THE COMPARISON OF CO-STIMULATORY SURFACE MOLECULE EXPRESSION ON HUMAN MONOCYTE-DERIVED DENDRITIC CELLS INFECTED WITH TWO STRAINS OF INFLUENZA VIRUS

Jessie M. Simmon
Northern Michigan University

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By

Jessie M. Simmon

THESIS

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In partial fulfillment of the requirements
For the degree of

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Graduate Studies Office

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This thesis by Jessie M. Simmon is recommended for approval by the student’s thesis committee in the Department of Biology and by the Dean of Graduate Studies.

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NAME: Simmon, Jessie M.

DATE OF BIRTH: June 13, 1984
ABSTRACT

THE COMPARISON OF CO-STIMULATORY SURFACE MOLECULE EXPRESSION ON HUMAN MONOCYTE-DERIVED DENDRITIC CELLS INFECTED WITH TWO STRAINS OF INFLUENZA VIRUS

By

Jessie M. Simmon

The early immune response against influenza is not well understood. The goal of the present work was to investigate co-stimulatory surface molecule expression between two strains of influenza virus. It has been demonstrated that foot-and-mouth-disease virus (FMDV) infection in mice causes a down-regulation of activation molecules in dendritic cells (DC) while inactivated FMDV induces expression of co-stimulatory surface molecules. Influenza virus and FMDV are cytopathic and both induce a T-cell independent response to clear infection. In this thesis, monocytes from whole blood were magnetically separated using a biotinylated antibody against the CD14 molecule. These monocytes were cultured with cytokines, and differentiated into DC, and were infected with two strains of influenza virus. Each sample was stained with a panel of antibodies specific for molecules involved in DC activation. The results indicated that a strain of influenza that currently infects the human population down-regulates activation molecules on human monocyte-derived DC. In contrast, DC infected with the avian-adapted strain demonstrated an up-regulation of activation molecules. The human adapted strain behaves similarly to infectious foot-and-mouth disease virus, while the avian adapted strain behaves like inactivated FMDV in human monocyte-derived DC. The implications of these findings for the understanding of the early protective immune response against different strains of the influenza virus are discussed.
DEDICATION

It is with great honor and love that I dedicate this thesis work to my dear father, who has unconditionally supported me in all aspects of my life. Through hard work and self sacrifice, he has enabled me to achieve my own dreams and aspirations. It is my hope that I continue to make him forever proud of my accomplishments, and that he is truly proud of the person I have become.
ACKNOWLEDGEMENTS

I owe my greatest appreciation to my advisor and mentor of 5 years, Dr. Osvaldo Lopez, of Boonshoft School of Medicine at Wright State University. As his graduate student, it is easy to remark on how much of an impact he has had on my life, and this thesis work. He is not only a commendable professor, but he is also an exemplary role model. Through his work, he demonstrates what it is to be a humanitarian minded academic scientist—something that is a premium in the complex world in which we live. He has constantly held me to a level of performance that will allow me to excel in my future endeavor as a physician and scientist. Dr. Lopez is without a doubt, the most influential teacher I have ever had. I am reminded of a comment I made in the fall of 2005 while we were in Buenos Aires, Argentina presenting at a virology conference. I had the pleasure of meeting his graduate student Dr. Matias Ostrowski, who is now currently working at the Curie Institute in Paris. Matias had asked me during dinner one evening what my future career plans were. I replied hastily, “I don’t want to get a PhD.” This surprised Matias, and he immediately inquired as to why. I joking replied “I don’t want to become like Dr. Lopez.” I now reflect on that comment, and I shake my head in dismay for being so naive. As I’ve progressed in my education, I have a vastly different perspective. I now strive to model myself after Dr. Lopez every day, in hopes that I may one day become even one iota of the scientist and person he is. Despite his early departure from this university, and my inability to retain the right to keep him as my thesis chair, he has overseen this work from beginning to end, and my training as a scientist. For this, I owe him my greatest gratitude.
I owe a great deal of thanks to Dr. Donna Becker of the Department of Biology, as she has acted as my thesis chair. Not only has she served as an inspiration to me as a woman in science, but she has always kept my best interests in mind. I sincerely appreciate that, as it has been difficult to find that at this institution. I have enjoyed working under her, and have also learned a great deal as a result. As her student during my undergraduate career, I had the pleasure of taking two of her classes, which further stemmed my interest in microbiology. The Department of Biology is truly fortunate to have such a gifted and caring professor in its midst.

Dr. Neil Cumberlidge, of the Department of Biology has been instrumental in polishing this thesis work. His critique and constructive criticism have greatly improved the quality of this thesis - which I deeply appreciate. He has also supported my best interests, and has done everything in his power to help me finish my endeavors here at Northern. Dr. Cumberlidge is a true scholar in academia, and it has been my honor to have him serve as my committee member.

As Medical Director of the Cytogenetics and Molecular Diagnostic Laboratory at Marquette General Hospital, Dr. Ramakrishman Sasi has done so much for me, and I cannot thank him enough. This professor deserves a significant amount of recognition. He does so much for everyone, and receives little credit and respect. His constant reinforcement and encouragement had ultimately kept my outlook exceptionally positive, despite the environment I was working in. He has worked with fervent dedication to ensure that I was able to finish my degree. It has been an absolute honor and privilege to work under him, and I look forward to continuing our collaboration.
This research would not have been possible had it not been for the collaboration of

**Marquette General Hospital.** Their generous use of the flow cytometer was key in this research. I can’t express my gratitude adequately enough to **Flo Namiotka** and **Martin Renaldi**, who were both influential in completing this thesis. Flo was absolutely wonderful when it came to processing my samples, no matter how many I had. She was also flexible, and provided a great deal of advice on how to improve my samples. She was of great help to me, and I will miss working with her. **Dr. John Weiss**, Medical Director of Pathology, also deserves recognition for allowing me the use of the hospital facility. His collaboration with our laboratory has been most respected.

**Dr. Thomas Froiland**, of the Department of Biology was of great help with his advice in maintaining our embryonated chicken eggs. His generous offer for the use of his incubator was also greatly appreciated. Without his collaboration, it would not have been possible to propagate influenza virus needed for my research. I also very much enjoyed his Histology class. He helped me to understand the histology of the lung, and only until after that was I able to fully understand the deadly pathology of influenza infection.

**Dr. Erich Ottem**, of the Department of Biology was very kind in allowing me to use his laboratory for the last few months of my experiments. He has done everything in his power to help me to finish my degree in the absence of my advice, and for this I owe him a great deal for his patience, and continued support. He has had a good sense of humor when it came to my antics, and I sincerely appreciate that!
**Dr. Mary Stunkard**, of the Department of Clinical Laboratory Sciences, was of great help to me during the phlebotomy portion of this thesis. Her advice was also appreciated with optimization of my flow cytometry protocol. And as always, her collaboration was greatly appreciated.

As Chief of the Influenza Molecular Genetics branch at the Centers for Disease Control, **Dr. Ruben Donis** kindly provided our laboratory with advice regarding the virology pertaining to this research. He has been a great source of inspiration, as he is a crucial researcher in the influenza field. I thank him for his advice, his collaboration with our lab, and his support.

**Dr. Matias Ostrowski** was a tremendous role model for me in this thesis work. As a member of our laboratory, his previous work has been important in providing me with direction and focus. His publications have provided me with tremendous insight into my own research. I also thank him for the fantastic time he showed me in Argentina! I wish him luck as he is now enjoying a research position at the Curie Institute in Paris, France. I look forward to continuing our collaboration.

**Ms. Susie Piziali** of the Department of Biology is an individual who I cannot thank enough for everything. She has served as a great source of encouragement during my education here at Northern. I wish her and her family the best, and I will miss her greatly after I graduate.
Dr. Frank Verley of the Department of Biology has been a great resource for me during this thesis work. His kindness and understanding allowed me to continue focusing on my work, and not on the political storm that I had been cast into.

Chris Sorelle was of great help to me with several aspects of my thesis work. He is a great friend, and a truly kindhearted individual. Thank you!

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I thank the Department of Biology for making the decision to hire Dr. Lopez. Had they not selected him for the microbiologist position, I would not have the great opportunities I have today, or the priceless education. Clearly, they know a true scientist when they see one.

I would like to express my greatest thanks to the Department of Biology Graduate Students. In particular, I would like to express my gratitude to Carla Serfas, Grant Slusher, Bill Severud, Steve Schaar, Tiffany Opalka, Kim Hardenbrook, Ericka McCarthy, Mike Peters, and Trisha Sippel. I cannot thank these dear people enough for their support, friendship, and humor during the completion of this thesis.

During my time at Northern Michigan University, I have had the honor of developing many friendships with Members of the Marquette community. These individuals have
been absolutely wonderful in supporting my research, and this has meant a great deal to me. I never expected to become so attached to this community, but with its tremendous support, love and friendship, this has certainly become the case. Without a doubt, I will feel a tremendous sadness when I graduate, and move away from the area. I will miss everyone very much.

**Dr. Ronald Parejko** of the Department of Biology encouraged my passion for microbiology and infectious diseases. Unfortunately, I was only able to take one course with Dr. Parejko before he retired. During the time that I took his course Medical Microbiology, I became completely entranced in the subject matter, and no longer viewed studying as a mundane chore. He is the individual who sparked my intense desire to study microbiology. He continues to serve as an inspiration, and I sincerely thank him for his tremendous impact on my academic aspirations. I hope he is enjoying his retirement!

I would like to express my dearest appreciation to **my Students**. As a teaching assistant here at Northern Michigan University, I have had the opportunity to teach both Medical Microbiology and General Microbiology. This job has brought me the upmost satisfaction, as I have always been fortunate to have very passionate and motivated students. Each class has always embraced the subject at hand, and was open to learning more. Not only where my students always eager to learn, but as they gradually learned about my own research, they were always encouraging me in every aspect. Many of my students, both former and present, showed their support in attending my thesis presentation, which also had meant very much to me.
Admittedly, the shear musical genius and innovation of Professor Satchafunkilus is what fueled my ability to stay up until 4 am many mornings in the laboratory working diligently on my experiments, as opposed to flying in a blue dream. His music is also what ultimately kept me motivated to complete my work even when I found myself in the most trying situations. Satch’s sound will continue to inspire and stimulate my desire to learn, and improve the world in every aspect that I can.

I also wish to thank two members of the Lopez laboratory in particular: Hope O’Donnell and Matthew Kortes. Hope greatly encouraged me in my research and was a true collaborator. It deeply saddened me that she left the university prematurely; however I am glad that she is continuing her work now as a PhD student at Cornell. Matthew Kortes has been a great friend throughout my research. I wish him luck as he pursues his medical degree from the Medical College of Wisconsin.

Lastly, it gives me a great sense of pride to thank my Family for all of their support in every aspect of my life. I have been given more opportunities than most because of their hard work and devotion to raising me. From raking blueberries as a summer job in high school, to caring for patients in my home health job, they have instilled in me always to give 100%, and to be appreciative of everything I have been given, no matter what the circumstance. Even when the climate at this university turned sour, they still encouraged me to finish the thesis work I had started. It is because of their support that I can truly give back to the world what it has been given to me. Now that I look to the future; I hope that I continue to make them proud of my accomplishments.
PREFACE

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This thesis follows the format prescribed by the *Journal of Immunology* (http://www.jimmunol.org/misc/authorfulllength.shtml) and the Department of Biology.
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<tbody>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
<td>Carbon Dioxide</td>
</tr>
<tr>
<td>CO₂</td>
<td>Carbon Dioxide</td>
<td>degrees Celsius</td>
</tr>
<tr>
<td>˚C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRBC</td>
<td>Chicken red blood cells</td>
<td></td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
<td></td>
</tr>
<tr>
<td>EID</td>
<td>Egg infectious dose</td>
<td></td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
<td></td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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<tr>
<td>FMDV</td>
<td>Foot-and-mouth-disease virus</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
<td></td>
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<tr>
<td>H1N1</td>
<td>Hemagglutinin 1 Neuraminidase 1</td>
<td></td>
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<tr>
<td>H3N2</td>
<td>Hemagglutinin 3 Neuraminidase 2</td>
<td></td>
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<tr>
<td>HAU</td>
<td>Hemagglutinin units</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
<td></td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
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</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
<td></td>
</tr>
<tr>
<td>MOI</td>
<td>Multiplicity of infection</td>
<td></td>
</tr>
<tr>
<td>ml</td>
<td>milliliter</td>
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</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Solution</td>
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PE.................................................................Phycoerythrin
PBMC..........................................................Peripheral Blood Mononuclear Cells
RPMI ......................................................Roswell Park Memorial Institute 1640 (culture media)
SS Log..........................................................Side Scatter Logarithm
U.................................................................unit
UV..............................................................ultra-violet
VSV.............................................................Vesicular stomatitis virus
INTRODUCTION

Immune response against a pathogen in mammalian hosts

Over the course of evolution, the immune systems of mammal species have been constantly challenged by microbial pathogens such as fungi, bacteria, and viruses, and the host species have developed immune responses against them. Most viruses infect their hosts through mucosa in the respiratory, gastrointestinal or urogenital tracts, and replicate in the tissues of their hosts and this in turn produces an immune response to oppose infection.

