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TRYPTOPHAN METABOLISM AND CD8+T-CELL FUNCTION

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Abstract

L-tryptophan is the least common essential amino acid and is necessary for cellular proliferation. In the context of immune regulation, lack of tryptophan in tissue microenvironments and the production of its bioactive metabolites has been implicated as an immunosuppressive phenomenon. The rate limiting enzyme in tryptophan degradation is indoleamine 2,3 dioxygenase (IDO), which metabolizes tryptophan along the L-kynurenine pathway. When induced by interferon signaling, IDO promotes anergy and apoptosis in naïve T-cells, and enables the differentiation and activation of T-regulatory cells. However, the role that IDO and tryptophan metabolism play in epitope specific $CD8^+$ T-lymphocyte (CTL) function has not yet been investigated. By using a pharmacological inhibitor (1-methyl-D-tryptophan) and knockout (KO) mice independently, IDO was depleted to examine its role in shaping the CTL responses to SV40 large T antigen (TAg) and influenza A virus (IAV). IDO KO and inhibition increased the frequency of $CD8^+$ T-cells specific for defined epitopes in both models, as measured by intracellular cytokine staining (ICS). This increase in cell frequency was mediated by a proliferative advantage exhibited by immunodominant IDO KO T-cells when compared with WT counterparts. Bioactive tryptophan metabolites such as L-kynurenine did not play a role in this system, and failed to decrease T-cell responses in either the TAg or IAV model. In addition, IDO KO potentiated the cytotoxic effector function of CTLs *in vivo*, but did not affect animal morbidity in a disease model of IAV infection.

Keywords: tryptophan metabolism, CD8+ T-cells, immunodominance, IDO, influenza

"Positive results aren't." – James Albertson

Corollary #1: "The reproducibility of a given experiment is inversely proportional to the excitement factor of the original results." – James Albertson

Corollary #2: "The important things are always simple, the simple things are always hard, and the easy way is always wrong." – James Albertson

Corollary #3: In reference to the above: "Never be the first, never be the last, and never volunteer to do anything" – James Albertson

Corollary #4: "If you think everything is going well, you have obviously missed something of the most vital importance and will soon pay for it with utter failure." – James Albertson

Corollary #5: "Smile, tomorrow will be worse" – James Albertson

Corollary #6: "Nobody ever reads the discussion anyway." – James Albertson

Corollary #7: "You should never have more than 6 corollaries to one quote. It makes you look like an a—hole." – James Albertson

"James Albertson was an a—hole." – Anonymous

"Jak sie nie bywa to sie nie istnieje."
[Translation] "If you don't attend you don't exist." – Cezary Krzyzanowski

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There were many wonderful people who helped me during the completion of this thesis, and without whose aid this would never have been written. They know who they are and they ask for no praise or thanks in return. To them I am eternally grateful.

"It is not the critic who counts; not the man who points out how the strong man stumbles, or where the doer of deeds could have done them better. The credit belongs to the man who is actually in the arena, whose face is marred by dust and sweat and blood; who strives valiantly; who errs, who comes short again and again, because there is no effort without error or shortcoming; but who does actually strive to do the deeds; who knows great enthusiasms, the great devotions; who spends himself in a worthy cause; who at best knows in the end the triumph of high achievement, and who at worst, if he fails, at least fails while daring greatly, so that his place shall never be with those cold and timid souls who neither know victory nor defeat."

-- Theodore Roosevelt

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List of Common Abbreviations

CTLs – CD8+ T-lymphocytes
TCR – T-cell receptor
MHC – major histocompatibility complex
ER – endoplasmic reticulum
APCs – antigen presenting cells
DCs – dendritic cells
IAV – influenza A virus
VV – vaccinia virus
TAg – SV40 large T antigen
BSA – bovine serum albumin
i.p – intraperitoneal
s.c – subcutaneous
i.v – intravenous
i.n – intranasal
L-kyn – L-kynurenine
1-MT – 1-methyl-D-tryptophan
IDO – indoleamine 2,3-dioxygenase
TDO – tryphophan deoxygenase
ICS – intracellular cytokine staining
IKA – *in vivo* killing assay
Pfu – plaque forming units

Tryptophan Metabolism and $CD8^+$ T-cell Function

Introduction

$CD8^+$ T-cell Antigen Recognition and Cytotoxicity

The main cytotoxic effectors of the adaptive immune system are $CD8^+$ T-lymphocytes (CTLs), which use their T-cell receptor (TCR) to recognize peptide antigens presented in the context of major histocompatibility complex class I (MHC I) molecules on the surface of all nucleated host cells. MHC I is composed of α and non-variable β_2-microglobulin polypeptide chains, with the α1 and α2 domains forming the peptide binding groove, and α3 acting as the transmembrane region (Figure i) (1,2). Newly synthesized MHC I α chains are assembled inside the endoplasmic reticulum (ER) of the host cell where they associate with calnexin, which is a membrane bound protein. Upon binding with β_2-microglobulin, the MHC I complex disassociates from calnexin, and interacts with a group of chaperone proteins to await delivery of an appropriate peptide (3).

The $CD8^+$ T-cell TCR which recognizes peptides presented in the MHC I binding groove is composed of two protein subunits, α and β, which together form a dimer that is embedded in the cellular membrane. The two subunits are further subdivided into variable and constant chains, which function to recognize antigen, and anchor the TCR into the cell membrane respectively. In addition, the TCR dimer is usually associated with at least six other signalling subunits such as various CD3 heterodimers (σ,γ, ε, etc.). In addition, CD8 molecules which bind to the non-variable

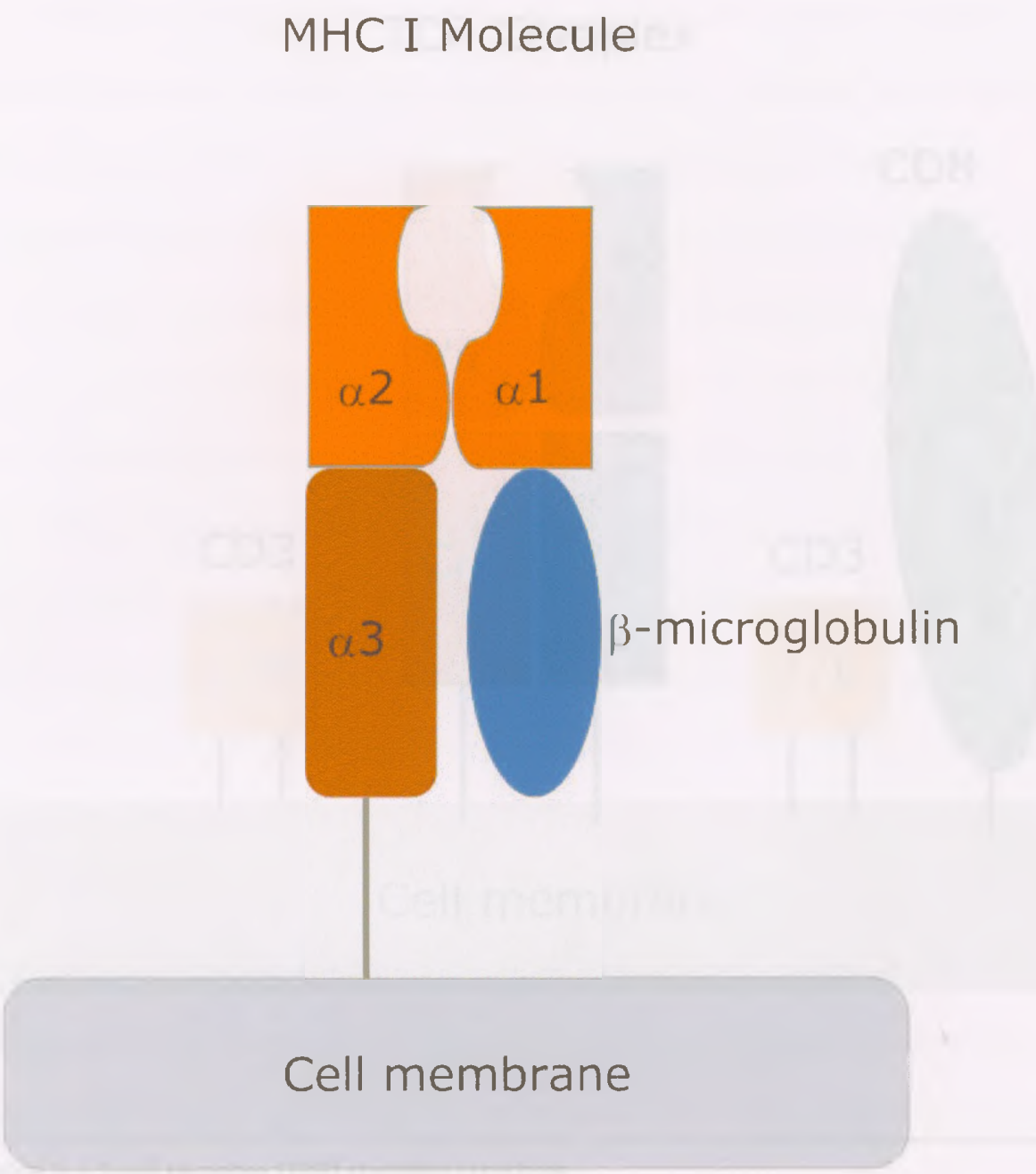


Figure i – Major histocompatibility complex I (MHC I) structure
The MHC I molecule is composed of α and β polypeptide chains. The α1+α2 subunits form the peptide binding pocket while α3 acts as the transmembrane domain. The β2-microglobulin domain is invariant and binds to CD8 co-receptors on the TCR.

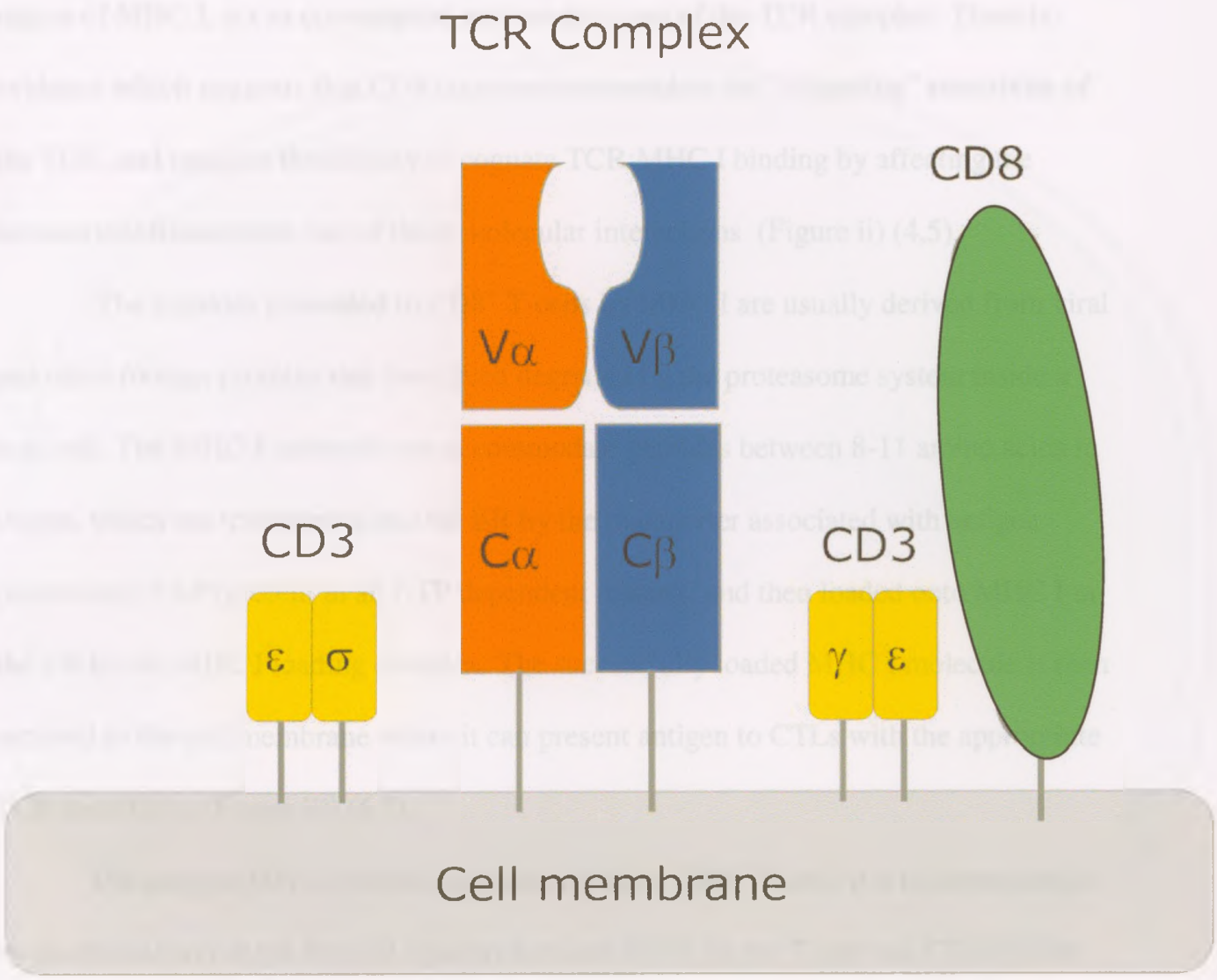


Figure ii – T-cell receptor (TCR) complex structure
The TCR is composed of variable and constant regions within α and β subunits, that together form the TCR dimer. This is accompanied by transmembrane heterodimeric CD3 receptors, as well as the CD8 co-receptor.

region of MHC I, act as co-receptors and are also part of the TCR complex. There is evidence which suggests that CD8 co-receptors modulate the "triggering" sensitivity of the TCR, and regulate the affinity of cognate TCR:MHC I binding by affecting the association/dissociation rate of these molecular interactions (Figure ii) (4,5).

