with your name and organism identification. Place tubes in racks for incubation.

14) **DISCARDING CLASSROOM MATERIAL** - All materials used in this class that are contaminated with culture (tubes, plates, pipettes, etc.) must be autoclaved before cleaning or disposal.

- a. **Used slides and cover slips** go in the metal can for contaminated glass waste. If working with nonpathogens, the slides can be cleaned with BonAmi and returned to desktop.
- b. **Used razor blades/pins/needles** go in a "sharps" container.
- c. **Plastic petri plates** go into the autoclave bag at the discard table.
- d. **Plastic transfer pipettes, pipette tips, swabs, etc** go in the autoclave bag at the discard table.
- e. **Culture tubes** go in wire baskets in metal coffins on the discard table. Loosen screw caps before autoclaving.
- f. **Glass bottles** go in metal coffins on the discard table. Loosen screw caps before autoclaving.
- g. **Uncontaminated paper** used to clean desk with LpH or lens paper can be placed in metal container on desktop. The metal container should be emptied in the main garbage can at the end of each lab.
- h. **Racks** are to be returned to the lab shelves.
- i. **Glass pipettes** are placed into plastic trays on desktop that contain bleach. Pipettes should all be placed in the same direction.
- j. **Organic solvents** such as phenol and chloroform should be discarded into properly labeled waste containers.

14) **LEAVING THE LAB** – Clear lab bench of all cultures, racks, and plates. Empty metal can of paper waste into a large garbage can. Clean desktop with LpH disinfectant. **Wash hands with soap.** Take off and store your lab coat (do not leave with it on!). Make sure you have all of your belongings before leaving lab.

PROPER USE OF EQUIPMENT

MICROSCOPE USE

The microscopes are valuable and must be cared for properly. Remove microscope from cabinet by rolling shelf all the way out, then lifting microscope to the table. Bumping the oculars into the top of the cabinet can damage them.

When replacing the microscope after each use:

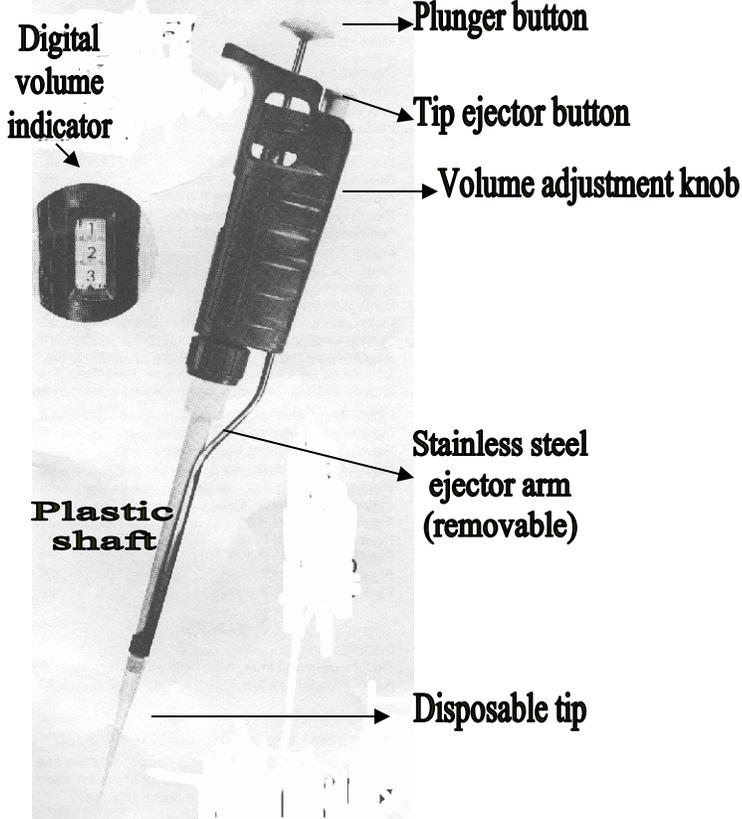
- a. Carefully clean oil off the 100X objective by wiping with flat lens paper, followed by flat lens paper saturated with lens cleaner. If lens paper is crumpled it will scratch the lens. If oil is left on the objective, it will destroy the seal and the lens will need to be replaced (>\$300).
- b. If oil is on the 45X lens, please let the instructor or TA know so it can have special cleaning.
- c. Remove dirt or oil from the stage, condensers or oculars, if needed.
- d. Store microscope with low power or blank objective in place and close to stage.
- e. Wrap the cord around the oculars neatly; replace in cabinet when shelf is completely rolled out.
- f. Sign and date the sign-out sheet in the cabinet.
- g. Have your TA check your microscope and initial sign-out sheet.

USE OF PIPETMAN

Place sterile tip onto pipetman by carefully opening sterile box of tips and seating plastic shaft down firmly onto tip. Place sterile tip into desired solution and push down to first stop on plunger button. Carefully release. Liquid should have been drawn up into tip. Put tip into the tube you want to dispense liquid into, press plunger button down to first stop and that should do it.

Never turn the numbers on the pipetman above the maximum amount. For example, with a P20 never turn dial past 20.0 or you will strip the gears. Place used disposable tips into plastic discard container.

PIPETMAN®



Pipetman Diagram

(bold number are in black on pipetman, nonbold numbers are in red on pipetman)

P 10

P 20

Range: 1 μ l to 10 μ l

1
0
0

= 10.0 μ l

Range: 2 μ l to 20 μ l

1
4
5

= 14.5 μ l

P 200

P 1000

Range: 20 μ l to 200 μ l

Range: 200 μ l to 1000 μ l

1
4
5

= 145 μ l

0
4
5

= 450 μ l

Immunology introduction:

Functioning immune systems are crucial for the survival of the species but their importance is sometimes forgotten when matters of evolution are discussed. All living plants and animals must be equipped with defense mechanisms against potential pathogens. For example, the unicellular amoeba produces anti-microbial peptides and many antibacterial and antifungal compounds have been isolated from terrestrial plants and algae. Invertebrates rely on innate immune systems whereas vertebrates have an adaptive system as well. The origin of the adaptive system is thought to have occurred with a serendipitous insertion of a recombination-activating gene (RAG) transposon into the genome of an ancestor to the jawed fishes before the divergence of cartilaginous fishes. Much research has been conducted on adaptive immune systems, in particular the mammalian system. However, in recent years more researchers have taken an interest in the innate system and the interactions that occur between the two different systems.

The innate immune system.

The innate immune system can be divided into first and second lines of defense. The first line takes the form of **anatomical and physiological barriers**. Examples of anatomical barriers in mammals are skin and mucosal membranes. Skin, being heavily keratinized and low in moisture content, is hard for pathogens to penetrate. This difficulty is also enhanced by the low pH of 3-5 that is created by lactic acid and certain fatty acids that are produced in the sebaceous glands. Microbial penetration of mucosal membranes is also difficult. Abundant mucus secretions can simply wash the pathogens away, and the cilia that line the airways of mammals beat constantly and transport the trapped pathogens so that they can be cleared.

The mucus that is released onto the body surface of teleosts also works as an anatomical and physiological barrier by making it hard for parasites, bacteria and fungi to enter or to start growing on the skin itself.

Antimicrobial proteins and peptides.

The second line of defense includes a wide range of molecules that inhibit the growth and spread of microbes by direct killing, or by facilitation of receptor-mediated phagocytosis. The skin and mucosal membranes produce antimicrobial proteins and peptides. **Lysozyme**, a hydrolytic enzyme that cleaves the peptidoglycan layer in the cell wall of Gram-positive bacteria, is found in tears and in the respiratory mucosa and is also produced in the Paneth cells of the intestinal mucosa of mammals. Lactoferrin, secreted in the mammalian respiratory system, also shows antimicrobial activity. Its activity seems to be confined to the first 25 amino acids of the N-terminus and this fragment gets cleaved off to form the active peptide. Antimicrobial peptides are also produced from the N-terminal of pepsinogenin from the bullfrog, *Rana catesbeiana*. Other antimicrobial peptides have been isolated, mainly from mammals and *Drosophila*, and they can be divided into **α and β defensins** depending on if their secondary structures mainly consist of an α -helix or a β -sheet.

The *Drosophila* antibacterial and antifungal peptides include cecropins, attacin, diptericin, drosocin and drocomycin. They are secreted from the fat body, analogous to the liver in mammals. The intracellular signaling that leads to transcription of the relevant genes is conducted by *relish*, which is homologous to the mammalian NF- κ B transcription factor. This factor is responsible for activation of many of the acute phase proteins in mammals. A couple of the α -defensins, human defensin-5 (HD-5) and HD-6, are constitutively expressed in the Paneth cells of the intestine; HD-5 is also secreted constitutively into the mucus of the vagina and cervix.

