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IL-10 and Suppression/ Susceptibility in MAIDS Model

by

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This paper was prepared

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Professor Sharon Stranford

for eight credits.

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ABSTRACT

Individuals infected with the Human Immunodeficiency Virus (HIV) typically progress to Acquired Immune Deficiency Syndrome (AIDS). AIDS is characterized by an increase in opportunistic infections like tuberculosis, as well as the development of certain cancers, like Kaposi's sarcoma. The debilitation of the immune system in AIDS patients usually presents a bleak prognosis of death. However, there exist a select few of HIV-positive individuals who do not progress to AIDS. These individuals, known as 'long-term nonprogressors' or LTNPs, remain an important area for scientific study because the activity of their immune system may provide important insight to the inhibition of AIDS.

Several animal models of AIDS have been developed over the years. One such model involves the induction of AIDS-like symptoms in mice via infection with the Murine Leukemia virus (MuLV). This model is termed Murine Acquired Immunodeficiency Syndrome (MAIDS). The MAIDS model is appropriate for studying AIDS because, like humans, some mice (e.g. BALB/c) present like LTNPs while others such as the C57BL/6, present like 'rapid progressors'.

We hypothesized that in mice, a potential contribution to this phenomenon may lie in the expression of the immunosuppressant IL-10 such that the more susceptible C57BL/6 mice produce increased levels of IL-10 relative to their less susceptible BALB/c counterparts. We tested this hypothesis via Enzyme-Linked Immunosorbent Assays (ELISA). Results suggest that prior to infection, both mice express relatively equal levels of IL-10. At three days post MuLV infection however, C57BL/6 susceptible mice begin to express close to three times as much IL-10 compared to BALB/c resistant mice. This suggests that the eventual development of MAIDS in C57BL/6 mice post MuLV infection may be driven by an early over expression of the immunosuppressant IL-10. This finding is of importance because it provides insight to the potential role of immunosuppresants like IL-10 not only in MAIDS models, but also in AIDS development in humans post HIV infection.

INTRODUCTION

Over the past 30 years alone, more than 25 million people have died from AIDS with an estimated 34 million people still living with the HIV infection as of 2010 (WHO, 2012). Despite these glaring numbers, however, close to 3 million people, of which 390,000 were children, were newly infected with the virus, in the same year, worldwide (WHO, 2012). This ever-recurring problem of new, annual HIV infections, despite global efforts to eliminate the disease suggests that the direction of resources towards finding a cure or vaccine is crucial, as efforts at eradicating the disease by prevention alone continue to prove challenging.

The geographical distribution of people who have been infected with HIV is even more alarming, and bolsters the above conclusion. For instance, in 2008, while close to 6 million people in South Africa, and 4 million people in Nigeria, were living with the HIV infection, the numbers in France and Germany were a little over 100,000 and 50,000, respectively (UNAIDS, 2012). These statistics indicate that relative to developed countries like France, more people in sub-Saharan Africa are likely to develop and die from AIDS. The discrepancy in these numbers also suggests that the prevalence of HIV is based on different factors in different parts of the world. For instance, in developing countries like those in sub-Saharan Africa, poverty, high incidences of untreated sexually transmitted diseases (STDs) and poor health care dissemination contribute greatly to the increasing rates of STDs, including HIV/AIDS (Corbett et al. 2002). In developed countries, however, fewer factors contribute to the development of AIDS; a major

factor is delaying antiretroviral therapy in patients suspected to be infected (Cohen, et al. 2011).

It would be short-sighted, however, to talk about the spread of HIV/AIDS without mentioning the advances we have made so far in the worldwide battle against the spread of the virus and development of this syndrome. Some praise-worthy progress reports include the fact that between 2003 and 2010, the number of HIV infected people receiving antiretroviral therapy in low and middle income countries increased by 16-fold, to 6.6million (WHO, 2012). Furthermore, the Joint United Nations Programme on HIV/AIDS (UNIADS) reports of a new initiative launching a novel treatment plan known as 'Treatment 2.0' (UNAIDS, 2010). Once in full swing, Treatment 2.0 is aimed at fulfilling a number of tasks including increasing the number of life-saving drugs used to treat HIV, reducing HIV infection treatment costs, and improving the overall quality of lives of HIV-infected individuals and of their families. Yet as exciting as they are, innovations like Treatment 2.0 remain a temporary relief for individuals living with HIV. Sooner or later, as indicated in earlier paragraphs, a cure or vaccine would have to be found in order to realize the much desired vision of UNAIDS: zero new HIV infections, zero discrimination (against infected individuals) and zero AIDS-related deaths (UNAIDS, 2010).

A major reason for the inability to develop a cure for HIV infections is the complexity of the mode of attack of the virus, which targets the human immune system. Logically therefore, to understand how HIV gets an upper hand over

humans, one must first appreciate the normal mode of functioning of the human immune system.

THE IMMUNE SYSTEM

The human body is made up of several lines of defense against infectious disease (Parham, 2009). The skin, sometimes considered the largest organ of the body (Goldsmith, 1990) is also known as the first line of defense of the body (Parham, 2009). Though it serves as a barrier, it can sometimes be broken by injuries, like cuts or insect bites, which can then serve as entry points for pathogenic microorganisms (Parham, 2009). Generally, however, the body is set up such that the skin is in close contact with blood vessels. As a result, such breaches trigger a coagulation system that facilitates the formation of blood clots (Parham, 2009). Blood clots, in turn, simultaneously impede entry of microorganisms as well as blood loss (Parham, 2009). Besides blood clots there are many other processes set in place by the body to minimize invasion by foreign matter. Mucosal surfaces of the respiratory tract coupled with cilia (tiny hair-like structures) help 'sweep out' microorganisms that may have been breathed in or swallowed. Additionally, secretions like sebum from hair follicle sebaceous glands inhibit bacterial growth on the skin, soluble defensins kill bacteria, fungi and enveloped viruses, lysozyme (found in sweat and tears) kills bacteria, and low pH environments in organs like the stomach destroy most ingested microorganisms (Parham, 2009).

As robust as these systems are, there are many occasions when pathogens (any microorganism that is capable of causing disease- OED, 2012) are able to circumvent established lines of defense. During these times, an immune response is triggered, due to the presence of the pathogen, by a set of cells and molecules (Parham, 2009). Before triggering such an immune response, however, the immune system needs a feature to be able to distinguish the body's 'self' from pathogenic 'non-self'. An example of such a characteristic feature is called the antigen. By definition, an antigen is that foreign substance that triggers the production of antibodies upon introduction into a living organism (OED, 2012). Being able to distinguish 'self' from 'non-self' is important in a normally functioning immune system because it ensures that in an immune response, 'self' is conserved while 'non-self' is destroyed. There are several organs, cells and soluble compounds responsible for protecting and defending the body from all kinds of harm: from the presence of foreign matter like allergens, to infections caused by intruding microorganisms (Parham, 2009). The cells that make up the immune system are made in the bone marrow and are called leukocytes, or white blood cells. They consist of B cells, T cells, Natural Killer cells (NK Cells), neutrophils, eosinophils, basophils, dendritic cells, mast cells and macrophages (Parham, 2009). Generally, the immune system can be divided into two main categories: the innate immune system and the adaptive immune system (Parham, 2009).

THE INNATE IMMUNE RESPONSE

The innate immune system is known to trigger a form of immunity called the 'nonspecific immune response'. It is so termed because it is spontaneously triggered at the onset of an infection but is unable to decipher between the minute differences that distinguish specific pathogens (Parham, 2009). For example, during an innate immune response, a distinction cannot be made between one type of RNA virus and another. However, a distinction can be made between pathogenic bacteria (due to the presence of molecules like lipopolysaccharides, on the bacterial coat) and RNA viruses, for example (Parham, 2009). Furthermore, a healthy immune system is able to absolutely discriminate between products of the self and the non-self so that self-cells or proteins are generally not destroyed in an innate immune response (Parham, 2009). Among the cells mentioned above, all but B cells and T cells are involved in innate immunity (Parham, 2009). In addition to cells, there are also certain soluble plasma proteins. The complement system, an example of a collection of proteins generated in the liver, plus other cell-secreted compounds like cytokines, circulate in the blood and therefore can quickly reach a site of infection in order to facilitate an innate immune response (Parham, 2009).

The mode of operation of the innate immune system is very primitive but also very fast. It begins with an abrasion of a line of defense, like a cut on the skin. This provides an entry point for pathogens like bacteria (Parham, 2009). Once the immune system recognizes the pathogen as foreign (due to the presence of a

recognizable structure) it initiates several processes to eliminate it. As mentioned above, one such process is complement fixation which involves complement proteins coating the pathogen to flag it for engulfment by phagocytozing cells like macrophages (Parham, 2009). This is termed opsonization (Parham, 2009). Alternatively, the complement proteins can also destroy the pathogen by perforating the pathogens' surface membrane (Parham, 2009).

Besides complement, there are other means utilized by the innate immune system to defend the body. For example, most extracellular pathogens possess certain unique molecular structures known as pathogen-associated molecular patterns (PAMPs) which are easily detected by the corresponding pattern recognition receptors (PRRs) of resident innate immune cells like macrophages (Parham, 2009). Most of these PRRs are signaling proteins that belong to the toll-like receptors (TLRs) family of proteins. TLRs are transmembrane in nature. The extracellular region is important for recognizing the pathogen and the intracellular domain transmits this signal inside the cell (Parham, 2009). There are several types of TLRs and these detect different types of pathogens, including bacteria, viruses and fungi (Parham, 2009).

Once a pathogen has been detected, cells involved in the innate immune response (collectively called effector cells once in action) are drawn to the site of infection to destroy the pathogen via various processes called effector mechanisms. These mechanisms include phagocytosis (by macrophages), or direct killing of virus infected cells by NK Cells (Parham, 2009). Such events are

typically associated with the release of cytokines that trigger inflammation and vasodilation (the enlargement of blood vessels). This is important because it allows more blood to rush to the area of infection, and the blood carries along more effector cells necessary to entirely eliminate the intruding pathogen (Parham, 2009). It is this swelling, or edema, due to the rush of blood to the site of infection that causes redness and places pressure on nerve endings, causing us pain! As one would expect, this entire process takes some time and in the event when the innate immune response is unable to eradicate the intruder, the adaptive immune response is activated (Parham, 2009).

THE ADAPTIVE IMMUNE RESPONSE

Two types of cells are the primary contributors to adaptive immunity: B lymphocytes and T lymphocytes (Parham, 2009). Lymphocytes are made, and mature, in structures known as primary lymphoid organs (Parham, 2009). There are only two primary lymphoid organs: the thymus and the bone marrow, which are the origin of mature T cells and B cells, respectively. All other organs within which lymphocytes are stored after maturation and during immune activation are called secondary lymphoid organs. These include lymph nodes, tonsils, Peyer's patches, and the spleen, among others (Parham, 2009).

The adaptive immune response is more complex than the innate immune response and differs from the latter in several ways. First, unlike innate immunity,

which is present in most organisms, only some vertebrates are able to mount an adaptive immune response against pathogens (Janeway, 1997). Secondly, the adaptive immune response is very specific: B or T cells which express receptors specific to parts of the invading pathogen (antigens) are selected for proliferation after exposure. This is known as clonal selection and clonal expansion, respectively, because all lymphocytes chosen to engage in a given adaptive immune response are clones of an initial select few which had receptors specific to antigens from the attacking pathogen (Parham, 2009). Furthermore, some lymphocytes that respond during an infection persist after the pathogenic threat has been eliminated in order to serve as memory cells for the immune system should the same pathogen reinvade the body (Parham, 2009). This kind of memory is termed acquired immunity and ensures that a stronger, faster immunological response is triggered against a pathogen upon subsequent attacks of the body (Parham, 2009). At this stage, the body is described as having protective immunity to that particular pathogen (Parham, 2009).

Many genres of pathogens remain a threat to the human race including species of viruses, bacteria, fungi and parasites. However, the two most common classes of organisms that disrupt our health over and again are bacteria and viruses (Smith and Sweet, 2002). Due to their different modes of attack, they trigger different arms of the adaptive immune system. For example, bacteria typically trigger humoral immunity, while viruses normally require cell-mediated immunity (Abedon, 1998) for elimination. Figure 1 gives a broad overview of the

cells that make up the innate and the adaptive immune system. Unlike the left half of the image which hints at the non-specific nature of defense of the innate immune response, the right half of the image, which depicts the adaptive immune response, shows that while certain cells (like B-cells) are designed to effectively attack pathogens via specific routes (antibody release, discussed later), T cells will efficiently eradicate a pathogen via (an) entirely different mechanisms (including apoptosis of infected cell post direct T-cell to infected-cell interaction, discussed later). This suggests that though all immune responses are generated for the greater good, the body typically utilizes that arm of the immune system which is more effective at extensively eradicating the invading pathogen.

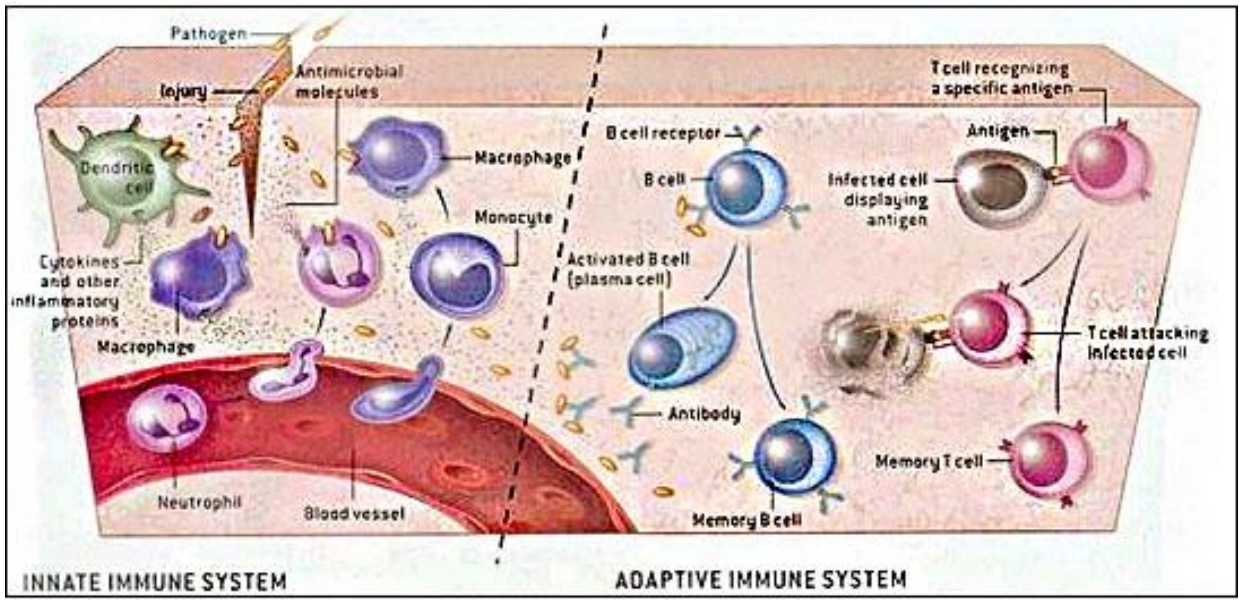

Fig.1: Connections between the innate and adaptive parts of the immune system. The left shows cells and molecules involved in the innate immune response while the right shows those primarily involved in the adaptive immune response, which is triggered when the innate response is unable to contain a pathogen. Source: Health Coach. Available at: http://valerietonnerhealthcoach. blogspot.co m/2011/04/immune-syste-part-one-what-is-immune.html. Accessed: April, 2012.