The peptides presented to $CD8^+$ T-cells by MHC I are usually derived from viral and other foreign proteins that have been degraded by the proteasome system inside a host cell. The MHC I molecule can accommodate peptides between 8-11 amino acids in length, which are transported into the ER by the transporter associated with antigen processing (TAP) protein in an ATP dependent manner, and then loaded onto MHC I in the ER by the MHC I loading complex. The successfully loaded MHC I molecule is then secreted to the cell membrane where it can present antigen to CTLs with the appropriate TCR specificity (Figure iii) (6,7).

The antigen:MHC complex can activate naive $CD8^+$ T-cells if it is accompanied by co-stimulation in the form of ligation between CD28 on the T-cell and CD80/CD86 molecules on professional antigen presenting cells (APCs) such as dendritic cells (DCs) (Figure iv). DCs engage in the 'cross priming' of naive $CD8^+$ T-cells when they take up and process antigenic material and express it in the context of their own MHC I molecules. This is in contrast to 'direct priming', which involves the direct presentation of viral peptides by virally infected host cells without the necessary involvement of DCs (8,9).

Once activated, CTLs migrate to the lymph node where they undergo clonal expansion and then disseminate throughout the body, killing cells that express the recognized antigen:MHC complex. Target cells can be eliminated by CTLs through two

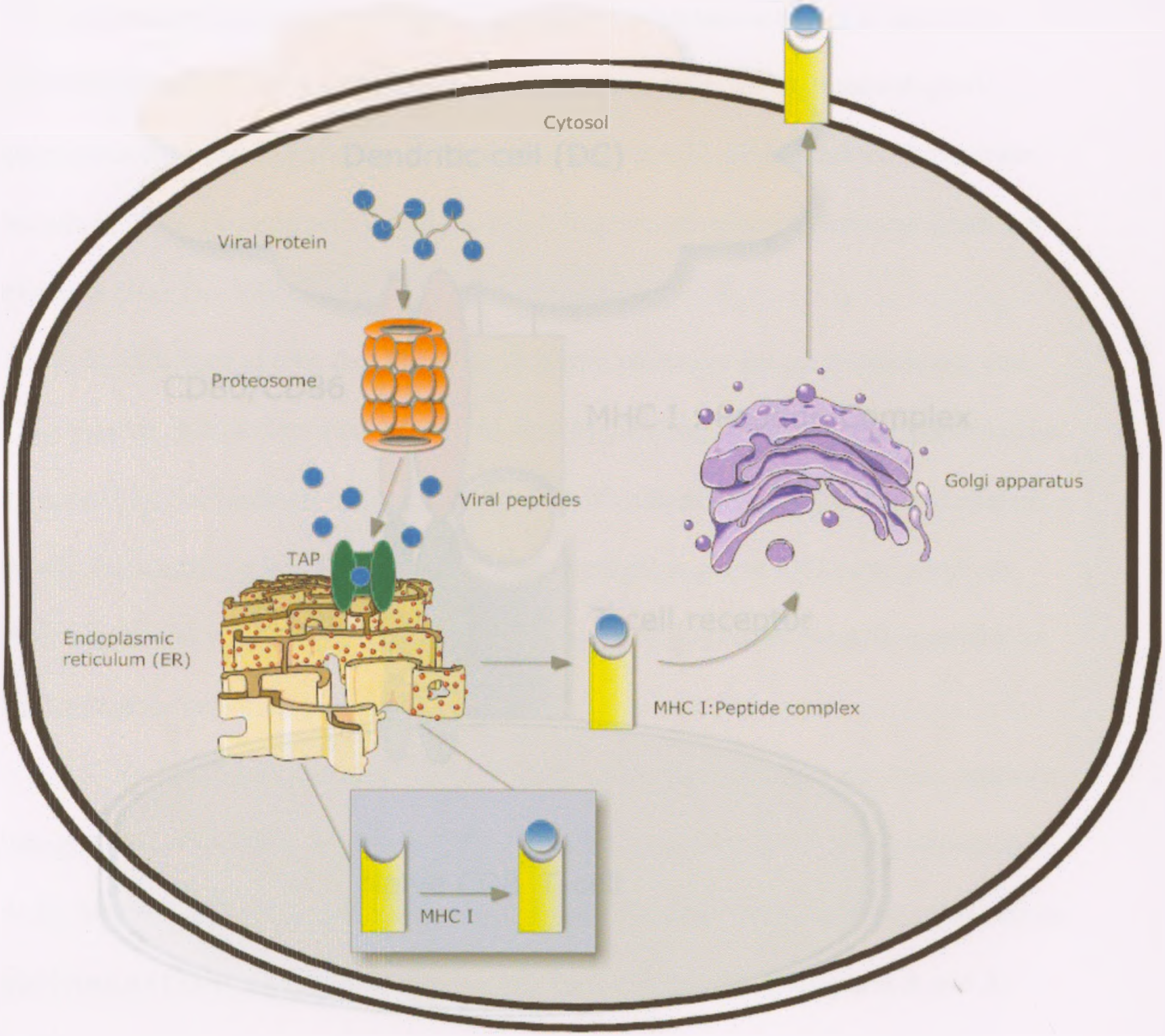


Figure iii – Antigen processing and presentation in host cell
Cytoplasmic viral proteins are processed into short length peptides by the proteasome system. These peptides are then transported into the ER by TAP, where they are loaded on MHC I molecules. MHC I:Peptide complexes are shuttled to the cell membrane where they present antigen to CTLs

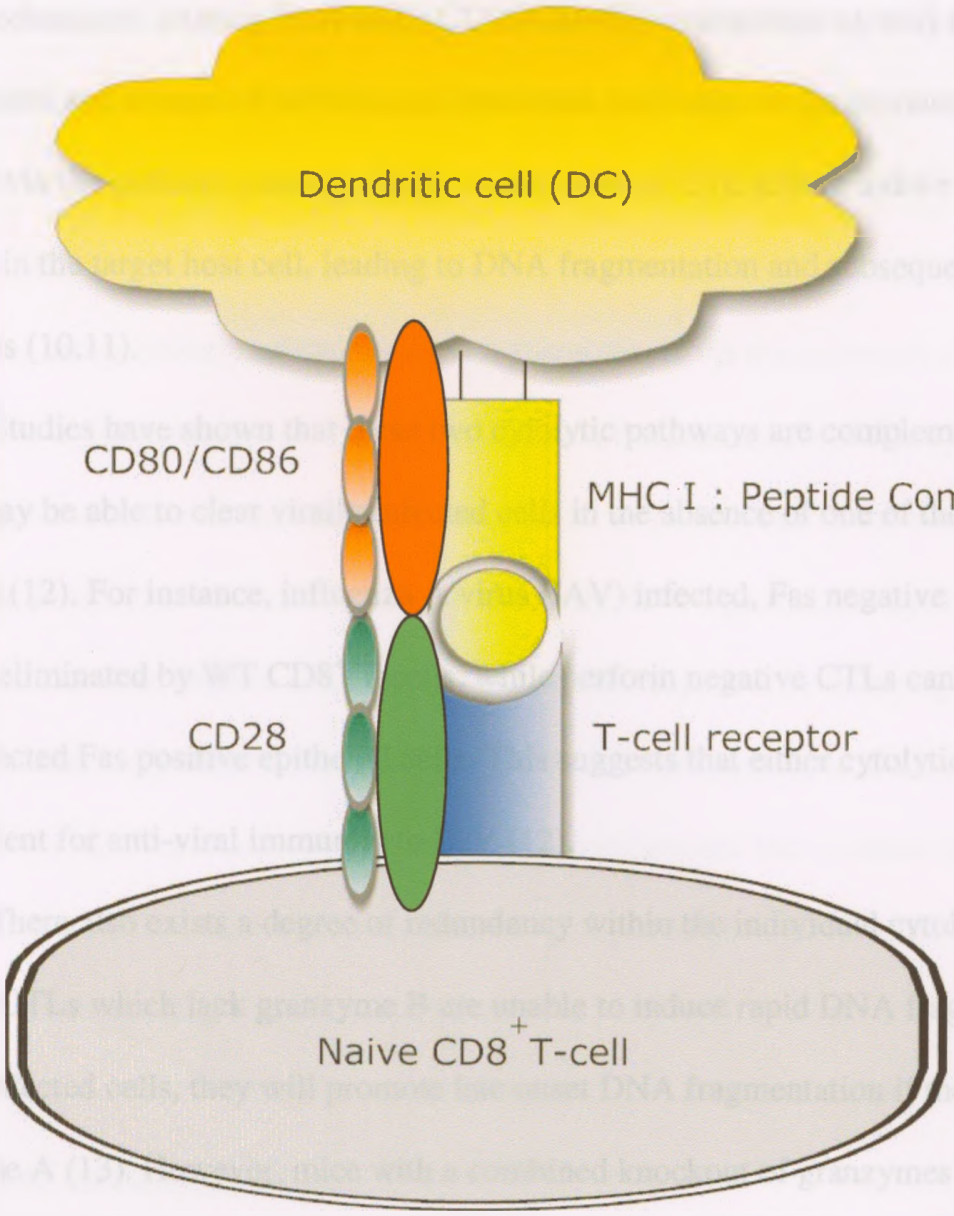


Figure iv – Activation of naive CD8$^+$ T-cells by DCs
Naive T-cells require both TCR recognition of MHC I:Peptide complexes, as well as costimulation in the form of CD28 ligation with CD80/CD86, in order to become activated

main mechanisms, namely Fas/Fas-L (CD95/CD95L) interactions as well as the polarization and release of perforin and granzyme molecules at the immunological synapse via the perforin pathway. Both mechanisms of CTL killing induce the caspase cascade in the target host cell, leading to DNA fragmentation and subsequent death via apoptosis (10,11).

Studies have shown that these two cytolytic pathways are complementary, and CTLs may be able to clear virally infected cells in the absence of one of the pathways but not both (12). For instance, influenza A virus (IAV) infected, Fas negative epithelial cells are still eliminated by WT $CD8^+$ T-cells, while perforin negative CTLs can eliminate IAV infected Fas positive epithelial cells. This suggests that either cytolytic mechanism is sufficient for anti-viral immunity to IAV (12).

There also exists a degree of redundancy within the individual cytolytic pathways. Though CTLs which lack granzyme B are unable to induce rapid DNA fragmentation in virally infected cells, they will promote late onset DNA fragmentation if they still express granzyme A (13). However, mice with a combined knockout of granzymes A and B exhibit a severe cytolysis defect, similar to perforin KO mice, which suggests that perforin alone is not sufficient to induce cell death and that a combination of perforin and granzyme A or B is required (13).

It is necessary to note that the perforin/granzyme pathway, though important for viral clearance, has also been linked to autoimmune CNS pathology during specific viral infections. WT mice actually exhibit a higher risk of demyelination following intracranial infection with a mouse coronavirus (JHMV) when compared with perforin KO mice. The animals lacking perforin also exhibit lower morbidity and mortality at doses of virus

that are lethal in WT mice. This suggests that although the perforin/granzyme pathway is important for the clearance of certain viral infections, it also represents a double edged sword that can exacerbate disease in certain contexts (14).

Another important manner through which activated $CD8^+$ T-cells regulate viral infections, independently of their cytotoxic functions, is via the secretion of immuno-modulatory cytokines such as IFN-γ. For instance, the production of this cytokine by CTLs has been linked to the increased recruitment of neutrophils to the site of antigen challenge at the level of the skin (15). Furthermore, IFN-γ production by $CD8^+$ T-cells also augments other aspects of the adaptive immune response, by inducing T-bet dependent and independent class switching in B-cells in response to alum adjuvanted vaccination (16). Importantly, IFN-γ also directly influences the outcome of certain viral infections, and CTLs require the secretion of this cytokine in order to efficiently clear borna disease virus (BDV) in mice (17).

$CD8^+$ T-cell Immunodominance

Despite the fact that thousands of potentially immunogenic peptides are produced inside host cells during a viral infection, only a small percentage of these actually induce detectable CTL responses *in vivo*. The relative immunogenicity of these epitopes varies and can be arranged in a so called immunodominance hierarchy, which ranks the antigenic determinants on the basis of their propensity to elicit CTL responses. Immunodominant determinants are so termed because they are recognized by and activate the largest percentage of CTLs, whilst subdominant epitopes induce responses in a smaller population of T-cells. The reasons behind the phenomenon of

immunodominance are an active field of research, with profound implications for CTL immunobiology and vaccine design. If factors that augment the immunodominance hierarchy of a given virus are identified, this will allow the generation of vaccines which induce a broader spectrum of CTL immunity, and prevent the creation of a bottleneck that will aid in viral escape (18).

