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EARLY DIFFERENTIAL IDO EXPRESSION PATTERNS IN THE MAIDS MODEL

by

Yemsratch Akalu

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Department of Biological Sciences
South Hadley, MA 01075

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ABSTRACT

Murine acquired immunodeficiency syndrome (MAIDS) is an animal model that is used to study AIDS. It is caused by an exposure to a defective retrovirus present in the LP-BM5 isolate of murine leukemia virus (MuLV) mixture. Strains of inbred mice differ in their resistance to MAIDS development. While susceptible strains of mice such as C57BL/6 develop progressive immune deficiency, other resistant strains such as BALB/c recover from infection and build protective immunity. Earlier studies, conducted in the MAIDS model system, indicate that knocking down or inhibiting enzymatic activities of indoleamine 2, 3-dioxygenease (IDO) suppresses viral replication, up-regulates Type I IFN mRNA levels, and increases plasmacytoid dendritic cell counts. Since IDO is a natural immunosuppressant molecule, its overactivation could contribute towards immune dysregulation and disease progression in MAIDS susceptible mice.

Preliminary experiments conducted in our lab showed significantly higher IDO mRNA expression levels in the secondary lymphoid organs of naïve and virus-infected disease-susceptible C57BL/6 mice than in resistant BALB/c mice. In this project, we developed our own ELISA based assay to measure mouse-IDO. Our results show an early upregulation of IDO protein following viral exposure, where differences of protein expression between the two strains of mice are highly pronounced 14-days post infection both in the spleen and lymph nodes of the animals. IDO-mediated immune activity in the susceptible C57BL/6 strain may perpetuate viral replication by dampening efficient immune anti-viral responses. Pilot experiments are being conducted to elucidate immune mechanisms that could mediate this MuLV-induced increase of IDO expression, such as immunoinhibitory CTLA-4/B7 interactions.

INTRODUCTION

Acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV), is a disease that primarily targets the human immune system. Since its first recognition in the early 1980s, the disease has been a major health problem in many parts of the world. According to the World Health Organization, an estimated 34 million people were living with HIV at the end of 2010, with 2.7 million new HIV infections per year. The report showed that improved access to antiretroviral treatment and care, coupled with educational efforts and preventative measures, have notably lowered annual new HIV infections and deaths from AIDS-related illnesses (UNAIDS, 2011). Since the peak of the epidemic in 1997, new HIV infections were reduced by 21% and AIDS-related deaths decreased by 21% since 2005 (UNAIDS, 2011).

The AIDS pandemic presents geographic disparities in its prevalence across the globe. By far, sub-Saharan Africa is the worst affected region in the world. At the end of 2010, an estimated 22.9 million people were living with HIV, with 5% of the adult population HIV-infected (WHO, 2011). Distinctive to this region is also the proportion of women living with HIV. Fifty-nine percent of all people living with HIV in sub-Saharan Africa are women (WHO, 2011).

Over the past decade, the lack of cures or HIV vaccines has driven the increased use of antiretroviral therapies. Antiretroviral treatments reduce both new infections and deaths from HIV/AIDS by controlling viral titers in HIV patients. Interestingly, long-term nonprogressors, rare individuals who have been

infected with HIV that can control the infection without antiretroviral therapy, present a unique phenotype that begs further investigation. Extensive research is being done to define the genetic basis underlying control of the progression of the disease by these individuals (Walker, 2007).

In line with this, a mouse model of AIDS (MAIDS) has been adopted to better understand the genetic basis of the disease and answer basic questions about its pathology. In this research project, we have sought to elucidate how differential expression of the enzyme indoleamine 2, 3- dioxygenase contributes to either resistance or susceptibility to MAIDS, during the first two weeks of infection. Understanding immunomodulation mediated through this enzyme may correspondingly influence our understanding of human immune susceptibility to HIV.

Organs of the Immune System

Secondary Lymphoid Organs: School for Lymphocyte activation and differentiation

The lymphoid organs are sites of development, maturation, function and proliferation of lymphocytes. Lymphocytes that are derived from progenitor cells in the bone marrow mature into B and T cells in the bone marrow and thymus, respectively (Parham, 2009). Thus, the bone marrow and thymus are called primary (central) lymphoid organs. Through a negative selection process, self-reactive lymphocytes are removed (Parham, 2009). B and T cells that pass this stage, termed naïve cells, will then circulate in the blood and lymphatic system in

search of foreign antigens. This circulation allows for regular passage through secondary (peripheral) lymphoid organs.

The secondary lymphoid organs are specialized tissues where lymphocytes encounter antigens to subsequently get activated and initiate an adaptive response (Parham, 2009). Secondary lymphoid organs include the spleen, lymph nodes, tonsils, adenoids, Peyer's patches and other mucosa-associated lymphoid tissues.

Spleen

The spleen is an intraperitoneal organ that is morphologically set up to filter the blood (Parham, 2009). Splenic arteries serve as highways for incoming blood-borne cells and various antigens that are heading towards anatomically distinct compartments of the spleen, namely the red and white pulp (Cesta, 2006).

The red pulp serves as a mechanical filter of the blood wherein foreign materials and damaged red blood cells are removed (Tarantino *et al.*, 2011). Trapped antigens and damaged cells are phagocytosed by resident macrophages. There is also a reserve of iron, red blood cells, platelets and plasma cells (mature B cells that produce and secrete large amounts of antibodies). The white pulp, on the other hand, is a site of active immune responses. Different leukocytes, including B and T-cells, traffic and concentrate in this region to "sample" antigens that transit through the spleen in search of foreign pathogens (Tarantino *et al.*, 2011). Upon encountering a foreign antigen, these lymphocytes become activated.

Lymph Nodes

Strategically situated at the junction of lymphatic networks, lymph nodes drain interstitial fluid of the local region, thereby promoting rapid interaction of trapped pathogens with resident immune cells (Randall *et al.*, 2008; Parham, 2009). Accordingly, the nodes provide structural platforms to transfer information from innate to adaptive immune cells. Thus, the specialized function of lymph nodes is supported by a dynamic, leukocyte and macrophage rich environment that is highly organized into functional compartments (Randall *et al.*, 2008).

The architecture of a lymph node can then be classified into three distinct regions: the cortex, the paracortex and the medulla. Below the fibrous capsule that encloses the lymph node lies cortex. The cortex hosts tightly packed B-cells and networks of follicular dendritic cells (von Andrian and Mempel, 2003). Hence, this area of the lymph node is called the B-cell area. Below the cortex, the paracortex is comprised of less densely packed T-cells that are constantly interacting with dendritic cells that have migrated from peripheral tissues to the node (Parham, 2009). The medullary cords, located in the innermost layer of the lymph node, are sparsely populated by macrophages, plasma cells, memory T cells (von Andrian and Mempel, 2003).

The Stages of Immune Response

Since pathogens rapidly evolve and adapt to circumvent detection and destruction by the immune system, the host employs various defense mechanisms that also evolve to recognize and destroy pathogens. In this race between host and pathogen, the host immune system extends two interdependent arms of response that chronologically and functionally differ from each other (Parham, 2009). These are the innate and adaptive immune responses.

Innate Immunity

The innate immune system is the first line of defense that gets activated when a pathogen, such a virus or bacteria, invades a host's system. The immune system has a hierarchical organization of function that starts with a passive form of defense. Physical barriers such as the skin and mucosal lining of the respiratory tract prevent pathogens from entering an organism. If a pathogen succeeds in breaching this barrier though, the innate immune system provides an immediate, non-specific, and fixed response.

Innate cells include monocytes, macrophages, dendritic cells, natural killer cells, mast cells and granulocytes (eosinophils, basophils, and neutrophils) (Parham, 2009). These cells detect the presence of foreign antigens through their evolutionarily conserved, germline-encoded surface and intracellular pattern-recognition receptors (PRRs) (Beutler, 2004; Parham, 2009). The PRRs specifically detect and bind to pathogen-associated molecular patterns (PAMPs)

that do not resemble self and are common to groups of pathogens. Since these PAMPs are usually integral to the existence of the pathogen, they are not subject to alteration by the pathogen (Akira *et al.*, 2006). Examples of PAMPs include lipopolysaccharides (LPS), endotoxins that are commonly found on the cell membrane of Gram-negative bacteria, and double stranded RNA (dsRNA), a nucleic acid variant that is present in viruses. Correspondingly, these structures are recognized by a family of PRRs called Toll-like receptors (TLRs) that upon activation initiate different pathways of intracellular signaling (Akira *et al.*, 2006).

The signaling cascades often lead to two outcomes. The first one is phagocytosis of infectious microorganism by macrophages and neutrophils (Akira *et al.*, 2006). Once a pathogen is taken up by a phagocyte, it gets destroyed by toxic peroxidases and other enzymes present in the lysosome (Parham, 2009). Supplementary to this response, pathogens are marked by a cascade of plasma proteins called complement. Subsequently, complement-marked antigens are ingested by phagocytic cells or degraded through direct lysis (Parham, 2009). Additionally, intracellular signaling activated at the TLRs triggers the secretion of soluble messenger molecules called cytokines. These cytokines elicit an inflammatory response by attracting other immune cells to infiltrate the site of infection (Kumar *et al.*, 2009; Sharon, 1998). For instance, viral infections induce the production and secretion of interferons (IFN-α, IFN-β and IFN-γ). IFNα/β can nonspecifically be produced by all infected cells, whereas IFN-γ is primarily secreted by lymphocytes (Boehm *et al.*, 1997; Fensterl and Sen, 2009). Secretion

of IFNs results in the apoptosis of infected cells and promotion of antiviral state in surrounding cells (Boehm *et al.*, 1997). Hence the release of IFNs and other cytokines during innate immune response facilitates transition to the next stage of defense called adaptive immune response.

Adaptive Immunity

If a pathogen successfully evades the innate immune system, pathogen or antigen specific adaptive immune responses are in place to provide protection. In fact, the immune system is tuned to refine or adapt its response during infection to improve its recognition of the pathogen. As a result, this arm of immunity requires some lag time after exposure to the pathogen or other foreign antigen, before a maximal response is mounted. Upon resolution of the infection, the successful antigen-specific response is retained in the form of immunological memory. A pool of antigen-specific memory cells will be kept in quiescence. Subsequent exposures to the same antigen will then result in a quicker and stronger immune response (Parham, 2009). As a result, a pathogen can often be dealt with in subsequent exposure (called secondary responses) very efficiently, before any detectable symptoms of disease are observed by the host. This is the principle behind the prophylactic use of vaccines.

The primary mediators of adaptive immunity are B and T lymphocytes. Hence, there exist two main classes of adaptive immunity. These are B-cell mediated (humoral) immunity and T-cell mediated (cellular) immunity. B and T

cells have unique and randomly determined antigen-binding sites called B-cell receptor (BCR) and T-cell receptor (TCR), respectively. These receptors allow these lymphocytes to bind to specific antigens, and are much more attuned to subtle differences than receptors of the innate immune system such as PRRs. Once naïve B-cells, cells that haven't encountered antigen before, recognize a foreign antigen they undergo clonal expansions to give rise to millions of cells, each with a slightly "tweaked" antigen binding BCR. Following recombination of antigen receptor genes, multiple forms of the same primary BCR are formed. This allows for the best version of a BCR to be positively selected, multiply and be released from the surface of the cell as soluble proteins. These soluble proteins termed antibodies will then flag their unique antigen thereby inactivating it. Neutralization and agglutination will then direct a foreign antigen towards a degradation pathway spearheaded by phagocytes. Consequently, this mode of response is designed to target free-existing, often large, extracellular antigens (Parham, 2009). For instance many bacteria and toxins are prevented from binding to host cells in this fashion.

In contrast, intracellular pathogens such as viruses require action by a different class of lymphocytes called T-cells. Based on the type of surface glycoprotein they use to recognize antigenic peptide loaded MHC molecules, T-cells can be further classified as CD4+ and CD8+ cells (Parham, 2009). Naïve CD4+ and CD8+ T-cells require activation by antigen presentation cells (APCs) before they become effector cells (which will be discussed further in the next

section). After CD8+ T-cells have "seen" specific antigens on the surface of APCs, they will get "educated" and will become effector cells. Effector CD8+ T-cells will then circulate in the blood and lymphatic vessels killing any host cells that display the specific foreign antigen on their surfaces. Because of their role in the direct apoptosis of infected cells, CD8+ T-cells are also called cytotoxic T lymphocytes (CTLs). Concomitantly, these effector cells will produce various cytokines and chemokines (chemotactic signaling molecules) to activate phagoctyic macrophages that can clean up dead cells left behind after cytotoxic lysis (Parham, 2009).

CD4+ T-cells, also known as T-helper cells, influence both humoral and cell-mediated immunity. The local milieu of cytokines secreted determines which type of immunity each CD4+ T-cell is involved in. Hence depending on which cytokines are present in the immediate environment, a CD4+ T-cell will differentiate into one of several types of helpers: Type I helper T (Th1) cells, Type II helper T (Th2) cells, Type17 helper T (Th 17) cells or T regulatory (T_{reg}) cells (Parham, 2009). Each subset of CD4+ T-cell will augment a different kind of immune response through characteristic cytokine and chemokine secretion. Th1 cells stimulate production of cytokines that recruit, activate and enhance macrophage function. Since these cells promote active recruitment of phagocytic macrophages and neutrophils, they enhance inflammatory responses at the site of infection. Alternatively, Th2 cells act as drivers of humoral immunity by inducing B-cell proliferation and differentiation. Consequently, there exists a class of

antigens to which B-cells can respond to only if they are activated by the Th2-cells. In fact, most antigens are T-dependent and will stimulate production of antibodies only in the presence of T-cells (Parham, 2009). Overall, Th1 cytokines suppress differentiation of precursor cells into Th2 cells, and vice versa. An additional subtype of helper CD4 T cells that seem to have pro-inflammatory functions are the Th17 cells. The hallmark for Th17 cells is the production of IL-17, a cytokine with inflammatory properties. These three subsets of CD4+ T-cells are finally kept in check or regulated by a closely related family of T_{reg} cells.

Antigen Presentation: Bridging Innate and Adaptive Immune Responses

Unlike B-cells, T-cells are incapable of binding to free antigen. They can only recognize antigens that have been processed and presented on the surface of APCs (Parham, 2009). Therefore, naïve T-cells are only equipped to scan antigens presented on the surface of APCs trafficking through secondary lymphoid organs such as the spleen and lymph nodes. APCs can fall into two categories — professional (pAPCs) and non-professional.

Professional APCs include dendritic cells (DCs), macrophages and B-cells. Because of their efficiency in internalizing and displaying fragments of antigens, dendritic cells are probably the most important class of pAPCs. Often DCs are best in presenting intracellular antigens such as viral particles. On the other hand, macrophages and B-cells are adapted towards presenting extracellular antigens (Parham, 2009). All APCs engulf and degrade pathogens, directing

processed fragments of these antigens into protein complex known as the major histocompatibility complex (MHC), of which there are two types, class I and II molecules. MHC molecules are surface molecules that bind fragments of processed antigen and present these to passing T cells. While MHC Class I molecules are expressed by all APCs, constitutive expression of class II molecules is restricted to pAPCs. The site at which an APC encounters an antigen determines the type of antigen loading complex that will be used to present the antigen on the surface of the cell. This will then dictate the type of T-cell activation, and therefore the type of response generated. Accordingly, recognition of fragments of intracellular antigens on MHC Class I molecules specifically activates CD8+ T-cells (CTLs) leading to the apoptosis of the target cell (van den Elsen *et al.*, 1998). Conversely, exogenous antigens displayed as MHC Class II: peptide complexes activate naïve CD4+ T-cells (van den Elsen *et al.*, 1998).

T-cell Activation

Activation of T-cells requires the binding of T-cell receptors (TCRs) to antigen presented on MHC molecules. Hence T-cell activation depends on the level of expression of MHC molecules. Enzymes present within APCs digest pathogens into smaller pieces containing epitopes, parts that are recognized by the immune system, which are then presented to T-cells (Alberts *et al.*, 2008). While CD4+ T cells recognize antigenic pieces presented on MHC class II molecules, the TCRs of CD8+ T-cells bind to peptides loaded on to MHC class I molecules.

