THE ROLE OF PLASMACYTOID DENDRITIC CELLS (PDCS) IN MURINE ACQUIRED IMMUNODEFICIENCY SYNDROME (MAIDS)

By

Barret Zimmermann

A Paper Presented to the
Faculty of Mount Holyoke College in
Partial Fulfillment of the Requirements for
the Degree of Bachelors of Arts with
Honor

Department of Biological Sciences
South Hadley, MA 01075

May 2011
This paper was prepared under the direction of Professor Sharon Stanford for eight credits.
For my mom and dad – my two biggest fans.
ACKNOWLEDGEMENTS

It is with immense gratitude that I thank Professor Sharon Stranford for the opportunity to do my thesis in her lab. From my first experience in immunology lab while taking the course junior year to the completion of this senior thesis, she has fostered my interest in immunology. She also encouraged me to work independently and think critically while working through problems, offering me some invaluable guidance along the way.

It gives me great pleasure in acknowledging the support and help of Professor Rachel Rink. She championed for my success throughout my thesis-work by inspiring my confidence with her wise words. I am indebted to Professor Janice Gifford for her help navigating the statistical portion of my thesis. Her patience with my impromptu visits are also much appreciated. I would also like to thank Amber Douglas for her support of me throughout thesis writing.

I am grateful to my many Stranford lab colleagues, past and present, for their support in and out of lab. I would like to especially thank Sonia Bakkour, Ph.D., with whom I took immunology class. It was her enthusiasm and eloquent discussion of immunology that first peaked my interest in the field. I cannot express enough thanks to Bing Miu for initially showing me how to perform flow cytometry and Macy Akalu for being there with me in lab for many of my experiments.

I would like to acknowledge the Mount Holyoke biology department as a whole. Over these past four years the biology department has become a family to me. Additionally, being a part of such an intellectually stimulating community has made my time here memorable and invaluable. I owe my deepest gratitude to the faculty and staff, who have showered me with their wisdom and compassion.

I cannot find words to express my gratitude to my parents. Their unconditional love and belief in my abilities has always been the basis of my academic resolve. They continue to give my life purpose and keep me grounded, as do the many individuals with whom I have grown close over these four years. Family and friendship are two of life’s greatest gifts.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>List of Tables</td>
<td>xi</td>
</tr>
<tr>
<td>Abstract</td>
<td>xii</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Immune System Overview</td>
<td>2</td>
</tr>
<tr>
<td>Immune Cell Types</td>
<td>2</td>
</tr>
<tr>
<td>The Innate Immune System</td>
<td>6</td>
</tr>
<tr>
<td>The Adaptive Immune System</td>
<td>7</td>
</tr>
<tr>
<td>T lymphocytes (T cells)</td>
<td>8</td>
</tr>
<tr>
<td>Antigen Presenting Cells</td>
<td>10</td>
</tr>
<tr>
<td>Major Histocompatibility Complex (MHC)</td>
<td>11</td>
</tr>
<tr>
<td>Cross-Presentation</td>
<td>12</td>
</tr>
<tr>
<td>Dendritic Cells (DCs)</td>
<td>13</td>
</tr>
<tr>
<td>The Site of DCs: Lymphoid Tissue</td>
<td>14</td>
</tr>
<tr>
<td>Plasmacytoid DCs (pDCs)</td>
<td>17</td>
</tr>
<tr>
<td>DCs and their Interferon production</td>
<td>19</td>
</tr>
<tr>
<td>Human <em>versus</em> Mouse pDCs</td>
<td>19</td>
</tr>
<tr>
<td>Retroviruses and Lentiviruses</td>
<td>21</td>
</tr>
</tbody>
</table>
Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS)……………………………………… 22
Murine Acquired Immunodeficiency Syndrome (MAIDS)…………… 25
MAIDS versus AIDS……………………………………………… 28
pDCs in AIDS and MAIDS………………………………………… 29
Fluorescence Activated Cell Staining (FACS) and
Monoclonal Antibodies……………………………………………. 31
Experiment Rationale/ Previous Studies……………………………. 33
Research Objective………………………………………………….. 35
**Materials and Methods**………………………………………….. 37
Animals……………………………………………………………….. 37
Virus Stock……………………………………………………………. 37
Infection and Dissection……………………………………………. 37
Obtaining Leukocytes from Spleen…………………………………… 40
Antibodies……………………………………………………………… 41
Immunostaining………………………………………………………. 45
Flow Cytometric Analysis…………………………………………. 48
Measurement………………………………………………………….. 48
Identifying Specific Cell Populations………………………………… 48
Color Compensation………………………………………………….. 53
Statistical Analysis…………………………………………………… 55
Results

Experiment Rationale

Proof of Concept Experiment

Antibody Dilution Determination

Color Compensation

Evaluation of pDCs in Infected Mice

Independent Groups t-Test: pDCs

Gated Subpopulation of Double Positive (CD317+CD45R+) Cells

Qualitative Analysis

Discussion

Previous Lab Work: A Motivation for Studying pDCs

A Unique Experimental Design

Part I

Using Antibodies to Define pDCs

Using staining Patterns to define pDCs

Part II

Sources of Variability

Part III

Potential functions of pDCs in MAIDS and AIDS

Comparing human and mouse pDCs

Future Studies

Conclusions
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.</td>
<td>Immune system cell lineages</td>
<td>3</td>
</tr>
<tr>
<td>Figure 2.</td>
<td>Dendritic Cells (DCs) are the most efficient T cell activators during viral infection</td>
<td>15</td>
</tr>
<tr>
<td>Figure 3.</td>
<td>Keep plasmacytoid dendritic cell (pDC) features and functions</td>
<td>18</td>
</tr>
<tr>
<td>Figure 4.</td>
<td>The murine AIDS (MAIDS) model uses a MAIDS susceptible and MAIDS resistant strain of mice</td>
<td>26</td>
</tr>
<tr>
<td>Figure 5.</td>
<td><em>Mus musculus</em> spleen location</td>
<td>39</td>
</tr>
<tr>
<td>Figure 6.</td>
<td>Treatment with Fc block prior to immunostaining can reduce background fluorescence</td>
<td>44</td>
</tr>
<tr>
<td>Figure 7.</td>
<td>Experimental system for labeling pDCs with antibodies</td>
<td>47</td>
</tr>
<tr>
<td>Figure 8.</td>
<td>Gating to collect viable leukocytes</td>
<td>49</td>
</tr>
<tr>
<td>Figure 9.</td>
<td>Creating quadrants in a dot plot to exclude nonspecific background staining</td>
<td>51</td>
</tr>
<tr>
<td>Figure 10.</td>
<td>Gating on a specific double-stained population</td>
<td>52</td>
</tr>
<tr>
<td>Figure 11.</td>
<td>Excitation and emission spectra of APC, PE, and Alexa 488</td>
<td>54</td>
</tr>
<tr>
<td>Figure 12.</td>
<td>Testing three anti-CD45R antibody dilutions for optimal staining</td>
<td>62</td>
</tr>
</tbody>
</table>
Figure 13. Testing three anti-CD317 antibody dilutions for optimal staining…………………………………………………………. 63

Figure 14. Compensation is required to remove fluorescence spillover between detector channels…………………………….. 65

Figure 15. Triple stained populations are very small, making data analysis difficult………………………………………… 67

Fig 16. Double color staining with anti-CD317-Alexa Fluor® 488 and anti-CD45R-PE antibodies………………………………… 71

Figure 17. Dot plot of adjusted double-positive (DP) (CD317+CD45R+) population from the spleen of each individual mouse, based on strain (N = 10 for both BALB/c and C57BL/6)………………. 77

Figure 18. A significantly larger double-stained (CD317CD45R) population is seen in the spleens of MAIDS susceptible mice at 7 days post infection………………………………………. 78

Figure 19. Gating a smaller DP (CD317+CD45R+) for comparison between strains……………………………………………………… 81

Figure 20. The MAIDS resistant strain has many distinct DP (CD317+CD45R+) populations, while the MAIDS susceptible strain has one larger diffuse DP population…. 83
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 1.</td>
<td>Antibodies used in Immunofluorescent Staining</td>
<td>42</td>
</tr>
<tr>
<td>Table 2.</td>
<td>Antibody Concentrations Tested for Optimization of Cell Staining Procedure</td>
<td>59</td>
</tr>
<tr>
<td>Table 3.</td>
<td>Percentage of spleen cells double-stained with anti-CD317 and anti-CD45R or with anti-CD317 and anti-CD11c</td>
<td>69</td>
</tr>
<tr>
<td>Table 4.</td>
<td>Percentage of spleen cells expressing both CD317 and CD45R</td>
<td>73</td>
</tr>
<tr>
<td>Table 5.</td>
<td>Group Statistics</td>
<td>75</td>
</tr>
<tr>
<td>Table 6.</td>
<td>Independent groups t-test for adjusted double-stained (CD317CD45R) cells</td>
<td>76</td>
</tr>
</tbody>
</table>
Murine AIDS is an animal model used to study human AIDS. Infection of susceptible strains of mice such as C57BL/6 with murine leukemia virus (MuLV) leads to MAIDS in a manner analogous to HIV-induced AIDS. Conversely, MAIDS resistant strains like BALB/c generate a successful immune defense against the virus after infection. Dendritic cells (DCs) play the largest role in viral particle processing and activation of naïve T cells, which elicit immune responses that can eradicate HIV or MuLV infection. The few published studies that have examined the role of a specific DC subset, plasmacytoid dendritic cells (pDCs), in AIDS, express opposing views; some argue that pDCs inhibit HIV spread, while others argue pDCs promote it. We used flow cytometry to compare pDC percentages in the spleens of BALB/c and C57BL/6 mice one week post-infection. Firstly, we identified a larger pDC (CD317+ and CD45R+) population in the susceptible strain after infection; this difference was statistically significant (p < .0001). These findings suggested that pDCs may be associated with disease vulnerability. Secondly, phenotypic differences were observed between strains, suggesting that these cell populations may be distinct. Nevertheless, the observed strain-specific differences in CD317+CD45+ population might influence disease susceptibility in MAIDs, and correspondingly could influence our understanding of human AIDS-susceptibility.
INTRODUCTION

Worldwide, chronic viral infections, like human immunodeficiency virus (HIV), cause major health problems with severe morbidity and mortality. In 2008 there were 33.4 million people living with HIV according to the UNAIDS/WHO AIDS Epidemic Update. An additional 2.7 million people were newly infected with HIV and there were 2 million acquired immunodeficiency syndrome (AIDS)-related deaths in 2008 (UNAID, 2009).

AIDS has rapidly developed into one of the largest pandemics in history, since its recognition by the Center for Diseases Control (CDC) in 1981 (Hall, 2008). Not only has AIDS been devastating due to its pandemic spread, it has also proven detrimental on an individual basis due in large part to its dynamic pathogenesis. HIV sabotages the immune system by targeting one of the cells responsible for fighting viral infections, CD4+ T helper cells (CD4+), rendering the immune system unable to eradicate the virus. While there have been great strides in the prevention of HIV transmission and care of HIV infected individuals through antiviral treatment, there is no known cure for HIV-AIDS or vaccine to prevent the spread of HIV. Thus, researchers continue to study the virus’s dynamic mode of infection and replication, hoping to discover better methods of treatment and prevention.
**Immune System Overview**

Throughout evolutionary history multicellular organisms have been settled and subsequently infected by pathogenic microbes. To limit the infection of these microbes, animals have evolved a basic defense system. Subsequently, humans, as well as other animals, have diverged from using this basic system to employing a multifaceted series of defenses against today’s parade of microbes (Parham, 2009). The immune system is composed of a highly organized network of cells and allied tissues often divided into two distinct, yet, highly related parts: the innate immune response and the adaptive immune response (Parham, 2009).

**Immune Cell Types**

The vertebrate immune system is principally composed of leukocytes, or white blood cells, and the tissues related to them. These, along with other blood cells are continually generated by the body through a process called hematopoiesis (from the Greek ‘hemato,’ blood, and ‘poesis,’ to make) (Parham, 2009). Hematopoietic stem cells (HSCs) reside in the bone marrow and have the unique ability to give rise to any and all of the different mature blood cell types: red blood cells (erythrocytes), white blood cells (WBCs), and megakaryocytes, the source of platelets (see Figure 1). HSCs also give rise to the myeloid and lymphoid cell lineages of WBCs (Chi et al., 2009) (see Figure 1). To clarify, a myeloid cell describes any leukocyte (white blood cell) that is not a lymphocyte, which originates in the bone marrow (Parham, 2009).
Figure 1. Immune system cell lineages

The myeloid progenitor cell divides and differentiates to produce at least six different cell types. The first three of these types are granulocytes: the neutrophil, the eosinophil, and the basophil (Parham, 2009) (refer to Figure 1). Granulocytes are so named for their prominent cytoplasmic granules, which contain reactive substances that kill pathogenic microbes and enhance inflammation. Neutrophils are the most abundant white blood cell and they specialize in a process called phagocytosis. As the most lethal phagocyte, neutrophils specialize in capturing, engulfing, and killing microbes (Parham, 2009). The second most abundant granulocyte, the eosinophil, defends against helminth worms and other parasites. Lastly, the less abundant and understood basophil is thought to aid in immune responses aimed at parasites (Sokol et al., 2009).

The mast cell, the macrophage, and the dendritic cell (DC), are the last of the six cells that differentiate from the myeloid progenitor (Parham, 2009). Mast cells, which take up residence in mucosal and connective tissues, also have granules, which upon activation contribute greatly to inflammation. Monocytes circulate in the blood, and are distinguished from other leukocytes due to their larger size and distinct single nucleus, hence the name monocyte (Geissman et al., 2010; Parham, 2009). As mobile progenitors, monocytes travel via blood to tissues, where they take up residence and mature into macrophages. Macrophage means ‘large phagocyte,’ and like the neutrophil, macrophages are capable phagocytes. The macrophage is the body’s general scavenger, and as such, it
phagocytoses and disposes of the bodies’ dead cells and related debris, in addition to invading microbes (Geissman et al., 2010). DCs are resident in the bodies’ tissues and although they share many qualities with macrophages, they function uniquely as cellular messengers that are sent to call upon the adaptive immune response when needed. At such times, DCs leave the infected tissue in which they reside, with their cargo of degraded pathogen, and migrate to one of several lymphoid organs capable of eliciting an adaptive immune response (Geissman et al., 2010).

The common lymphoid progenitor divides and differentiates to give natural killer (NK) cells, B lymphocytes (B cells), and T lymphocytes (T cells) (see Figure 1). Until recently, NK cells were generally thought of as large effector lymphocytes of the innate immune system, however, they are now considered to be components of the innate and adaptive immune system (Vivier et al., 2011). NK cells are important in the defense against viral infection; they enter infected tissues and prevent the spread of infection by killing virus-infected cells and secreting cytokines, small cell-signaling protein molecules, to inhibit viral replication in infected cells (Parham, 2009; Vivier et al., 2011). B cells and T cells are small lymphocytes of the adaptive immune system with almost no cytoplasm. They are small so that they may circulate in a functionally inactive and immature form. Yet, recognition of a pathogen drives their selection, growth, and differentiation: B cells divide and differentiate into plasma cells, whereas T cells differentiate into various types of effector T cells. B and T cell activation
leads to a wonderfully powerful response after 1-2 weeks, highly specific for the invading pathogen (Parham, 2009).

