Introduction

Immunosenescence is a functional and phenotypic exhaustion observed in adaptive and innate immune cell subsets, leading to an increased rate of infection, decreased vaccine responsiveness, and autoimmunity (Aw, Silva, & Palmer, 2007). Immunosenescence occurs with advancing chronological age and in individuals with a chronic viral infection. These changes ultimately result in increased mortality rates (Aw et al., 2007).

Many studies have investigated the profound remodeling that occurs to immunosenescent cells. Wherry et al. (2007) explored the ‘exhausted’ CD8+ T cell phenotype that occurs with chronic viral infection. Phenotypic changes, such as increased PD-1 expression, as well as functional changes, such as impaired cytokine signaling pathways, were observed. Altered levels of gene products involved in chemotaxis, adhesion, and migration were also detected. Remondini et al. (2010) investigated patterns of gene expression in T cell immunosenescence and observed changes in the expression of genes involved in T cell receptor signaling, cytokine-cytokine receptor interactions, metabolic pathways, and transcriptional regulation. Understanding the changes in certain immune cell subsets at a transcriptional level is critical to understanding the process of immunosenescence. Further identifying these pathways can help lead to the development of improved immunotherapies to target immunosenescent cells.

Immunosenescence leads to poor responses to vaccines. Elderly individuals and those with a chronic viral infection are often unable to mount an effective immunological response to the influenza vaccination (Malaspina et al., 2005). These individuals are not only vulnerable to infection by this virus, but they are at an increased risk of developing complications (Malaspina et al., 2005). Despite receiving an influenza vaccination, individuals with HIV often still acquired the influenza virus, leading to an increased number of visits to health care providers and life-threatening complications (Klein, Lu, DelliBalso, Cote, & Boivin, 2007). Understanding the role of different genes and gene products that are needed to mount an effective response to vaccine, can ultimately help gain insight to prevent infection and disease in vulnerable populations.

The purpose of this research project is to gain a greater understanding of the mRNA profile of immune cell subsets in individuals infected with HIV and how the mRNA profiles contribute to a good or poor response to vaccination. The objective of the study was to compare the B cell, CD8+ and CD4+ T cell mRNA profile of ‘high-responders’ and ‘low-responders’ to the influenza vaccine in order to examine differences at a transcriptional level that may contribute to a good or poor response. Using this chronic viral infection as a model of accelerated aging may also help gain insight as to why the human immune system becomes less effective with age.

My role as a summer student assisting with this research project was to identify individuals infected with HIV to be tested as ‘high-responders’ and ‘low-responders’ to the 2015/2016 influenza vaccine. My role was also to develop and optimize a protocol for the manual isolation of good quality RNA from pure populations of separated B cells, CD4+ T cells, and CD8+ T cells. Ultimately, this RNA would be sent to The Centre for Applied Genomics for transcriptome analysis. It was hypothesized that more than half of the 25 individuals infected with HIV would be classified as ‘low-responders’ to the influenza vaccine. This hypothesis is a reflection of the poor response to vaccine that is often seen in individuals that are aged or infected with a chronic viral infection (Malaspina et al., 2005). Additionally, it was hypothesized that a sufficient quantity of good quality RNA (greater than 500 ng) could be extracted from an initial starting cell population of about 25.0x10^6 cells.

Methods

(1) Determining Response to Influenza Vaccine

A hemagglutination inhibition (HAI) assay was used to determine whether an individual was a 'high-responder' or 'low-responder' to the 2015/2016 strain of influenza vaccine. HAI assays measure the level of
antibodies in an individual's plasma against the influenza envelope protein hemagglutinin (HA) (Noah, Hill, Hines, White, & Wolff, 2009). Serial dilutions of previously isolated plasma samples from 25 individuals infected with HIV, both pre- and post-influenza vaccine, were tested. The HAI titer was reported as the greatest dilution of plasma at which hemagglutination inhibition still occurs.