CELL-MEDIATED IMMUNITY

Cell-mediated immunity is orchestrated by T lymphocytes only. T cells are produced in the bone marrow but mature in the thymus, a primary lymphoid organ (Parham, 2009). There are several types of T cells including regulatory T cells, that suppress immune responses (discussed further in later paragraphs); cytotoxic T cells, that control viral infections, and helper T cells, that fight against both intracellular and extracellular infections (Parham, 2009). Each of these cells

carries a T cell receptor (TCR) which it uses to detect the presence of pathogenic proteins in the body (Parham, 2009). In addition, however, cytotoxic T cells possess a co-receptor called CD8 while helper T cells possess the CD4 co-receptor (Parham, 2009). Therefore, these cells are often referred to as $CD4^+$ T_H (helper) cells and $CD8^+$ T_C (cytotoxic) cells. The term 'helper' is used to describe those T cells that help other cells (like B cells) perform their functions (e.g., antibody production).

The receptors of T cells function uniquely in that their activation depends on interactions with specific cells known as professional antigen presenting cells (APCs). These include dendritic cells, B cells and macrophages (Parham, 2009). While APCs themselves usually do not get infected with pathogens, they are able to gather and display peptides from pathogens in a process called antigen processing and presentation. The process of antigen presentation and activation is outlined in Figure 2. A phagocytosing cell, for example a dendritic cell, encounters a pathogen like a bacterium, engulfs it, and then degrades it to the peptide level. Next, these peptides are moved to the surface of the dendritic cell and displayed in the binding cleft of a molecule called the major histocompatibility complex (MHC) (Parham, 2009). Antigen presentation is carried about, not only by APCs, but also all infected cells that interact with T cells. There are two types of MHC molecules: MHC class I and MHC class II (Parham, 2009). MHC class I molecules present to cytotoxic T cells ($CD8^+$ T cells) while MHC class II molecules present to $CD4^+$ T cells or T_H (helper) cells

(Parham, 2009). All cells, except red blood cells, possess the MHC class I molecule and can therefore engage $CD8^+$ T cells. However, only APCs possess MHC class II molecules (Parham, 2009) and therefore can present to, and activate, $CD4^+$ T cells. In order to fully activate the $CD4^+$ T cell into a functioning effector cell, however, there must be an additional stimulating signal delivered to the T cell by the APC (Parham, 2009). This is called a co-stimulatory signal and in its absence the T cell will be non-responsive (even in the presence of the appropriate, specific peptide:MHC complex). It is the combination of the co-stimulatory signal and the signal from a specific peptide:MHC complex that activates $CD4^+$ T cells and enables them move around in the body in an attempt to activate other potential effector cells like B cells (discussed later), and macrophages, towards completely eliminating pathogens (Parham, 2009). As such, $CD4^+$ T cells are important not only because they contribute to eliminating infectious pathogens but also because they are able to stimulate other adaptive immune cells to do the same. This is an important point to remember.

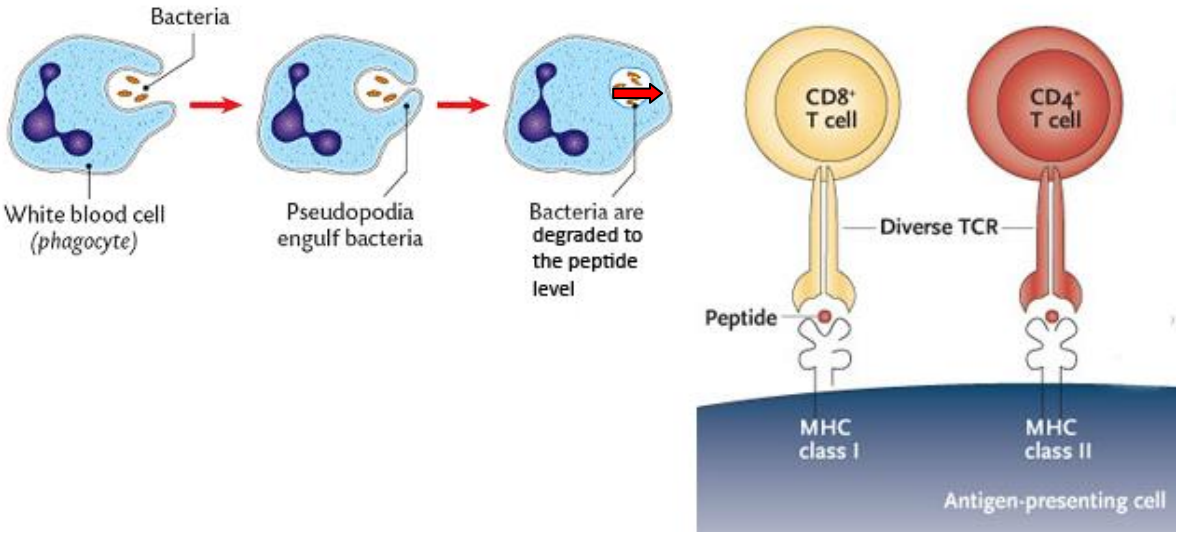

Fig. 2: The process of antigen presentation: A phagocytosing cell, like a macrophage, engulfs a pathogen and degrades it. Next, the peptide is positioned in the binding cleft of an MHC molecule and displayed on the cell surface. Interaction of the peptide-MHC molecule complex with an appropriate T cell helps activate the T cells with receptors that recognize this peptide derived from the pathogen. Source: pathology outlines.com (available at: http://www.pathologyoutlines.com/topic/ cdmarkersd.html) and leavingbio.net (available at: http://appsgroup.blog.com/2010201.archive.html) Accessed: April, 2012.

Different pathogens elicit different responses from the immune system. If the pathogen is a virus, then any cell that is infected with it will flag itself for destruction by presenting the degraded proteins from the virus in its MHC class I molecule cleft, which interacts with $CD8^+$ T cells (Parham, 2009). Only APCs can activate $CD8^+$ T cells. Once activated, a $CD8^+$ T cell with the right receptor can

interact with the MHC class I- bound antigen on the infected cell, to form a complex through which the T cell destroys the infected cell via lysis or by inducing apoptosis (Parham, 2009). The soluble molecules that facilitate intercellular communication and T cell effector functions are known as cytokines and cytotoxins, with the latter being specific to cytotoxic ($CD8^+$) T cells and natural killer cells (Parham, 2009). T cell cytokines (also called interleukins, abbreviated IL) bind to specific receptors on target cells and alter their behavior (Parham, 2009). Cytotoxins, on the other hand, help destroy infected target cells (Parham, 2009). Helper T cells ($CD4^+$) therefore rarely destroy pathogens, or pathogen infected cells, but rather mediate their destruction via communication with other effector cells, namely macrophages and cytotoxic T cells (Parham, 2009).

HUMORAL IMMUNITY

All pathogens that are extracellular in nature, like fungi, are kept at bay via humoral immunity, also known as antibody mediated immunity (Parham, 2009). Between the two lymphocytes that make up adaptive immunity, humoral immunity is controlled primarily by B cells (Abedon, 1998; Parham, 2009). B cells are so called because they undergo their maturation processes in the bone marrow. When B cells are produced, they undergo a series of developmental processes in primary lymphoid organs or the bone marrow in order to attain special receptors, known as B cell receptors, which are unique to specific

pathogens (Parham, 2009). This is to say, the development of B cells (and T cells) is usually designed to ensure that there is an array of cells, each with a unique receptor that responds only to the presence of a particular antigen, and not self proteins. Except, of course, in the case of autoimmune diseases where the immune system begins to attack oneself (Parham, 2009).

Once B cells have reached maturity they circulate through lymphoid tissue via the blood and lymph, as mature but naïve (unexposed) B cells, awaiting pathogens that manage to evade the immune response (Parham, 2009). Such pathogens normally present antigens on their surfaces (Abedon, 1998; Parham, 2009). If a B cell has a receptor that is specific to the antigen in question, it binds to it tightly but is often not activated until it comes into contact with helper T cells ($CD4^{+}$) as mentioned above, or another secondary signal (Parham, 2009). Only when this recognition process has occurred are more copies of the successful B cell produced by the body (Parham, 2009). The cloned B cells release their receptors to identify and bind to more antigens of the invading pathogen (Parham, 2009). At this point when they are releasing receptors they are called plasma cells (Parham, 2009). The released receptor is known as an antibody or an immunoglobulin, and its interaction with an antigen flags the pathogen for destruction (Parham, 2009). At this point, an adaptive immune response has been triggered and the pathogen is destroyed by various effector mechanisms similar to those adopted in an innate immune response. For instance, antibodies can bind to the region of a pathogen needed for its growth, host cell binding or

reproduction/replication. This is termed neutralization (Parham, 2009). Additionally, they can opsonize the pathogen: coating it so that it is targeted for phagocytosis by pathogen engulfing cells like macrophages (Parham, 2009). Antibodies can also activate complement proteins, described in earlier paragraphs. It is worth noting that not all the cloned B cells release their receptors as antibodies. Some remain for very long periods of time with their surface receptors intact, and these are termed memory B cells (Parham, 2009). Memory cells trigger a faster immune response during a second bout of infection by the same (or very similar) pathogen (Parham, 2009). The entire process of humoral immunity is summarized in Figure 3.

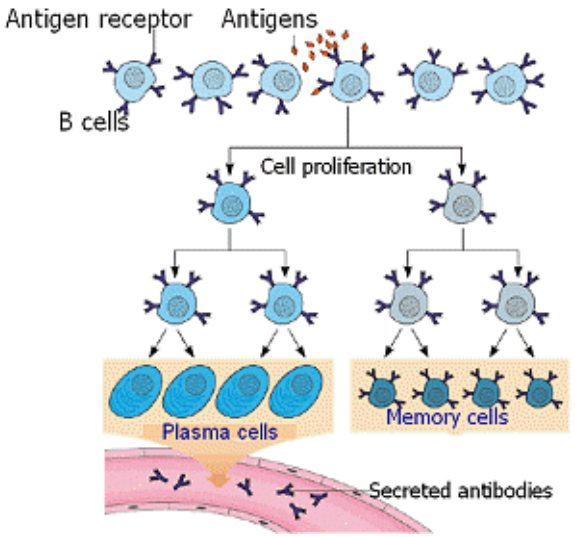

Fig. 3: A Diagramatic represenataion of humoral immunity. B cells possess a B cell receptor and each receptor is specific to a particular antigen present on a pathogen. Once the right receptor binds tightly with an antigen and the B cell receives appropriate secondary signals, it becomes activated and proliferates. Some of the proliferating B cells become plasma cells (left) and continue to release antibodies that circulate throughout the body and help protect from subsequent infections. Other B cells maintain the successful B cell receptor and become memory cells for use in a later infection by the same pathogen (right). Source: Encarta Reference Library. Available at: http://www.eduspace.org/ necc2005/antigen-antibody.htm. Accessed: April, 2012.

STOPPING AN IMMUNE RESPONSE

Detecting and destroying a pathogen is vital to our survival; but leaving this destructive machinery on and unchecked can be detrimental to humans as well. For this reason the immune system has conventional mechanisms through

which to shut itself down after a pathogen has been completely destroyed and is therefore no longer a threat. One group of cells known to regulate the immune system in this manner is the regulatory $CD4^+$ T cells, also known as Tregs (Sakaguchi et al., 2009). Tregs regulate a broad array of immune cells including APCs, like dendritic cells and macrophages, as well as adaptive immune response cells, like B cells, $CD4^+$ and $CD8^+$ T cells (Shevach, 2009). Tregs can be identified usually by their expressing of certain proteins necessary for their function, including the transcription factor Foxp3 and the surface protein CTLA-4, an abbreviation for Cytotoxic T-Lymphocyte Antigen 4 (Sakaguchi et al., 2009). Additionally, Tregs express certain anti-inflammatory or immunosuppressive cytokines, namely IL-4, IL-10 and TGF-βeta (TGF-β) (Parham, 2009).

CTLA-4 is a cell surface receptor (Sakaguchi et al., 2009; Shevach, 2009) that inhibits immune responses by downregulating the expression of costimulatory ligands like CD80 and CD86 on APCs (Shevach, 2009). Since these ligands are vital for producing co-stimulatory signals for T cells, their downregulation limits naïve T cell activation by these APCs, and hence suppresses the immune response (Shevach, 2009). Furthermore, Treg cells which express high levels of CTLA-4 are known to be able to induce dendritic cell expression of an enzyme called indoleamine 2,3-dioxygenase, abbreviated IDO (Sakaguchi et al., 2009; Shevach, 2009). Once IDO is expressed, Tryptophan, an amino acid essential to T cell activity, is converted to a more toxic form called kynurenine, rendering the T cells inactive (Sakaguchi et al., 2009). Another Treg

immunosuppression facilitator worth mentioning is LAG-3 (Shevach, 2009). LAG-3 mediates immunosuppression by binding tightly to MHC class II molecules on dendritic cells (Shevach, 2009). This interaction triggers an inhibitory response that prevents the maturation and stimulatory capacities of dendritic cells (Shevach, 2009). Furthermore, Neuropilin (Npr-1), another important Treg surface receptor, inhibits immune response activation by literally competing with effector cells for immature dendritic cell interactions. Specifically, when the Npr-1 receptor binds to immature dendritic cells, they prevent other effector cells from binding to that dendritic cell, and so prevent immune response activation (Shevach, 2009). Finally, when Tregs come into contact with other effector T cells (for example, cytotoxic T cells) or B cells, they are capable of triggering anti-inflammation by generating adenosine from adenosine triphosphate (ATP) via the A2a immune cell adenosine receptor (Sakaguchi et al., 2009). All these methods of immune suppression are summarized in Figure 4, adopted from Shevach (2009).

