ASTARTE IN ACTION

Using a Recall Antigen Assay as a Tool for Understanding Immunity
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Introduction

While there is no industry-accepted protocol for measuring the functionality of peripheral blood mononuclear cells (PBMC), it’s an important test that should be conducted for quality control.

We needed a reliable, reproducible way to measure the functionality of our cryopreserved PBMC, so we developed a custom assay using recall antigens to understand the in vitro activity of our cells. By testing PBMC for immune response to several different antigens over time, we intended to better understand an acceptable range of variation in recall response from PBMC samples from the same donor over time.

The recall antigen testing assay needed to effectively evaluate the quality of important immune cell types, including T cells, B cells, and monocytes. We strategically designed the assay to use several antigens:

- Phytohaemagglutinin (PHA): As a positive control
- Lipopolysaccharide (LPS): To stimulate B cells and monocytes
- Cytomegalovirus (CMV): As a natural, chronic infection for a subset of the CMV-infected population
- Tetanus Toxoid: Due to its widespread use in vaccinations

Methods

1. Cryopreserved PBMC were thawed in a 37°C water bath and the concentration adjusted to 2.5 x 10^6 per mL in X-VIVO™ 15 medium.
2. Cells were added to a round bottom, 96 well plate at 100 uL per well.
3. Dilutions of antigens and mitogens were prepared in X-VIVO 15 medium. Tetanus toxoid and Cytomegalovirus (CMV) antigens were diluted to 2 ug/mL. Lipopolysaccharide (LPS) was diluted to 200 ng/mL. Phytohemagglutinin (PHA) was titrated to determine the optimal concentration, which we found to be 1 ug/mL.
4. Antigens were added to the well plate at 100 uL per well in triplicate. 100 uL of medium was added to three wells as a control.
5. Samples were incubated at 37°C, 6% CO₂ for four days. Note: Day four is not the optimal time to measure cytokine concentrations for all of the antigens and mitogens in this assay. Day four was chosen to maximize the chance of seeing a response to all four antigens.
6. 150 uL of medium was removed from each well and used for cytokine analysis.

Results

The Rate of Cytokine Production Varies Based on Antigen and Time

Cytokine analysis can show us how immune cells react to a stimulus or change in the immune system and is a great measure of cell quality. To assess the overall quality of PBMC samples using the antigen recall assay, we measured production of 10 different cytokines over time in response to CMV and tetanus toxoid.

We found that IFNγ had the most significant increase over background production in response to both antigens used. The other cytokine that was increased for both recall antigens was TNFα.

We chose to track these two cytokines routinely. While IFNγ is sufficient to detect responses to CMV and tetanus, TNFα was a logical choice for monitoring the stimulation of monocytes by LPS. TNF is detectable within 4 hours of LPS stimulation, but with antigen stimulation, TNF did not increase until day 3.
This difference between the rate of cytokine production means it is not possible to measure cytokine at the optimal time for T cell antigens and the LPS and PHA mitogens. Instead, we chose to collect media for cytokine analysis after 4 days, which gave some margin for detection of positive responses to the antigens.

<table>
<thead>
<tr>
<th>Cytokine Production Over Time</th>
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<tbody>
<tr>
<td>IFNγ</td>
</tr>
<tr>
<td>CMV</td>
</tr>
<tr>
<td>Day 1</td>
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<tr>
<td>Day 2</td>
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<tr>
<td>Day 3</td>
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<tr>
<td>Tetanus</td>
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<tr>
<td>Day 1</td>
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<tr>
<td>Day 2</td>
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<td>Day 3</td>
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Figure 1. Note: ND = Not Detectable

✔ Limitations in Cell Proliferation Measurement

When using CellTiter-Glo®, a reagent based on adenosine triphosphate (ATP) bioluminescence content, increased cell metabolism and higher ATP per cell is interpreted as proliferation. However, increased cell metabolism can also increase ATP per cell, which may complicate interpretation.

Another method for measuring proliferation is the uptake of bromodeoxyuridine (BrdU), which labels cells replicating their DNA.

We measured cell proliferation using both the ATP and BrdU uptake methods, which yielded different results. Figure 2 shows that cell proliferation was higher for both donor samples used in the recall assay when stimulated with PHA than for any other antigen. Figure 3 tells a different story with more variable proliferation measurements depending on donor and antigen. Lot number 1406 had proliferation above control for all 4 stimuli (tetanus, PHA, LPS and CMV) with the highest proliferation observed in response to CMV.

Figure 2. PBMC proliferation measured by ATP content using CellTiter-Glo®.