Co-stimulatory Molecules

Aside from engagement of the TCRs, activation of T-cells is contingent on co-stimulation of yet another receptor present on the surface of T-cells called CD28. Without CD28 co-stimulation, signaling carried out through TCRs alone results in anergy (Parham, 2009). An anergic T-cell will then fail to respond to its specific antigen.

It is known that the surface of activated pAPCs is marked by the presence of co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86), both of which can bind to CD28. When a pAPC interacts with a T-cell, these molecules bind to CD28 on T-cells. This activates the cell, enhancing T-cell proliferation and cytokine production thereby generating an effective immune response (Thompson *et al*., 1989). CD28-mediated co-stimulation is thought to promote T-cell activation by lowering TCR signaling threshold, or by lowering the amount of time that a T-cell needs to be in direct contact with an APC to induce T-cell proliferation (Iezzi et al., 1998; Gett and Hodgkin, 2000).

In contrast, B7 molecule engagement of the CD28's inhibitory counterpart cytotoxic T-lymphocyte antigen 4 (CTLA-4) produces opposite outcomes (Sharp and Freeman, 1994). Signaling carried out through CTLA-4 downregulates cytokine production and inhibits activation of T-cells in the early stages of infection (Alegre et al., 1998). In fact, CTLA-4 deficient mice exhibit autoimmune pathology, demonstrating how important this molecule is at

inhibiting the immune response (Tai *et al.*, 2007). Hence, as shown in Figure 1, it is known that the primary functional role of CTLA-4 is to down-regulate CD28 signaling (Walker and Sansom, 2011).

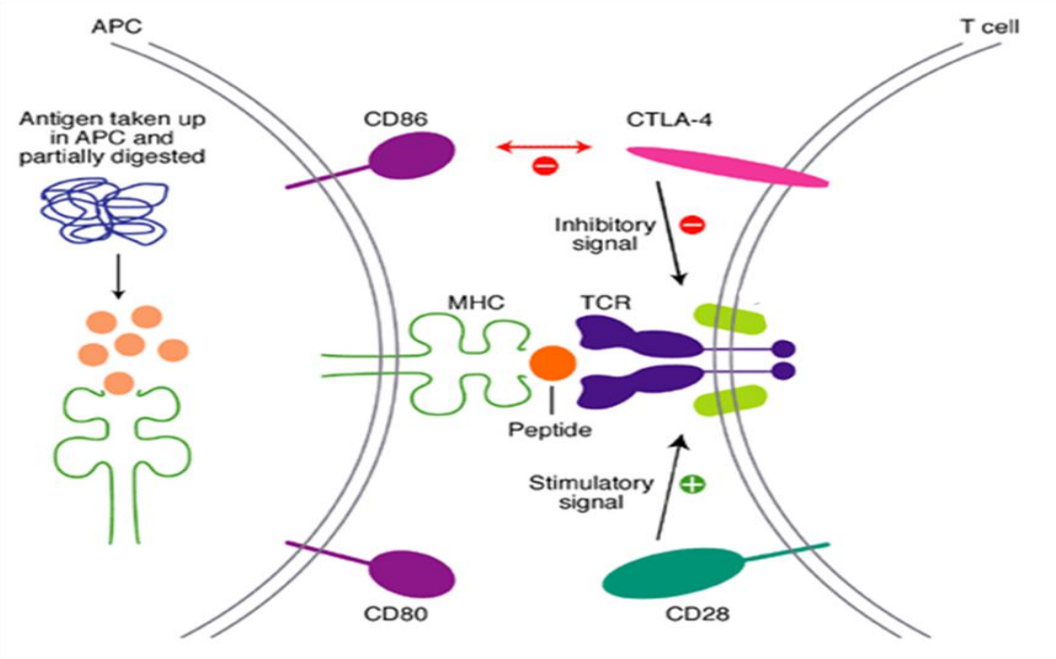


Figure 1. Two signal model of T-cell activation.

The outcome of stimulation through the T cell antigen receptor (TCR) is determined by two critical costimulatory receptors— CD28 and CTLA-4. While CD28 mediated signaling is excitatory and promotes naïve T-cell differentiation into an effector cell, CTLA-4 engagement produces inhibitory signals that block T-cell activation. Consequently, T-cells integrate TCR stimuli with costimulatory signals of CD28 and inhibitory signals of CTLA-4 to launch an appropriate response to antigens they encounter. This image was adapted from Expert Reviews in Molecular Medicine, 2005 Cambridge University Press.

Although CTLA-4 and CD28 are both dimers and bind the same ligands—B7-1 (CD80) and B7-2 (CD86) present on the surface of pAPCs — they differ in their aptitude to bind ligands. CTLA-4 interacts with both ligands with a higher affinity and avidity than does CD28 (Walker and Sansom, 2011). As indicated in Figure 1B, while interaction between CTLA-4 and CD80 (a dimer) is the strongest, binding of CD28 with CD86 (a monomer) is the weakest (Collins *et al.*, 2002). Although CD28 itself is a dimer its interaction with both ligands is monovalent binding. This is thought to be a result of steric hinderance.

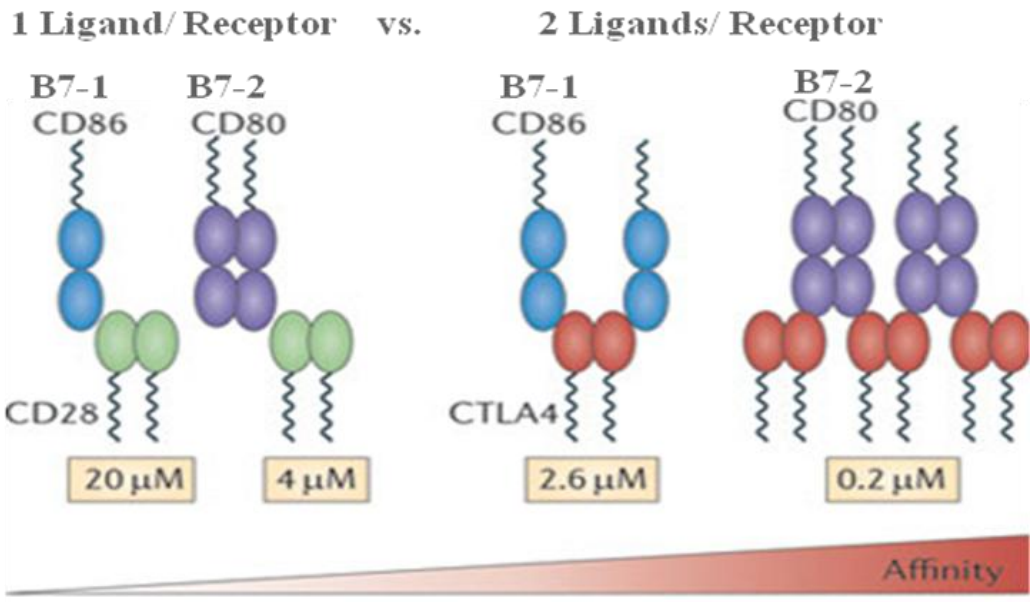


Figure 2. Interactions between T-cell co-receptors and their ligands.

The four distinct interactions shown between the co-receptors and their mutual ligands are important mediators of T-cell activation. The numbers shown are values of dissociation constants (K_d). K_d represents the propensity of a complex falling apart into its component molecules. The CTLA-4- CD80 (B7-1) interactions have the lowest K_d. The higher avidity observed in this particular complex is likely a result of the dimer- dimer interactions between the receptor and ligand. Image adapted from Walker and Sansom, 2011.

Even low levels of CTLA-4 expression on the surface of a cell are enough to induce negative signals in a T-cell. Consequently, surface CTLA-4 expression is tightly regulated. A key feature of CTLA-4 expression is the constant trafficking of the receptor to and from the plasma membrane (Zhang and Allison, 1997). Independent of ligand binding, CTLA-4 constitutively undergoes endocytosis; it gets cycled between storage and surface expression. Internalization of cell surface CTLA-4 is very rapid. Within 5 minutes, greater than 80% of surface CTLA-4 gets internalized (Walker and Sansom, 2011). It has a short half-life of ~2 hrs and it undergoes a rapid lysosomal degradation (Egen *et al.*, 2002). However, localization of CTLA-4 in the lysosome does not necessarily make it dysfunctional. Specialized secretory lysosomes may actually regulate CTLA-4 trafficking to the cell surface (Iida et al., 2000). Thus, the trafficking and expression levels of CTLA-4 may be regulated by lysosomal targeting rather than transcription (Egen *et al.*, 2002). As a result, at any given time, low levels of CTLA-4 are expressed on the surface of activated T-cells and much of the protein instead builds up in endocytic vesicles (Rudd, 2008). Linsley et al., report that following T-cell activation, both intracellular and surface CTLA-4 get directed towards activation sites on the T-cell receptor complex. Whether cytoplasmic or surface expressed, exactly how CTLA-4 molecules are responsible for the inhibitory actions that follow is unclear.

Although both CD28 and CTLA-4 are glycoproteins expressed by both CD4+ and CD8+ T-cells, differential control of their expression is provided by

differences in activation of T-cells. CD28 is constitutively expressed on the surface of both resting and activated CD4+ and CD8+ T-cells. On the other hand, CTLA- 4 is not expressed by resting CD4+ and CD8+ T-cells (Walker and Sansom, 2011). In both humans and mice, it is detected approximately 2 days post activation (Walunas et al., 1994; Linsley et al., 1992). In summary, although all activated T-cells express CTLA-4, in the absence of an activating agent, constitutive expression of CTLA-4 is confined to T_{reg} cells. In addition, activated T_{reg} cells further upregulate expression of this receptor (Walker and Sansom, 2011). The following section discusses this regulatory cell type in greater detail.

The Importance of Brakes

When the body is faced with harmful stimuli, it is important for it to initiate an active immune response. At the beginning of infection, there will be increased movement of leukocytes from the blood into affected tissues. Cascades of biochemical events promote inflammatory responses that involve not only the immune system but also the local vascular system and various cells at the site of infection. If needed, there will be a progressive shift in the types of cells needed at the site of inflammation. Often this collaborative effort to clear a harmful stimuli or a pathogen is successful. Therefore, once pieces of antigens that are stimulating an immune response are removed, all active immune responses should come to a halt. In the absence of a stimulus or residual antigen, antigen-specific effector B- and T-cells undergo apoptosis leaving only memory cells behind (Parham, 2009).

A normal immune system maintains a tight check and balance of its activation and suppression responses. During infection, the balance is slightly tipped to favor activation. In contrast, at the conclusion of infection, it is necessary to turn "off" immune responses. Immune responses that remain engaged after a pathogen has been cleared may cause damage to the host and even autoimmunity. Hence, terminating an immune response is as important as launching it.

The immune system employs several mechanisms to stop adaptive immune responses. These include cell-contact dependent mechanisms and soluble mechanisms, or production of natural immunosuppressant molecules. In the case of T-cells, cell-contact dependent suppression of effector cells is primarily mediated by T_{regs} (Fehérvari and Sakaguchi, 2004). One inhibitory glycoprotein that is constitutively expressed on the surface of T_{regs} is CTLA-4. Concomitantly, T_{regs} also produce an array of anti-inflammatory cytokines that influence activation of other T-cells (Fehérvari and Sakaguchi, 2004). Aside from regulating the phenotype of other effector T-cells through direct interactions, T_{regs} can indirectly suppress activation of other T-cells by inducing production of immunosuppressive molecules in APCs. One example of this is that CTLA-4 expressed on T_{regs} triggers production of a number of immunosuppressive compounds, such as indoleamine 2, 3- dioxygenase (IDO) in dendritic cells (DCs) (Fehérvari and Sakaguchi, 2004). This immune suppressing enzyme is the topic of the following section.-

Indoleamine 2, 3- dioxygenase

General Background

Indoleamine 2, 3- dioxygenase (IDO) is a heme-containing enzyme that catalyzes the first and rate limiting step of the major L-tryptophan catabolism pathway in mammals. It cleaves the indole moiety of the amino acid to initiate the production of kynurenines (Figure 3). IDO is predominantly expressed in epithelial cells, neutrophils, macrophages, and plasmacytoid dendritic cells (Mellor and Munn, 1999; Boasso and Shearer, 2007; Zelante *et al.*, 2008). When a pathogen breaches the first line of defense, leukocytes migrate to the site of infection. As a part of an inflammatory response, innate cells start to secrete interferons (IFNs). IDO is then upregulated by both type I and type II IFNs as well as by different agonists of Toll-like receptors (Taylor and Feng, 1991; Mellor and Munn, 2004). IFNs act in an autocrine and paracrine fashion to trigger IDO production in the same or other type of cells, respectively (Grohmann *et al.*, 2003). Subsequently local depletion of tryptophan will initiate antimicrobial resistance in the host. The lack of tryptophan, the least available essential amino acid, inhibits growth of various pathogens, including viruses (Grohmann *et al.*, 2003).

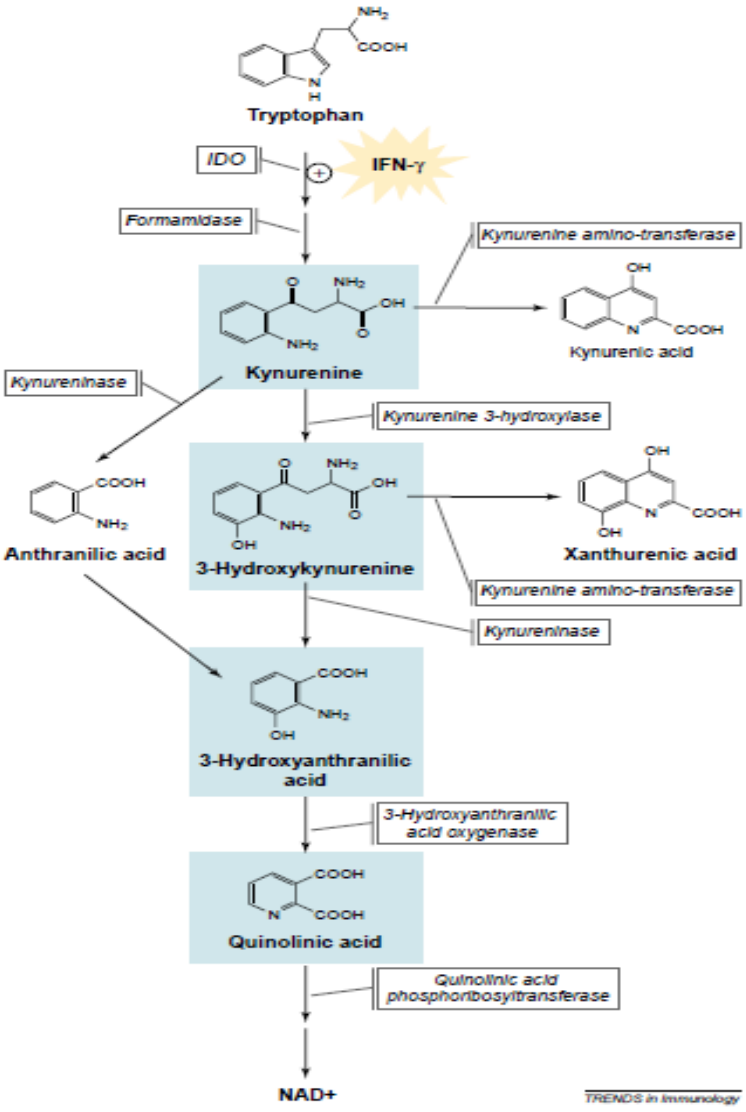


Figure 3. Major tryptophan metabolism pathway.

