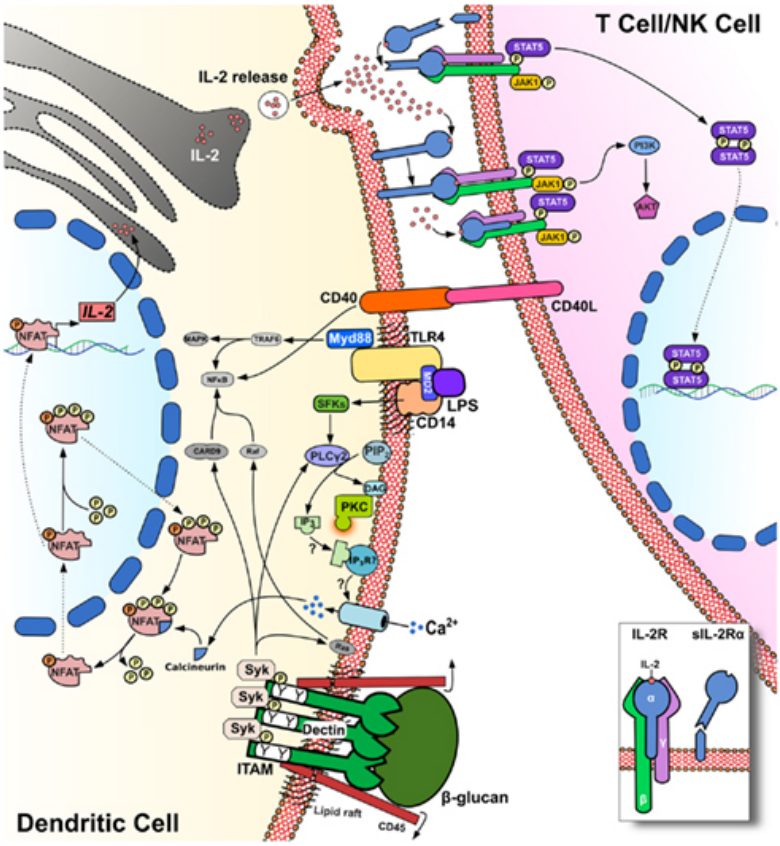
IMMUNOLOGY

BIO 463

LABORATORY EXERCISE

FALL 2018



IL-2 Production in mouse splenocytes

INTRODUCTION

Interleukin-2 (IL-2) is a potent cytokine stimulating T-cell proliferation. As such IL-2 was originally identified as a T-cell growth factor. This cytokine is produced by $CD4^+$ T helper cells, to a lesser extent by $CD8^+$ cytotoxic T cells and natural killer cells in secondary lymphoid tissues (Boymen and Sprent, 2012). Regulation of IL-2 expression occurs by multiple mechanisms, with silencing by the BLIMP1 gene. BLIMP1 is repressed by IL-2, and thus provides negative feedback signaling such that overexpression does not occur.

Dendritic cells, an important link between the innate and adaptive immune systems, are also strong producers of IL-2 in peripheral tissues. Pathogen-associated molecular patterns (PAMPs) are recognized by the pattern recognition receptors (PRRs) on dendritic cells to stimulate cytokine production. By binding to PRRs microbes stimulate the production of IL-2 in DCs, most likely through the nuclear factor of activated T-cells or NFAT pathway (Granucci *et al*. 2001). However, some microbes, such as enteropathogenic *E. coli* (EPEC) inhibit IL-2 production as part of the infection process (Malstrom and James, 1998).

IL-2 elicits many responses, from driving T-cell proliferation, the activation of natural killer cells, (NK) to stimulating apoptotic pathways. IL-2 is also responsible for maintenance of $Foxp3^+$ regulatory T cells (T_{regs}) and thus controls homeostasis (Abbas *et al*., 2018). IL-2-associated therapy has been suggested as a means to limit autoimmune disorders such as inflammatory bowel disease (Malek and Castro, 2010).

