IMMUNOLOGY

BIO 463

LABORATORY EXERCISE

FALL 2016
IL-2 Production in mouse splenocytes

INTRODUCTION

Interleukin-2, or IL-2, is a potent cytokine produced by T-cells, stimulating their proliferation. As such IL-2 was originally identified as a T-cell growth factor. IL-2 is produced by CD4+ T helper cells, to a lesser extent by CD8+ T cells and natural killer cells in secondary lymphoid tissue (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), those found on bacteria and viruses, are recognized by the PRRs on dendritic cells to stimulate cytokine production. By binding to PRRs microbes stimulate the production of IL-2 in DCs, most likely through stimulation of the nuclear factor of activated T-cells or NFAT pathway (Granucci et al. 2001). IL-2 elicits many responses, from driving T-cell proliferation, the activation of natural killer cells, (NK) to stimulating apoptotic pathways. Thus IL-2 controls not only immune activation but also homeostasis. As such, 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, or ELISA. Mouse splenocytes contain a number of leukocytes, including T-cells, B-cells, monocytes and dendritic cells.

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 antiserum 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 readings listed below, other literature you might pursue, 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 whole bacteria, purified LPS, anti-CD3 antisera, iomomycin, PMA (phorbol 12-myristate 13-acetate) 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.
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:
- E. coli bacteria, prototypical EPEC strain E2348/69 (overnight culture – use 1 to 2 µl)
- Purified LPS, 1 mg/ml, 100 µl aliquot
- Concanavalin A (ConA), 1 mg/ml, 200 µl aliquot
- Anti-CD3 antiserum, 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

96-well microtiter plates, 96-well ELISA plates

ELISA assay reagents:
- 96-well ELISA plate pre-coated with antibody to IL-2 (“capture antibody”) and pre-blocked
- IL-2 standard 31 ng/ml, 50 µl aliquot
- Assay Diluent, 40 ml aliquot
- Wash buffer in 500 ml squirt bottle
- Detection antibody (biotinylated anti-mouse IL-2), 11 µl
- Streptavidin-HRP, 44 µl
- Substrate Reagent A (H₂O₂ 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, November 1

1. Develop a testable hypothesis concerning the production of IL-2 by mouse splenocytes. How might whole bacteria or purified LPS affect IL-2 production? Will antisera directed against the CD3 receptor stimulate or inhibit expression of this cytokine? Splenocytes only, with no treatment, and concanavalin A (ConA), which stimulates IL-2 expression non-specifically, will be used as controls. Read through the entire procedure for Days 1 and 3, and state your hypothesis to the instructor prior to continuing with the next steps.

2. Carefully design your experiment. 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.

3. Illustrate your experimental design in your lab notebook. If it is helpful, you can find a microtiter plate image blank in pdf format 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 3

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 3.

1. Perform this step in B219. Without disturbing cells at the bottom of the wells, carefully transfer 100 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 B219.
Day 0 – Wednesday, November 9

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 B219, 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 B219. 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 3x with wash buffer from a squirt bottle. Leave the last wash in until you are ready to add your samples.

2. Prepare 600 ul of IL-2 standard at 200 pg/ml by diluting a stock aliquot of IL-2 standard (31 ng/ml) into Assay Diluent in a 1.7ml microfuge tube.

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

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

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 4-5 times to mix (don’t make bubbles!!).

   • Remove 100 µl from row B, dispense into row C and mix. 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).
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 antiserum 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 B219.

Day 1 – Thursday, November 10

We will use a full 90-minute class period to complete the assay in B219. 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 11 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 as before. Bang plate on paper towels.

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 H2O2). 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. 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, then add 50 µl Stop Solution (1M H3PO4) to each well. 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:

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<td>Formulate hypothesis, set up experiment</td>
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<td>Freeze samples at -20°C</td>
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<td>Place samples in pre-coated ELISA plate</td>
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<td>Perform ELISA</td>
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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.
4. **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.
5. **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.
6. **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.
7. **Literature cited.**

Lab reports are due Tuesday, November 22nd.