There are a variety of explanations why only a select number of viral epitopes are actually presented to and activate CTLs in an immunodominant hierarchy, and one of the main contributors is peptide affinity for MHC I allomorphs which are loaded with antigen in the ER. Less than 1% of all peptides generated via proteasomal degradation bind to MHC I with the adequate affinity required to stabilize the antigen presentation complex. For instance, out of 175,000 peptides generated during a pox virus infection, only 150 are able to bind to MHC I. This phenomenon applies to all organisms, even the extremophile *Methanococcus jannaschii*, which shows that the propensity of MHC I to limit the amount of peptides that bind it is an evolutionarily favoured and ancient event (19).

In addition, proteolytic liberation, TAP transport and TCR recognition are all steps along the antigen presentation pathway that limit the amount of viral peptides which eventually elicit detectable CTL responses. The number of antigenic determinants which are susceptible to and undergo each step steadily decreases from the original pool of viral peptides (18). Regulatory factors of immunodominance outside the core antigen presentation mechanism have also been identified, and mouse studies conducted with vaccinia virus (VV) have shown that the degree of dominance of the immunodominant determinant is dependent upon the route of immunization (e.g., intraperitoneal vs

intradermal). This is particularly important for vaccinology because it directly implicates route of administration as crucial for the generation of a particular CTL response (20).

However, there is a degree of discord between mouse data and the human system, and CTL responses to VV antigens in humans appear to be more "immunodemocratic". In addition, the particular immunodominant determinants recognized in a human cannot be predicted solely on that person's HLA genotype, because the CTL repertoire is also influenced by other HLA allomorphs to which the VV peptides are not restricted (21,22). Furthermore, humans obviously exhibit greater genetic variability than inbred strains of laboratory mice, and mutations or substitutions in key elements of the antigen presentation pathway (i.e., TAP1 and TAP2) have been shown to influence the repertoire of viral peptides which are presented to CTLs (23). Nevertheless, mouse models of immunodominance remain important tools to study this phenomenon *in vivo*, because they can still yield important mechanistic data that can then be evaluated in the human system.

Models of Immunodominance

Two particularly well studied models of immunodominance other than VV are the simian vacuolating virus 40 (SV40) large T antigen (TAg) system, and the influenza A virus (IAV) system. SV40 is a member of the Papovavirdae family of DNA tumour viruses which came to prominence following the discovery that SV40 could induce mesothelioma in hamsters. This, combined with the fact that thousands of infants received an SV40 contaminated polio vaccine during the 1950s-60s, promoted a significant degree of research into the oncogenic capacity of the virus (24). It was

subsequently found that the large TAg of SV40 interacts with tumour suppressor genes in the host cell (i.e., p53) and by deregulating their function promotes oncogenesis. SV40 TAg exhibits four peptides which may elicit measurable CTL responses in the C57BL/6 mouse, and these include Site I, II/III, IV, and V, with IV being the immunodominant determinant. Site V is sometimes considered immunorecessive because it will only induce a CTL response in the absence of the other antigenic epitopes (25).

Influenza a virus (IAV) is a prominent human pathogen that induces seasonal morbidity in humans on a global scale. In the C57BL/6 mouse, seven MHC I restricted immunogenic IAV peptides have been identified, and NP_{366} and PA_{147} are the immunodominant determinants in that particular system. Many prominent investigators, including Peter Doherty, utilize the IAV model to delineate the mechanistic details behind the immunodominance phenomenon. Applying the IAV to study immunodominance is useful because it is an actual human pathogen, and if administered intranasally (i.n) in mice also induces morbidity. Therefore using an i.n IAV model can be used to determine whether any changes in epitope specific CTL responses influence disease progression or outcome (26-28).

Tryptophan Metabolism and its Effects on the Immune System

Indoleamine 2,3-dioxygenase (IDO) is the endogenous rate limiting enzyme in tryptophan metabolism, and degrades L-tryptophan (L-Trp) along the L-kynurenine (L-Kyn) pathway (Figure v). This metabolic route was first described in 1947, but the actual IDO enzyme was not identified until Osamu Hayaishi's group accomplished the feat in

1963, by isolating what was then termed D-tryptophan pyrrolase from the rabbit intestine (29,30).

IDO is an ancient molecule, one that has been conserved through the last 600 million years of evolution (31). It is a heme containing cytoplasmic enzyme with two folding domains, the larger of which (C-domain) is composed of 15 α-helices and harbours the catalytic pocket. IDO binds L-Trp and promotes the oxidative cleavage of its pyrrole ring through the insertion of an oxygen molecule or superoxide anion, thus forming N-formylkynurenine which is then hydroylzed or spontaneously degraded into other by-products (32).

Unlike tryptophan 2,3-dioxygenase (TDO) which is a housekeeping enzyme mainly confined to the liver, IDO expression can be induced by immunological stimuli (e.g., IFN-γ) in a variety of tissues and cell types including fibroblasts, epithelial cells, and vascular smooth muscle cells (33). The Ido1 gene promoter contains two interferon stimulated response elements (ISREs), as well as an interferon activated site (GAS), which induce transcription by responding

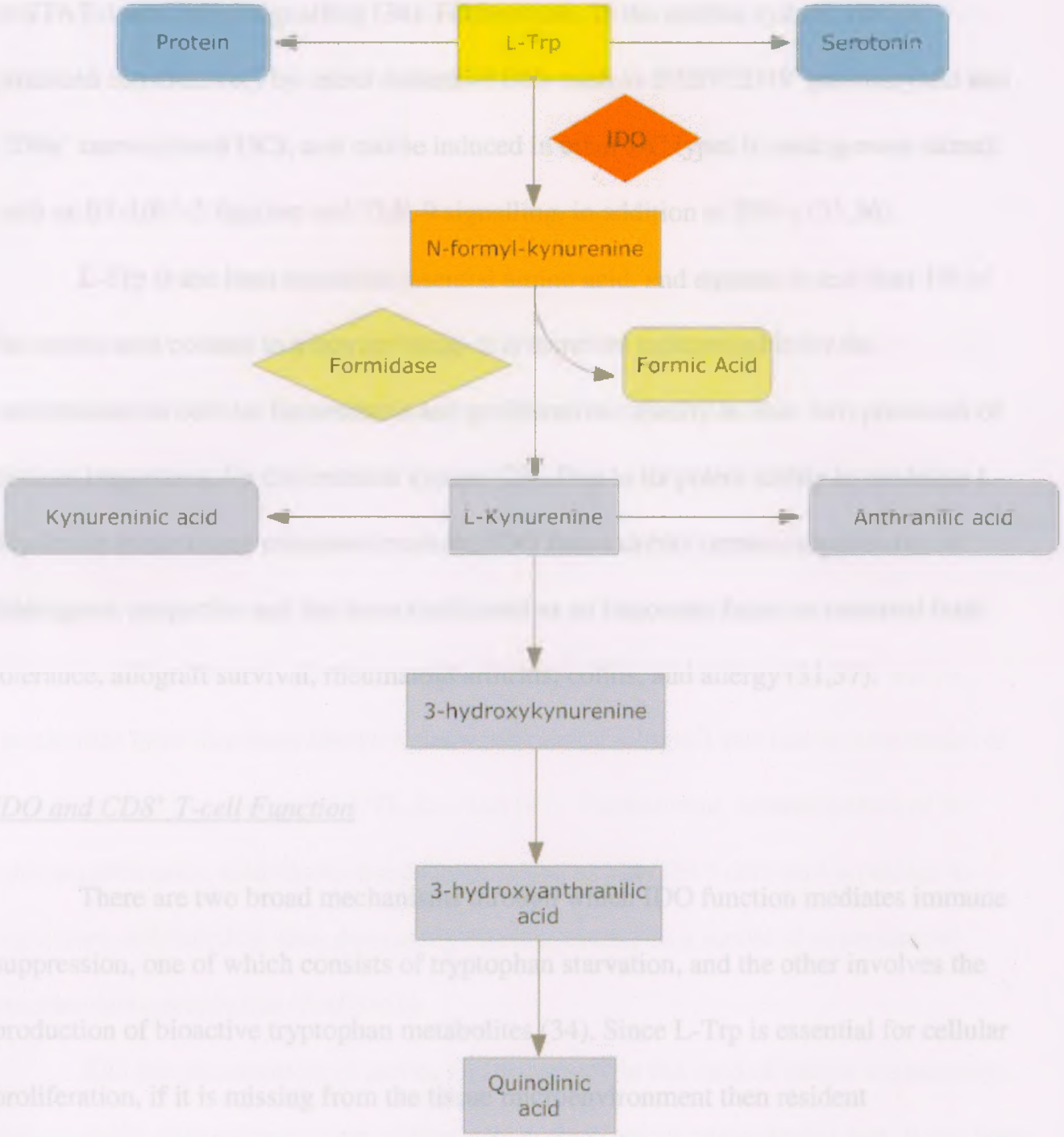


Figure v – L-Tryptophan metabolism along the L-kynurenine pathway
L-tryptophan can be incorporated either into protein, or the neurotransmitter serotonin. In the presence of IDO, it is metabolised into N-formyl-kynurenine, and then quickly converted to L-kynurenine by formidase. L-kynurenine is itself broken down into a variety of bioactive metabolites further along the pathway.

to STAT-1 and IRF-1 signalling (34). Furthermore, in the murine system, IDO is produced constitutively by select subsets of DCs such as $B220^+CD19^+$ plasmacytoid and $CD8\alpha^+$ conventional DCs, and can be induced in other DC types by endogenous stimuli such as B7-1/B7-2 ligation and TLR-9 signalling, in addition to IFN-γ (35,36).

L-Trp is the least abundant essential amino acid, and equates to less than 1% of the amino acid content in a human being. It is therefore indispensable for the maintenance of cellular homeostasis and proliferative capacity *in vivo*, two processes of distinct importance for the immune system (29). Due to its potent ability to modulate L-Trp levels in the tissue microenvironment, IDO thus exhibits immunosuppressive or tolerogenic properties and has been implicated as an important factor in maternal fetal tolerance, allograft survival, rheumatoid arthritis, colitis, and allergy (31,37).

IDO and $CD8^+$ T-cell Function

There are two broad mechanisms through which IDO function mediates immune suppression, one of which consists of tryptophan starvation, and the other involves the production of bioactive tryptophan metabolites (34). Since L-Trp is essential for cellular proliferation, if it is missing from the tissue microenvironment then resident immunocytes such as $CD8^+$ T-cells will be unable to expand and execute their functions. For instance, it has been established that the CTL cell cycle stops at a mid-G1 arrest point when the cellular microenvironment is devoid of tryptophan (38). In addition, when IDO expressing DCs interact with CTLs *in vivo* and *in vitro*, they induce GCN2 kinase signalling within the T-cells, thus leading to anergy and proliferative arrest (39). The GCN2 kinase pathway is activated in response to cellular stress (e.g., L-trp starvation),

and the proliferative arrest of CTLs in this system was reversed by supplementing the cells with tryptophan (39). Furthermore, IDO mediated activation of the GCN2 pathway within tumour draining lymph nodes can block the conversion of $FoxP3^+$ T-regulatory cells into a pro-inflammatory Th17 like phenotype (40).

It has also been established that L-kynurenine, and its immediate downstream metabolites like 3-hydroxykynurenine, 3-hydroxyanthranilic acid, and quinolinic acid exhibit bioactive properties that are in some cases detrimental to immune function (41-43). For instance, 3-hydroxyanthranilic acid actually ameliorates morbidity in an experimental model of asthma, by inducing the apoptosis of T-cells (44). The induction of CTL apoptosis by tryptophan metabolites corresponds with caspase-8 activation and cytochrome C release from mictochondria (45,46). In addition, L-kynurenine and its metabolites have also been shown to improve cardiac allograft survival in a rat model of transplantation by suppressing CTL function (47). Furthermore, administration of 3-hydroxyanthranilic acid blocks the differentiation of Th1/Th17 cells and promotes T-regulatory cell function, thus decreasing disease severity in a model of experimental autoimmune encephalitis (EAE) (48).

IDO has proven to be of particular importance in the field of cancer immunology, and a steadily expanding volume of literature on the subject attests to this fact. It has been established that when tumour cells are transfected with IDO, they use its properties to induce a zone of immunologic privilege surrounding the neoplasm, despite pre-existing T cell immunity to the cancer (49). In addition, constitutive IDO expression by human colorectal and ovarian cancer cells is linked to poor clinical prognosis (50,51). Furthermore, B16F10 tumour cells which constitutively express IDO have been found to

secure their own growth by inducing T-cell apoptosis in an IDO dependent manner. This phenomenon can be remedied by treating the tumours with IDO targeting siRNA, which reduces tumour induced T-cell apoptosis and inhibits cancer growth *in vivo* (52).

Not only do IDO expressing tumours possess the ability to modulate the immune system, but IDO^+ tolerogenic DCs have also been implicated in the development and maintenance of cancer. DCs which express IDO have been isolated in the tumour draining lymph nodes of humans with invasive melanoma, as well as mice with B16 melanoma tumours. Furthermore, IDO^+DCs isolated from breast, gastric, and colon peritumoral regions also express CD25, which adds to their immunosuppressive functions and helps promote tumour growth (53,54).