In this inquiry-based laboratory exercise you will develop hypotheses concerning IL-2 production in mouse splenocytes, and test your prediction using an enzyme-linked immunosorbent assay (ELISA). Mouse splenocytes contain a number of leukocytes, including T-cells, B-cells, monocytes and DCs.

We will use a "sandwich ELISA" in a 96-well microtiter plate format in order to test your hypothesis. For this assay, an antibody against IL-2 is coated onto the microtiter well, washed, and then the sample is placed over the antibody to bind. Excess sample is washed out, and then the same initial antibody is placed over the IL-2 bound antisera such that a "sandwich" is made. This second anti-IL-2 antibody is conjugated to biotin. The biotin is then bound to streptavidin conjugated to the enzyme horseradish peroxidase (HRP), and the quantity of IL-2 bound to the antibody can be determined after the addition of substrate using absorbance values, and comparing data to a standard curve.

Based on the references listed, other literature, and our in-class discussions, formulate ***one*** testable hypothesis concerning the production of IL-2 in mouse splenocytes. Components you will have available for testing include anti-CD3 antisera, ionomycin, PMA (phorbol 12-myristate 13-acetate), cultured EPEC bacteria, and the lectin Concanavalin (ConA) as a positive control. Make sure to include the positive and

negative controls in your experimental design, and at least three replicates per condition so that statistical analyses can be performed.

References

1. Abbas *et al*. 2018. Revisiting IL-2: Biology and therapeutic prospects. *Science Immunology*. DOI: 10.1126/sciimmunol.aat1482
1. Boyman and Sprent. 2012. The role of interleukin-2 during homeostasis and activation of the immune system. *Nature Reviews Immunology* 12: 180-190.
2. Granucci *et al*. 2001. Inducible IL-2 production by dendritic cells revealed by global gene expression analysis. *Nature Immunology*. 2, 882–888.
3. Malstrom and James. 1998. Inhibition of murine splenic and mucosal lymphocyte function by enteric bacterial products. *Infection and Immunity* 66 :3120-7.
4. Malek and Castro. 2010. Interleukin-2 receptor signaling: at the interface between tolerance and immunity. *Immunity* 33, 153–165.

This procedure was adapted from a laboratory exercise initially developed by Professor Lynn Hannum of Colby College.

MATERIALS AND METHODS – work in **pairs**

Mouse splenocytes
(stockroom will provide 96-well plates containing 200,000 cells/well mouse splenocytes)

Treatments:
Concanavalin A (ConA), 1 mg/ml, 200 µl aliquot; final concentration 10 µg/ml
Anti-CD3 antisera, 1 mg/ml, 10 µl aliquot
Ionomycin, 1 mg/ml, 25 µl aliquot
PMA (phorbol 12-myristate 13-acetate) 1 mg/ml, 50 µl aliquot
EPEC bacteria lysate, prototypical strain E2348/69 (grown in DMEM – use 10 µl of 1 µg/µl stock)

96-well ELISA microtiter plates

ELISA assay reagents:
ELISA plate pre-coated with antibody to IL-2 (“capture antibody”) and pre-blocked
Wash buffer in 500 ml squirt bottle
IL-2 standard 63 ng/ml, 50 µl aliquot
Assay Diluent, 40 ml
Detection antibody (biotinylated anti-mouse IL-2)
Streptavidin-HRP
Substrate Reagent A (H_2O_2 in buffer), 6.0 ml
Substrate Reagent B (3,3’,5,5’-tetramethylbenzidine, TMB), 6.0 ml
Stop solution, 5 ml
Microtiter plate reader, absorbance at 450 nm

PROCEDURE

Day 1 – Tuesday, October 30

1. Make sure you have a **testable hypothesis** concerning the production of IL-2 by mouse splenocytes. Will antisera directed against the CD3 receptor stimulate or inhibit expression of this cytokine? How might pre-treatment with lysates from a bacterial pathogen affect IL-2 production? Ionomycin alters calcium levels in cells, and PMA is a potent mitogen. Splenocytes only, with no treatment, and with concanavalin A (ConA), which stimulates IL-2 expression by cross-linking the TCR, will be negative and positive controls, respectively. You will need to figure out final concentrations of all the additions except ConA and the bacteria. Read through the entire procedure for **Days 1 and 3 prior to continuing with the next steps**.