The immune system of mammals comprises two main sub-systems: innate and adaptive. The innate immune system comprises several cell types and molecules that non-specifically recognize the presence of a virus in the host. The result of this non-specific recognition is the activation of cells involved in the first response against viral infections. These cells secrete a number of molecules that mediate communication between cells. These mechanisms are collectively named non-specific recognition because even though the presence of the infection is sensed, the specific virus is not fully recognized. As a first responder, the innate immune system blocks the replication of viruses and contains the spread of infections until a stronger and more specific response can be produced by the adaptive immune system. The adaptive immune response is not universal, and the strategy of infection of the virus will determine the appropriate adaptive immune response against it.
In the last ten years, it has become clear that the innate immune response is also responsible for determining the appropriate adaptive immune response against viral infection. Several cells are involved in the adaptive response host. The influenza virus is a common pathogen that infects mammals and birds. The viral receptors are recognized by the immune system of the host as antigens. Initial recognition of a pathogen by a group of specific cells of the immune system called “antigen presenting cells” initiates the adaptive immune response. The cells that present antigens include dendritic cells, B cells, and macrophages. Dendritic cells are the most efficient antigen presenting cells that activate other immune system cells by providing specific and non-specific stimuli. T cells, in particular, become activated when dendritic cells express surface major histocompatibility complexes (MHC) on their cell membrane (Figure 1) and other co-stimulatory molecules are expressed as well.

Figure 1. Illustration of a MHC molecule expressed on the outside of a dendritic cell membrane. The antigen exposed by the molecule is of the influenza virus. MHC molecules are crucial for the clearance of pathogens infecting a host.

When an immature dendritic cell interacts with a pathogen such as an influenza virus particle, they undergo a maturation process that includes the expression of surface molecules and the release of pro-inflammatory cellular messengers known as cytokines.
Once this maturation process is complete, the dendritic cell then acts to activate certain cells of the immune system.

Cells that produce antibodies are called B lymphocytes (B cells) and they can be divided in three different cell populations. The most numerous are the follicular B cells or B2 cells that circulate permanently in the body, contacting pathogens in the lymph nodes. The second group are the B1 cells, that are located predominantly in the body cavities, and these cells produce antibodies against conserved molecules of pathogens, especially sugars present on the cell walls of bacteria. B cells can be activated to produce antibodies in two ways. The first way is through a T-cell dependent response: peptides from the pathogen are expressed in association with MHC molecules on the cell membrane of antigen presenting cells. This name refers to the concept that in order to induce the most efficient and effective antibody response from B cells, T cells must be involved. T cells secrete cytokines and express activating molecules on their cell membrane to activate the B cell. This activation includes proliferation of the B cells into plasma cells, isotype
switching, and memory cell generation. A model for the typical adaptive T cell dependent immune response against a pathogen is shown in Figure 2.

Some bacteria and viruses induce a response in which there is no T cell involvement. These responses are called T-cell independent responses (Figure 3). Here, T cells are bypassed, and the antigen presenting cell directly activates B cells to produce antibodies. These immune responses are faster than the T-cell dependent responses.

Figure 3. T-cell independent response. Dendritic cells are loaded with influenza virus. As a result, the dendritic cell activates the B cell, and by-passes the T cell.

**Early immune response against cytopathic viruses**

The pathology associated with cytopathic viruses that induce acute infections is increased by the interference of the normal physiology of the host cell. The virus rapidly replicates, causing extended tissue injury in only a few days, so the induction of a protective immune response must be rapid. After infection of naïve individuals, both neutralizing antibodies and cellular immunity responses may be necessary to clear the virus. With some viruses, neutralizing antibodies seem to be the main branch of the immune response involved in viral clearance. Indeed, a strong protective neutralizing antibody-mediated
response against several cytopathic viruses is induced early after infection without the involvement of CD4 T helper cells, by a T cell independent response.

For example, in 1986, Charan and Zinkernagel demonstrated that three days after infection with vesicular stomatitis virus, neutralizing antibodies with high affinity are detected in blood, and the vesicular stomatitis virus is cleared (1). In addition, immune response against polyoma virus, rotavirus, and influenza virus are also T cell independent (2-4). It has been demonstrated that the immune response against infectious foot-and-mouth disease virus is T cell independent while the immune response against inactivated FMDV is T cell dependent (5,6). However, CD4 T helper cells are necessary to establish long-lasting immunological memory after infection (7).

The lack of CD4+ T cell involvement at the beginning of infection could be thought as an escape mechanism of these viruses since T cells are one of the two main populations of the adaptive immune system. It is hypothesized that this a physiological mechanism in mammals that evolved to induce a rapid induction of neutralizing antibodies to decrease the viral load at the peak of the infection while T cell dependent response matures and induces a long-lasting protective response.

**Immune response against influenza virus**

Influenza virus is a cytopathic virus belonging to the family Orthomyxoviridae. It has a lipid membrane (envelope) and a segmented negative single-stranded RNA genome. The current inactivated (non-infectious) vaccine against influenza induces immunity around 15 days after vaccination and the immunity conferred is short-lasting. The goal for this
vaccine is to induce neutralizing antibodies against the prevalent strains of influenza in
the season, but the antibodies only last for a few months. In contrast, infection with
influenza induces neutralizing antibodies more rapidly and the immunity is long-lasting.
Thus, although the vaccine and the infection produce antibodies against the virus, the
kinetics and the outcomes are significantly different, suggesting different pathways. The
mechanisms involved in these different pathways of the immune response are only
partially known.

The basic parameters of the immune response against infection with influenza have been
very well characterized in humans and mice. Although infection of mice does not
produce the same disease in humans, the availability of reagents and of genetically
modified mice provides a bountiful supply of information regarding the pathogenicity
and immune response induced. In 1998, Epstein et al. demonstrated that CD4+ and CD8+
cells generated by intra-nasal infection of C57BLK6 mice with influenza A/PR/8/34
(H1N1) were sufficient to protect from re-infection (8). Roman et al. confirmed this in
1986 by demonstrating protection of irradiated mice adoptively transferred with CD4+
and CD8+ cells followed by a challenge with influenza (9,10).

However, the absence of B cells is not protective, even in the presence of CD8+ specific
T cells, indicating that antibodies are necessary for protection against this virus (11). The
early immune response against influenza is T-cell independent. This was shown in T
helper cell deficient mice and in CD40 knock-out mice (11,12). Moreover, isotype
switching from IgM to IgG was also found in these mice, and these antibodies protected
the mice from re-infection up to 60 days post-infection (13). These results demonstrated
that there is a T cell independent pathway at the beginning of infection but T helper cells are necessary for the establishment of immunological memory. These results are similar to the ones obtained by Ostrowski et. al. in 2005 using a mouse model for foot-and-mouth-disease virus (6).

Bender et. al. demonstrated in 1998 that macrophages, but not dendritic cells undergo apoptosis following infection with influenza. Using monocytes from blood and cytokine-derived dendritic cells, it was demonstrated that only very low levels of virus were actually produced from infected dendritic cells. Bender et. al. concluded that influenza virus infection in dendritic cells is non-toxic and unproductive (14).

Oh et. al. demonstrated in 2000 that there was a decrease in allogenic T cell proliferation with influenza infected murine dendritic cells at a multiplicity of infection of 12.5 or greater. However, at a lower multiplicity of infection, there is an increase in T cell response, whereby dendritic cells were loaded with UV inactivated virus. Although relevant to this study, Oh et al. did not investigate the role of interference particles from influenza infection, and so it is difficult to comment on the significance of the differences seen with a lower multiplicity of infection. However, influenza infected dendritic cells down-regulate T cell responses due to the decrease in proliferation that accompanies higher multiplicity of infection. These researchers did not investigate co-stimulatory molecules expressed on influenza infected dendritic cells, but their findings suggest that there could be a decrease in the expression of co-stimulatory molecules as has been found to be the case with dendritic cells infected with foot-and-mouth disease virus (15).
Dendritic cells loaded with UV inactivated foot-and-mouth disease virus up-regulate activation markers and induce a typical T-cell dependent response, while dendritic cells infected with active foot-and-mouth disease virus down-regulate activation molecules and induce a T cell independent response (5,6). These results suggest that two different pathways are utilized to induce neutralizing antibodies- one for infectious virus and the other for inactivated foot-and-mouth disease virus.

Oh et al. also demonstrated that A/PR/8/34, an H1N1 influenza virus, abortively infects human monocyte-derived dendritic cells which fail to induce a strong Th1 immune response. Up-regulation of co-stimulatory molecules, cytokine secretion, and expression of several genes involved in the IFN-α/β response and in dendritic cell activation were investigated in this work (15).

In 2006, Fernandez-Sesma et al. found that influenza-infected dendritic cells exhibit a down-regulation of MHC Class II and CD86 molecules. These researchers concluded that this is a mechanism of viral evasion from the immune system (16). Based on the results with foot-and-mouth-disease virus, as well as those of other researchers working in other cytopathic viruses, it is hypothesized that the down-regulation reported by Fernandez-Sesma et al. is indeed a mechanism of the immune system to decrease inflammation induced by activation of T cells by dendritic cells at the peak of the infection, rather than a mechanism of escape for the virus (16).

If this hypothesis is correct then it is predicted that a current human strain of influenza virus such as A/Memphis/102/72 (H3N2) will decrease activation molecules expressed
by infected dendritic cells while an avian adapted strain (such as A/WSN/33 (H1N1))
will increase activation molecules on dendritic cells.

**Project Significance**

The influenza strain responsible for the 1918 pandemic killed 50 million people
worldwide over the course of two years. To date, this is the worst-ever influenza
pandemic. It is of particular interest that many affected by this strain were mostly middle
aged individuals, ranging from 20 to 40 years of age. Many researchers consider that
another pandemic is inevitable, and could happen at any time.

In the United States, seasonal influenza causes more than 36,000 deaths and more than
200,000 hospitalizations every year. This type of influenza commonly affects
immunocompromised individuals, so it is important to understand the early protective
immune response against influenza in humans. In addition to the risk of another
pandemic, and the constant burden of seasonal influenza, strains of the virus to which the
human population has no immunity constitute a high risk for pandemic activity. As seen
with the Spanish flu of 1918, another pandemic could be capable of killing millions of
people due to high pathogenicity, and the impact on the global economy could be
catastrophic.

This research provides an insight into the early protective human immune response
against influenza virus, and will help to understand the early rapid protective response
achieved after infection with influenza. Ultimately, this knowledge could be beneficial
for the engineering of effective vaccines that may provide longer immunity to influenza.
This research could lead to an explanation as to why differences are noted between the pathogenicity of different strains of influenza in humans.
CHAPTER 1: OPTIMIZATION OF PROTOCOLS

1.1 Methods/Materials

1.1.1 Propagation of influenza virus in embryonated chicken eggs

Stock influenza virus was used to carry out several key experiments in this thesis. The following protocol was used to propagate influenza virus used in subsequent experiments. Embryonated chicken eggs were kept at 37°C and 5% humidity in an incubator. Each day at 10:00 am, prior to infection, the eggs were turned to ensure viability.

On the tenth day of incubation, the eggs were candled to mark the air sacs by holding it up to a candlebox. The clear area seen through the shells was marked with a pencil (Figure 4). Working in sterile conditions each egg was sprayed with 70% ethanol and punched along the line of the air sac using an 18 gauge needle, and 0.1 mL of a 5000 U-µg penicillin-streptomycin (Gibco) solution was injected (Figure 4). The eggs were once again sprayed with 70% ethanol and injected with 0.2 mL dilution of influenza virus.

Influenza viruses A/Memphis/102/72 (H3N2) and A/WSN/33 (H1N1) were provided by Dr. Rubin Donis of the Centers for Disease Control (Atlanta, GA) and were diluted in 1X PBS solution adjusted to pH 7.4. The stock influenza virus was stored at -80°C.

After viral infection, the puncture holes on the eggs were taped, and eggs were incubated for 48 hr at 37°C and 5% humidity. After incubation eggs were put at either -20°C for 30 minutes or 4°C overnight to clot the blood. The eggs were opened in sterile conditions, and allantoic fluid was collected from each egg. All fluid was centrifuged at 4°C at 12,000 RPM for 15 minutes. The supernatants were collected in sterile conditions and aliquoted in 1 mL amounts. Once aliquoted, all samples were stored at -80°C.
Figure 4. Top Left: Diluting influenza virus using 1X PBS pH 7.4 in sterility. Top Right: Boring the injection site in the window area of egg. Middle Left: Injecting egg with 5000 U-μg penicillin-streptomycin solution. Middle Right: Injecting each egg with 0.2 mL virus dilution diluted in 1X PBS. Bottom Left: Wiping each egg with 70% ethanol, then taping and labeling. Bottom Right: Incubating eggs after infection 48 hours at 37°C and 5% humidity. After 48 hours, all eggs were held at -20°C for 30 minutes prior to harvesting the allantoic fluid.
1.1.2 Blood collection and storage

The experiments in this thesis required the use of infectious influenza virus. To effectively design these experiments, influenza virus propagated in embryonated chicken eggs needed to be quantified by hemagglutination titration that allowed the virus to be quantified in terms of hemagglutination units (HAU). In this method, chicken red blood cells (CRBC) are used to show a HAU. It is necessary to have $1 \times 10^6$ virus particles to make one HAU.