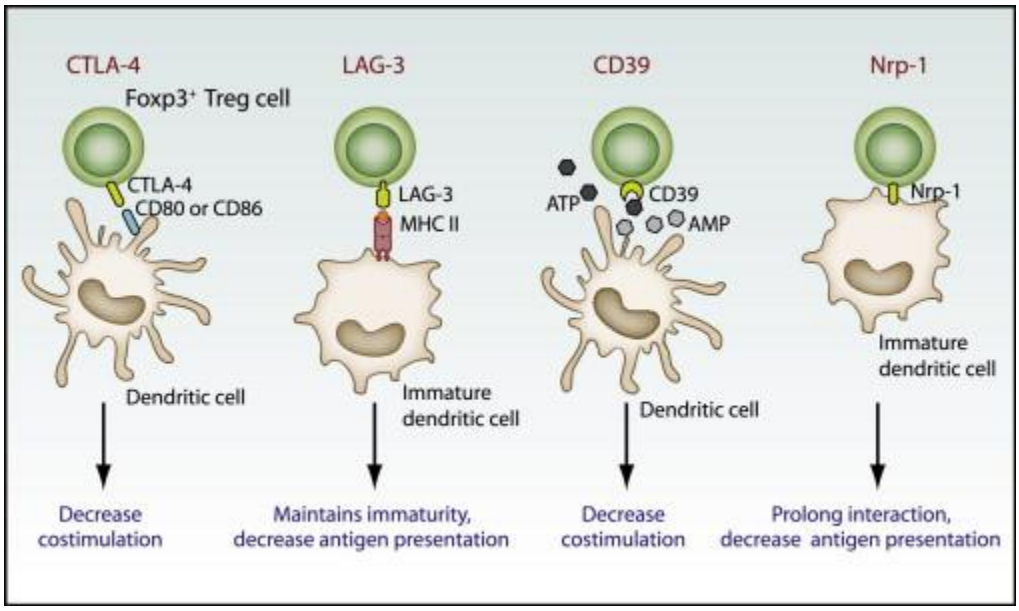

Fig.4: Four proposed mechanisms of APC (dendritic cell) immune suppression by T –regulatory cells.

It is worth noting that Treg cells (and indeed many other cells) are also capable of immunomodulation via cytokine secretion. Two major cytokines made by Tregs are TGF-β and IL-10 (Grutz, G. 2005; Sakaguchi et al., 2011). Several reviews have suggested that TGF-β may regulate immune suppression by transforming $Foxp3^{-}$ T cells into $Foxp3^{+}$ Treg cells (Anderson et al., 2008; Sakaguchi et al., 2011) On the other hand, IL-10 inhibits several immune system activating cytokines: TNF-α and IL-16, involved in innate immune response; IL-12 and IL-18, which activate helper T cells; and IL-8, which recruits immune cells to the site of infection (Grutz, G., 2005). This anti-inflammatory suppressive loop induced by cytokines like TGF-β and IL-10 is depicted in Figure 5. Note, that this figure sheds light on the production of IL-10 and TGF- β by Treg cells.

However, there are many other cells that can also secrete both cytokines. These include macrophages and dendritic cells, which form part of the innate arm of immune responses (Mousakas et al., 2002; Blount et al., 2008). This is not surprising. In fact during immune suppression, dendritic cells (which serve as a link between the innate and adaptive immune responses) are also able to produce IL-10 which in turn regulates their own production of cytokines (autocrine signaling) to ultimately prevent the differentiation of naïve T cells (Blount et al., 2008). Hence these suppressor cytokines, though regulators of T cells (adaptive arm of immune responses), can be secreted by innate cells even before an adaptive immune response begins. They could therefore be involved in regulating the extent to which adaptive responses are activated.

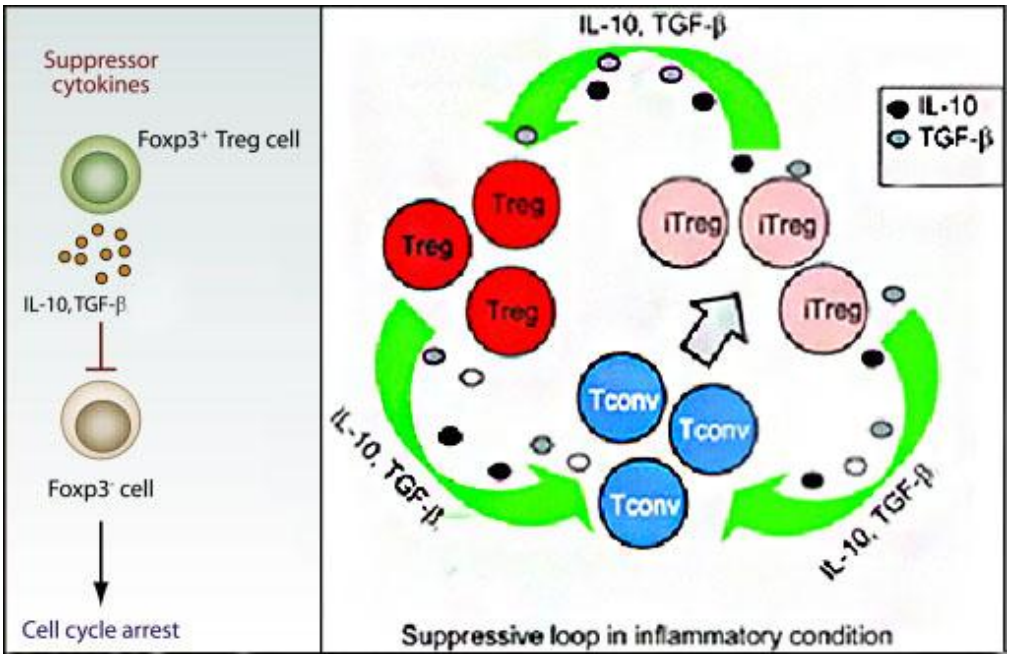

Fig.5: Mechanisms of immune system suppression via anti-inflammatory cytokines IL-10 and TGF-β. In summary, on the left, the secretion of IL-10 and TGF-β suppress Foxp3^{-} T cells which would otherwise mediate an immune response in the presence of a pathogen. On the right, the suppressive loop of Treg activity is shown. Treg cells secrete IL-10 and TGF-β. These cytokines induce the conversion of naive T cells into induced Treg cells (iTreg). More Treg cells (induced or original) results in the production of more IL-10 and TGF-β, hence the loop is maintained and immunosuppression facilitated. Images adapted from Shevach, E. (2009) and Sakaguchi et al., (2011).

THE HUMAN IMMUNODEFICIENCY VIRUS (HIV) AND ACQUIRED IMMUNODEFICIENCY SYNDROME (AIDS)

There are two types of HIV: HIV-1 and HIV-2 (Ghandi and Campbell-Yesufu, 2011). While both viruses gain access via the same modes (sexual contact, blood exposure and mother-to-child transmissions), several differences exist between them (Ghandi and Campbell-Yesufu, 2011). For example, while HIV-1 is prevalent around the world, HIV-2 is said to be more common in and among west Africans (Ghandi and Campbell-Yesufu, 2011). Furthermore, HIV-1 is recognized to be more pathogenic than HIV-2 (Kanki et al., 2007). This may be due to the fact that viral RNA levels (which result in the production of viral proteins) are higher in HIV-1 infected individuals relative to HIV-2 infected individuals (Kanki et al., 2007; Ghandi and Campbell-Yesufu, 2011). An additional explanation for the differences in HIV1/HIV2 pathogenicity is that the $CD4^+$ T cell counts in HIV-2 infected individuals are higher relative to HIV-1 infected individuals (Kanki et al., 2007; Ghandi and Campbell-Yesufu, 2011). This automatically implies that individuals infected with HIV-2 will have a stronger immune system. Interestingly enough, however, even though HIV-2 is less virulent than HIV-1, individuals infected with HIV-2 have a higher mortality rate than those infected with HIV-1 when they progress to advanced immunodeficiency (Ghandi and Campbell-Yesufu, 2011).

Whether infected with HIV1 or HIV2, however, it is important that infected individuals receive antiretroviral treatments (ARTs). Though these do not eliminate HIV they are usually successful at suppressing the replication rate of the

virus if administered in a timely manner. This is important because slower viral replication rates result in slower progression to AIDS (Siegfried and Uthman, 2010). In fact, recent studies have shown that relative to the conventional form of administering ART, where drugs are given to infected individuals when $CD4^+$ T cell counts have fallen to dangerously low levels, early delivery of ART to infected individuals decreased incidence of clinical difficulties due to the onset of immune deficiency by 4-fold (Cohen et al., 2011). However, these ARTs must be taken consistently for decades to yield favorable results; and yet not everyone can afford them, especially for so long (MSF, 2008). As a result, there remains the ever increasing need to obtain a cure for AIDS or a vaccine against HIV. To achieve this, it is important to understand what drives the development of immune deficiency. To date, studies aimed at this have focused on HIV-1 since this is the virus type that is most common among infected individuals and the most studied (Ghandi and Campbell-Yesufu, 2011). Thus, hence forth, references to HIV will refer to HIV-1.

STRUCTURE OF HIV

HIV classification can get more complex than just branching into type 1 and type 2. For example, as an RNA virus which requires reverse transcriptase, HIV belongs to the familiy retroviridae and is therefore able to change its RNA to cDNA in order to be integrated into the host cell DNA (NCBI, 2009). HIV also belongs to the genus of lentiviruses, a type of retrovirus which is slow to cause

overt disease. Particularly, lentiviruses are able to set up a lifelong infection by remaining latent in the host cell genome for extended periods of time as a provirus; only resurfacing with the activation of the target cell (Hunt, 2009). Further classifications of HIV-1 include being broken down into groups M, O, N and P. Group M (‘major’) is the predominant cause of the AIDS pandemic and group O (‘outlier’) is restricted to west-central Africa, especially Cameroon (Baveja and Rewari, 2004). Group P is relatively new and believed to be derived from the simian immunodeficiency virus found in gorillas (Plantier, 2009). Yet as complex as this classification seems, HIV itself encodes only 15 proteins (Frankel and Young, 1998). As a result, most of its complexity is derived from its human host (Goff, 2007). In fact, recent studies have shown that HIV depends on over 250 factors which are solely provided by the host cell (Brass et al. 2008).

On the outside, HIV has a fatty outer coat called the viral envelope (Baveja and Rewari, 2004). Arranged on this coat are 72 spikes made of glycoproteins (abbreviated gp). Specifically, two types of spikes exist: gp120 (on the outer surface) and gp41 which is embedded in the lipid matrix (Baveja and Rewari, 2004). The inner section of the virus is called the core, and it contains only 2 copies of single stranded ribonucleic acid (RNA) enclosed in a case called the capsid (Baveja and Rewari, 2004). Important to note, however, is that all viruses (including HIV) are inactive outside a host cell. In humans, the body functions on proteins made (translated) from messenger RNA (mRNA) which is originally generated or transcribed from DNA in the nucleus (Clancy and Brown,

2008). Consequently, any protein that is needed in the body must first come from DNA, and yet HIV only contains RNA (Baveja and Rewari, 2004). To solve this problem, the virus carries other important enzymes including reverse transcriptase, integrase, and proteases (Brass et al. 2008) discussed below. The basic structure of HIV is highlighted in Figure 6.

Indeed, even though HIV has plagued the human race for years, it remains an enigma to the scientific community because of its ability to rapidly mutate certain variable regions of its structure while maintaining others as highly conserved (Baveja and Rewari, 2004).

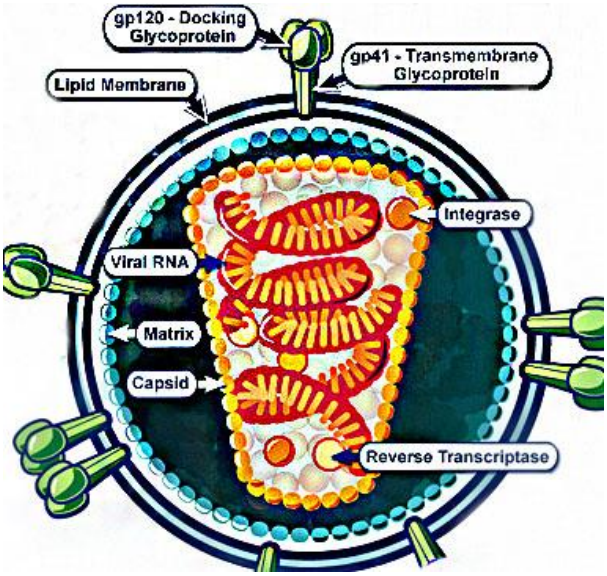

Fig. 6: Basic HIV structure showing some of the important proteins necessary for host infection and viral replication. Source: The Body (The Complete HIV/AIDS Resource) Available at: http://www.niaid.nih.gov/topics/HIV/ AIDS/ Understanding/Biology/pages/structure. aspx. Accessed: April, 2012.

HIV MODE OF INFECTION

Though HIV infects very few cells in the body, it does infect an important subset. Over the years, studies have shown that the virus infection occurs in cells that possess specific surface receptors, namely CD4, as well as either CCR5 or CXCR4 co-receptors (Baveja and Rewari, 2004, Brass et al. 2008). These include, but are not limited to, a number of adaptive immune cells including all $CD4^+$ T cells, and some macrophages and dendritic cells (Baveja and Rewari, 2004). Being a retrovirus, HIV employs basic steps adopted by retroviruses to attack its human host cell (Fig.7) using specific proteins to its advantage. First, to enter the

host cell, HIV attaches to the CD4 receptor and either the co-receptor CXCR4 or CCR5 using its gp120 receptor protein (Brass et al. 2008). After fusing its membrane with the host cell membrane, it releases the contents of the viral core into the host cell's cytoplasm where it makes use of two important enzymes: reverse transcriptase facilitates synthesis of viral double-stranded cDNA and integrase allows the transcribed cDNA to be incorporated into the host genomic DNA as a provirus (Brass et al. 2008). At this point the host cell is 'hijacked' and transcription of host DNA results in transcription of viral DNA. The viral messenger RNA produced moves into the cytoplasm of the host where a third important enzyme, protease, facilitates the building of new viron capsids which eventually lyse the infected cell as they bud off to maturity from the infected cell's plasma membrane in order to infect new $CD4^+$ T cells (Frankel and Young, 1998).