**The Innate Immune System**

The body’s first line of defense from infection is the innate immune system. Innate immune mechanisms are fast and fixed in their mechanism of action, and in most cases effective at terminating infections at an early stage (Parham, 2009). This is in large part due to the ability of the cells and molecules of innate immunity to identify common classes of pathogens, such as virus, and destroy them. Any organism with the potential to cause disease is known as a pathogen (Parham, 2009).

The cells of the innate immune system, such as granulocytes, macrophages, and DCs recognize molecules on the surface of pathogens called antigens rather than the entire pathogen. Upon being recognized by the immune system, antigens are identified as belonging to foreign microbiological invaders rather than self, and they produce an immune response (Parham, 2009). This recognition system is mediated by non-specific receptors on the surface of and within innate immune cells. Pattern recognition receptors (PRRs) recognize molecular patterns common to viruses, bacteria, parasites, and fungi (van der Aar et al., 2007). For instance, viral nucleic acids, of either RNA or DNA origin, can trigger intracellular PRRs such as Toll-like receptor (TLR) 3, TLR7, TLR8, and TLR9. Other surface PRRs, such as TLR4, can recognize viral envelope proteins
When PRRs are triggered the innate immune system will respond with its powerful predetermined defenses (Parham, 2009).

Innate cells are able to function and communicate by releasing soluble molecular messages known as cytokines (Parham, 2009). Most infections are efficiently cleared by the innate immune response, and this is due in part to the production of early-stage cytokines. In the case of a viral infection the most common of these are Interferon (IFN) α and β. The production of IFN α and β by infected cells and interferon-producing cells, mainly DCs, induces a “antiviral state” in the cells, which is characterized by inhibition of both viral replication and cell proliferation, and also enhancement of the ability of natural killer (NK) cells to lyse virally infected cells (Parham, 2009; Björck, 2001). IFNs can also influence the activity of more antigen-specific lymphocytes, should the infection escape innate immunity, at which point the virus faces the combined forces of innate and adaptive immunity (Parham, 2009).

**The Adaptive Immune System**

Although it is slower to start, the adaptive immune response builds on the mechanisms of innate immunity to provide a powerful response that is tailored to the pathogen at hand. That is, the adaptive immune system recognizes and responds to pathogens in a highly specific manner (Parham, 2009). Pathogen recognition and destruction involves T and B cells, which express receptors of a single and unique binding type. A particular pathogen stimulates only a small
subset of lymphocytes that are able to bind to a specific portion of antigen, known as an epitope. This receptor-epitope binding event is akin to a key fitting into a lock. From this point on in the immune response, the stimulated B lymphocyte subpopulation will expand while its receptors and simultaneously evolve an increased affinity for the pathogen (Parham, 2009). T and B cells play a paramount role in fighting infection and retaining memory for later exposures.

*T Lymphocytes (T cells)*

T lymphocytes or T cells are a type of white blood cell that plays a principal role in cell-mediate immunity. These cells circulate in the body in a naïve state until they encounter their specific antigen. If this occurs, they are activated to divide and differentiate into effector T cells, of which there are two main types: CD8+ T cells and CD4+ T cells. CD8+ T cells or cytotoxic T cells (CTLs) are so named because they express the CD8 glycoprotein at their surface. They destroy virally infected cells and carry out the main form of cell-mediated immunity that leads to viral clearance during infection (Parham, 2009).

On the other hand, CD4+ T cells express the CD4 protein on their surface. Upon activation they predominately become one of four or more subtypes of helper T cells: T_{H1}, T_{H2}, T_{H17}, and Tregs (Parham, 2009). These subtypes facilitate different types of immune responses, some of which are mediated by their secretion of different cytokines. T_{H1} cells activate macrophages by delivering cytokine and ligand singles (a molecular feature that binds to a
receptor), at which point the intracellular pathogens inhabiting macrophages can be eliminated. Using a different set of cytokines and ligands, T<sub>H</sub>2 cells activate B lymphocytes or B cells, which carry out humoral or antibody-mediated immunity; this targets extracellular pathogens. Activated CD4<sup>+</sup> T cells also have the ability to activate CD8<sup>+</sup> T cells, a function that plays an important role in viral immunity (Parham, 2009).

As with the other helper T cells, T<sub>H</sub>17 cells and Tregs are derived from a common progenitor cell; their differentiation is dependent upon the stimulation of mucosal DCs and macrophages by microbial, parasitic, or fungal products, as well as common cytokines (Kanwar et al., 2010). Pro-inflammatory T<sub>H</sub>17 cells are critical in the defense against bacteria and fungi. Tregs differ from other T cells by the expression of a unique surface marker, CD25, and by the use of a transcriptional repressor protein called FoxP3 to exert anti-inflammatory functions and control self-reactive T cells, including T<sub>H</sub>1, T<sub>H</sub>2, and T<sub>H</sub>17 cells. By maintaining this active form of tolerance and preventing autoimmunity, Tregs are one of the main mechanisms for protecting the integrity of the body’s tissues and organs (Parham, 2009; Kanwar et al., 2010). Interestingly, Tregs and T<sub>H</sub>17 cells share common chemokine receptors and tissue homing properties, indicative of their reciprocal functions (Kanwar et al., 2010).
Antigen Presenting Cells

Unlike B cells, which can recognize many antigenic molecules in their native form, human T cells can only recognize antigenic molecules being presented by antigen-presenting cells (APCs). There are three professional antigen-presenting cell types: DCs, macrophages, and B cells (Parham, 2009). For a pathogen to be recognized by a T cell, pathogen-derived proteins must be degraded into smaller peptide pieces intracellularly and bind a class of glycoproteins known as major histocompatibility complex (MHC) molecules on the surface of APCs. A T cell can only recognize antigen being presented in this MHC: peptide complex. Thus, in order for a T cell to be activated from its naïve state, it must recognize the MHC: peptide complex, while simultaneously binding to a co-stimulatory molecule on the surface of the APC (Parham, 2009).

APCs are specialized white blood cells that play a paramount role in initial immune responses. They bridge the immune system’s innate and adaptive responses by activating T cells (Clark & Kupper, 2005). Of the three major APC classes, DCs play the largest role in viral particle processing and introduction to naïve T cells. DCs are also the most efficient APC at presenting peptides; for instance, they can use several pathways to process and present viral protein antigens. Macrophages are most efficient at presenting tissue-derived antigens, while B cells specialize in presenting soluble antigens such as microbial toxins or free virus (Parham, 2009). Due to the unique presence of co-stimulatory
molecules and the expression of all types of MHC molecules on their surfaces, APCs are the only cells capable of activating naïve T cells.

**Major Histocompatibility Complexes (MHC)**

MHC molecules are used by the immune system to survey the body for the presence of intracellular and extracellular pathogens. There are two main classes of MHC glycoproteins: MHC class I and MHC class II (Parham, 2009). MHC class I molecules are typically used to present antigens or peptides originating from cytosolic pathogens, such as viruses. All of the body’s nucleated cells constitutively express MHC class I molecules, since they are susceptible to viral infection. On the other hand, MHC class II molecules present antigens originating from extracellular pathogens. These MHC class II molecules are only found on the surface of APCs (Parham, 2009).

The MHC molecule by which a peptide antigen is presented, informs the immune system about the nature of the invading pathogen. If a T cell binds antigen presented by MHC class I on an APC, the T cell will ‘recognize’ the antigen’s cytosolic origin. These activated T cells can now eradicate virally infected cells. If a naïve T cell binds antigen peptide presented by MHC class II of an APC, the T cell will recognize the antigen’s extracellular origin. MHC class I molecules are only recognized by CD8+ T cells, while MHC class II molecules are only recognized by CD4+ T cells.
**Cross-Presentation**

The initiation of a cytotoxic CD8\(^+\) T cell response to a virus requires MHC class I mediated presentation of a virus-derived peptide by an APC. Based on the above descriptions of class I and II pathways, if a virus does not infect an APC (i.e. is not in the cytosol), it cannot be presented on an MHC class I molecule. To reiterate, only APCs can present antigen to activate naïve T cells, though MHC class I molecules are present on most cells of the body. In such situations, a CD8\(^+\) T cell response can be generated by a third, distinctive antigen presentation pathway – cross-presentation (Crotzer & Blum, 2010).

Cross-presentation is a sensitive mechanism that ensures T cells be given the correct information about the origin of a pathogen. The name implies a connection between class I and II pathways (Parham, 2009). Typically, APC digestion of an extracellular antigen would lead to presentation on MHC class II molecules, whereas APC digestion of intracellular viral antigen would lead to presentation on MHC class I molecules. If an extracellular-derived viral antigen was presented by MHC class II, T cells would be misinformed about that environment in which the virus is residing, since viruses reside within intracellular environments. Thus, cross-presentation ensures that the phagocytosis of extracellular material from virus-infected cells by an APC will lead to antigen presentation on MHC class I, rather than MHC class II, correctly informing the T cell and leading to an appropriate adaptive immune response (Mizushima et al., 2008). DCs are the principal APCs endowed with the capacity to cross-present
antigens, and therefore, are especially adept at presenting virus derived peptides to naïve T cells (Crotzer & Blum, 2010).

**Dendritic Cells (DCs)**

DCs are innate immune cells that function as the most important APCs in activating adaptive immune responses to viruses. Additionally, DCs are known to regulate immune responses by inducing both central and peripheral tolerance (Parham, 2009). DCs play a vital role in negative selection of developing T cells by deleting those with high-affinity for self-peptide-MHCs. In the periphery, DCs mediate peripheral tolerance by promoting Treg cell development, induction of T cell unresponsiveness, and deletion of activated T cells. In this way, DCs govern the choice between tolerance (do not react with self-cells) and immunity of the T cells they encounter, depending on the development stage of the T cell (Parham, 2009).

As professional APCs, DCs are capable of presenting antigen on MHC molecules and activating CD4 and CD8 T cell-responses. Furthermore, DCs are superior to the macrophage at stimulating naïve T cells and initiating a primary immune response. This superiority is in large part due to the migratory nature of DCs: they carry their load of antigens from sites of infection to secondary lymphoid tissues, where they meet and activate naïve T cells (Parham, 2009). They also form an extremely heterogeneous group of cells based on phenotype, function and location. Although their developmental pathways of differentiation
are highly complex and unresolved, it is clear that they are key coordinators of immune responses (Crotzer & Blum, 2010; Kamath et al., 2000; Parham, 2009; Tewfik et al., 2010).

*The Site of DCs: Lymphoid Tissue*

The current paradigm pictures DCs as residents in the tissue on the patrol for pathogens. Upon activation by the uptake of foreign antigen, DCs become migratory traveling through the lymphatic system to draining lymph nodes or lymphoid organs (Swiecki & Colonna, 2010). Primary lymphoid tissues include the bone marrow and thymus, and constitute the initial site of lymphocyte production and selection. All other lymphoid tissues, including the spleen, adenoids, tonsils, appendix, lymph nodes, and Peyer’s patches, are known as the secondary or peripheral lymphoid organs, and are the site at which lymphocytes encounter pathogens and become stimulated to respond to foreign pathogens by APCs (Parham, 2009).

As residents in tissue, DCs are in their immature state (See Figure 1). After uptake of foreign antigen, they become activated, and change from being efficient antigen processors to potent T cell activators and migrate to local lymphoid tissues like the spleen (see Figure 2). At this point, DCs are found only in the T cell areas of secondary lymphoid tissues, where their sole function is to activate T cells (Parham, 2009).
Figure 2. Dendritic Cells (DCs) are the most efficient T cell activators during viral infection

As shown here, immature DCs reside in infected tissues, where they pick up foreign virus particles. They then migrate via lymphatic vessels to local lymphoid tissues, like the spleen. Mature DCs take up permanent residence in T cell areas of lymphoid tissues, where their sole function is to activate T cells, by presenting digested virus antigen to them. Naïve T cells that become activated by DCs will differentiate into effector cells, which play a key role in fighting viral infection.

DCs also display a myriad of other functions such as generating regulatory T cells, activating NK cells, and producing type I IFNs in response to virus-infected target cells (Crotzer & Blum, 2010; Kamath et al., 2000). However, despite these common biological functions, DCs have highly heterogeneous surface phenotypes, and at least some of these different subsets differ in developmental origin. Furthermore, different DC subtypes confer different developmental fates on the T cells they activate (Kamath et al., 2000).
**Plasmacytoid DCs (pDCs)**

A subset of DCs, plasmacytoid DCs (pDCs), constitutes a minority of DCs in the whole body and yet appears to play a key role in innate immune response to viral infection (Swiecki & Colonna, 2010). Based on our current understanding, pDCs circulate through blood and lymphoid tissues and are characterized by the production of type I IFN upon activation (Wong et al., 2010) (see Figure 3). Additionally, they constitute < 0.4-0.5% of peripheral blood mononuclear cells (Tversky et al., 2008; Asselin-Paturel, 2003). The developmental pathways and differentiation relationship of this DC subset remains unclear, although it is recognized that immature DC precursors have great potential for development into a host of DC subsets. However, it is important to point out that there are many mature DC subsets with similar features and yet distinct developmental pathways (Diao et al., 2004).
Figure 3. Key plasmacytoid Dendritic Cell (pDC) features and functions


After pDCs detect viruses they stimulate and call to arms other cells. pDCs detect viruses in the acidified endosomes by means of Toll-like receptors (TLRs 7 and 9); they also engulf viral particles. Upon viral infection pDCs produce copious amounts type I IFNs (IFN-α and IFN-β), which induces a “antiviral state” characterized by inhibition of both viral replication and cell proliferation. IFNs also enhance the ability of natural killer (NK) cells to lyse virally infected cells. Finally, pDCs use MHC class II and I molecules to present virus, sometimes through cross-presentation, to naïve T cells in order to activate them and elicit their anti-viral effector functions.

In particular, murine pDCs are characterized by three surface antigens: CD11c, CD45R (B220), and CD317 (plasmacytoid DC antigen-1 (PDCA-1)). Human pDCs also express CD317, although it is referred to as bone marrow stromal cell antigen 2 (BST2).
Interferon (IFN) \( \alpha \) and \( \beta \), type I IFNs, play an important role in the first-line defense against viral infections and can modulate cytokine responses by T-helper cells. These IFNs are clinically important in infectious diseases, such as HIV, and in the treatment of leukemia and lymphomas (Bjorck, 2001). Many different cell types have the capacity to produce IFN-\( \alpha \) after encounter with virus and bacteria, however, the major natural type 1 IFN-producing cell in humans was recently described as the pDC (Bjorck et al., 2001; Gill et al., 2010; Robbins et al., 2008) (see Figure 3). Since pDCs are vital in the early production of IFN-\( \alpha \) and IFN-\( \beta \), they have the ability to activate other innate effector cells such as NK cells. Thus, pDCs are critical for defense against infections, as they are specially suited for the early detection of pathogens, the rapid development of effector functions, and the triggering of downstream responses in other innate and adaptive immune cells (Robbins et al., 2008).