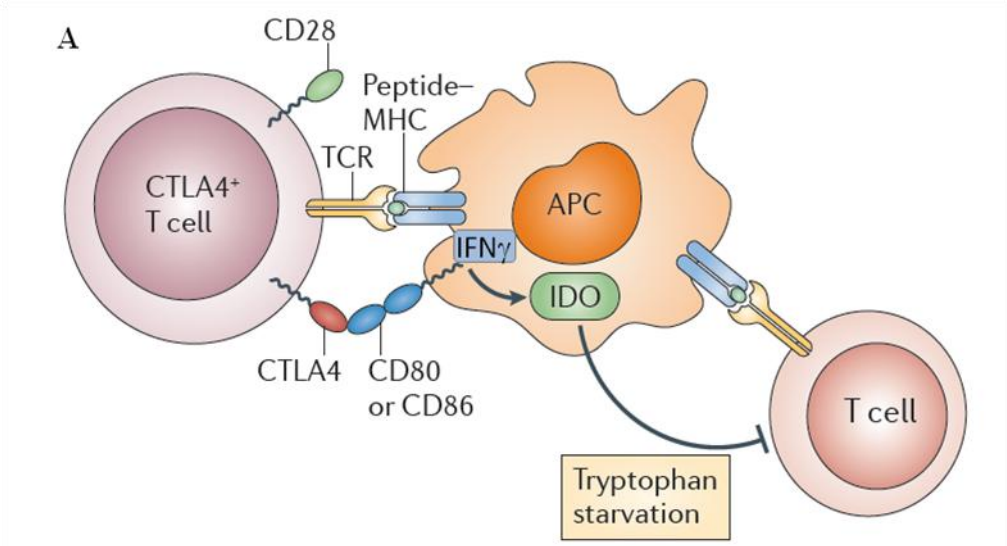
Of the total tryptophan that is not used in protein synthesis, 99% is metabolized along the kynurenine pathway pictured above. The first and rate-limiting step of this tryptophan degradation pathway is catalyzed by indoleamine 2, 3- dioxygenase (IDO). Depletion of tryptophan and generation of toxic metabolites, such as 3-hyroxyanthranilic acid and quinolinic acid, contributes towards a suppressive immune environment. Image taken from Grohmann *et al.*, 2003.

While enacting its antimicrobial activity, IDO also inhibits the activation and proliferation of T-cells (Mellor and Munn, 2004). In the past, several mechanisms of initiating T-cell tolerance, or the host's ability to suppress responses to self proteins, have been described. One suggested mechanism is the blockade of trypthophan sensitive G1 cell cycle checkpoint in T-cells (Munn and Mellor, 2004). Activation of the GCN2-mediated stress response pathway interrupts T-cell clonal expansion (Boasso and Shearer, 2007). GCN2 (general control nonrepressed 2) is a serine/threonine- protein kinase that is activated by elevated levels of uncharged tRNA, and it modulates amino acid metabolism in response to nutrient deprivation (Munn *et al.*, 2005). In addition, IDO-dependent apoptosis of T-cells is regulated by various toxic metabolites that are collectively called kynurenines (Terness *et al.*, 2002). While DCs are not affected by kynurenine-mediated induction of apoptosis, T-cells, B-cells and natural killer (NK) cells are highly suppressed (Terness *et al.*, 2002). At relatively low concentrations, kynurenine derivatives 3- hydroxyanthranilic and quinolinic acids induce selective apoptosis of murine thymoctes (hematopoietic progenitors of T-cells) *in vivo* and pro-inflammatory Th1 cells *in vitro* (Fallarino et al., 2002; Grohmann et al., 2003). However, these kynurenine derivatives do not cause apoptosis in anti-inflammatory Th2 cells (Grohmann et al., 2003). Consequently, this can shape the outcome of a host's immune response when it is under assault from pathogens.

Because of its immunosuppressive role, IDO is involved in a number of disparate activities. Some examples include tolerance induction, immunosuppression of anti-fetal responses during pregnancy, inhibiting anti-graft responses during transplantation and the development of autoimmunity (Takikawa, 2005). For instance, mammalian placental cells producing IDO protect the fetus from maternal T-cell driven rejection (Mellor et al., 2001). Similarly, IDO has been shown to suppress T-cell mediated rejection of allografted pancreas islets in mice and thus prolong graft survival significantly (Takikawa, 2005).

Activity of IDO is modulated by regulatory instructions present in the environment. In particular, IFN-γ is the most potent activator of IDO production (Taylor and Feng, 1991). A pathway that triggers IDO activity through the release of IFN-γ is initiated by the inhibitory glycoprotein CTLA-4 (Orabona *et al.*, 2004). Possible roles of CTLA-4 can be divided into T-cell-intrinsic and T-cell-extrinsic (Walker and Sansom, 2011). While intrinsic functions involve direct inhibitory action of CTLA-4 on the cell that expresses it, extrinsic functions include inhibition carried out through other cells (Walker and Sansom, 2011). One proposed model of T-cell extrinsic CTLA-4 function is the activation of IDO (Grohmann, 2003; Orabona *et al.*, 2004; Walker and Sansom, 2011). Engagement of B7 molecules on dendritic cells with CTLA-4 expressed on T_{reg} cells induces expression of the enzyme IDO in DCs (Figure 4). That is, reverse signals through the B7 molecules on DCs activate production of IFN-γ that will, in turn, drive

IDO production (Grohmann, 2003; Orabona *et al.*, 2004; Walker and Sansom, 2011). However, it has been reported that CTLA-4 can also activate IDO independent of IFN-γ (Walker and Sansom, 2011).



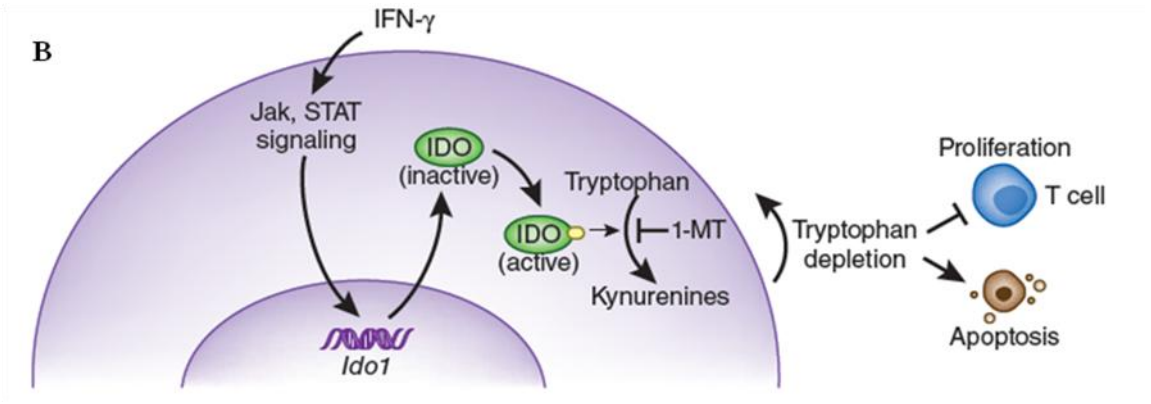


Figure 4. Induction of IDO through CTLA-4 initiated signaling

(A) CTLA-4 initiated reverse signaling through B7 molecules (CD80 and CD86) present on antigen presenting cells (APCs) results in the induction of IDO via IFN-γ-mediated signaling. (B) IFN-γ signals through Jak/STAT molecular pathways to induce IDO production from *Ido1* gene. IDO can inhibit T-cells by depleting local tryptophan and accumulating immunomodualatory metabolites. Image (A) adapted from Walker and Sansom, 2011 and (B) taken from Chen, 2011.

IDO: New Role for an Old Enzyme

For several years, IDO's role in halting potentially harmful hyperinflammatory response in the host immune system has been thought to be purely enzymatic. Recently, however, Pallota *et al.*, characterized IDO as a signal transducer in the TGF-β-IDO axis of immunosuppresion. IDO is produced in many cells of the immune system including macrophages and DCs (Mellor and Munn, 2004). Of note, IDO is also expressed in plasmacytoid dendritic cells (pDCs) which represent a very small yet very important subset of DCs that secrete Type I interferons (IFNα and β) in response to viruses (Pallota *et al.*, 2011) . In their experiment, Pallota *et al*. preconditioned a culture of pDCs with either IFN-γ or TGF-β and subsequently cocultured them with naïve $CD4^+CD25^-$ T cells (a subset of T helper cells expressing the IL-2 receptor). While the IFN-γ preconditioned pDCs suppressed T-cell expansion by inducing apoptosis, TGF-β preconditioned pDCs transformed the naïve $CD4^+$ T-cells into T_{reg} cells (Pallota *et al.*, 2011). When the cocultures were treated with 1-methyltryptophan, an inhibitor of IDO's enzymatic activity, IFN-γ induced T-cell inhibition and apoptosis were blocked (Pallota *et al.*, 2011). Surprisingly, treatment with the IDO inhibitor did not produce any observable effect on the generation of T_{reg} cells by TGF-β preconditioned pDCs (Pallota *et al.*, 2011). Taken together, this suggests that following TGF-β stimulation of pDCs, IDO acts as a signal transducer to facilitate upregulation of its own expression and production of more

TGF-β (Figure 5). This will then "instruct" pDCs to generate T_{reg} cells that may initiate, sustain and amplify immunosuppressive environments (Chen, 2011). Interestingly, these findings align with previously proposed T-cell extrinsic functions of CTLA-4. That is, CTLA-4 present on T_{reg} cells stimulates production of regulatory cytokines such as TGF-β, which can act to inhibit downstream DCs and T-cells which have numerous cytokine receptors (Walker and Sansom, 2011). In light of Pallota et al.'s findings, it's possible that IDO induced by CTLA-4-B7 signaling can function as a signaling molecule in pDCs.

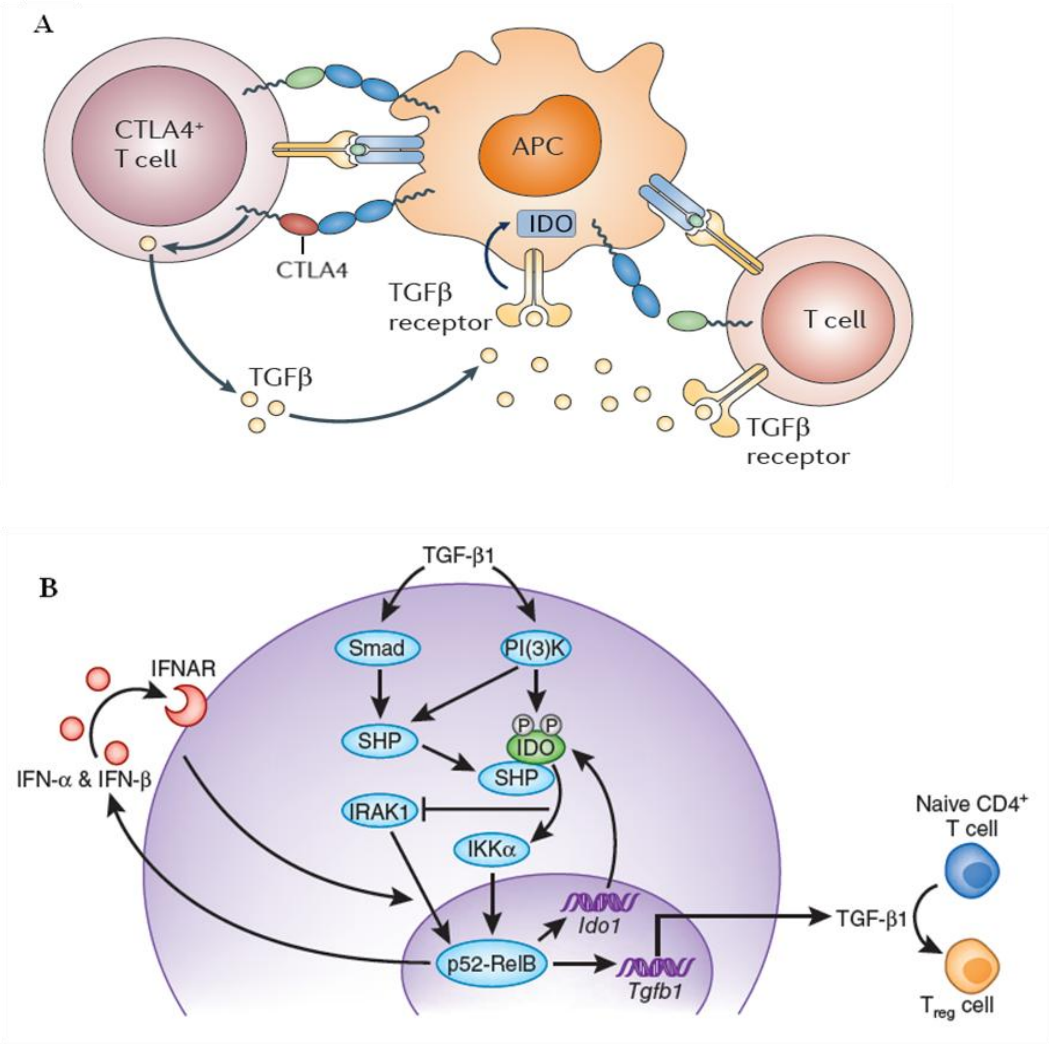


Figure 5. IDO functions as an intracellular signal transducer.

(A) Inhibitory CTLA-4 signals stimulate the production of TGF-β, a regulatory cytokine which can act to inhibit antigen presenting cells (APCs) or other T-cells. (B) TGF-β binds to receptors present on pDCs to initiate signaling through a PI(3)K-dependent pathway. IDO then binds to SHP to activate the nancanonical NF-κB pathway by inducing phosphorylation of the kinase IKKα and nuclear translocation of p52-RelB to its target genes. Activation of the noncanonical NF-κB pathway will then promote activation of the *Ido1* and Tgfb1 genes. TGF-β released by pDCs will finally transform CD4+ T cells into T_{reg} cells. Image (A) adapted from Walker and Sansom, 2011 and (B) taken from Chen, 2011.

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

Course of Infection

Human immunodeficiency virus (HIV) is a lentivirus that induces the onset of acquired immunodeficiency syndrome (AIDS), a condition where the depletion and inhibition of immune cells causes severe disruption of the homeostasis of the host immune system (Weiss, 1993). The virus particularly infects and destroys crucial CD4+ T- helper lymphocytes, macrophages and dendritic cells (Liang *et al.*, 1996). Aside from using CD4 surface receptors as entry sites to cells, it infects cells by also binding to either one of the co-receptors CCR5 or CXCR4 (Parham, 2009).

There are two types of HIV that have been isolated: HIV-1 and HIV-2. HIV-1 is the cause of the majority of infections worldwide and it is more virulent as well as more infective than HIV-2. Because of its low infectivity and hence its poor rate of transmission, HIV-2 is mostly confined to Western parts of Africa (Reeves and Doms, 2002). HIV is a positive sense, single-stranded, enveloped RNA virus. As a result, once the virus gets into a cell, its viral genome is reverse transcribed into complementary DNA (cDNA) by a virally encoded reverse transcriptase. The resulting cDNA is then integrated into the host's genome by the actions of viral integrase. Host cells are taken over as factories for producing new RNA genomes and viral proteins, thereby, allowing for new virions to be

assembled (Parham, 2009). New virus particles will then bud from infected cells to infect and replicate in uninfected CD4+ cells anew.