Many antimicrobial peptides are cytotoxic to both Gram negative and Gram-positive bacteria, and some peptides, such as Metchnikowin from *Drosophila*, have both antifungal and antimicrobial activity. While some are expressed constitutively, other

peptides are inducible. Antimicrobial peptides are also found in neutrophil granules and recently they have been found to interact with the adaptive immune system by attracting dendritic cells and T-cells to sites of infection.

The hepatic (liver) acute phase response.

Humoral components of innate immunity are not limited by constitutive expression of fixed rates. Indeed increases and decreases in protein synthesis in the liver during the early phase of an infection or immediately following a traumatic event help protect the individual from opportunistic pathogens. These so-called **acute phase proteins** (APPs) are secreted into the plasma. Those that increase in quantity are considered to be positive APPs whereas those that decrease are negative APPs.

More than thirty-five APPs have been identified in mammals. There are three major mammalian APPs: **C-reactive protein (CRP), Serum Amyloid A (SAP) and Serum Amyloid A (SAA)**. Others include **complement components, serum proteinase inhibitors, coagulation factors and metal binding proteins**.

Cells in innate immunity

Granulocytes (neutrophils, eosinophils and basophils), **dendritic cells**, **monocytes** and **tissue macrophages** are important components of the secondary line of defenses in innate immune systems. Most are phagocytic and can secrete molecules (interleukins) that orchestrate the inflammatory response. Of the granulocytes, neutrophils and eosinophils can phagocytose but basophils cannot. The granules in neutrophils stain with both acidic and basic dyes, eosinophil granules stain with acidic dyes and the granules in basophils, as the name implies, stain with basic dyes.

Neutrophils, or polymorphonuclear leukocytes (PMNs) as they are also called, are important in the diagnosis of acute infections in mammals, since their numbers increase rapidly early in an infection. In mammals, the neutrophil granules contain collagenase and other proteolytic enzymes, lysozyme, lactoferrin, peroxidase, and

bactericidal/permeability-increasing protein, as well as several α -defensins. These granules fuse with phagosomes and help in the digestion of engulfed particles. Neutrophils also produce different oxygen and nitrogen radicals such as superoxide and nitric oxide that are highly damaging to pathogens.

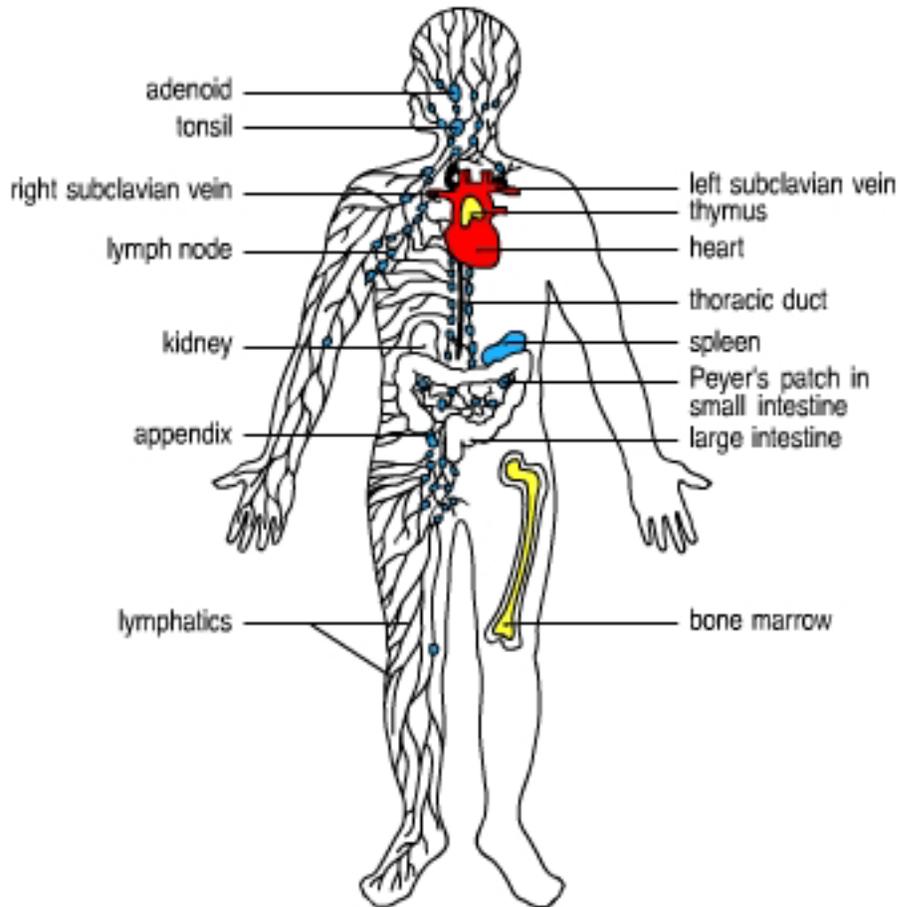
Eosinophils are involved in defense against parasites and also play a role in asthma and allergies. The roles of basophils are not well defined. However, both eosinophils and basophils, by their release of histamines and other vasoactive substances, are part of the pathology seen in allergies and asthma.

Macrophages and dendritic cells (DC) have many functions. They are capable of opsonin-dependent and opsonin-independent phagocytosis. Secondly, they produce cytokines after they are “activated” by phagocytosed particles and thirdly, they degrade captured particles and present some of the resulting peptides, in the context of MHC II, on the cell surface. The antigen, held within a groove in the MHC molecule, is then recognized by T-helper cells. This event, which involves several signals eventually leads to expansion of the specific T-cell clones. These T-cells trigger B-cells to produce antibody. **Monocytes** circulate in the blood and, when needed outside the vasculature, become activated and migrate into the affected tissue as macrophages.

Lymphocytes, B and T-cells, clonal expansion, memory, self-recognition, antibody diversity, primary and secondary responses are hallmarks of the adaptive immune system. B-cells, like macrophages and DCs, are antigen-presenting cells (APCs) with the main difference between the cell types being that B-cells can differentiate into plasma cells that secrete antibodies. A large diversity of antibody specificities can be achieved by rearranging immunoglobulin gene segments.

LAB #1 HISTOLOGY OF LYMPH ORGANS

Cells of the immune system are located in highly organized compartments in lymphoid tissue. Particularly the spleen and lymph nodes (LN) contain areas where certain lymphocytes are found and others are usually not. The advantage to this type of arrangement allows trafficking of cells to the appropriate sites of the tissue. In particular, the **cortex** is the outer most area of lymph nodes and the inner portion is the **medulla**. B lymphocytes are mainly organized into follicles and T lymphocytes and dendritic cells are contained in **paracortical** areas. This type of organization allows Ag to be carried into the lymph node by migrating dendritic cells which can then activate T helper cells. The T helper cells can then interact with B cells and mediate their activation. When B cells become activated they will usually develop into **plasma cells** which secrete large quantities of Ig. However, an activated B cell can enter a follicle and begin to proliferate vigorously as well as somatically mutate their Ig genes. This site is referred to as a **germinal center**. Astonishingly, there are documented studies which show that a single B cell can clonally expand to encompass an entire germinal center.



Slides are on display in the microscopes. Please look at the slides and identify some of the structures seen.

1. Lymph nodes, Peyer's patch, tonsil and spleen – identify lymphocytes, dendritic cells and macrophages (can be tricky). Also look for blood vessels.
2. Bone marrow - please identify myeloblasts, reticulocytes and band neutrophils.
3. Do one drawing of an organ with some cells labeled for your lab report. You can choose what you want to draw from the slides on display.

DIFFERENTIAL COUNT

1. You will have 3 slides that have been stained with wrights stain of human blood smears. One is normal blood from a healthy individual, and will be marked as normal. The other two will be coded as B and C, and are unknown pathologies of human blood. The goal is to determine how the unknown blood samples differ from the normal control. By counting the percentages of white blood cells in each category, you can determine the likely pathology. Today the unknowns are derived from patients that have either a viral infection resulting in mononucleosis, or have a nasty case of allergies or possibly infection with parasites. To figure out which condition you have, you have to determine what cells would be increased in each type of infection.