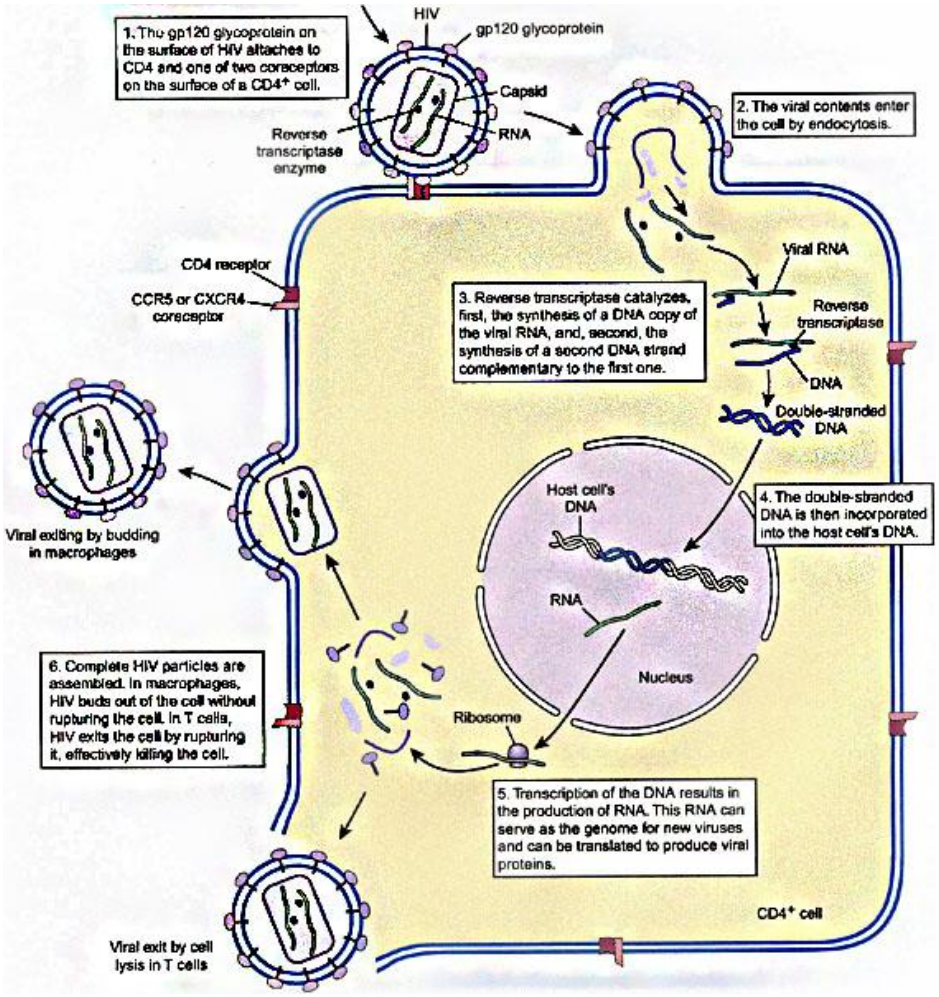

Fig.7: Replication life cycle of HIV in a human CD4+ T cell. Virus membrane merges with host membrane via the interaction between viral surface proteins (gp120 and gp41) and host surface proteins (CD4 and CCR5 or CXCR4). Once viral RNA enters the host cell, reverse transcriptase transcribes it into cDNA and integrase enables integration within host cell DNA. At a later stage, viral proteins are assembled with the help of the protease enzyme and new virons bud off of host cell, taking away part of the host cell membrane. Alternatively virons may exit the host cell by lysing it. Source: Dr. George **Johnson's Backgrounders. Available at:** http://www.txtwriter.com/Backgrounders/Aids/aids3.html. Accessed: April, 2012.

HIV infection is a paradox, triggering a complex response from the body that both inhibits and stimulates viral replication and survival (Baveja and Rewari, 2004). During the primary phase of HIV infection, the virus enters the body and is trapped by APCs like dendritic cells (Baveja and Rewari, 2004). The problem, however, is that many such cells also possess receptors for the virus that make them susceptible to infection (Baveja and Rewari, 2004). Furthermore, as dendritic cells travel to lymph nodes to activate other adaptive immune cells like $CD4^+$T cells, they transfer the virus on to them (Baveja and Rewari, 2004). Loss of $CD4^+$ T cells by lysis as well as bystander inactivation (where uninfected CD4+ T cells stop functioning properly) is problematic because $CD4^+$ T cells are important for stimulating other cells like B cells (Parham, 2009). As a result, there is a constant 'battle' in the lymph node to produce enough cells to ward off the virus, and yet it is this rapid replication of adaptive immune cells that stimulates the high levels of virema in the blood (Baveja and Rewari, 2004) - an unfortunate paradox.

Of course, at a point, the body is able to mount strong immune responses against the virus via the humoral (antibodies from B cells against HIV) and cell mediated ($CD8^+$ cytotoxic T cells) immune responses. However, the latter involve eliminating cells germane to the immune system; not to mention that both humoral and cell mediated immunity are rarely enough to eliminate the virus completely, particularly because the provirus could be latent in other non-dividing, reservoir cells (Fauci et al., 1996; Baveja and Rewari, 2004). Hence, the patient

only goes into a chronic, asymptomatic stage of infection: $CD4^+$ T cell counts are high enough (above or at 500 cells/ µl of blood) to keep the immune system active but the virus is quietly replicating or latent in reservoir cells of the body (Fauci et al., 1996; Baveja and Rewari, 2004). Eventually, an HIV-infected patient gets to the point where the $CD4^+$ T cell count drops below the 200 cells/µl of blood. Such an individual can no longer effectively fight off the virus. Additionally, the contraction of opportunistic infections like the common cold or TB only serves to stimulate the already weakened immune system. Such stimulation also triggers viral replication (Fauci et al., 1996; Baveja and Rewari, 2004). The continuous release of inflammatory cytokines to ward off the virus and the presence of diverse opportunistic pathogens contribute to destroying the architecture of the lymphoid tissue (Baveja and Rewari, 2004, Connor, 2011) which begins to be replaced by fibroid tissue (Baveja and Rewari, 2004). At this stage, the individual is diagnosed with AIDS and is likely to die, eventually. This death is hastened in the absence of ARTs (Ray et al., 2010). However there exists a group of individuals who do not progress to AIDS, even in the absence of ARTs. Such individuals are called long-term nonprogressors (LTNPs) or elite controllers (Okulicz et al., 2009). These unique individuals have become a hotspot for scientific research since they may hold important clues to further understanding the progression to AIDS and strategies for the design of effective therapies (Saez Cirion et al., 2007).

THE MAIDS MODEL

In an effort to understand the enigma presented by HIV/AIDS, several model systems have been used to study specific aspects of infection and development of immune deficiency. One such useful model is the mouse model in which certain strains of mice infected with the LP-BM5 strain of a Murine Leukemia Virus (MuLV) develop AIDS-like symptoms including immunodeficiency, susceptibility to opportunistic infections and swollen lymph nodes and spleens (Jolicoeur, 1991; Casabianca et al., 2003). This is termed murine AIDS, or MAIDS, and certain mouse strains, like BALB/c, are resistant to developing MAIDS post MuLV infection. Other mice, like the C57BL/6, are susceptible to developing MAIDS post MuLV infection (Pantaleo and Walker, 2001).

The MuLV strain used to induce MAIDS is a mixture of three viruses (Casabianca et al., 2003; Green and Li, 2006): the disease inducing replication-defective virus (BM5D) and two other helper viruses, namely the B-tropic ecotropic (BM5e) and the B-tropic mink cell focus-inducing (MCF) viruses (Casabianca et al., 2003). According to Chattopadhyay et al. (1991), although the helper viruses are not required for the development of MAIDS, their presence facilitates the cell-to-cell spreading of the disease inducing defective virus.

Differences in genetic makeup may yield differences in the immune system of the various mouse strains which in turn could confer either partial or full resistance to MuLV infections (Pantaleo and Walker, 2001). For example,

since the MuLV consists of a retroviral cocktail including defective and helper viruses, it must compete with other endogenous murine retroviruses in order to infect host cells (Pantaleo and Walker, 2001). This suggests that mice with high amounts if endogenous retroviruses may be less susceptible to MuLV infections (Pantaleo and Walker, 2001). Similarly, it is more likely that mice with a higher percentage of endogenous retroviruses will have immune cells harboring endogenous retroviral infection. This contributes to the reduced susceptibility of such mice to MuLV infections because host cells already infected with endogenous retroviruses cannot be re-infected with the MuLV; termed a super infection (Pantaleo and Walker, 2001). Furthermore, adult mice that express specific MHC class I or MHC class II loci may develop an immune response that elicits more effective cytotoxic, helper or antibody responses, hence allowing them to eliminate, either partially or fully, an MuLV infection (Pantaleo and Walker, 2001). Also, variations in the growth rate of MuLV target cells in different mouse strains can make some mice more susceptible to MuLV infection and others less susceptible. Particularly, mice that have a faster MuLV target cell growth rate will most likely be more susceptible to MuLV infections, and vice versa (Pantaleo and Walker, 2001).

Unlike HIV, MuLV does not target the CD4 receptor of T cells but rather the Fv-1 receptor of B cells and macrophages. The *Fv-1* receptor gene has different alleles (alternate forms of the same gene) and possessing a particular allele ($Fv\text{-}1^b$) confers susceptibility to MuLV infection. Both BALB/c and

C57BL/6 are *Fv-1*$^{b+}$ and so are equally susceptible to MuLV infection (Pantaleo and Walker, 2001). Susceptibility to the development of MAIDS, however, post MuLV infection effects depend on both MHC and non-MHC genes of the different mouse strains. Over time it has been shown that mice that carry an H-2^{d} MHC allele (eg. BALB/c) are more likely to be resistant to developing MAIDS post MuLV infection (Pantaleo and Walker, 2001). The C57BL/6 MAIDS susceptible mice do not possess the H-2^{d} MHC haplotype; these mice instead carry the H-2^{b} haplotype (Pantaleo and Walker, 2001). As mentioned above, other non-MHC genes are also important for resistance of, or progression to, MAIDS post MuLV infection. For example, non-MHC loci like *Rmcf*, are able to limit the spread of the virus by mechanisms not related to the functioning of the immune system (Hartley et al., 1990). Generally, MAIDS infected immunocompromised mice (like the C57BL/6) die within 16-22 weeks post infection due to their susceptibility to opportunistic infections; (Beilharz et al., 2004) with 100% mortality observed within 24 weeks post infection (Morse et al., 1992). MAIDS resistant mice, however, including the BALB/c mice, can live for many months post infection (Gilmore, 1997) and will typically die from natural causes, as opposed to the opportunistic infections observed in the C57BL/6 MAIDS susceptible mice.

As earlier indicated, there are several similarities between the symptoms observed in MAIDS and AIDS. However, it is also worth noting that there exist several differences between the two diseases, especially with regard to the cellular

orchestration of the two diseases. For instance, although both are retroviruses, HIV is a lentivirus (Jolicoeur, 1991) while MuLV is a C-type retrovirus (Beilharz et al., 2004). Furthermore, while the main cell population for attack in HIV infections are cells that possess the CD4 receptor (including $CD4^+$ T cells, dendritic cells and macrophages), the main cell population targeted by MuLV infection is B cells and macrophages (Beilharz et. al, 2004; Green and Li, 2006). As a result, while $CD4^+$ T cell numbers and function is drastically reduced in humans prior to progression to AIDS, the $CD4^+$ T cell count does not drop in murine-induced AIDS, although these cells do experience inhibited function (Morse et al., 1992).

PREVIOUS WORK IN THE LAB AND PROPOSED STUDY

Thus far, several discoveries have been made concerning potential factors involved in AIDS progression using the MAIDS model. Although all these findings cannot be discussed in this thesis, the results obtained from a few cytokine-related MAIDS studies are worth noting. One of these suggests that there is an interesting interplay between pro- and anti- inflammatory cytokines of the immune system. Some of the cytokines released by a mouse infected with MuLV and progressing towards MAIDS include the immunosuppresants IL-4 and IL-10, known to favor a Th2 (humoral) mediated immune response. IL-4 is a cytokine made principally, but not exclusively, by $CD4^+$ T cells (King and Mohrs, 2009). It is important for immunoglobulin (antibodies) type E and G1 switching

(King and Mohrs, 2009). It is also essential in inducing the T_H2 (humoral, anti-inflammatory) arm of the immune response and does so by downregulating the expression of pro-inflammatory genes like interferon gamma (IFN-γ; King and Mohrs, 2009). The cytokine IL-10, like IL-4, also exhibits anti-inflammatory properties (Saraiva and O'Garra, 2010). It achieves this by limiting T_H1 (pro-inflammatory) immune responses as well as inducing the differentiation of Tregs, another cell type that produces more IL-10 in a positive feedback loop mechanism, as shown described in Fig. 5 (Saraiva and O'Garra, 2012). IL-10 is produced by an array of cells that make up the immune system, including Tregs, dendritic cells, and B cells, among others (Saraiva and O'Garra, 2012).

According to Morse et al. (1995), during the initial response to the MuLV infection, some cytokines in the spleens of MAIDS-resistant BALB/c and -susceptible C57BL/6 mice exhibit similar patterns of mRNA expression. Elevated levels of IL-4 and IL-10 are seen in both one week post MuLV infection, which drops to baseline by 3 weeks post infection. Beyond 3 weeks, however, IL-10 and IL-4 mRNA expression remains high in infected C57BL/6 mice, but drops in BALB/c mice. The high expression of these cytokines (along with IFN-γ) yields a deficiency in co-stimulatory activity when APCs interact with T cells, which eventually become anergic or unresponsive to antigen presentation, and an effective adaptive immune response is stalled (Morse et al., 1995). This profile is observed in C57BL/6 mice (Morse et al., 1995). Furthermore, Hoshi et al. (2010) demonstrated that in the absence of the anti-inflammatory enzyme IDO, and in the

presence of pro-inflammatory cytokines, like the type-1 interferons, there is a reduction in the replication of the virus in MuLV infected C57BL/6 mice. This suggests that the absence of IDO facilitated an increase in pro-inflammatory cytokines which mediated reduced viral replication (Hoshi et al., 2010). However, as (is) with all (of) scientific research, more work needs to be done in order to understand the important but complex role IDO, and other anti-inflammatory proteins, may play in immunomodulation of immunocompromised mice.