Infection with HIV occurs through transfer of bodily fluids from an infected person to an uninfected individual. During primary or acute stages of infection, a person is likely to have flu-like symptoms such as sore throat, fever, lymphadenopathy (lymph node enlargement) and muscle pain (Coffin *et al.*, 1997). Because of the non-specific nature of these symptoms, they are often mistaken for signs of other common infectious diseases. During this stage, there is a rapid rate of viral replication that results in a marked drop in the numbers of CD4+ T-cells. Acute viremia, or viral replication, also results in the activation of CD8+ T-cells that kill infected cells, as well as seroconversion, or production of anti-HIV antibodies (Coffin *et al.*, 1997). Although it does not eliminate the virus, good CD8+ T-cell effector activity has been shown as an important component in the control of viral replication and, subsequent, disease progression (Migueles et al., 2002). Following this initial stage of infection, infected individuals enter a period of clinical latency with few or no symptoms. This stage can last anywhere between a few months to twenty years or more. Meanwhile, the virus continues to infect and replicate in CD4+ T-cells. When the number of CD4+ T-cells declines in blood to below 500cells/ μL, the individual will start to suffer from opportunistic infection, or disease from common microbes that do not normally cause pathology (Parham, 2009). Finally, when the CD4+ T-cell counts in blood fall below a critical level of 200cells/ μL, individuals are said to have AIDS

(Parham, 2009). In this stage, cell-mediated immunity is lost and infections with a variety of opportunistic microbes becomes common and life-threatening (Coffin *et al.*, 1997). Eventually, these illnesses lead to death.

Resistance to HIV/AIDS

Studies of HIV/AIDS have identified groups of HIV-exposed people who remain uninfected and others that can avoid acquisition of AIDS, despite lack of prior antiretroviral therapy. These groups are referred to as exposed-uninfected and long-term nonprogressors (LTNPs), respectively. The former group is a very small subset of exposed individuals, observed particularly among commercial sex workers, drug users that have been exposed to the virus with contaminated needles, and individuals having regular unprotected sex with seropositive partners (Marmor *et al.*, 2006). On the other hand, LTNPs compose 5-10% of individuals infected with HIV (Marmor *et al.*, 2006). These individuals suppress viral replication and maintain stable CD4+ T-cell counts (Sajadi et al., 2007). To date, it has not been determined whether LTNPs have the ability to never acquire AIDS or merely experiencing prolonged latency. However records show that many LTNPs have been seropositive for 20+ years without manifesting symptoms of immunodeficiency (Marmor *et al.*, 2006).

Finding resistance-associated factors that shield repeatedly exposed individuals from initial infection and infected individuals from acquiring immunodeficiency syndromes has been on an intense area of research that may

provide key insights for the development of therapeutics and vaccines. Some of the factors that have been associated with resistance include differential expression of receptors such as CCR5 and high level of specific CD8+ T-cell responses (Stranford et al., 1999; Marmor *et al.*, 2006; Hersperger *et al.*, 2011). CCR5 is a chemokine receptor that is initially utilized by many forms of HIV to infect host cells. Mutation in this receptor, in the form of a 32-nucleotide deletion, closes off one of the routes of HIV infection. However, factors such as these account for a small part of a very complicated response to the virus.

Interestingly, a clinical study called HPTN 052 has shown the promising benefit of using antiretroviral drugs (ARVs) to greatly reduce HIV-1 transmission between serodiscordant partners (Cohen *et al.*, 2011). The study enrolled 1763 discordant couples in which one person was HIV-1-positive and the other was uninfected. The participants were from 9 countries, namely Botswana, Kenya, Malawi, South Africa, Zimbabwe, Brazil, India, Thailand, and the United States. To enroll in the study, the infected partner could not be taking any ARVs and needed to have a CD4+ T-cell count between 350 and 550 cells per cubic milliliter of blood (Cohen *et al.*, 2011). This meant that all those infected had relatively unimpaired immune systems and they had yet to develop AIDS. Of the HIV-1 infected partners, 50% were men. Half of the infected individuals were randomly assigned to early therapy with ARVs, while the other half received delayed treatment, starting only after their CD4+ T-cell count dropped to less than 250 cells per cubic milliliters or they had developed symptoms associated with the

onset of AIDS (Cohen *et al.*, 2011). Treatment with a cocktail of ARVs lowered the rate of infection of the HIV-negative partners by 20-fold and improved the quality of life for the infected individuals (Cohen *et al.*, 2011). Specifically, of the 28 people who became infected with HIV and were diagnosed with the same viruses as those found in their long-term partners, only one was in the early treatment group (Cohen *et al.*, 2011).

Murine Leukemia Virus (MuLV) and Murine Acquired Immunodeficiency Syndrome (MAIDS)

The need to understand the pathology of HIV/AIDS has initiated the development of several small animal models. In particular, the murine AIDS (MAIDS) model system has been used to produce significant results in laboratory research conducted to better understand HIV-induced AIDS. Interestingly, not all strains of mice are equally susceptible to MAIDS (Jolicoeur, 1991). Susceptible mouse strains infected with the LP-BM5 isolate of murine leukemia virus (MuLV), a C-type retrovirus, develop MAIDS, a disease with similarities to human AIDS (Mosier, 1996).

The MAIDS model system employs two strains of mice (*Mus musculus*), C57BL/6 and BALB/c. Although both strains of mice are susceptible to MuLV infection, only BALB/c mice can launch an effective immune response to eradicate the virus and avoid acquisition of MAIDS (Mosier, 1996). C57BL/6 mice, once infected with the LP-BM5 mixture of MuLV, develop progressive

immune compromise and eventually die of opportunistic infections 17-24 weeks post infection (Mosier, 1996).

Both HIV-induced AIDS and MAIDS are characterized by immunosuppression, an increased susceptibility to opportunistic infections, hypergammaglobulinemia (increased antibody production), impaired B- and T-cell functions, neoplasia (cancer) and, at later stages, neurological dysfunction (Mosier, 1996; Liang *et al.*, 1996; Jolicoeur, 1991). MAIDS is also characterized by a massive proliferation of B-cells. However, this polyclonal expansion is not antigen-specific (Liang *et al.*, 1996). As a result, differentiation of these B-cells into plasma cells that produce antibodies is not adapted to clear the virus. This is complemented by a switch from a Th1 to Th2-biased cytokine response to the virus. MAIDS-susceptible mice exhibit abnormally high levels of Th2 cytokines (Liang *et al.*, 1996). This switch from an inflammatory Th1 response to an antibody-mediated Th2 response may facilitate an immunosuppressive immune environment that contributes to the poor prognosis of MAIDS-susceptible mice. Furthermore, both CD4+ and CD8+ T-cells in animals with MAIDS become anergic and are unresponsive to antigens (Liang *et al.*, 1996).

Aside from the factors described above, additional features that recommend MAIDS as a model system for studying AIDS are the ability to use a larger quantity of literature on mouse immunology and virology, a lower biohazard risk, and cost efficiency (Mosier, 1996).

AIDS versus MAIDS

Although the MAIDS model system provides accessible and sophisticated genetic backgrounds to investigate the pathogenesis of AIDS, there exist some features that prevent MAIDS from being a perfect model of AIDS. MAIDS and AIDS are not identical and they are induced by two different classes of retroviruses. HIV is a lentivirus that can infect cells of the immune system, including those that are non-dividing and terminally differentiated, with high efficiency (Fassati, 2006). On the other hand, the etiologic agent in MAIDS is a different class of retrovirus, a C-type retrovirus. Unlike HIV, MuLV targets B-cells and macrophages instead of CD4+ T-cells (Mosier, 1996). Although CD4+ T-cells are not primarily infected by MuLV in MAIDS, their presence is required for the development of immunodeficiency.

Nevertheless, clinical disease progression associated with MAIDS is strikingly similar to HIV pathogenesis observed in AIDS (Mosier, 1996). For instance, stages of immunodeficiency in both syndromes are characterized by severe inflammation of secondary lymphoid organs, lymphomas as well as a number of opportunistic infections (Liang et al., 1996). At the cellular level, polyclonal expansion of B-cells and impaired T-cell responses are observed (Liang *et al.*, 1996).

Microarray Analysis Identifies Immunosuppressive Genes

The Stranford laboratory has sought to identify resistance and susceptibility associated lymphoid organ genes that are differentially expressed in the MAIDS model during the first week of infection. To this end, DNA microarray technology was utilized to generate gene expression data from the spleen and a pool of lymph nodes (consisting of the axillary, brachial, mesenteric and inguinal lymph nodes) of MuLV-infected BALB/c and C57BL/6 mice (Tepsuporn *et al.*, 2008). The greatest strain-specific differences were found in the lymph nodes of 3-day MuLV infected animals (Tepsuporn *et al.*, 2008). At 7-days post infection, among many other differentially expressed sequences, the gene encoding the immune inhibitory glycoprotein CTLA-4 was expressed at a higher level in susceptible C57BL/6 mice than in resistant BALB/c mice. The role of CTLA-4 in inducing the expression of the immunosuppressant protein IDO has been discussed previously.

Relevant Previous Works

IDO in AIDS

HIV-infected patients tend to present reduced concentration of tryptophan and an increased expression of kynurenines or other products of tryptophan degradation in their plasma (Boasso and Shearer, 2007; Schroecksnadel *et al.*, 2007). This is indicative of enhanced IDO activation in these patients. Peripheral blood mononuclear cells (PBMCs) isolated from HIV+ patients showed elevated

IDO mRNA levels when compared to uninfected healthy controls (Boasso *et al.*, 2007).

It has been reported that the HIV virion can also induce IDO expression in pDCs and macrophages (Boasso and Shearer, 2007). This was illustrated by exposing uninfected PBMCs to HIV (Boasso *et al.*, 2007). Increased level of IDO in lymph nodes was then shown to contribute to the impairment of CD4+ T-helper cells in an IFN-independent manner (Boasso *et al.*, 2007). Moreover, *in vitro* inhibition of IDO's enzymatic activity with 1-methyl tryptophan resulted in increased CD4+ T-cell proliferation in PBMCs taken from HIV+ patients (Boasso *et al.*, 2007).

As discussed earlier, CD4+ T-cells are classified into different subsets, including Th1, Th2, T_{reg}, and Th17 cells (Parham, 2009). Past studies have shown that progressive immune dysregulation in HIV+ individuals is associated with the loss of Th17cells, cells that can protect the mucosal surface against invading pathogens, and a reciprocal increase in immunosuppressive T_{reg} cells (Favre *et al.*, 2010 and Clark *et al.*, 2011). It was found that induction of *Ido1* gene, which encodes IDO, by myeloid antigen-presenting dendritic cells is associated with the disturbance of balance between Th17 and Treg cells in HIV+ patients (Favre *et al.*, 2010). The loss of balance between Th17 and Treg cells is directly mediated by 3-hydroxyanthranilic acid, a downstream metabolite in the kynurenine pathway of typtophan catabolism (Favre *et al.*, 2010).

Therefore, since IDO may contribute to perpetuation of HIV infection and favors chronic infection by interfering with anti-viral responses a better understanding of this immune suppressive mechanism is needed. Perhaps a therapeutic approach aimed at modifying IDO's activities can be considered in the context of HIV/AIDS.

IDO in MAIDS

Studies have only recently been focused on understanding IDO's role in the murine model of AIDS. In 2010, Hoshi *et al*. reported on the role of IDO after LP-BM5 MuLV infection. They used *IDO1* gene deficient (IDO-/-), 1-methyl-D-L-trypthophan-treated (an inhibitor of IDO's enzymatic activity) and wild type (WT) mice of C57BL/6 background (Hoshi *et al.*, 2010). They observed significantly suppressed viral replication, up-regulated Type I IFN mRNA levels, and an increased count of pDCs in the spleens of the knockout and IDO inhibitor treated mice. The WT mice, however, did not exhibit any of these phenotypes. In addition, the group recorded a prolonged survival rate in IDO-/- and IDO inhibitor treated animals infected with LP-BM5 (Hoshi *et al.*, 2010). These findings indicate IDO may be inappropriately expressed in the MAIDS susceptible mice, thereby, suppressing their ability to clear this viral infection.

Based on the findings of Hoshi *et al.*, studies were conducted in the Stranford laboratory to measure IDO mRNA expression levels in LP-BM5 MuLV- infected BALB/c and C57BL/6 mice. It was found that higher IDO

mRNA levels are expressed in MAIDS susceptible C57BL/6 mice compared with resistant BALB/c strain during the first week of infection (L. Sceats, Thesis, 2011). Significant differences between the two strains were particularly observed in the lymph nodes of naïve and 3 day infected animals.

Proposed Studies

This project will expand upon work done by Lindsay Sceats in defining IDO mRNA expression patterns in the murine model of AIDS. This preliminary study had generated results that indicated that MAIDS-susceptible mice may be suppressing well-directed inflammatory responses, instead promoting suppressive immune environments that cannot control widespread viral replication.

This research will aim to compare IDO protein expression levels between the two strains of mice. I hypothesize that MAIDS-susceptible C57BL/6 mice may inappropriately suppress proinflammatory Th1 responses, thereby, favoring susceptibility to immune deficiency. On the other hand, MAIDS-resistant BALB/c mice launch a controlled and directed immune response to eradicate the virus and develop protective immunity. To test my hypothesis, I will focus on designing a mouse-IDO ELISA assay that will allow me to compare expression levels of the T-cell tolerogenic and immunosuppressive enzyme IDO in the two relevant strains of mice.

The majority of work done in the MAIDS model system is at later time points in the course of infection. My study will instead follow the expression of IDO in the first two weeks of infection, during which time innate and adaptive

immunity are first engaged and the virus is either eradicated or immune deficiency begins to develop. I want to determine whether there is an early differential expression of IDO between MAIDS susceptible and resistant mice. Furthermore, I seek to understand how this can influence the innate and/or adaptive arm of the immune system, consequently leading the two strains of mice to respond differently to the virus and select different immune response paths, one leading to virus destruction and protective immunity and the other resulting in persistent virus and MAIDS- susceptibility.

Findings that illustrate differences in IDO expression levels between the two strains of mice in this model can direct us towards important questions relevant to HIV/AIDS and may guide us in the production of treatment strategies or vaccine designs that can be most effective against this devastating virus.

MATERIALS AND METHODS

Animals

Eight to ten week old female BALB/c and C57BL/6 mice (*Mus musculus*) were purchased from Taconic Labs, New York and housed at Mount Holyoke College Animal Facility. All animals were handled according to the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Infection and Tissue Isolation

Infected mice from each strain were intraperitoneally injected with 1mL of the LP-BM5 isolate of MuLV at 3.6 x 10^4 PFU/mL, and compared with age-matched uninfected control (naïve) mice. Animals were sacrificed at 3, 7 or 14 days post-inoculation. Spleens and a pool of lymph nodes, including axillary, brachial, and inguinal nodes were isolated. The location of these secondary lymphoid organs is indicated in Figure 6.

