I. Identify a Problem

In order for cells to communicate within each other, they transit chemical signals. When a cell receives a chemical signal, it often triggers a pathway that produces some reaction appropriate for the cause of the initial signal. For example, when an organism’s immune system has been compromised by some injury, white blood cells (leukocytes) begin to release proteins that signal for other cells to begin the inflammation process. The inflammatory response is an attempt to remove the malignant stimulus and begin healing. However, if inflammation continues unchecked, it can do more harm to an organism than good, and thus in addition to pro-inflammatory signaling proteins, called pro-inflammatory cytokines, the immune system also responds to anti-inflammatory cytokines, which are produced in order to reduce inflammation and to allow an organism to continue functioning. Thus, the overall magnitude of the immune response at any given time is driven by the balance between pro-inflammatory cytokines and anti-inflammatory cytokines, but how?

1. Why is inflammation important for preventing and/or fighting an infection?

*Students should know that inflammation occurs when they are injured. Even when they experience minor cuts, they experience localized inflammation. They should be able to relate inflammation to swelling and redness, which they might connect to increased blood flow to the site of the injury. They may or may not understand that this is intended to bring additional resource to the area through the blood to fight the infection.*

2. Why do you think it is necessary to have an anti-inflammatory response?

*Students, knowing the definition of homeostasis, should understand that no change in an organism’s internal environment can persist forever without having serious consequences for that organism. Therefore an anti-inflammatory response would be the body’s homeostatic mechanism to provide negative feedback against the pro-inflammatory response, thus returning the body to an equilibrium state.*

3. What do you think happens after an anti-inflammatory response is initiated?

*Following the answer from the previous question, inflammation should decrease, since the pro-inflammatory response is being counteracted, and the body should return to an equilibrium.*

4. How is homeostasis relevant for understanding inflammatory and anti-inflammatory immune responses?

*Homeostasis governs all aspects of an organism’s internal environment, and the presence of inflammation would be one of those infinite numbers of aspects. Pro-inflammatory and anti-inflammatory responses work against each other in a negative feedback mechanism, which many teachers incorporate into their lessons on homeostasis for advanced biology students.*
II. Design an Experiment

You have decided you want to see how the presence of different cytokines changes over time after an organism’s immune system has been compromised. You know that because an organism requires homeostasis for survival, eventually a pro-inflammatory response ought to succumb to an anti-inflammatory response, but you must design an experiment to determine precisely when or how this happens. You decide to infect mice with *Pseudomonas aeruginosa*, a common bacterium found in soil, and you decide that you want to measure the concentrations of different cytokines present in blood serum across various time points. To save time and resources, you decide to limit yourself to analyzing the concentration of Interleukin 2 (IL-2), which is a pro-inflammatory cytokine that encourages the creation of T-Cells, and Interleukin 10 (IL-10), which is an anti-inflammatory cytokine that stops the output of IL-2.

5. Why do you think you would only want to analyze the blood serum, as opposed to the blood plasma, or the entire blood sample? (The plasma is what is left when all cells are removed from the blood, and the serum is what is left when all cells AND all clotting factors are removed from the blood.)

In practice, this is because the presence of blood cells and clotting factors will obscure the data. However, students would likely not produce such an accurate response, but they may offer that the proteins would be present in the fluid, rather than in the cells.

6. What units would you expect to use when measuring these concentrations? Why?

Students in advanced biology courses at the high school level will usually have taken a general course in chemistry already, so they may already be familiar with the idea that concentrations are measures of solute per unit of solvent. They may think of this in a mass/volume relationship, or perhaps mass/mass, or mass/volume. (Later in the activity, measurements will be in mass/vol.)

7. What sort of trends in your data would you expect to see?

Students would likely expect to see some initial inflammation, characterized by high concentrations of pro-inflammatory cytokines, and they should expect inflammation to go down over time due to increased concentrations of anti-inflammatory cytokines.

8. Draw how you would imagine a graph displaying this data would look.

Students would likely represent the trend they offered for the above question in a graphical format.
Collecting Data with an ELISA

To measure these protein concentrations, you decide to use an enzyme-linked immunosorbent assay (ELISA), which is a fairly common assay in biochemistry laboratories. This technique can detect the presence of a protein or compound in a liquid sample through the use of color-changing proteins.