2. You will be provided with **6 rows, and 8 columns**, or a total of **48 microtiter plate wells** containing ~**200,000** mouse splenocytes in each well. This will include wells for both **cells only (3 wells)** and **positive (ConA) controls (3 wells)**, and those necessary to test your hypothesis **(42 wells)**. Add ConA to the wells at a final concentration of 10 μg/ml. Carefully design your experiment!

3. Illustrate your experimental design in your lab notebook. If it is helpful, you can find a microtiter plate image blank in pdf, and additional information about the reagents and assay components on the Courses Server.

4. Aliquots of each treatment are STERILE and should only be opened in the STERILE LAMINAR FLOW HOOD using PROPER STERILE TECHNIQUE. Add the appropriate components to your microtiter plate wells containing the mouse splenocytes in either the **BSL-2 level Biosafety cabinet** in Jay's lab, **B120**, **or** with Greta using the **cell culture cabinet in the Biology Stockroom**. See Figure 1 below for the overall plating scheme.

5. Incubate your microtiter plate in the tissue culture incubator in **B120** for ~48 hours, until your next class period, **Day 3**.

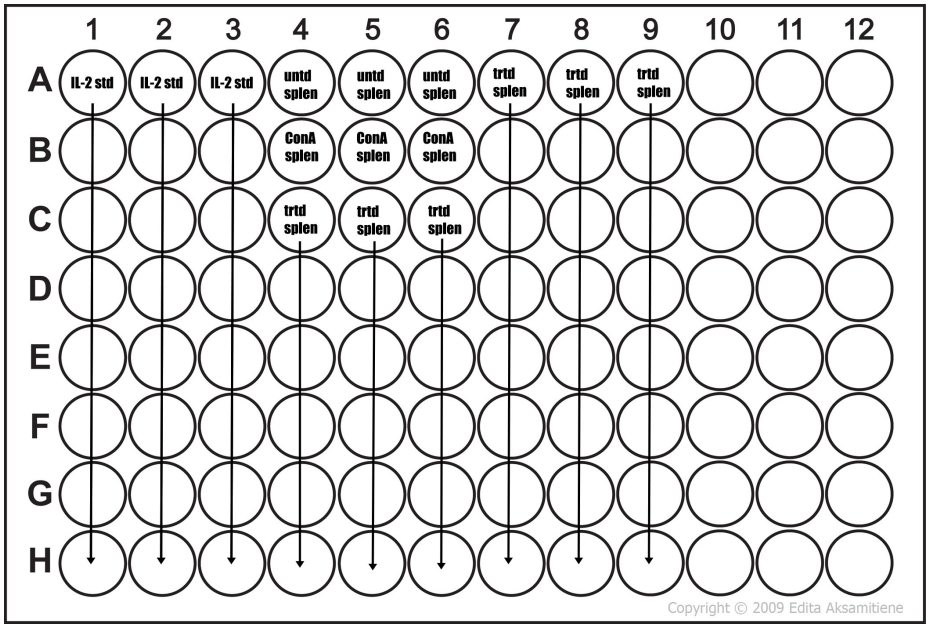


Figure 1. Use of untreated cells and **3 wells** of the ConA controls (triplicate samples) and up to **42 wells** of treated splenocytes to test your hypothesis, corresponding to columns 4 through 9, rows A through H above. You will also use the above plate scheme for the ELISA next week **in a separate microtiter plate**. Then you will use an additional **24 wells (**columns 1 through 3, rows A through H) for the IL-2 standard concentrations in order to construct a standard curve.