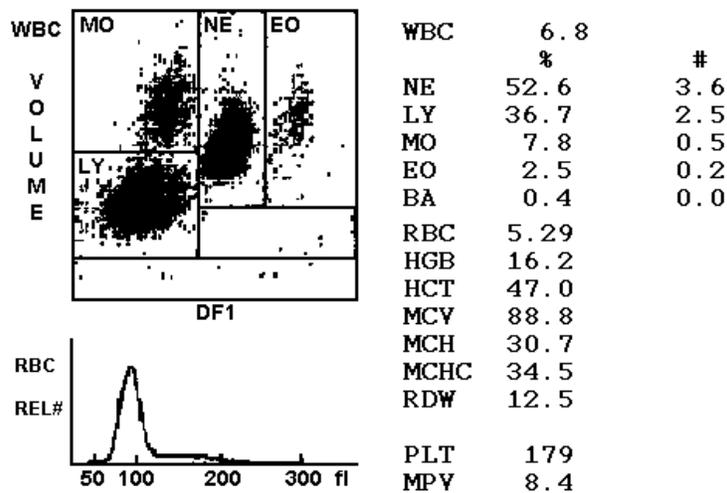
2. Count 100 “white cells” (lymphocytes and granulocytes, monocytes etc.) and categorize them into lymphocytes, neutrophils, eosinophils, monocytes and basophils. Do this for the control slide, the B and C slides and then you can make comparisons between them. Each person in the lab pair will need to make the same counts, and then the data can be shared in the lab pair. Make a judgment on the cause of infection in your discussion of your results showing the differential counts you observed.

Differential counts are done often in the hospital or at the doctor’s office if any kind of infection is suspected. What can a differential count tell you about the status of the patient? Of course it can tell you if an infection or other condition is affecting the immune system. What we are doing today is what was done in clinics before the

advent of flow cytometry to determine the health status of a patient. It is still done often for more detailed analysis if abnormalities are observed.

Pictures of a CBC and also the morphology of various white blood cell types follow on the next few pages.

Sample scan of a CBC (cellular blood count) or complete blood count done in a clinic by flow cytometry.

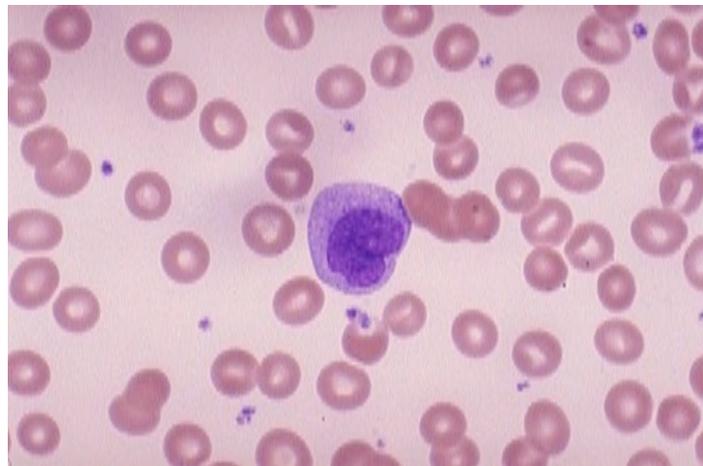


The bottom graph is a sedimentation rate calculation on RBC's. This reflects the activation of clotting factors and acute phase proteins triggered by inflammation or damage. This measurement is also called "seds" for short in the hospital. You can impress your doctor next time by asking what your CBC counts showed and what your "seds" were the next time you get a blood draw.

We will be doing flow cytometry later in the quarter.



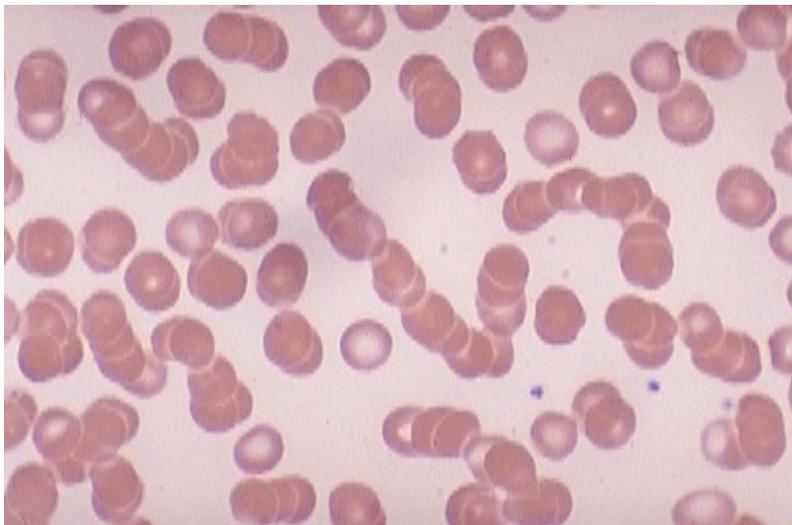
A normal mature lymphocyte is seen on the left compared to a segmented PMN on the right. An RBC is seen to be about 2/3 the size of a normal lymphocyte.



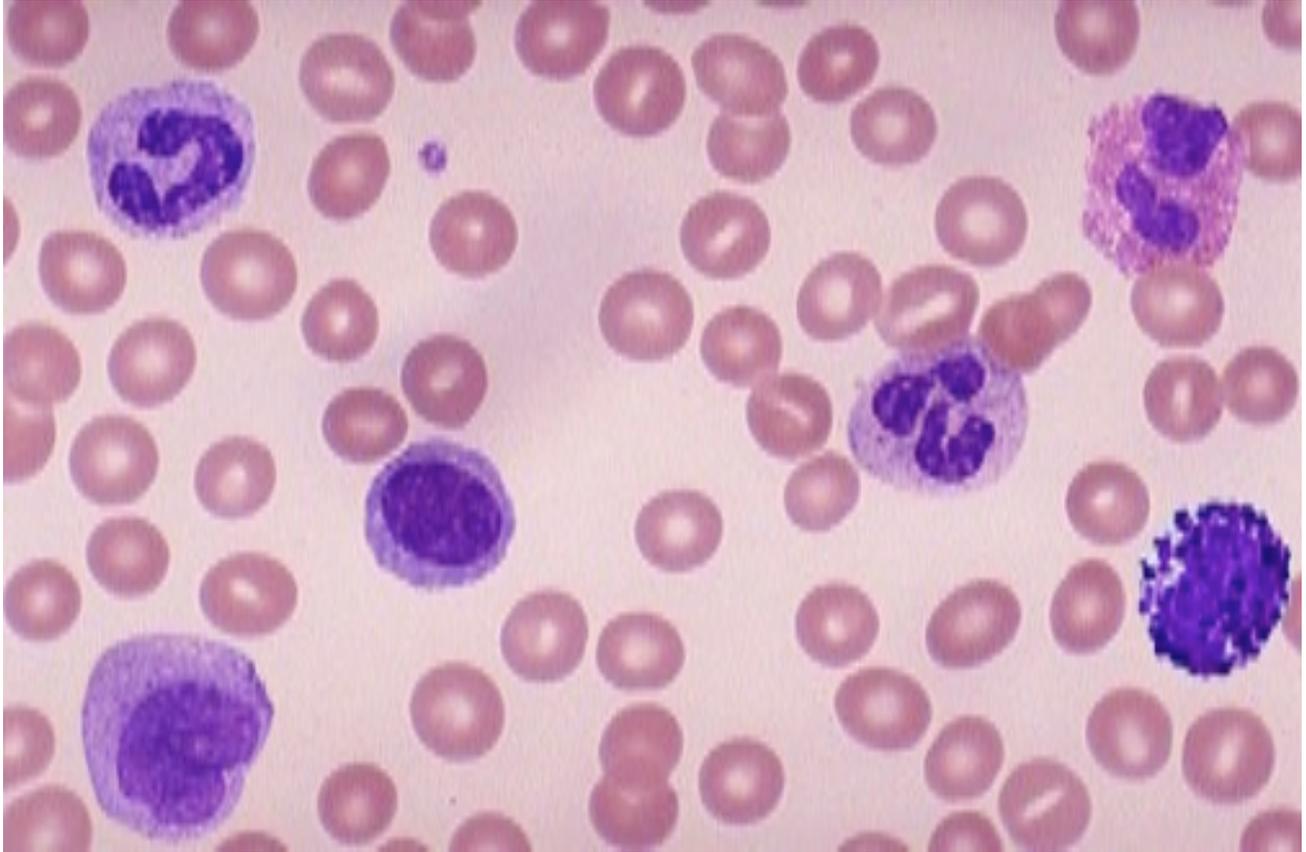
Here is a monocyte. It is slightly larger than a lymphocyte and has a folded nucleus. Monocytes can migrate out of the bloodstream and become tissue macrophages under the influence of cytokines. Note the many small smudgy blue platelets between the RBC's



In the center of the field is an eosinophil with a bilobed nucleus and numerous reddish granules in the cytoplasm. Just underneath it is a small lymphocyte. Eosinophils can increase with allergic reactions and with parasitic infestations.



The RBC's here have stacked together in long chains. This is known as "rouleaux formation" and it happens with increased serum proteins, particularly fibrinogen and globulins. Such long chains of RBC's sediment more readily. This is the mechanism for the sedimentation rate, which increases non-specifically with inflammation and increased "acute phase" serum proteins.