The HAI titers for the individual's plasma samples taken pre- and post-influenza vaccine were compared in order to categorize the individuals into a 'high-responders' or a 'low-responders' category. Individuals with total influenza specific antibody titers 2-fold above their pre-vaccine levels were categorized as 'high-responders' and individuals with less than a 2-fold increase in their total influenza specific antibody titers were categorized as 'low-responders'. The HAI assay was completed off-site.

(2) Optimizing Protocol for Cell Separation and RNA isolation

Peripheral blood mononuclear cells (PBMCs) from uninfected controls were isolated from whole blood. The isolated PBMCs were used to optimize a protocol for the manual separation of B cells, CD8+ T cells, and CD4+ T cells. Initially, 'The Big Easy' EasySep™ magnet and three positive selection kits from STEMCELL Technologies were used to separate the populations. The isolation was done in the order of CD19, CD8, and then CD4 positive selection. The protocol was subsequently modified to be CD19 positive selection, CD4 positive selection, and finally a CD8+ T cell enrichment. 'The Big Easy' EasySep™ magnet was replaced with the EasySep™ magnet. Ultimately, upon arrival of the RoboSep-S Automated Cell Separator to the lab, the EasySep™ magnet was replaced with the RoboSep-S and PBMCs from individuals infected with HIV were used with the optimized protocol.

The isolated immune cell subsets were assessed for purity by staining with fluorescent antibodies and using flow cytometry. The B cells, CD4+ T cells, and CD8+ T cells were assessed for purity using fluorescent anti-human CD19, CD4, and CD8 antibodies, respectively. All cells were also stained with a fluorescent anti-human CD3. The B cells, CD4+ T cells, and CD8+ T cells were identified as CD19+/CD3- cells, CD3+/CD4+ cells, and CD3+/CD8+ cells, respectively. Figure 1 depicts the gating strategy that was used to identify the immune cell subsets. With each population, single cells were selected, followed by lymphocytes, and finally the percentage of purified cells was identified.

After the populations were confirmed to be greater than 90% pure using flow cytometry analysis, total RNA was extracted and isolated using the Qiagen RNeasy® Mini Kit. Additional DNase treatments were performed to ensure there was no contaminating DNA. The RNA was assessed for concentration and purity using spectrophotometry. Agarose gels were run to visualize the RNA integrity.

Results

(1) Determining Response to Influenza Vaccine

From a group of 105 individuals infected with HIV, 25 individuals were selected for this study. Twenty males and five females between the ages 33 and 73 who were mono-infected with HIV were chosen. These individuals had CD4+ T cell counts greater than 200 cells/μL and an HIV viral load of less than 50 copies/mL at the time of vaccination. Plasma from these 25 individual's pre- and post-vaccine blood draws were selected and sent off to a collaborator for an HAI assay. The results from the HAI are still pending.

(2) Optimizing Protocol for Cell Separation and RNA isolation

Three immune cell subsets were separated using a magnetic bead-based separation system. Several approaches were compared to identify optimal cell viability, purity, and recovery after separation. Major changes made to the protocol include replacing the 'The Big Easy' EasySep™ Magnet with the EasySep™ Magnet, increasing the concentration of selection cocktail, and replacing the CD8 positive selection kit with a CD8+ T cell enrichment kit.

Initially, the separation of uninfected control PBMCs using a sequential CD19, CD8, and CD4 positive selection led to an unusual distribution of the CD8+ T cells when analyzed using flow cytometry. The CD8+ T cells presented with an abnormally high side scatter (SSC) (Figure 2). The cells within the population with a higher SSC (SSC hi) were 98.9% CD3+/CD8+, whereas the cells with a low SSC (SSC lo) were only 71.8% CD3+/CD8+. The CD8+ T cells were within the SSC hi population. Switching from a CD8 positive selection isolation method to a CD8+ T cell enrichment evaded the abnormally high SSC of the population (Figure 3). The purity of the CD8+ T cell lymphocyte population increased from 87.9% to 92.5% with the switch to the CD8+ T cell enrichment method (Figure 3).
The purities of the separated populations were relatively high while using ‘The Big Easy’ EasySep™ Magnet, but the yields of the cells were low. There was a much lower yield of CD4+ T cells than hypothesized. Beginning the separation with 21.5x10^6 PBMCs recovered 1.7x10^6 CD4+ T cells. Switching to the EasySep™ magnet improved the recoveries. Starting with 22.6x10^6 PBMCs recovered 8.6x10^6 CD4+ T cells. Additionally, the switch to the EasySep™ magnet led to populations that were over 90% pure. Figure 4 is a representation of the pure populations that were isolated using the optimized protocol. The B cells (CD19+) were 93.9% pure, the CD4+ T cells were 98.4% pure, and the CD8+ T cells were 92.5% pure.