**Human versus Murine pDCs**

There are many functional similarities between human and murine pDCs. Like human pDCs, murine pDCs have been described as the natural IFN-\( \alpha \)-producing cell, capable of producing large quantities of interferons in response to viral infection (Bjorck, 2001; Crotzer & Blum, 2010; D’Amico, 2003). Additionally, both human and murine pDCs, exhibit their maximal type 1 IFN-producing capacity at a precursor stage; pDCs isolated from bone marrow
responded to viral stimulation with higher IFN-α production than cells of the same phenotype isolated from spleen. (Bjorck, 2001)

Of the human DCs, pDCs are particularly recognized for their ability to cross-present (Crotzer & Blum, 2010). In mice, pDCs constitute a similar minority of cells, yet, a significant population of the total DCs found in the spleen (Tewfik et al., 2010). Additionally, in mice, pDCs have been shown to enhance CD8 T cell responses to various viruses like VSV, belonging to a family of single-stranded negative sense RNA viruses (Swiecki & Colonna, 2010). CD8 T cells are activated by pDCs through cross presentation, namely, pDCs display antigen via MHC I and activate CD8 T cells, which elicit protective immune responses to viral infections (Tang et al., 1997).

The murine pDC shares morphologic features with its human counterpart, although it has some distinct phenotypical characteristics. Murine pDCs can be differentially isolated based on their expression of CD11c, B220 (CD45R), Thy1.2 (CD90), BST-2 (mPDCA or CD317) and Siglec-H (Bjorck, 2001; Gill et al., 2010). They lack expression of myeloid (i.e., CD11b) antigens and CD8α, a marker used to isolate lymphoid DCs (Bjorck, 2001). In humans pDCs express the surface markers CD123, BDCA-2(CD303), BDCA-4(CD304), and bone marrow stromal cell antigen 2 (BST2/CD317), but do not express CD11c or CD14, which distinguishes them from conventional DCs or monocytes, respectively (Cao et al., 2009; Gill et al., 2010).
Retroviruses and Lentiviruses

HIV is a type of retrovirus. Retroviruses comprise a diverse family of enveloped RNA viruses. They store their single-stranded RNA in a nucleocapsid, surrounded by a protective glycoprotein outer envelope. Specifically, HIV belongs to the genus of slow retroviruses, or lentiviruses, so classified because of their exceptional ability to replicate in non-dividing cells, making them extremely efficient at delivering genetic information into host cell DNA. Lentiviruses cause disease principally by killing or inducing loss of function of specific cells and tissues (Coffin, 1997).

Like other retroviruses, HIV follows the usual transcription and translational processes at a later stage of its life cycle; this is because they perform an additional reverse-transcription step converting RNA to DNA, hence the term “retrovirus” (Coffin, 1997). The first step of the retrovirus lifecycle involves the interaction of viral surface glycoproteins with a specific receptor on the susceptible host cell, which causes fusion of the membranes and release of the viral nucleocapsid into the host’s cytoplasm. Once in the host cell, the viral RNA strands undergo reverse transcription, generating a double-stranded DNA copy of RNA genome (Coffin, 1997). The retroviral DNA, or provirus, is transported into the nucleus and integrated into the host’s genomic DNA. At this point, the provirus is transcribed by cellular RNA polymerase II into mRNAs as well as full-length progeny virion RNA (Coffin, 1997). Upon hijacking the host’s cellular machinery, located in the cytoplasm, the viral mRNA is translated into
viral proteins. These proteins and progeny RNA assemble at the cell periphery into progeny virus. Progeny virions are released from the cell by budding from the host’s plasma membrane and subsequently mature into infectious virus (Coffin, 1997).

**Human Immunodeficiency Virus (HIV) and Acquired Immunodeficiency Syndrome (AIDS)**

Infection by HIV typically leads to acquired immune deficiency syndrome, or AIDS. HIV is transmitted through bodily fluid containing HIV, such as blood. It can be spread through sex or by the sharing of needles, as well as from mother to child during pregnancy, birth, or breast-feeding. During the initial stage of the infection, called the acute stage, many individuals experience flu-like symptoms for one to two weeks (Coffin, 1997). This period is marked by a crucial increase in viral replication and significant decrease in specific blood cells: CD4+ T cells. The normal range of 600–1200 CD4+ T cells drops to below 500 cells/μl blood (Coffin, 1997). Approximately 3 to 6 weeks after infection both humoral (B cell mediated) and cellular (T cell mediated) antiviral immune responses can be detected. This antiviral response is enough to temporarily stabilize the CD4+ T cell population, as well as result in a decrease in viral load (viral RNA copies per μl of blood plasma) (Coffin, 1997).

HIV damages a person’s body by destroying or altering CD4+ T cells, which are crucial to helping the body fight disease (WHO, 2009). The acute
phase of infection is followed by a long-term asymptomatic phase, termed clinical latency, during which there is continual, rapid viral replication and a steady decline in the numbers of CD4$^+$ T cells (Coffin, 1997). This asymptomatic period generally lasts for about 8 to 12 years; however, this period is much longer for a small portion of the population. Long-term nonprogressors have been shown to survive with a reasonably normal and stable CD4$^+$ T cell count for 20 or more years.

AIDS is the late stage of HIV infection, when a person’s immune system is severely damaged and is vulnerable to opportunistic infections and certain cancers (WHO, 2009). The transition from chronic HIV infection to a state of AIDS occurs when the CD4$^+$ T cell count falls below 200 cells/μl. At this point the patient reaches a state of immunodeficiency: becoming susceptible to AIDS-defining opportunistic infections and neoplasms (Coffin, 1997). Opportunistic pathogens are those that do not typically trouble immune competent individuals. Thus, death is usually the result of one or more opportunistic infections, rather than the direct result of HIV infection (Parham, 2009).

The primary target of the HIV virus is CD4$^+$ T cell population, while macrophages have been identified as the other main target (Coffin, 1997). Although the exact mechanism of CD4$^+$ T cell depletion is unknown, the HIV virus is known to enter the cell via its CD4 surface molecule. In addition to its role as the principal receptor for HIV, CD4 is crucial for the generation of immune responses because it is the natural ligand for MHC class II molecules.
When APCs display processed peptide antigens in association with the MHC class II molecules to the T-cell receptor complex on CD4\(^+\) T cells, CD4 serves as an adhesion molecule that stabilizes the stimulatory interaction (Parham, 2009). Even prior to depletion, T cells in HIV-infected individuals enter an anergic state, leaving them less responsive to stimulatory signals. Thus, the binding of HIV to CD4 may be an important component of HIV-1 pathogenesis (Coffin, 1997).

CD8\(^+\) T cell populations are also affected by HIV infection. These cells experience periods of decline and increase, though a loss of function renders their increase during the latency period functionally ineffective. Similarly, B cells proliferate non-specifically to abnormally high levels during infection (Coffin, 1997).

While there have been great strides in the prevention of HIV infection and in disease care, there is neither a protective vaccine nor a curative therapy available for HIV infection. The mechanism by which efficient viral replication occurs in the face of a strong host immune response early on is still unresolved. However, the virus is recognized to rapidly mutate and camouflage its surface with glycosylation sites. Thus, evading the immune response and causing damage to the immune system may preclude a protective immune response (Coffin, 1997). The real difficulty with understanding HIV and AIDS is that the virus is difficult to study; it only infects humans and a few non-human primates. Experimentation in primates is costly and time-consuming due to the slow progression from HIV infection to AIDS, making good animal models hard to come by.
Murine Acquired Immunodeficiency Syndrome (MAIDS)

Murine Acquired Immunodeficiency Syndrome (MAIDS) is one mouse model of human AIDS (see Figure 4). MAIDS is inducible by the LP-BM5 strain of murine leukemia virus (MuLV) (Liang et al., 1996). However, the degree of susceptibility to MAIDS varies among strains (Jolicouer, 1991). Two strains that differ greatly in their susceptibility to disease following infection are C57BL/6 and BALB/c. BALB/c mice are susceptible to infection with MuLV, but they generate a healthy immune response that successfully eliminates the virus within the first week of infection (Jolicouer 1991) (refer to Figure 4). These mice generate an immune memory for the virus and demonstrate resistance to subsequent exposure. Conversely, the immune response produced by the susceptible strain, C57BL/6 mice, is unable to eradicate the viral infection (see Figure 4). These C57BL/6 mice instead develop an AIDS-like syndrome characterized by immunosuppression that renders them susceptible to opportunistic pathogens and can prove lethal by approximately 14 weeks post infection (Jolicouer 1991; Liang et al., 1996).
Figure 4. The murine AIDS (MAIDS) model uses a MAIDS susceptible and MAIDS resistant strain of mice

Both strains are treated with the LP-BM5 strain of murine leukemia virus (MuLV). After virus treatment, both strains become infected. However, the MAIDS resistant BALB/c mice generate an immune response that successfully clears the infection within a first week, rendering the mice resistant to disease. Conversely, the immune response produced by the MAIDS susceptible strain, C57BL/6 mice, is unable to clear the infection, rendering them susceptible to disease.
Substantial proliferation and differentiation of non-antigen specific B cells is characteristic of MuLV infection in disease susceptible mouse strains (Mosier, 1996). B cell proliferation and immunodeficiency occurs within 4 weeks of infection for the sensitive strains, like C57BL/6, while resistant strains such as BALB/c recover from infection and develop protective immunity (Tang et al., 1997; Tepsuporn et al., 2008). T cells do not appear to be directly infected by the virus, although the presence of CD4\(^+\) T cells is required for the development of MAIDS (Mosier, 1987). In each strain, the immune responses initiated are antigen dependent, and the resistance or susceptibility associated with infection are tied to the initial immune responses.

CD8\(^+\) T cells have also been shown to contribute to MAIDS resistance by preventing abnormal B cell proliferation; they have antigen-specific cytotoxic activity against infected targets (Kastrukoff, et al., 2010). Since infected APCs may be less effective at sensitizing CD8\(^+\) T cells, they might cause impaired selection of T cells required for the protective response to the virus (Tang et al., 1997). For instance, there is an increased rate of T cell activation and apoptosis in the disease-susceptible C57BL/6 strain after infection. Correspondingly, there is an initial rise in CD4\(^+\) T cell dependent polyclonal B cell activation, producing a high concentration of IgG1, IgM, and IgE immunoglobulin. The immune response elicited also causes macrophage activation (Mosier et al., 1996). Thus, the MuLV infection of susceptible mice is characterized by enlarged secondary lymphoid organs, including the spleen and lymph node (Liang et al. 1996).
Following prolonged T cell stimulation, these cells become anergic and a subsequent decrease in the number of B cells becomes noticeable (Mosier et al., 1996).

**MAIDS versus AIDS**

MuLV and HIV are both retroviruses that induce similar symptomatic progression and ultimate immunodeficiency (Liang et al., 1996). These similarities make MAIDS an invaluable research model for the study of human AIDS. Yet, there are significant differences between HIV and MuLV. MuLV is a type-C retrovirus rather than a lentivirus like HIV (Mosier et al. 1996). Specifically, the ability of MuLV to elicit immunosuppression is due to its defective coding of a gag fragment precursor protein (Jolicouer, 1991). The cells infected by MuLV are primarily B cells and macrophages, with very few T cells, unlike infection with HIV, which chronically infects CD4\(^+\) T cells and macrophages (Mosier et al. 1996). Although the HIV virus targets T cells while the MuLV virus targets B cells, both infections cause T cell anergy and depletion of the CD4\(^+\) T cell population, as well as abnormal polyclonal proliferation of B cells (Mosier, 1996). Therefore, differences aside, MAIDS continues to be a convenient and applicable model for the study of human AIDS.
pDCs in AIDS and MAIDS

Intense focus has been placed on studying the function of pDCs in human viral infection, particularly HIV. Loss of pDCs due to HIV infection has been correlated with high viral loads, decreased numbers of CD4 T cells, and the onset of opportunistic infections, the cause of which is unclear (Donaghy et al., 2001; Meyer et. al., 2007; Swiecki & Colonna, 2010). pDC-derived type I IFN has been shown to limit HIV replication in CD4 T cells, as well as stimulate CTLs to mount a viral response, suggesting that pDCs may be capable of mounting a protective immune response to HIV (Meyer et. al., 2007; Nascimbeni et al., 2009; Swiecki & Colonna, 2010).

Human pDCs have been identified as one of the two main peripheral DC subsets (Conry et al., 2009). Immature human pDCs are generally found in the human spleen localized in the marginal zone and the peri-arteriolar region. In human HIV-infected patients, the enduring pDC population is similarly found in these areas of the spleen (Nascimbeni et al., 2009). Relationally, in HIV infected patients, pDC numbers in blood were found to be reduced, correlating with a rapid influx of pDCs into peripheral tissues. One explanation is that HIV could induce changes in the migratory properties of pDCs inducing their migration to peripheral tissues like the spleen (Swiecki & Colonna, 2010). However, despite pDC accumulation in spleens from HIV infected patients with high viral loads, as well as increased IFN-α production in the marginal zone, it is still unclear whether pDCs are the main IFN-α producer (Nascimbeni et al., 2009).
After pDCs detect viruses they stimulate and call to arms other cells. pDCs detect viruses in the acidified endosomes by means of Toll-like receptors (TLRs) (see Figure 3). Yet, pDC responses to certain single-stranded RNA (ssRNA) viruses occur only after live viral infection. Recognition of such viruses by TLR7 or TLR9 requires transport of cytosolic viral replication intermediates, which serve as pathogen signatures, into the lysosome by the process of autophagy (Lee et al., 2007). Upon activation, pDCs produce type I IFNs, IL-12 and chemokines such as MIP-1α and β that attract naïve CD8+ T cells to T cell areas of the spleen, where they become virus-specific CTLs (Swiecki & Colonna, 2010). These virus-specific CTLs proliferate and migrate to the periphery where they produce IFN γ and kill-virus infected cells via perforin and granzyme, proteases that induce apoptosis of virus-infected cells. pDCs promote CD8+ T cell responses primarily by secreting chemoattractants; pDCs may also help CD8+ and CD4+ T cells by secreting IFNs and IL-12 directly or indirectly by inducing the maturation of other DCs (Swiecki & Colonna, 2010).

Both human and mouse pDCs function to control viral infections by the use of a particular surface molecule: CD317 (see Figure 3). CD317 (also called BST2, tetherin, HM1.2 antigen, bone marrow stromal antigen (BMSA) 2, or PDCA-1) is a transmembrane glycoprotein with a molecular mass of 29-33 kD (Cao et al., 2009; Douglas et al., 2009; Swiecki & Colonna, 2010). Although it is predominantly expressed on pDCs in naïve mice, it is also up-regulated on most cell types following stimulation with type I IFNs and type II IFN-(gamma).
Additionally, it is highly expressed on terminally differentiated normal pDCs (Cao et al., 2009; Douglas et al., 2009). CD317 is a recently identified, IFN-induced cellular response factor that appears to block release of HIV-1 and other retroviruses like MuLV from infected cells. In particular, it was recently shown to restrict the release of HIV by trapping virions at the plasma membrane of certain tetherin-producer cells. Its tethering activity critically depends on its density at the cell surface, which is antagonized to some extent by the HIV-1 accessory protein Vpu (Habermann et al., 2010; Swiecki & Colonna, 2010). Specifically, CD317 expression was correlated with, and induced, a requirement for Vpu during HIV-1 and MuLV (Neil et al., 2008). This newly recognized antiviral tactic is yet further support that pDCs play a critical role in the viral immune response, even when infected by HIV in humans or MuLV in mice.