Day 3 – Thursday, November 1

After 48-hour incubation of the splenocytes at 37˚ C in **B120** you will need to freeze your samples so that they can be assayed for IL-2 production next week. You will perform this step toward the end of the regularly scheduled lecture period on Thursday, November 1.

1. Perform this step in **B107.** Without disturbing cells at the bottom of the wells, carefully transfer 150 μl of culture medium from each well to a new microtiter plate. Remember to label the new plate as "cell supernatants," and with the information about which wells contain which treatments.

2. Wrap the plate containing the supernatants in parafilm, store in a freezer box, labeled with your group name, in the -20˚ C freezer in **B107**.

Day 0 – Wednesday, November 7

The sandwich ELISA is used to quantify the amount of antigen that is present in a sample- the amount of IL-2 produced by mouse splenocytes under the tested conditions. Retrieve your samples from the -20˚ C freezer in **B107**, and place the plates on the bench top in order for them to thaw.

Note: **It is essential that the wells of an ELISA plate never completely dry out. Do not remove fluid from the wells until you are ready to put more fluid back in!**

1. Perform this step of the assay in **B107**. Obtain a 96-well ELISA plate pre-coated with anti-IL-2 antisera, and pre-blocked. Flick the liquid out of the wells of your ELISA plate (**not the microtiter plate containing your mouse splenocytes samples!**) into a sink. Wash the plate with wash buffer from a squirt bottle; **make sure all of the wells are thoroughly filled**! Flick the liquid out as before, to remove wash **completely** and repeat 2X for a total of 3 washes. **Leave the last wash in until you are ready to add your samples**.

2. Prepare 800 ul of IL-2 standard at 200 pg/ml by diluting the IL-2 standard (63 ng/ml) into Assay Diluent in a 1.7ml microfuge tube. You should have a **total** volume of 800 ul at a final concentration of 200 pg/ml.

3. When you are ready to add the IL-2 standard, remove the wash buffer from **columns 1-3, row A**. Add 200 ul of the 200 pg/ml IL-2 standard dilution to each well in **columns 1-3, row A**.

4. Next, remove wash buffer from **columns 1-3, rows B-H**. Add 100 µl Assay Diluent to each well in **columns 1-3, rows B-H**.

5. You will now create a serial dilution series starting from the 200 pg/ml wells, resulting in the following dilutions in **columns 1-3, rows B-G**: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64.

- Carefully remove 100 µl from **row A**, then dispense it into **row B**. Pipet up and down, thoroughly, 6-10 times to mix (minimize bubbles, but they will form).

- Remove 100 µl from **row B**, dispense into **row C** and mix, 6-10 times. Make sure to change pipette tips between each well!

- Continue down plate until you have removed 100 µl from **row G** –now discard the solution remaining in your pipet tips. **Row H** should be your "no cells" control, and contain only 100 µl Assay Diluent.

6. Next, remove wash buffer and add 100 ul of your samples (supernatants) to wells in **columns 4-9, rows A-H**. (Add 100 µl of supernatant from untreated splenocytes to **columns 4-6, row A,** as a negative control; add 100 µl of supernatant from

splenocytes treated with ConA to **columns 4-6, row B**, as a **positive control**). **Note: if you are using a multi-channel pipettor make sure that ALL the tips are on thoroughly. Observe the liquid that comes in to the disposable tip, and make sure it seems correct.**

- **Use remaining wells (columns 4-6, rows C-H and/or columns 7-9, rows A-H) to test your hypothesis.** Add 100 µl of your splenocyte samples to these wells. Refer to your plate map plan for correct placement. Remember that the splenocyte-containing plate and the one coated with anti-IL-2 antersera are two different microtiter plates!

7. Seal the ELISA plate with a sticky film sheet. Incubate the ELISA plate overnight at 4˚ C, in the refrigerator in **B107**.