In the context of viral infection, IDO and tryptophan metabolism play a more paradoxical and ill defined role. In one study, the presence of IDO was shown to potentially benefit patients with dengue virus infection, and the authors claim that the gamma interferon response to dengue virus is partially dependent on IDO (55). Along those same lines, IDO has been found to mediate the gamma interferon response to hepatitis B virus in human hepatocyte derived cells (56). This clearly conflicts with previously cited literature about the immunosuppressive functions of IDO, but there are also studies which support a suppressive role for IDO in a viral system. For instance, it has been established that the absence of IDO potentiates the type I IFN response to a murine leukemia virus (LP-BM5), and that IDO KO mice infected with this virus exhibit a lower viral load and better survival than their wild type counterparts (57).

Hypothesis and Specific Aims

Clearly the role of IDO in viral infection remains to be elucidated and thus represents a field for exciting further research. The purpose of the following study was to determine the role that IDO plays in epitope specific $CD8^+$ T-cell responses to viral antigens, and what the functional consequences of this are *in vivo*. It was hypothesized that IDO would suppress $CD8^+$ T-cell responses and be detrimental to their cytotoxic function. The specific aims were 1) to determine the epitope specific $CD8^+$ T-cell responses to TAg and IAV in IDO KO vs WT mice, 2) to elucidate the mechanistic details behind this phenomenon (e.g., cross priming, proliferation, anergy, etc.), 3) to explore the effects of IDO KO on the cytotoxic responses of epitope specific CTLs and 4) to illustrate whether IDO KO is protective against disease inducing viral challenge.

Materials and Methods

Animals

Seven to twelve week old male and female C57Bl/6 were purchased from Charles River Canada and housed in the West Valley Barrier facility at the University of Western Ontario. Two pairs of $IDO^{-/-}$ mice were purchased from Jackson Laboratories and a breeding colony was established at the same West Valley Barrier facility. Mice in control and experimental groups were always sex matched, aged matched to within +/- 1 week of each other, and moved to the experimental housing room at least three days prior to treatment, allowing them to acclimatize to the new environment.

Propagation of PR8 and X31 influenza A virus (IAV)

Embryonated chicken eggs were purchased and placed in an egg incubator at 33-35°C. The eggs were then "candled" to determine which had healthy embryos and thus were suitable for virus inoculation. This was achieved by visualizing each egg in front of a bright light in a dark room and identifying those with well defined egg sacs and blood vessel morphology. The healthy eggs were then inoculated with 1:1000 dilution of PR8 (H1N1) or X31 (H3N2) strain of IAV, by inserting a syringe with a 1 inch needle at a 45 degree angle into the allantoic cavity. One hundred microlitres of virus was injected in this manner into each egg, which was then returned to the incubator for a further 48 hours. At the completion of the incubation period, the top of the shell was removed from the eggs and the allantoic fluid was collected and pooled into 50 ml tubes. This fluid was then spun down at 400g for 4 minutes and the supernatant was aliquoted into 1 ml glass tubes for freezing.

Preparation and intraperitoneal injection of IAV

IAV (H1N1 PR8 and H3N2 X31) virus stock was kept at -80°C in 1 ml aliquots in PBS. Before injection the virus was thawed and 1 ml of PBS was added to the aliquot for a final volume of 2 mL per tube. Each mouse received 0.5 ml of the diluted virus via intraperitoneal (i.p) injection, using a 500µl insulin syringe. For experiments focussing on the acute response, each mouse received a single i.p injection of PR8 and intracellular cytokine staining (ICS) was conducted on Day 7. However, if the memory recall response was to be enumerated, then each mouse received an i.p injection of PR8 on Day 0, followed by a "booster" i.p injection of X31 on Day 30. X31 (H3N2) was used instead of PR8 (H1N1) in order to avoid a neutralizing antibody response against the second viral challenge. The ICS was then conducted on Day 37.

Preparation and intranasal innoculation of IAV

A final pfu of 6-8 pfu per mouse was prepared by serial dilutions of PR8 virus stock in PBS. Mice were anesthetised with a 70:30 ratio of isofluorane and propylene glycol, and were considered properly subdued when the toe pinch reflex was absent. A total of 50 µl of PR8 virus solution was pipetted into the nose of each animal, which corresponded to 6-8 pfu per mouse in each experiment. Each mouse was kept in an upright position for at least a minute following inoculation, to ensure that the virus did not leak back out of the nostrils or mouth. The animals were then weighed each day to monitor their infection and health status.

Preparation and intraperitoneal injection of C57SV and KD2SV cells

C57SV cells are a transformed fibroblast cell line from C57Bl/6 mice that also stably express the large T antigen (TAg) of SV40 virus (58). When injected into C57Bl/6 mice, C57SV cells induce MHC I restricted $CD8^+$ T-cell responses to TAg. KD2SV cells express TAg as well, but this is a kidney epithelial cell line from BALB/c mice and thus exhibits a different MHC haplotype: H2d. When injected into C57Bl/6 mice, KD2SV cells promote T-cell responses against TAg, but also induce an alloreactive response due to the MHC incompatibility (59).

Regardless of cell line, on Day 0 mice were injected intraperitoneally with $20x10^6$ cells using a 1 ml insulin syringe. The cells were cultured prior to injection in DMEM medium with 10% fetal calf serum (FCS) and no antibiotics. The desired pre-injection confluence of 90-100% was achieved by seeding $2.0x10^6$ cells in a $175cm^3$ culture flask for three days.

1-Methyl-D-Tryptophan (1-MT) preparation and intraperitoneal injection

1-MT was purchased from Sigma in several different batches. Twenty five milligrams of 1-MT was dissolved in 2 ml of 0.1 M NaOH. The solution was brought up to a neutral pH by drop wise addition of 12 M HCL and then filtered through a syringe top filter after addition of 2.98 mL of PBS. Thus, the final concentration of 1-MT in the pH adjusted solution was 5mg/ml. The control solution consisted of an ion matched PBS preparation (155mM NaCl). Each mouse was treated with 0.5 ml of 1-MT solution via the i.p route, which equates to 2.5 mg of 1-MT, per day for two days prior and two days

following C57SV injection. Age and sex matched controls received the same volume of vehicle over the same injection schedule.

L-Kynurenine (L-Kyn) preparation and intraperitoneal injection

L-Kyn was purchased from Sigma. Fifty milligrams of L-Kyn was dissolved in 5 ml of PBS in order to produce a solution with a concentration of 10mg/ml. Each mouse received 10mg of L-Kyn in 1 ml injections on the day of and two days following C57SV or IAV injection. Control mice received 1ml of PBS as vehicle in 1ml injections over the same schedule.

Intracellular cytokine staining (ICS)

Mice were euthanized via cervical dislocation on either Day 7 (IAV) or Day 9 (C57SV) post injection. Following retraction of the skin on the abdominal region, peritoneal exudates cells (PECs) were collected by flushing the inside of the peritoneal cavity with 10 ml of PBS; a 10 ml syringe attached to a 19 gauge needle was used to achieve this. The spleen from each animal was then excised and disrupted with a glass homogenizer. After centrifugation at 400 rcf for 4 minutes, the pelleted homogenates were subjected to ACK lysis buffer, thus removing most red blood cells from the suspension.

Rotational filtration was then employed to clear the fat and other organ debris from the homogenates. This entailed slowly decanting each suspension into a clean 15ml falcon tube, whilst rotating to ensure that fat remained on the walls of the parent tube. These splenocytes were counted using a haemocytometer and the volume of the

suspensions was adjusted to a final concentration of $20x10^6$ cells per ml in 1 ml of complete RPMI (cRPMI) medium.

Synthetic peptides corresponding to the antigenic determinants of IAV or TAg were prepared at 1μM concentrations in 2 ml of cRPMI medium. A medium only control was also prepared, as well as a control using an irrelevant peptide. In all cases the irrelevant peptide was GB_{498} because it exhibits the appropriate MHC I restriction and is also the most immunodominant epitope in the HSV-1 system. C57SV cells and a DC2.4 cell line infected with IAV were used to enumerate the overall T-cell response to all antigenic epitopes of either TAg or IAV, and functioned as a positive control.

The splenocytes were plated on a non-tissue culture treated 96 well plate, with $2.0x10^6$ splenocytes per well. One hundred microlitres of the synthetic peptides and controls were then added to the appropriate wells, thus ensuring a final volume of 200 μl per well. The plate was incubated for 2 hours at 37°C and 6% CO_2. At the end of 2 hours, 20 μl of 0.1mg/ml Brefeldin A was added to each well in order to attenuate protein secretion and thus retain any synthesized cytokines inside the cell. The plate was then incubated under the same conditions for a further 3 hours.

At the 3 hour interval, the plate was centrifuged at 2100 rpm for 5 minutes at 4°C. Following removal of the supernatant and a quick vortex to disrupt the pellets, Fc block (2.4G2 hybridoma supernatant) was added to the wells and the plate was incubated on ice for 15 minutes. An anti-mouse CD8 monoclonal antibody conjugated to allophycocyanin (APC) was then added to each well and incubated in the dark on ice for a further 30 minutes. Following three washes with PBS and PBS with 0.5% bovine serum albumin (BSA), the cells were fixed for 20 minutes using 1% paraformaldehyde at room

temperature. A solution containing 1% saponin, PBS and an anti-mouse IFN-γ monoclonal antibody conjugated to fluorescein (FITC) was prepared and then added to each well after two washes with PBS and PBS+0.5%BSA. The plate was sealed with parafilm and incubated at 4°C overnight. The next morning the wells were washed with PBS and PBS 0.5% BSA, brought up to a final volume of 200 µl in PBS BSA, and enumerated via flow cytometric analysis.

Synthetic peptides used for ICS

The synthetic peptides used for splenocyte restimulation were provided by Dr. Jonathan Yewdell's lab at the NIH in Bethesda, Maryland. Stock solutions were prepared at a concentration of 1mM in DMSO and stored at -30°C. See Figure vi for details.

Figure vi – Synthetic Peptides from SV40 and IAV

SV40 TAg Peptides + MHC Restriction	**IAV PR8 Peptides + MHC Restriction**
SV40 Site I: $TAg_{206-215}$ D^b	IAV $NP_{224-233}$ D^b
SV40 Site II/III: $TAg_{223-231}$ D^b	IAV $PA_{366-374}$ D^b
SV40 Site IV: $TAg_{404-411}$ K^b	IAV $PB1_{703-711}$ K^b
SV40 Site V: $TAg_{498-497}$ D^b	IAV $PB1\text{-}F2_{62-70}$ D^b
	IAV $M1_{128-135}$ K^b
	IAV $NS2_{114-121}$ K^b
	IAV PB2

Staining for Intracellular and Extracellular Markers

Splenocytes were prepared, plated, and restimulated with peptides as described above in the ICS section. However, in addition to using anti-mouse CD8-APC and anti-mouse IFN-γ-FITC antibodies, the following antibodies were also included as appropriate: anti-human Ki67-FITC, anti-mouse programmed death one (PD1)-PE, anti-mouse CHOP-FITC, anti-mouse IFN- γ-PE. All of these additional antibodies were prepared using a standard 1:100 dilution. Ki67-FITC and CHOP-FITC are intracellular markers and so were added in conjunction with IFN-γ-PE in parallel wells. PD1-PE is extracellular and was included with the CD8-APC antibody cocktail.

Tetramer Staining

Site IV-PE and Site I-APC tetramers were acquired from Todd Schell at the Penn State. Splenocytes from C57SV challenged mice were prepared as described in the ICS section, and $1.0x10^6$ cells were transferred to two tubes for tetramer staining. The cells were spun down at 400 rcf for 4 minutes and Fc blocked for 10 minutes on ice. The respective tetramer cocktails were prepared using a 1:100 dilution in PBS+0.5%BSA, and a 1:100 dilution of anti-mouse CD8-FITC antibody was included in the mix (e.g., Site IV-PE + CD8-FITC). These tetramer cocktails were then added to the appropriate tube for 15 minutes at room temperature, and then the cells were washed, fixed with 1.0% paraformaldehyde, and enumerated using flow cytometry.

In vivo killing assay (IKA)

Naive C57Bl/6 mice were sacrificed via cervical dislocation and their spleens were excised and processed as described in the ICS section. The splenocytes were then partitioned into two tubes and pulsed with either Site IV peptide or the GB_{498} irrelevant peptide at 1uM concentrations. These suspensions were incubated for 45 minutes at 37°C and 6% CO_2, and then washed twice with PBS.

The two splenocyte pools, pulsed with Site IV and GB_{498}, were then labelled with a high (5μM) and a low (1.5 μM) concentration of CFSE dye respectively, so that they could be distinguished later by flow cytometry. After the dye was added at the appropriate concentration, the cells were incubated for 20 minutes at 37°C and 6% CO_2, then washed, counted, equalized, and mixed into one tube (a count of 4×10^6 cells in each group was the minimum acceptable number, for a total of at least 8×10^6 cells total).

The mixed splenocyte pool was then injected intravenously into mice that had been treated with C57SV cells nine days previously, as well as two naive control mice. All mice were warmed under infrared light for at least an hour before injection to ensure adequate vasodilation, and the tail was dipped in warm water immediately prior to injection. A 500μl syringe with a 27 gauge needle was used to inject 200μl of the cell suspension into each mouse. Instantly following the injection of each mouse, a timer was started for 45 minutes, the time corresponding to the desired in vivo killing time in the animals.