**Fluorescence Activated Cell Staining (FACS) and Monoclonal Antibodies**

Fluorescence Activated Cell Scanning (FACS), also called flow cytometry, is a powerful method used to analyze multiple parameters of a heterogeneous cell population. During FACS, individual cells tagged with specific fluorochromes are passed through laser beams at a rate of up to a couple of thousand cells per second, causing light to scatter and fluorochromes to emit light at various frequencies. A flow cytometer collects fluorescent signals in one of several channels, which correspond to different laser excitation and fluorescence emission wavelengths. The fluorescence signals are converted into
electrical signals and cell data is collected about heterogeneous cell sub-
populations (Davey and Winson, 2003). FACS generates three types of data:
forward scatter (FSc) or approximate cell size, side scatter (SSc) or cell
complexity and granularity, and fluorescent labeling used to investigate cell
surface structures (Bonetta, 2005).

FACs requires the use of specific antibodies (Abs). In most cases, these
are commercially-produced monoclonal Abs, made highly specific for a particular
antigen using hybridoma technology (Pandey, 2010). This technology fuses a
myeloma cell, a cancerous immune cell, with a splenic plasma cell derived from
an animal immunized with the relevant antigen (Ag). This fusion produces a
highly Ag-specific hybridoma, capable of producing large amounts of monoclonal
Ab (Pandey 2010). These Abs exist naturally as the immune system’s mechanism
for humoral immunity and antigen recognition.

Monoclonal Abs can be readily coupled with fluorochromes and utilized
by FACS analysis to detect the presence or absence of surface markers on cells
(Herzenberg, 2004). First a cocktail of fluorescently labeled monoclonal
antibodies and the heterogeneous cell population of interest is made. This
cocktail may consist of single antigen-specific antibodies, each specific to a
particular surface marker, or multiple antigen-specific antibodies, each with a
different colored tag (Herzenberg, 2004). Some flow cytometers can detect up to
18 colors, thereby examining 18 markers simultaneously. Although this ability
increases the resolving power for analyzing cell population subsets with subtle
differences, it also adds complexity to the later analysis of results (Bonetta, 2005). It is important to choose surface markers that will almost, if not exclusively, represent the target population of interest.

**Experiment Rationale/ Previous Studies**

Previously in the Stanford lab, flow cytometry experiments were completed in order to look for differences in the frequency of activated antigen presenting cells (APCs) between MAIDS resistant BALB/c and MAIDS susceptible C57BL/6 mice at different post-MuLV infection time points (Miu, 2010). Results from these studies demonstrated a higher frequency of activated B cells (CD86+CD45R+) in the lymph nodes of MuLV-infected BALB/c mice at 7 days post-infection.

When staining for B cells, antibodies specific for CD86 (B7.2) and CD45R (B220) surfaces markers were used. Since CD45R is also found on pDCs, in addition to CD86, the marker selected for activated APCs, we anticipated a small portion of the B cell population observed in resistant BALB/c mice to be composed of pDCs. As a result, this study endeavors to answer that very question: was a portion of the B cell population observed in resistant BALB/c mice composed of pDCS?

Bing’s B cell work lends itself to the hypothesis that more pDCs will be observed in resistant BALB/c. I hypothesize that the ability of BALB/c mice to develop an effective immune response to MuLV may be due, in part, to a higher
frequency of pDC’s present during infection, as well as their role in fighting infection. pDCs have the ability to promote a strong CD8$^+$ T cell response, primarily by secreting type I IFNs to induce their virus-specific activation; they also have a tethering-surface molecule, CD317, which may serve to restrict MuLV virion spread from infected cells. The antiviral activity of pDCs could have profound effects on the immune system’s ability to eradicate MuLV infection.

One of the major functional characteristics of murine pDCs, like their human counterparts, is the expression of pathogen recognition receptors, such as Toll-like receptor (TLR)-7 and -9, which endow these cells with the ability to rapidly produce large amounts of type I interferons (IFNs) following encounter with RNA or DNA viruses (Bocharov et al., 2010). Hence, by providing a first wave of antiviral IFN, pDCs have been observed to limit viral replication within peripheral target organs, like the spleen.

Not only do pDCs immediately limit viral spread, but they also set the stage for antigen-specific adaptive immune responses (Bocharov et al., 2010). CD317, a recently identified, IFN-induced cellular response factor that blocks release of HIV-1 and other retroviruses from infected human cells, has also been observed on the surface of mouse pDCs, where it can presumably serve a similar antiviral function (Swiecki & Colonna, 2010). It is further recognized that pDCs are the primary activator of CD8$^+$ T cells (Swiecki & Colonna, 2010). Interestingly, previous studies have indicated that virus-specific CD8$^+$ T cell
responses are important for the control of MuLV infection and suppression of MAIDs development (Tang et al., 1997).

Murine pDCs are characterized by a particular set of phenotypic markers and special functional properties (Bocharov et al., 2010). Past studies have utilized plasmacytoid DC antigen-1 (PDCA-1) for specific identification of pDCs (Bjorck, 2001; Blasius et al, 2006; Gill et al., 2010). Although PDCA-1 (CD317) is one of the most specific antigens for pDCs, a similar splenic population of DCs was recently identified as mutually capable of expressing PDCA-1. Murine CD11c+ cells expressing the pDC markers B220 (CD45R), PDCA-1 (CD317), and CD11b (not expressed on pDCs) was found to be highly related to pDCs (Bierly et al., 2008). To reiterate, murine pDCs can be differentially isolated based on their expression of CD11c, B220 (CD45R), Thy1.2 (CD90), BST-2 (mPDCA or CD317), and Siglec-H; they are also CD8α and CD11b negative (Bjorck, 2001; Gill et al., 2010). Nonetheless, multiple studies have identified the murine pDC-specific phenotype as the following combination of surface markers: CD11c^low^B220^PDCA-1^+ (Bierly et al., 2008, Blasius et al, 2006, Swiecki & Colonna, 2010, Tewfik et al., 2010, Wong et al., 2010 and Workman et al, 2009).

**Research Objective**

During infection, HIV interacts with a few key players of the immune system, one of which is pDC. Although the exact mechanism of this interaction is unknown it is clear that the pDC could play a paramount role in the eradication of
this virus. pDCs release protein-signals that trigger immune system defenses, such as limiting HIV replication in CD4+ T cells; pDCs also upregulate a surface molecule that appears to restrict the release of HIV by trapping virions at the plasma membrane.

The goal of this investigation is to look for the presence of pDCs in the MAIDS model. This may help clarify their role in responding to the human infection with HIV and later disease state, AIDS. In order to investigate the presence and possible role of pDCs, they will be identified in the spleens of MuLV- infected mice. FACS analysis will used to measure pDC population quantity and composition at 7 days post-infection, making comparisons within two strains of mice: (MAIDS susceptible) C57BL/6 and (MAIDS resistant) BALB/c strains. The following three fluorochrome-conjugated Abs will be used: APC-conjugated CD11c (far red), Alexa 488-conjugated CD317 (green), and PE-conjugated CD45R (orange). Results from this study will hopefully offer new insights into potential pDC behavior and function in HIV infection. Elucidating the role of pDCs in HIV pathogenesis and progression to AIDS, may contribute to the development of important new AIDS therapies.
MATERIAL AND METHODS

Animals

C57BL/6 and BALB/c mice (*Mus musculus*) used in this study were purchased from Taconic (NY) and housed in the Mount Holyoke College Animal Facility according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Virus Stock

Murine leukemia virus (MuLV) mixture of the LP-BM5 isolate was harvested from MuLV-LP-BM5 infected SC-1 cell cultures. SC-1 cells are derived from fetal mouse embryonic fibroblasts. The protocol for creating cell cultures, collecting, and storing the virus stocks were previously described by a past lab member (Tepsuporn, 2005). Virus titers for 2009 and 2010 were $2 \times 10^4$ and $3.2 \times 10^3$ PFU/ml, respectively. Therefore, all mice received 1 ml of viral supernatant, containing between $10^3$ and $10^4$ PFU of virus.

Infection and Dissection

Six to eight week old female C57BL/6 and BALB/c mice were infected by 1 ml intraperitoneal injection of the LP-BM5 isolate of MuLV ($10^3$ to $10^4$ PFU/ml), from infected SC-1 cell cultures, and were sacrificed through carbon dioxide inhalation at 7 days post-infection. For each of the animals, the date of birth, infection and sacrifice are recorded in Appendix 1. Immediately after
sacrifice, spleens were collected (see Figure 5) and submerged in phosphate buffered saline, with a pH of 7.2, along with 2% heat inactivated fetal bovine serum (PBS/2% FBS).
Figure 5. *Mus musculus* spleen location

This image was adapted from Dunn, 1954.
Obtaining Leukocytes from Spleen

To achieve this separation, spleens were placed in 70µm Nylon mesh cell strainers (Fisher) in small petri dishes containing 2ml room temperature PBS/ 2% FBS, and were ground with a sterile rubber plunger removed from a 3µl syringe, creating a single cell suspension. Physical grinding and cell collection was repeated to produce a total of 6ml of suspension from each spleen. Purification of leukocytes was achieved by using room temperature Histoplaque-1083 (SIGMA), also known as ficoll. Ficoll separation is a centrifugation technique for separating leukocytes from other blood elements based on a density gradient. Each splenic cell suspension was carefully layered on top of 6ml ficoll in a 15ml conical tube and then centrifuged at 2000 rpm for 20 minutes at room temperature with the brake and acceleration off. After centrifugation, the leukocytes were collected from the buffy coat (interface between the blood plasma layer and ficoll), and washed with 10 ml of PBS/ 2% FBS. Cells were centrifuged at 1800 rpm for 10 minutes at room temperature to remove residual ficoll. Supernatant was carefully aspirated inside the cell culture hood leaving the cell pellet, which was then resuspended in 10 ml of PBS/ 2% FBS and centrifuged at 1200 rpm for 10 minutes at room temperature. After this second wash, the supernatant was again removed, and isolated spleen cells were resuspended in 10ml of cold PBS/ 2% FBS. From this point on, cells were kept at 4°C. A cell count of the leukocytes was obtained using a light microscope and hemocytometer.
Antibodies

Antibodies were purchased from BD Pharmingen, (San Jose, CA) and eBioscience (San Diego, CA) and stored at 4°C. A list of all antibodies used, the company, and catalogue number is shown in Table 1. Antibodies were specific for: CD11c-APC Hamster Anti-Mouse, CD45R-PE Anti-Human/Mouse (B220), and CD317- Alexa Fluor® 488 Anti-Mouse (BST2, PDCA-1). Matching isotype controls recommended by the distributors were also purchased: CD11c-APC Hamster IgG1, λ1 Isotype Control; CD317- Alexa Fluor® 488 Rat IgG2b κ Isotype Control; and CD45R-PE Rat IgG 2a κ Isotype Control.
Table 1: Antibodies used in Immunofluorescent Staining

<table>
<thead>
<tr>
<th>Antibody Specific for</th>
<th>Catalog #; Company</th>
<th>Conjugation</th>
<th>Stock Concentration (mg/ml)</th>
<th>Staining Concentrations (µg/10^6 cells in 100µl volume)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45R</td>
<td>12-0452; eBioScience</td>
<td>PE</td>
<td>0.2</td>
<td>0.25</td>
</tr>
<tr>
<td>CD317</td>
<td>53-3172; eBioScience</td>
<td>Alexa488</td>
<td>0.5</td>
<td>0.50</td>
</tr>
<tr>
<td>CD11c</td>
<td>550261; BD BioSciences</td>
<td>APC</td>
<td>0.2</td>
<td>0.25</td>
</tr>
<tr>
<td>CD16</td>
<td>553142; BD BioSciences</td>
<td>-</td>
<td>0.5</td>
<td>-</td>
</tr>
</tbody>
</table>

Based on previous work in the lab by Bing Miu that demonstrated reduced background fluorescence levels could be achieved by staining cells with Fc block, a similar protocol was followed (see Figure 6). Prior to immunostaining purified rat anti-mouse CD16 2.4G2; IgG 2b K was used to reduce non-specific staining from Fc receptor binding by antibodies of interest. Cells were either stained with isotype control or antigen-specific antibodies after this treatment.
**Immunostaining**

One million cells in 100 µl were transferred to 4mL FACS staining tubes, which were centrifuged at 1000 rpm 4°C for 5 minutes. Supernatant was disposed of and the tubes were vortexed prior to adding 1 µl of anti-CD16 mAb; cells were incubated for 10 minutes to prevent nonspecific binding, in which the anti-CD16 Ab is allowed to attach to the FcR on leukocytes, blocking any subsequent antibodies from nonspecifically binding to this receptor (see Figure 6).
Figure 6. Treatment with Fc block prior to immunostaining can reduce background fluorescence

Leukocytes extracted from the spleen of a 3-day MuLV infected BALB/c animal. (A) Pre-treated with 1 µl of purified Rat Anti-Mouse CD16 /Fc block. (B) No Fc block treatment. Samples were subsequently treated with 0.06µg of FITC Rat IgG 2a Ab, the isotype control antibody for CD86-FITC. The results showed a 10 fold decrease in background fluorescence after Fc treatment (0.3% vs. 3.0%).

(Miu, B., 2010. Senior Honors Thesis)
The following Abs conjugated with fluorochromes were added directly to the tubes after the 10 minute Fc-block: APC-conjugated CD11c, Alexa 488-conjugated CD317, and PE-conjugated CD45R. The optimized antibody staining concentration, listed in Table 1, was selected from antibody dilutions that gave the best staining cell population with minimum background. Isotype controls were used at the same concentration as their corresponding antibody to detect for background non-specific staining.

Samples were initially single-stained and triple-stained, although in the majority of the subsequent experiments samples were single and double stained. Single color staining was performed using one specific fluorochrome-conjugated antibody or one isotype control, and these samples were used for color compensation. Triple staining was performed using a cocktail of APC-conjugated CD11c, Alexa 488-conjugated CD317, and PE-conjugated CD45R. Double staining was performed using a cocktail of CD317 specific for pDCs with either CD11c or with CD45R, specific for antigen presenting cell populations. Double staining for CD317 and CD45R is illustrated in Figure 7. The antibody concentrations used were the same for single, double, and triple staining. The negative control tubes were made by staining cells with isotype matched Abs, with parallel single, double, and triple staining and at identical concentrations.

Staining consisted of a 30-minute incubation period in the dark at 4°C. After the incubation on ice, cells were washed three times with 1ml of PBS/2% FBS and collected by centrifugation (1200 rpm for 5 minutes). Each sample was
resuspended in 500µl PBS/2% FBS. Cells were kept cold and dark until data
collection.
pDCs were labeled with two antibodies. In part A, a group of isolated leukocytes, including a pDC, is depicted. In Part B, the isolated WBCs were then stained with pDC specific antibodies which fluoresce green and orange, in order to tag them for fluorescent recognition by flow cytometry. The fluorochrome-tagged antibodies were: PE-CD45R (orange) and Alexa488-CD317 (green). After a 30-minute incubation period at 4°C, the antigen specific antibodies have bound their respective surface pDC antigens, as shown in part C. Part D represents the tube filled with stained cells, which are ready for flow cytometric analysis.

Figure 7. Experimental system for labeling pDCs with antibodies.