To preserve collected chicken blood it is necessary to dilute it in Alsevers solution that was made in sterile conditions prior to blood collection. Five mL whole blood was collected from 6 month old female chickens using 5 mL sterile Alsever’s solution and 23-gauge needles. Each drawing site was thoroughly wiped with alcohol. After collection, the sites were again cleaned with alcohol and cotton was placed between the wing and the body of the chicken for 5 minutes to control bleeding. The blood was then transferred to sterile conical tubes and stored at 4°C for up to 30 days.

1.1.3 Hemagglutination unit titration

To perform the titration, 2.5 mL of chicken blood stored in Alsevers solution was rinsed three times with sterile 10 mL 1XPBS pH 7 at 2000 RPM for 5 minutes. After the last rinse the supernatant was removed and 0.4 mL of pooled chicken red blood cells was collected and diluted in 39.6 mL sterile 1X PBS pH 7. Fifty microliters of 0.5% bovine serum albumin (BSA) in 1X PBS pH 7 was added to each well in a 96 well plate, excluding the first column.
One hundred microliters of undiluted propagated virus (A/WSN/33 and A/Memphis/102/72) was added to appropriate wells in the first column. A fifty microliter sample from this column was then collected and pipetted into the next column using a multi-channel pipettor. The well contents were then mixed 10 times by pipetting up and down slowly. Fifty microliters of column 2 were then pipette into column 3, and mixed. This was repeated for all columns excluding the last. Fifty microliters of CRBC solution was then added to each well starting with the last column as demonstrated in Figure 5. The contents of the last column contained only 0.5% BSA solution and CRBC solution. The plate was then incubated at room temperature for 30 minutes in sterile conditions. Wells were then noted for agglutination.

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Figure 5. Diagram illustrating organization of influenza viral titration determined by hemagglutinin units. The diagram above represents a 96 well plate. Each well of column 1 received 50 µl of influenza virus and 50 µl of 1% CRBC solution, each well of column 2 received 50 µl of influenza diluted 1:2 with 0.5% BSA solution, and 50 µl of 1% CRBC solution, etc, up to column 11. Column 12 did not receive influenza virus, and served as the internal control.

1.1.4 Determination of the egg infectious dose of propagated influenza virus

The HAU titer of a virus indicates the number of virus particles (infectious and non-infectious) in a volume of influenza virus suspension. For the purposes of this thesis, it was necessary to use an egg infection dose (EID) method in order to determine infectivity of the propagated viruses. Embryonated chicken eggs were kept at 37°C and 5% humidity. Every day prior to infection eggs were turned at approximately 10 a.m.
On the tenth day of incubation, the eggs were candled to mark the air sac. Dilutions of A/WSN/33 (HAU 1:256) were made using sterile 1X PBS. The dilutions were then inserted in 1 mL syringes. Dilutions of A/Memphis/102/72 (HAU 1:512) were made in 1X PBS. The eggs were sprayed with 70% ethanol and punched along the line of the air sac using an 18-gauge needle. One hundred microliters of a 1000 U/ml penicillin-streptomycin (Gibco) solution was injected into each egg. The eggs were once again sprayed with 70% ethanol and were injected with 0.1 mL of virus dilution.

After viral infection, puncture holes on the eggs were taped. Eggs were incubated for 48 hr at 37°C and 5% humidity. After incubation, eggs were held at either -20°C for 30 minutes or at 4°C overnight to clot the blood. The eggs were opened in sterile conditions, and allantoic fluid was collected from each egg. All fluid was centrifuged at 4°C at 12,000 RPM for 15 minutes. Two and a half mL of chicken blood was stored in Alsevers solution was rinsed three times with sterile 10 mL 1X PBS pH 7 at 2000 RPM for 5 minutes. After the last rinse the supernatant was removed and 0.4 mL of pooled CRBC was collected and diluted in 39.6 mL sterile 1X PBS pH 7. Two 96 well plates were used to determine of the EID of each virus strain. Figure 6 shows the design for the titration of A/WSN/33. Figure 7 shows the design for the titration of A/Memphis/102/72.
Figure 6. The plate design for the determination of EID of propagated A/WSN/33. The first column from A1-C1 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-4}$. Column A2-C2 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-5}$. Column A3-C3 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-6}$. Column A4-C4 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-7}$. Column A5-C5 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-8}$. Column A6-C6 contains 50 microliters of 1% CRBC solution and 50 microliters of control allantoic fluid that was not infected with virus. Rows F through H were used to verify the titer of A/WSN/33. Columns 7A-7D, 8A-8D, 9A-9D, 10A-10D, 11A-11D, and 12A-12D were not used.
Figure 7. The plate design for the determination of EID of propagated A/Memphis/102/72. The first column from A1-C1 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-4}$. Column A2-C2 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-5}$. Column A3-C3 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-6}$. Column A4-C4 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-7}$. Column A5-C5 contains 50 microliters of a 1% CRBC solution and 50 microliters of allantoic fluid from the set of eggs infected with a dilution of $10^{-8}$. Column A6-C6 contains 50 microliters of 1% CRBC solution and 50 microliters of control allantoic fluid that was not infected with virus. Rows F through H were used to verify the titer of A/Memphis/102/72. Columns 7A-7D, 8A-8D, 9A-9D, 10A-10D, 11A-11D, and 12A-12D were not used.

1.1.5 Separation of peripheral blood mononuclear cells

Whole human blood was collected via venupuncture in heparinized Vacu-Jet® tubes. Histopaque 1077 (Sigma-Aldrich) was aliquoted by amounts of 3 and 15 mL into sterile conical tubes, and stored at -20°C. Before use, tubes were thawed and kept at room temperature. Three mL of whole blood was carefully layered on top of the Histopaque. Each tube was then centrifuged for 30 minutes at room temperature at 1600 RPM. The plasma layers were then discarded, and the peripheral blood mononuclear cell (PBMC) layers were collected, and resuspended in cold 10 mL 5% FCS in RPMI. The cells were rinsed 3X at 1200 RPM for 10 minutes with 10 mL 5% FCS in RPMI. After the final rinse, the cells were resuspended in 5 mL 5% FCS in RPMI and quantified. Fifty
microliters of cell suspension was added to 445 microliters of 5% FCS in RPMI. An
additional 5 microliters of trypan blue was added. Cells were quantified using a
hemacytometer and light microscope. Cells were counted three times, and then averaged.
The average was then multiplied by 2X the dilution factor and them by 10,000. This
provided the concentration of cells per milliliter.

1.1.6 Fluorescence microscopy

Whole human blood was collected via venupuncture in heparinized Vacu-Jet® tubes
(Becton-Dickinson). At the time of use, tubes containing Histopaque 1077 (Sigma-
Aldrich) were thawed and kept at room temperature. Three mL of whole blood was
carefully layered on top of the Histopaque under sterile conditions. Each tube was then
centrifuged for 30 minutes at room temperature at 1600 RPM.

The plasma layers were then discarded, and the PBMC layers were collected and
resuspended in cold 10 mL 5% FCS in RPMI. The cells were rinsed 3X at 1200 RPM for
10 minutes with 10 mL 5% FCS in RPMI. After the final rinse, the cells were
resuspended in 5 mL 5% FCS in RPMI and quantified. Fifty microliters of cells
suspension was added to 445 microliters of 5% FCS in RPMI. An additional 5
microliters trypan blue was added. Cells were quantified using a hemacytometer and
light microscope. Cells were counted three times, and then averaged. This provided the
concentration of cells per milliliter. Peripheral blood mononuclear cells were
quantified and subsequently tagged with fluorescent biotinylated antibodies
(eBioscience). Markers included anti-human CD4, CD14, CD21, CD40, and a rat isotype
control. PBMC were aliquoted into 5 Eppendorf tubes suspended in 5% FCS in RPMI
with 0.5 mM EDTA.
The antibodies were diluted in an amount of 1 microgram per million cells in 5% FCS in RPMI with 0.5 mM EDTA, including the secondary antibody Streptavidin-FITC. Cells were pelleted at 5000 RPM for 30 seconds at room temperature and resuspended in the appropriate antibody dilutions. The cells were then incubated on ice for 30 minutes with gentle agitation every 10 minutes, and then rinsed three times using 500 microliters of 5% FCS 0.5 mM EDTA in RPMI. The cells were then resuspended in the secondary antibody dilutions (50 microliters per marker). The cells were then incubated again for 30 minutes on ice in dark conditions. After incubation, all cells were rinsed again three times with 500 microliters 5% FCS in RPMI with 0.5 mM EDTA each time and then fixed with 1% paraformaldehyde and stored at 4°C in dark conditions.

1.1.7 Flow cytometry

Whole human blood was collected via venipuncture in heparinized Vacu-Jet® tubes. Fifteen mL whole blood was layered on top of 15 mL sterile Histopaque 1077 (Sigma-Aldrich) warmed to room temperature. Each blood suspension was then centrifuged for 30 minutes at room temperature at 1600 RPMs. The plasma layers were then discarded, and the PBMC layers were collected and resuspended in cold 10 mL 5% FCS in RPMI. The cells were rinsed three times at 1200 RPM for 10 minutes with 10 mL 5% FCS in RPMI. After the final rinse, the cells were resuspended in 5 mL 5% FCS in RPMI and quantified. Fifty microliters of cell suspension was added to 445 microliters of 5% FCS in RPMI. An additional 5 microliters trypan blue was added. Cells were quantified using a hemacytometer and light microscope. Cells were counted three times, and then averaged. This provided the concentration of cells per milliliter.
Cells were subsequently stained with the following FITC conjugated antibodies: CD4, CD4, CD19, and rat isotype control. Cells were kept on ice while the antibodies were diluted in 5% FCS 0.5 mM EDTA RPMI. The amount of cells used per antibody totaled 5 x 10^5. The cells were aliquoted into Eppendorf tubes, and pelleted at 5000 RPM for 30 seconds and resuspended in 50 microliters of antibody dilution. The cells were then incubated on ice in dark conditions. Each sample was agitated every 10 minutes to ensure antigen-antibody contact. At the end of incubation each sample was rinsed 3 times in 500 microliters 5% FCS 5 mM EDTA in RPMI. After the final rinse each sample was resuspended in 500 microliters 1% paraformaldehyde and stored at 4°C in darkness.

### 1.1.8 Identification of CD11c and CD14 positive cells from human blood

Whole human blood was collected via venupuncture in heparinized Vacu-Jet® tubes. Whole blood was layered onto 15 mL sterile Histopaque 1077 tubes (Sigma-Aldrich) under sterile conditions and warmed to room temperature. Each blood suspension was then centrifuged for 30 minutes at room temperature at 1600 RPM. The plasma layers were then discarded, and the PBMC layers were collected and resuspended in cold 10 mL 5% FCS in RPMI. The cells were rinsed three times at 1200 RPM for 10 minutes with 10 mL 5% FCS in RPMI. After the final rinse the cells were resuspended in 5 mL 5% FCS in RPMI and quantified. Fifty microliters of this cell suspension was added to 445 microliters of 5% FCS in RPMI, and additional 5 microliters of Trypan blue was added. Cells were quantified using a hemacytometer and light microscope. This provided the concentration of cells per milliliter.

The cells were subsequently pelleted and resuspended in FITC conjugated CD11c antibody, FITC conjugated CD14, and biotinylated rat isotype control. All antibodies
were diluted in 5% FCS in RPMI. The cells were incubated for 30 minutes on ice. After incubation, the cells were rinsed three times with 5 mL cold 5% FCS in RPMI. After the third rinse the samples that were treated with FITC CD11c and FITC CD14 were resuspended in 250 microliters 1% paraformaldehyde. The sample treated with biotinylated rat isotype control antibody was resuspended in diluted FITC-Streptavidin antibody. The sample was kept on ice in dark conditions for 30 minutes. After incubation, the cells were rinsed with 5 mL cold 5% FCS in RPMI. After the third rinse the sample was resuspended in 250 microliters 1% paraformaldehyde and stored at 4°C in dark conditions.