Day 1 – Thursday, November 8

We will use a full 90-minute class period to complete the assay in **B107**. On this day, you will wash the microtiter plate wells, incubate with a secondary antibody conjugated to biotin. The enzyme horseradish peroxidase (HRP) is conjugated to streptavidin, binding tightly to biotin after addition. You will then add the substrate and collect the results using a microtiter plate reader.

7. Prepare Working Detector (this is a combination of biotinylated anti-mouse IL-2 and streptavidin-HRP). Dilute the Detection Antibody 1:1000 into **12 ml** of Assay Diluent in a reagent reservoir. To this, add the Enzyme Reagent at 1:250. When the Working Detector is ready, wash plates 5x with Wash Buffer. Bang plate on paper towels between each rinse.

8. Add 100 µl of the Working Detector per well (biotinylated anti-mouse IL-2 and streptavidin-HRP).

9. Incubate the plate at room temperature for 20 minutes.

10. At the end of this incubation period, prepare the Substrate Solution (TMB in H_2O_2). Mix 6.0 ml of Substrate Solution A and 6.0 ml of substrate solution B into a **new reagent reservoir**. Be sure to mix well by pipetting up and down 6-10 times. Cover with foil.

12. Wash the plate 7x with wash buffer as before, bang plate on paper towels, then add 100 µl Substrate Solution to each well.

13. Incubate in the dark for 30 minutes (a drawer will work fine for this), then add 50 µl Stop Solution (1M H_3PO_4) to each well. Pipette up and down 3-4 times to mix. Absorbance at 450 nm will be measured using the BioRad 680 microplate reader, instructions provided.

Instructions for plate reader:

Power “on” with switch at back right, near the plug.
~15 second initialization period,\then login:
user: common user
password: 00000
wait ~3 minutes before first run, to allow thermal equilibrium in machine

press “Memory Recall” button
select “Protocol” then press enter
select “End Point” then press enter
use up/down arrows to find End point #14, press enter
should look like this:

14: End point #14
M450(2) R570(6)
Shake 3 s,Mid

Place ELISA plate into tray, close door, press “Start”

SCHEDULE SUMMARY FOR IL-2 ELISA LABORATORY EXERCISE:

		Task
Week 9	**Day 1 – Tuesday, Oct 30**	Formulate hypothesis, set up experiment
	Day 3 – Thursday, Nov 1	Freeze samples at -20˚ C
Week 10	**Day 0 – Wednesday, Nov 7**	Place samples in pre-coated ELISA plate
	Day 1 – Thursday, Nov 8	Perform ELISA

Report

Your lab report for this assignment will be in the format of a scientific journal article, and should be 5 to 6 single-spaced pages in length. The Introduction will provide information, previous experimentation that supports your hypothesis. Make sure to include the standard curve for IL-2 concentration as part of your Results. Is the curve linear over the entire range of IL-2 concentrations? Include statistical analysis in order to support or refute your hypothesis. The standard curve should include error bars, an equation of the line, and over what concentration range you consider the response to be linear.

Work on the assignment with your lab partner. Please provide a statement just below the Abstract as to the individual contributions to the assignment.

The format is listed below.

1. Title- Concise, appropriately descriptive title.
2. Abstract - Brief description of your project.
3. Introduction- Pertinent, properly referenced, background information. The *hypothesis* should be clearly stated within the Introduction.
5. Materials and Methods – How did you answer the question(s) posed? This does not need to be exhaustive, but should be detailed enough so that other researchers could reproduce your results.
6. Results – Results of your experimentation, and whether they support or refute your hypothesis. Any graphs should have properly labeled axes, and all figures must have informative legends.
7. Discussion - Discuss the significance of your results as they pertain to the current literature, any difficulties you encountered, and suggest alternate approaches and/or future experiments to address the question posed.
8. Literature cited.

Lab reports are due Tuesday, November 20th.