The recipient mice were euthanized via cervical dislocation at the 45 minute time point in the same order they were injected, to ensure that each animal experienced the same in vivo killing time. Their spleens were excised and processed in the manner

discussed in the ICS section, and then immediately enumerated via flow cytometric analysis. All steps were conducted in the dark to ensure the preservation of the CFSE dye in the cell suspension.

Flow Cytometry

Events were acquired using a BD FACS Canto II machine in conjunction with BD FACS Diva software. Analysis was performed using Flow Jo software. When required, compensation libraries were prepared with single stained splenocytes using the BD FACS Diva software options.

Statistical analysis

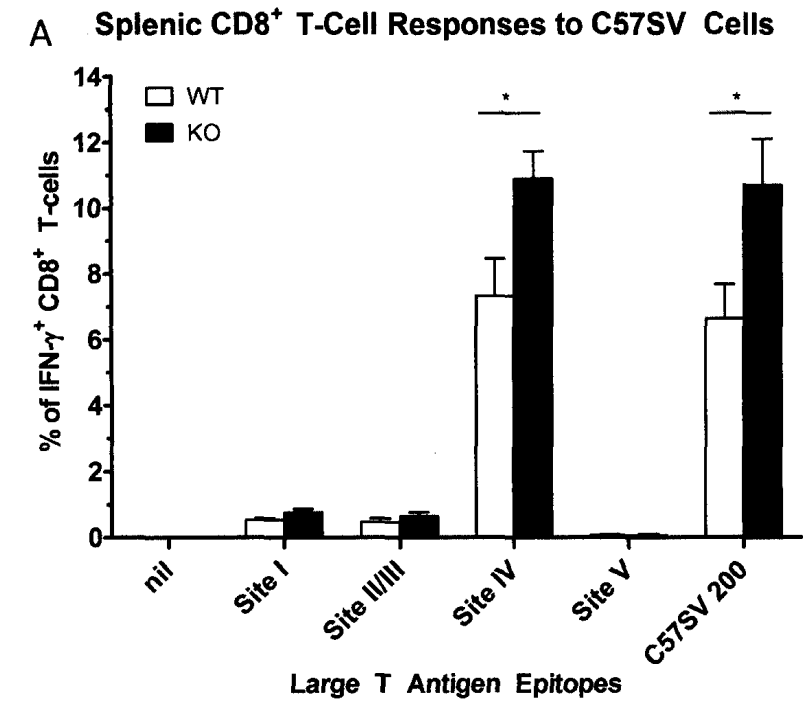
The data was analysed in most cases using the Student's t test in Microsoft Excel 2007. P values less than 0.05 were considered significant (* <0.05, ** < 0.01, *** < 0.001)

Results

IDO suppresses CD8$^+$ T-cell responses to the immunodominant epitopes of SV40 TAg and IAV

IDO has already been shown to suppress T-cell activity in a variety of contexts (39), but its effect on epitope specific CTL responses to viral antigens has not been investigated. To that end, three separate ICS experiments in IDO KO and WT mice treated with C57SV cells i.p, showed that the absence of IDO increased the percentage and absolute number of IFN-γ^+ CD8$^+$ T-cells specific for the immunodominant site IV epitope of TAg. This cell population corresponds to CTLs that were activated *in vivo* and secreted IFN-γ upon re-stimulation with synthetic peptides *ex vivo*. In addition, IDO KO did not affect CTL responsiveness to the other antigenic determinants, but did increase activity against C57SV cells *ex vivo*, a response that is largely driven by the site IV epitope itself (Figure 1). These results suggest that IDO exhibits an apparently selective CTL inhibition, whereby it only affects the T-cells responding to the most immunodominant epitope of TAg.

A similar phenomenon was seen in mice treated i.p with KD2SV cells, and two ICS experiments again enumerated a statistically significant difference in the percentage and absolute number of activated CD8$^+$ T-cells specific for the site IV epitope (Figure 2). This confirmed the observation was not limited to treatment with one cell type, and also implicated cross priming as a possible mechanistic detail in the process. Since KD2SV cells express an H2^d MHC restriction, any CTL responses to TAg in a C57Bl/6 mouse must have been driven by cross priming via endogenous DCs. C57Bl/6 T-cells would not



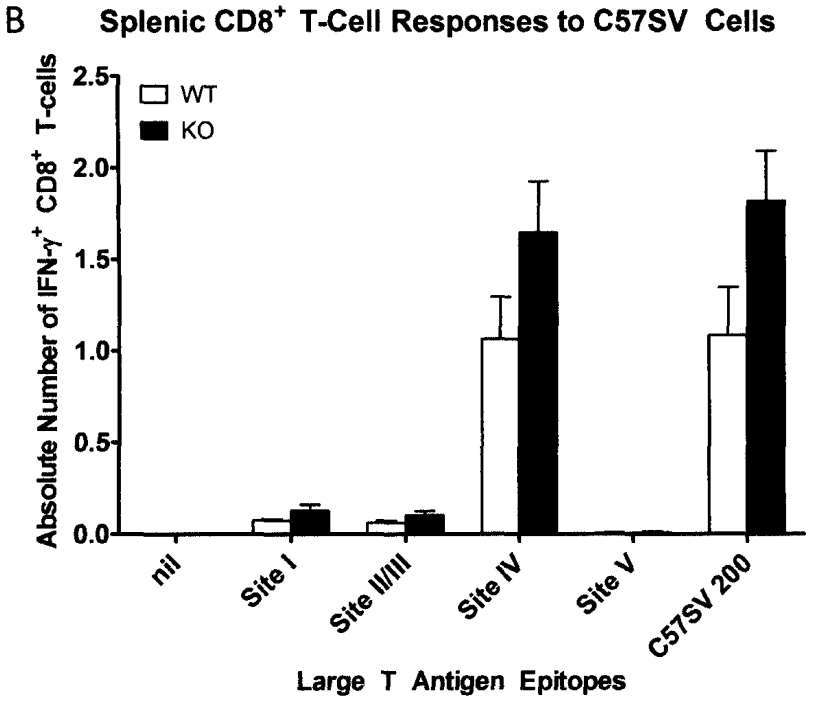
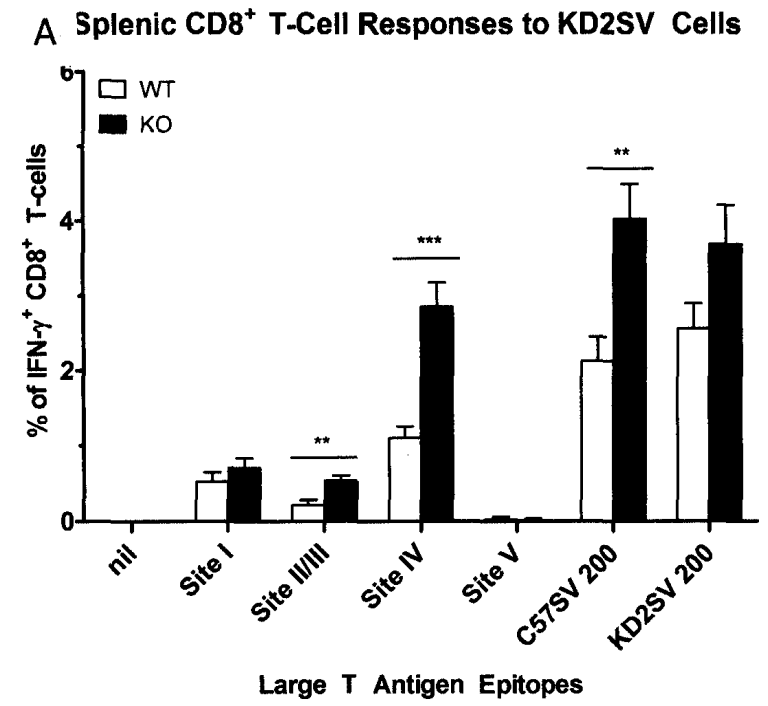


Figure 1 – Epitope specific T-cell responses to C57SV in IDO KO vs WT mice
Mice were injected with C57SV cells i.p and ICS for IFN-γ was conducted on splenocytes on Day 9 following injection. The figure shows A) the percentage of IFN-γ^+CD8$^+$ T-cells specific for TAg epitopes in IDO KO vs WT mice and B) the absolute number of IFN-γ^+CD8$^+$ T-cells specific for TAg epitopes in IDO KO vs WT mice; numbers calculated based on bulk splenocyte count. Data from pooled experiments: N = 12 WT & 10 IDO KO



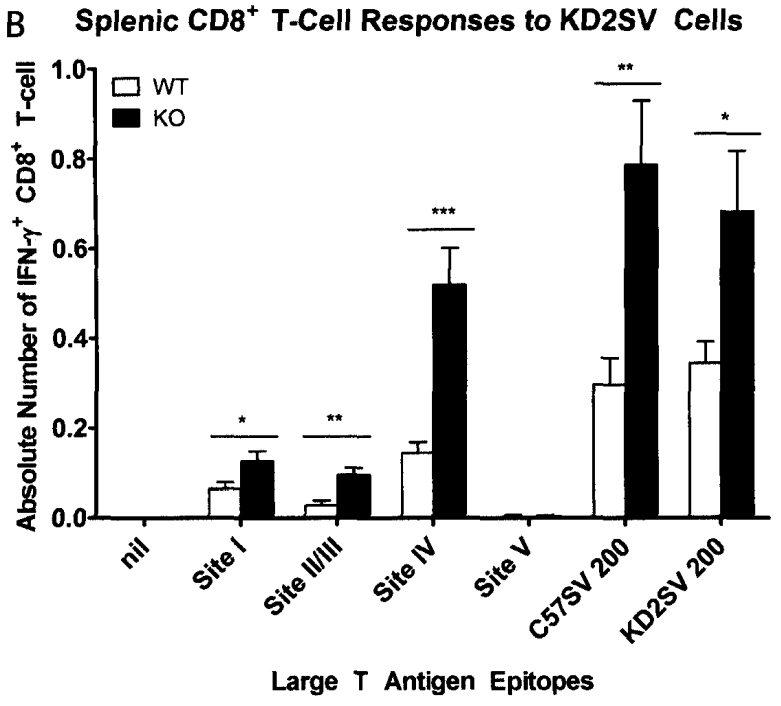


Figure 2 – *Epitope specific T-cell responses to KD2SV in IDO KO vs WT mice*

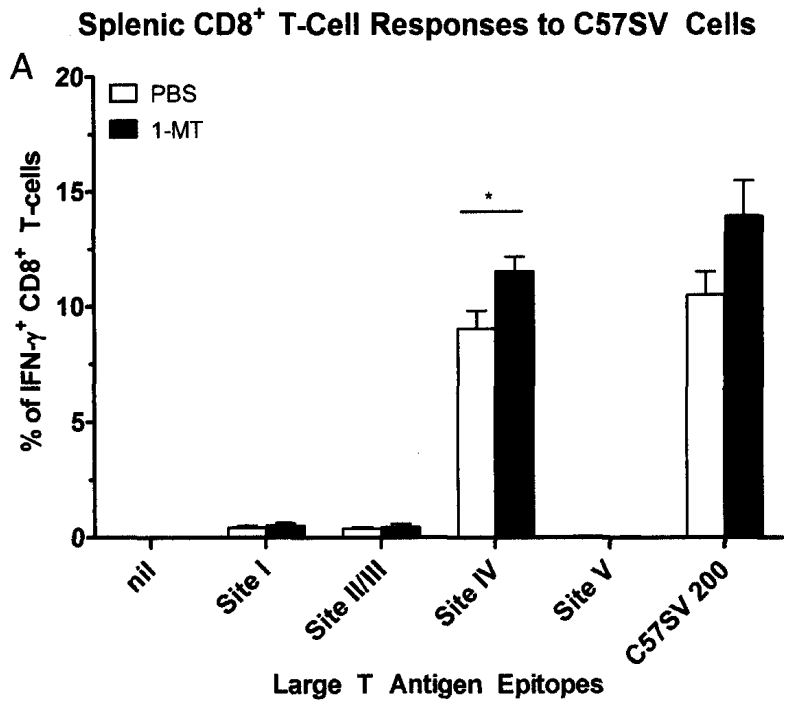
Mice were injected with KD2SV cells i.p and ICS for IFN-γ was conducted on splenocytes on Day 9 following injection. The figure shows A) the percentage of IFN-γ^+CD8$^+$ T-cells specific for TAg epitopes in IDO KO vs WT mice and B) the absolute number of IFN-γ^+CD8$^+$ T-cells specific for TAg epitopes in IDO KO vs WT mice; numbers calculated based on bulk splenocyte count. Data from pooled experiments: N = 8 WT & 8 IDO KO

have been able to directly recognize TAg epitopes presented in the context of $H2^d$ MHC molecules and thus would have had to rely on host DCs to present the antigenic material.

It is possible that the difference in CTL responsiveness between IDO KO and WT mice was due to an intrinsic T-cell phenotype or repertoire defect in the knockout mice themselves. To address these concerns, WT mice were treated with either 2.5 mg/day of 1-MT (a competitive inhibitor of the IDO enzyme) or vehicle for two days prior and post C57SV injection, and the ICS was again conducted as described. The results of these 3 experiments exactly paralleled those seen in the knockout studies, and mice treated with 1-MT exhibited a higher percentage and absolute number of T-cells specific for site IV of TAg (Figure 3). This shows that the results are replicable in WT mice treated with an IDO inhibitor, and ascribes a causational role for the IDO enzyme in this system, while eliminating the possibility of confounding variables in the knockout mice themselves.