1.1.9 Enrichment of monocytes using magnetic bead separation

Whole human blood was collected via venupuncture in heparinized Vacu-Jet® tubes. Samples of whole blood were layered onto 15 mL sterile Histopaque 1077 tubes under sterile conditions (Sigma-Aldrich), and warmed to room temperature. Each blood suspension was then centrifuged for 30 minutes at room temperature at 1600 RPM. The plasma layers were then discarded, and the PBMC layers were collected and resuspended in cold 10 mL 5% FCS in RPMI. The cells were rinsed three times at 1200 RPM for 10 minutes with 10 mL 5% FCS in RPMI. After final rinse, the cells were resuspended in 5 mL 5% FCS in RPMI and quantified. Fifty microliters of cells suspension was added to 445 microliters of 5% FCS in RPMI. An additional 5 microliters trypan blue was added. Cells were quantified using a hemacytometer and light microscope. Cells were counted three times, and then averaged. This provided the concentration of cells per milliliter.

Biotinylated CD14 antibody was diluted in 5% FCS in RMPI and kept on ice. The total number of PBMC isolated was pelleted and resuspended in the diluted antibody and
incubated on ice for 30 minutes. Streptavidin beads (Miltenyi-Biotect) were diluted in cold 0.5% BSA solution and kept on ice. After incubation, cell suspension was pelleted, and resuspended in the streptavidin bead dilution. The cells were incubated on ice for 30 minutes. After incubation, 10 mL of cold 0.5% BSA solution was added to the cell suspension, and was centrifuged for 10 minutes at 1000 RPM. After centrifugation, the supernatant was removed, and the cells were resuspended in 500 microliters of separation buffer.

A magnetic column apparatus was prepared and kept at -20°C until use. A sterile column was opened and rinsed with 1 mL cold separation buffer. Five hundred microliters of cell suspension was then added to the top of the column and allowed to flow through the column. All buffer filtered through was collected in a sterile tube. After all cell suspension was filtered, 1 mL of additional separation buffer was added to rinse the column. Five milliliters of cold 5% FCS in RPMI was added to the cell suspension. Another 1 mL of cold separation buffer was added to the column and quickly plunged. All fluid was collected in another sterile conical tube. Five mL cold 5% FCS in RPMI was added to this tube.

FITC anti CD11c antibody was diluted in 5% FCS in RMPI and stored on ice in dark conditions. Cells in both conical tubes were pelleted and resuspended in antibody dilution. Both samples were incubated for 30 minutes on ice in dark conditions. After incubation, cells were rinsed three times using 500 microliters 5% FCS in RPMI. After final rinse, the pellets were resuspended in 250 microliters of a 1% paraformaldehyde solution. The samples were stored at 4°C in dark conditions.
1.1.10 Propagation of dendritic cells derived from monocytes

Whole human blood was collected via venupuncture in heparinized Vacu-Jet® tubes. Under sterile conditions, amounts of whole blood were layered onto 15 mL sterile Histopaque 1077 tubes (Sigma-Aldrich) warmed to room temperature. Each blood suspension was then centrifuged for 30 minutes at room temperature at 1600 RPM. The plasma layers were then discarded, and the PBMC layers were collected, and resuspended in cold 10 mL 5% FCS in RPMI. The cells were rinsed 3X at 1200 RPM for 10 minutes with 10 mL 5% FCS in RPMI. After final rinse, the cells were resuspended in 5 mL 5% FCS in RPMI and quantified. Fifty microliters of cells suspension was added to 445 microliters of 5% FCS in RPMI. An additional 5 microliters trypan blue was added. Cells were quantified using a hemacytometer and light microscope. Cells were counted three times, and then averaged. This provided the concentration of cells per milliliter.

Biotinylated CD14 antibody was diluted in 5% FCS in RPMI and kept on ice. The total number of PBMC isolated was pelleted and resuspended in the diluted antibody. A small amount of cells was separately tagged for with FITC-CD14 using the protocol in section 1.1.6. The sample was read the following day using flow cytometry. The cells suspension was then incubated on ice for 30 minutes. Streptavidin beads (Miltenyi-Biotec) were diluted in cold 0.5% BSA solution and kept on ice. After incubation, the cell suspension was pelleted and resuspended in the streptavidin bead dilution. The cells were incubated on ice for 30 minutes.

After incubation, 10 mL of cold 0.5% BSA solution was added to the cell suspension and was centrifuged for 10 minutes at 1000 RPM. After centrifugation the supernatant was
removed and the cells were resuspended in 500 microliters of separation buffer. A magnetic column apparatus (Miltenyi Biotech) was prepared and kept at -20°C until use. A sterile column was opened and rinsed with 1 mL of cold separation buffer. Five hundred microliters of cell suspension was then added to the top of the column, allowed to flow through the column, and collected in a sterile tube. After all cell suspensions were filtered 1 mL of additional separation buffer was added to rinse the column. Five mL of cold 5% FCS in RPMI was added to the cell suspension. Another 1 mL of cold separation buffer was added to the column and quickly plunged. The fluid was collected in another sterile conical tube. Five milliliters of cold 5% FCS in RPMI was added to this tube. Cells that were collected last from the column were resuspended in 15 mL dendritic cell culture medium. In a sterile 12-well tissue culture plate, the cell suspension was distributed to 9 wells. The plate was then incubated for 6 days at 37°C and 5% humidity. On the 6th day of incubation the cell suspensions were collected and pooled in a 50 mL conical tube. Each well of the plate was rinsed 3 times with 1 mL 5% FCS in RPMI. The cells were then tagged with FITC-CD14 and CD11c according to protocol 1.1.6. The samples were processed the following day.

1.2 Results

1.2.1 Propagation of influenza virus in embryonated chicken eggs

Embryonated chicken eggs were infected with influenza strains A/Memphis/102/72 (H3N2) and A/WSN/33 (H1N1). Allantoic fluid was collected after 30 minutes of incubation at -20°C and clarified via centrifugation. A hemagglutination titration was run to quantify virus particles. After titration plates were prepared and incubated at room
temperature for 30 minutes, each column was observed for hemagglutination.

The strain A/Memphis/102/72 was found to have a HAU of 1:512 (Figure 8). The last column (A12-H12) was used as an internal control, it received only BSA solution and CRBC solution, and so was negative for agglutination. Columns 1-9 show positive agglutination, ending with the dilution of 1:512. Columns 10 (1:1096) and 11 (1:2192) were negative for agglutination. Thus, the propagated virus strain A/Memphis/102/72 (H3N2) was shown to have a HAU of 1:512.

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<td>1:64</td>
<td>1:128</td>
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<td>1:512</td>
<td>1:1096</td>
<td>1:2192</td>
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</tbody>
</table>

Figure 8. Results of A/Memphis/102/72 HAU titration. Columns 1-9 showed agglutination. Columns 10 and 11 demonstrated no agglutination. Column 12 received no virus, and was negative for agglutination.

The strain A/WSN/33 was found to have a HAU of 1:256 (Figure 9). The last column (A12-H12) was used as an internal control, and received only BSA solution and CRBC solution and was negative for agglutination. Columns 1-8 show positive agglutination, ending with the dilution of 1:256. Columns 9, (1:512), 10 (1:1096) and 11 (1:2192) were negative for agglutination. Thus, the propagated virus strain A/WSN/33 (H1N1) was shown to have a HAU of 1:256.
Figure 9. Results of A/WSN/33 HAU titration. Columns 1-8 showed agglutination. Columns 9-11 demonstrated no agglutination. Column 12 received no virus, and was negative for agglutination.

### 1.2.2 Determining the egg infectious dose of propagated virus

Egg infectious dose cultures were determined using 10 day old embryonated chicken eggs and propagated influenza from the experiment in 1.2.1. Dilutions were made of each virus strain and eggs were infected for 48 hours. After incubation eggs were kept at -20°C. Allantoic fluid from the eggs was collected and clarified via centrifugation. Ninety-six well plates were prepared according to the protocol used in 1.1.3. The plates were incubated at room temperature for 30 minutes and were then observed for agglutination. As seen in Figure 10, A/Memphis/102/72 was shown to have an egg infectious dose of $10^7$ EID50%. Columns containing allantoic fluid from eggs treated with dilutions less than $10^{-7}$ were shown to be positive for agglutination. This indicates that eggs inoculated with dilutions $10^{-7}$ or less were infected. There was no infection in eggs treated with dilution $10^{-8}$.
A/WSN/33 was shown to have an EID50% of $10^{-5}$ and an HAU of 1:256. As seen in Figure 11, the first 2 columns should be positive for agglutination. The columns containing allantoic fluid from eggs treated with dilutions $10^{-6} - 10^{-7}$ were negative for agglutination. The control column was negative for agglutination as well. The stock virus from both strains was kept at –80°C for future experiments.
<table>
<thead>
<tr>
<th></th>
<th>10^4</th>
<th>10^3</th>
<th>10^6</th>
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</table>

Figure 11. A/WSN/33 EID and titration results. The top portion of the plate demonstrates agglutination in the first 2 columns. Dilutions 10^{-6} and 10^{-7} were negative for agglutination. The column that contained virus diluted to 10^{-8} was positive for agglutination. The control wells were negative for agglutination. The repeated titration reaffirmed that the propagated virus has an HAU of 256. Columns 7 through 12 were unused (marked by an X) in the top half the plate.

1.2.3 Separation of peripheral blood mononuclear cells

Human peripheral blood mononuclear cells were separated from whole blood using the protocol described in 1.1.4. The goal of these experiments was to optimize the technique of separation, and to obtain a consistent amount of cells. Two different types of rinsing reagents were used. From this, it was determined that using 5% fetal calf serum diluted in RMPI medium yielded the higher amount of cells after all washing steps had been completed. It was determined that from 3 mL of whole blood, more than a million PMBC could be obtained as seen in Table 1.
Table 1. The results of the initial PMBC isolation.

<table>
<thead>
<tr>
<th>Experiment Date</th>
<th>Blood Volume</th>
<th>[PBMC]</th>
<th>Total cells</th>
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<tr>
<td>6/13/07</td>
<td>3 mL</td>
<td>6.5 x 10^6</td>
<td>1.90 x 10^7</td>
</tr>
<tr>
<td>6/20/07</td>
<td>3 mL</td>
<td>6.2 x 10^6</td>
<td>1.86 x 10^7</td>
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<tr>
<td>6/29/07</td>
<td>3 mL</td>
<td>4.8 x 10^6</td>
<td>1.44 x 10^7</td>
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<td>7/2/07</td>
<td>3 mL</td>
<td>6.8 x 10^6</td>
<td>2.00 x 10^7</td>
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<tr>
<td>7/9/07</td>
<td>3 mL</td>
<td>3.0 x 10^6</td>
<td>9.00 x 10^6</td>
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<td>7/12/07</td>
<td>3 mL</td>
<td>1.9 x 10^6</td>
<td>5.70 x 10^6</td>
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</tbody>
</table>

1.2.4 Fluorescence microscopy

After separation of PBMC from whole blood had been optimized, the cells were tagged with various markers and then observed under fluorescence microscopy. These experiments determined if certain cell populations could accurately be quantified using fluorescent antibodies. These experiments were used to optimize flow cytometry analysis.

Using the protocol listed in 1.1.6, cells were separated and stained. Images were taken using both brightfield microscopy and fluorescence microscopy. Cells were enumerated in both fields to determine the positive fluorescence in each sample.

Table 2. Results from quantifying PBMC tagged with fluorescent antibodies viewed under 400X magnification.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Total Cells</th>
<th>Positive Cells</th>
<th>Percentage of Positive Cells</th>
<th>Expected Percentage</th>
</tr>
</thead>
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<tr>
<td>Isotype</td>
<td>116</td>
<td>4</td>
<td>3%</td>
<td>0%</td>
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<td>CD4</td>
<td>107</td>
<td>20</td>
<td>19%</td>
<td>25%</td>
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<td>CD14</td>
<td>106</td>
<td>10</td>
<td>9%</td>
<td>3-7%</td>
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<tr>
<td>CD40</td>
<td>125</td>
<td>34</td>
<td>27%</td>
<td>23-33%</td>
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<tr>
<td>CD21</td>
<td>113</td>
<td>34</td>
<td>30%</td>
<td>18-43%</td>
</tr>
</tbody>
</table>

As seen in Table 2, cells were tagged with 5 markers and quantified. The rat isotype control demonstrated a 3% positive population of cells. A low percentage of cells are expected with a sample treated with this marker which was used as the internal control in these sets of experiments. The sample of cells treated with anti-CD4 antibodies indicated
a positive population of cells amounting to 19%. In a healthy individual, the number of CD4 positive cells is close to 25%. Samples treated with CD14 and CD40 were within range of normal levels to be expected. Cells stained with marker CD21 showed a positive population of 30%, which is also within range. All antibodies used in these experiments were purchased from eBioscience. Below are shown typical results obtained after optimization of the technology.

Figure 12. The images above demonstrate peripheral blood mononuclear cells stained with a rat isotype control antibody conjugated to FITC-Streptavidin. The left image shows cells under 400X brightfield microscopy. The image to the right demonstrates no fluorescent binding. This sample served as an internal control.