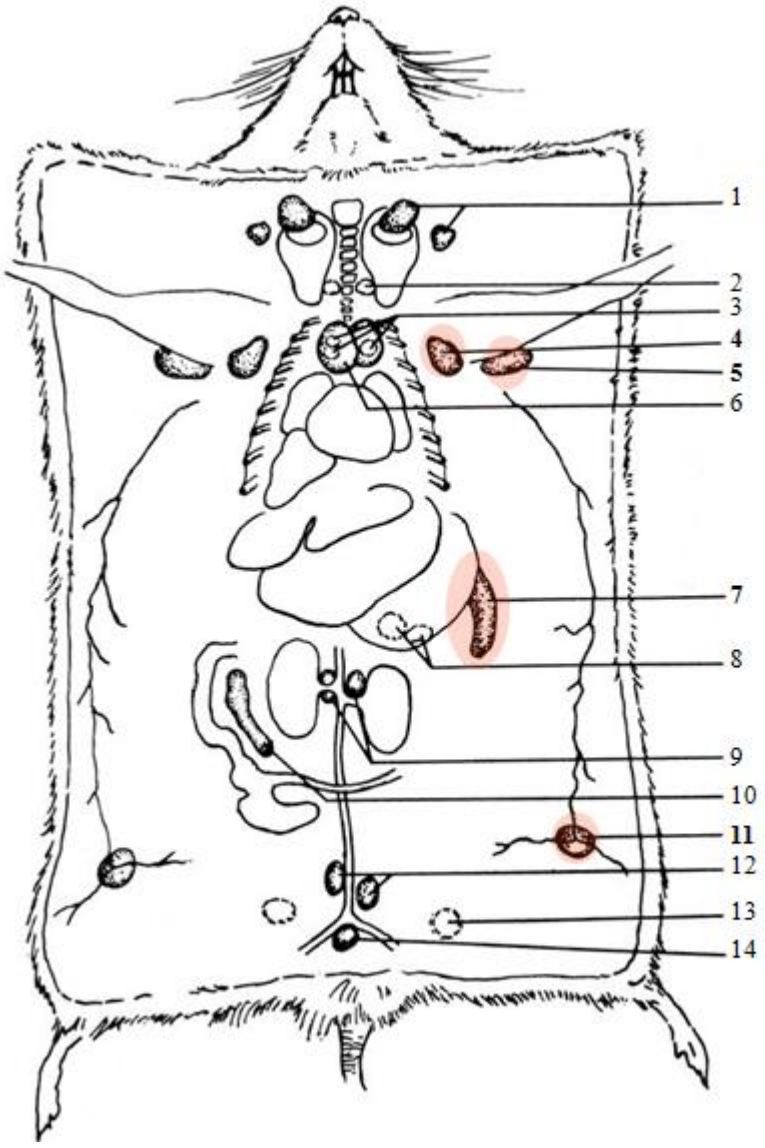


Figure 6. Schematic representation of the locations of lymph nodes in *Mus musculus*

Pictured here are the locations of all the lymph nodes present in a normal mouse (*Mus Musculus*). The numbers correspond to the spleen (7) and different types of lymph nodes. All the peripheral lymph nodes are bilateral and they are, therefore, found symmetrically on both sides of the body. Of special interest to this study are the peripheral axillary lymph nodes (4), brachial lymph nodes (5), and inguinal lymph nodes (11). The image was adapted from Dunn, 1954.

ELISA Sample Preparation

A protocol for homogenizing rat liver was adapted by Oana Ursu to harvest selected secondary lymphoid organs collected from naïve and infected animals (Borovikova *et al.*, 2000). Following isolation, pooled lymph nodes and spleens were homogenized in 0.5 mL or 1 mL ice-cold lysis buffer, respectively, for 30 sec at ¾ speed with a Polytron homogenizer. The lysis buffer was made of 1x BD BaculoGold Protease Inhibitor Cocktail (BD catalog # 554779) and 0.5% Triton X-100 in sterile PBS at a pH of 7.2. The homogenate samples were centrifuged at 12,000xg and 4 °C for 15 minutes. Clear supernatants were then aliquoted into small volumes and stored at -80 °C for later use.

IDO ELISA

A plate based assay known as an indirect ELISA was employed to detect and quantify differential expression of IDO in the secondary lymphoid tissues of naïve and MuLV- infected BALB/c and C57BL/6 mice. In this assay, an antigen of interest is immobilized to the surface of a microtiter plate and complexed with a primary antibody. Once the antigen adsorbs to the assay plate, its presence can be detected indirectly by adding a secondary enzyme-conjugated antibody. Subsequent incubation with a chromogenic substrate will produce a measurable product that can be detected and quantified by using a UV-Vis spectrophotometer. Figure 7 outlines the basic steps involved in setting up an indirect ELISA.

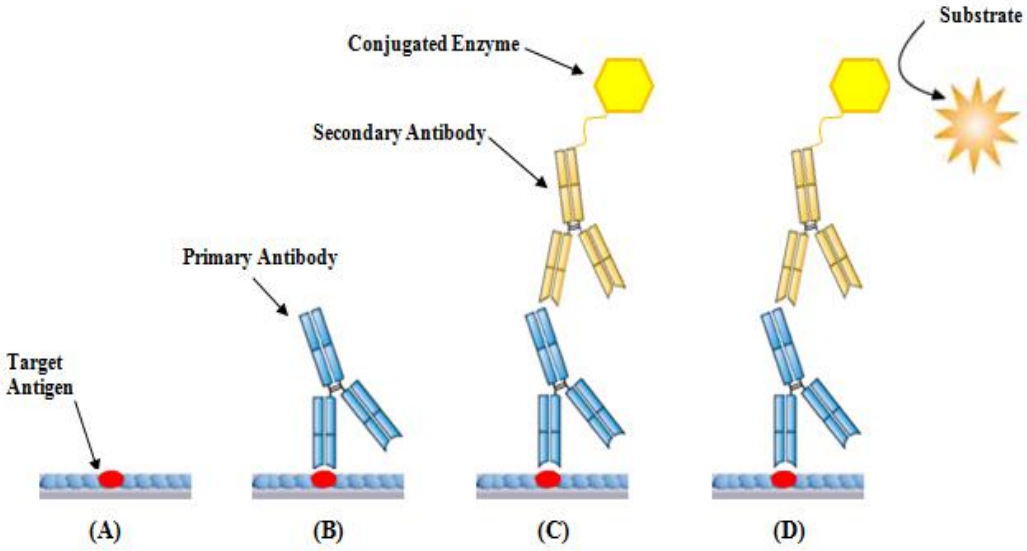


Figure 7. Illustration of an Indirect ELISA Platform.

In this assay (A) samples are added first allowing the antigen of interest to be immobilized on the microtiter plate through direct adsorption. (B) A specific unlabeled primary capture antibody binds to the target antigen. (C)A matched enzyme-conjugated secondary antibody is added to the plate. (D) Detection is accomplished when the enzyme reacts with the substrate to produce a chromogenic product. Through the use of a UV-Vis spectrophotometer, the results can be quantified. Image by Yemsratch Akalu.

Developing and Optimizing an Indirect IDO ELISA Platform

All reagents and enzymes were freshly prepared and kept cold (4 °C) throughout the experiment. The viability of the new IDO ELISA platform was first tested by constructing a standard curve using serial dilution of known-concentrations of the standard target protein. That is, recombinant IDO (rIDO) protein, 1 mg/mL, obtained from Enzo Life Sciences (Catalog # ALX-201-335-C050) was adsorbed to microtiter wells of BD Falcon 96 wells high affinity binding plates (Catalog # 353915) overnight at 4 °C. Nine two-fold serial dilutions of rIDO, ranging from 50,000 pg/mL to 97 pg/mL, were prepared in coating buffer (0.159 g Na_2CO_3, 0.35 g $NaHCO_3$ in 100mL of diH_2O, pH 9.6) and 100 µL aliquots of the solution were added to triplicate wells. Similarly 100 µL of coating buffer was added to the standard control wells.

Alternatively, spleen and lymph node samples produced a detectable signal that fell well within the range of the standard curve when diluted at 1:10 and 1:100, respectively. All test samples were diluted in the coating buffer before they were added in triplicates. All samples were run in triplicates to provide enough data for statistical validation of the results. Appropriately diluted lysis buffer was used as a negative control for the test samples.

The coated wells were washed three times with PBST (1x PBS and 0.05% Tween-20; 1x PBS = 0.156 g of NaH_2PO_4, 8.5 g of NaCl and 1.6 g of Na_2HPO_4 in 1 L of diH_2O, pH 7.0-7.2). The wells were blocked with 100 µL of 1x R&D

Assay Diluent (PBS + 1%BSA) diluted with distilled water from 10x stock solution, obtained from R&D Systems (Catalog # DY995) and incubated at 37 °C for 2 hours. Plates were washed three times again with PBST after incubation. One hundred microliters of purified Rabbit anti-Mouse IDO IgG from Enzo Life Sciences (Catalog # ALX- 210-432-C100) that had been diluted to 2 μg/mL with 1x R&D Assay Diluent were added to each well. The plates were then incubated at 37 °C for 1 hour. Plates were again washed three times with PBST before 100 μL of horseradish peroxidase (HRP) conjugated secondary antibody (Goat anti-Rabbit IgG) from Enzo Life Sciences (Catalog # ADI-SAB-300-J) that was prepared at 1:5000 dilution with 1x R&D Assay Diluent was added to each well. Following an hour long incubation at 37 °C, the plates were finally thoroughly washed five times with PBST and 100 μL of substrate solution (1:1 mixture of TMB I and II, pH 6.0 from R&D Systems, Catalog # DY999) was added to each well. Unsealed plates were then incubated in the dark at room temperature for 20 minutes. Finally, 50 μL of stop solution (1 M H_3PO_4) was added to stop the action of the enzyme on the substrate. To ensure complete mixing of the stop solution with the existing enzyme-substrate complexes, the plates were gently swirled. The absorbance for each sample was read within 5 minutes on an ELISA Plate Reader (VersaMax microplate reader) measuring absorbance at 450 nm, with the correction wavelength set at 570 nm.

Data Analysis

Once optical density (O. D.) values were obtained for all samples, triplicate wells that were not within 20% of their mean O.D. value were masked and taken out of consideration. The concentration of IDO in our unknown samples was then determined from the standard curve. Statistical analysis was done using a univariate analysis of variance (ANOVA). As a collection of statistical models, the ANOVA partitions variance observed in a particular variable into components that arise from different sources of variation. The ANOVA statistically determines whether the means of multiple sample groups are all equal. It does so by testing the null hypothesis that the error variance of the dependent variable is equal across groups. Hence the null hypothesis states that there are no differences in the expression of IDO protein between the different groups that differ by strain and time post-infection.

In order to meet this assumption of homogeneous variance amongst different groups, the mean concentration values of IDO present in the spleen were transformed into their corresponding natural log values. However, IDO mean concentration levels obtained from lymph node samples were not transformed. In fact, the presence of two outliers (2 naïve C57BL/6 mice) did not allow the lymph node data set to pass Levene's test for equality of variances. As a result, ANOVA was carried out on day 3, 7 and 14 lymph node samples only. Since the inclusion of such outliers does not interfere with the evaluation of statistical differences,

however, analysis for statistical significance was done with the entire data set. The residuals, defined as differences between observed mean concentrations of IDO and values estimated by the regression equation were also used to test the assumption of the analysis. Plots of estimated values vs. residuals should lack specific trends.

Since we have one dependent variable (mean concentration of IDO protein) and three independent variables (strain, days post-infection and tissue type), a three-way ANOVA was performed. This allowed us to test the effect of strain, time post-infection and tissue type on the level of IDO protein expressed. More specifically, the ANOVA allowed us to access whether there are any interactions that exist between strain and time post-infection. The data was evaluated with an N of 3 for samples collected from naïve, day 3 and day 7 animals, while an N= 4 was used for samples collected at day 14 post- infection.

Two tailed *t*-tests were also conducted to determine whether there were significant differences between a set of specified conditions. At day 14, a t-test was used to test for significant differences in IDO expression between MuLV-infected BALB/c and C57BL/6 mice, both in the spleen and lymph nodes. Additionally, *t*-tests were performed by time in the same tissue, within strain. Statistical significance was ascribed to p values less than 0.05. 5% is an arbitrary significance level (alpha). In order to reduce the problem of multiple comparisons, a Bonferroni correction was done that alters the alpha value. This

conservative measure gave us a cutoff value of 0.01, thereby, reducing the chance of making Type I errors that assume an effect when there isn't one. Therefore, statistical significance was evaluated with two parameters — according to alpha ($p<0.05$) and Bonferroni ($p<0.01$).

Finally the standard error of the mean (SEM) was used as a tool of assessing error. The SEM is a measure of the accuracy of the sample mean by comparing it to the true population mean. It is calculated by dividing the standard deviation (STD) by the square root of the number of samples ($\sqrt{N}$). Therefore, an increase in sample size will lower the SEM.

RESULTS

Detecting Changes in IDO Protein Levels using an Indirect ELISA

Generating a Standard Curve

One of the primary objectives of this study was to design an optimal and reproducible mouse IDO ELISA system that would allow the comparison of protein levels in naïve and MuLV infected mice. To the authors' knowledge, there is currently no mouse IDO ELISA platform that is commercially available. Upon the recommendation of Enzo Life Sciences, an indirect ELISA system was set up to determine detectable concentration range of the standard protein. Starting with recombinant IDO concentration of 50,000 pg/mL a two-fold serial dilution was carried out to obtain a final concentration of 97.5 pg/mL. A working standard curve such as the one shown in Figure 8 was generated. The dynamic standard concentration range was then determined to be 50,000- 781.5 pg/mL.

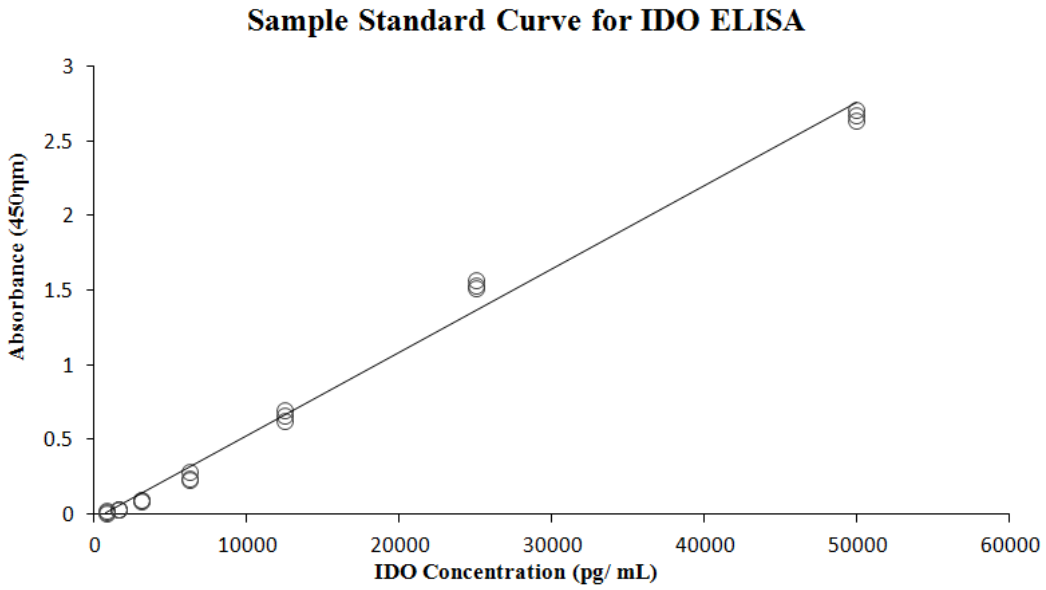


Figure 8. Sample standard curve for IDO indirect ELISA.

A standard curve was constructed by plotting known concentrations of IDO protein against corresponding absorbance readings. Results presented above are for each standard concentration that was tested in triplicate wells. The concentrations of unknown samples can be extrapolated from the linear fit line of this graph. The line is represented by a linear fit equation of y= 6E-05x - 0.0384 where R^2 value equals 0.992.

Differential Expression of IDO

Analysis of IDO protein expression was done on tissue samples obtained from the spleens and lymph nodes of naïve and virus-infected animals. IDO concentrations in the secondary lymphoid organs of MuLV-infected mice were assessed 3 days, 7 days and 14 days post-infection. Each condition (defined by strain, tissue and time after infection) was represented by three animals. At Day 14 post-infection, however, four animals instead of three were used. Once O.D. readings were recorded from the samples, the standard curve was utilized to extrapolate corresponding IDO concentrations in pg/mL. Dilution factors were then taken into account (1:10 for spleen samples & 1:100 for lymph node samples) to finally determine protein concentration in our original extract.

Spleen

Mean IDO concentration values obtained from the spleen were transformed into their natural log equivalents to meet the test for equality of variances. Results obtained from the ANOVA test showed interaction between strain and time post-infection. Figure 9 represents the mean concentration of IDO in the spleens of naïve and virus-infected animals as a function of time post-infection. During the first three days of infection, significant changes in IDO expression level are not observed in both BALB/c and C57BL/6 mice. By day 7 post-infection, however, IDO expression is significantly upregulated in the spleens of both resistant and susceptible mice. The level of increase in IDO

protein concentration observed between day 3 and day 7 in the resistant BALB/c strain is significant by α ($p< 0.05$) and Bonferroni ($p< 0.01$), whereas in the susceptible C57BL/6 mice, it is significant by α ($p<0.05$) only. During the second week of infection, susceptible C57BL/6 mice maintain their level of IDO expression, while resistant BALB/c mice significantly downregulate IDO expression by day 14 ($p< 0.01$).

