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Human Immune Cells: A Closer Look

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Introduction

The immune system is a system of biological structures and processes that play a vital role in protecting the body from infections and foreign pathogens. Each component of the immune system, (i.e. cells or molecules) has a specialized task that helps the body to recognize, eliminate, and remember invading microbes. My project focused on two cell populations that play an important role in the adaptive (or aquired) immunity (which is important for the development of immunological memory): B and T lymphocytes. These cells develop from stem cells found within the fetal liver and bone marrow. B cells mature in the bone marrow while T cells migrate to the thymus for maturation.[1]

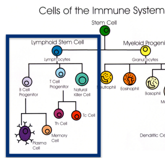

Figure 1: A snapshot of the cells of the immune system, with specific focus on B cells and T cells

B cells produce immunoglobulins, which are antigen-specific antibodies responsible for eliminating invading microorganisms. T cells can be subdivided in two subtypes: helper T cells, which activate or direct other immune cells (for example: B cells), and cytotoxic T cells, which eliminate pathogens by killing cells that are infected.[1] In order to investigate these lymphocytes in experiments or assays, it is first necessary to isolate them from whole blood.

Along with B cells and T cells, I have also used Ramos cells in my assays. Ramos cells are a specific B cell line that is derived from Human Burkitt's Lymphoma. A cell line is an immortal cell population that given adequate space and media, will continue to grow indefinitely.[2] The use of Ramos cells in our experiments allow us to optimize our assays without risking valuable patient or volunteer samples. An example where I've used Ramos cells is in optimizing a novel flow cytometry assay: the BD Violet Fluorescent Cell Barcoding Kit. This kit allows 16 samples to be treated differently and mixed into one tube, barcoded with combinations of two different fluorescent colours, and then separated during analysis using flow cytometry

Flow cytometry is a laser-based technology used for biomarker detection, counting and sorting. Using flow cytometry, properties of cells (size, structure, biomarkers) can be measured by suspending them in a fluid stream and passing them by lasers. Lymphocytes and Ramos cells possess specific biomarkers. Using antibodies conjugated to a fluorescent dye that bind to these specific markers, it is possible to separate a sample of cells into different subpopulations based on their markers by flow cytometry. With antibody staining experiments, we have examined lymphocyte populations.

My summer project has focused on aquiring various techniques to study cells of the human immune system, specifically B and T cells. I have processed whole blood in order to isolate peripheral blood mononuclear cells (PBMCs), which contain B and T cells. Subsequently, antibody staining experiments were performed on these PBMCs to study the frequency of these cells within a sample. In addition, I have learned to culture Ramos cells for use in optimizing assays that will eventually involve B cells from patient samples.

[1] Delves PJ, Roitt IM. The Immune System- First of Two Parts. New Engl J Med; 2000: 37-49
[2] Cell Lines. Biology Online. 3 October, 2005. 9 August, 2012. http://www.biology-online.org/dictionary/Cell_lines

Methods: Blood Processing

PBMCs were isolated from whole blood samples. The whole blood sample was spun in the centrifuge in order to separate the plasma from the rest of the blood. This plasma layer was then removed and stored for later use. The remaining volume of blood was diluted using R-10 media (5mL of Penicillin-Streptomycin + 50mL of FBS + 500mL of RPMI media) and then layered on top of Ficoll Plaque. The sample was then placed into the centrifuge again and the spin separated the blood into four distinct layers (*see Figure 2*).

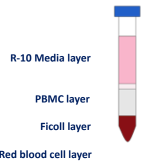

Figure 2: Blood separated into it's components after Ficoll spin

The PBMC layer was then removed. The cells were washed with R-10 twice to remove any remaining Ficoll and then resuspended in a 10% Dimethyl sulfoxide solution in FBS. This solution allowed the white blood cells to be frozen in the liquid nitrogen tank safely, without the risk of them all dying. It is also possible to isolate the red blood cell layer, but for my experiments I only needed the PBMCs.