Figure 3. PBMC proliferation measured by uptake of bromodeoxyuridine.
When measuring proliferation, note that both methods have their limitations. We found the ATP method had a poor signal to noise ratio — the increase in ATP following antigen stimulation was not significantly higher than the relatively large amount of ATP in the absence of stimulation — and the BrdU uptake incorporation assay to be cumbersome.

These two methods can produce vastly different results, especially with immune cell populations in which a small subset may proliferate with the majority simply maintaining their metabolism. With these limitations in mind, we decided to use the cytokine production (see Figure 1) as the optimal measure of primary immune cell response.

**Recall Response is Highly Reproducible, But Some Variation is Expected**

Repeated testing of a reference lot of PBMC using the recall antigen assay resulted in varying levels of cytokine production, even when the lot of antigen, culture medium, and other conditions were kept constant.

Due to this variability, the recall antigen assay should not be used to compare separate runs or experiments. Although recall patterns seem to be stable across experiments, the variation makes it difficult to compare results quantitatively.

However, comparisons of results within the same run or experiment are acceptable. We have used this recall antigen assay to evaluate different lots of antigen with high reproducibility.

See Figures 4 and 5 for results from vial-to-vial reproducibility testing using tetanus toxoid and a comparison of CMV antigen lots.

**Figure 4.** The response to tetanus toxoid was measured using the same lot of PBMC and the same lot of antigen on five separate occasions. Each experiment reported an immune response to tetanus toxoid, but IFNγ production varied from less than 100 pg/mL to 700 pg/mL.

**Figure 5.** The recall antigen assay was used to measure cytokine production of the PBMC samples using two different lots of CMV at varying concentrations. This graph shows lot-to-lot variability for CMV.
Recall Antigen Assay Results Are Consistent Over Time

An examination of the longitudinal data from running the recall antigen assay consistently on multiple donors revealed that the results of the assay are fairly consistent over time. Figures 6 and 7 are just two examples of our longitudinal data, although we have examined the assay on other donors with similar results and continue to run these longitudinal studies.

PBMC from donor 369 produced IFNγ when stimulated with either CMV or tetanus toxoid. The responses have been relatively steady since May 2017. A pipetting error may have caused the large standard deviation observed in July 2017. PBMC collected in February 2017 had much lower cytokine output, which raises questions as to what may have affected the cells at that time.

We have noticed that some donors produce TNFα in response to tetanus toxoid or CMV while others do not. There is also a tendency for donors to produce higher (>4,000 pg/mL) or lower (<2,000 pg/mL) TNFα following stimulation with LPS, a substance known to activate monocytes. TNFα levels do not necessarily relate to the percentage of monocytes present; it seems to be a donor-specific characteristic. We are continuing to monitor this characteristic to determine if we can relate it to other donor characteristics.

Looking at the recall responses over time for another donor, we found that PBMC from donor 290 were positive for CMV response as measured by TNFα production. However, donor 290 produced little to no response to TNFα when stimulated with tetanus toxoid. The low response does not indicate that donor 290 is unresponsive to tetanus toxoid because there was IFNγ found in the sample.

Recall Antigen Testing: Donor 290

Figure 7. PBMC from donor 290 were tested in the recall antigen assay over a year. This graph depicts the TNFα production observed. IFNγ production in response to CMV was off scale in every batch.

Recall Antigen Testing: Donor 369

Figure 6. PBMC from donor 369 were evaluated in the recall antigen assay over a year. The response to tetanus toxoid had greater fluctuation than the response to CMV antigen.
The Recall Antigen Assay Can Be Manipulated to Increase or Decrease the Immune Response

There is great excitement surrounding the progress of targeted therapies that use the immune system to attack cancer cells. The activity of one such drug, Nivolumab, can be demonstrated in our recall antigen assay by measuring the immune response to CMV antigen.

Nivolumab works by intensifying the immune response to cancer. Figure 8 shows that the cultures in our assay containing Nivolumab had a greater response to CMV antigen than the control cultures.

Similar approaches can also be tested using this assay to monitor the impact of drug candidates on the in vitro immune response. Whether you want to boost the response as in cancer or vaccine design or blunt the immune attacks as in autoimmune disease or allergy drug candidates, this assay can be used as a model.

Figure 8. The response of donor 349 to CMV antigens was measured when Nivolumab (Opdivo®) was added to the culture. IgG4 was used as a negative control for Nivolumab. The secretion of IFNγ was increased 100% or more by this checkpoint inhibitor.

Conclusion

The assay we developed is a useful measure of immune cell quality. Our customers have found the data from the assay helpful in reviewing cell quality prior to purchase to select lots that will work well in their assays.

While we did not set out to measure donors longitudinally or compare cytokine profiles between donors, this data has been beneficial in understanding the variability between donors and in a single donor over time. Our recall antigen assay is also used as a model for monitoring the effects of test compounds on immune cell activation.