First, samples containing the protein whose concentrations you want to quantify are loaded into wells on a 96-well plate using a micropipette. Then, antibodies that are designed to attach to these specific proteins are also loaded into the wells with samples. These antibodies have attached to them certain enzymes. Lastly, you load into the wells the protein that will change colors if it comes into contact with an enzyme that has been properly connected by means of an antibody to your protein sample. The samples with the highest concentrations of your original proteins will produce a more visible color change.

After completing the assay, your results are displayed on the right. Row A contains your control samples; each well in Row A has a specific concentration of antibodies that you already know. For the samples in Row B, you tested for the presence of IL-2, your pro-inflammatory cytokine, and in Row C, you tested for the presence of IL-3, your anti-inflammatory cytokine. You can determine the concentrations of cytokines in Row B and Row C by comparing their color change to the shades expressed by your controls in Row A.

9. Why is it important to establish a set of controls (or standards of comparison) for your assay?

Students will have been told that you need a set of control for any experiment you conduct. However, they may not realize how this actually plays a role in experimental design. Students should be able to see that through this particular experiment, without a set of controls, there is nothing to which the experimental groups can be compared.

10. Draw what you would expect the arrangement of molecules to look like based on the description of the assay above. Be sure to label your cytokines, your antibodies, your enzymes, and your color-changing protein. (Hint: The molecules are arranged like links in a chain.)

Students will of course not know the specific structures for these molecules, but they will be able to tell by the introduction that the alignment of molecules will be something like this:

(sample with detectable proteins) + (antibodies) + (enzymes) + (color-changing protein)
How fast does my body heal from a serious injury?

III. Analyze an Experiment

You collected blood from a patient who has experienced an insult to their immune system, and you measured the concentrations of two cytokines in the serum across the following time points.

You decided to measure interleukin-2, which is a pro-inflammatory cytokine, and interleukin-10, which is an anti-inflammatory cytokine.

<table>
<thead>
<tr>
<th>Time</th>
<th>Concentration of IL-2 (pg/μL)</th>
<th>Concentration of IL-10 (pg/μL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 hr</td>
<td>37</td>
<td>19</td>
</tr>
<tr>
<td>3 hr</td>
<td>63</td>
<td>17</td>
</tr>
<tr>
<td>6 hr</td>
<td>92</td>
<td>18</td>
</tr>
<tr>
<td>12 hr</td>
<td>113</td>
<td>25</td>
</tr>
<tr>
<td>24 hr</td>
<td>123</td>
<td>40</td>
</tr>
<tr>
<td>36 hr</td>
<td>94</td>
<td>63</td>
</tr>
<tr>
<td>48 hr</td>
<td>61</td>
<td>80</td>
</tr>
<tr>
<td>60 hr</td>
<td>37</td>
<td>97</td>
</tr>
<tr>
<td>72 hr</td>
<td>18</td>
<td>121</td>
</tr>
<tr>
<td>84 hr</td>
<td>15</td>
<td>123</td>
</tr>
<tr>
<td>96 hr</td>
<td>21</td>
<td>98</td>
</tr>
</tbody>
</table>

11. Use a scatterplot to display the above data. Remember to include useful labels and an accurate scale!

![Cytokine concentrations v. Time since immune insult](image)

12. Why might you not have a time point at immediately after the insult (i.e. at 0 hrs)? In reality, if this were data from human burn victims, it would take time to transport a patient to a hospital where tests could be performed, especially because burn clinics are few and far between in many parts of the country. Students may offer any number of answers.

13. What quantitative trends do you initially notice based on your graph? Students should see an immediate increase in pro-inflammatory cytokines and a delayed increase in anti-inflammatory cytokines, which coincides with a decrease in pro-inflammatory cytokines.
14. If you were to fit this data to a mathematical function, what type of function would be the best fit? If one regression may fit part of a graph better than another, give the relevant domain (x values), and explain why.

a. Polynomial Students may see a parabola at the peaks of each response.
b. Linear Students may see intersecting lines in between the peaks of response.
c. Sinusoidal In general, students may see that the graph behaves like a sine or cosine function.
d. Exponential Students may see exponential growth in the initial increase in anti-inflammatory cytokines, or exponential decay in the initial decrease in pro-inflammatory cytokines.
e. Logarithmic Students may see logarithmic growth in the initial increase in pro-inflammatory cytokines.