The biotinylated antibody Rat IgG2a Isotype control is used as an isotype control immunoglobulin. It is expected that a sample of human PBMC treated with this antibody would show a minimal population of cells, if any. A population higher than 10% would indicate a poor protocol. As shown in Figure 12, no cells are positive for fluorescence. Cells are shown however under brightfield microscopy.
Figure 13. The images above demonstrate peripheral blood mononuclear cells stained with CD4 FITC. The left image shows the cells under 400X brightfield microscopy. The image to the right shows a group of cells positive for CD4 viewed under 400X UV.

The CD4 marker is expressed on many cells including most T-cells, and a minute population of T-helper cells. It can also be found in low levels on monocytes. CD4 is the main receptor for the human immunodeficiency virus as well. As seen in Figure 13, the cells in the PMBC population positive for CD4 were visible via FITC. Figure 13 shows cells treated with CD4 in brightfield. In that same view (right), under fluorescence microscopy, only 3 cells are seen. These 3 cells enclosed in the red box were considered positive for expression of marker CD4.
Figure 14. The images above demonstrate PBMC stained with CD14 FITC. The left image shows cells under 400X brightfield microscopy. The image to the right shows a group of cells positive for CD14 viewed under 400X UV.

The CD14 marker is expressed generally on monocytes as well as macrophages and some dendritic cells. As seen in Figure 14, the cells in the PBMC population positive for CD14 were visible via FITC. Figure 15 shows cells treated with CD14 in brightfield. In that same view (right) under fluorescence microscopy only 3 cells could be seen, these (enclosed in the red box) were considered positive for expression of marker CD14.

Figure 15. The images above demonstrate more PMBC stained with PE conjugated CD21 antibody. The left images show cells under 400X brightfield microscopy. The images to the right demonstrate CD21 positive cells under fluorescence microscopy.
CD21 is a marker expressed by mature B cells, follicular dendritic cells and a small population of epithelial cells. This marker also serves as a receptor for complement component C3d and Epstein-Barr virus. CD21 is also involved in B cell activation by T cells. Demonstrated in Figure 15, images using brightfield and fluorescence microscopy show cells positive for expression of CD21. Cells positive for fluorescence are seen in the images to right enclosed in red boxes. Those same cells are viewed with brightfield microscopy on the left in Figure 15.

Figure 16. The images above demonstrate more PBMC stained with FITC conjugated CD40 antibody. The left images show a group of cells enclosed 400X brightfield microscopy. The images to the right show the same population of enclosed cells as CD40 positive.

The biotinylated anti-CD40 antibody is expressed on several populations of cells. It is primarily expressed on B lymphocytes, and follicular dendritic cells. It can be expressed at low levels on thymic epithelium, and a subset of peripheral T cells. Engagement of CD40 with CD40L, in addition with IL-4, acts to ensure B cell development and maturation by initiating Ig isotype switching. It also serves to protect B cells from surface Ig-induced apoptosis and promotes proliferation. CD40 has also been found to play a role in cross-talk between B cells and T cells, and costimulation and immune regulation.
Figure 16 shows images taken under brightfield and fluorescence microscopy using an anti-CD40 antibody. CD40 positive cells are shown enclosed in red boxes to the right. Those same cells can be seen in the images to the left under brightfield microscopy.

1.2.5 Flow cytometry

In order to conduct the crucial experiments in this thesis, the technique of flow cytometry had to be optimized. The objective of these experiments was to successfully stain PBMC with selected anti-marker antibodies. Once stained, these cells would be analyzed using flow cytometry technology. After staining, cells were fixed with paraformaldehyde and incubated with the selected antibody and kept at 4°C in dark conditions. These conditions are necessary in order to preserve fluorescence until reading the results using flow cytometry technology. Samples were generously analyzed at Marquette General Hospital. Below are shown typical results obtained after optimization of the technology.

1.2.5.1 Determination of flow cytometry using an irrelevant antibody

Baseline readings were done using the irrelevant antibody, a biotinylated rat isotype antibody, that served as a negative control. Figure 17 shows the output of flow cytometry using the irrelevant antibody, whereby positive cells for the rat isotype antibody were found, indicating that flow cytometry outputs for the other samples are reliable and valid.
Figure 17. The above diagrams represent the flow cytometry analysis for the cell sample treated with the irrelevant antibody. The above diagram to the left is the output of the side scatter (SS Log) versus fluorescence intensity (Iso-fitc). The box does not show a positive cell population, which is to be expected, as this sample was negative. The diagram to the right shows the output for the number of cells versus fluorescence (Iso-fitc).

1.2.5.2 Determination of flow cytometry using an anti-CD19 antibody

Samples stained with biotinylated antibody CD19 were also processed through flow cytometry. The scatters seen in Figure 18 show the intensity of the cells positive for fluorescence, as well as how many cells were positive for the CD19 marker. One-hundred thousand cells were processed in the sample. A low percentage of those, were found to be CD19 positive. CD19 is commonly found on B cells, at any stage of maturation. Follicular dendritic cells also express CD19 but they are not found in blood.
Figure 18. Flow cytometry analysis for the cell sample treated with CD19. The diagram on left is the output of side scatter (SS Log) versus fluorescence intensity (CD19-fitc). The cells within the box are considered positive for the expression of CD19. The diagram on the right is the output of the number of cells versus fluorescence intensity (CD19-fitc).

1.2.5.3 Determination of flow cytometry using an anti-CD45 antibody

The antibody CD45 specifically binds to leukocyte common antigens (LCA) that are expressed on all hematopoietic cells, except for platelets and erythrocytes. This antigen is important in the activation of lymphocytes. CD45 is expressed prolifically on many cells, with the expectation that a sample of peripheral blood mononuclear cells would mostly be CD45 positive. Figure 19 shows that almost all cells processed via flow cytometry were CD45 positive.
1.2.5.4 Determination of flow cytometry using an anti-CD4 antibody

One-hundred thousand cells were stained with CD4 antibody, and roughly a third of these cells were found to be CD4 positive (Figure 20), a quantification that is within range of the normal CD4 T cell population in circulation.
1.2.5.5 Determination of flow cytometry using an anti-CD14 antibody

There is a very low population of dendritic cells in circulation in the blood. To propagate dendritic cells from humans, monocytes from blood must be isolated and stimulated for DC maturation. Monocytes in the blood can amount from 2-10% in a healthy individual. The basis for these sets of experiments was to quantify the percentage of monocytes in the donors blood. CD14 is mainly expressed on monocytes and dendritic cells.

Figure 21. Flow cytometry analysis for the samples stained with CD14. The diagram on left is the output of side scatter (SS Log) versus fluorescence intensity (CD14-fitc). The cells in the box are CD14 positive. The diagram to the right shows the output for the number of cells versus fluorescence intensity (CD14-fitc).

Figure 21 shows a very small percentage of cells stained with the CD14 antibody were identified as positive, as would be expected because very few cells in circulation would display the CD14 marker. When stimulated, monocytes from blood mature into dendritic cells. There is a dramatic increase in CD14 marker expression, as well as CD11. This increased expression of markers indicates maturation has occurred in the population.
1.2.5.6 Determination of flow cytometry using an anti-CD11c antibody

Another molecule of interest in relation to monocytes in the blood is marker CD11c. This molecule is also mainly found on monocytes and dendritic cells. Thus, it was critical to determine baseline numbers in the donor’s blood for the two markers.

![Flow cytometry analysis](image)

Figure 22. Flow cytometry analysis for the samples stained with CD11c. Cells displaying CD11c mainly include monocytes, the cell of interest in this experiment. The diagram on left is the output of side scatter (SS Log) versus fluorescence intensity (CD11c-fitc). The cells in the box are considered CD11c positive. The diagram to the right shows the output for the number of cells versus side fluorescence intensity (CD11c-fitc).

Figure 22 shows the scatter plots for the cell sample stained with CD11c. There are very few monocytes in circulation. Thus, it is expected that this sample would not demonstrate a high population of CD11c positive cells.

1.2.6 Enrichment of monocytes using magnetic bead separation

Specific cell populations can be enriched using magnetic beads. Large amounts of dendritic cells were needed, but this population is in low frequency in the blood. Monocytes from blood can be stimulated to mature into dendritic cells, if exposed to certain cytokine cues. Therefore, monocytes were isolated from blood and cultured *in vitro* to differentiate in dendritic cells. The separation of the monocytes from peripheral
blood mononuclear cells needed to be optimized. In these experiments, PBMC were incubated with biotinylated CD14 antibody, and subsequently with streptavidin conjugated magnetic beads. Subsequently, the cells were then run through a magnetic column. The cells expressing the CD14 marker remained in the column, while the CD14 negative cells were flushed through. The cell population remaining in the column would then be enriched with marker CD11c. The cell population that passed through the column would be depleted of the CD11c marker.

Table 3. Two samples of cells were processed via flow cytometry to determine monocyte enrichment. The separated population was collected after all cell suspension was run through the column. This population would be considered CD11c-enriched. The sample not separated shows a CD11c+ population of 6.05%. After the cells were separated there was an increase in CD11c+ cells.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total Cells counted</th>
<th>CD11c + Cells</th>
<th>% CD11c + Cells</th>
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</thead>
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<tr>
<td>Separated</td>
<td>5317</td>
<td>1399</td>
<td>26.31</td>
</tr>
<tr>
<td>No separation</td>
<td>10000</td>
<td>6052</td>
<td>6.05</td>
</tr>
</tbody>
</table>

Table 3 shows that a sample of cells was not magnetically separated. The amount of cells in this population was 6.05%. This provides a baseline for the amount of cells expressing CD11c in the blood. Figure 23 shows that the scatter plots for this sample also depicted the low population occurring in circulation.
Cells labeled with CD14 were bound to magnetic beads with anti-CD14 antibodies. Therefore when the sample was run through the column, cells positive for the CD14 marker were retained, while the cells in the CD14 negative population were not. Figure 24 shows that the labeled cells (CD14 enriched) had a higher percentage of CD11c positive cells, as to be expected. It is also noteworthy that only a very small population of CD11c cells was able to be quantified, which is to be expected because there are normally very few monocytes in circulation in the blood.
1.2.7 Production of monocyte-derived dendritic cells

Successful propagation of monocyte-derived dendritic cells can be demonstrated with an increase of the marker CD14. As seen in Table 4, an initial sample of cells later stimulated with cytokines was stained with molecule CD14. This initial enumeration of CD14 positive cells provided a baseline. After culture with cytokines, it is expected that a sample of cells stained again with CD14 antibody would show a higher percentage.

Table 4. Before monocytes were incubated with cytokines GM-CSF and IL-4, cells were tagged with FITC-conjugated antibodies CD14 and CD11c (specific for monocytes and dendritic cells). After a 6 day incubation period with cytokine treatment, cells were tagged again with CD14 and CD11c to note an enrichment of the population. Figure 27 shows the initial sample of cells stained with CD14 antibody with a very low percentage of positive cells. This is expected, because the cells had not yet been exposed to stimulatory cytokines in vitro.

<table>
<thead>
<tr>
<th>MARKER</th>
<th>Total Cells</th>
<th>Total FITC + Cells</th>
<th>Total Percentage FITC + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before Cytokine Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>100000</td>
<td>6064</td>
<td>6.06</td>
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<tr>
<td>After Cytokine Treatment</td>
<td>54337</td>
<td>18938</td>
<td>34.85</td>
</tr>
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</table>

Figure 25 demonstrates a smaller population of CD14 positive cells. This is to be expected because the cells had been stained before cytokine treatment was given. An increase in CD14 expression is only expected to be observed after the cells had been treated with IL-4 and GM-CSF.
Figure 25. Flow cytometry scatter plots of cells initially stained with CD14 antibody. These cells had not yet been incubated with cytokines that would stimulate dendritic cell maturation. A small subset of cells from the sample expressed the molecule CD14. The diagram on left is the output of side scatter (SS Log) versus fluorescence intensity (CD14-fitc). The diagram to the right shows the output for the number of cells versus fluorescence intensity (CD14-fitc).

Figure 26 shows the flow cytometry analysis for cells stained after incubation with cytokines. It is demonstrated here that there was a higher percentage of cells expressing the CD14 molecule. With this increase, it can be concluded that the monocytes separated with blood had successfully matured into dendritic cells.

Figure 26. Flow cytometry scatter plots of cells stained with CD14 antibody after a 6-day incubation with cytokines IL-4 and GM-CSF. After this incubation, a higher percentage of cells were expected to be CD14 positive. As shown, a higher percentage of cells cultured with the cytokine express the molecule. The diagram on the left is the output of side scatter (SS Log) versus fluorescence intensity (CD14-fitc). The diagram to the right shows the output for the number of cells versus fluorescence intensity (CD14-fitc).
CHAPTER 2: Co-stimulatory surface molecule expression on human monocyte-derived dendritic cells infected with two strains of influenza virus

2.1 Materials/Methods

Whole human blood was collected via venupuncture in heparinized Vacu-Jet® tubes. Under sterile conditions, whole blood was layered onto 15 mL sterile Histopaque 1077 tubes (Sigma-Aldrich) warmed to room temperature. Each blood suspension was then centrifuged for 30 minutes at room temperature at 1600 RPM. The plasma layers were then discarded, and the PBMC layers were collected, and resuspended in cold 10 mL 5% FCS in RPMI. The cells were rinsed 3X at 1200 RPM for 10 minutes with 10 mL 5% FCS in RPMI. After final rinse, the cells were resuspended in 5 mL 5% FCS in RPMI and quantified. Fifty microliters of cells suspension was added to 445 microliters of 5% FCS in RPMI. An additional 5 microliters trypan blue was added. Cells were quantified using a hemacytometer and light microscope. Cells were counted three times, and then averaged. The average was then multiplied by 2X the dilution factor and then by 10,000. This provided the concentration of cells per milliliter.