Identify the segmented neutrophil, band neutrophil, lymphocyte, monocyte, eosinophil, basophil, and platelet in the image above:

Answers:

Band neutrophil: upper left corner.

neutrophil: right middle

lymphocyte: left middle

monocyte: lower left corner

eosinophil: upper right corner

basophil: lower right corner

LAB #2 PHAGOCYTOSIS

In this lab we will examine the role of **macrophages**. In addition to being important in the initiation of a specific immune response (antigen processing and presentation, secretion of factors), macrophages are also potent effector cells. In their capacity as phagocytic cells, macrophages actively ingest and digest such varied antigens as whole pathogenic bacteria and parasites as well as injured and dead host cells. The process of phagocytosis involves several steps. The macrophage is attracted to and moves toward the foreign body by a process called chemotaxis. Following attachment of the antigen to the macrophage cell membrane, it is engulfed within a membrane-bound structure called a phagosome. As the phagosome moves toward the cell interior it fuses with a structure called a lysosome, which releases a variety of destructive agents, including hydrogen peroxide, oxygen free radicals, nitric oxide, peroxidase and lysozyme. Once these compounds have destroyed the contents of the phagolysosome, they are eliminated by exocytosis. Although this process is highly effective for dealing with most pathogens, there are certain intracellular pathogens which have adapted and can even multiply within phagocytic cells (ex. *Listeria monocytogenes*, *Salmonella typhimurium*, *Neisseria gonorrhoea*, *Mycobacterium sp.*).

There are a wide variety of assays for examining the killing activity of macrophages. They include detection of the products of respiratory burst (hydrogen peroxide, superoxide anion), migration and chemotactic assays, measurement of a chemiluminescent response (from reactive oxygen species) and assessment of actual killing efficiency. In this lab we will use a microscopic measurement of phagocytosis - ingestion of microorganisms to determine phagocytic efficiency. We will be using a macrophage cell line (RAW 264.7) and assessing phagocytic activity by monitoring the uptake of stained yeast cells and we will also examine phagocytosis of IgG-opsonized

sheep red blood cells. The yeast particles will also be either uncoated or treated with complement rich serum to examine the impact of complement on phagocytosis. The sheep RBC serve as a model particle that when recognized as foreign by antibody will be taken up by Fc receptors for IgG expressed on macrophages. We will examine uncoated versus IgG-coated RBC to demonstrate how antibody opsonization of particles works as an immune mechanism of clearance of foreign particles.

Protocol:

1. Obtain a 24 well plate with macrophages already seeded and growing in the bottom of the wells on glass coverslips. The macrophages are adherent cells and will stay on the coverslips as long as they do not dry out during washes.

2. A. To 2 wells add 2 μ L of yeast cells stained with Congo Red (concentration 9×10^8 yeast cells/ml). To a second set of wells add yeast that have been incubated with complement rich serum.

B. Sheep RBCs have been prepared in advance as either uncoated or opsonized with IgG. Add 10 μ l of uncoated RBCs to two wells, and 10 μ l of IgG-opsonized RBCs to a second set of wells. Make sure to label the wells with the type of particle that was added by writing on the cover of the plate. At this point the plates are spun gently to settle the particles nearer to the macrophages on the bottom. The plates will be spun at 850 rpm in the allegra centrifuge. See a TA or instructor so we can load several plates at a time.

3. Incubate the mixture of macrophages and particles at 37°C for 30 minutes.

Aspirate off the liquid and wash the wells with 400 μ l PBS to remove excess particles.

For the yeast containing wells, place 8 μ L of Trypan Blue (0.2%) on two spots on a microscope slide. Place the coverslip on the trypan blue liquid on the microscope slide

using fine forceps with the cells facing down, and then seal the edges of the coverslip using nail polish. Place on the microscope stage and count the cells (40X).

4. You and your partner should then work with the RBC containing samples. For these wells, after being washed with PBS as above, you need to remove extracellular RBC. This is done with a quick hypotonic lysis of the RBC and not the macrophages, due to structural differences between the cells. You must use a timer for this step and act quickly or the samples will be lost. Set the timer for 30 seconds, then aspirate the PBS, and add 400 μ l of pure distilled water to the wells for 30 seconds. Aspirate *gently* and add back PBS to restore the osmotic balance. The coverslip can now be removed with forceps and placed on a 8 μ l drop of PBS on a slide, as done in the yeast samples. *No* trypan blue is added to these samples. Make sure to seal the coverslip to the slide using nail polish so it does not dry out. On a microscope, look for red RBCs within the macrophages to observe phagocytosis at 40X.

5. Count 100 cells randomly to determine the % exhibiting phagocytic activity, and at the same time count the number of yeast cells or RBCs per phagocyte to determine the phagocytic index. The phagocytic index is the number of particles internalized by 100 macrophages that you counted.

6. Data analysis is up to you on this one! Think of the best way that you can express the differences. Remember, more is not better; be succinct and thorough! (Hint-a graph might be nice!)

Materials:

24 well plates with RAW 264.7 cells
37°C incubator
allegra centrifuge
alcohol squirt bottle
rack - 1/pr Eppendorf tubes and rack
P20,P200, tips, and p1000 tips
Microscope slides
Round glass coverslips
Phase microscopes
Trypan Blue (0.2%),
Microfuges
yeast and RBC particles
phosphate buffered saline
distilled water

LAB #3 NITRIC OXIDE PRODUCTION IN RESPONSE TO MICROBIAL STIMULI

Macrophages and other cells of the innate immune system often respond to pathogens by inducing various anti-microbial defense mechanisms. We previously have examined phagocytosis, which is an important means of ingesting and clearing microbes by macrophages. In this lab we will examine one of the antimicrobial defenses used by macrophages to kill pathogens, namely production of nitric oxide. Nitric oxide is highly toxic to most microbes and is produced in the macrophage by nitric oxide synthase in conjunction with NADPH and oxygen. Production of nitric oxide is often associated with phagocytosis or endocytosis, but can also be induced by recognition of pathogen associated molecular patterns (PAMPS). These molecular patterns recognized by innate immunity are often structural components of microbes that are not found in mammals, such as LPS of gram-negative bacteria, peptidoglycan of gram-positive bacteria, or dsRNA of viruses. One important family of receptors found on macrophages that can recognize these patterns is the Toll-like receptor family. These receptors can stimulate macrophages to induce anti-microbial defenses and increase other immune responses that lead to clearance of the pathogen. We will test in this lab how different PAMPS trigger nitric oxide as compared to whole pathogens. One of the interesting concepts of this system is that different pathogen types may trigger a response tailored to the unique attributes of the pathogen. Thus in this lab we will test whether there are differences in how various PAMPS induce nitric oxide production.

Protocol: Note- this is a two day lab. Today will be less than 2 hours. You will need to come back on Friday and analyze the samples which also should take less than 2 hours time.

Wednesday:

1. Obtain a 24 well plate that contains macrophages already growing in media in the wells.

2. Label 6 wells with the appropriate name of the stimulus used. They will be as follows:

- A. control-nothing added
- B. heat killed yeast
- C. heat killed bacteria
- D. LPS
- E. Peptidoglycan
- F. DsRNA mimic poly-IC

3. Obtain the appropriate tubes of PAMPS used to stimulate the macrophages. You will need to add the PAMPS using sterile technique in the flow hood. If in doubt about a pipet, get a new one. Never go back into the stock solutions with a used pipet.

- A. nothing added
- B. 10 μ l of heat killed yeast
- C. 10 μ l of heat killed bacteria
- D. final concentration of 1 μ g/ml LPS
- E. final concentration of 10 μ g/ml peptidoglycan
- F. final concentration of 10 μ g/ml poly-IC

Depending on the stock concentration of the PAMPS, you may need to make dilutions in separate tubes prior to adding to the cells. The stock concentrations will be given out in class. Ask a TA or instructor for the concentrations, and for guidance if needed in making the correct dilutions.

5. Once the PAMPS have been added to the cells, the plate will be incubated at 37 C in the cell culture incubator for two days. You will need to come back in on Friday afternoon to finish the lab and do analysis.

Friday: The Greiss reaction for detection of nitric oxide products

1. Get your plate from the cell culture incubator and take it to your bench. Label six microfuge tubes corresponding to the PAMPS used per well. To the tubes, add 250 μ l of media from the top of the well to the corresponding labeled tube.
2. Spin the microfuge tubes in a centrifuge for 1 minute to pellet any cell debris that may be present. The clear liquid supernatant will then be used to measure nitric oxide production that is released or exocytosed from the macrophages.
3. To measure nitric oxide we will detect the stable breakdown product of nitric oxide, nitrite (NO_2^-). In order to quantify the amount we need to make a standard curve using sodium nitrite. The standard samples will be sodium nitrite diluted into fresh culture media, which in this case is RPMI to match the media the cells were grown in. The stock nitrite is 0.1M, and you will need to make a dilution series starting at 50, 25, 12.5, 6.25, 3.13, 1.56, and 0 μ M. This is done by diluting the 100 μ M tube by 1/2 concentration steps in RPMI. The 0 standard will be RPMI alone. The minimum volume of each standard must be 200 μ l, but can be more depending on how you do the dilution series. If you are stumped on the standards, you can ask for help.
4. Obtain a 96 well plate and designate the left 3 columns by 8 rows for the standards, labeling the lid with the concentration of the standards. You will do each standard in triplicate, each row has one standard for 3 wells across. Add 50 μ l of standard per well across 3 wells in a row. Follow this for each standard. DO NOT PUT BUBBLES INTO THE WELLS! Do NOT press past the stop on the pipette so as to avoid bubbles which will throw off the plate reader absorbance.