The above paragraph suggests that high mRNA levels of IL-10 and IL-4 may be important for the progression of MAIDS post MuLV infection in C57BL/6 mice. On the part of IL-4, these findings are supported by other researchers like Kanagawa et al. (1993) whose work demonstrate that IL-4 is required for a rapid progression to MAIDS in susceptible mice, since knock-out mice for this cytokine demonstrate slower progression. Yet Green et al. (2008) show that for the same doses of LP-BM5 MuLV isolate, IL-10 knockout C57BL/6 mice exhibit a more exaggerated MAIDS condition compared to wild type mice that produce IL-10 normally.

These results are not in isolation from observations made in human studies. In fact there seems to be an interesting interplay between anti-inflammatory cytokines and T-cell responses in chronic HIV progression. According to studies by Elrefaei at al. (2009), different subpopulations of $CD4^+$ and $CD8^+$ T cells produce different cytokines that may create an immunosuppressive environment in the immune systems of individuals with chronic HIV infections. For example,

they mention that the presence of a distinct group of TGF-β and IL-10 producing $CD8^+$ T cells inhibit HIV-specific T- cell effector responses and HIV-specific cytolysis, respectively (Elrefaei et al., 2009). Additionally, they suggest that these two subpopulations of $CD8^+$ T cells may be regulated by the expression of CTLA-4 such that the blockade of CTLA-4 leads to the production of pro-inflammatory cytokines like IFN-γ but a decrease in production of TGF-β and IL-10.

Overall these findings suggest that there is a complex pattern in the expression of inflammatory and immunosuppressing cytokines in both MuLV infected mice and HIV infected individuals. Additionally, it is important to point out that the MAIDS related studies looked at MuLV infected mice no earlier than one week post-infection, and more typically after symptoms of MAIDS had already began to surface in susceptible mice and resistant mice had begun to recover. Additionally, expression levels of the above mentioned cytokines were typically measured via RT-PCRs. While these experiments give an accurate representation of the cytokine mRNA levels, they are unable to provide information on how much of this mRNA, for example, is actually translated into protein. Furthermore, the results from all of the studies cited were derived from studies utilizing mouse spleens or human blood. However, as described in the pathology of HIV/AIDS, initial interactions between virus and host cells likely occur in the lymph nodes (Baveja and Rewari, 2004). Therefore, in addition to

looking at the spleens of infected mice, the lymph nodes may also be an interesting organ to study, as would the earliest stages of MuLV infection.

The purpose of this thesis project is therefore to study the protein expression level of specific immunosuppressant cytokines in both MuLV resistant (BALB/c) and susceptible (C57BL/6) mice. Specifically, I will be studying the protein levels of IL-10 in the lymph nodes of both mouse strains at very early time points (within, but not after, the first week of MuLV infection). This choice of an early time point for investigation is important because it will provide insight into the direction taken during the innate to adaptive transition in the immune response to this virus in each mouse strain, which could influence later susceptibility to the development of MAIDS. This information is important because it can potentially provide insight into the factors inducing human vulnerability to AIDS following HIV infection.

MATERIALS AND METHODS

Organ Isolation, Cell Lysis and Sample Preparation

Mice from both strains (BALB/c and C57BL/6) were injected intraperitoneally with 1ml of the LP-BM5 isolate of MuLV at three days and seven days prior to sacrificing via carbon dioxide inhalation (n=4). The viral titre was 3.6 x 10^4 PFU/ml. An additional 4 mice from each strain were sacrificed uninfected, to use as naïve controls. Spleens as well as brachial, axial and inguinal lymph nodes were isolated from each mouse. Mesenteric lymph nodes were not included for this study owing to their differences in gene and cytokine expression relative to the afore mentioned peripheral lymph nodes. Each spleen and lymph node was cut into tiny pieces to facilitate homogenizing at a later stage. Spleen or pooled lymph nodes were then placed separately in sterile, labeled, round bottom tubes containing cold lysis buffer (1x protease inhibitor cocktail from BD BaculoGold in PBS with 0.05% Triton X-100 at pH 7.1) and homogenized carefully for approximately 30 seconds with a polytron homogenizer set at ¾ speed. Between each homogenizing session, the polytron rod was washed twice with PBS and once with 70% ethanol (followed by air drying) to prevent contamination between samples. Next, the samples were transferred into 2ml centrifuge tubes and centrifuged at 12,000g for 10 minutes in a cold room. After centrifugation, supernatants from each sample were transferred into newly labeled 1.5ml centrifuge tubes. Spleens were divided into 50µl aliquots and lymph nodes were divided into 25µl aliquots. All aliquots were stored at -80^{o}C until needed.

Sandwich ELISA:

To ensure the IL-10 ELISA assay was viable, the kit (Mouse IL-10 ELISA set Cat. No. 555252, BD Biosciences) was first tested with the standard chemokine included. Only after affirming the viability of the kit and accuracy of the standard curve were samples also prepared and tested.

Plate Preparation

Non-sterile, BD Falcon polystyrene 96 well ELISA plates (Cat. No. 353279) were used in these experiments. Wells were coated with 100µl of a 1:250 dilution of IL-10 capture antibody (BD Biosciences Cat. No. 555252) diluted in coating buffer (12.49g Na_2HPO_4, 15.47g NaH_2PO_4, pH 6.5; used within 7 days of preparation). The capture antibody was brought to room temperature immediately before dilution, and then quickly returned to storage at 4^0C. The dilution ratio was 1:250. Next, the plate was sealed with parafilm and incubated overnight at 4^oC. The following day, wells were washed 5x with greater than 300µl of wash buffer (0.05% tween-20 in PBS stored at room temperature). This buffer content was dumped carefully but forcefully into a waste container and the plate blotted efficiently over clean paper towels to remove any excess buffer or air bubbles. Coated wells were finally blocked with 300µl of assay diluent (10%FBS in PBS at pH 7, stored at 4^oC but brought to room temperature prior to each use). The plate was covered with parafilm and incubated for 3.5 hours at room temperature (extended blocking times did not adversely affect results). After this incubation step, the wells were aspirated and washed 5 times as previously described.

Sample Preparation

About 30 minutes before the blocking step was completed, standards and samples were prepared. The IL-10 standard, reconstituted to 120ng/ml in 1ml of de-ionized water and stored at -80^{0}C in 50µl aliquots, was diluted to 2000pg/mls (this amounts to a 1:60 dilution) in assay diluent (PBS with 10% FBS, pH 7.0; used within 3 days of preparation). From this, two-fold serial dilutions between 1000pg/mls and 31.25pg/mls were prepared. To ensure pipetting accuracy, serial dilutions were prepared to recover 500ul of sample per tube, as shown in Figure 8.

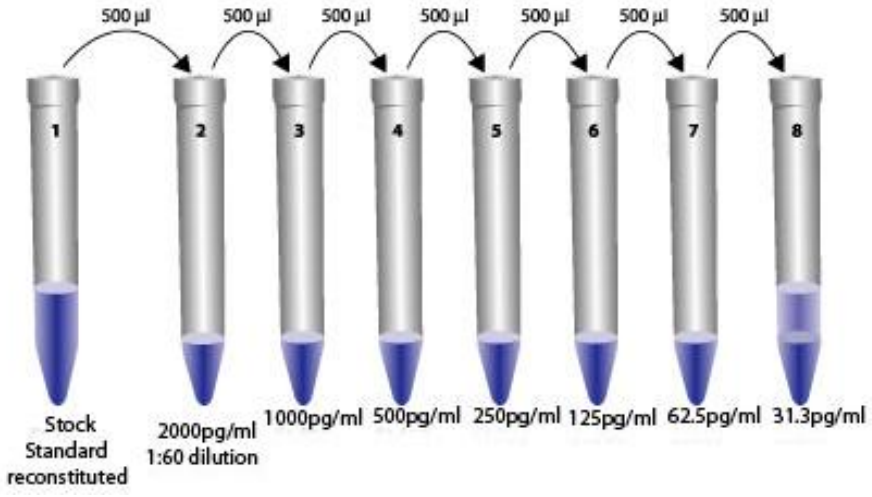

Fig.8: Diagrammatic Representation of Serial Dilution of the IL-10 Standard. After diluting the reconstituted stock at 1:60 in assay diluent, 500ul of IL-10 standard at 2000pg/ml was pipetted from tube 2 and added to 500ul of assay diluent in tube 3, diluting the original 2000pg/ml by half. This was repeated until the lowest concentration of 31.3pg/ml was obtained. Adapted from: Cayman Chemical. Available at: http://www.caymanchem.com/app/template/analysis,EIA.vm/promo/analysis.eia. Accessed: April, 2012.

Experimental samples were diluted at 1:10, 1:50 and 1:100, in assay diluent in order to determine the appropriate dilution factor that yielded IL-10 protein concentrations within the limits of the IL-10 standard curve. All samples and standards were stored on ice until needed. Additionally, all standards and samples were tested in triplicates to account for well-to-well variations and dispensed at 100µl per well after sufficient vortexing. The plate was sealed and incubated for 2hrs, at room temperature after which the aspiration and washing step described was repeated 5 times. For each experiment, thawed samples were discarded and none were re-used to avoid problems with protein degradation due to a freeze-thaw cycle.

In the next step, 100µl of Working Detector antibody (Biotinylated anti-mouse IL-10 detection antibody with streptavidin-horseradish peroxidase) was dispensed into each well. Dilution for the Working Detector was prepared as follows: first, the detection antibody was quickly brought to room temperature and diluted at 1:250 in assay diluent. Then, 15 minutes before the Detector was used, the streptavidin-horseradish peroxidase was also quickly brought to room temperature and diluted in the same solution, also at a 1:250 dilution. (It was important to make sure that both the Detector antibody and the streptavidin-horseradish peroxidase were returned to 4^0C immediately after use to prevent protein degradation). This mixture was vortexed sufficiently (without creating too many bubbles) before wells were filled with 100µl aliquots. Again the plate was

sealed and incubated for 1hr at room temperature. This was followed by 10, slow, deliberate, 30 second aspiration/washing steps with the wash buffer.

Next, freshly prepared substrate solution at a 1:1 ratio of solution A and B was then added to each well at 100µl per well. The plate was incubated, in the dark, for 30 minutes without a parafilm seal after which the reaction was stopped with 50µl of stop solution (1M H_3PO_4). It is important not to incubate beyond this 30 minute period as this may result in an overdeveloped signal, especially for standard dilutions at higher concentrations. Finally, within 30 minutes of stopping the reaction, the plate was read with an ELISA Plate Reader (SpectraMax, Molecular Devices) set at 450nm with a wavelength correction of 570nm, and pre-warmed to about 20^{o}C. The latter wavelength is included to correct for optical imperfections that may occur in the plate.

Time and sample quantity constraints did not allow for a BioRad assay to be performed in order to ascertain the total protein concentration for lymph nodes and spleens isolated from each of the eight mice used. However, the IL-10 concentrations for each well of the ELISA plate for each experiment was evaluated. This can be determined via the following procedure: first, to generate the proper controls, “blank” wells were designated as those containing appropriately diluted lysis buffer rather than just assay diluent. This completely eliminates the triplicate wells that contained assay diluent as blank from the experiment template in the SoftMax Pro software. Lysis buffer is set as a blank, instead of the assay diluent, because our samples (lymph nodes and spleens) are

homogenized in the former. Once a blank has been designated, its value is automatically subtracted from every other well containing a sample reading on the template. After a plate is read, a table is generated. This table contains true OD readings (red), and corresponding optical corrections highlighted in green (Fig. 9). The optical correction value is the OD reading at 570nm subtracted from the 450nm OD reading for each well. A set of triplicates is considered if they do not differ by more than 0.02. In the event where the comparison of replicate wells yields one among three with a difference greater or less than 0.02, that well is termed an outlier and is not considered in further calculations. Its value is, however, imputed with the average of the remaining two wells that are within range.

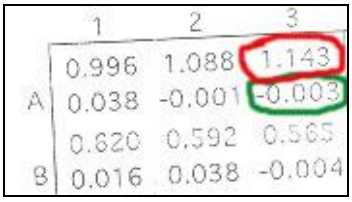

Fig.9: A section of a template table generated from the SoftMax Pro after an ELISA plate has been read. The value highlighted in green is subtracted from that highlighted in red. This calculation is done for every well that is part of a set of triplicate wells. Resulting differences among a set of triplicates that differ by more 0.02 are considered experimental outliers.

Once the corrected values have been obtained, they are entered into the linear equation obtained from the IL-10 standard curve (Fig. 10) as Y-values in

order to solve for the concentration (X variable) of IL-10 per sample, per well. Since each sample was prepared at a dilution (and the final dilution used for these experiments in which lymph node homogenates were tested was 1:50), the resulting X value is multiplied by 50 to yield the total IL-10 protein concentration in each original sample. For each set of triplicates, the average of these concentrations is obtained. This is considered the estimated total IL-10 protein concentration in the spleen or lymph node of each original mouse sample (n=4).