Flow Cytometric Analysis

Measurement

Extracted leukocytes were stained with fluorochrome-conjugated antibodies that are specific to the target pDC population. Cell fluorescence was measured by running each of the samples, the negative controls first, in the Accuri C6 Flow Cytometer. A total of 500,000 events (cells) were counted for each sample. Cell populations of interest were gated and analyzed using CFlow Accuri software. Detectors FL1 (530 ± 15nm), FL2 (585 ± 20nm), and FL4 (675 ± 12.5nm) were used to collect green, orange-red, and far-red fluorescence of the Alexa488, PE, and APC dyes respectively.

Identifying specific cell populations

An Accuri C6 flow cytometer was used to measure multiple parameters of individual cells within the heterogeneous target cell population including: forward scattered light, side scattered light, and colored fluorescence. Forward-scatter, or low-angle light scatter, is the amount of light scattered in a forward direction as the laser collides with an event (or cell); it is proportional a cell’s diameter. Side-scatter refers to the light scattered at all angles, caused by the granularity inside a cell or cell surface irregularity. Since cellular debris including dead cells have lower forward-scatter and higher side-scatter than do live cells, a forward and side-scatter plot was used to select for viable leukocyte populations (see Figure 8).
Figure 8. Gating to collect viable leukocytes.

The primary gate (in red) was set on the forward and side light scatter to target the leukocyte population and remove cellular debris. Leukocytes extracted from the spleen of an infected BALB/c and C57BL/6 mouse were used to set gates on the viable cells; the BALB/C mouse is shown here. A total of 50,000 cell events were collected. Each dot represents a single event (or cell); its position indicates its forward-scatter (FSC) and side scatter (SSC) intensity values. Cells concentrated to the lower left corner of the P1 gate of the FSC and SSC plot are primarily leukocytes, while those cells on the very left bottom corner of the plot are dead cells and cellular debris (black arrow). The P1 gate accounts for most of the cell events (80.7%), which falls within the typical 75-85% range.
By setting this primary gate, the CFLOW Accuri software was subsequently configured to exclusively collect data in fluorescence signals for x number of cells within this gate. The PI gate accounted for ~80.7% of the total cell events.

Using a scatter plot display, four quadrants were created using the isotype control staining to differentiate nonspecific background staining levels; background staining levels were kept at or below 0.5% of the total target cell population (see Figure 9). Fluorescent signal compensation was applied later to identify the desired pDC double-stained population and remove any non-specific color bleed through between channels. Additional quadrant gates in combination with fluorescent compensation were used to analyze a more specific pDC double-stained population (see Figure 10).
Isotype Control Antibodies

A)

CD317 + CD45R Specific Antibodies

B)

Figure 9. Creating quadrants in a dot plot to exclude nonspecific background staining

Isotype controls were used to establish quadrants, which identify positively versus negatively stained cell populations. Leukocytes extracted from the spleen of each infected BALB/c and C57BL/6 mouse were stained separately with A) antibody isotype controls and with B) antigen-specific antibodies; a C57BL/6 mouse stained with A) Alexa488-rat IgG2b and PE Rat IgG 2a K and B) CD317-Alexa488® and CD45R-PE is shown here. A) Isotype control staining was used to differentiate nonspecific background staining levels from B) positively stained cell populations. The background staining levels in the upper left (UL) and lower right (LR) quadrants were kept at or below 0.5% of the total target cell population and was kept at or below 1.0% in the upper right (UR); the same quadrants were maintained for all experiments.
Leukocytes were extracted from the spleen of each BALB/c and C57BL/6 mouse 7-days post MuLV infection, and were stained separately with A) antibody isotype controls and with B) antigen-specific antibodies; a C57BL/6 mouse stained with A) Alexa488-rat IgG2b and PE Rat IgG 2a K and B) CD317-Alexa488® and CD45R-PE is shown here. Quadrants were set based on isotype control staining. An R2 gate was added to measure a more specific double-positive (CD317+CD45R+) population within UR region. 
**Color Compensation**

Color compensation is an electronic calculation that removes signal overlap that the optical system cannot remove. The inherent overlap between fluorescent channels of emission spectra from antibody fluorescent labels makes compensation necessary when making simultaneous immunofluorescence measurements from multiple cell-bound antibodies (2, 3, or more color analysis). Compensation was completed to correct the fluorescence “spillover” created by the overlapping emission spectra of APC, PE and Alexa 488 (see Figure 11).

Fluorescence compensation works by subtracting spill over between specific pairs of fluorescent parameters; for example, FL1 and FL2. Spectral emission overlap between Alexa 488 and PE produced light which was detected by both the FL1 and FL2 detectors. The amount of Alexa 488 fluorescence being detected by the FL2 detector (i.e. PE detector) was regarded as excess non-specific fluorescence and therefore was compensated out according to the CFlow User Guide. Quadrants were set for the single color staining in order to separate double negative and single positive populations into the lower and upper left quadrants respectively, of the 2D-plot. Color compensation was set so that the mean fluorescence channel values for the two quadrants are approximately equal. Mean channel fluorescence is the mean fluorescence intensity value for a population of events (cells) within a particular gate.
Figure 11. Excitation and emission spectra of APC, PE, and Alexa 488

The figure illustrates the phenomena of fluorescence spectral overlap between the excitation (Ex) and emission (Em) spectra of PE and FITC, which will result in fluorescence bleed through from one fluorochrome to a different detection channel, if color compensation is not performed (adapted from BD Biosciences).
Statistical Analysis

Data on double positive (DP) (CD317+CD45R+) pDC populations were analyzed using an independent groups T test via analytical computer software called SPSS. T-tests are used to assess whether the means of two groups are statistically different from each other. In this case two groups were the two strains of mice: C57BL/6 (disease susceptible) and BALB/c (disease susceptible).

For this project, a total of 20 mice, 10 for each strain were all tested 7-days post MuLV infection. The null hypothesis of the t-test was that there would be no difference in the percentage of spleen pDCs between the two strains of mouse.

An independent variable is the presumed cause of any statistically significant effect of measured response. In this design strain of mouse was the independent variable. A dependent variable is the presumed effect or measured response. In this experiment adjusted double positive (DP) population was the independent variable. For each double stained antibody treatment tested, a corresponding antibody isotype control was also tested to serve as a ‘check’ for non-specific background staining. Thus, the adjusted DP population for each mouse was acquired by taking the difference between the percentage staining in specific antibody treatment and isotype control. Thus, a single observation was made for each mouse.
RESULTS

Experiment Rationale

Previously in lab, flow cytometry experiments were conducted in order to look for differences in the frequency of activated antigen presenting cells (APCs) between MAIDS resistant BALBc and MAIDS susceptible C57BL/6 mice at different post-MuLV infection time points (Miu, 2010). Most prominent of her results were those that demonstrated a higher frequency of activated B cells (CD86+CD45R+) in the lymph nodes of MuLV-infected BALB/c mice at day 7 post-infection.

When staining for B cells, she used antibodies specific for CD86 (B7.2) and CD45R (B220) surfaces markers. Since CD45R is also found on pDCs, in addition to CD86, an activation marker, she expected that a small portion of her observed cell population might composed of pDCs. As a result, this study endeavored to answer that very question: was a portion of the expanded B cell population after MuLV infection that is observed in resistant BALB/c mice composed of pDCs?

The research objectives of this study were (1) to determine whether pDCs could be identified in mouse spleen via flow cytometry, and if successful, (2) to look for a strain-specific difference in pDC percentage at 7 days post infection (a time point selected based on Bing’s results). Bing’s B cell work lent itself to the hypothesis that more pDCs would be observed in resistant BALB/c mice. The
rationale being that an increase in the number of pDCs in the spleen might lead to an increase in activated T cells, thus facilitating eradication of the pathogen.

**Proof of Concept Experiment**

The first step was replication and confirmation of prior findings to demonstrate the reliability of laboratory practice and technique. This proof of concept experiment was a replicate of one of Bing’s experiments. Peripheral lymph nodes including axillary, brachial, and inguinal lymph nodes were used from both a BALB/c and a C57BL/6 mouse, 7 days post MuLV-infection, as well as from two naïve mice. Cells were both single and double-stained with FITC-CD86 antibody, and APC-CD45R antibody. Control samples were stained with the respective isotype controls. FITC-CD86 and APC-CD45R antibodies, specific for particular B cell surface antigens, were used to evaluate relevant B cell populations at the chosen time point.

This experiment successfully replicated those of Bing’s original experiment, in that differences between the strains were observed in terms of the percentage of activated CD45R+ cells (presumably B cells), and the observed trend was of more CD45R+ cells in BALB/c mice was maintained (data not shown).
**Antibody Dilution Determination**

The company that manufactures the antibodies used in this study (eBioscience) recommended optimal antibody dilutions for both CD45R and CD317 (Table 2). However, two additional dilutions were tested for each antibody to ensure that the recommended antibody concentrations gave the best results.

The optimal concentration for CD11c had been previously determined by a past student, Bing Miu, and was 0.25 µg/10^6 cells in 100uL volume (Table 3). The three concentrations tested for CD45R were determined by titrating up and down from the recommended stain concentration provided by EBioscience, 0.25 µg/10^6 cells in 100uL volume (Figure 12). The three concentrations tested for CD317 were determined by titrating down from the recommended stained concentration, 0.50 µg/10^6 cells in 100uL volume, as recommended by EBioscience (Figure 13).
Table 2: Antibody Concentrations Tested for Optimization of Cell Staining Procedure

<table>
<thead>
<tr>
<th>Antibody Specific for</th>
<th>Conjugate</th>
<th>Stock Concentration (mg/mL)</th>
<th>Antibody Concentrations Tested (ug/10^6 cells in 100uL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45R</td>
<td>PE (orange)</td>
<td>0.2</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.25*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.50</td>
</tr>
<tr>
<td>CD317</td>
<td>Alexa488 (green)</td>
<td>0.5</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.50*</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
<tr>
<td>CD11c</td>
<td>APC (red)</td>
<td>0.2</td>
<td>0.25**</td>
</tr>
</tbody>
</table>

* Staining concentrations chosen based on the present experiment.
** Staining concentration chosen based on previous experiment done by Bing Miu, 2010.
The three antibody concentrations tested for anti-CD45R-PE versus corresponding isotype controls gave the following staining results in terms of percentage of leukocytes stained: 1/40 (47.4% vs. 0.2%), 1/80 (50.8% vs. 0.2%), and 1/160 (60.8% vs. 0.1%), (see Figure 12). For the PE-rat IgG2a isotype control antibody there was not a significant difference in different in background staining at different antibody dilutions (compare Figure 12. D, E, F). At the first two dilutions of CD45R-PE antibody there was a clear separation between the stained and unstained cell populations; this was less true for the last dilution (see Figure 12. A, B, C). Using 0.25 ug/10^6 cells in 100uL gave clear positive staining, clear separation between the positive (UR) and negative (LL) stained population (see Figure 12. B) and still no significant background staining in the isotype control. Thus, 0.25 ug/10^6 cells was chosen as the staining concentration for CD45R-PE.

The three antibody concentrations tested for anti-CD317-Alexa Fluor® 488 versus corresponding isotype controls gave the following: 1/50 (3.1% vs. 0.3%), 1/100 (2.6% vs. 0.3%), and 1/200 (2.4% vs. 0.2%), (see Figure 13). For the Alexa Fluor® 488 RatIgG2bK isotype control antibody, there was also not a significant difference in background staining at different antibody dilutions (compare Figure 13. D, E, F). However, 0.50 ug/10^6 cells in 100uL, was the best staining companion to CD45R, and showed low non-specific staining. Therefore, this concentration was chosen as the staining concentration for Alexa488-CD317 (data not shown).
A similar analysis had been previously performed for CD11c-APC by Bing Miu. For this antibody the optimal concentration was set at 0.25 ug/10^6 cells in 100uL (see Table 3).
Figure 12. Testing three anti-CD45R antibody dilutions for optimal staining

Leukocytes from spleen stained with CD45R-PE and an isotype control antibody at three antibody dilutions. Leukocytes were extracted from a C57BL/6 animal 7-days post MuLV infection. Cells were stained with either CD45R-PE (A, B, C) or the corresponding PE-rat IgG2a isotype control antibody (D, E, F) at three dilutions. For each 2D plot, the x-axis and y-axis represent two detectors, in this case, the FL2 and FL4. Gates were set so that the UL quadrant would express the fluorescent cells that were detected by channel FL2 (CD45R-positive).
Figure 13. Testing three anti-CD317 antibody dilutions for optimal staining.

Leukocytes from spleen stained with Alexa488-CD317 and an isotype control antibody at three antibody dilutions. Leukocytes were extracted from a C57BL/6 animal 7-day post MuLV infection. Cells stained with either Alexa488-CD317 (A, B, C) or corresponding Alexa488-rat IgG2b isotype control antibody (D, E, F) at three dilutions. For each 2D plot, the x-axis and y-axis represent two detectors, in this case, the FL1 and FL4. Gates were set so that the UL quadrant expresses the fluorescent cells that were detected by channel FL1 (CD317-positive).
**Color Compensation**

Color compensation was performed after triple staining with CD45R-PE, CD317- Alexa Fluor® 488, and CD11c-APC and their isotype controls. Double staining was performed with CD45R-PE and CD317- Alexa Fluor® 488 and for CD11c-APC and CD317- Alexa Fluor® 488 and their respective isotype controls. The procedure for color compensation is detailed in the previous section under *Materials and Methods*. The need for color compensation varied slightly between strains and mice, as well as for triple-stained samples versus double-stained samples. For example, in the end compensation was required to remove from 2.4 – 2.7% of fluorescence from detector FL2 that spilled into FL1, and from 5 – 9% of fluorescence from detector FL 1 that spilled into FL2. This corrected the problem of fluorescence spillover and allowed more accurate detection of the double positive populations (Figure 14). Note the shifted population in the LR quadrant now has a mean fluorescence closer to that seen in the LL quadrant.
Compensated Double-stained Cell Population

Uncompensated Double-stained Cell Population

FL1

FL2

Figure 14. Compensation is required to remove fluorescence spillover between detector channels.

Leukocytes extracted from the spleen of a 7-day MuLV infected C57BL/6 mouse. (A) Double-stained cells, whose fluorescent spillover was removed by compensation. (B) The same population of double-stained cells, which show fluorescent spillover due contamination of FL2 into FL1. The UR quadrant expresses the double-stained cell population of interest; there is noticeable clear difference in staining percentage, where A) depicts accurate detection and B) depicts inaccurate detection of double-stained cells.
Evaluation of pDCs in Infected Mice

The initial study was performed using CD45R-PE, CD317- Alexa Fluor® 488, and CD11c-APC specific antibodies on spleen samples from 7-day MuLV infected BALB/c and C57BL/6 mice. In each condition, 1 animal from each strain was used (N=1). For this experiment three-color compensation was performed to eliminate fluorescent spill-over between samples (see Figure 15).
Figure 15. Triple stained populations are very small, making data analysis difficult

Leukocytes extracted from the spleen of 7-day MuLV infected BALB/c and C57BL/6 mice. Cells were triple stained with CD317- Alexa488®, CD45R-PE, and CD11c-APC specific antibodies (A and B) or Alexa488-rat IgG2b, CD45R-PE Rat IgG 2a K, and APC- Hamster IgG1, λ1 isotype control antibodies (C and D). Quadrants were set based on isotype control staining. The UR quadrant of A) and B) show the triple-stained (CD317+CD45R+CD11c+) population of interest. Results were first gated on the CD45R+ cells and then from these, the CD317+ and CD11c+ subpopulation is shown.
Due to the unusually large degree of spill-over and very small target population size, it proved impractical to fully compensate the aggregate spill-over between three fluorophores. This resulted in extremely small target population size, and limited the ability to interpret the results and identify a unique population. The compensation calculations made for this experiment were sent to an Accuri company representative who confirmed the accuracy of the compensation technique performed and had no further suggestions for collecting data on this population, besides using only two colors. Therefore, double staining using only two target antigens was employed for all subsequent experiments.