The use of 1-MT was then validated by excluding the possibility of any potential off target effects of the inhibitor itself. This was accomplished by treating WT and IDO KO mice with either 1-MT or vehicle, and enumerating activated CTL frequency via ICS. Though an increase was observed in the 1-MT treated WT mice when compared with vehicle treated controls, no concomitant increase was detected between the IDO KO groups, suggesting that 1-MT is indeed specific to the IDO enzyme (data not shown).

In order to determine whether these finding were applicable to other model systems, the same ICS experiments were performed using IDO KO and WT mice treated with i.p. IAV. Again, the results of these investigations show that in the absence of IDO, the animals developed a more robust CTL response (both percentage and absolute number) to the two immunodominant epitopes (NP and PA) of IAV in both a primary and



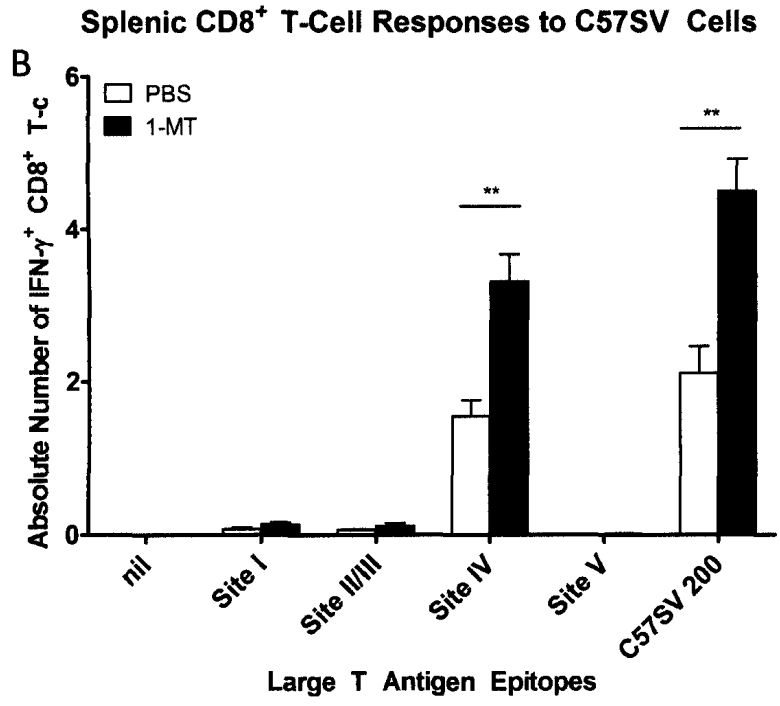
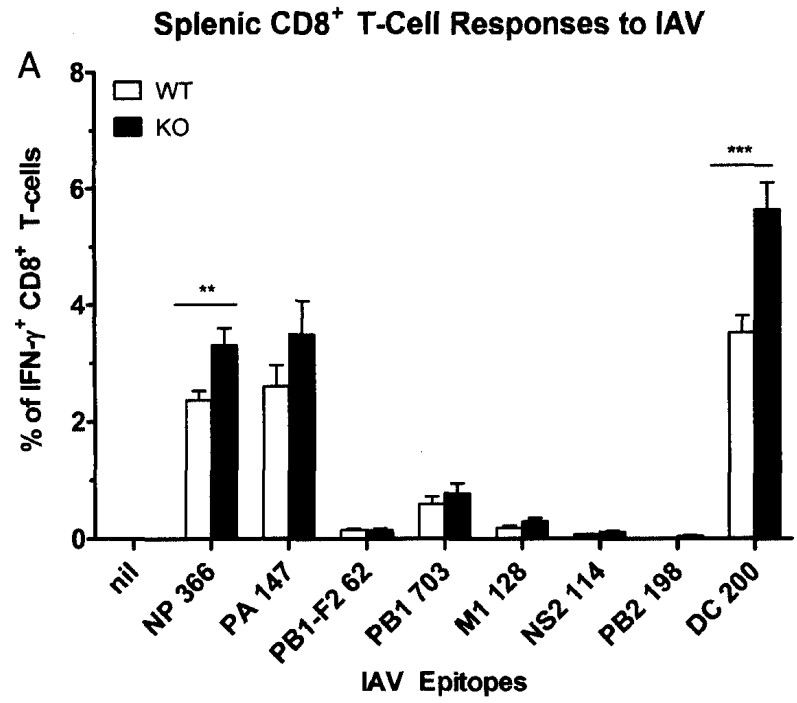


Figure 3 – Epitope specific T-cell responses to C57SV in 1-MT treated vs PBS treated mice
Mice were injected with 2.5mg/day of 1-MT for 2 days prior and 2 days post C57SV injection. ICS for IFN-γ was conducted on splenocytes on Day 9 following cell injection. The figure shows A) the percentage of IFN-γ^+CD8$^+$ T-cells specific for TAg epitopes in IDO KO vs WT mice and B) the absolute number of IFN-γ^+CD8$^+$ T-cells specific for TAg epitopes in IDO KO vs WT mice; numbers calculated based on bulk splenocyte count. Data from pooled experiments: N = 7 PBS & 7 1-MT



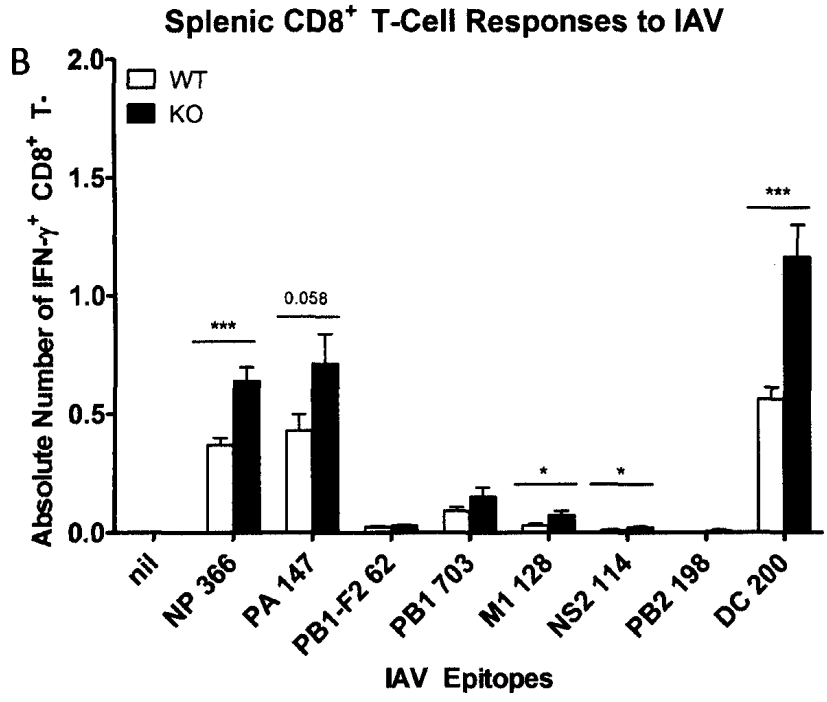
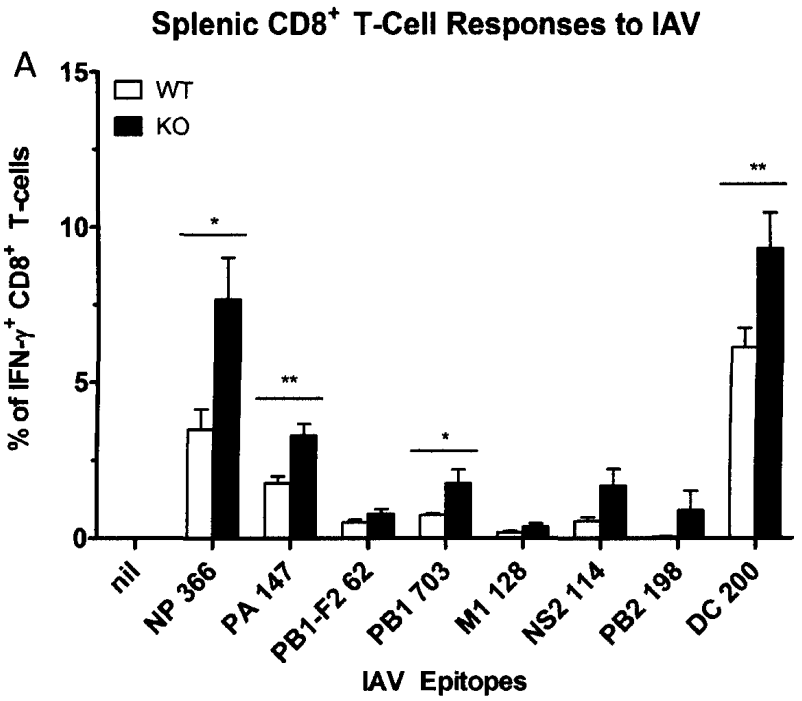


Figure 4 – Epitope specific primary T-cell responses to IAV in IDO KO and WT mice
Mice were injected with IAV i.p, and ICS for IFN-γ was conducted on splenocytes on Day 7 following injection. The figure shows A) the percentage of IFN-γ^+CD8$^+$ T-cells specific for IAV epitopes in IDO KO vs WT mice and B) the absolute number of IFN-γ^+CD8$^+$ T-cells specific for IAV epitopes in IDO KO vs WT mice; numbers calculated based on bulk splenocyte count.
Data from pooled experiments: N = 11 WT & 11 IDO KO



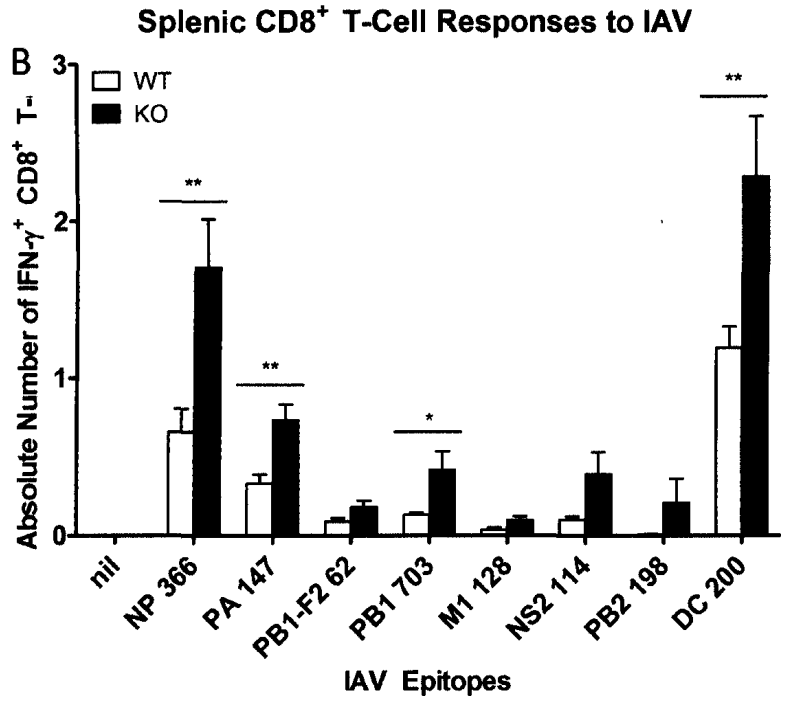
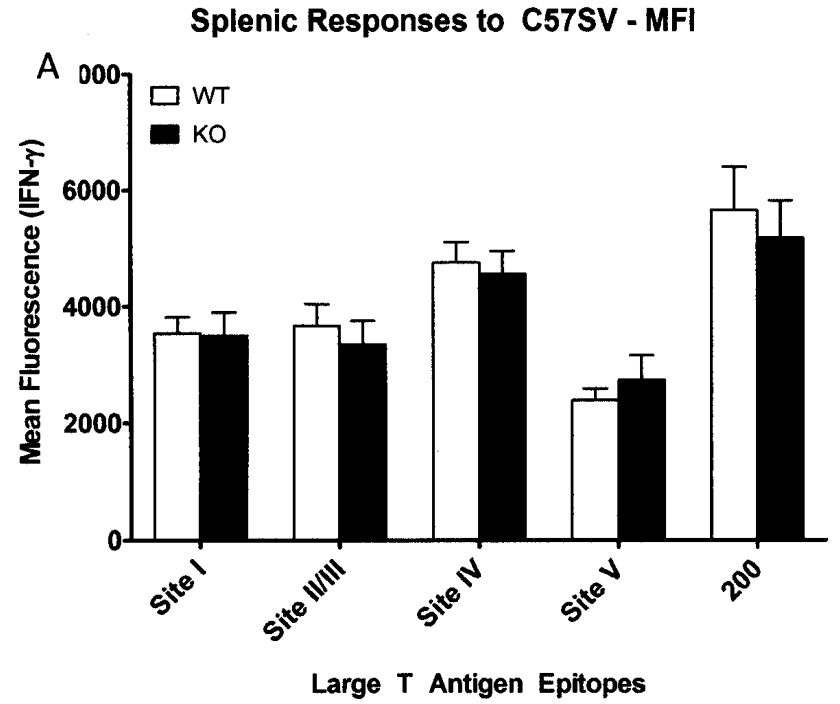


Figure 5 – Epitope specific memory T-cell responses to IAV in IDO KO and WT mice
Mice were injected with IAV (PR8) i.p on Day 0 and IAV (X31) i.p on Day 7. ICS for IFN-γ was conducted on splenocytes on Day 30. The figure shows A) the percentage of IFN-γ^+CD8$^+$ T-cells specific for IAV epitopes in IDO KO vs WT mice and B) the absolute number of IFN-γ^+CD8$^+$ T-cells specific for IAV epitopes in IDO KO vs WT mice; numbers calculated based on bulk splenocyte count. Data from pooled experiments: N = 8 WT & 8 IDO KO

memory recall context (Figure 4 and 5). This is important because it illuminates a more general role for IDO in the modulation of CTL responses to viral antigens, and that the phenomenon is not only confined to one model system.