Mark an area of 18 wells total for the experimental samples, using 3 columns and 6 rows for this area. You will be doing triplicates of each of your 6 samples, thus the 18 wells. Take 50 μ l of sample and add it to a row of wells, 50 μ l per well for 3 wells of the same sample. Then move down a row and do the same for each new sample of the 6 total. Label the rows with the stimulus used on the lid of the plate.

4. Add 50 μ l of the sulfanilamide solution to each sample well, then incubate under foil or in a drawer away from light for 5 minutes.
5. After 5 minutes, add 50 μ l of the NED solution to the sample wells and incubate in the dark for 15 minutes.
6. Look at the plates, you should be getting a magenta color in the concentrated standards and also some color in the experimental samples. The colored product will start to fade after about 35 minutes, so keep the plate in the dark after checking it and before it is run on the plate reader. The plate is ready to read in the plate reader which will scan the absorbance at the 520nm wavelength and quantify the amount of product produced.

7. After getting the numbers back, you can generate a standard curve using the average of the three wells for the absorbance vs. nitrite concentration. After finding the linear fit for the standards, you can then determine the amount of nitrite in the experimental samples. This is best done by graphing the results, either by hand or with excel. The graphs will be important for your lab report.

LAB #4 MACROPHAGE INTRACELLULAR KILLING OF BACTERIA

Macrophages are tissue resident cells that deal with maintaining tissue homeostasis and providing immune defenses against invading microorganisms. One important function that we have observed in Lab#2 is phagocytosis, where macrophages engulf particles like yeast, IgG coated cells, and other particles for clearance from the body. The second part of the process is the actual breakdown of the particle, and in the case of bacteria involves several mechanisms designed to kill off the bacteria. One mechanism that we have measured in Lab#3 is the production of nitric oxide, a nitrogen based free radical that is very effective in killing most bacteria. Today, we are looking at the end point of all the mechanisms induced during the phagocytosis and clearance of bacteria, including nitric oxide, superoxide, antimicrobial peptides, acidic pH, and enzymatic digestion in terms of the effect on killing bacteria. We will measure the ability of macrophages to kill bacteria that have been phagocytosed over 2 hours time, and then enumerate the surviving bacteria by lysing the macrophages and plating the suspension on LB agar plates to allow bacterial growth to occur.

The experimental hypothesis that we will test is what is the effect of cytokines that act on the macrophage externally to modify the killing ability against bacteria. The cytokine that we will be using is Interferon gamma, or IFN- γ . IFN- γ is a cytokine produced by activated CD4 T helper lymphocytes, CD8 cytotoxic lymphocytes and Natural Killer cells generally. It acts on these cells and other cells such as macrophages to induce several anti-microbial mechanisms such as superoxide, nitric oxide, and phagosome maturation on several levels. We will compare the bacterial killing of macrophages treated with or without IFN- γ in today's experiment.

Protocol:

1. You will receive a 24 well plate with macrophages growing on the bottom layer. There will be 6 wells with cells in them, 3 will be primed with IFN and three will be untreated. Make sure you pay attention to which ones are marked IFN.
2. You will get a tube with E. coli bacteria in PBS at a known concentration. You will need to make a dilution in complete cell culture media with bacteria to achieve the desired number of bacteria per well of macrophages. Each well of macrophages will be cultured with 1ml of media with bacteria added, so you will need 7mls of media and calculate the number of bacteria for 7 wells so you have extra for the cells.

The concentration of the E.coli and the number needed will be given out the day of class.

3. Using a P1000 pipette, remove the existing media from the wells. Quickly add 1ml of media containing the correct number of E. coli per well. Cover the plate with the lid after adding the media.
4. The plate now needs to be spun in a centrifuge to allow the bacteria to concentrate at the bottom of the well on top of the macrophages. This allows more efficient phagocytosis to occur. The plates are spun at 1000rpm for 4 minutes. Following the spin, the plates are put into the 37C CO2 incubator to allow phagocytosis to proceed for 1 hour.
5. After 1 hour, you will get your plate out and remove the media with a P1000 pipette. Then add 1 ml PBS to wash the wells gently, to remove excess unbound bacteria.

6. After removing the PBS wash with a pipette, add 1ml of complete media with 50ug/ml gentamicin antibiotic in it. Cover with the lid, and return the plate to the 37C CO₂ incubator for 1 hour. This kills off bacteria that are not inside of macrophages, but does not effect internalized bacteria
7. Get the plate, remove the media with a pipette, and add 1ml of PBS to wash the cells. Remove media and repeat the wash again with PBS.
8. You now need a tube of 0.1% Triton x-100 in water. Remove the PBS wash, and then add 300ul of 0.1% Triton x-100 to each well of the plate. Rock the plate to mix, and let stand for 10- 15 minutes minutes to lyse the cells and release any surviving bacteria.
9. You now need a tube of LB bacterial growth media and some LB agar plates. Add 700ul of LB liquid media to the wells to bring the volume up to 1 ml total in each well. Get your Bunsen burner on and flame your spreader, or use a sterile disposable spreader.
10. You will now need 12 eppendorf tubes. Add 900ul of LB to each tube. Label 6 tubes IFN and three control. For each well in the experiment, you will have a 1:10 tube and a 1:100 tube for dilutions. You will make 2 dilutions: the first is 1:10, and the second is 1:100. Now add 100ul of the lysate from the well in step 9 to each of the corresponding labeled tubes of 1:10 dilution. After mixing, plate 100ul of this on an LB agar plate. Then take 100ul of the 1:10 dilution and add that to a corresponding 1:100 tube. Mix and plate 100ul for the 1:100 tube.

11. Put the agar plates in a 37C standard incubator for bacteria. Next time you will count the colonies on the plates to determine the surviving CFU/ml of bacteria for the control versus the IFN treated cells.

This assay is a classic method used to determine killing ability in all types of animals and humans. It is known as an intracellular killing assay. The assay can tell you if there are factors present such as cytokines, drugs, or toxins etc. that are either activating the immune system or suppressing it. A similar assay using whole blood can also be done to measure these same parameters, but it does not focus on intracellular killing. Make sure you understand the steps in the protocol that make this an intracellular killing assay as opposed to extracellular.

Materials:

24 well plate with macrophages on bottom

E. coli bacteria in PBS.

P1000 and p200 pipette and tips

Waste bucket for media

RPMI media

RPMI media complete for cell growth

RPMI media + gentamicin

Eppendorf tubes

LB liquid media

0.1% Triton X-100 in water.

LB agar plates

LAB #5 SDS-PAGE AND WESTERN BLOTTING

Antibodies are widely used in industrial, clinical, and academic research as tools for the detection, quantification and analysis of specific protein molecules or complexes. The widespread use of antibodies in research is a direct consequence of the specificity of an antibody for its antigen and its ability, once secreted, to interact with that antigen in the absence of other components of the immune system. One of the most powerful analytical procedures involving antibodies is **immunoblotting** (sometimes called **Western blotting**). In this procedure, a complex mixture of proteins (e.g., serum, lysates of specific cell types or lines, lysates of virus-infected cells, gene-manipulated cells, bacteria, and virus particles) is separated by electrophoresis through a polyacrylamide gel. Once separated the proteins are transferred to a nitrocellulose sheet, again by electrophoresis. After transfer to the nitrocellulose sheet, the sheet, or filter can be incubated with specific antibody, which will bind only to the protein antigen under investigation. The bound antibody can then be visualized by a variety of techniques.

Molecules which carry a net charge at the pH of the buffer system used will migrate when an electric current is applied across a matrix (electrophoresis). The rate of movement will depend on the charge density (the ratio of charge to mass); the higher the ratio of charge to mass, the faster the protein will migrate. In addition, the direction of migration will depend on the polarity of the charge; a protein with a net positive charge will migrate towards the anode, and a protein with a net negative charge will move towards the cathode. As different proteins will vary considerably in their charge density and polarity at a given pH, these features will interfere with separation on the basis of molecular weight. To counter this, protein mixtures are boiled in buffer containing sodium dodecyl sulfate (SDS) prior to electrophoresis. SDS is a powerful

ionic detergent which will bind polypeptides in a constant weight ratio (1.4 g SDS to 1 g of protein). SDS carries a very large negative charge density, and the density and polarity of the charge intrinsic to the polypeptide is negligible in comparison. Thus proteins dissolved in SDS in this fashion will migrate towards the cathode in an electric field and will be separated on the basis of molecular weight alone.