Methods: Cell Culture

In order to also use Ramos cells for experiments, the cells had to be cultured first. Ramos cells were removed from the liquid nitrogen tank, thawed, and washed with FBS and R-10. Subsequently, the cells were counted on a MACSQuant Flow Cytometer and cultured in a six well plate in a concentration of 1 000 000 cells/well in R-10 media. Three times a week, the R-10 media was refreshed. Every week, cells were collected, counted and transferred to new plates.

Methods: Barcoding Ramos Cells

In order to limit use of reagents and thus the cost of a large staining experiment, we are optimizing the protocol for the BD Cell Barcoding Kit. In this protocol, two colours, Dye 450 (CBD 450), and Dye 500 (CBD 500) are used to stain 16 tubes of samples. Each sample is first prepared with paraformaldehyde to fix the cells, soaked in methanol to permeablize them, and then stained with the colours in different amounts. For instance, one tube may receive a high amount of CBD 500, but no CBD 450, while another receives medium CBD 450 and low CBD 500. Once all the possible staining combinations have been completed, the tubes are then combined in one 2mL tube. After the excess dye has been washed off and the cells have been suspended in a small amount of Barcoding Wash Buffer, they are run through the flow cytometer. In order for the protocol to be perfectly optimized, sixteen very distinct populations should appear when we analyze the combined sample using FlowJo software.

Methods: Staining Ramos Cells and PBMCs

After culturing the Ramos cells, an antibody staining was peformed in order to phenotype them. First 1 000 000 cells were removed from the plate and split between two new tubes. These samples were spun on the centrifuge and the excess media was dumped from each tube. Cells were resuspended in FACS Staining Buffer (970mL of PBS+20mL of FBS+10mL 10% sodium azide) and four antibodies were added to each sample tube (anti-CD19-eF450, anti-IgM-APC, anti-CD22-FITC, and anti-CD27-PE). After the cells incubated for 20 minutes in the dark at four degrees Celsius (which gave the antibodies time to bind), the cells were washed with FACS staining buffer and the excess volume was dumped from each tube, therefore removing the antibodies that did not bind. Finally the cells were re-suspended in 100µL of FACS staining buffer, and were ran on the MACSQuant.

After practicing the staining experiment on Ramos cells, an antibody staining experiment was then performed on isolated PBMCs (lymphocytes) from healthy volunteers. This staining involved four different antibodies (anti-CD19-PE, anti-CD3-APC, and anti-CD4-FITC, and anti-CD8-VioBlue). These antibodies bind to surface markers on lymphocytes, allowing the identification of the different types of cells, and their frequency. CD19 is expressed on B cells, whereas CD3 is expressed on T cells. CD4 and CD8 define helper T cells and cytotoxic T cells, respectively.

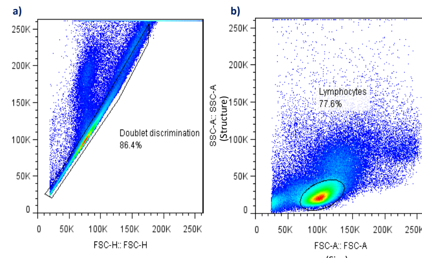

Figure 3: Gating Strategy. For all experiments, it was first necessary to perform the following gating strategy on the dot plots. This is a critical step towards identifying the cell population of interest. **(a)** A doublet discrimination is necessary to eliminate any cells that were stuck together while passing through the cytometer. **(b)** A gate was created based on size and structure to include viable lymphocytes (or Ramos cells).

Results

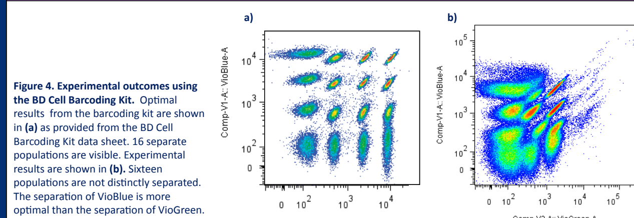

Figure 4. Experimental outcomes using the BD Cell Barcoding Kit. Optimal results from the barcoding kit are shown in **(a)** as provided from the BD Cell Barcoding Kit data sheet. 16 separate populations are visible. Experimental results are shown in **(b).** Sixteen populations are not distinctly separated. The separation of VioBlue is more optimal than the separation of VioGreen.