Intracellular cytokine staining is a versatile experimental modality, because it also allows the enumeration of cytokine production on a per cell basis via mean fluorescence intensity (MFI). In this case, in both the TAg and IAV systems, even though IDO KO mice had a higher percentage and number of IFN-γ^+CD8^+ T-cells specific the immunodominant epitope following i.p treatment with C57SV cells or IAV, there was no difference in IFN-γ production on a per cell basis (Figure 6). This means that in the absence of IDO, there exists a higher frequency of activated immunodominant T-cells, but the individual IDO KO cells do not become more highly activated than WT cells.

Collectively, these experiments show that there is an increased number of activated immunodominant CTLs when IDO is absent, which seems to implicate a proliferative phenomenon as the mechanistic mediator behind these results. This can be logically reconciled with the purported function of IDO, which is to inhibit the proliferation of T-cells through localized tryptophan deprivation and/or the production of toxic, tryptophan derived metabolites. It is thus possible that in the steady state, site IV specific CTLs proliferate more than their sub dominant counterparts, due to their singular nature as immunodominant responders. In the absence of IDO, these highly proliferating immunodominant CTLs are able to expand to an even higher degree, because they are more sensitive than subdominant T cell clones to tryptophan degradation and microenvironment toxicity when IDO is present.



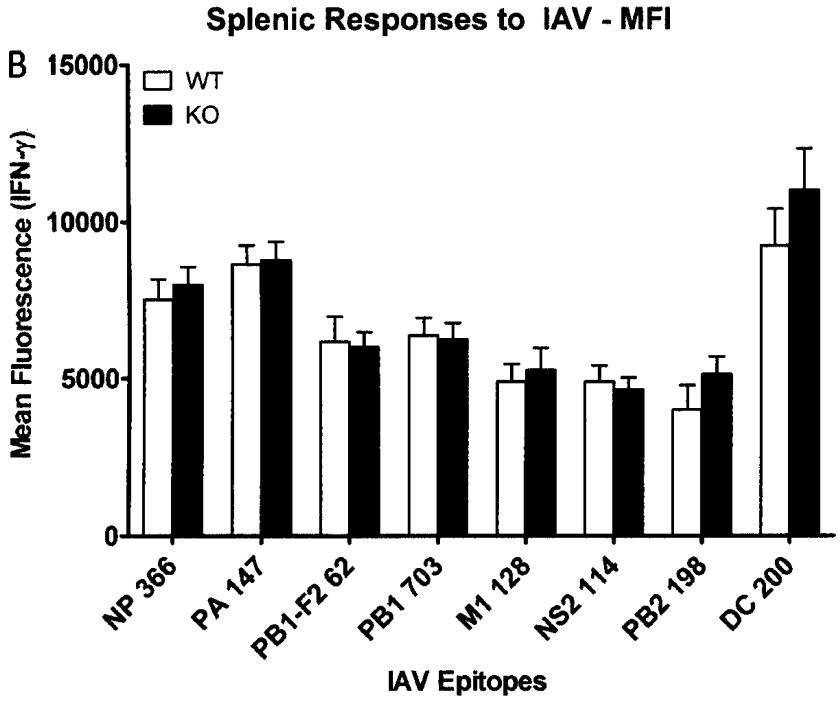


Figure 6 – Mean fluorescence intensity of IFN-γ in epitope specific T-cells (C57SV and IAV) in IDO KO vs WT mice

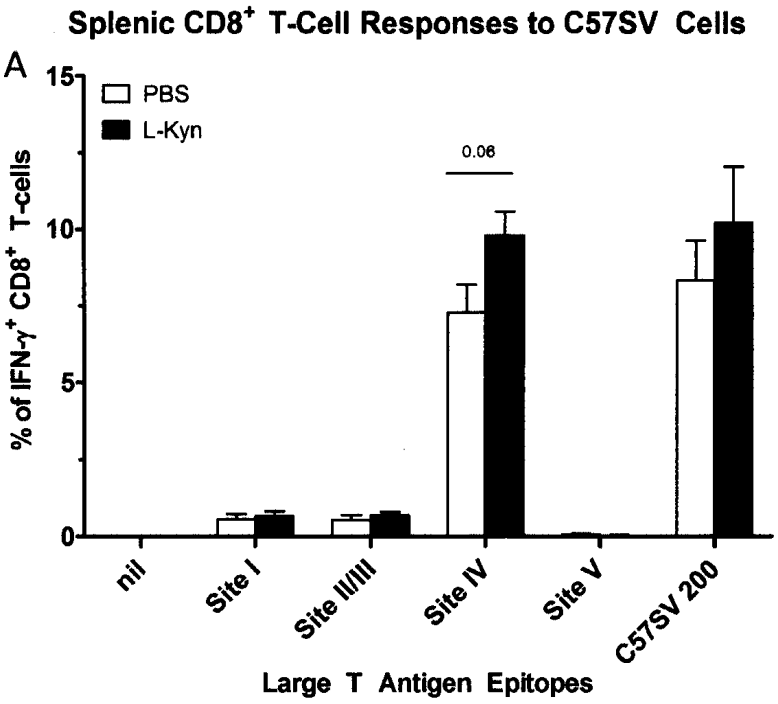
Mean fluorescence intensity is a measure of IFN-γ production on a per cell basis. A) MFI of IFN-γ in IFN-$\gamma^{+}CD8^{+}$ T-cells specific for TAg epitopes in IDO KO vs WT mice and B) MFI of IFN-γ in IFN-$\gamma^{+}CD8^{+}$ T-cells specific for IAV epitopes in IDO KO vs WT mice. Data from pooled experiments: TAg N = 12 WT & 10 IDO KO, IAV N = 11 WT & 11 IDO KO

In order to address this hypothesis, it therefore became important to question whether treating mice with L-kynurenine, the most immediate stable downstream metabolite of L-tryptophan, would in fact suppress site IV specific T-cell responses in WT mice. This would show whether immunodominant CTLs are more sensitive to tryptophan metabolites than subdominant clones.

Treatment of mice with L-kynurenine does not suppress specific T cell responses to TAg or IAV

It is well established in the literature that L-kynurenine and its downstream metabolites exhibit immunosuppressive properties *in vivo* (42,60,61). This is why the generation of toxic metabolites is considered one of the mechanisms through which IDO modulates immune function.

Given the hypothesis that immunodominant CTLs are more sensitive to the toxic microenvironment created by tryptophan catabolism, WT mice were treated with i.p L-kynurenine and C57SV cells. The subsequent ICS experiments yielded surprising results, because they recapitulated the same trend seen in the IDO KO and inhibition experiments: an increase in the frequency of IFN-γ^+CD8^+ T-cells specific for site IV (Figure 7). In addition, there was an L-kynurenine dose dependent increase in the frequency of site IV specific cells (Figure 8). This was contrary to all expectations in the published literature, and may speak to some putative negative feedback loop through which high concentrations of L-kynurenine actually inhibit IDO. This however is all speculative and based upon a similar phenomenon with TDO, a housekeeping enzyme in the liver that also degrades tryptophan (62,63).



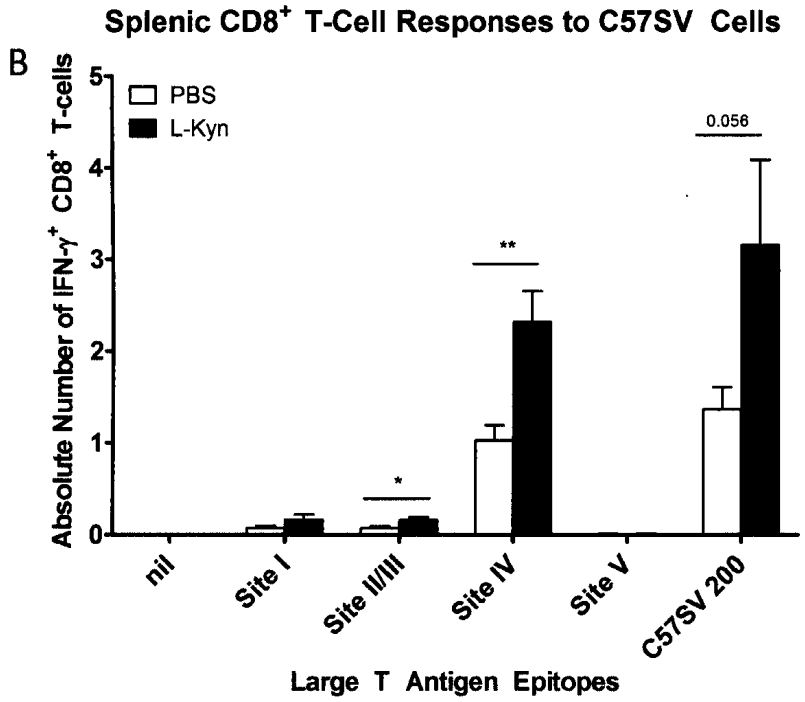


Figure 7 – Epitope specific T-cells responses to C57SV cells in L-Kyn treated vs PBS treated mice Mice were injected i.p with either vehicle or 10mg/day of L-Kyn on the day of, and two days following C57SV i.p injection. ICS was conducted on splenocytes on Day 9 post injection and the figure shows A) the percentage of IFN-γ$^+$CD8$^+$ T-cells specific for TAg epitopes in L-Kyn vs PBS treated mice and B) the absolute number of IFN-γ$^+$CD8$^+$ T-cells specific for TAg epitopes in L-Kyn vs PBS treated mice. Data from pooled experiments: N = 8 PBS & 8 L-Kyn

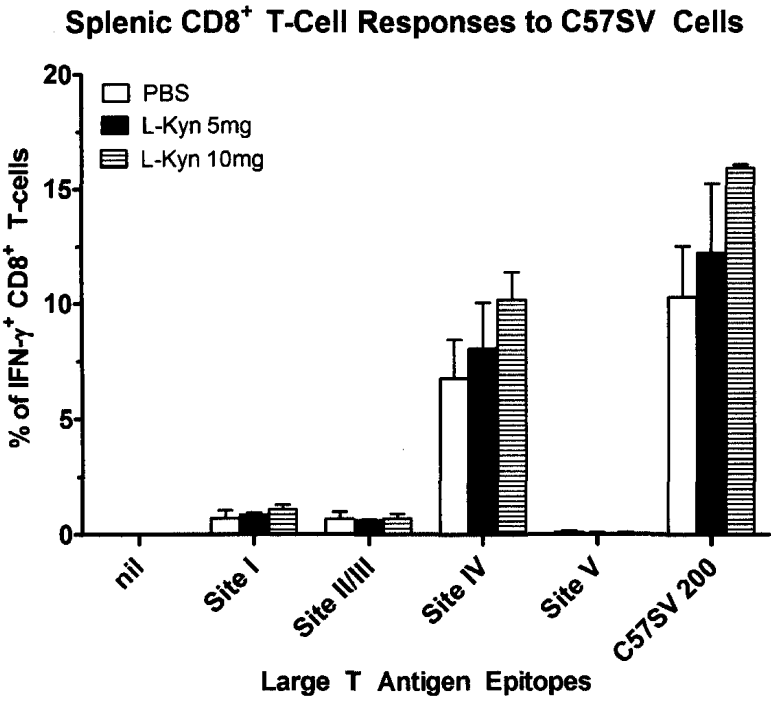


Figure 8 – Epitope specific T-cells responses to C57SV cells in mice treated with different doses of L-Kyn vs PBS treated mice

Mice were injected with either vehicle or 5mg/day or 10mg/day of L-Kyn on the day of and two days after C57SV injection. The figure shows the percentage of IFN-γ^+CD8$^+$ T-cells specific for TAg epitopes in L-Kyn vs PBS treated mice. N = 4 PBS & 2 L-Kyn 5 mg & 2 L-Kyn 10 mg