15. If you were a doctor, and you wanted to prescribe your patient a drug which would reduce the expression of inflammatory cytokines, why might it be important to consider the type of function, or the rate, at which the cytokines are increasing? (Remember that drugs are chemicals, and every chemical reaction has its own reaction rate as well.)

Teachers should make students aware that many of these models appear in many aspects of science, and that it is useful to be familiar with them here in order to identify them elsewhere. In this case, students may suggest that the drug should decrease the rate of pro-inflammatory cytokines at a rate similar to that which would happen when anti-inflammatory cytokines are introduced. Otherwise, the body may experience some reaction that moves it further away from equilibrium.

16. Given the total amount of cytokines present in the samples at each given time point, determine what concentrations you would expect to have if the proportion between pro-inflammatory cytokines and anti-inflammatory cytokines remained 1:1. (Hint: If the concentrations are proportional in a 1:1 ratio, half of the cytokines will be pro-inflammatory, and half of the cytokines will be anti-inflammatory.)

<table>
<thead>
<tr>
<th></th>
<th>1hr</th>
<th>3 hr</th>
<th>6 hr</th>
<th>12 hr</th>
<th>24 hr</th>
<th>36 hr</th>
<th>48 hr</th>
<th>60 hr</th>
<th>72 hr</th>
<th>84 hr</th>
<th>96 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total concentration of cytokines</td>
<td>56</td>
<td>80</td>
<td>110</td>
<td>138</td>
<td>163</td>
<td>157</td>
<td>141</td>
<td>134</td>
<td>139</td>
<td>138</td>
<td>119</td>
</tr>
<tr>
<td>Expected concentration of IL-2 at a 1:1 ratio</td>
<td>28</td>
<td>40</td>
<td>55</td>
<td>69</td>
<td>81.5</td>
<td>78.5</td>
<td>70.5</td>
<td>67</td>
<td>69.5</td>
<td>69</td>
<td>59.5</td>
</tr>
<tr>
<td>Expected concentration of IL-10 at a 1:1 ratio</td>
<td>28</td>
<td>40</td>
<td>55</td>
<td>69</td>
<td>81.5</td>
<td>78.5</td>
<td>70.5</td>
<td>67</td>
<td>69.5</td>
<td>69</td>
<td>59.5</td>
</tr>
</tbody>
</table>
17. Use a chi-square goodness-of-fit test for each of the following time points with the pro-inflammatory cytokines and anti-inflammatory cytokines. Use your experimental data for your observed values, and compare these to the expected concentrations you calculated above.
(Hint: For all time-points, your degree of freedom is 1.)

Null Hypothesis: \( H_0 \): Cytokine concentrations are equal
Alternative Hypothesis: \( H_1 \): Pro-Inflammatory > Anti-Inflammatory

\[ X^2 = \sum \left( \frac{\text{observed} - \text{expected}}{\text{expected}} \right)^2 \]

1 hour: \[ X^2 = \left( \frac{(37-28)^2}{28} + \frac{(19-28)^2}{28} \right) = 5.79 \]

P-Value: \( 0.05 < p < 0.10 \) \quad P-Value Comparison: \( p \gg a \)

3 hours: \[ X^2 = \left( \frac{(63-40)^2}{40} + \frac{(17-40)^2}{40} \right) = 26.45 \]

P-Value: \( \_ \_ \_ \_ < p < 0.001 \) \quad P-Value Comparison: \( p \ll a \)

6 hours: \[ X^2 = \left( \frac{(92-55)^2}{55} + \frac{(18-55)^2}{55} \right) = 49.78 \]

P-Value: \( \_ \_ \_ \_ < p < 0.001 \) \quad P-Value Comparison: \( p \ll a \)

36 hours: \[ X^2 = \left( \frac{(94-78.5)^2}{78.5} + \frac{(63-78.5)^2}{78.5} \right) = 6.12 \]

P-Value: \( 0.001 < p < 0.005 \) \quad P-Value Comparison: \( p \ll a \)

Null Hypothesis: \( H_0 \): Cytokine concentrations are equal
Alternative Hypothesis: \( H_1 \): Anti-Inflammatory > Pro-Inflammatory

48 hours: \[ X^2 = \left( \frac{(61-70.5)^2}{70.5} + \frac{(80-70.5)^2}{70.5} \right) = 2.56 \]

P-Value: \( 0.10 < p < 0.15 \) \quad P-Value Comparison: \( p \gg a \)