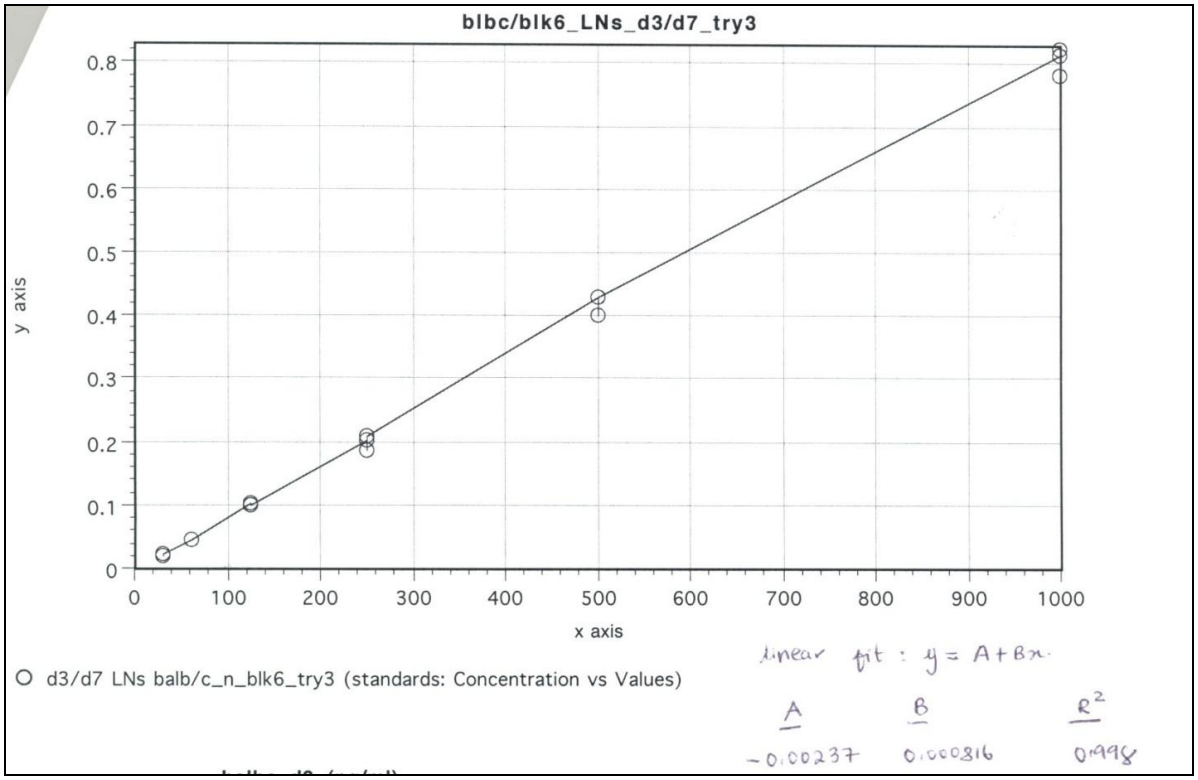

Fig.10: Sample of a standard curve from an IL-10 ELISA. Linear fit represents the equation used to calculate final IL-10 concentration per well.

Once generated, the average IL-10 concentrations within strains and across time points were used to plot a scatter graph of mean IL-10 protein concentration (see Results section). In these graphs, standard error bars were used to elucidate the statistical significance, if any, in differential IL-10 protein expression between strains and across time points. In the absence of a larger number of mice (n=4 in this experiment), two ELISA experiments were conducted with the same samples and the concentrations obtained were combined for corresponding strains and time points. This was done in order to obtain sufficient power for ANOVA statistical analysis.

Analysis of Variance (ANOVA)

Using the program Statistical Package for the Social Sciences (SPSS), a 3 x 2 full factorial Analysis of Variance (ANOVA) was used to determine the statistical significance of IL-10 protein concentration differences between mouse strains (C57BL/6 and BALB/c; this is where the '2' in the factorial comes in) and across tested time points (naïve=day 0, day 3, and day 7 post infection; this is where the '3' in the factorial comes in). Preliminary attempts at ANOVA uncovered unequal within group variances. This violates the underlying assumptions of ANOVA with regards to this experiment that mice of a particular strain (which are also biological clones of each other) should not vary significantly among themselves. Thus in order to meet the underlying assumptions of ANOVA, the natural log of concentration was created and used as

the dependent variable. The main effect of mouse type and time, as well as an interaction term, were tested in order to consider how time and mouse type (strain) affected concentration levels of IL-10 protein. Additionally, via ANOVA, an outlier was obtained among the C57BL/6 susceptible mice. Since this mouse was not an outlier by biological standards, i.e. corrected OD readings within the replicate set of wells did not vary by 0.02, two sets of statistical tests were performed: one including the statistical outlier, and another excluding it to ensure significance obtained from results (if any) were not due to the presence of this one outlier mouse.

RESULTS

The main technique utilized for experiments in this thesis was the Enzyme Linked Immunosorbent Assay (ELISA). ELISA is a preferred method for attaining the level of protein expression in an organ of interest post mRNA expression studies. This is because while mRNA studies tell how much of a gene is being transcribed at a particular time, they do not give an accurate description of how much protein is actually translated from the transcribed mRNA.

Using the IL-10 kit from BD Biosciences (see Materials and Methods), initial trials were run on lymph node homogenate samples using the BD Sciences IL-10 standard as guide. Standard curves that accurately depicted IL-10 protein concentration levels were compared to a lymph node homogenate diluted at 1:50. An example of an accurate standard curve can be seen in Fig. 10 (Materials and Methods section). Two ELISA experiments were used to perform data analysis on IL-10 protein concentrations in MAIDS resistant (BALB/c) and MAIDS susceptible (C57BL/6) mice. In each ELISA there were three time points (naïve or Day 0, Day 3 and Day 7) and a total of four animals (designated Animal 1, Animal 2, Animal 3and Animal 4) per time point. Each experiment yielded three data points per animal, per time point, and 12 data points per strain, per time point. Consequently, the combination of both trials yielded 6 data points for each animal number (three from trial 1 and three from trial 2), and 24 data points per strain, per time point (Fig. 11). As a result, even though the number of mice used per time point (n= 4) was rather few compared to other studies where the sample size

can be as large as 12, the increased number of data points from the combined experiments served to increase the power of results during statistical analysis via the Analysis of Variance (ANOVA). No data could be obtained for C57BL/6 (susceptible) mouse # 4 for only trial one at D3 post MuLV infection as there was not enough of this sample to be tested. Fortunately, the combination of data from both ELISA trials yielded 9 data points for ANOVA.

Table 1: Tabular representation of OD readings and corresponding concentration values obtained from ELISA tests 1 and 2. OD values are typically tight within triplicate wells. Red values indicate examples of methodological outliers which differ from triplicate replicates by more or less than 0.02. In each experiment, there are 3 data points per animal, per time point and four animals for each time point. As a result, there was a total of 24 data points to be used for BALB/c mice sacrificed at 3 days post MuLV infection, instead of 12 that would have been obtained from one experiment. This is true for all mice at all time points except C57BL/6 susceptible mice in which there was not enough D3 sample to be tested for Animal 4.

	O.D Readings Trial 1 Day 3 Triplicates Balb/c			Corresponding Concentrations (pg/ml) Balb/c Day 3		
	Well	**Well2**	**Well 3**	**Well**	**Well2**	**Well 3**
Animal 1	0.069	0.086	0.077	2502.273	3054.221	2762.013
Animal 2	0.038	0.048	0.043	1495.779	1820.455	1658.117
Animal 3	0.020	0.014	0.019	911.3636	716.558	878.896
Animal 4	0.072	0.073	0.063	2599.675	2632.142857	2307.468

	O.D Readings Trial 2 Day 3 Triplicates Balb/c			Corresponding Concentrations (pg/ml) Balb/c Day 3		
	Well	**Well2**	**Well 3**	**Well**	**Well2**	**Well 3**
Animal 1	0.034	0.027	0.026	2228.554	1799.632	1738.358
Animal 2	0.017	0.011	0.021	1186.887	819.240	1431.985
Animal 3	0.014	0.009	0.008	1003.064	696.691	635.417
Animal 4	0.024	0.024	0.022	1615.809	1615.809	1493.260

The data obtained was rather consistent for both experimental trials so that combining both experiments was not an issue of concern. Additionally, box and whisker plots were generated to shed light on the general distribution of data for BALB/c resistant mice (Fig. 11) and C57BL/6 susceptible mice (Fig. 12).

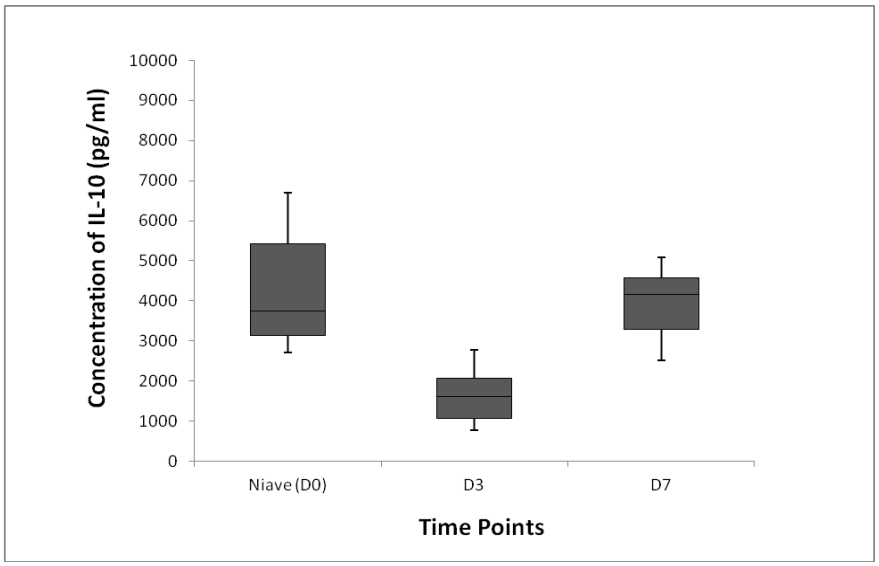

Fig. 11: Box and whisker plot for BALB/c (resistant) mice. Data is taken from both trials1 and 2. The box and whisker plot gives a general distribution of the data without reference to statistical significance across time points. Whiskers represent the range of data distribution. Black solid line in each box represents the median (50^{th} percentile IL-10 concentration at each time point). Area above it represents IL-10 concentrations between 50^{th} and 75^{th} percentile and that below represents IL-10 concentrations between 50^{th} and 25^{th} percentile. Data seems to be skewed at naïve (D0) and D7 with a larger value of IL-10 above the median (3743.5pg/ml) at D0 and some lower values of IL-10 below the median (4158.7pg/ml) at D7. In D3, there seems to be an equal distribution of concentration values above and below the median (1616.5pg/ml).

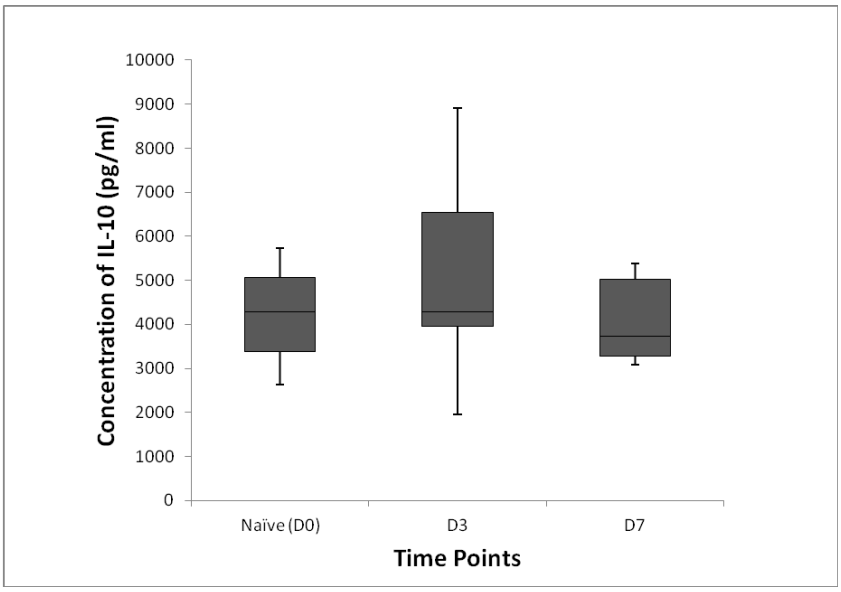

Fig. 12: Box and whisker plot for C57BL/6 (susceptible) mice from trials 1 and 2. Similar to that of BALB/c, the box and whisker plot gives a general distribution of the IL-10 concentration data but with no references to statistical significance. Again, whiskers represent the range of data distribution and solid lines in each box represents the median (50^{th} percentile IL-10 concentration at particular time point). For C57BL/6, data seems to be homogenous at naïve (D0) but skewed at D3 and D7 with a larger value of IL-10 above the median at both time points. Median IL-10 concentration was 4291.5pg/ml at D3 and 3733.7pg/ml at D7. Median at D0 was 4281.25pg/ml.

Data was analysed using univariate ANOVA to determine significant differences in IL-10 between both strains and across the three time points. Additionally, IL-10 protien concentration values were log-transformed before proceeding with univariate ANOVA analysis, in order to meet the assumption of equal variances. A statistically significant interaction ($p<0.001$) was detected,

indicating that the expression of IL-10 depends on both factors: strain and time. With a significant interaction term, one cannot compare one strain versus the other, without also including information on time. Follow-up t-tests were therefore conducted to evaluate where these differences occurred.

Naive mice (day 0) from both strains produced large amounts of IL-10 protein, which can be presumed to be the basal level of protein expression since day 0 mice were not infected with MuLV. This basal level expression, if it may be called as such, was observed, again in day 7 mice. There was no statistically significant difference between the amount of IL-10 produced between strains at either of these time points. At day 3, however, a differential expression of IL-10 was observed between BALB/c (resistant) and C57BL/6 (susceptible) mice with BALB/c producing a significantly lower amount of IL-10 relative to its C57BL/6 counterpart which seems to produce IL-10 above the basal level expression ($n= 15$, $df= 13$, $p= 0.001$). Data on the log-transformed average amount of IL-10 produced between mouse strains and across time points was plotted on a line graph via ANOVA (Fig. 13). A graph of means and their standard errors (Fig. 14) was also generated in Excel®.