Biotinylated CD14 antibody was diluted in 5% FCS in RPMI and kept on ice. The total number of PBMC isolated was pelleted, and resuspended in the diluted antibody. A small amount of cells was separately tagged with FITC-CD14 using the protocol in section 1.1.6. The cell suspension was then incubated on ice for 30 minutes. The sample was read the following day using flow cytometry.

Streptavidin beads (Miltenyi-Biotec) were diluted in cold 0.5% BSA solution and kept on ice. After incubation, cell suspension was pelleted, and resuspended in the streptavidin bead dilution. The cells were incubated on ice for 30 minutes. After incubation, 10 mL
of cold 0.5% BSA solution was added to the cell suspension, and was centrifuged for 10 minutes at 1000 RPM. After centrifugation, the supernatant was removed, and the cells were resuspended in 500 microliters of separation buffer. A magnetic column apparatus was prepared and kept at -20°C until use. A sterile column was opened and rinsed with 1 mL cold separation buffer. Five hundred microliters of cell suspension was then added to the top of the column and allowed to flow through the column. All buffer that filtered through was collected in a sterile tube. After all cell suspension was filtered, 1 mL of additional separation buffer was added to rinse the column. Five mL of cold 5% FCS in RPMI was added to the cell suspension. Another 1 mL of cold separation buffer was added to the column and quickly plunged. All fluid was collected in another sterile conical tube. Five milliliters of cold 5% FCS in RPMI was added to this tube.

Cells that were collected last from the column were resuspended in 15 mL dendritic cell culture medium. In a sterile 12-well tissue culture plate, the cell suspension was distributed to 9 wells. The plate was then incubated for 6 days at 37°C 5% CO₂. On the 6th day of incubation, the cell suspensions were collected and pooled in a 50 mL conical tube. Each well of the plate was rinsed 3X with 1 mL 5% FCS in RPMI. All contents were pooled, and then split into three sterile tubes in sterile conditions. Tube 1 was pelleted and resuspended in 7 mL 1X trypsin in RPMI, and put on ice. This tube served as the mock infected tube. This suspension was split into two tubes equally. Both tubes were pelleted. The first tube was resuspended in 1 mL undiluted A/WSN/33 (HAU 1:256) and 200 microliters 1X trypsin. The second tube was resuspended in 200 microliters A/WSN/33 (HAU 1:256) and 1.8 mL 1X trypsin. These cells were put on ice.
The third tube was pelleted, and resuspended. The contents were equally split into three tubes. All tubes were pelleted subsequently. The first tube was resuspended in a dilution of 1:10 A/Memphis/102/72 in 1X trypsin. The second tube was resuspended in a viral dilution of 1:100 in 1X trypsin. The final tube was resuspended in a 1:1000 dilution of the virus in 1X trypsin. All tubes were then gently agitated, and put in a 37°C water bath for 40 minutes. After incubation, 10 mL 10% FCS in RPMI was added to each tube. Each tube was pelleted, and resuspended in 5 mL 10% FCS in RPMI. Each infection was performed in a separate tissue culture plate for 4 hours at 37°C in 5% CO₂.

After incubation, the contents from each plate were collected. The wells were rinsed 3X with 10% FCS in RMPI. The 3 treatments were then pelleted, and resuspended in eppendorf tubes, and rinsed again with 1 mL 10% FCS in RPMI. The cell suspensions were then split equally into 9 Eppendorf tubes per treatment. Antibodies rat isotype control, CD11b, CD40, CD86, MHC Class II, MHC Class I, and BAFF were diluted in 5% FCS in RPMI accordingly, and kept on ice. Each tube was pelleted and resuspended in the appropriate antibody. The cell samples were resuspended in antibody, diluted and coded, as seen in Table 5.
Table 5. The table shows codes indicating what treatment each cell suspension received during the final experiment. Initially, two groups of cells were infected with two strains of influenza virus: A/WSN/33 and A/Memphis/102/72. A third group was mock infected with 1X trypsin. After incubation, the three groups were then split equally into 9 Eppendorf tubes and stained with antibodies MHC Class II, MHC Class I, CD40, CD86, CD11b, BAFF, and an irrelevant rat isotype control. A total of 54 samples were prepared, and processing using flow cytometry.

<table>
<thead>
<tr>
<th></th>
<th>A/WSN/33</th>
<th>A/Memphis/102/72</th>
<th>Mock-Infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MHC Class II</td>
<td>CD40</td>
<td>CD86</td>
</tr>
<tr>
<td>MOI 0.1 A/WSN/33</td>
<td>A2-1</td>
<td>A2-2</td>
<td>A2-3</td>
</tr>
<tr>
<td>MOI 1 A/Memphis/102/72</td>
<td>2-1</td>
<td>2-2</td>
<td>2-3</td>
</tr>
<tr>
<td>MOI 0.1 A/Memphis/102/72</td>
<td>3-1</td>
<td>3-2</td>
<td>3-3</td>
</tr>
<tr>
<td>Mock-Infected</td>
<td>C-1</td>
<td>C-2</td>
<td>C-3</td>
</tr>
</tbody>
</table>

Each tube was incubated on ice at 4°C in dark conditions for 30 minutes. After incubation, each cell suspension was rinsed 3X with 5% FCS in RMPI. Upon final rinse, cell pellets were resuspended in 500 microliters 1% paraformaldehyde solution. The cells samples were stored at 4°C in dark conditions until processing.

2.2 Results

2.2.1 Rat isotype marker expression

After infection each group was stained with an irrelevant antibody used in previous experiments in Chapter 1. Table 6 shows that cells infected with A/WSN/33 (MOI of 1 and 0.1), and A/Memphis/102/72 (MOI of 1 and 0.1). A group of cells was also mock infected with diluted Trypsin in RPMI. After infection, each group of infected cells was stained with a rat isotype control antibody, and fixed in 1% paraformaldehyde.
Table 6. Cells stained with a rat isotype control antibody. Two groups of cells were infected with A/WSN/33 MOI of 1 and 0.1. Another set of cells was infected with an A/Memphis/102/72 MOI of 1, and 0.1. The last group was mocked infected with 1X trypsin only. The second column shows the total amount of cells enumerated by the flow cytometry machine. The 3rd column shows the number of cells expressing the irrelevant rat marker. The far right column denotes the percentage of cells positive for expressing the irrelevant marker.

<table>
<thead>
<tr>
<th>Rat Isotype Control</th>
<th>Total Cells Counted</th>
<th>Marker + Cells</th>
<th>% Marker + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>6934</td>
<td>4</td>
<td>0.06</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>4907</td>
<td>4</td>
<td>0.08</td>
</tr>
<tr>
<td>A/Memphis/102/72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>8487</td>
<td>4</td>
<td>0.05</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>4346</td>
<td>4</td>
<td>0.09</td>
</tr>
<tr>
<td>Mock Infected</td>
<td>7991</td>
<td>10</td>
<td>0.13</td>
</tr>
</tbody>
</table>

The group of cells infected with the viral strains A/WSN/33 and A/Memphis/102/72 showed no marker expression of the rat isotype (Figures 27-31, Appendix A). This is to be expected, as this antibody is designed to act as an internal control. Cells containing fluorescent antibody would demonstrate background due to staining technique. Figure 31 shows a small subset of cells that was mock infected was essentially negative for expression of the isotype marker indicating that there was no significant background due to staining technique, and validating of the results from the other samples.

2.2.2 CD40 marker expression

Groups of cells infected with A/WSN/33 (MOI of 1 and 0.1), A/Memphis/102/72 (MOI of 1, and 0.1) were stained with an antibody directed against the cellular marker CD40. A group of cells was also mock infected with diluted Trypsin and then stained with the antibody. The marker CD40 is expressed by B lymphocytes, follicular dendritic cells,
thymic epithelium, and a small subset of peripheral T cells. This marker serves to regulate B cell maturation and development by initiating antibody isotype switching. This marker stimulates proliferation in collaboration with chemokines such as IL-4, and protects B cells from apoptosis. CD40 is also crucial for the cross talk of other immune system players. Table 7 shows cells processed through flow cytometry quantified based on fluorescence.

Table 7. Cells stained with CD40 antibody. Two groups of cells were infected with A/WSN/33 MOI of 1 and 0.1. A second set of cells was infected with an A/Memphis/102/72 MOI of 10, 1, and 0.1. The last group was mock infected with 1X trypsin only. The second left column shows the total amount of cells enumerated by the flow cytometry machine. The middle column shows the number of cells expressing the CD40. The far right column denotes the percentage of cells positive for expressing the CD40 marker.

<table>
<thead>
<tr>
<th>CD40</th>
<th>Total Cells Counted</th>
<th>Marker + Cells</th>
<th>% Marker + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>33387</td>
<td>1607</td>
<td>4.81</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>11075</td>
<td>84</td>
<td>0.76</td>
</tr>
<tr>
<td>A/Memphis/102/72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>10334</td>
<td>19</td>
<td>0.18</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>12003</td>
<td>106</td>
<td>0.88</td>
</tr>
<tr>
<td>Mock Infected</td>
<td>12532</td>
<td>12</td>
<td>0.10</td>
</tr>
</tbody>
</table>

In Figure 32, cells infected with A/WSN/33 H1N1 multiplicity of infection (MOI) of 1 showed a small amount of CD40+ cells. Groups of cells infected with the viral strains A/WSN/33 moi 0.1 and A/Memphis/102/72 moi 0.1 showed no marker expression of CD40 (Figures 33-36, Appendix A). Cells mock infected with 1X Trypsin also showed no expression of CD40. There was no expression found in the mock infected group of
cells. However, expression of CD40 was found with cells infected with MOI 1 A/WSN/33.

2.2.3 CD86 marker expression

Cells infected with A/WSN/33 (MOI of 1 and 0.1), A/Memphis/102/72 (MOI of 10, 1, 0.1) were stained with an antibody directed against the cellular marker CD86. A group of cells was also mock infected with diluted trypsin and then stained with the antibody. CD86 can be found on B cells, macrophages, and dendritic cells at low levels. It tends to be up-regulated on B cells when stimulated with the BCR complex, CD40, and some cytokine receptors. CD86 also plays a crucial role in T-B crosstalk, T cell co-stimulation, autoantibody production, and Th-2 mediated Ig production. The kinetics of up-regulation of CD86 upon stimulation supports its major contribution during the primary phase of an immune response. Table 8 shows the cells processed using flow cytometry and enumeration based on fluorescence.
Table 8. Cells stained with CD86 antibody. Two groups of cells were infected with A/WSN/33 MOI of 1 and 0.1. A second set of cells was infected with an A/Memphis/102/72 MOI of 1, and 0.1. The last group was mocked infected with 1X trypsin only. The second left column shows the total amount of cells enumerated by the flow cytometry machine. The middle column shows the number of cells expressing the CD86 marker. The far right column denotes the percentage of cells positive for expressing the CD86 marker.

<table>
<thead>
<tr>
<th>CD86</th>
<th>Total Cells Counted</th>
<th>Marker + Cells</th>
<th>% Marker + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>MOI 1</td>
<td>22633</td>
<td>6069</td>
<td>26.81</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>10631</td>
<td>6210</td>
<td>58.41</td>
</tr>
<tr>
<td>A/Memphis/102/72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>10751</td>
<td>4695</td>
<td>43.67</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>10403</td>
<td>5033</td>
<td>48.38</td>
</tr>
<tr>
<td>Mock Infected</td>
<td>125507</td>
<td>3812</td>
<td>30.48</td>
</tr>
</tbody>
</table>

Groups of cells infected with the viral strains A/WSN/33 and A/Memphis/102/72 showed CD86 marker expression (Figures 37-41 Appendix A). Cells mock infected with 1X trypsin also showed expression of CD86 (Figure 41, Appendix A). The cells infected with A/WSN/33 MOI 1 showed a positive percentage of 26.81. In contrast, the cells infected with A/Memphis/102/72 MOI 1 showed a percentage of 43.67. The cells infected with the H3N2 strain expressed CD86 at higher levels. In comparison with both infections using a multiplicity of infection of 0.1, there is an increased expression in the marker with A/WSN/33. Cells infected with A/Memphis/102/72 demonstrated a lower level of expression. The mock infected cells expressed CD86 at a percentage of 30.48 (Table 10). Therefore, there are differences in marker expression in each strain infection, as compared to the control group, and to each other.
2.2.4 MHC Class II marker expression

Cells infected with A/WSN/33 (MOI of 1 and 0.1), A/Memphis/102/72 (MOI of 1, and 0.1) were stained with an antibody directed against the cellular marker MHC Class II. A group of cells was also mock infected with diluted trypsin and then stained with the antibody. This marker is expressed on antigen presenting cells including B cells, monocytes, macrophages, dendritic cells, and activated T cells. MHC Class II ultimately plays an important role in the presentation of pathogen peptides to CD4\(^+\) T lymphocytes. After infection, the cells were stained, and processed using flow cytometry. Table 9 shows cell samples that were enumerated based on fluorescence.