Total RNA from the separated immune cell subsets was isolated and purified using the RNeasy® Mini Kit. The mass of RNA that was purified was proportional to the number of cells that were isolated. Using a representative sample separation, 1.5x10^6 CD19+ cells yielded 1.05 ug of RNA, while 8.6x10^6 CD4+ T cells and 1.9x10^6 CD8+ T cells yielded 5.5 ug and 1.2 ug of RNA, respectively. The A260/A280 ratios were greater than 2.0. The A260/A230 ratios were greater than 2.3. When run on a gel, the RNA migrated into distinct bands.

Transferring the optimized protocol for the manual separation to the automated RoboSep-S lead to similar results. All three immune cell subsets had purities that were greater than 90% with recoveries that were comparable to the protocol using the EasySep™ magnet. These results were achieved with both uninfected control samples and samples from individuals infected with HIV (data not shown).

**Discussion**

(1) **Determining Response to Influenza Vaccine**

Due to unforeseen circumstances with our collaborators, this data has not yet been received. Upon arrival of this data, PBMCs from 5 of the ‘high-responder’ and 5 of the ‘low-responders’ will be separated into B cells, CD4+ T cells, and CD8+ T cells. The RNA will subsequently be isolated and purified, and then sent to The Centre of Applied Genomics in Toronto for transcriptome analysis.

(2) **Optimizing Protocol for Cell Separation and RNA isolation**

After many protocol modifications and attempts, an optimized protocol for the manual and automatic separation of immune cell subsets and extraction of good quality RNA has been developed. This protocol enables the separation of populations of B cells, CD4+ T cells, and CD8+ T cells that are over 90% pure. Additionally, the protocol ensures that a sufficient quantity of cells can be isolated from a starting population of approximately 25.0x10^6 PBMCs. The RNA extraction ensures that pure and intact RNA can be effectively isolated from these immune cell subsets.

An unusual SSC of CD8+ T cells was observed when visualizing the single cells that were separated with the CD8 positive selection method (Figure 2). This abnormally high SSC is likely a reflection of a larger, more granular population of CD8+ T cells that occurs following activation of the cells (Cell Size and Granularity, 2016). It was hypothesized that the activation of the CD8+ T cells was a reflection of the antibodies used in the separation process. A CD8+ T cell enrichment kit, as opposed to the CD8 positive selection kit, was consequently used to test this hypothesis. A very different SSC distribution was observed. The SSC of the separated cells did not indicate large and granular cells; it was much more representative of CD8+ T cells (Figure 3). It is critically important for this subset of cells, as well as the other subsets, to be undisturbed by the separation process. The granulation would likely lead to alterations in the mRNA profile, which would be reflected in the transcriptome analysis. It is likely that using a CD8 positive selection method in the protocol would lead to modifications in the CD8+ T cell mRNA profile.

The purities of the populations were quite consistent with each control sample of PBMCs that were tested. B cells, CD4+ T cells, and CD8+ T cells had average purities of 94%, 96%, and 86%, respectively. Because a population with a greater than 90% purity was needed before isolating and extracting RNA, the EasySep™ Magnet was tested in lieu of ‘The Big Easy’ EasySep™ Magnet to test if the purity of the CD8+ T cells would increase. This modification to the protocol yielded much pure populations. The CD8+ T cells purity increased to 92.5%. This was an important change to implement. Not only did the EasySep™ magnet yield a much more pure population, it also contributed to isolating a larger number of cells. The proportion of PBMCs that were isolated from the CD4 positive selection increased from 8% to 38%, while keeping a similar purity. CD4+ T cells comprise about 30-45% of PBMCs, hence this is a much more complete isolation (Miyahira, 2011). This protocol modification was also beneficial because it led to a larger quantity of isolated RNA, which is important for the transcriptome analysis.
The optimized protocol tested on the RoboSep-S yielded similar results to the EasySep™ magnet. Because the RoboSep-S yielded a high recovery of the purified populations, this protocol was used on PBMCs from individuals infected with HIV. The RoboSep-S is the preferred method of separation because it minimizes the prospect for human error.