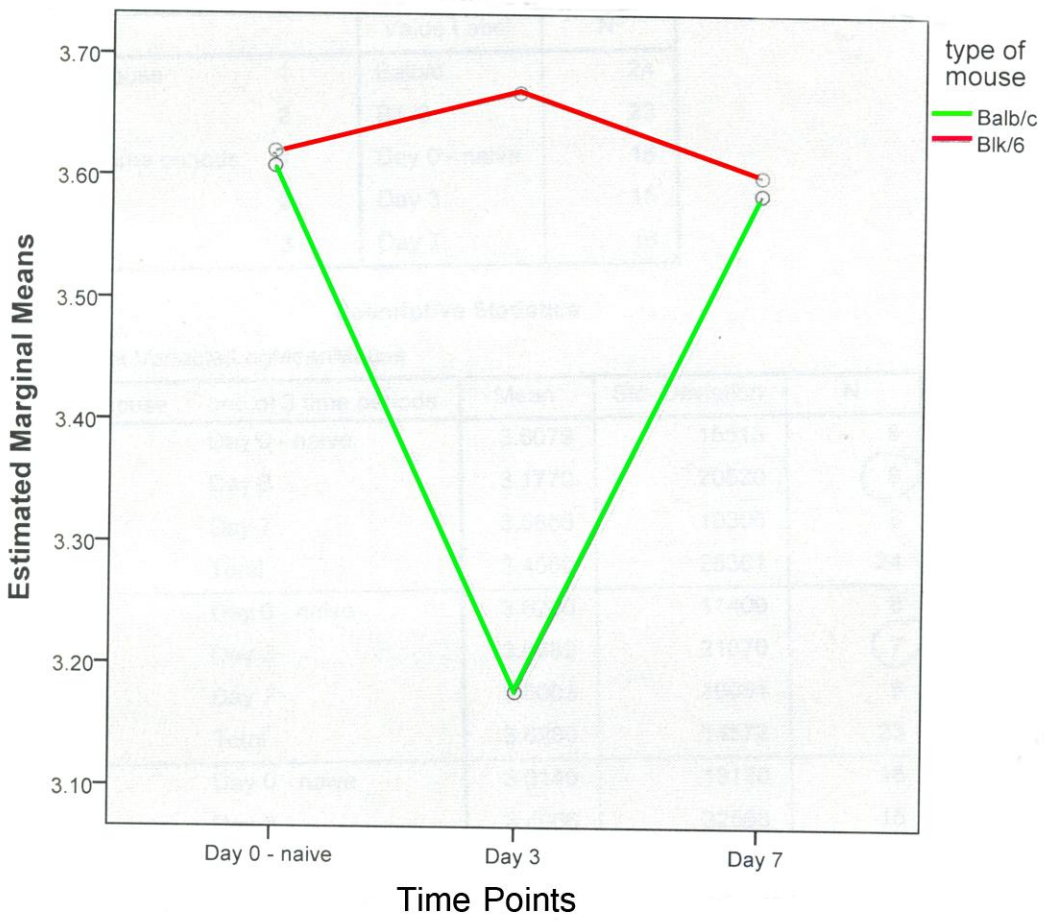

Fig. 13: Log transformed line graph representation of the average IL-10 concentration (pg/ml) produced by both mouse strains across the 3 time points. This is not longitudinal data i.e., the same mouse was not used at naïve (D0), D3 and D7. Instead different mice were sacrificed and used at each time point, for each strain. Linear graphs are therefore chosen for the purposes of visual comprehension only. A significant differential expression (p=0.0001) was observed in IL-10 protein concentrations between the two strains at D3.

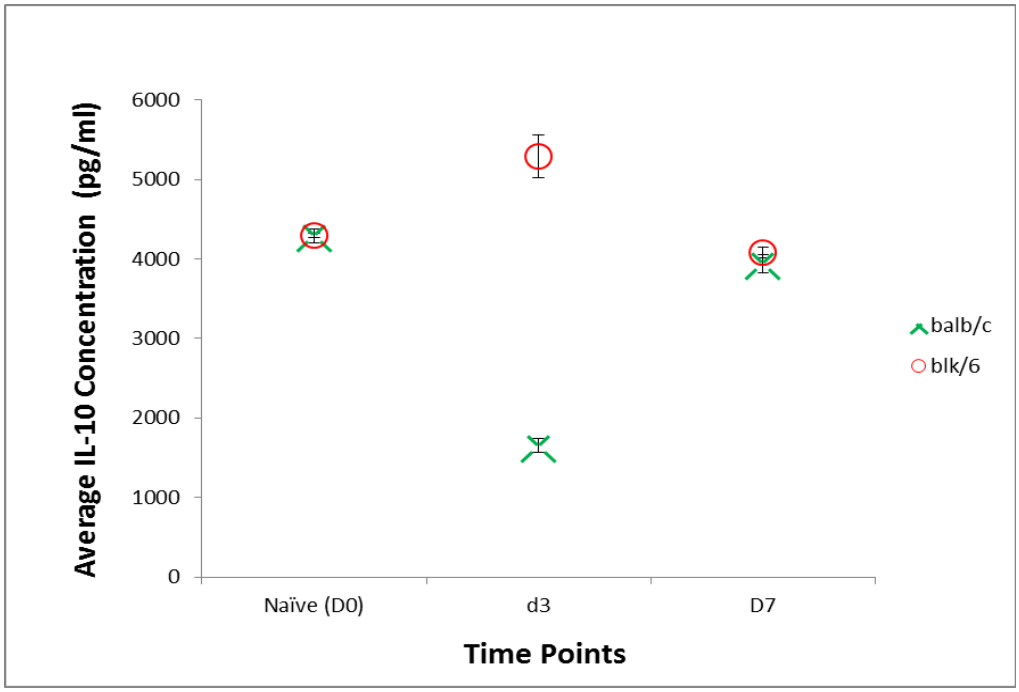

Fig. 14: Mean and standard error of IL-10 concentration (pg/ml) produced by both mouse strains across 3 time points. Error bars were small indicating minimum deviation. Additionally, IL-10 expression levels were not significantly different at the naïve (D0) and D7 time points, and so overlap in this graph. However, differential expression of IL-10 is observed between mouse strains, at D3 time point, $p < 0.0001$.

An exploration of outliers found an unusually high concentration of IL-10 in C57BL/6 (susceptible) mouse number 1 at D3 time point. This statistical anomaly was observed in both trials. In order to consider the possibility that this outlier was responsible for the significant differential expression ($p < 0.0001$, Figs. 13

and 14) of IL-10 between mouse strains at D3, ANOVA was conducted without this data point and similar conclusions were reached. Mean concentration values for IL-10, were also calculated after removing this outlier and a corresponding linear graph on a log scale was derived (Fig. 15).

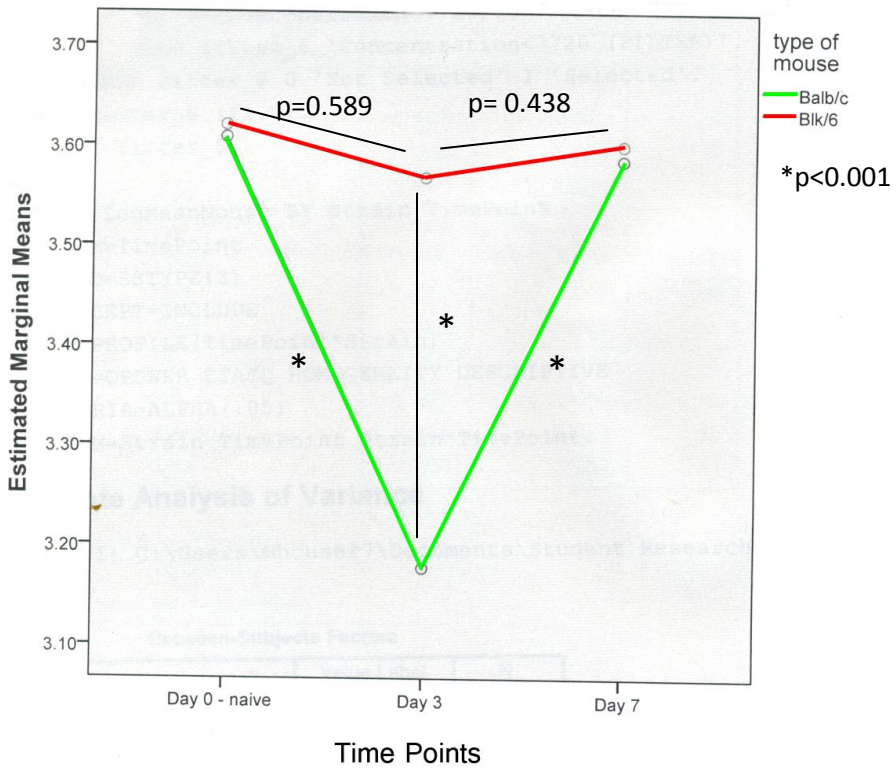

Fig. 15: Log transformed line graph representation of the average IL-10 concentration (pg/ml) produced by both mouse strains across 3 time points. The dip observed in C57BL/6 (susceptible) is due to the removal of the observed outlier. Again, a significant differential expression (p= 0.001) was observed in IL-10 protein between the two strains at D3. This is a linear graph, it is not longitudinal data i.e., the same mouse was not used at naïve (D0), D3 and D7 time points. Instead, different mice were sacrificed at each time point, for each strain. Linear graphs are therefore chosen for the purposes of visual comprehension only.

Mean concentration of IL-10, standard deviation and the number of mice used with and without this outlier are displayed in table 2. It was observed that without the outlier, there was still a significant interaction between strain and time of IL-10 albeit, at a higher p-value (*p= 0.002).

Table 2: Tabular representation of the mean IL-10 protein concentration (pg/ml) expressed by mice of both strains at all three time points. M stands for 'mean', S, represents 'standard deviation' and N means 'number of wells combined from two trials'. C57BL/6 and **C57BL/6 represents data for susceptible mice with and without the outlier, respectively. There is no difference in IL-10 expressed in C57BL/6 and **C57BL/6 at D0 (naïve) and D7 because at these time points, there is no outlier, hence no values were eliminated. At D3, differential expression is still observed in C57BL/6 and **C57BL/6 but standard deviation and IL-10 concentration expressed is smaller in **C57BL/6 than C57BL/6 due to removing outlier from data points.

	Day 0 (Naïve)		
	M	S	N
BALB/c	4290.911743	1567.288	8
C57BL/6	4294.804399	1102.004	8
**C57BL/6	4294.804399	1102.004	8
	Day 3		
	M	S	N
BALB/c	1650.15279	733.1333107	8
C57BL/6	5164.5462	2415.186841	7
**C57BL/6	3892.13798	1228.24927	5
	Day 7		
	M	S	N
BALB/c	3942.827434	868.2051196	8
C57BL/6	4078.951012	958.0907629	8
**C57BL/6	4078.951012	958.0907629	8

As observed in Figs. 13 and 15, IL-10 protein concentrations in both mice at the naïve time point (D0) were very similar to those obtained at the D7 time point. This suggests that mice are behaving in a similar manner at these times. To determine if MuLV viral load at D0 (naïve) and D7 were also identical, a graph of average IL-10 concentration was superimposed with data of viral load mRNA obtained by Dr. S. Bakkour (unpublished data). Results showed that although IL-10 concentrations appeared to return to basal level at D7 post-MuLV infection in both mouse strains, the viral mRNA was actually still increasing in the C57BL/6 (susceptible) mice but had decreased to zero in the BALB/c resistant mice. This is shown in Fig. 15.

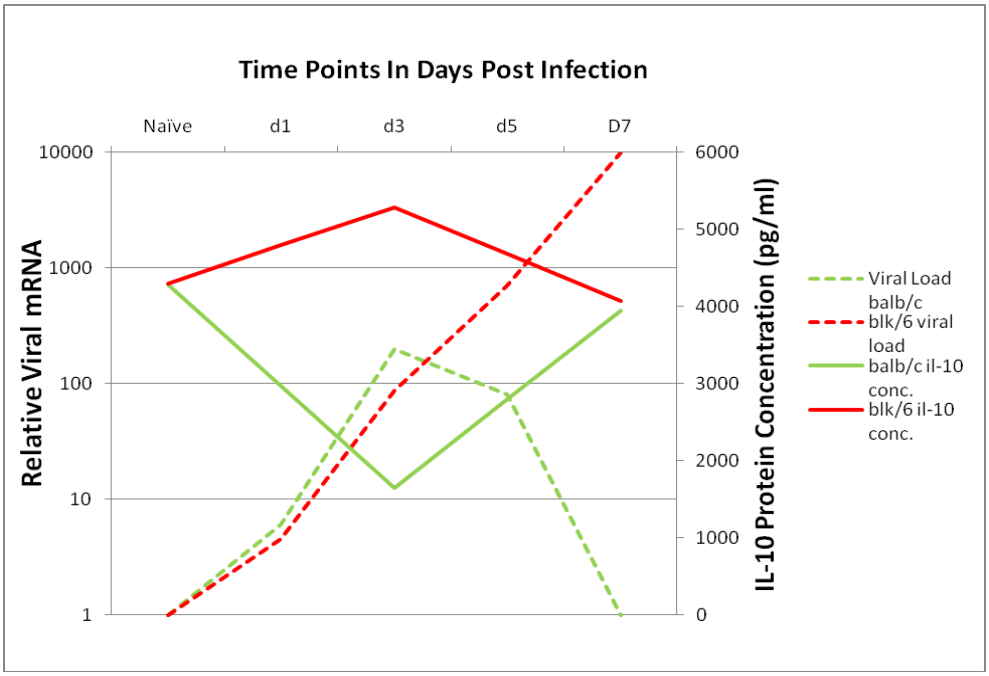

Fig. 16: Graphical representation of the overlay of relative viral mRNA (Dr. S. Bakkour, unpublished data) and IL-10 protein (pg/ml) production at different times before (D0, naïve) and after MuLV infection. Viral mRNA is highest at D3 post infection in both mouse strains. However, at that time, IL-10 concentration is highest for C57BL/6 susceptible mice and lowest for BALB/c mice (resistant). Thereafter, there is an observed decrease in viral load in BALB/c mice to close to zero by D7, but the viral mRNA in C57BL/6 mice continues to increase.

DISCUSSION

To this day, HIV/AIDS remains an issue of global concern, inspiring scientists to continually strive towards going beyond antiretroviral drugs to finding a cure or vaccine. In line with this effort several model systems have been developed over the years to study various aspects of HIV infection and subsequent AIDS immunodeficiency developments. One such useful model is the murine model in which certain strains of mice infected with the MuLV develop AIDS like symptoms termed murine AIDS or MAIDS (Casabianca et al., 2003).

The MAIDS model has been instrumental in facilitating a better understanding of the pathology involved in AIDS. For instance, in 2005, Baliga et al. made an important discovery stating that the increase in life expectancy of HIV infected individuals also resulted in an increase in an array of other health complications, including cardiovascular disease, with no direct relationship between these complications, and immunodeficiency or opportunistic infections. Using the MAIDS model, they demonstrated for the first time a definite functional vasculopathy with endothelial involvement that was associated with, and correlated to, increased oxidative stress and specific endothelial activation. Their findings were echoed in a relevant population of HIV infected individuals. Furthermore, in other investigations, results have shown that there is an interesting interplay between pro- and anti-inflammatory cytokines in the immune system of MAIDS susceptible mice. For example, Hoshi et al. (2010) demonstrated that there is a reduction in MuLV replication rates in C57BL/6

susceptible mice in the presence of pro-inflammatory cytokines like type-1 inteferons, but in the absence of anti-inflammatory proteins, like the enzyme IDO. In both studies (and this seems true for the large majority of MAIDS research), only mice with a C57BL/6 background were studied. Additionally, mice were typically sacrificed within a time frame of one to five weeks post MuLV infection. Furthermore, where necessary, the spleen was the organ of interest for the study of differential expression patterns of pro-inflammatory and anti-inflammatory cytokines post MuLV infection.