For each individual animal, a total of 2 separate staining assays were performed; each including the reasonably pDC-specific marker, CD317, and another defining marker, either CD11c or CD45R. Additionally, a total of 2 separate experiments were completed in order to have 4 animals (N=4) in each staining condition and strain. After analyzing these data for double-stained percentage differences between strains, CD45R was identified as a better second marker to use in combination with CD317 (see Table 3).
Table 3: Percentage of spleen cells double-stained with anti-CD317 and anti-CD45R or with anti-CD317 and anti-CD11c.

<table>
<thead>
<tr>
<th>Mouse #</th>
<th>CD317+</th>
<th>CD45R Adjusted % DP*</th>
<th>CD11c Adjusted % DP**</th>
<th>Mouse #</th>
<th>CD317+</th>
<th>CD45R Adjusted % DP*</th>
<th>CD11c Adjusted % DP**</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>0.3</td>
<td></td>
<td>5</td>
<td>1.5</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.9</td>
<td>0.6</td>
<td></td>
<td>6</td>
<td>2.4</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>0.5</td>
<td>0.5</td>
<td></td>
<td>7</td>
<td>2.1</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>1.2</td>
<td>0.4</td>
<td></td>
<td>8</td>
<td>3.2</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Mean</td>
<td>0.775</td>
<td>0.450</td>
<td></td>
<td></td>
<td>2.3</td>
<td>0.325</td>
<td></td>
</tr>
<tr>
<td>Stdev</td>
<td>±0.340</td>
<td>±0.129</td>
<td></td>
<td></td>
<td>±0.707</td>
<td>±0.171</td>
<td></td>
</tr>
</tbody>
</table>

*DP = double positive (CD317CD45R)
**DP = double positive (CD317CD11c)

Adjusted double positive (DP) percentage refers to the percentage of double stained cells out of the total spleens cells collected. This adjusted value was obtained by subtracting the double isotype control stained percentage (not shown) from the double specific antibody stained percentage (not shown).
A final experiment was completed in order to test an additional 6 mice with just this double (CD317CD45R) antibody combination, giving a total of 10 animals (N=10) for each strain.

Figure 16 shows an analysis that was done for one CD317- Alexa Fluor® 488 and CD45R-PE staining experiment. One animal from each strain was selected as a representative of its group. A 2-D plot is shown. A percentage was calculated and is shown in each quadrant based on the number of events (or cells) inside that quadrant, compared to the total events. The Upper Right quadrant (UR) represented the double positive (CD317+ CD45R+) population. The Lower Right quadrant (LR) represented cells that were CD45R+ CD317-, which were likely B cells, while the Upper Left quadrant (UL) represented CD317+CD45R- cells, of an ambiguous identity. The Lower Left quadrant (LL) represented the double-negative population, which were cells that did not express either CD317 or CD45R.

Quadrants were set according to isotype controls as described in the Materials and Methods; the background level for UL and LR quadrants was set at or below 0.5% and for the UR quadrant was set at or below 1.0% of the total cell population for all antibodies. These quadrants were maintained for all samples in all experiments.
Fig 16. Double color staining with anti-CD317-Alexa Fluor® 488 and anti-CD45R-PE antibodies

Leukocytes were extracted from the spleens of both mouse strains (BALB/c and C57BL/6) 7-days post MuLV infection. Cells were stained with both Alexa488-CD317 and CD45R-PE (A and B) specific antibodies or both Alexa488-rat IgG2b and PE-rat IgG2a isotype control antibodies (C and D). Quadrants were set based on isotype control staining. The UR quadrant of A) and B) show the double-stained (CD317+CD45R+) population of interest.
Multiple 2-D plots were generated from separate double-staining experiments. The plots revealed the frequency of double-stained cells, presumably pDCs, in both mouse strains. The raw data for CD317+CD45R+ cells from each individual animal is included in Table 4. Group mean for percentage of double positive cells and standard deviations were calculated for these cells in both conditions (antibody treatment and isotype control) for both strains (BALB/c and C57BL/6), 7 days post-infection.
Table 4. Percentage of spleen cells expressing both CD317 and CD45R

<table>
<thead>
<tr>
<th>Experiment Day</th>
<th>Mouse #</th>
<th>Adjusted % DP*</th>
<th>Mouse #</th>
<th>Adjusted % DP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>0.5</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>0.9</td>
<td>6</td>
<td>2.4</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.5</td>
<td>7</td>
<td>2.1</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>1.2</td>
<td>8</td>
<td>3.2</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>0.9</td>
<td>15</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>1.1</td>
<td>16</td>
<td>1.7</td>
</tr>
<tr>
<td>3</td>
<td>11</td>
<td>1</td>
<td>17</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>12</td>
<td>1.3</td>
<td>18</td>
<td>1.4</td>
</tr>
<tr>
<td>3</td>
<td>13</td>
<td>0.8</td>
<td>19</td>
<td>3.8</td>
</tr>
<tr>
<td>3</td>
<td>14</td>
<td>0.6</td>
<td>20</td>
<td>3.1</td>
</tr>
<tr>
<td>Mean</td>
<td>-</td>
<td><strong>0.880</strong></td>
<td>-</td>
<td><strong>2.370</strong></td>
</tr>
<tr>
<td>Stdev</td>
<td>-</td>
<td>±0.282</td>
<td>-</td>
<td>±0.792</td>
</tr>
</tbody>
</table>

*DP = double positive (CD317CD45R)
Independent Groups t-Test: pDCs

An independent groups t-test was performed (described in the Material and Methods) to measure the significance of the difference in pDC (CD317+CD45R+) frequency between strains after MuLV infection. Group statistics including group mean for percentage of double positive cells, standard deviation and standard error, were calculated for both strains (BALB/c; N=10 and C57BL/6; N=10), 7 days post-infection (see Table 5).

After running the data in an independent groups t-test, a statistically significant difference in the adjusted double-positive (DP) (CD317CD45R) percentage of cells in spleen was found between strains at 7 days post infection (see Table 6). The adjusted DP was greater for the susceptible C57BL/6 strain; this difference was statistically significant whether equal variances for strain populations were assumed or not, at a value of p< 0001. The variation in adjusted DP % for each mouse is provided in Figure 17, while the mean adjusted DP% for the two strains, along with standard deviation are provided in Figure 18.

A confidence interval is a type of interval estimate of a population parameter, used to indicate the reliability of an estimate. Here, strain DP differences was the population parameter, and the 95% refers to the frequently that the parameter of interest would be included in the interval, if the experiment is repeated, thus, a 95% confidence interval of the difference is very good (see Table 6).
Table 5: Group Statistics

<table>
<thead>
<tr>
<th>Strain</th>
<th>N</th>
<th>Mean</th>
<th>Std. Deviation</th>
<th>Std. Error Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjusted DP BALB/c</td>
<td>10</td>
<td>.8800</td>
<td>.28206</td>
<td>.08919</td>
</tr>
<tr>
<td>Adjusted DP C57BL/6</td>
<td>10</td>
<td>2.3700</td>
<td>.79169</td>
<td>.25036</td>
</tr>
</tbody>
</table>

Here the groups are identified, their sample sizes (N), their means, their standard deviations, and their Std. Error Means are given.
Table 6: Independent groups t-test for adjusted double-stained (CD317CD45R) cells

<table>
<thead>
<tr>
<th></th>
<th>t</th>
<th>df</th>
<th>Sig. (2-tailed)</th>
<th>Mean Diff.</th>
<th>Std. Error Diff.</th>
<th>95% Confidence Interval of the Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equal variances</td>
<td>-5.606</td>
<td>18</td>
<td>.000</td>
<td>-1.49000</td>
<td>.26577</td>
<td>-2.04836 to -.93164</td>
</tr>
<tr>
<td>Equal variances</td>
<td>-5.606</td>
<td>11.248</td>
<td>.000</td>
<td>-1.49000</td>
<td>.26577</td>
<td>-2.07338 to -.90662</td>
</tr>
</tbody>
</table>

Here are the main results that we want. Because the assumption of homogeneity can be assumed, we follow along the top line. The crucial number is in the "Sig. (2-tailed)" column and tells us that the observed difference in the means (-1.49) is significant. The Confidence Interval information is also given. Notice the null hypothesis value (i.e., zero) does not fall within this interval.
Figure 17. Dot plot of adjusted double-positive (DP) (CD317+CD45R+) population from the spleen of each individual mouse, based on strain (N = 10 for both BALB/c and C57BL/6).

Data illustrate the frequency of CD317+ CD45R+ cells in the spleen of C57BL/6 and BALB/c mice 7-days post MuLV infection. For individual mouse adjusted DP percentage values, difference scores, and strain means, refer back to Table 4.
Figure 18. A significantly larger double-stained (CD317CD45R) population is seen in the spleens of MAIDS susceptible mice at 7 days post infection.

The frequency of pDCs in the spleen of susceptible C56BL/6 (BL/6) mice compared to in the spleen of resistance BALB/c (BA/c) mice, at day 7 post MuLV-infection. The red column indicates the mean % adjusted DP leukocytes in the spleens of BA/c mice (N =10). The blue columns indicates the % adjusted DP leukocytes in the spleens of BL/6 mice (N =10). Means and error bars (stdev.) are included. * = p <0.0001.
**Gated Subpopulation of Double Positive Cells (CD317+CD45R+)**

In addition to the double stained pDC population in the entire UR quadrant, a smaller, more distinct DP population was designated using a more restricted gate. For these observations this smaller CD317+CD45R+ population was separated using an R2 gate.

Figure 19 shows an analysis conducted for CD317- Alexa Fluor® 488 and CD45R-PE staining in splenocytes. One animal from each strain was selected as a representative of its group. A 2-D plot is shown. A percentage was calculated and is shown in each quadrant based on the number of events (or cells) inside that quadrant, compared to the total events. An additional (R2) gate was also set for the distinctive double-positive cell population with higher CD317 staining (see Figure 19). Background staining levels were kept consistent with previous data. The Upper Right quadrant (UR) represents the double positive (CD317+CD45R+) population, which the smaller DP target population shown within the R2 gate. The Lower Right quadrant (LR) represented cells that were CD45R+CD317-, which were likely B cells, while the Upper Left quadrant (UL) represented CD317+CD45R- cells, whose identity remains ambiguous. The Lower Left quadrant (LL) represented the double-negative population, which were cells that did not express either CD317 or CD45R.

After analyzing these data for a mean DP % difference, it was clear that the percentage of these cells was not different between strains and an independent
groups t-test would not find statistically significant results, so no further statistical analysis was completed on these data.
Leukocytes were extracted from the spleens of both mice strains (BALB/c and C57BL/6) 7-days post MuLV infection. Cells were stained with both Alexa488-CD317 and CD45R-PE (A and B) or both Alexa488-rat IgG2b and PE-rat IgG2a isotype control antibodies (C and D). Quadrants were set based on isotype control staining. The UR quadrant of A) and B) expressed the double-positive (CD317+CD45R+) population, and contained the smaller R2-gated DP % from total leukocytes extracted from spleens.
**Qualitative Analysis:**

There were noticeably different phenotypic DP staining profiles for the two mouse strains. In the resistant BALB/c strain, there is a distinct population of double stained cells (CD317\text{medium}CD45R\text{medium}), whereas in the susceptible C57BL/6 strain, the CD317+CD45R+ cell profile is more heterogeneous or diffuse (see Figure 20).
Figure 20. The MAIDS resistant strain has many distinct DP (CD317+CD45R+) populations, while the MAIDS susceptible strain has one larger diffuse DP population.

Leukocytes were extracted from the spleens of both mice strains (BALB/c and C57BL/6) 7-days post MuLV infection. Cells were stained with both Alexa488-CD317 (x-axis) and CD45R-PE (y-axis) (A and B). Quadrants were set based on isotype control staining. The UR quadrant of A) has High, Medium, and Low cell populations. Specifically, there is a Low/ Low, Medium/ Medium, and Low/ Medium population; there is no High/ High population. The UR quadrant of B) has a *single diffuse, heterogeneous population.
**DISCUSSION:**

DCs function as the most important APC in activating adaptive immune responses to viruses, a function which extends somewhat to the pDC subset. Some scientists argue that this APC function is used specifically by pDCs to elicit the T cell-mediated immune responses necessary for HIV or MuLV eradication (Habermann et al., 2010; Swiecki & Colonna, 2010). Yet, interestingly, this same mechanism is argued by others, to cause more damage than good. By presenting viral antigen to naïve T cells, pDCs may inadvertently transmit the virus to CD4+T cells, thereby promoting its spread rather than extinction. On the other hand, pDCs are unique effectors at the interface of innate and adaptive immunity, endowed with other more powerful mechanisms for viral repression, such as early IFN production, which both directly and indirectly limits viral spread (Crotzer & Blum, 2010; Bjorck et al., 2001; Gill et al., 2010; Robbins et al., 2008). pDCs play a highly complex and ambiguous role in HIV and MuLV disease pathogenesis.

To date, there have been no published studies examining the involvement of pDCs in murine AIDS. The goal of this investigation was to study the presence of pDCs in the MAIDS model, in order to further elucidate their potential function in human AIDS and HIV pathogenesis.
Previous Lab Work: A Motivation for Studying pDCs

Previous microarray studies done in the Stranford laboratory found higher expression of DC related genes (DC-SIGN, DCAR, and OCIPRP2) in BALB/c mice at 3 days post MuLV infection as compared to C57BL/6 mice (Tepsuporn et al., 2008). This upregulation may have been related to an increased expression of their molecules by DCs, simply an increase in the number of DCs expressing the molecule, or somewhat attributed to both causes. Other studies in the Stranford laboratory used RT-PCR to quantify the strain-specific differences in the DC related mRNA expression, though that project was abandoned due to contamination (Giles, 2007). Subsequent studies using immunofluorescent staining revealed randomly distributed DC staining in the lymph nodes. These data, however, showed extreme variability between biological and experimental replicates (Giles, 2007).