Additional of PBMCs from individuals infected by HIV in both the 'high-responder' and 'low-responder' groups are ready to be separated with the RoboSep-S and analyzed. Eventually, a comparison of the transcriptome from 'high-responders' and 'low-responders' can help us learn and see if there is a unique mRNA profile in these defined subsets of immune cells in individuals that respond well to influenza vaccinations. The information that will be obtained from this study can hopefully contribute to having a greater understanding of which genetic pathways play an important role in enabling an individual to have an effective response to vaccination. This information could aid in the development of improved vaccine formulations or immune modulation drugs. The results from these investigations could benefit not just those with chronic viral infections, such as HIV, but the ageing population as a whole.
Figure 1. Gating strategy used to determine the purity of the isolated cell populations. Representative plots showing the three-step gating strategy is presented. Initially, single cells were selected on a FSC-A vs. FSC-H plot to exclude any doublet cells. Next, the tight population of lymphocytes was selected to exclude any debris that was present in the sample. Lastly, the population of interest was selected to determine the purity of the population. Above, the CD4+ T cells were assessed for purity by selecting single cells, and then lymphocytes, and subsequently identifying the percentage of cells that were both CD3+ and CD4+.

Figure 2. CD8+ T cell populations isolated using a CD8 positive selection strategy have an abnormal SSC distribution. The CD8+ T cell population presented with an abnormally high SSC when analyzed using flow cytometry after a positive selection of these cells. After selecting single cells, the lymphocyte population was analyzed to assess the CD3+/CD8+ population. By gating SSChi and SSClo on an SSC-A vs FSC-A plot, it was evident that the CD8+ T cells (CD3+/CD8+) had a higher SSC. This higher SSC indicates larger and more granular cells, suggestive of CD8+ T cell activation.
Figure 3. Replacing CD8 positive selection with CD8+ T cell enrichment reduced the SSC of the lymphocytes and increased the purity of the cell population. (A) Using a CD8 positive selection method led to an abnormally distributed lymphocyte population with an extremely high SSC-A. The purity of the CD8+ T cell population was 87.9%. (B) Switching to a CD8+ T cell enrichment method reduced the SSC of the lymphocyte population on the SSC-A vs FSC-A plot. The purity of the CD8+ T cell population increased to 92.5%.

Figure 4. Representative plots of the purities achieved through the separation of the B cells, CD4+ T cells, and CD8+ T cells using the optimized protocol and described gating strategy. (A) The B cell (CD3-/CD19+) population was determined to be 93.9% pure. (B) The isolated CD4+ T cell populations had a purity of 99.4%. (C) The CD8+ T cell population that was isolated was 92.5% pure.
References


Personal Reflection

I am very grateful to have received the CFID Undergraduate Summer Student Research Award. I have had the opportunity to develop a new set of skills including cell separation, flow cytometry analysis, and nucleic acid isolation. These skills will enable me to contribute to the completion of this project and will also support my honours project work this fall. Furthermore, my time in the lab has helped me gain experience and appreciation for the way a lab works. I have a greater appreciation for the importance of teamwork in the lab and the roles of the lab staff. The protocol would not have been finalized without the guidance and support of Dr. Lisa Barrett, Dr. Drew Slauenwhite, and Krista Arseneault; I truly appreciate their assistance. Conclusively, this Research Award has given me newfound skills that have prepared me for my ongoing role in the lab and through which we have identified a protocol that is required for the successful completion of this project.