Most MAIDS research is conducted using C57BL/6 susceptible mice because they progress to MAIDS post MuLV infection and therefore present as an ideal system to observe in order to understand the pathogenesis of MAIDS (and potentially AIDS). For this same reason, a later time point is preferred in order to be able to pinpoint changes that may have occurred in the immune system during the progression of the disease. Although research using these parameters has been insightful, much remains to be understood regarding the mechanisms at play in what eventually leads to the failure to clear the virus and development of AIDS or MAIDS. Additionally, considering that the spleen is the site for blood filtration and a secondary lymphoid organ that responds to blood-borne pathogens and antigens (Parham, 2009), it is an easy and appropriate organ to monitor during AIDS and MAIDS progression. However HIV and MuLV infect many leukocytes, including T cells, B cells and macrophages (Pantaleo and Walker, 2001). Since these cells spend much of their time in lymph nodes and ultimately form a part of

lymph (extracellular fluid and cells carried in lymphatic system-Parham, 2009), these secondary lymphoid organs may serve as a hot spot for viral activity post infection. As a result, they are also appropriate organs to study, although research using lymph nodes in humans is quite difficult.

The aim of this thesis, therefore, was to study differential cytokine expression patterns in the lymph nodes and spleens of MAIDS resistant and susceptible mice at the very onset of MuLV infection. This is being done in order to determine the direction taken by the immune system of each mouse at time points during which innate to adaptive immune response transitions occur and adaptive pathways are established. Particularly, the anti-inflammatory cytokine IL-10 was studied in an attempt to understand its potential role in immune suppression that might influence susceptibility to MAIDS.

Three time points post MuLV infection were studied: day 0 (D0, or naïve), day 3 (D3) and day 7 (D7). To reiterate our observed results, at D0, both susceptible (C57BL/6) and resistant (BALB/c) mice expressed a relatively high basal level of IL-10. At D3, however, there was a differential expression of IL-10, with resistant mice (BALB/c) producing significantly less than C57BL/6 susceptible mice. IL-10 expression levels at D7 were similar to those observed at D0: both BALB/c (resistant) and C57BL/6 (susceptible) mice expressed a similar, but relatively high amount of IL-10.

Several studies have identified IL-10 as a key anti-inflammatory immune system regulator. Though mainly produced in Treg cells (part of the adaptive arm of the immune response), IL-10 is not a cell-type specific cytokine. That is to say, it can be expressed by many other immune cells, like monocytes and macrophages, which make up the innate arm of an immune response (Commins et al., 2010; Saraiva and Garra, 2010). IL-10 works via the IL-10R1 and IL-10R2 receptor complex (Commins et al., 2012) and functions with a half-life of just about one hour (Trong et al., 1997).

Overall, IL-10 tames an active immune system by inhibiting inflammatory responses in a number of ways. For example, it can inhibit the production of pro-inflammatory cytokines like IFN- γ and TNF-α (Commins et al., 2012) and can also prevent the expression of receptors required for T cell co-stimulatory signaling (Ding et al., 1994). Furthermore, in order to ensure that immune cells do not produce an exaggerated response to usually harmless particles, like allergens, IL-10 is constitutively expressed in the respiratory tracts of healthy individuals to induce tolerance in immune cells towards these allergens (Borish et al., 1996). Though IL-10 is in itself an immunoregulatory cytokine, it belongs to a larger IL-10 family which consists of many other cytokines, including IL-19, IL-20, IL-24 and IL-26. While these cytokines share regions of homology with IL-10, the latter (IL-10) is unique in its ability to significantly inhibit the synthesis of other cytokines (Commins et al., 2010).

The time frame for this study was one week post MuLV infection. However from previous investigations, it is expected that C57BL/6 susceptible mice will die from opportunistic infections within 16-24 weeks post MuLV infection, while BALB/c resistant mice may live for many months and typically die from natural causes (Morse et al., 1992; Beilharz et al., 2004). Based on these expected outcomes, the observed results may suggest that at the very onset of an MuLV infection (by D3), before an adaptive immune response has been completely mounted against the virus, cells from the innate immune response in the C57BL/6 susceptible mice produce high levels of IL-10 relative to BALB/c resistant mice, which produce significantly lower amounts of IL-10. By producing much lower levels of IL-10, the immune system of BALB/c resistant mice shifts from anti-inflammatory to pro-inflammatory; a response that may be more effective at eliminating the virus. This may explain why BALB/c mice are able to resolve the viral infection as early as two weeks post infection (Gilmore, 1997). The susceptible C57BL/6 mice however, by expressing higher amounts of IL-10, shift the weight of an MuLV-induced immune response towards anti-inflammation, as opposed to an pro-inflammation, resulting in persistent infection, susceptibility to MAIDS and death.

There are several reasons why the same MuLV infection elicits different responses in the two mouse strains. As described in the introduction, the mode of HIV infection is a paradox in which the launch of an active immune response against the virus only serves to facilitate the replication of the virus, leading to a

chronic infection. Such chronic infections like HIV are able to evade the immune system because they eventually wear it out, a phenomenon termed immune exhaustion (Emory University, 2009). Immune system exhaustion occurs in an adaptive immune response. During immune system exhaustion, previously high levels of pro-inflammatory cytokines, aimed at eliminating the pathogen now aid in the shutting down of the immune response via a negative feedback mechanism (Barber et al., 2006) that includes hallmark anti-inflammatory cytokines such as IL-10. Transposing this over the current results under discussion, a question to consider is whether the production of IL-10 in susceptible mice in the presence of the virus may be a form of immune exhaustion. However, this is unlikely, especially considering the fact that observed results in differential expression of IL-10 occurred as early as D3, at which point the innate immune response is still active and the adaptive arm of immunity has not yet been fully activated (Parham, 2009).

Another possible explanation for differential expression of IL-10 between the mouse strains across the three time points, particularly at D3, could be that while the immune system of the C57BL/6 susceptible mouse is unable to detect the presence of the MuLV post infection, BALB/c resistant mice are able to more accurately decipher the type of pathogen assault (based on PAMPs, say). As a result, at D3, BALB/c resistant mice reduce the amount of IL-10 produced so as not to trigger anti-inflammatory responses, while C57BL/6 susceptible mice produce more IL-10 and hence activate an anti-inflammatory response, rendering

the immune system ineffective at controlling the virus. If this is the case, it could be due to the genetic background of the two mouse strains, and could be based on either MHC or non-MHC genes.

Another plausible explanation for the differential expression of IL-10 may lie in the initial basal level expression of IL-10. It is unclear why there is such a high, consistent expression of IL-10 in both mouse strains before MuLV infection (D0), since conventional wisdom suggests that IL-10 is produced to attenuate a pathogen-induced pro-inflammatory response. Nevertheless, this high basal level of IL-10 may be the spring board via which the paths of susceptibility and resistance are charted. As already mentioned, IL-10 is not a cell specific cytokine (Saraiva and Garra, 2010). However, cell type specific signals do exist (Saraiva and Garra, 2010). For certain cells, like B cells and macrophages, the pathway that yields IL-10 production is not clearly understood (Saraiva and Garra, 2010). However for other cells the pathway is better understood. For example, Fig. 16 shows the level of IL-10 expression yielded by different cells of the innate immune system. Of particular interest is the fact that IL-10 expression levels can be induced by TLR-dependent and TLR-independent pathways. It is quite unlikely that the high levels of IL-10 at the naïve stages of both mice are triggered by the more common TLR-dependent pathway, since TLR 7 and TLR 8 are two of few TLRs that do not induce IL-10, but that can detect single stranded viral DNA (Parham, 2009) which MuLV (and HIV) possess prior to fusing with host cell DNA.

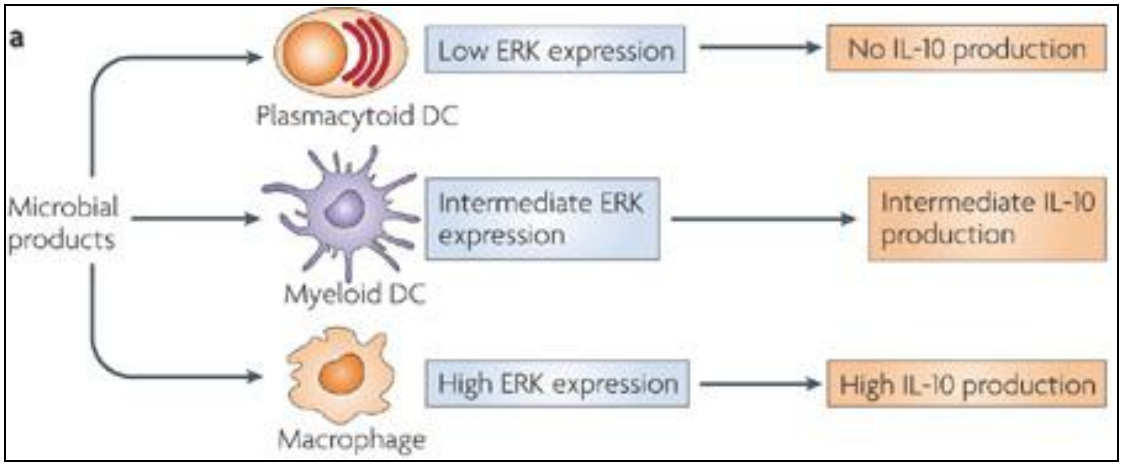

Fig. 17. Diagramatic representation of IL-10 induction of immune cells in the innate arm of an immune response. The presence of microbial products yields different IL-10 expression responses from different immune cells with plasmcytoid dendritic cells producing no amount of IL-10 and macrophages producing the higest amount of IL-10. Adapted from Saraiva and Garra, 2010.

IFN-γ, however, is known to interact with IL-10 via a number of pathways. One such pathway that is not TLR dependent is the Phosphoinositoide 3-kinase (PI3K)-AKT pathway. The PI3K-AKT pathway is important for triggering apoptosis in normal cells (Morgensztern and McLeod 2005). The role IFN-γ in this pathway is to interfere with this pathway by the triggering of the production of a particular kinase (amino acid phosphorylating agent) called glycogen synthase kinase 3 (GSK3). GSK3 in turn blocks IL-10 production by acting on a number of vital proteins including the cAMP response element-binding protein (CREB) and activator protein 1 (AP1) (Saraiva and Garra, 2010). This goes to suggest that although IL-10 may be produced at this high basal level to prevent exaggerated immune responses to harmless particles (like certain allergens), it

may serve as the ‘pitfall’ for C57BL/6 mice in the event where the immune system of the susceptible mice is unable to produce the appropriate antagonizing cytokine to bring these dangerously high levels of IL-10 down during an attack by a virus like MuLV which causes immunodeficiency.

Furthermore, it is worth remembering from the results section, that although there is a differential expression of IL-10 between strains at D3, and within the BALB/c resistant strain at D0, D3 and D7 (Fig. 15), there is no significant differential expression of IL-10 within the C67BL/6 susceptible strain at D0, D3 and D7. Connecting this result to the above discussion, a possible reason for the observed differential expressions may be that although both mice produce a similar amount of IL-10 before infection and at D7 post MuLV exposure, the presence of the MuLV post infection may induce the production of an appropriate amount of IFN-γ which down-regulates the expression of IL-10 via the PI3K-AKT pathway using GSK3 and hence encourages inflammatory responses in BALB/c mice (MAIDS resistant). On the other hand, while the C57BL/6 susceptible mice may produce IFN-γ post MuLV infection, perhaps not enough is produced to block IL-10 production. In such a case, the presence of the pro-inflammatory cytokine will dominate and can contribute to the eradication of the MuLV in BALB/c resistant mice. On the other hand, the absence of these cytokines could facilitate the continual replication of MuLV in C57BL/6 susceptible mice (Fig. 16).

In conclusion, therefore, results seem to suggest that the presence of IL-10 in both strains of mice, may initially not be pathogenic, but may become pathogenic, due to the presence of a virus, like MuLV, that induces immune deficiency in mice.

SOURCES OF ERROR

The entire protocol required for this project was rather long: right from infecting mice with the MuLV, to sacrificing, to homogenizing isolated organs through to running the final samples on ELISA plates. As a result, there is the tendency for error to occur, albeit this was avoided as often as possible.

The first error to consider is the possibility that some mice received less, or more, of the designated 3.6×10^4 PFU/ml viral titre during intraperitoneal injections (injections in their stomach). This may have occurred if a small amount of the virus remained in the syringe during an injection, or if, a slightly larger titre was injected into a mouse at a particular time, respectively.

A second source of error may have occurred during the organ isolation and homogenizing stages. For example, while the spleen is a single large organ –easy to identify and homogenize, the lymph nodes are fewer in number and a little more difficult to isolate. As a result, there may have been times when fewer than the expected 6 lymph nodes were isolated or a small piece of lymph node was lost during homogenization. In fact, there may even have been times when a glob of

fat was isolated, with the false notion that it was a lymph node. All these would typically result in a smaller sample size for lymph nodes and could have affected obtained data. Such errors were carefully avoided, however.

A final source of error may have occurred while running the samples in the ELISA plate itself. Since this required filling each well at a quick enough rate that the plate did not dry up, there was the potential risk of performing pipetting errors during the ELISA protocol.

FUTURE DIRECTIONS

In the near future, this project can go in a number of different directions. For one, studies can be conducted to determine if IL-10 is the sole contributing factor to MAIDS susceptibility. That is to say, in the absence of IL-10, will MAIDS resistance be conferred to the C57BL/6 susceptible mice? And if not, then a good question to consider next could be what other cytokines may be involved in not just susceptibility to MAIDS, but resistance as well. Irrespective of the direction taken, however, a major factor to consider in future experiments will be the sample size. While this was not a source of error, a major challenge during data analysis in this project was the fact that the study was centered on a very small mouse sample size number (n=4). In statistical analysis, large sample sizes are always key to detecting slight nuances that may be present in obtained

results. Consequently, larger sample sizes may be needed to enable high levels of accuracy in subsequent tests.

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