In subsequent studies, the effect of MuLV infection on lymphangiogenesis response in MAIDS was studied by Vidya Raghavan (2009). She used immunofluorescence staining and found increased vessel growth in the BALB/c mice after MuLV infection, versus delayed lymphangiogenesis in the C57BL/6 mice under the same treatment (Raghavan, 2009). To clarify, the growth of lymphatic vessels promotes the migration of APCs to the draining lymph nodes, which seemed to facilitate a more robust immune response in the MAIDS-resistant BALB/c strain.
This APC trend was also illustrated by the flow cytometry work of Bing Miu (2010). She observed a higher frequency of activated B cells (CD86+CD45R+) in the lymph nodes of MuLV-infected BALB/c mice at day 7 in particular. Conversely, across organs (lymph node and spleen), the MAIDS-susceptible C57BL/6 mice were found to have a higher frequency of activated classical DCs (CD86+CD11c) pre- and post-MuLV infection. Bing used CD86 (B7.2) and CD45R (B220) specific antibodies to stain for B cells. However, since CD45R and CD86 is also found on pDCs, she expected a small portion of her observed B cell population to be pDCs. These flow cytometry results lead to the following questions: was a portion of the larger activated B cell population observed in resistant mice composed of only B cells, or partially of pDCs?

Basically, the experimental goals of this study were to identify pDCs using flow cytometry, and if successful, look for a strain-specific difference in their percentage after infection with MuLV. Bing’s work lent itself to the hypothesis that more activated pDCs would be observed in resistant BALB/c. The rationale being, that an increase in the number of pDCs in the spleen should lead to an increase in activated T cells, thus facilitating eradication of the pathogen. This hypothesized increase in the number of pDCs would contribute to the observed increases in the expression of the molecules mentioned above.
A Unique Experimental Design

There were a number of factors which altered the experimental design from that proposed by Bing in her *Future Directions*, to the design executed in this study. Briefly, since pDCs constitute < 0.4-0.5% of peripheral blood mononuclear cells steady-states (not infection) it would be highly improbable to find them in the lymphoid tissue of naïve mice (Tversky et al., 2008). For this reason, only infected mice were studied in this project. Additionally, based on the current paradigm of pDC migratory patterns, it was clear that pDCs were more likely to be found in mouse spleens than lymph nodes (Tewfik et al., 2010). Therefore, splenocytes from infected mice were analyzed for pDC presence. pDCs express CD45R and intermediate levels of CD11c, whereas the conventional lymphoid DCs Bing observed do not express B220 and express high levels of CD11c. Additionally, murine pDCs differentially express CD317 (BST-2 or mPDCA) (Bjorck, 2001; Gill et al., 2010; Segura et al., 2010). Therefore, CD11c, CD45R, and CD317 were chosen as antibody targets to distinguish pDCs from other DC subsets, as well as other leukocytes in general.

Flow cytometric analysis is able to detect rare cell types, including pDCs. In order to investigate the presence and possible role of pDCs, leukocytes were isolated from the spleens of MuLV infected mice. FACS analysis was then used to measure the size and composition of the pDC population at 7 days post-infection, making comparisons within two strains of mice: (MAIDS susceptible) C57BL/6 and (MAIDS resistant) BALB/c strains. The following three
fluorochrome-conjugated Abs were originally used: APC-conjugated CD11c (far red), Alexa 488-conjugated CD317 (green), and PE-conjugated CD45R (orange). In later experiments, pDCs were defined as CD317+CD45R+ cells.

Discussion Outline: Deciphering the Significance of Experimental Results

The results from this study indicated that the percentage of spleen pDCs (CD317+CD45R+) were significantly different post-MuLV infection between MAIDS-resistant and MAIDS-susceptible strains. These changes were strain and condition dependent. The increase in pDCs occurred in the spleen of the susceptible strain one week post MuLV infection. The higher observed percentage of CD317+CD45R+ cells were more abundant in the susceptible strain spleens, 2.37±0.792% versus 0.88±0.282% in the resistant strain. However, some ambiguity remains as to whether this CD317+CD45R+ population can be interpreted as ‘true’ pDCs. The pDC field is in its infancy and there is great debate about how to functionally, define pDCs.

Therefore, the first part of the discussion will focus on the data obtained, explaining the choice of antibodies and phenotypic differences in the double-stained pDC populations. The second part of the discussion will focus on potential error from analysis. The third part of the discussion will explore the potential roles of the pDCs in the MAIDS-susceptible and resistant strains, as well as whether the phenotype and function of murine pDCs can really be compared to that of human pDCs. Finally, the conclusion will shed some light on possible future studies for this research.
Part I

Using Antibodies to Define pDCs

As described in the Introduction, murine pDCs are characterized by a particular set of phenotypic markers (Bocharov et al., 2010). To reiterate, they can be differentially isolated based on their expression of CD11c, B220 (CD45R), Thy1.2 (CD90), BST-2 (mPDCA or CD317), and Siglec-H; they are also CD8α and CD11b negative (Bjorck, 2001; Gill et al., 2010). Nonetheless, multiple studies have identified the murine pDC-specific phenotype as the following combination of surface markers: CD11c^{low}CD45R+CD317+ (Bierly et al., 2008, Blasius et al, 2006, Swiecki & Colonna, 2010, Tewfik et al., 2010, Wong et al., 2010 and Workman et al, 2009). Hence, this combination was used for the current study.

CD45R is a surface antigen expressed by pDCs, whereas conventional lymphoid DCs do not express it (Segura et al., 2010). Apart from pDCs, it is expressed on B cells of all developmental stages, but is down-regulated on terminally differentiated plasma cells, which do not participate in antigen presentation. CD45R seems to be a physiologically irrelevant molecule, present on some cell surfaces and used by researchers as a defining cell marker, rather than playing a functional role as receptor or ligand. Additionally, CD11c, the common marker selected for DCs, is highly expressed on all DC subtypes,
including pDCs, where it is expressed in lower levels, making use of the marker complex (Villadangos & Schnorrer, 2007).

CD317 or BST2, (plasmacytoid DC antigen-1 (PDCA-1)), tetherin, HM1.2 antigen, or bone marrow stromal antigen (BMSA) is the most commonly used surface marker specific to the identification of pDCs (Bjorck, 2001; Blasius et al, 2006; Cao et al., 2009; Douglas et al., 2009; Gill et al., 2010; Swiecki & Colonna, 2010). Its multiple names can be attributed to different classification systems. This study has used the clusters of differentiation (CD) classification, which is based mainly on flow cytometry data (Salganik et al., 2005). CD317 is a transmembrane glycoprotein with a molecular mass of 29-33 kD (Cao et al., 2009; Douglas et al., 2009; Swiecki & Colonna, 2010). Unlike other pDC-specific surface markers, like SiglecH or Thy1.2 (CD90), CD317 is also found on human pDCs (Bjorck, 2001; Bocarsly & Jacobs, 2010, 2010; Gill et al., 2010).

Although BST2 (CD317) is one of the most specific antigens for pDCs, it appears that other splenic populations can also express this antigen. A murine population of cells expressing B220 (CD45R), BST2 (CD317), CD11c, and CD11b (not expressed on pDCs) was recently found to be highly related to pDCs (Bierly et al., 2008). This highly related yet distinct population illustrates the difficulties encountered when first trying to choose pDC-specific antibodies. A few studies found that BST2 (CD317) is predominantly expressed on pDCs in naïve mice and that it is also up-regulated on most cell types to some extent, following stimulation with type I IFNs and type II IFN-(gamma) (Cao et al.,
2009; Douglas et al., 2009). However, these observations should be taken with a grain of salt, as they both characterize pDCs differently than later studies and use anti-BST2 antibodies other than CD317.

Under ideal circumstances it would be advantageous to compare pDC numbers in naïve versus infected mice. Logistically this is not really possible. Under steady state (non-infection conditions) the current paradigm pictures pDCs as circulating through blood and lymphoid tissues in search of pathogens. It is not until the uptake of foreign antigen, that pDCs become activated and migrate through the lymphatic system to the spleen or other secondary lymphoid organs, where they will remain activating T cells and serving other anti-viral functions for the duration of infection (Swiecki & Colonna, 2010).

In humans and mice, pDCs may use BST2 (CD317) to help control viral infection, discussed in further detail in Part 3 of this Discussion. Briefly, BST2 is also known as tetherin is a recently identified, IFN-induced cellular response factor that appears to block release of HIV-1 and other retroviruses like MuLV from infected cells (Habermann et al., 2010; Swiecki & Colonna, 2010). In particular, it was recently shown to restrict the release of HIV by trapping virions at the plasma membrane of certain tetherin-producer cells. Its tethering activity critically depends on its density at the cell surface, which is antagonized to some extent by the HIV-1 accessory protein Vpu (Habermann et al., 2010; Swiecki & Colonna, 2010).
Using staining Patterns to define pDCs

As previously mentioned in the Results section, due to difficulties with color compensation of fluorescent signals from the three antibodies used for staining (CD317, CD45R, and CD11c) triple-staining was logistically impractical. Thus, early on the protocol was altered to measure double-stained populations using CD317/CD45R and separately using CD317/CD11c. Based on initial findings that showed larger CD317+CD45R+ populations in both strains, as well as a larger population difference between strains (see Table 3, in Results), the CD317/CD45R antibody combination was utilized to define and stain for pDCs for the remainder of the experiments.

Upon quantitative analysis, the double-positive (CD317+CD45R+) cell population accounted for a mean value 0.88±0.282% in the MAIDS resistant BALB/c spleen and 2.37±0.792 % in the MAIDS susceptible C57BL/6 strain, of the total stained spleen cell population (see Table 4, in Results). Murine pDCs constitute around 0.4-0.5% of peripheral blood mononuclear cells (Tversky et al., 2008; Asselin-Paturel, 2003). Upon infection pDCs migrate to the spleen. Thus, in infected animals, pDCs might be expected to compose a slightly higher percentage of total cells in the spleen. Yet, by 7 days after infection pDC activation and proliferation may be by altered by the virus. Since pDC physiological norms are currently unknown, quadrants were set based on the background isotype staining.
Upon qualitative analysis, there were noticeably different phenotypic staining profiles for the two strains. In the resistant BALB/c strain, there is a distinct population of double stained cells (CD317<sup>medium</sup>CD45R<sup>medium</sup>), whereas in the susceptible C57BL/6 strain, the (CD317+CD45R+) cell profile is more heterogeneous (see Figure 20). To reiterate, the ‘medium’ in the CD317<sup>medium</sup> staining phenotype, refers to ‘medium’ fluorescent intensity, presumably due to comparable expression of CD317 on individual cells. In an attempt to characterize a ‘truer’ (CD317+CD45R+) pDC population, a R2 gate was created to capture the CD317<sup>medium</sup>CD45R<sup>medium</sup> cell population seen in the BALB/c mice (see Figure 19). This gate was created based on all 4 mice tested per strain. Fluorescent compensation values were set for a single mouse to determine whether the working gate would include its CD317<sup>high</sup>CD45R<sup>high</sup> cell population, and was adjusted accordingly. If an adjustment was made the process was repeated for all mice, ensuring the gate best represented the CD317<sup>high</sup>CD45R<sup>high</sup> cell population of all mice.

Although the pDC (CD317<sup>medium</sup>CD45R<sup>medium</sup>) percentages defined by this R-2 gate were not statistically different between strains, the trend matched previous results. There was a higher percentage of CD317<sup>medium</sup>CD45R<sup>medium</sup> cells in the MAIDS susceptible C57BL/6 strain than there were in the MAIDS resistant BALB/c strain (see Figure 19). These values were closer to the percentage of pDCs that have been observed in peripheral blood (0.4-0.5% of all leukocytes).
To stay consistent, a similar gate (R5) was established for the other double staining antibody pair (CD317+CD11c+) (data not shown). In this case, there was a higher percentage of pDCs in the MAIDS resistant BALB/c strain than there were in the MAIDS susceptible BALB/c strain, however, the CD317^{high}CD11c^{low} cell percentages between strain, as defined by this R-gate, showed even less statistical different from one another (percentages not given).

To come full circle, it is difficult to characterize the double-stained (CD317+CD45R+) population as ‘true’ pDCs, due to the phenotypic differences observed between strains. The double-stained CD317+CD45R+ population observed in UR quadrant of susceptible mice is heterogeneous (see Figure 20, plot B), which could be the result of pDCs with varying CD317 and CD45R antigen expression or a mixture of ‘true’ pDCs and other cells. Since CD317 is an induced IFN-induced cellular response factor that appears to block release of HIV or MuLV from infected cells, it is logical that less might be present on pDCs in susceptible mice. Perhaps, in susceptible mice, CD317 is more highly antagonized by a viral protein similar to the HIV-1 accessory protein Vpu, which antagonizes CD317 expression in human pDCs. (Habermann et al., 2010). Conversely, in the resistant mice there is a clear CD317^{medium}CD45R^{medium} population, which may signify a ‘truer’ pDC population. When an R-2 gate was added for further analysis of the CD317+CD45R+ population, the trend of observing a higher cell percentage in the susceptible strain was maintained, although the results were not statistically significant due to the extremely low cell
percentages being compared. Therefore, based on the current pDC phenotypic model, it seems reasonable to characterize double-stained (CD317+CD45R+) populations as pDCs, since there is currently no other DC category in which to place these cells.

**Part II**

*Sources of Variability*

The variability of the data set most likely comes from a variety of sources. The entire experimental procedure, from leukocyte isolation to the analysis of stained cells via flow cytometry, involves many steps. Therefore, there is room for error and sources of variability at many points along the way. However, certain steps were taken to control experimental variability, as well as biological variability.

Firstly, as described in the *Results*, a proof of protocol experiment was performed to demonstrate reliability of laboratory practice and technique. This experiment was a replicate of part of an experiment previously done by Bing Miu. Thus, it was possible to compare the results with those obtained by Bing. They were similar in nature (data not shown).

Several different parameters were considered to minimize experimental variability. Firstly, the experimental procedure described in the *Material and Methods*, was carefully following for every experiment. Reagents were carefully
labeled and color-coded to maintain maximum consistency. For each staining treatment measured, a complementary isotype control was run to allow for precise comparisons between experimental conditions. The isotype control was also used to ensure that non-specific background staining was present only in minimal levels.

Prior to using the flow cytometer for data analysis, the machine was calibrated with alignment beads to ensure optimal functioning. Flow cytometry alignment beads facilitate the calibration of a flow cytometer's lasers, optics and stream flow. They are highly uniform with respect to both size and fluorescence intensity; in addition, they are designed to approximately replicate the size, emission wavelength and intensity of biological samples, dismissing the need to waste valuable experimental material. Subsequently, when using the flow cytometry, a consistent amount of events (50,000) were run for each experimental sample. Additionally, the isotype controls were used to set background staining level at or below 0.5% in the UL and LR quadrants, and at or below 1% in the UL quadrant whenever possible. When gates were used to analyze smaller double-stained populations, the same gate was maintained for every mouse sample. Finally, similar color compensation procedures, as described in the Material and Methods, were upheld for every experiment.

Fluorescent color compensation was used to correct the fluorescence “spillover” created by the overlapping emission spectra of fluorochrome signals between APC, PE, and Alexa 488 (see Figure 7). A large portion of this part of
the project involved protocol development and optimization. The entire procedure was very involved and used a three-color compensation technique that had never been used before in the Stanford laboratory. Therefore, there was a significant learning curve for the experimenter. The process included making electronic calculations to remove signal overlap that the optical system cannot remove. These calculations are order-dependent and must be made using single-stained samples, in order to correctly analyze contingent multi-stained target samples.