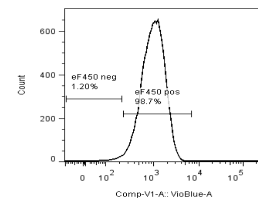

Figure 5: Ramos cell staining of the B cell surface marker CD19 (eF450). As Ramos cells are derived from a B-cell line, they will be positive for the B cell surface marker, CD19. In this particular sample, 98.7% of total Ramos cells expressed this marker.

Results (continued)

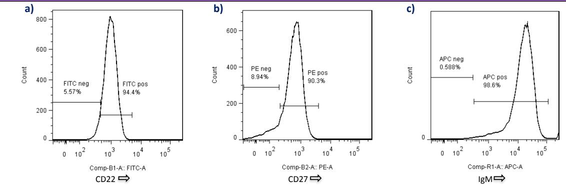

Figure 6: Ramos cell staining of surface markers CD22-FITC, CD27-PE and IgM-APC within the CD19+ population. (a) 94.4% of cells are positive for FITC, which means that 94.4% of cells have the CD22 surface marker. **(b)** 90.3% of cells are positive for PE, which means that 90.3% of the cells have the CD27 surface marker. **(c)** 98.6% of cells are positive for APC, which means that 98.6% of the cells have the IgM surface marker.

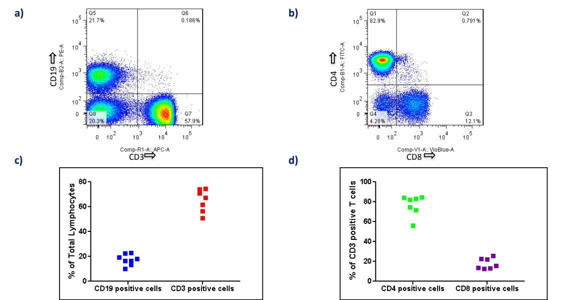

Figure 7: Identifying B and T cells within total lymphocyte population in PBMC samples (a) Gating Strategy to identify B and T cells. Antibodies against CD19-PE and CD3-APC were used to gate on the CD19 positive B cells and the CD3 positive T cells within the lymphocyte population. **(b) Gating Strategy to identify T cell subsets.** Antibodies against CD4-FITC and CD8-VioBlue were used to gate on the CD4 helper T cells and the CD8 positive cytotoxic T cells within the CD3 positive population. **(c) Percentage of B and T cells within the total lymphocyte population.** Among the 8 PBMC samples measured, the percentage of CD19 positive B cells within the total lymphocyte population ranged from 10.1%-22.7% and the percentage of CD3 positive T cells ranged from 51.0%-74.3%. (d) **Percentage of T cell subsets within the CD3 positive T cell population.** Among the 8* PBMC samples measured, the percentage of CD4 positve helper T cells within the total CD3 positive T cell population ranged from 56.0%-84.4% and the percentage of CD8 positive cytotoxic T cells ranged from 12.5%-25.7%.

*Staining for CD3, CD4, and CD8 positive cells have an n=7 because one sample was not stained with the CD3 antibody, which prevented further T cell subsets from being identified.

Summary

In summary, whole blood was processed in order to separate it into its component layers. The PBMC layer was removed and used in antibody staining experiments to examine the percentages of various lymphocyte populations. From these results it can be concluded that within lymphocytes, T cells are found in higher percentages than B cells and helper T cells are more common than cytotoxic T cells. Ramos cells were also cultured and used in antibody staining experiments in order to practice staining protocols, and ultimately, to phenotype these cells. From our results, we can conclude that the majority of Ramos cells in our sample expressed all four surface markers: CD19, CD22, CD27, and IgM. These Ramos cells were also used to optimize the BD Cell Barcoding Protocol. After the first attempt, the 16 populations were not optimally separated, although the separation was better for VioBlue than for VioGreen. Reasons for this could include problems with the compensation during analysis or even technical error, such as non-consistency of the samples. The next steps will involve repeating this experiment: making sure all tubes are treated identical and also taking single stained samples of every colour combination and using these to adjust the voltage of both colours, VioBlue and VioGreen.

Acknowledgements

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