When comparing spleen IDO levels across strains before infection, the two strains of mice have comparable levels of IDO (Figure 10). Similarity in the two strain's levels of expression of IDO is still observed 3 days post-MuLV exposure. Although at significantly higher concentrations, both strains maintain this trend at day 7. By day 14 though, resistant BALB/c mice have a significantly lower concentration of IDO in their spleens ($p<0.05$).

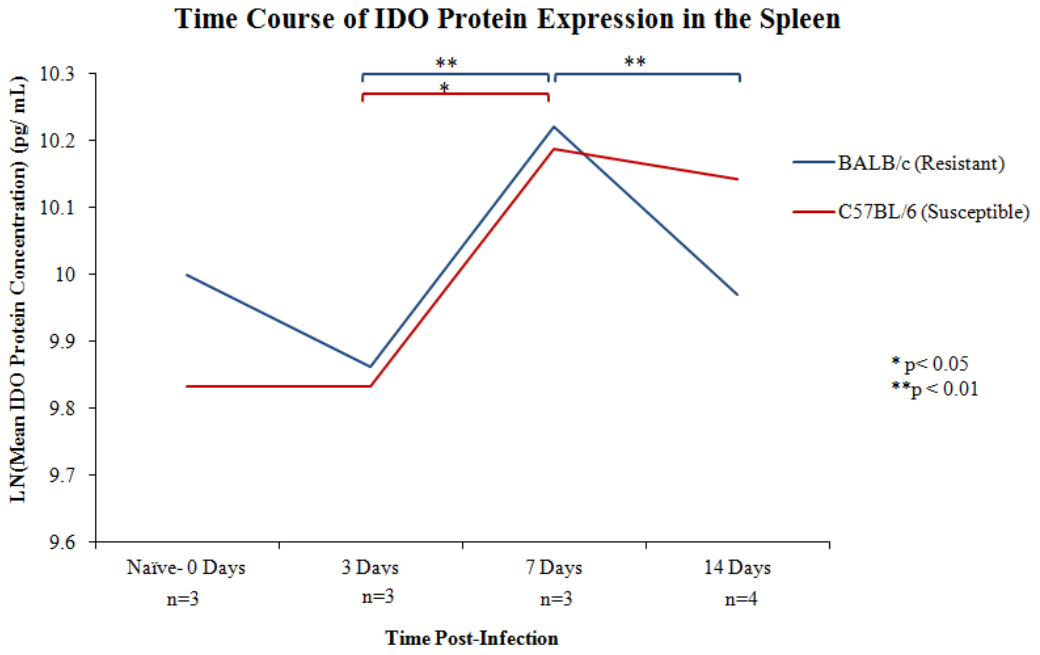


Figure 9. IDO protein expression differs significantly across time in the spleens of MuLV-infected mice.

Up until the end of the first week of MuLV-infection, the two strains of mice maintain comparable levels of IDO protein in their spleens. However, between days 3 and 7 post-infection, both strains significantly upregulate the concentration of the protein ($p<0.05$ for C57BL/6 and $p<0.01$ for BALB/c). After day 7, only resistant BALB/c mice are observed to significantly repress IDO ($p<0.01$). Note that the data is not longitudinal. Each mean concentration of protein was recorded from a unique set of animals.

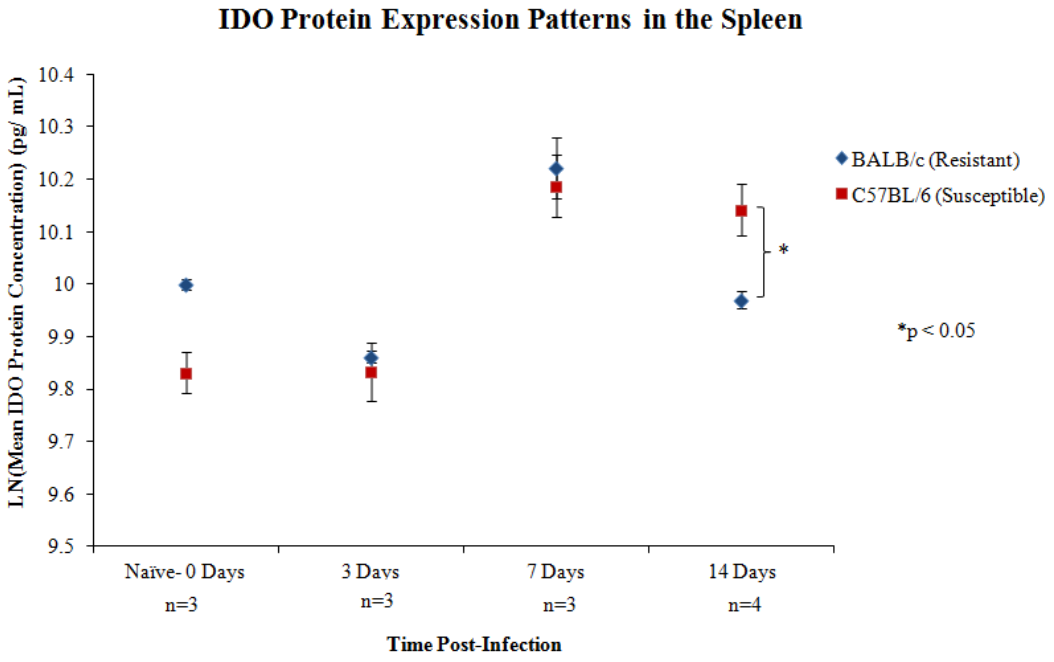


Figure 10. Differential expression of IDO protein between BALB/c and C57 BL/6 mice is observed in the spleen.

Naïve, 3 day infected and 7 day infected mice of different strains do not exhibit statistically significant IDO expression differences. On the other hand, at day 14, significantly higher levels of IDO protein are recorded in the susceptible C57BL/6 mice ($p<0.05$).

Lymph nodes

Data gathered from pooled lymph nodes of naïve and virus-infected animals was not transformed to meet the assumptions of the null hypothesis. Instead it was sufficient to utilize mean IDO concentration values to plot and observe an interaction between strain and time post-infection in lymph nodes. Overall, the patterns of IDO expression in the lymph nodes were very similar to that of the spleen. As shown in Figure 11, when compared to mice that have not been exposed to the virus, infected animals of both strains maintain similar levels of IDO throughout the two weeks following infection. The only exception is recorded between days 7 and 14 when the resistant BALB/c mice significantly decrease IDO expression in the peripheral lymph nodes ($p < 0.05$).

When the same set of data is evaluated for strain-specific differences of IDO expression in Figure 12, it is apparent that the most significant differences are detected at day 14. IDO concentration is significantly higher, as evaluated by both α ($p < 0.05$) and Bonferroni ($p < 0.01$). Of note, two outliers have contributed towards an increase of the basal level of IDO recorded in the naive C57BL/6 group.

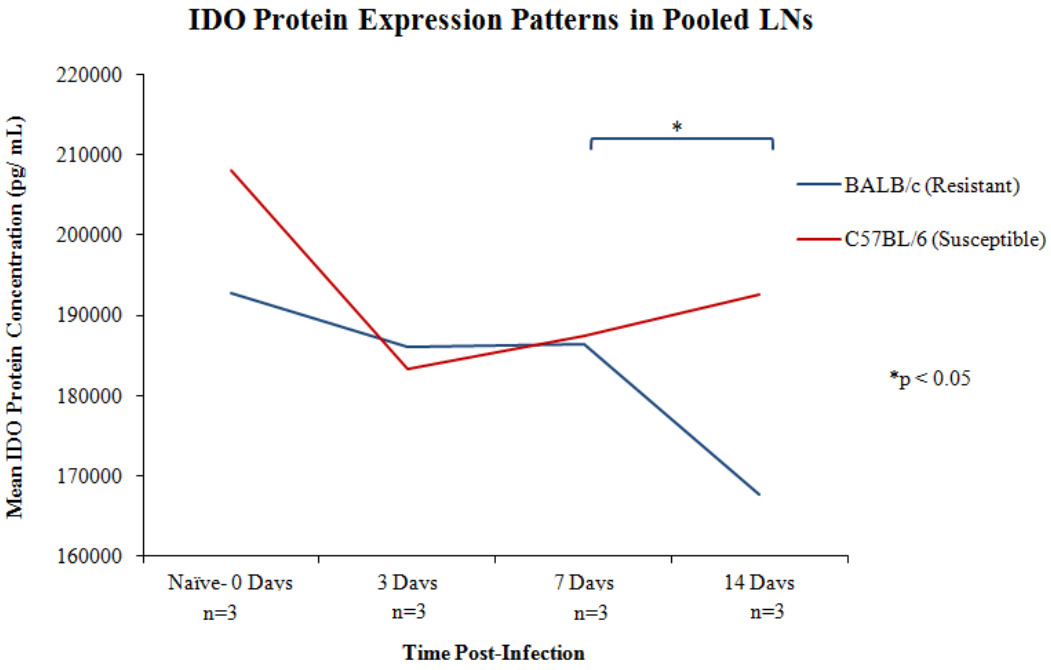


Figure 11. Over the course of the second week of infection, IDO protein expression differs in the lymph nodes of MAIDS-resistant BALB/c mice.

Until day 7 post-infection, relatively similar levels of IDO protein were expressed by both strains of mice. After day 7 post-infection, MAIDS-susceptible C57BL/6 mice continued to maintain similar levels of IDO concentration in the lymph nodes, while MAIDS-resistant BALB/c mice significantly dampened their expression of the protein ($p<0.05$). Note:- As repeated observations of the same variable (i.e. mean IDO concentration) were not made, the data is not longitudinal.

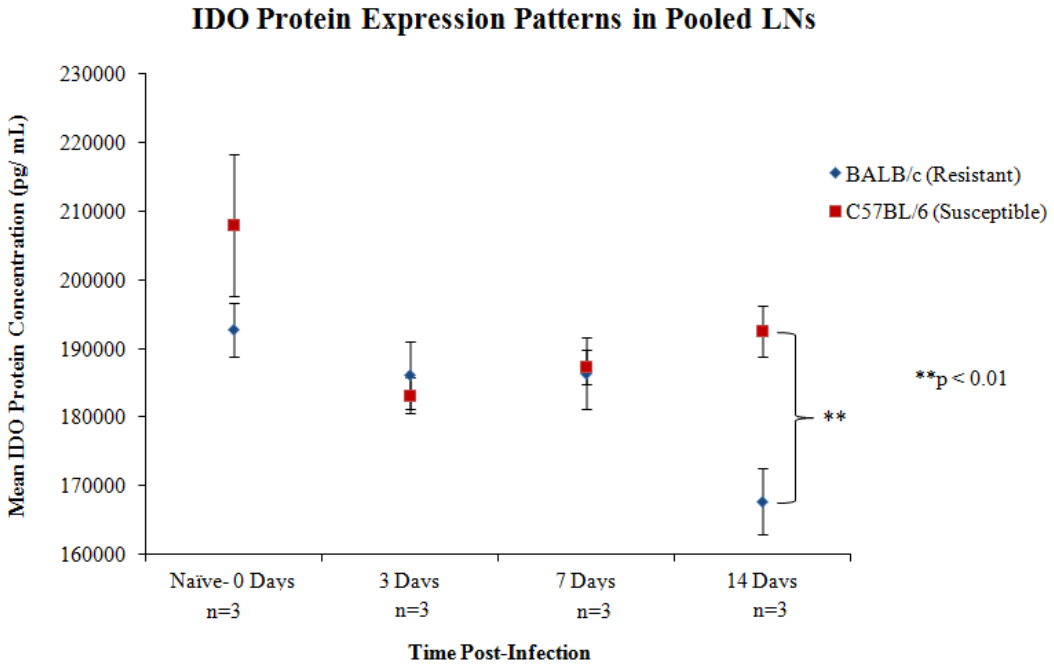


Figure 12. The highest concentration differences in IDO between strains in the lymph nodes of virus infected animals were recorded at D14.

At day 14 C57BL/6 mice express significantly more IDO than the corresponding BALB/c cohorts ($p<0.05$). At all other times of observation, differences of protein expression across strains are not statistically significant.

DISCUSSION

In normal individuals, primary infections are cleared by the combined efforts of the innate and adaptive immune systems. Once a pathogen evades targeting by the innate immune response, a transition to a more specific and robust adaptive immune response is required. After a pathogen has successfully been cleared from the host's system and an infection has concluded, active immune responses need to come to an end. In other words, maintaining balance between active pathogen-fighting responses and negative regulatory signals is crucial towards having a properly functioning immune system. If an immune response is turned off too soon, or the wrong arm of the immune system is suppressed during infection, the host will likely mount an inefficacious response that fails to eliminate target pathogens. Natural immunosuppressant molecules play a considerable role in maintaining a fine balance between activation and suppression of the immune system. Over the course of an infection, they are induced to dampen or altogether shut down active responses to various stimuli. One mechanism that the immune system utilizes to control local immune response is to deploy key proteins that are involved in various metabolic pathways to influence innate and adaptive immunity, as well as the transition in between.

In recent years, more focus has been given to studying the basis of using IDO mediated catabolism of tryptophan as an immunoregulatory mechanism in the body. A significant amount of research has been done to understand how expression of IDO in placental cells prevents rejection of mammalian fetuses.

Pioneering work done by Munn *et al*. in 1998 had demonstrated that IDO prevents maternal T-cells from attacking the fetus by degrading the essential amino acid tryptophan. Additionally, IDO's role in inhibiting graft rejections in various transplant models has solidified IDO's place as an immunomodulatory protein (Takikawa, 2005). Although links between inflammation and induction of IDO have been drawn for years, well defined mechanisms that outline its role in fighting against invading pathogens are still lacking.

A broad range of cell types produce IDO and different molecular cues control its activity during inflammation. The inhibitory receptor CTLA-4 expressed on T-cells induces IDO production by binding B7 molecules present on the surface of DCs (Grohmann *et al.*, 2002). Signaling down B7 molecules activates IFN-γ expression which in turn initiates IDO production. CD4+CD25+ T_{regs} initiate signaling through CTLA-4 to trigger IDO production in DCs (Fallarino *et al.*, 2003). In contrast, ligation of B7 molecules by stimulatory CD28 receptors on T-cells inhibits IFN-γ mediated IDO production and instead facilitates interleukin-6 (IL-6) expression (Orabona *et al.*, 2004). Hence, CTLA-4-B7 interactions generate tolerogenic DCs (T-cells that recognize their antigen on these DCs develop tolerance for the displayed antigens), whereas CD28-B7 interactions produce stimulatory DCs. This can be detrimental in the context of a chronic infection as inappropriate expression of IDO, and by extension predominant expression of tolerogenic DCs, can interfere with the type of T-cell immunity launched. That is, different subsets of DCs express a variety of surface

molecules on their surface that determines what type of T-cell polarizing signal they display (Steinman *et al.*, 2003; Grohmann *et al.*, 2003). Thus, a Th1, Th2, T_{reg} or Th17 response can be generated. In the case of MAIDS and as a corollary HIV/AIDS, the subsets of T-cells that are employed by the adaptive immune system to fight off the viral infection are crucial in determining the type of outcome obtained.

The MAIDS model provides an ideal platform to compare different responses to the same virus in two strains of MuLV-infected mice that lead to either acquisition or prevention of immune deficiency. This will allow us to investigate if there is an early preferential expression of immunosuppressant molecules in the MAIDS susceptible mice strain and how a misdirected and ill-timed production of these molecules affects subsequent immune responses by promoting a Th1, Th2, T_{reg} or Th17 cell-based immunity. In an effort to build upon the work of Hoshi *et al.*, 2010, this research project will evaluate if there are differences in the expression of IDO protein between BALB/c and C57BL/6 mice during the first two weeks post- MuLV infection. Hoshi and colleagues had demonstrated that the absence of IDO or inhibition of its enzymatic activity results in the upregulation of Type I interferons, suppression of viral replication and increase of pDC count. To the best of our knowledge, this project is the first to measure IDO protein concentration levels in the secondary lymphoid organs using an ELISA platform in the MAIDS model system. Other groups generally

tend to investigate IDO enzymatic activity, instead of protein concentration, and they utilize western blots to quantitatively determine IDO expression levels.