Fluorescence compensation works by subtracting spill over between specific pairs of fluorescent parameters; for example, FL1 and FL2. Isotype controls were used to set quadrants for the single color staining in order to separate double negative and single positive populations into the lower and upper left quadrants, respectively, of the 2D-plot. Color compensation was set so that the mean fluorescence channel values (mean fluorescence intensity) for the two quadrants are approximately equal, which was essentially accomplished by trial and error. Thus, determining the spillover percentages was the most variable and temperamental part of the process. On the other hand, once the experiment was switched to double-staining, and the method involved in color compensation was fully worked out, this part of protocol was the most consistent and dependable.

In addition to considering experimental variability, biological variability was also considered in order to make sure that the cell population being measured and later identified as pDCs was representative of the target population. Minute
details were considered, experiments were always begun in the morning or early afternoon, in addition to the obvious, experiments were always conducted 7 days post-infection. Although the mice varied in age slightly between the three main experiments, they were all tested in an age range of 6 to 10 weeks. Finally multiple biological replicates were performed. Ten mice from each strain were tested in each condition.

**Part III**

*Potential functions of pDCs in MAIDS and AIDS*

There have been few studies investigating pDCs in mice, and there have not been any studies published examining the role of pDCs in murine AIDS. By first isolating splenocytes, then staining for pDCs, their presence could first be established. Further, since double-stained CD317+CD45R+ cells, assumed to be pDCs, were found in a greater percentage in the susceptible C57BL/6, several consequences can be imagined. First, pDC viral inhibiting functions will be discussed. Next, an argument for pDCs as virus transmitters will be provided in order to cover all bases. Ultimately, one side will be taken, based on the data acquired from this study.

DCs function as the most important APC in activating adaptive immune responses to viruses, a function which extends to the pDC subset. Many articles argue that this APC function is used by pDCs to elicit the T cell-mediated
immune responses necessary for HIV eradication (Bocharov et al., 2010; Swiecki & Colonna, 2010; Habermann et al., 2010). Yet, interestingly, this same mechanism is argued by others to cause more damage than good. By presenting viral antigen to naïve T cells, pDCs may actually be spreading viral infection, instead of promoting its extinction. On the other hand, pDCs are unique effectors at the interface of innate and adaptive immunity, endowed with other more powerful mechanisms for viral repression, such as early IFN production, which both directly and indirectly limits viral spread. pDCs play a highly complex and ambiguous role in HIV and MuLV disease pathogenesis.

Following encounter with RNA viruses, like MuLV or HIV, both murine and human pDCs endowed with pathogen recognition receptors (Toll-like receptor (TLR)-7 and -9) rapidly produce copious amounts of type I interferons (IFNs) (Bocarsly & Jacob, 2010; Bocharov et al., 2010). Although the majority of other IFN-producing cell types require viral gene expression and formation of replicative intermediates before being able to detect virus presence, pDCs produce IFN-α simply in response to the presence of many viruses, such as inactivated HSV-1, influenza virus, MuLV or HIV-1, even before viral gene expression occurs (Bocarsly & Jacobs, 2010; Bocharov et al., 2010). pDC-derived type I IFN has been shown to limit HIV replication in CD4+ T cells, as well as stimulate CD8+ T cells (cytotoxic T cells) to mount a viral response, suggesting that pDCs are capable of encouraging a protective immune response to HIV (Meyer et. al., 2007; Nascimbeni et al., 2009; Swiecki & Colonna, 2010). Thus, pDCs not only
produce these IFNs earlier in the immune response than other cells capable of IFN production, but they also produce ten- to 1000-fold more than these other cell types, thus limiting viral replication (Bocarsly & Jacobs, 2010).

Furthermore, CD317, a recently identified IFN-induced cellular response factor, blocks the release of HIV-1 and other retroviruses from infected human cells. CD317 has also been observed on the surface of mouse pDCs, where it can presumably serve a similar antiviral function (Swiecki & Colonna, 2010).

CD317 appears to block HIV-1 release by tethering newly manufactured virions into the intracellular cell surface, hence the name tetherin (Habermann et al., 2010). CD137, observed by fluorescent light microscope, was found to localize at both the plasma membrane and intracellularly around virions (Masuyama et al., 2009). The intracellular CD317 exhibited partial co-localization with trans-Golgi network and early, recycling endosomes. Microscopy and biochemical studies seemed to suggest that CD317 constantly cycles between these cellular membranes, as well as being internalized by clathrin-mediated endocytosis from the cell (Masuyama et al., 2009). Using immunoelectron microscopy (EM), it was further determined that the majority of CD317 localized to the plasma membrane in uninfected cells and was significantly redistributed to the endosomal compartment upon wt-HIV infection, though CD317 levels at the cell surface were deemed critical for the inhibition of virus release (Habermann et al., 2010).

Not only do pDCs directly limit viral spread, but they also set the stage for antigen-specific adaptive immune responses (Bocharov et al., 2010). Early on,
there was no convincing evidence that human pDCs could crosspresent antigens. However, human pDC crosspresentation was observed by Hoeffel et al., (2005), who further suggested that this form of presentation was enhanced by stimulation with influenza virus, and thus, somewhat dependent on type I interferon. Human pDCs are now the principal APCs recognized for their capacity to cross-present antigen, and therefore, are especially adept at presenting virus derived peptides to naïve T cells (Swiecki & Colonna, 2010). Murine pDCs have also been shown to crosspresent soluble antigen to transgenic T cells in vitro upon viral stimulation, which is dependent IFN-α secretion (Shinohara et al., 2006). Additionally, pDCs are the primary activator of CD8+ T cells, which elicit protective immune responses to viral infections in humans, as well as control MuLV infection and suppress MAIDs development (Crotzer & Blum, 2010; Kastrukoff, et al., 2010; Tang et al., 1997; Swiecki & Colonna, 2010).

Yet, due the crucial role of pDCs in anti-viral immunity, many viruses have developed the ability to inhibit and otherwise interfere with their functioning. For instance, the tethering activity of CD317 (tetherin) critically depends on its density at the cell surface, which is antagonized by a HIV-1 accessory protein Vpu (Habermann et al., 2010; Swiecki & Colonna, 2010). CD317 has an unusual structure, including a transmembrane domain and a lumenal anchor, which was conjectured to retain budding enveloped virions on the plasma membranes, thus, providing a mechanism of viral retention (Van dammel et al., 2008). It was recently clarified that tetherin dramatically inhibits
the release of Vpu-defective HIV-1 virions, but has only modest effects on wild-
type Vpu-expressing HIV-1 (Habermann et al., 2010). Moreover, Vpu co-
localizes with tetherin intracellularly, including the trans-Golgi network, and in so
doing prevents the co-localization of tetherin with budding virions (Neil et al.,
2008). Furthermore, Vpu also downregulates tetherin from the cell surface,
thereby reducing cell levels of tetherin, at least under conditions of short-lived
over-expression (Bieniasz, 2009).

In HIV infection, pDCs are known to interact with HIV and play
potentially key roles in transmission of the virus to CD4+ T cells. For instance, it
was previously known that Toxoplasma exploited a pDC-related cell as Trojan
horses, first targeting them for early infection and suppressing their cytokine
effector function, then using them as a means of dissemination to new host cells.
These pDC-related cells were similar in function and phenotype, although they
differed by expression of key molecules like, CD11b (Bierly et al., 2008).
Interestingly, a loss of pDCs due to HIV infection had been correlated with high
viral loads, decreased numbers of CD4+ T cells, and the onset of opportunistic
infections, the cause of which is unclear, although some argue that infected pDCs
may spread HIV to CD4+ T cells (Donaghy et al., 2001; Meyer et. al., 2007;
Swiecki & Colonna, 2010).

In conclusion, human pDCs have been identified as both HIV
disseminators and viral repressors. They exemplify key antigen presenting
functions, especially in the form of cross-presenting to activate CD8+ T cells.
pDCs uniquely produce large amounts of type I IFNs early in infection, as well as use CD317 (tetherin) to inhibit viral spread. Conversely, pDCs infected with HIV, upon interaction with naïve T cells, may cause death of CD4+ T cells by cytotoxic mechanisms, or indirectly by presenting viral antigen to CD4+ T cells, eventually causing their apoptosis. Additionally certain HIV accessory proteins, such as Vpu, are known to counteract the tethering mechanisms of CD317.

Similarly, murine pDCs have been implicated in performing both functions in response to similar viruses, like influenza or MuLV. Murine pDCs are also copious type I IFNs producers, which seem to use CD317 (tetherin) to inhibit viral spread; they also cross-present to activate CD8+ T cells (Bocharov et al., 2010; Swiecki & Colonna, 2010). CD8+ T cells have also been shown to contribute to MAIDS resistance by preventing abnormal B cell proliferation; they have antigen-specific cytotoxic activity against infected targets (Kastrukoff, et al., 2010). CD4+ T cells do not appear to be directly infected by the virus, although the presence of CD4+ T cells is required for the development of MAIDS (Mosier, 1987). Thus, although there have not been similar studies done for murine pDCs, in regards to the role they may play in the death of CD4+ T cells by antigen-presenting mechanisms or indirectly by pDC-activated cytotoxic cells (CD8+ T cells or NK cells), based on the data observed in this experiment, that a greater percentage of (CD317+CD45R+) pDCs were observed in susceptible C57BL/6 mice, this phenomenon seems likely.
Comparing human and mouse pDCs

Due to all the differences between human and mouse immunology, any given response in mice may not occur in precisely the same way in humans. However, mice are generally accepted as good *in vivo* models to aid our understanding of basic immunologic mechanisms in the human. The use of inbred strains reduces individual genetic variability and allows us to study trends in immune regulations upon infection. The resistance-associated factors that protect BALB/c from MAIDS development, for example, may reveal similar factors related to HIV/AIDS resistance.

Early on in the pDC field, the mouse and human pDC were regarded as being remarkably similar in surface phenotype, as well as in the way that they respond to pathogenic stimuli (Hubertus et al, 2002). Few, but noticeable differences were recognized. Human pDCs were commonly marked with CD123, while murine pDCs lacked an equivalent surface marker and were commonly stained with CD11c, which human pDCs lacked (Gilliet et al., 2002). Furthermore, the identification of murine pDCs had been obscured due to their high expression of CD45R (B220), which is also found on the surface of B cells, and some NK cells. However, distinct B cells markers, such as CD19, made separating B cells from pDCs possible (Vremec et al., 2000). Interestingly other pDC antigens used for antibody tagging, such as Ly-6G (GR-1) differed in expression by mouse strain: in BALB/c mice, a majority of pDCs could be
isolated or stained by Ly-6G, while pDC from C57BL/6 mice barely stained (Hochrein et al., 2002).

Currently, murine pDC are recognized to share some morphologic features with their human counterparts, although they also have some distinct phenotypical characteristics. To reiterate, murine pDCs can be differentially isolated based on their expression of CD11c, B220 (CD45R), Thy1.2 (CD90), CD317 (mPDCA, BST-2, or tetherin) and Siglec-H (Bjorck, 2001; Gill et al., 2010). They lack expression of myeloid (i.e., CD11b) antigens and CD8α, a marker used to isolate lymphoid DCs (Bjorck, 2001). In humans pDCs express the surface markers CD123, BDCA-2 & -4 (tetherin molecules), but do not express CD11c or CD14, which distinguishes them from conventional DCs or monocytes, respectively (Gill et al., 2010). More importantly, the tetherin (CD317) molecule is currently the one pDC unique marker recognized on both human and mice pDCs.

Early on it was realized that pathogens were recognized by the innate immune system via pathogen associated molecular patterns (PAMPs), which bind special receptors called the pattern-recognition receptors (PRR) on innate immune cells. As mentioned in the Introduction, toll-like receptors (TLRs), a type of PRR, can be found on both human and mouse pDCs (Hochrein et al., 2002). Expression patterns for the TLRs were thought to differ between human DC subsets, with human pDCs principally expression TLR7 and TLR9 (Hochrein et al., 2002). Recently, murine pDCs were also found to express TLR7 and TLR9 (Lee et al., 2007).
As previously mentioned murine pDCs are recognized as responding to viral infection, such as that produced by MuLV, in a manner similar to human pDCs. Thus, the striking phenotypic and functional similarities shared by human and mouse PDC suggest a very high level of conformity in their roles in immune responses. However, considering the complexity of immune and non-immune cells in vivo, and interspecies differences such as differential expression of certain surface molecules, whose functions have not yet been defined, care must be taken when translating murine studies into the human system.

**Future Studies**

In future experiments, several steps should be taken to decrease variability, such as keeping mouse age even more consistent, and perhaps continuing to run multiple mice per experiment. Despite the logistical and biological impossibility of finding a significant number of pDCs in naïve mice, it would be beneficial to run at least a few experiments with naïve mice in each strain; thus, making sure that results still remain statistically significant.

Results from this study posed important questions regarding the presence of pDCs and susceptibility in MAIDS, 1 week post infection, as well as the functional importance of the surface markers chosen to define pDCs. Future experiments might utilize fluorescence-activated cell sorting (FACS), a specialized type of flow cytometry, to separate out CD317+ CD45R+CD11c+ from rest of the leukocytes. This is especially critical, as the initial intent of the
experiment was to staining cells with all three antibodies, and might identify a ‘truer’ pDC population. Again, further experimenters should search for other functional surface markers, as they are identified. Since the pDC field is still in an early state, the major obstacle for testing pDCs remains best defining this DC subpopulation with antibodies. In addition, pDCs are extremely rare and difficult to isolate. Finally, future experiments should try to determine what, if any, role pDCs play in the MuLV immune response, such as whether they present MuLV to naïve T cells.

Conclusions

We are on the cusp of better defining the pDC phenotype, as well as deciphering the now elusive roles of plasmacytoid dendritic cells, which have already been identified as an important cell in all types of anti-viral immunity. Specifically, pDCs are able to present virus pathogen through a special cross-presentation process, which allows CD8+ T cells to be properly activated. Due to their critical role in the anti-viral immune response, many viruses, including HIV, have evolved ways to interfere with pDC functioning, such as by down-regulating CD317 (tetherin) on the pDC surface. HIV is known to interact with and bind pDCs, it appears, leading to the ultimate death of CD4+ T, although the exact mechanisms of this interaction remain ambiguous. The role of pDCs is an important focus in HIV research; however, many of the studies have reported
contradictory results. Surprisingly, this study seems to be the first to date regarding the role of pDCs in murine AIDS.

MAIDS is a simple murine model system used to study human AIDS. HIV/AIDS are logistically difficult to study, since only humans and some non-human primates are susceptible to HIV infection. Thus, examining the role of pDCs in MuLV infection and MAIDS pathogenesis many provide important insights and offer new research directions for understanding the human immune response to HIV infection and the development of AIDS.

Additionally, these studies may also have implications in HIV medical treatment. For example, drugs that inhibit Vpu function and consequently mobilize of tetherin’s (CD317) antiviral activity could be a potential therapeutic strategy in HIV/AIDS (Bieniasz, 2009; Swiecki & Colonna, 2010). Conversely, given the unique ability of pDCs to cross-present and prime T cells and their potential role in HIV pathogenesis, pDC or DC-directed treatments or vaccines may inhibit HIV spread.

The role in pDC function and viral inhibition or spread in MAIDS is worth continued investigation as it may offer valuable insights into the behavior of cells in human HIV and AIDS, ultimately leading to new therapeutic treatments, as well as promising vaccine options.
REFERENCES


Miu, Bing. 2010. Early activation of professional antigen presenting cells in the MAIDS system, Mount Holyoke College Thesis.