Table 9. Cells stained with MHC Class II antibody. Two groups of cells were infected with A/WSN/33 MOI of 1 and 0.1. A second set of cells was infected with an A/Memphis/102/72 MOI of 10, 1, and 0.1. The last group was mocked infected with 1X trypsin only. The second left column shows the total amount of cells enumerated by the flow cytometry machine. The middle column shows the number of cells expressing the MHC Class II marker. The far right column denotes the percentage of cells positive for expressing the MHC Class II marker.

<table>
<thead>
<tr>
<th>MHC Class II</th>
<th>Total Cells Counted</th>
<th>Marker + Cells</th>
<th>% Marker + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>22846</td>
<td>6587</td>
<td>28.83</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>11729</td>
<td>3197</td>
<td>27.26</td>
</tr>
<tr>
<td>A/Memphis/102/72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>12342</td>
<td>895</td>
<td>7.25</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>12156</td>
<td>895</td>
<td>7.36</td>
</tr>
<tr>
<td>Mock Infected</td>
<td>7571</td>
<td>540</td>
<td>7.13</td>
</tr>
</tbody>
</table>

Differences between both infections are summarized in Figures 42-46, Appendix A.

A/WSN/33 infection MOI 1 showed a population of 28.83% MHC Class II positive cells.

In contrast, A/Memphis/102/72 infection MOI 1 showed a population of 7.25% positive
cells. The same difference is noted in the infection using a MOI of 0.1, as seen in Table 11. The cells infected with the H1N1 strain have a higher expression of the marker than do the H3N2 infected cells. The mock infected cells show an expression percentage of 7.13. It can also be noted that the cells infected with A/Memphis/102/72 MOI 10 showed an expression of 5.9%.

2.2.5 MHC Class I marker expression

The cells infected with A/WSN/33 (MOI of 1 and 0.1), A/Memphis/102/72 (MOI of 10, 1, 0.1) were stained with an antibody directed against the cellular marker MHC Class I. A group of cells was also mock infected with diluted Trypsin and then stained with the same marker. This antigen is expressed by all human nucleated cells, and these are key in cell-mediated immune response and tumor surveillance. After infection, the cells were stained, and processed using flow cytometry. As seen in Table 10, the cell samples were enumerated based on fluorescence.
Table 10. Cells stained with MHC Class I antibody. Two groups of cells were infected with A/WSN/33 MOI of 1 and 0.1. A second set of cells was infected with an A/Memphis/102/72 MOI of 1, and 0.1. The last group was mocked infected with 1X trypsin only. The second left column shows the total amount of cells enumerated by the flow cytometry machine. The middle column shows the number of cells expressing the MHC Class I marker. The far right column denotes the percentage of cells positive for expressing the MHC Class I marker.

<table>
<thead>
<tr>
<th>MHC Class I</th>
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<th>Marker + Cells</th>
<th>% Marker + Cells</th>
</tr>
</thead>
<tbody>
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<td>A/WSN/33</td>
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<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>19613</td>
<td>15308</td>
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<tr>
<td>MOI 0.1</td>
<td>8953</td>
<td>4758</td>
<td>53.14</td>
</tr>
<tr>
<td>A/Memphis/102/72</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>8881</td>
<td>5453</td>
<td>61.4</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>6785</td>
<td>3104</td>
<td>45.75</td>
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<tr>
<td>Mock Infected</td>
<td>9537</td>
<td>5522</td>
<td>57.90</td>
</tr>
</tbody>
</table>

As seen in Figures 47 through 51 in Appendix A, the group of cells infected with the viral strains A/WSN/33 and A/Memphis/102/72 showed differences in marker expression. A/WSN/33 infection MOI 1 showed a very high percentage of cells expressing MHC Class 1 antigen. In contrast, A/Memphis/102/72 showed a lower expression of 61.4%. With MOI of 0.1 for each virus, A/WSN/33 shows yet a higher percentage of expression than does A/Memphis/102/72 infection. As seen in Figure 51 in Appendix A, a sample was mock infected, and showed a 57.90% population of MHC Class I positive cells. From these results, it can be concluded that cells infected with the strains H3N2 and H1N1 showed differences in the incidence of expression for MHC Class I marker.
2.2.6 CD11b marker expression

The cells infected with A/WSN/33 (MOI of 1 and 0.1), A/Memphis/102/72 (MOI of 10, 1, 0.1) were stained with an antibody directed against the cellular marker CD11b. A group of cells was also mock infected with diluted trypsin and then stained with the same marker. CD11b is an antigen that binds to a subset of molecules on neutrophils and monocytes after stimulation with chemokines. This then plays a role in adhesive cell interactions. After infection the cells were stained and processed using flow cytometry. Cell samples were enumerated by fluorescence using flow cytometry (Table 11).

Table 11. Cells stained with CD11b antibody. Two groups of cells were infected with A/WSN/33 MOI of 1 and 0.1. A second set of cells was infected with A/Memphis/102/72 MOI of 1, and 0.1. The last group was mocked infected with 1X trypsin only. The second left column shows the total amount of cells enumerated by the flow cytometry machine. The middle column shows the number of cells expressing the CD11b marker. The far right column denotes the percentage of cells positive for expressing the CD11b marker.

<table>
<thead>
<tr>
<th>CD11b</th>
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<th>Marker + Cells</th>
<th>% Marker + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>6190</td>
<td>3</td>
<td>0.05</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>6834</td>
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<td>0.06</td>
</tr>
<tr>
<td>A/Memphis/102/72</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
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<td>3</td>
<td>0.05</td>
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<tr>
<td>MOI 0.1</td>
<td>6516</td>
<td>2</td>
<td>0.03</td>
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<tr>
<td>Mock Infected</td>
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<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The group of cells infected with the viral stains A/WSN/33 and A/Memphis/102/72 showed no difference in CD11b marker expression (Figures 52-56, Appendix A). The mock-infected sample showed no expression of CD11b (Figure 56, Appendix A). From these results, it can be concluded that cells showed no difference in the level of CD11b expression.
2.2.7 BAFF marker expression

Cells infected with A/WSN/33 (MOI of 1 and 0.1), A/Memphis/102/72 (MOI of 1, and 0.1) were stained with an antibody directed against the cellular antigen BAFF. A group of cells was also mock infected with diluted trypsin and then stained with the same marker. BAFF is a type 11 membrane protein that exists in both membrane-bound and soluble forms. Expression of membrane-bound BAFF on monocytes is regulated by interferon gamma. The antigen acts as a potent B cell growth factor and co-stimulator of Ig production. After infection, the cells were stained, and processed using flow cytometry. Cell samples were enumerated by fluorescence using flow cytometry (Table 12).

Table 12. Cells stained with the BAFF antibody. Two groups of cells were infected with A/WSN/33 MOI of 1 and 0.1 A second set of cells was infected with an A/Memphis/102/72 MOI of 10, 1, and 0.1. The last group was mock infected with 1X trypsin only. The second left column shows the total amount of cells enumerated by the flow cytometry machine. The middle column shows the number of cells expressing the BAFF marker. The far right column denotes the percentage of cells positive for expressing the BAFF marker.

<table>
<thead>
<tr>
<th>BAFF</th>
<th>Total Cells Counted</th>
<th>Marker + Cells</th>
<th>% Marker + Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/WSN/33</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>26803</td>
<td>6</td>
<td>0.02</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>10548</td>
<td>4</td>
<td>0.04</td>
</tr>
<tr>
<td>A/Memphis/102/72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MOI 1</td>
<td>13068</td>
<td>2</td>
<td>0.02</td>
</tr>
<tr>
<td>MOI 0.1</td>
<td>9955</td>
<td>6</td>
<td>0.06</td>
</tr>
<tr>
<td>Mock Infected</td>
<td>10314</td>
<td>3</td>
<td>0.03</td>
</tr>
</tbody>
</table>

The group of cells infected with the viral strains A/WSN/33 and A/Memphis/102/72 showed no difference in BAFF marker expression (Figures 57-61, Appendix A). The mock infected sample showed no expression of BAFF (Figure 61, Appendix A). From
these results, it can be concluded that cells showed no difference in the level of BAFF expression in any of the groups.
Discussion

In this thesis work, dendritic cells were cultured from blood monocytes and infected with two strains of influenza and the expression of activation molecules on the cellular membranes were studied. A/WSN/33 is a strain of influenza virus belonging to the subtype group H1N1 that was isolated in 1933 and is a descendant of the virus responsible for the 1918 pandemic. A/Memphis/102/72 is another strain of influenza virus that belongs to the H3N2 subtype that was responsible for the 1967 pandemic. Although A/Memphis/102/72 (H3N2) continues to infect humans and is the cause of most acute cases of influenza infection today, while A/WSN/33 (H1N1) does not pose a risk for infection in humans. This is due to many passages in culture, and subsequently A/WSN/33 has lost its pathogenicity as a result.

The immune response against cytopathic viruses in response to infection with A/Memphis/102/72 H3N2 was shown to decrease activation molecule expression on the cell membranes of dendritic cells, using A/WSN/33 H1N1 virus as a control. This hypothesis was tested by quantifying two strains of influenza virus and optimizing the technology to detect the presence of activation molecules on the membranes of cells.

Embryonated chicken eggs were used to propagate both strains of influenza virus. A/Memphis/102/72 H3N2 grew well in the embryos and the titer for this stock was $5 \times 10^7$ EID50% per mL and 512 by hemagglutination (Figures 9, and 11). A/WSN/33 H1N1 grew less effectively in chicken embryos, and had a EID50% of $5 \times 10^5$ per mL and a hemagglutination titer of 256 (Figures 10, and 12), indicating the presence of interferent particles in this viral stock.
After influenza virus propagation and quantification, peripheral blood mononuclear cells were characterized by membrane molecules using fluorescence microscopy. Cells were separated from whole blood and stained using biotinylated antibodies with specificity to certain molecules conjugated to fluorescent dyes. After the cells were incubated with the antibody, they were observed using an epifluorescent microscope, and the percentage of fluorescent cells was recorded (e.g., cells stained with an anti-CD4 antibody can be seen in Figure 14). To determine background staining, a biotinylated rat isotype control antibody conjugated to fluorescein was used. As seen in Figure 13, no cells were identified as positive due to fluorescent staining. This validated the protocol used because no cells are expected to be positive with this antibody.

After fluorescent cell staining was optimized the same protocol was then applied to characterize cells using flow cytometry. Cells were tagged with antibodies specific for membrane molecules, and processed. To determine background fluorescence using this technology, a biotinylated rat isotype control antibody conjugated to fluorescein was used. This provided a negative control. In Figure 18, it is shown that no cells were identified as positive due to fluorescent staining using this antibody as expected for the negative isotype control used. As seen in Figure 19, cells stained with an antibody specific for cellular molecule CD19 were quantified using flow cytometry. CD19 is a molecule found mostly on B cells in humans. Positive cells in the sample demonstrated a high intensity of fluorescence due to the expression of this molecule. Eight and a half percent of positive cells were found using this antibody. This percentage is within the range of expected B cell frequency in peripheral mononuclear cells.
After this technology had been optimized, it was possible to proceed onto another crucial aspect of the project: production of dendritic cells by differentiation of monocytes. Dendritic cells are not as abundant in circulation as other immune system cells. Blood-derived monocytes differentiate into immature dendritic cells when cultured with cytokines IL-4 and GM-CSF. The percentage of monocytes in blood is 2-10% depending on the individual but it is possible to enrich this population using magnetic bead separation with antibodies against a molecule present on the monocytes cell membrane such as CD14. Therefore, it was necessary to optimize this technology for enrichment of monocytes from blood. As seen in Figure 23 before separation of monocytes, the occurrence of this population in blood was 6.07%. Optimization of the magnetic bead separation allowed the monocyte population to be enriched and was 26.24% (Figure 24).

Once this protocol was optimized, high numbers of monocytes were stimulated using IL-4 and GM-CSF to develop into immature dendritic cells. It was possible to increase the dendritic cell population from 6.6% before cytokine treatment (Figure 25) to 34.85% after cytokine treatment (Figure 26).

Monocyte-derived dendritic cells were then infected with influenza strains A/WSN/33 H1N1 and A/Memphis/102/73 H3N2. Two different multiplicities of infection were used (MOI=1 and MOI=0.1), and dendritic cells were incubated for 4 hours after infection. An aliquot of cells were mock infected with 10% trypsin in RPMI, as a negative control. The cells were then stained with a panel of antibodies specific for co-stimulation molecules expressed on dendritic cells. To determine background fluorescence within the experiment, a biotinylated rat isotype control antibody conjugated to fluorescein was
used. In Figure 18, no cells were identified as positive due to fluorescent staining using this antibody. This is expected, as this is an isotype control used.