IDO expression differs in BALB/c and C57BL/6 mice

An indirect ELISA was designed to measure the amount of IDO protein present in selected secondary lymphoid organs (spleen and some peripheral lymph nodes) of BALB/c and C57BL/6 mice. Results obtained from spleen samples showed that there is a significantly greater and sustained expression of IDO in C57BL/6 mice than there is in corresponding cohorts of BALB/c mice between 7 and 14 days post-infection. The differences in protein expression across the two strains were most pronounced 2 weeks post-MuLV-exposure. When these results were evaluated against relative viral load at various time points, it was clear that the higher levels of immunosuppressive IDO protein maintained in the MAIDS-susceptible C57BL/6 mice correlated with exponential expression of viral mRNA (Figure 13). In contrast, after eradicating the virus, BALB/c mice suppress their IDO levels significantly.

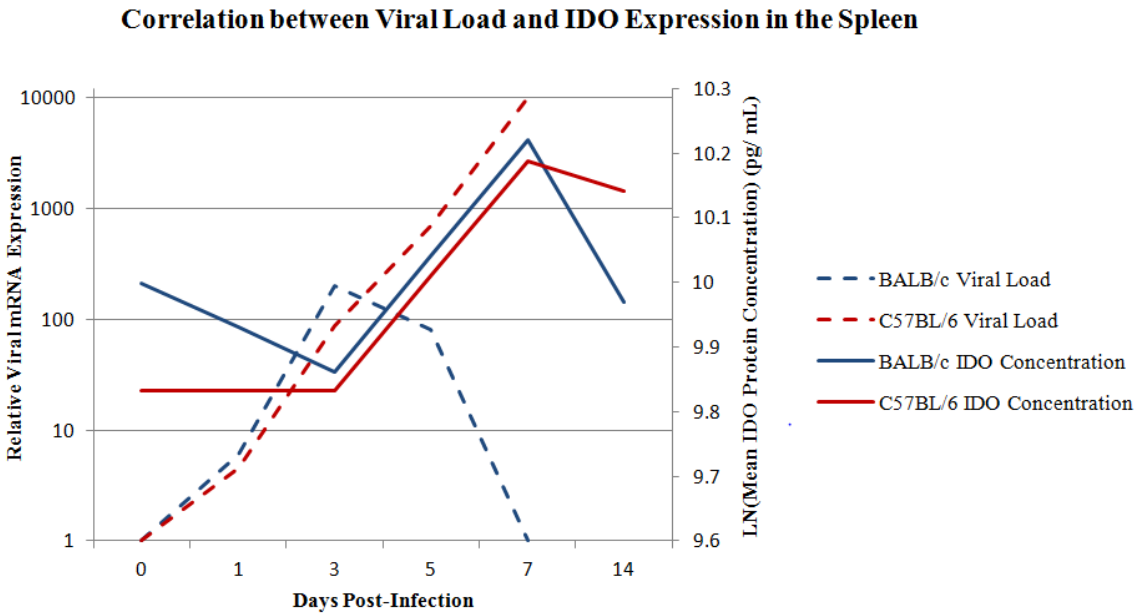


Figure 13. Correlation between viral loads and IDO expression in the spleens of each strain of mice.

The data above indicates relative viral loads present in the spleens of MuLV-infected BALB/c and C57BL/6 mice. Simultaneously shown are corresponding mean IDO concentration levels (in pg/mL). IDO expression data for days 1 and 5 were extrapolated by connecting day 0, 3 and 7 data points by a line. As viral load continues to exponentially grow in MAIDS-susceptible C57BL/6 mice, higher IDO levels are maintained. Conversely, in MAIDS-resistant BALB/c mice, following clearance of the virus, IDO expression is suppressed. Credit for gathering viral mRNA expression data goes to Sonia Bakkour, Ph.D.

The IDO protein expression patterns recorded in the spleens of these mice can perhaps be explained in terms of balancing antimicrobial and immunosuppressive activities by IDO. IDO's enzymatic action against tryptophan has been primarily understood as a mechanism of reducing microbial growth (Pfefferkorn, 1984; Grohmann *et al.*, 2003). The same mechanism is also employed in suppressing the immune system (Mellor and Munn, 2004). This dual use of a single mechanism introduces a delicate balance that requires the immunosuppressant molecule to be upregulated to fight a pathogen, while avoiding inhibition of strong immune responses that are required to protect the host's system.

After exposure to the virus for 3 or 7 days, both strains of mice achieve homogeneity in their expression of IDO in the spleen. This could be the period in which the respective immune systems of these two strains of mice receive optimal stimulation from the presence of the virus. Secretion of various cytokines such as IFNα/β and IFN-γ can induce significant production of IDO in both strains of mice. Hence, IDO activity will be present at the beginning of the inflammatory process. IDO can then exercise its anti-microbial function by starving pathogens through its degradation of trypthophan. Additionally, the presence of IDO will inhibit proliferation of T-cells. Of note, toxic tryptophan metabolites can induce selective apoptosis of activated T-cells (Terness *et al.*, 2002). So in MAIDS-resistant BALB/c mice, IDO activity could be significantly dowregulated in the second week of infection to allow activated T-cells to circulate in the blood and

clear viral infection. In contrast, MAIDS-susceptible C57BL/6 mice may be maintaining immunosuppressive levels of IDO that are selectively killing their pro-inflammatory Th1 cells, leaving their immune systems vulnerable to a variety of opportunistic infections. If this is true, it is important to identify which IDO-mediated mechanisms inappropriately suppress effective immune responses.

Of the known functions of IDO, the two most likely mechanisms that the protein could be using to suppress immune responses in the C57BL/6 mice are catabolism of tryptophan and accumulation of toxic kynurenine metabolites. In cell-culture medium, tryptophan concentration needs to be less than 0.5-1μM to produce an immunosuppressive environment (Munn *et al.*, 1999). In the context of the extracellular environment *in vivo*, where there is a constant diffusion of molecular products, it is unlikely for low tryptophan concentrations required for inhibition of T-cell proliferation to be maintained. Instead, it is more plausible for tryptophan metabolites to contribute towards immunosuppression in the C57BL/6 mice by inhibiting T-cell proliferation. As mentioned before, two downstream metabolites of kynurenine, 3-hydroxy-anthranilic acid and quinolinic acid, cause depletion of murine thymocytes (Fallarino *et al.*, 2002). Moreover, these two toxic metabolites were shown to induce selective death of Th1 cells (Fallarino *et al.*, 2002).

Patterns of mean IDO expression in samples obtained from peripheral lymph nodes (axillary, brachial and inguinal) of uninfected and virus-infected

animals closely resembled results acquired from corresponding spleen samples of the same sets of mice. Unlike the pattern of IDO expression in the spleen, however, there was no significant increase of protein expression observed in either strain of mice, during the first week of infection. Exposure to the virus doesn't induce detectable increase of IDO in both BALB/c and C57BL/6 mice. Instead, both strains of infected mice maintain levels of IDO that are comparable to recorded concentrations of IDO in uninfected animals. In fact, data gathered at days 3 and 7 is very homogeneous as both strains of mice express very similar levels of IDO. This could mean that molecular signals required for initiation of IDO production are not present around immune cells in peripheral lymph nodes. Thus the balance is tipped towards maintaining lymphocyte proliferation instead of launching IDO-mediated anti-microbial activity. However, this mechanistic hypothesis does not explain why C57BL/6 mice that are keeping the immunosuppressive IDO protein at a basal level progress to immunodeficiency while BALB/c mice, that significantly downregulate IDO expression between days 7 and 14, avoid acquisition of MAIDS. Alternatively, we can speculate that there are distinct subsets of immune cells that are primarily responsible for producing IDO during the healthy (uninfected) state and viral infection. It has been shown that a subset of IDO-producing human- monocyte driven DCs could be induced to have tryptophan catabolic function but not have T-cell suppressive activity (Munn *et al.*, 2002; Terness *et al.*, 2005). The surfaces of these non adherent DCs are marked by the presence of CD123 and CCR6 (Munn *et al.*,

2002; Terness *et al.*, 2005). Consequently, after infection, the types and functions of subsets of IDO-producing immune cells may be markedly different in the two strains of mice. As a result, it may be the case that MAIDS-resistant mice suppress IDO expression to successfully control viral replication, while MAIDS-susceptible mice leave selective killing of T-cells unchecked.

Immunosuppressive axis in the MAIDS model system

The ability of APCs to orchestrate an immune response largely comes from external cues from the environment. These external signals include the local cytokine milieu. Specifically, if there are pro-inflammatory Th1 cytokines around, then APCs such as DCs will receive instruction to present antigen. Alternatively, anti-inflammatory and immunosuppressive Th2 associated cytokines will give tolerogenic potential to DCs (Grohmann *et al.*, 2003; Steinman *et al.*, 2003). Interleukin-10 (IL-10), an anti-inflammatory cytokine, restricts the activity of Th1 cells and potentiates stable expression of IDO in DCs (Munn *et al.*, 2002). Findings in the Stranford laboratory show that, when compared to MAIDS-resistant BALB/c mice, MAIDS-susceptible C57BL/6 mice express significantly higher levels of IL-10 in their lymph nodes (A. Kwaa, Thesis, 2012). Since this significant difference in IL-10 expression was observed at day-3 post-infection, it is likely that IL-10 is influencing the direction taken by the innate immune systems of the two strains of mice. More importantly, these differences in IL-10 expression were recorded from tissue samples that were also used to test for

differential IDO expression. Hence early inappropriate upregulation of IL-10 in lymph nodes of the C57BL/6 mice may be promoting stable expression of IDO, whereas, the low levels of IL-10 present in the BALB/c mice may be facilitating subsequent dowregulation of IDO expression between day 7 and 14 post-infection. Hence, during the first two weeks after infection, IDO may be regulated differentially in the two animals.

Lastly as mentioned before, DCs have T-cell polarizing signals that allow them to determine the type of T-cell response induced during an immune response (Grohmann *et al.,* 2003). If DCs indeed assume tolerogenic phenotypes in MAIDS susceptible C57BL/6 mice, then they can induce T_{reg} cell activity (Grohmann *et al.,* 2003; Steinman *et al.*, 2003). It's important to recall that CTLA-4 mediated signaling on T_{reg} cells as well as release of TGF-β by T_{reg} cells induces IDO activity in subsets of DCs (Pallota et al., 2011; Walker and Sansom, 2011). Thus it is possible that an immunosuppressive environment is further facilitated in the C57BL/6 mice by an imbalance between Treg cells and other CD4+ T-cell subtypes.

Future Studies

In this project we have managed to answer our primary question of interest regarding early differential expression of IDO following MuLV infection of MAIDS-susceptible and -resistant mice. However, this study has raised a number of basic questions that we would like to address in the future. Before initiating

experiments to answer related questions though, the results obtained in this project should be replicated. Since we have additional tissue samples from animals used in this study, expression levels of IDO can be re-tested in these samples. This would decrease variability within statistical replicates and impart statistical power to observed trends of IDO expression. Addition of biological replicates to the study will also increase the confidence level in the observed patterns of protein expression.

Currently analysis of IDO expression only evaluates protein concentration in pg/mL of assayed supernatant. Future experiments should first assess IDO concentrations relative to each lysates' total protein concentration in pg IDO/mg total protein. Total protein concentrations of the lymph node and spleen supernatants can be obtained by running a Bradford assay.

Once each animal is intraperitoneally injected with the LP-BM5 isolate of MuLV, free virus should become systemic. Thus, free virus will be present in the blood as well as the lymphatic system. However, the cell to cell viral load may not be the same in the lymph nodes and the spleen. Nevertheless, the pattern of viral burden should be similar. Throughout this experiment, we have been consistent in our isolation of lymph nodes. Our approach has been standardized so that we only collect specific peripheral lymph nodes. Since all isolated lymph nodes are bilateral and the spleen can be cut longitudinally into halves, a future experimenter can take lymph nodes present on one side of the body and halves of

the collected spleens to measure viral expression, while using the rest of the samples to run a protein assay. Direct correlation between viral load and IDO levels can then be evaluated. In addition, it would be interesting to measure IDO expression in central lymph nodes that are present in the body's central cavity. Samples obtained from these sites may or may not show the patterns of IDO expression observed in the peripheral lymph nodes.

Since IDO's role as an immunosuppressant protein has been well documented, it would be useful to determine mechanisms that allow it to inhibit immune responses. This would involve studies of other proteins such as the inhibitory glycoprotein CTLA-4 and immunosuppressive cytokine TGF-β. In addition, it is worth investigating the role of suppressive T_{reg} cells in the MAIDS model. Alternatively, we can measure kynurenine metabolites in the secondary lymphoid organs of virus-infected animals. This will elucidate IDO activity following MuLV infection in the two strains of mice.

Finally it is well known that IDO can be expressed by a wide variety of immune and nonimmune cells (Mellor and Munn, 2004). Therefore it would be interesting to investigate what the consequences of IDO production in nonimmune cells are. If only certain subpopulations of APCs use IDO to shut off lymphocyte expansion and activity, what is the immunoregulatory function of non-immune cells that produce IDO? Such lines of investigation will allow us to determine

potential pathways through which tolerogenic immune cells may be exerting their function.

Conclusion

In normal individuals the immune system is successful in maintaining a fine balance between activation and suppression of its different arms. Sometimes, in the presence of invading pathogens, this balance is disturbed to dangerous levels. Recent research has clearly highlighted how increased expression of natural immunosuppressant molecules such as IDO can contribute towards progression to immunodeficiency by interfering with the launching of a robust and specific immune response against murine leukemia virus (Hoshi *et al.*, 2010).

Our results from this study indicate that there is an early differential expression of IDO in BALB/c and C57BL/6 mice. Differences of protein expression between the two strains of mice are highly pronounced 14-days post infection both in the spleen and lymph nodes of the animals. Increased expression of IDO in the susceptible C57BL/6 strain may perpetuate viral replication by dampening efficient immune anti-viral responses. Hence, the differences in IDO expression between the two strains of mice and its correlation with the efficiency of pathogen eradication, revealed in this study, may have similar relevance in HIV/AIDS. Therefore, the level and role of IDO protein expression, and immune signals that induce its production after an animal's exposure to MuLV, command

further investigation as it may offer important insights into the function of IDO and other closely related immunosuppressant molecules in HIV/AIDS.

REFERENCES

Akira, S., Uematsu, S., and O. Takeuchi. 2006. Pathogen recognition and innate immunity. *Cell*. 124:783-801.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., and P. Walter. 2008. *Molecular Biology of the Cell*. 5th ed. New York: Garland Science.

Alegre M., Shiels H., Thompson C. B., and T. F. Gajewski. 1998. Expression and function of CTLA-4 in Th1 and Th2 Cells. *J. Immuno.*161: 3347-4456.

Beutler, B. 2004. Innate immunity: an overview. *Mol. Immunol.* 40(12):845-849.

Boasso, A., Herbeuval, J., Hardy, A. W., Anderson, S. A., Dolan, M. J., Fuchs, D. and G. M. Shearer. 2007. HIV inhibits CD4+ T-cell proliferation by inducing indoleamine 2, 3-dioxygenase in plasmacytoid dendritic cells. *Blood.* 109: 3351-3359.

Boasso, A. and G. M. Shearer. 2007. How does indoleamine 2, 3- dioxygenase contribute to HIV-mediated immune dysregulation. *Curr. Drug Metab.*8:217-223.

Boehm, U., Klamp, T., Groot, M., and J. C. Howard. 1997. Cellular responses to interferon-gamma. *Annu. Rev. Immunol*. 15:749-795.