Electrophoresis of proteins is most commonly carried out in a gel matrix of polymerized acrylamide (polyacrylamide), hence the term **SDS-PAGE (SDS PolyAcrylamide Gel Electrophoresis)**. In addition to SDS, the buffer in which the samples are boiled (sample buffer) usually contains b-mercaptoethanol (2 ME) or dithiothreitol (DTT). These agents are termed thiol reagents and cleave the disulfide bonds that stabilize the tertiary structure of some polypeptides and maintain the associations of polymeric proteins. This is usually an important feature of sample buffers because a tertiary folded structure can distort the migration of a protein through the gel, precluding accurate molecular weight determination.

Once the proteins in the mixture have been separated by SDS-PAGE, the gel can be laid flat against a sheet of PVDF. If an electric field is established across the gel and PVDF, running from negative to positive from the gel to the PVDF, the separated proteins in the gel will transfer to PVDF, forming an identical pattern on the PVDF sheet to that found on the original gel. The proteins bind to the PVDF sheet by nonspecific hydrophobic interactions. The remaining capacity of the PVDF to bind proteins nonspecifically can be taken up by incubation with an excess of an irrelevant protein such as gelatin or milk. Subsequently the PVDF filter (or blot) is incubated with antibody recognizing the protein of interest. If the protein was present in the initial sample that was applied to the gel, the antibody will bind to it. In most western procedures, a second antibody, which specifically recognizes the first and which has been conjugated to an enzyme such as alkaline phosphatase or horseradish peroxidase is applied. The second antibody binds specifically to the first, and in the final step a

chromogenic substrate for the conjugated enzyme is applied. Color or luminescence develops only at the points on the blot where the conjugated enzyme is bound.

In this lab you will determine whether the antibody you have is specific for HEL (hen egg lysozyme) or OVA (chicken ovalbumin).

Protocol: SDS-PAGE and Western Blot

1. Gels for SDS-PAGE are already cast and assembled in the gel holders.
2. Solutions of MW (molecular weight markers), HEL and OVA are already dissolved in reducing sample buffer and have been boiled.
3. Each gel has 15 wells. Each group will have 3 wells; therefore, only 4 groups will use any given gel. Do not use the outermost well on either side. Load **5 μ l of MW standard** into the left-most available well. Load **10 μ l of HEL** into the adjacent well to the right and **10 μ l of the OVA** in the well to the right of that. **Check with an instructor before you load the wells.** Everybody will be extremely unhappy with you if you load in the wrong ones.
4. When the gels are loaded they will be connected to the power packs and electrophoresed for 40 minutes to 1 hour (200V).
5. When the gels have run to completion the instructors will remove them from the gel boxes and assemble them into the western blot apparatus.
6. The western blots will be electrotransferred overnight at 4C onto the membranes, after which the gels will be discarded. The PVDF membranes will be cut to give each group one filter which contains the three lanes they loaded. After the western blot apparatus is opened, the filters must not be allowed to dry out. If they dry out before the entire procedure is complete, they can be rewetted in methanol.

TAs will do this: The strips will be blocked for you by the TA's. IF you needed to do this yourself you would have done the following:

7. Obtain a plastic box, add a small amount of **TBS buffer** and bring this to the front collect your PVDF strip. Pour off the buffer into the sink and add more from the bottle (~10 ml). Wash on the shaker or by rocking for 2 minutes.

8. Pour off the buffer into the sink and add 10 mls of TBST with 5% nonfat milk for use as a blocking agent. Close and label the box and place it at 4°C until the next lab period.

Next Lab period:

9. Pour off the blocking solution (or transfer to a fresh box) and wash the filter twice in **10 ml of TTBS** for 1 minute each time on the shaker.

10. Pour off the last TTBS wash and add 5 ml of TTBS to the box. Get your tube of affinity purified antibody and add TTBS+5% milk to the tube to reach a 15ml volume. Pour off the TTBS in the box and add **the full 15ml of antibody to the blot**. Place the box on the shaker for 1 hour.

11. Pour off the antibody solution and wash the filter with **TTBS** five times for 5 minute each time on the shaker.

12. Pour off the last TTBS wash and add **15 ml of second-step antibody solution** (1:10,000 dil.) diluted in blocking buffer to the box. Place on the shaker for 45 minutes.

13. Wash three times for 10 minutes in **TTBS** on the shaker.

15. Leave the blot in TTBS, and get ready to fieldtrip to the darkroom.

16. If you were doing this part you would do the following: I will do this with you:

The ECL reaction is rapid, so don't mix the two solutions together until you have plastic wrap ready, a film cassette handy, and we are ready to go to the darkroom to load film into the cassettes. Once the solutions are mixed, soak the blot in a plastic tray for 2-5 minutes and then remove it from the solution and place it flat onto plastic wrap and cover it. Load the blots into the cassette, and then we will head to the darkroom to load film, and develop our results. From the results, determine which specificity of antibody you have.

Materials

6 10% polyacrylamide gel + gel box

Power pack

Western blot apparatus

MW markers (5 μ l/pair, in 25 μ l aliquots)

Ova (10 μ l/pair, in 50 μ l aliquots, 1 mg/ml, in sample buffer)

HEL (10 μ l/pair, in 50 μ l aliquots, 1 mg/ml, in sample buffer)

PVDF filters

p200, p20 and tips, gel loading tips

5 ml pipettes - canisters and Incubation trays

SDS-PAGE running buffer (192 mM Glycine, 25 mM Tris, 0.1% SDS, pH 8.5)

Western transfer buffer (48 mM Glycine, 39 mM Tris, 20% methanol)

TBS (20 mM Tris, 500 mM NaCl, pH 7.5) - 30 ml

TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween-20, pH 7.5) - 100 ml

BCIP/NBT solution - 15 ml

saran wrap

Lab #6: T CELL & B CELL ACTIVATION AND ANALYSIS BY FLOW CYTOMETRY

Lymphocyte activation is critical for a functioning immune system. What do T cells look like when they get activated? This is the question that we are asking this week. What are some of the changes that T and B cells undergo for appropriate activation? The easiest way to attack this issue is to think about the circumstances where T and/or B cells become activated. For example, what happens during a viral or bacterial infection? Lets examine the common cold. A Rhinovirus will infect cells in your upper respiratory tract and lead to symptoms that we all know!

How does the immune system respond to this situation? The first thing that happens is that the viral proteins are processed and presented by the many cells that line the respiratory tract. Antigen presenting cells (APC) can migrate into LN in order to activate T cells. These cells can then present viral peptides to CD8 bearing T cells in the context of MHC class I and to CD4 T cells in the context of MHC class II. Thus, as T cells circulate through the blood and lymph they will be exposed to APC. If the T cell receptor (TCR) that they bear can interact with the MHC molecules on the APC with high enough affinity, then activation will ensue. What happens when T cells get activated?

CD4 T cells begin to make cytokines which promote growth and differentiation. B cells begin to secrete immunoglobulins (Ig), mainly IgM, and also begin to undergo somatic hypermutation. Additionally, they will also undergo a class switch. CD8 T cells, on the other hand, will become activated and begin to destroy the virally infected cells as long as they can recognize the viral peptide that is presented by the cell. Moreover, all three cell populations will undergo **Blast Transformation**. This is a state when the cell becomes larger than normal and is an excellent indication of activation.

Of course this may be an oversimplification, but in general this is an accurate account. The details of these events are constantly being examined and rewritten.

Another aspect of activation that is very critical is the fact that as lymphocytes become activated they do not return to the LN, but instead penetrate into tissues. Think carefully about this situation: why would they return to the LN when all of the action in this circumstance is in the upper respiratory tract? Therefore, one of the first events after activation is the downregulation of a molecule that is used for LN homing. CD62-L is referred to as a "Selectin" molecule that is used by lymphocytes to bind to high endothelial venules which allow penetration into the LN.

One of the most important events during lymphocyte activation is the secretion of growth factors. For lymphocytes to clonally expand it is essential that growth factors are produced. In this experiment we will examine the expression of CD25. This molecule is one of the three components of the interleukin-2 receptor (IL-2R). You will stain cells today before and after activation for expression of this molecule and the cells will be analyzed by flow cytometry. How do you think the cell populations will compare?