Antibodies specific for CD11b, and BAFF did not show a positive population of cells indicating that these two molecules were not expressed by the infected and mock-infected dendritic cells (Figures 52-61). CD11b is a typical marker for monocytes-macrophages and BAFF is a membrane molecule belonging to the family of Tumor Necrosis Factor that has been shown to be involved in activation of B cells by dendritic cells (17-19). Therefore, infection with these strains did not reverse the differentiation of dendritic cells to a monocyte-macrophage-like phenotype as it was described previously with foot-and-mouth disease virus (6).

Proteins from virus infected cells are processed in the cytoplasm by proteasomes and expressed on the cell membrane in association with MHC Class I. Although these molecules are normally expressed on the cell membranes, infection increases expression. Therefore, expression of the MHC Class I molecule on dendritic cells can be used as an indication of virus replication. This molecule was expressed higher in cells infected with a MOI of 1 of A/WSN/33 H1N1 (78.05%) as compared to the mock infected dendritic cells (57.9%) (Table 12). However, cells infected with a MOI of 0.1 did not show an increase in expression (53.14%). The titer of A/WSN/33 H1N1 is $5 \times 10^5$ EID$_{50}$/ml but the titer by hemagglutination is high (256). This difference between a high HA and low EID strongly suggest the presence of interferent particles. Although the use of one infectious virus per cell clearly increases expression of MHC I, indicating the
presence of viral peptides due to viral infection, a lower MOI (0.1) does not increase MHC I, which is consistent with lack of viral replication.

This result is congruent with the data showing high levels of interferent particles in this virus stock. Infection with A/Memphis/102/72 H3N2 that has a MOI of 1 or 0.1 does not induce expression of MHC I compared to the mock infected control. This is consistent with previous data in the literature indicating that the influenza virus induces an abortive infection (14,16); blocking replication of the human strain of influenza may be due to the expression of α interferon by the dendritic cells.

Interestingly, activation markers such as CD40, CD86 and MHC II (Tables 8, 10, and 11) increase expression in cells infected with A/WSN/33 H1N1 but not in cells infected with A/Memphis/107/72H3N2. Thus, dendritic cells seem to block replication of A/Memphis/107/72 but at the same time decrease the expression of activation molecules on the cell membrane. In contrast, A/WSN/33 H1N1 was able to replicate (as demonstrated by increase of MHC I expression on the cell membrane of the dendritic cells) and at the same time increased the expression of activation markers.

The results obtained with infection with the human influenza virus A/Memphis/107/72 H3N2 are similar to the ones obtained with foot-and-mouth disease virus infecting mouse dendritic cells. The results obtained with the avian adapted A/WSN/33 H1N1 are similar to the those obtained with inactivated FMDV: either infection with this strain or uptake of inactivated FMDV increases expression of activation markers (6).
Two explanations could account for this behavior by A/WSN/33 H1N1: 1) the high proportion of interferent particles that encourage this virus to act as an inactivated virus, or 2) though there is replication of the virus, the level of replication is not enough to alert “danger-signal” receptors in the cytoplasm of the dendritic cells. Nonetheless, this strain is sensed by the dendritic cell as a particular non-infectious antigen. Subsequently dendritic cells drive the response into a classical T-cell dependent pathway.

In the case of strain A/Memphis/107/72H3N2, dendritic cells block replication (abortive replication) but the level of infection is still enough to activate the danger signal molecules in the cytoplasm of the infected dendritic cell. This drives the dendritic cell to a pathway in which no T cells will be activated (T cell independent) as it is the case for influenza (4). This pathway is similar to the one described in FMDV-infected dendritic cells (6) and allows for the suggestion that a similar mechanism is in place for both viruses: infection with a pathogenic influenza virus strain is sensed by the human dendritic cell which is alerted by danger receptors.

As a result, virus replication is aborted, possibly by α-interferon. This mechanism leads to a decrease of activation markers which will not activate T cells, thus decreasing inflammation. In conclusion, infected dendritic cells directly activate B cells to produce antibodies by a different mechanism other than BAFF-APRIL engagement. The overall results of the cross-talk are a decrease in inflammation at the beginning of the infection with a rapid induction of neutralizing antibodies (17-19).
REFERENCES


Figure 27. Flow cytometry analysis for cells infected with A/WSN/33 MOI 0.1, and then stained with rat isotype control antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 28. Flow cytometry analysis for cells infected with A/WSN/33 MOI 1, and then stained with rat isotype control antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 29. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 0.1, and then stained with rat isotype control antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 30. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 1, and then stained with rat isotype control antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 31. Flow cytometry analysis for cells mock infected with 1X trypsin, and then stained with rat isotype control antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 32. Flow cytometry analysis for cells infected with A/WSN/33 MOI 1, and then stained with CD40. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 33. Flow cytometry analysis for cells infected with A/WSN/33 MOI 0.1, and then stained with CD40. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 34. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 1, and then stained with CD40. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 35. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 1, and then stained with CD40. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 36. Flow cytometry analysis for cells mock infected with 1X tryps, and then stained with CD40. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 37. Flow cytometry analysis for cells infected with A/WSN/33 MOI 1, and then stained with CD86. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 38. Flow cytometry analysis for cells infected with A/WSN/33 MOI 0.1, and then stained with CD86. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 39. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 1, and then stained with CD86. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 40. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 0.1, and then stained with CD86. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 41. Flow cytometry analysis for cells mock infected with 1X trypsin, and then stained with CD86. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 42. Flow cytometry analysis for cells infected with A/WSN/33 MOI 1, and then stained with MHC Class II. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 43. Flow cytometry analysis for cells infected with A/WSN/33 MOI 0.1, and then stained with MHC Class II. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 44. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 1, and then stained with MHC Class II. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 45. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 0.1, and then stained with MHC Class II. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 46. Flow cytometry analysis for cells mock infected with 1X trypsin, and then stained with MHC Class II. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 47. Flow cytometry analysis for cells infected with A/WSN/33 MOI 1, and then stained with MHC Class I. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 48. Flow cytometry analysis for cells infected with A/WSN/33 MOI 0.1, and then stained with MHC Class I. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 49. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 1, and then stained with MHC Class I. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 50. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 0.1, and then stained with MHC Class I. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 51. Flow cytometry analysis for cells mock infected with 1X trypsin, and then stained with MHC Class I. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 52. Flow cytometry analysis for cells infected with A/WSN/33 MOI 1, and then stained with CD11b. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 53. Flow cytometry analysis for cells infected with A/WSN/33 MOI 0.1, and then stained with CD11b. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 54. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 1, and then stained with CD11b. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 55. Flow cytometry analysis for cells infected with A/Memphis/102/72 MOI 0.1, and then stained with CD11b. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 56. Flow cytometry analysis for cells mock infected with 1X trypsin, and then stained with CD11b. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 57. Flow cytometry analysis for cells infected A/WSN/33 MOI 1, and then stained with BAFF antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 58. Flow cytometry analysis for cells infected A/WSN/33 MOI 0.1, and then stained with BAFF antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 59. Flow cytometry analysis for cells infected A/Memphis/102/72 MOI 1, and then stained with BAFF antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.

Figure 60. Flow cytometry analysis for cells infected A/Memphis/102/72 MOI 0.1, and then stained with BAFF antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
Figure 61. Flow cytometry analysis for cells mock infected with 1X trypsin, and then stained with BAFF antibody. The left scatter plot diagram shows the output of cells stained with fluorescence. The diagram on the right indicates the output for the number of cells versus intensity of fluorescence.
APPENDIX B

Preparation of solutions used in this work

1. Alsevers solution
20.5 grams Dextrose
7.9 grams Sodium Citrate · 2H₂O (27 mM)
4.2 grams NaCl (71 mM)
Dissolve reagents in 50 mL distilled water. Adjust pH with 1M Citric Acid Solution.
Filter sterilize. Store at -20°C.

2.) 1 M Citric Acid Solution
Dissolve 48.0325 grams in 250 mL distilled water. Store at room temperature.

3.) 5% FCS-RPMI
5 mL sterile fetal calf serum
95 mL sterile RMPI (Invitrogen)
Add 95 mL RMPI to 5 mL FCS in autoclaved glass bottle in sterility. Store at 4°C

4.) 10X PBS pH 7.4
2.62 grams NaH₂PO₄
11.5 grams Na₂HPO₄
43.84 grams NaCl
Combine all ingredients in 250 mL distilled water. Adjust pH to 7.4 Add 250 mL distilled water. Autoclave

5.) 1X PBS pH 7.4
100 mL 10X PBS pH 7.4
900 mL distilled water
Combine 100 mL 10X PBS and 900 mL distilled water in glass bottle. Autoclave. Store at room temperature.

5.) 2% BSA solution
10 mL of 10X PBS
90 mL distilled water
2 grams BSA
Add 90 mL distilled water to 10 mL 10X PBS. Add 2 grams BSA. Stir to mix. Filter sterilize. Aliquot in 10 mL amounts. Store at -20°C.
6.) 1% Paraformaldehyde solution
0.1 grams paraformaldehyde
100 mL 1X PBS
Add 0.1 grams paraformaldehyde to 50 mL 1X PBS. Raise pH to 10. Heat to dissolve. Add additional 50 mL 1X PBS. Lower pH to 7. Store at room temperature.

7.) Separation buffer
0.2 mL 5 M EDTA Solution (Gibco)
1X PBS
Biotin free BSA
Add 0.2 mL EDTA solution to 499.6 mL 1X PBS. Add 2.5 grams BSA to solution. Filter sterilize. Aliquot into 5 and 10 mL amounts. Store at -20°C.

8.) Human Dendritic Cell Culture Medium
89.5 mL sterile RPMI
10 mL FCS
12 microliters Recombinant Human GM-CSF (eBioscience)
12 microliters Recombinant Human IL-4 (eBioscience)
400 microliters Penicillin-Streptomycin (Gibco)
Add 89.5 mL RMPI to sterile glass bottle. Add antibiotics, and cytokines. Add 10 mL FCS and pipette to mix. Store at 4°C.

9.) 1X Trypsin
10 mL Trypsin
90 mL sterile RPMI
Add 90 mL RPMI to 10 mL trypsin in sterile bottle. Store at 4°C.
APPENDIX C

Approval for Vertebrate Use

April 19, 2007

TO: Dr. Osvaldo Lopez
    Biology Department

FROM: Cynthia A. Prosser, Ph.D.
      Dean of Graduate Studies & Research

RE: Application to use Vertebrate Animals
    Application # IACUC 57

The Institutional Animal Care and Use Committee approved your modification of an application currently approved for your project to use vertebrate animals in research entitled “Transient immunosuppression due to infection with influenza virus”.

If you have any questions, please contact me.

kjm

cc: Biology Department
APPENDIX D

Approval for use of human subjects

May 11, 2007

TO: Dr. Osvaldo Lopez
    Biology

FROM: Cynthia A. Prosen, Ph.D.
     Dean of Graduate Studies & Research

RE: Human Subjects Proposal # HS07-107
    “Early protective immune response against infection with influenza virus”

The Human Subjects Research Review Committee has reviewed your proposal and has given it final approval. To maintain permission from the Federal government to use human subjects in research, certain reporting processes are required. As the principal investigator, you are required to:

A. Include the statement “Approved by HSRRC: Project # (list above) on all research materials you distribute, as well as on any correspondence concerning this project.

B. Provide the Human Subjects Research Committee letters from the agency(ies) where the research will take place within 14 days of the receipt of this letter. Letters from agencies should be submitted if the research is being done in (a) a hospital, in which case you will need a letter from the hospital administrator; (b) a school district, in which case you will need a letter from the superintendent, as well as the principal of the school where the research will be done; or (c) a facility that has its own Institutional Review Board, in which case you will need a letter from the chair of that board.

C. Report to the Human Subjects Research Review Committee any deviations from the methods and procedures outlined in your original protocol. If you find that modifications of methods or procedures are necessary, please report these to the Human Subjects Research Review Committee before proceeding with data collection.

D. Submit progress reports on your project every 12 months. You should report how many subjects have participated in the project and verify that you are following the methods and procedures outlined in your approved protocol.

E. Report to the Human Subjects Research Review Committee that your project has been completed. You are required to provide a short progress report to the Human Subjects Research Review Committee in which you provide information about your subjects, procedures to ensure confidentiality/anonymity of subjects, and the final disposition of records obtained as part of the research (see Section II.C.7.d).

F. Submit renewal of your project to the Human Subjects Research Review Committee if the project extends beyond three years from the date of approval.

It is your responsibility to seek renewal if you wish to continue with a three-year permit. At that time, you will complete (D) or (E), depending on the status of your project.

kjm