Borovikova, L. S., Ivanova, S., Zhang, M., Yang, H., Botchkina, G.I., Watkins, L.R.,Wang, H., Abumrad,N., Eaton, J.W. and K. J. Tracey. 2000. Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin. *Nature.* 405:458-462.

Cesta, M. F. 2006. Normal structure, function, and histology of the spleen. *Toxicol. Pathol.* 34:455-465.

Chen W. 2011. IDO: more than an enzyme. *Nat. Immunol.* 12 (9): 809-811.

Clark, S., Page, E., Ford, T., Metcalf, R., Pozniak, A., Nelson, M., Henderson, D. C., Asboe, D., Gotch, F., Gazzard, B. G., and P. Kelleher. 2011. Reduced T(H)1/T(H)17 CD4 T-cell numbers are associated with impaired purified protein derivative-specific cytokine responses in patients with HIV-1 infection. *J. Allergy Clin. Immunol*. 128(4):838-846.

Coffin, M. J., Hughes, H. S., and E. H. Varmus. 1997. *Retroviruses*. Plainview, NY: Cold Spring Harbor Laboratory Press.

Cohen, M. S., Chen, Y. Q, McCauley, M., Gamble, T., Hosseinipour, M.C, *et al*., (29). 2011. Prevention of HIV-1 infection with early antiretroviral therapy. *N. Engl. J. Med.* 365(6):493-505.

Collins, A. V. , Brodie, D. W., Gilbert, R. J. C., Iaboni, A., Manso- Sancho, R., Walse, B., Stuart, D. I., Anton van der Merwe, P. and S. J. Davis. 2002. The interaction properties of costimulatory molecules revisited. *Immunity.* 17: 201-210.

Dunn, T. B. 1954. Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. *J. Nat. Cancer Inst.* 14 (6): 1281-1433.

Egen, J. G., Kuhns, M. S. and J. P. Allison. 2002. CTLA-4: new insights into its biological function and use in tumor immunotherapy. *Nat. Immunol.* 3(7): 611-618.

Fallarino, F., Grohamann, U., Hwang, K. W., Orabona, C., Vacca, C., Bianchi, R., Belladonna, M. L., Fioretti, M. C., Alegre, M.-L and P. Puccetti. 2003. Modulaton of tryptophan catabolism by regulatory T cells. *Nat. Immunol.* 4:1206-1212.

Fallarino, F., Grohmann, U., Vacca, C., Bianchi, R., Orabona, C., Spreca, A., Fioretti, M.C. and P. Puccetti. T cell apoptosis by tryptophan catabolism. *Cell Death Differ.* 9: 1069–1077.

Fassati, A. 2006. HIV infection of non-dividing cells: a divisive problem. *Retroviriology* 3(74): 1-15.

Favre, D., Mold, J., Hunt, P. W., Kanwar, B., Loke, P., Seu, L., Barbour, J. D., Lowe, M. M., Jayawardene, A., Aweeka, F., Huang, Y., Douek, D. C., Brenchley, J. M., Martin, J. N., Hecht, F. M., Deeks, S. G., and J. M. McCune. 2010. Tryptophan catabolism by indoleamine 2, 3-dioxygenase 1 alters the balance of T_H17 to regulatory T Cells in HIV disease. *Sci. Transl. Med.* 2(32):32ra36.

Fehérvari, Z., and S. Sakaguchi. 2004. CD4+ Tregs and immune control. *J. Clin. Invest*. 114(9):1209-1217.

Fensterl, V. and G. C. Sen. 2009. Interferons and viral infections. *Biofactors*. 35(1):14-20.

Gett, A. V. and P.A. Hodgkin. 2000. A cellular calculus for signal integration by T cells. *Nature Immunol.* 1:239-244.

Grohmann, U., F. Fallarino and P. Puccetti .2003. Tolerance, DCs and Tryptophan: Much Ado About IDO. *Trends Immunol.*24 (5):242-248.

Grohmann, U., Orabona, C., Fallarino, F., Vacca, C., Calcinaro, F., Falorni, A., Candelor, P., Belladonna, M. L., Bianchi, R., Fioretti, M. C. and P.

Puccetti. 2002. CTLA-4-Ig regulates tryptophan catabolism *in vivo. Nat. Immunol.* 3:1097-1101.

Hersperger, A. R., Martin, J. N., Shin, L. Y., Sheth, P. M., Kovacs, C. M., Cosma, G. L., Makedonas, G., Pereyra, F., Walker, B. D., Kaul, R., Deeks, S. G. and M. R. Betts. 2011. Increased HIV- specific CD8+ T cell cytotoxic potential in HIV elite controllers is associated with T-bet expression. *Blood.* 117(14):3799-3808.

Hoshi M., Saito, K., Hara, A., Taguchi, A., Ohtaki, H., Tanaka, R., Fuigaki, H., Osawa, Y., Takemura, M., Matsunami, H., Ito, H. and M. Seishima. 2010. The absence of IDO upregulates type I IFN production, resulting in suppression of viral replication in the retrovirus-infected mouse. *J. Immunol.* 185:3305-3312.

Iezzi, G., Karjalainen, K. and A. Lanzavecchia. 1998. The duration of antigenic stimulation determines the fate of naïve and effector T-cells. *Immunity*. 8:89-95.

Iida, T., Ohno, H., Nakaseko, C., Sakuma, M., Takeda-Ezaki, M., Arase, H., Kominami, E., Fujisawa, T. and T. Saito. Regulation of cell surface expression of CTLA-4 by secretion of CTLA-4-containing lysosomes upon activation of CD4+ T cells. *J Immunol.* 165(9): 5062- 5068.

Jolicoeur, P. 1991. Murine acquired immunodeficiency syndrome (MAIDS): an animal model to study the AIDS pathogenesis. *FASEB J.* 5:2398-2405.

Kumar, H., T. Kawai and S. Akira. 2009. Toll-like receptors and innate immunity. *Biochem. Biophys. Res. Commun*. 388(4):621-625.

Kwaa, A. 2012. Honors Thesis. *In house publication*. Mount Holyoke College. South Hadley MA-01075.

Liang, B., J. Y. Wang, R. R. Watson. 1996. Murine AIDS, a Key to Understanding Retrovirus-induced immunodeficiency. *Viral Immunol.* 9(4):225:239.

Linsley, P. S., Bradshaw, J., Greene, J., Peach, R., Bennett, L. and R. S. Mittleer. 1996. Intracellular trafficking of CTLA-4 and focal localization toward sites of TCR engagement. *Immunity.* 4:535.

Linsley, P. S., Greene, J. L., Tan, P., Bradshaw, J., Ledbetter, J. A., Anasetti, C. and N. K. Damle. 1992. Coexpression and functional cooperation of CTLA-4 and CD28 on activated T lymphocytes. *J. Exp. Med.* 176: 1595-1604.

Marmor, M., Kertzmark, K., Thomas, S. M., Halkitis, P. N. and M. Vogler. 2006. Resistance to HIV infection. *J. Urban Health*. 83:5-17.

Mellor, A. L. and D. H. Munn. 1999. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation?. *Immunol. Today*. 20(10): 469:473.

Mellor, A. L. and D. H. Munn. 2004. IDO expression by dendritic cells: tolerance and tryptophan catabolism. *Nat. Rev. Immunol*. 4: 762-774.

Mellor, A. L, Sivakumar, J., Chandler, P., Smith, K., Molina, H., Mao, D. and D. H. Munn. 2001. Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. *Nat Immunol.* 2: 64-8.

Migueles, S. A., Laborico, A. C., Shupert, W. L., Sabbaghian, M. S., Rabin, R., Hallahan, C. W., Van Baarle, D., Kostense, S., Miedema, F., McLaughlin, M., Ehler, L., Metcalf, J., Liu, S. and M. Connors. 2002. HIV-specific CD8+ T cell proliferation is coupled to perforin expression and is maintained in nonprogressors. *Nat Immunol.* 3(11):1061-1068.

Mosier, D. E. 1996. Small Animal Models for Acquired Immune Deficiency Syndrome (AIDS) Research. *Lab. Anim. Sci.* 46(3):257-264.

Munn, D. H. and A. L. Mellor. 2004. IDO tolerance to tumors. *Trends Mol. Med.* 10(1):15-18.

Munn, D. H., Shafizadeh, E., Attwood, J.T., Bondarev, I., Pashine, A. and A. L. Mellor. 1999. Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J. Exp. Med.* 189: 1363-1372.

Munn, D. H., Sharma, M. D., Baban, B., Harding, H.P., Zhang, Y., Ron, D. and A. L. Mellor. 2005. GCN2 Kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2, 3-dioxygenase. *Immunity*. 22(5): 633-642.

Munn, D. H., Sharma, M. D, Lee, J. R, Jhaver, K. G, Johnson, T. S, Keskin, D. B, Marshall, B., Chandler, P., Antonia, S. J, Burgess, R., Slingluff, C. L Jr. and A. L. Mellor. 2002. Potential regulatory function of human dendritic cells expressing indoleamine 2, 3-dioxygenase. *Science.* 297(5588):1867-70.

Munn, D.H., Zhou, M., Attwood, J. T., Bondarev, I., Conway, S. J., Marshall, B., Brown, C. and A. L. Mellor. 1998. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science.* 281:1191-1193.

Orabona, C., Grohmann, U., Belladonna, M. L., Fallarino, F., Vacca, C., Bianchi, R., Bozza, S., Volpi, C., Salomon, B. L., Fioretti, M. C., Romani, L. and P. Puccetti. 2004. CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. *Nat Immunol.* 11:1134-42.

Parham, P. 2009. *The Immune System*. 3rd ed. New York: Garland Science, Taylor & Francis Group.

Pfefferkorn, E. R. 1984. Interferon gamma blocks the growth of *Toxoplasma gondii* in human fibroblasts by inducing the host cells to degrade tryptophan. *Proc. Natl. Acad. Sci. USA*. 81:908-912.

Randall, T. D., Carragher, D. M. and J. Rangel-Moreno. 2008. Development of secondary lymphoid organs. *Annu. Rev. Immunol*. 26:627-50.

Reeves, J. D., and R. W. Doms. 2002. Human Immunodeficiency Virus Type 2. *J. Gen. Virol.* 83(6):1253-1265.

Rudd, C.E., 2008. The reverse stop-signal model for CTLA4 function. *Nat. Rev. Immunol.* 8: 153-160.

Sajadi, M. M., Heredia, A., Le, N., Constantine, N. T. and R. R. Redfield. 2007. HIV-1 natural viral suppressors: control of viral replication in the absence of therapy. *AIDS.* 21:517-528.

Sceats L. 2011. Honors Thesis. *In house publication*. Mount Holyoke College. South Hadley MA- 01075.

Schroecksnadel, K., Zangerle, R., Bellmann-Weiler, R., Garimorth, K., Weiss, G. and D. Fuchs. 2007. Indoleamine 2, 3- dioxygenase and other interferon-γ-mediated pathways in patients with human immunodeficiency virus infection. *Curr.Drug Metab*. 8:225-236.

Sharon, J. 1998. *Basic Immunology*. 1st ed. Baltimore: Williams & Wilkins.

Sharp, A. H. and G. J. Freeman. 1994. The B7-CD28 superfamily. *Nat. Rev. Immunol.* 2:117-126.

Steinman, R. M., Hawiger, D. and M. C. Nussenzweig. 2003. Tolerogenic dendritic cells. *Annu. Rev. Immunol.* 21:685–711.

Stranford, S. A., Skurnick, J., Louria, D., Osmond, D., Chang, S. Y., Sninsky, J., Ferrari, G., Weinhold, K., Lindquist, C. and J. A.Levy.1999. Lack of infection in HIV-exposed individuals is associated with a strong CD8(+) cell noncytotoxic anti-HIV response. Proc Natl Acad Sci USA. 96(3):1020-1035.

Tai, X., Van Laethem, F., Sharpe, A. H. and A. Singer. 2007. Induction of autoimmune disease in CTLA-$4^{-/-}$ mice depends on a specific CD28 motif that is required for in vivo costimulation. *Proc. Natl Acad. Sci. USA* 104: 13756-13761.

Takikawa, O. 2005. Biochemical and medical aspects of the indoleamine 2, 3-dioxygenase-initiated L-tryptophan metabolism. *BBRC.* 338: 12-19.

Tarantino, G., Savastano, S., Capone, D. and A. Colao. 2011. Spleen: A new role for an old player? *World J. Gastroenterol.* 17(33): 3776-3784.

Taylor, M.W. and G.S. Feng. 1991. Relationship between interferon-gamma, indoleamine 2.3-dioxygenase, and tryptophan catabolism. *FASEB J.* 5: 2516- 2522.

Tepsuporn, S., J. N. Horwitt, G. W. Cobb, S. A. Stranford. 2008. MAIDS resistance- associated gene expression patterns in secondary lymphoid organs. *Immunogenetics*. 60:485-494.

Terness, P., Bauer, T. M., Rose, L., Dufter, C., Watzlik, A., Simon, H. and G. Opelz. 2002. Inhibition of allogeneic T cell proliferation by indoleamine 2, 3- dioxygenase-expressing dendritic cells: mediation of suppression by tryptophan metabolites. *J. Exp. Med.* 196: 447-457.

Terness, P., Chuang, J., Bauer, T., Jiga, L., and G. Opelz. 2005. Regulation of human auto- and alloreactive T cells by indoleamine 2, 3-dioxygenase (IDO) - producing dendritic cells: Too much ado about IDO? *Blood.* 105: 2480- 2486.

Thompson, C., Lindsten, T., Ledbetter, J. A., Kunkel, S. L., Young, H. A., Emerson, S. G., Leiden, J. M. and C. H. June. 1989. CD28 activation pathway regulates the production of multiple T cell-derived lymphokines/cytokines. *Proc. Natl Acad. Sci. USA* 86: 1333-1337.

UNAIDS. 2011. UNAIDS World AIDS Day Report. <http://www.unaids.org/en/media/unaids/contentasets/documents/unaid-publication/2011/JC2216_WorldAIDSday_report_2011_en.pdf> . Accessed 5 January 2011.

Van den Elsen, P. J., Gobin, S. J., van Eggermond, M. C. and A. Peijnenburg. 1998. Regulation of MHC Class I and II gene transcription: differences and similarities. *Immunogenetics*. 48(3): 208-221.

Von Adrian, U. H. and T. R. Mempel. 2003. Homing and cellular traffic in lymph nodes. *Nat. Rev. Immunol.* 3:867-878.

Walker, B. D. 2007. Elite control of HIV infection: implications for vaccines and treatment. *Top. HIV Med.* 15 (4): 134- 136.

Walker, L. S. K. and D. M. Sansom. 2011. The emerging role of CTLA4 as a cell-extrinsic regulator of T cell responses. *Nat. Rev. Immun*. 11:852-863.

Walunas, T. L., Lenschow, D. J., Bakker, C. Y., Linsley, P. S., Freeman, G. J., Green, J. M., Thompson, C. B., and J. A. Bluestone. 1994. CTLA-4 can function as a negative regulator of T cell activation. *Immunity*. 1:405-413.

Weiss, R. A. 1993. How does HIV cause AIDS? *Science*. 260:1273-1279.

WHO/ UNAIDS. 2011. Fast Facts on HIV/AIDS. World Health Organization. <http://www.unaids.org/en/media/unaids/contentassets/documents/fact-sheet/2011/20111121_FS_WAD2011_global_en.pdf>. Accessed 5 January 2011.

Zelante, T., Fallarino, F., Bistoni, F., Puccetti, P. and L. Romani. 2008. Indoleamine 2,3-dioxygenase in infection: the paradox of an evasive strategy that benefits the host. *Microbes Infect.* 11:133-141.

Zhang Y., and J. P. Allison. 1997. Interaction of CTLA-4 with AP50, a clathrin-coated pit adaptor protein. Proc. Natl. *Acad. Sci. USA*. 94: 9273-9278.