The idea of this experiment is to examine what T and B cells look like before and after activation. In these experiments we are using several ways to activate lymphocytes: bacterial lipopolysaccharide (LPS), and concanavalin A (Con A). You will be staining T cells with CD4 and B cells with CD19 using fluorescein isothiocyanate-(FITC) labeled antibodies and either CD25 or CD62-l that is conjugated to phycoerythrin (PE). The anti-CD19 antibody recognizes part of the B-cell receptor complex found only on B cells and B cell precursors. CD4 of course recognizes part of the TCR on T-cells. The cells will be examined by flow cytometry. Follow the directions carefully and once again please cooperate with the other groups in sharing tubes and reagents.

Flow cytometry

Flow cytometry involves sensing particles as they move in a liquid stream through a laser beam. When particles pass through a beam, they scatter light, and if a fluorescent dye has been attached the particle will give off its own light. The scattered and fluorescent light is collected by detectors which translate the light into an electronic signal proportional to the amount of light collected. This information is displayed by the attached computer so that the populations can be analyzed. Flow cytometry analysis has become a routinely used procedure for determining whether or not a population of cells is expressing a given molecule or marker on the cell surface. It is also used to determine the proportion of cells in the population expressing the marker and to sort the expressing cells from the non-expressing cells to give a population of 100% positive or 100% negative. In addition, it can be used to distinguish and sort cells on the basis of relative levels of expression of a given marker. Also, flow cytometry can be used to analyze cell cycle and apoptosis.

Generally, but not always, flow cytometry analysis involves the use of an antibody specific for a cell-surface marker; however, this is not the only connection the procedure has with immunology. The procedure has been incredibly important in immunological research since the early 1980's when the procedure was developed. Flow cytometry analysis has facilitated the recent explosion of data that has been obtained on both T cell and B cell biology and development through its ability to tell us what markers are expressed on what immune cells, and when they are expressed during development. It has furthermore allowed us to correlate expression of given markers with the function of given cell types. Using the flow cytometer, we can isolate and purify cells expressing a marker for which we have an antibody, so that the function and physiochemical characteristics of the cells can be studied in isolation from other cells. For this experiment we will only analyze the cells and will not collect them.

I.) Flow cytometry lab

Today we are asking, "What is the phenotype of unstimulated T and B cells versus those that are activated by different methods."

1. Your group will receive one tube that have a mixture of spleen cells already in them. The tubes will be labeled A, or B,. The identity of treatment of the cells you have is unknown. Remember to write down the letter code of the tube you have, it is **very important**.

2. You will need to aliquot 100 μ l of cells into 5 tubes per letter code. One person will stain 3 tubes, the other 2 tubes. Person 1 will stain with the following set of antibodies:

1. CD4 + CD62L
2. CD19 + CD62L
3. unstained control

Person 2 of the group will stain with the following antibodies:

4. CD4 + CD25
5. CD19 + CD25

To stain the cells, first the cells will need to be spun down in the microcentrifuge at 3000rpm for 2 minutes. Make sure to team up with other groups so we can spin several tubes per run. After spinning down the cells, use a pipetman and by hand remove the media from the well, making sure not to take the cells with the media. You can leave some liquid in the bottom of the tube, it is better to do so than to suck out the cells which ends your lab experiment at that point. Add 100 μ l of staining buffer to the wells, and gently pipet up and down a few times to put the cells in suspension.

3. Now add 2 μ l of the appropriate SET of antibodies, 1 μ l of each antibody to the tubes of cells. Notice that one antibody is FITC conjugated while the other of the pair is

PE conjugated. How will FITC and PE be identified by the cytometer, and why do you use separate colors? These questions will be answered during our presentation on flow cytometry.

4. The cells will be incubated with the antibodies on ice, and wrapped in foil to preserve the fluorescent tags of the antibodies. We will incubate for approximately 45 minutes to 1 hour. There will be a presentation on flow cytometry at this time.

5. Spin the tubes down again, then remove the liquid by hand as before with a pipetman. Add 100 μ l of staining buffer to wash the cells, and gently resuspend as before. Spin down again, remove liquid by hand, then resuspend the cells in 400 μ l of PBS staining buffer. The cells will be transferred to FACS tubes at this point. Label 5 FACS tubes with your letter code, and the number code (1-4) corresponding to the antibodies you stained with. Store the tubes on ice for our trip over to the FACS machine in ALS or to the one in Nash.

6. Your data is to be shared with everyone. The raw data will be posted in Canvas with the A or B treatment code next to group names. You will determine by looking at the different plots what type of cells were activated in your group and make an informed conclusion of what the stimulus was in the cells you stained. **The stimuli used act differently, with ConA activating mainly T-cells and LPS activating mainly B-cells.** The focus of this lab is to gain experience in drawing conclusions based on data you gain from an experiment. This reflects the real world in terms of how science is practiced, since there are many unknowns, yet experimental evidence can help solve these unknowns. Enjoy the mystery.

Stimuli used: Concanavalin A or Lipopolysaccharide

LAB #7 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Prior to the development of the **enzyme-linked immunosorbent assay**, or **ELISA**, most quantitative immunoassays involved the use of radiolabeled antibodies or antigens. Instead of radioisotopes, ELISAs use an enzyme substrate system in which either the antibody or antigen is chemically conjugated to an enzyme. The enzyme reacts with a substrate to give a colored product. As with western blots, the enzyme conjugates generally used are alkaline phosphatase or horseradish peroxidase. However, the substrates differ between the two procedures. In western blots a substrate is used which generates either an insoluble colored product or a luminescent product that localizes at the exact location of the bound antibody on the nitrocellulose filter. For ELISAs a substrate that generates a soluble colored product is used. In solution this distributes evenly through the sample, permitting the absorbance or optical density of the sample to be read in a spectrophotometer or a colorimeter with appropriate filters.

In this lab we will perform an ELISA with the purpose of detecting important cytokines produced by lymphocytes.

Proteins in solution tend to adsorb non-specifically to plastic and at a pH close to neutral, the association is essentially irreversible. Thus the bound antibody will not come off the plate during the subsequent procedures. To take up the excess binding capacity of the plate, it has been further incubated with an irrelevant protein (fetal calf serum), to preclude nonspecific association between the antibodies that you will use and the polystyrene matrix of the plate. The method we will use is known as a sandwich ELISA, since we use first a capture antibody coating the plate, then we incubate with the cytokine containing cell culture medium resulting in binding of the

capture antibody to the cytokine. Then we add a secondary antibody that can recognize the cytokine, to which a labeling group is attached, such as biotin. We then add an enzyme such as HRP coupled to avidin which binds the biotin on the secondary antibody. A substrate is then added as the final step for the HRP enzyme to catalyze a color change that we read in a plate reader to quantify the amount of bound cytokine.

CYTOKINES

A dominant feature of an immune response is the ability of cells to communicate with each other by interaction through soluble proteins called cytokines. These are molecules that are secreted by a wide variety of cell populations. Many of the cells of the immune system as well as other cells like fibroblasts, epithelial, endothelial and neurons produce these factors. Cytokines influence cell growth, differentiation, survival and death. Cytokines secreted by one cell can have effects on other cell populations as well as the cell that secreted the cytokine. This is referred to as autocrine production of the cytokine, i.e., the cell uses the factor which it secretes.

There are numerous examples of cytokines which function in the immune system. Many of these factors are divided into families and even sub families. For example, the interleukin-2 (IL-2) family of cytokines includes: IL-2, IL-4, IL-7, IL-9 and IL-15. Interestingly, these factors all use a similar receptor complex. The receptors are made up of two or three chains, an α/β and a common γ chain which is utilized in all receptors for these cytokines. The importance of the common γ chain is borne out in studies which have shown that mice deficient for this molecule produce very few lymphocytes in early development. Thus, these data and other data have shown that the IL-2 family is critical for cell growth. Other families of cytokines include the Tumor Necrosis Factor/Nerve Growth Factor (TNF/NGF) superfamily of receptors. This family is responsible for the many cell death and survival signals in the immune and nervous

systems. The interferon (IFN) receptor superfamily includes: IFN α/β , and IFN γ . The IFN molecules are intimately involved in viral resistance and inflammatory responses. IFN γ is important in activating cellular immunity and is a hallmark of Th1 responses.

Many cells of the immune system synthesize these factors, but T helper cells are clearly the main producers. The name "T helper" is derived from the fact that these cells provide help for other cells to respond appropriately. The best example of this situation is the polarization of Th1 and Th2 cells. In 1986 a pinnacle paper published by Drs. Timothy Mosmann and Robert Coffman (Journal of Immunology 1986, **136**:2348-2357) showed that T helper cells could be divided into two distinct groups: Th1 cells, which mainly secrete IFN γ and IL-2, and Th2 cells, which produce IL-4, IL-5 and IL-10. It is now very clear that Th1 cells support activating macrophages and other inflammatory cells and can stimulate B-cell to make antibodies.. On the other hand, Th2 cells provide cytokines for optimal B cell growth and differentiation and are often associated with allergic responses. Thus, this type of cytokine cross-regulation is very important for the immune system.