60 hours: \[ X^2 = \left( \frac{(37-67)^2}{67} + \frac{(97-67)^2}{67} \right) = 26.87 \]

P-Value: \( \_ \_ \_ \_ < p < 0.001 \) \quad P-Value Comparison: \( p \ll a \)

96 hours: \[ X^2 = \left( \frac{(98-59.5)^2}{59.5} + \frac{(21-59.5)^2}{59.5} \right) = 49.82 \]

P-Value: \( \_ \_ \_ \_ < p < 0.001 \) \quad P-Value Comparison: \( p \ll a \)

18. At what time points can you say that there is not a statistically significant difference between the concentrations of the two cytokines? What does this imply about the immune response for these time points?

Based on p-values, there are significant differences from 3 hours to 36 hours, and from 60 hours to 96 hours. Thus there is a significant pro-inflammatory response, then a period of transition, then a significant anti-inflammatory response.
19. Why might you decide to use a 1:1 ratio between cytokines for your Chi-Square test? How might this relate to an organism maintaining homeostasis?

If the levels of pro-inflammatory cytokines and anti-inflammatory cytokines are roughly equal, then there is no significant inflammatory or anti-inflammatory response occurring at that time, and thus a homeostatic equilibrium would be maintained. For a chi-square test, experimental values must be compared to expected values, and for homeostasis we might expect our cytokines to exist in a 1:1 ratio.

20. At what time point would you assert that the condition of the patient made a discernible change? What was that change?

At the 48 hour time point, which did not have a statistically significant difference between the pro-inflammatory cytokine concentration and the anti-inflammatory cytokine concentration, would be the transition between the inflammatory response and the anti-inflammatory response.

21. Assuming your data is still valid, what sort of additional data would be able to disprove your hypothesis?

This response will vary widely, but it encourages students to think about the possibility of follow up experiments that could lead him or her in a different direction. One example of additional data that could disprove the hypothesis is described in Number 22.

22. How might your interpretation of the data change if you decided to measure the concentrations of additional cytokines?

In this experiment, one pro-inflammatory cytokine was chosen among the many that actually exist, and one anti-inflammatory cytokine was chosen among the many that actually exist, each representing their respective immune responses. However, they may each be outliers among their respective classes, so perhaps the patient experiences a delayed inflammatory response, or is undergoing an anti-inflammatory response at the initial time points. You would need to look at all cytokines that determine responses to be sure.

23. Cell signals such as cytokines can produce cascades, meaning that when one cell receives the signal, that cell then sends that signal out to more cells. What do you think happens inside the cell between the time the cell receives a protein signal on its membrane and the time the cell releases its own protein signals?

This will require students to think about how cells transmit signals, but also how cells manufacture proteins. If the signal is not one that is waiting to be released from the cell, the cell will have to transcribe the genes necessary for producing the proteins before having the chance to release them.
24. You know that a patient will become severely septic if their inflammation becomes too excessive. What would you expect the cytokine concentrations to be in such a patient? Supply hypothetical values, and explain your reasoning for choosing such values.

<table>
<thead>
<tr>
<th>Concentration of IL-2 (mg/μL)</th>
<th>t = 1hr</th>
<th>t = 6hrs</th>
<th>t = 12hrs</th>
<th>t = 24hrs</th>
<th>t = 36hrs</th>
<th>t = 48hrs</th>
<th>t = 72 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of IL-10 (mg/μL)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The response to this question is highly variable, but seeing that inflammation is too excessive, students may pick larger numbers for the pro-inflammatory cytokine concentrations than were listed in the original data, and they may choose smaller concentrations for the anti-inflammatory response.

25. At what time point in your new data would you, as a doctor, become concerned by the results being returned from this lab?

Students now have to realize that for actual patients in actual hospitals, doctors do not get the chance to see laboratory results 90 hours out from that patient’s admittance. Therefore doctors have certain standard thresholds established that will give them justification for acting on particular changes in that patient’s status. The student must pick a time point where they would feel comfortable claiming that the patient is undergoing, or about to undergo, serious inflammation. This varies based on the student’s data, but

26. Biologically speaking, what do you think you would need to do as a doctor to prevent this patient from becoming severely septic?

Students will have to think about what sorts of symptoms of inflammation a doctor may want to treat before the inflammatory response leads to sepsis. They will probably suggest using some drug that elicits an anti-inflammatory response.