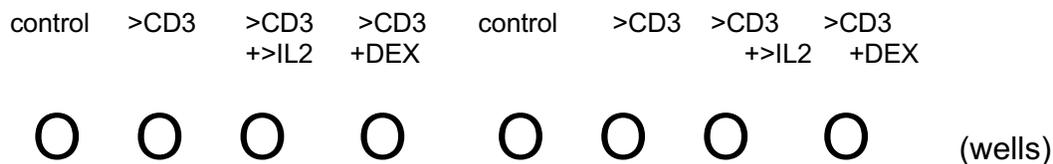
What are the critical factors that enhance T cell-derived cytokine production? This question boils down to two elements: Antigen and costimulation. T cells need antigen to recognize the fact that they should become activated, and they need costimulation which provides them with the necessary internal signal to produce cytokines. The major costimulatory signal is clearly CD28. This molecule is expressed on all T cells and its expression is upregulated when T cells "see" antigen. With antigen and CD28 ligation T cells can go from a naive resting state to an activated state. It has been shown by several laboratories in vitro and in vivo that two signals lead to activation, whereas one signal does not. It is worth mentioning that the ligand for CD28 is B7 which is mainly expressed on antigen presenting cells. What about T cells that have already been activated? This is more complex and is something that we are going to test in the experiment for this lab.

Today you will receive splenocytes from mice that are undergoing a Th1 response versus mice that are undergoing a Th2 response. The Th1 response was generated by injecting mice with complete Freund's adjuvant containing portions of mycobacterial cell walls. Mycobacteria are obligate intracellular pathogens that potently induce Th1 responses. For a Th2 response, mice were injected with irradiated schistosome eggs, which should induce potent Th2 responses. You will re-stimulate these already primed cells with nothing or anti-CD3 and examine IFN γ and IL-4 production. Of course, the mystery will be which type of T-helper cells you are working with, which the ELISA will help to answer. We will also test what effect depleting IL-2 has on re-stimulation, as well as the effect of dexamethasone treatment. Please follow the directions carefully and take your time!

NOTE: This is a two-part lab over 2 weeks. Part 1 Wednesday, part 2 next Wednesday

1. The set up for this experiment is straightforward and should not take a lot of time, but mistakes will be costly since these reagents are not cheap!

2. You will receive a tube of cells labeled A or B on ice. These contain spleen cells and this lab requires sterile technique in the flow hood. Remove PBS from all the wells. Place 200 μ l of the cells in each of 8 wells. The wells are labeled as follows:



3. Add 2 μ l of anti-IL2 antibody to the appropriate wells and remember to change tips each time. Now add 2 μ l of Dexamethasone to the appropriate wells and record how much Dex is in your wells. Remember write your name on your plate.

4. The cells will be cultured at 37°C in 5% CO₂ for 48 hours by the TAs.

5. You will then need to get another 96 well plate to prepare an ELISA plate for Friday. We will start this process by adding the capture antibody to the plates. The capture antibodies will be anti-IFN γ and anti-IL4. See below for the correct scheme. These will incubate overnight to coat the plate. You will make 8 wells with anti-IFN γ , and a second set of 8 with anti-IL4. The antibodies will be diluted 1:250 in coating buffer. Make sure to label the wells that you coat with each antibody. You coat the wells with 100ul per well the capture antibody solution. After doing this, the plates will be stored at 4C by the TA's, and will be washed and blocked by the TA's Thursday.

PART 2 next week: (This is a long lab so be prepared)

1. Get your ELISA plate, remove the sealing film, and make sure the wells are marked for the correct antibodies used. Then dump out the liquid and pat the plate on a paper

towel stack to remove the liquid. Add 300ul PBS-T to the wells using a pipette and let that soak until you are ready to add the supernatants from the cells.

2. Get your culture plate (was frozen after 2 days) that contains the spleen cells you set up on Wednesday. The plates will already have been spun down by the TA's, and you will use the clear supernatant above the cells for the ELISA. Your group's ELISA plate should have two rows for IL-4 detection and two rows for IFN detection. They should be clearly labeled. You will then add 100 μ L of the supernatants in row A directly to the plate. For row B, add 10 μ of supernatant to tubes first. Make up the total volume of the tubes to 100 μ l using 90 μ l PBS. You should actually pre-mix the supernatant and PBS in the tube first before adding the volume to the wells on the plate. After mixing, add the total volume of 100 μ l from the tube to the appropriate matching well shown in the scheme below.

control	>CD3	>CD3 + >IL2	>CD3 +DEX		control	>CD3	>CD3 + >IL2	>CD3 +DEX			
α IL4	α IL4	α IL4	α IL4		IFN	IFN	IFN	IFN			A
α IL4	α IL4	α IL4	α IL4		IFN	IFN	IFN	IFN			B

3. The plates are incubated at room temperature for 1 hour. After the incubation remove the supernatant by flicking the plate (See the TA for a demonstration) and blotting on paper towels. Immediately add 300 μ l of PBS/T using a pipette, followed by removing the buffer by flicking the plate-repeat this 3 times . Make sure the final wash stays on for about 1 minute before flicking. (CAUTION: try not to scratch the plate with your pipette tip, this can increase nonspecific binding). However, leave the last 300 μ l in the wells until you are ready for the next step. Do not let the plate dry out

4. Remove the PBST and add 100 μ l of the biotinylated secondary reagent IFN γ and the biotinylated secondary reagent IL4 to the appropriate wells. The antibodies are diluted 1:250 in PBS 10%FCS and the avidin –horseradish peroxidase is added at a concentration of 1:125. Incubate the plate for 45 minutes at room temperature.
5. After the incubation, wash the wells 5 times, this wash is very important. Make sure to have 2 washes soak for at least 1 minute each.
6. Remove the PBST and then add 100 μ l of the color development reagents (substrate) and immediately place the plate at room temperature in the dark. Always **avoid** introducing bubbles into the wells at all points from here on in the protocol. After 15 min or so examine the plate for color change. This can be quite variable and can take 30 minutes.
7. When you and the TAs have decided that there has been optimal color change add 50 μ l of the stopping reagent (2 N H $_2$ SO $_4$).
8. The plate should be read on an ELISA reader and your data will be compared to the standard curve which was generated by the TAs earlier. Thus, you will receive a list of standard concentrations vs. OD values and likely the excel file with the equation for the line. To do this standard curve you probably need to use a log scale to straighten out your line. With this information you will be able to calculate the amount of cytokine in each of your samples-if you need help ask the TAs or instructor.
9. The people that used cells A should now exchange data with the people that used cells B. What type of Th cells did you have? What were the effects of DEX, anti-CD3 and anti-CD3 with anti-IL-2? Please prepare your results in a bar graph, one with

cells A and one with cells B under the experimental conditions. Be concise in your lab report. Record the results and interpret them for the discussion.

Team events: Immuno-Olympics Challenge

Now when you are more familiar with the language and concepts of immunology, it should be a lot of fun to explore some of the current issues addressed in immunology research. You will work in **groups**. Topics from different areas within the field of immunology will be provided by the instructor.

These are the challenges facing the teams in Immuno-Olympics: All scores are tabulated to determine the winning team. This event is sanctioned by the International Committee for Immuno-Olympics and will award medals and prizes in accordance with governing organization rules. This is the premier event of the ICIO.

1. **Team concept drawing:** A topic from class will be given to the team in advance. When your team is called, you will have colored markers and will have 5 minutes to draw out on a large sheet of paper the concept given. All team members must draw some portion of the concept. Artistry is taken into consideration, as well as how well the drawing conveys the concept to the class. Following the 5 minutes, the team will explain the concept to the class.

2. **Expression session:** The team must now provide some of the following to enhance the entertaining delivery of the concept. Your team must do a minimum of 2 of the categories, of which Poetry is required. You can blend aspects of the categories together if that suits your presentation style. The categories include:

A. Theatrical performance of the concept using team members. Think one or two act play style event.

B. Poetry: Create a poem\limerick using the concept. Last year had some incredible examples, one of which will be provided for inspiration. Poetry slam freestyle concepts also allowed. Haiku's are also encouraged. 2 poems per team are required. No more than 4 poems per team allowed.

C. Song and dance routines: Musical props are allowed, as well as multimedia. Could use choreography, etc to convey the concept. Creativity is encouraged.

3. Team challenges continued: Feats of athletic ability and mental toughness.

A. Team challenge: details provided in class only.

B. Speed tip box loading: each team will be timed as to how fast they can load a single yellow pipette tip box to completion in a 2 person relay race challenge. This challenge requires dexterity and mental toughness as there are fabulous prizes on the line.

Prize categories:

Individual Awards:

1. Bravery Award

2. MVP Award

3. Team awards: Gold, Silver, and Bronze Immuno-Olympic medals