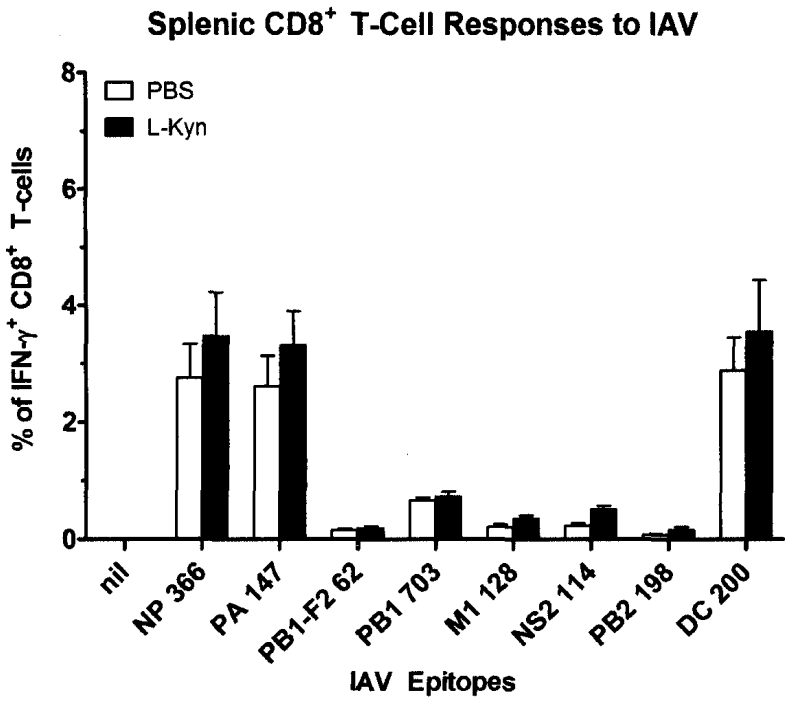


Figure 9 – Epitope specific T-cells responses to IAV in L-Kyn treated vs PBS treated mice
Mice were injected with either vehicle or 10mg/day of L-kyn on the day of and two days after IAV injection. The ICS was conducted on Day 7 post injection and the figure shows the percentage of IFN-γ^+CD8$^+$ T-cells specific for IAV epitopes in L-Kyn vs PBS treated mice. N = 4 PBS & 4 L-Kyn

Since the results of the L-Kyn TAg experiments were relatively unexpected and controversial, they were repeated in the IAV system where a similar trend was observed (Figure 9). Although it would be inappropriate to unreservedly claim L-kynurenine treatment actually increased CTL responses (because the data did not reach statistical significance), it is safe to conclude that the tryptophan metabolite did not suppress them. Taking these results into account, the hypothesis was augmented accordingly: IDO inhibits the responsiveness of immunodominant CTLs by suppressing their proliferation, but does so in an L-kynurenine independent manner.

With the role of tryptophan metabolites in this system illuminated, it was thus of importance to elucidate whether the absence of IDO actually affects the proliferative capacity of immundominant and subdominant CTLs, as the previous ICS data seemed to suggest.

IDO supresses the proliferative capacity of activated epitope specific CTLs

In order to determine whether activated CTLs specific for the site IV epitope proliferated more robustly in the IDO KO condition than WT, the ICS protocol was modified to include markers of proliferation (Ki67) and anergy (PD1 and CHOP). The classic CD8 and IFN-γ antibodies were used as before to identify activated epitope specific CTLs, whilst Ki67, PD1, and CHOP provided insight into the proliferative mechanisms involved in this phenomenon.

The main cell population of interest was the $CD8^+$ pool that also expressed IFN-γ and Ki67. These cells represented epitope specific (either site IV or site I) CTLs that

were actively proliferating *in vivo* at the time of euthanasia (64). In accordance with the hypothesis, IDO KO mice exhibited a higher percentage of IFN-γ^+Ki67$^+$ CTLs specific for site IV when compared with WT mice on Day 9 following i.p injection of C57SV cells. The same was true for site I specific CTLs, but the increase in frequency between KO and WT was much smaller than for the immunodominant clones (Figure 10 and 11). This means that these activated cells were proliferating more robustly in the absence of IDO, and explains why there was a higher frequency of activated immunodominant CTLs in IDO KO animals in the previous experiments. Site IV IDO KO CTLs also expressed more Ki67 on a per cell basis than their WT counterparts, as seen via MFI (Figure 12).

In addition, there was a higher frequency of PD1$^+$IFN-γ^+ Site IV specific CTLs in IDO KO animals, and though PD1 is a marker of anergy, it has been reported that highly proliferating cells express higher levels of this cell surface molecule (Figure 13) (65). By taking this finding into account, and combining it with the Ki67 expression data, it becomes clear that IDO suppresses the proliferation of activated immunodominant CTLs *in vivo*.

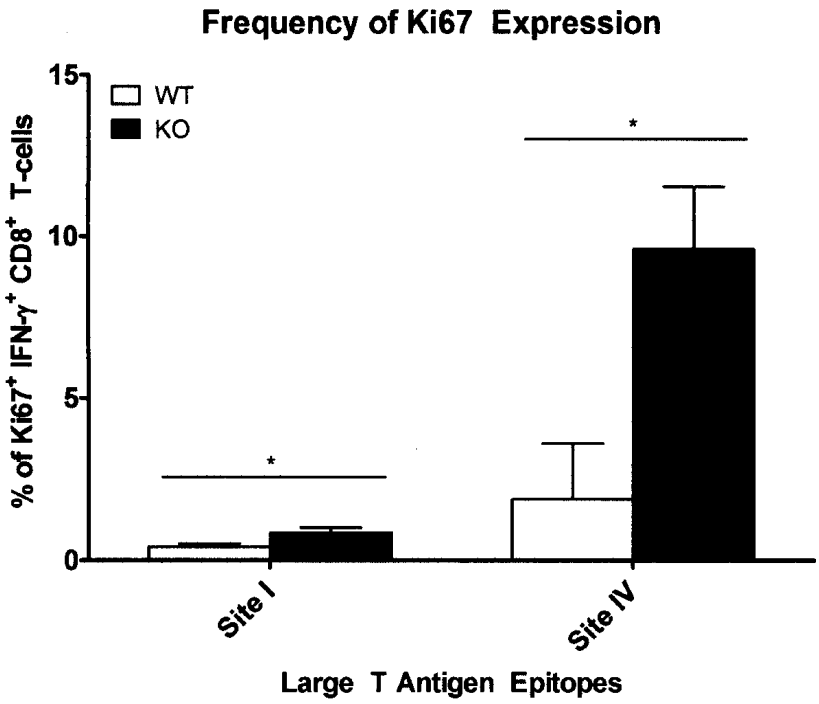


Figure 10 – Percentage of Ki67$^+$ IFN-γ$^+$ CD8$^+$ T-cells specific for Site I and Site IV in IDO KO vs WT mice

Mice were injected with C57SV cells i.p on Day 0 and splenocytes were extracted on Day 9 post injection for marker staining. Data representative of two experiments: N= 7 WT & 7 IDO KO

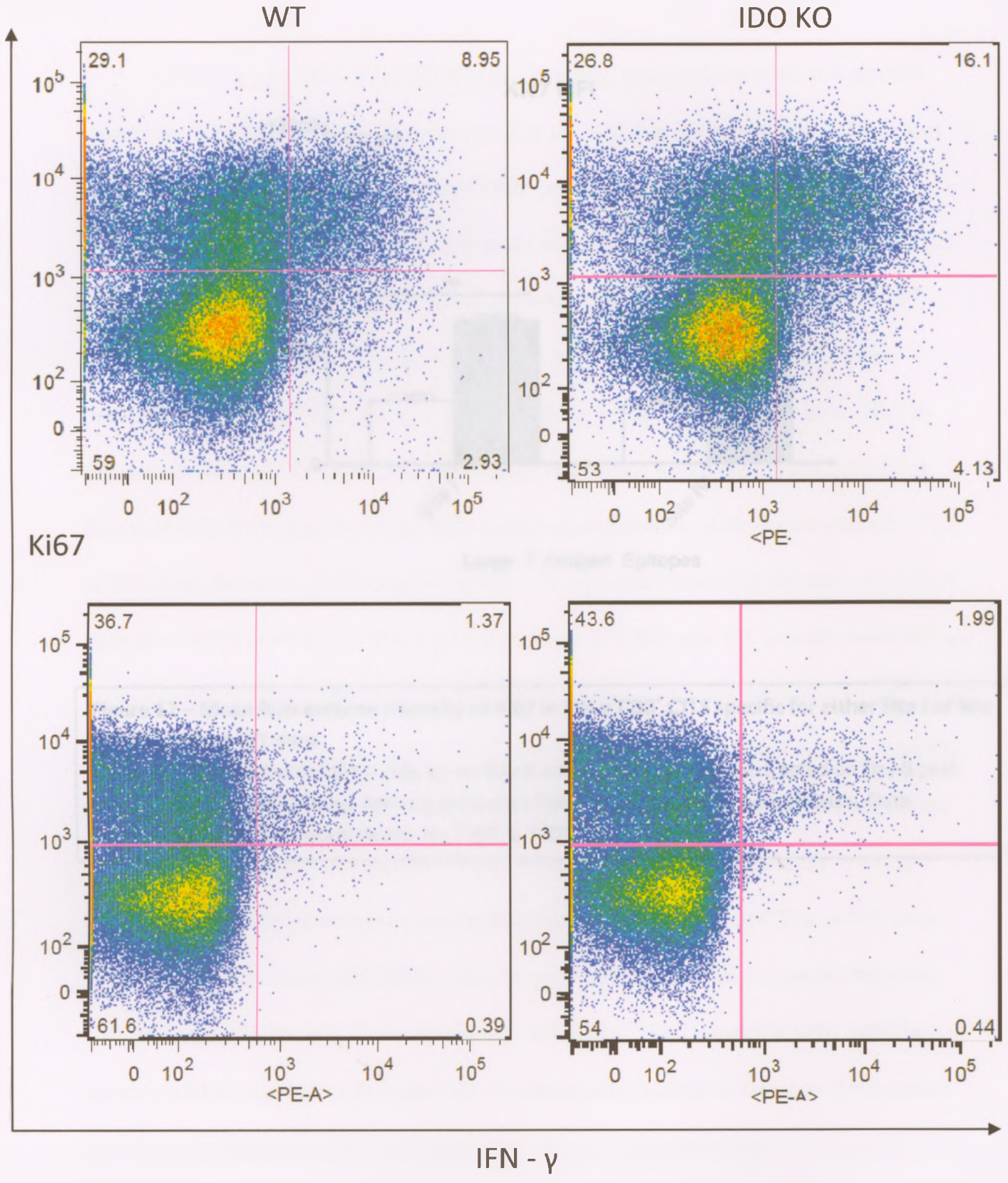


Figure 11 – Flow cytometry plots showing Ki67 and IFN-γ expression in a $CD8^+$ T-cell population specific for either Site IV (top panel) or Site I (bottom panel)

Flow cytometry gating strategy for Ki67 expression. Data representative of two experiments: N= 7 WT & 7 IDO KO

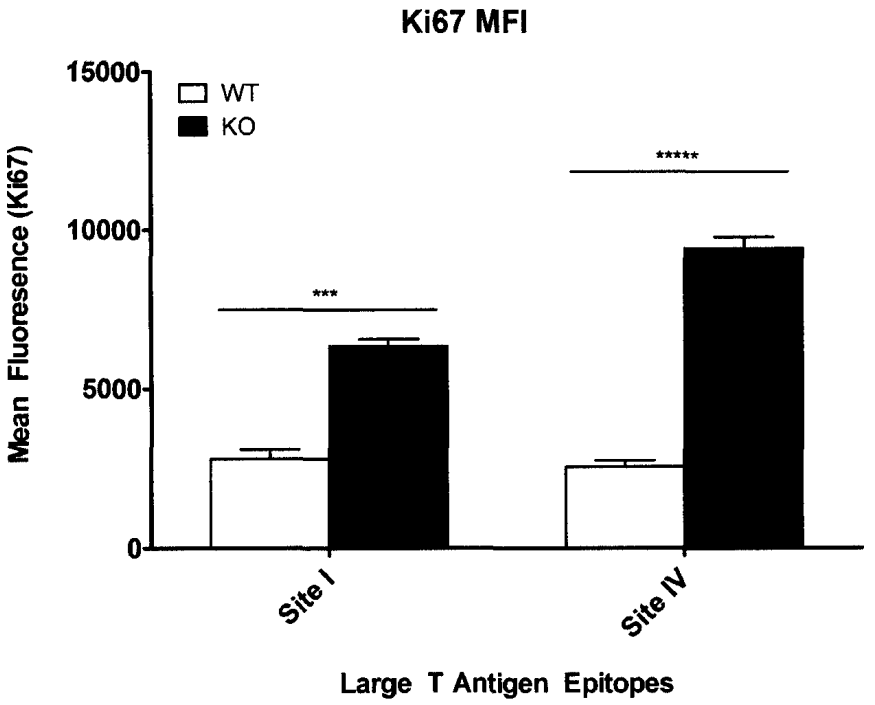


Figure 12 – Mean fluorescence intensity of Ki67 in IFN-$\gamma^{+}CD8^{+}$ CTLS specific for either Site I or Site IV in IDO KO vs WT mice

Mice were injected with C57SV cells i.p on Day 0 and splenocytes were extracted on Day 9 post injection for marker staining. MFI is a measure of Ki67 production on a per cell basis. Data representative of two experiments: N= 7 WT & 7 IDO KO

CHOP is a product of GCN2 kinase signalling, and has been used as a reporter protein for IDO function (39). Previous studies showed that IDO producing DCs induce GCN2 kinase activity (as measured by CHOP production) in T-cells, which in turn become anergic or upregulate their suppressor functions. Therefore it was of mechanistic interest to determine whether there was a difference in frequency of activated $CHOP^+$ immunodominant CTLs in IDO KO vs WT mice. The hypothesis was that a lower proportion of IDO KO CTLs would express CHOP, because there would be no IDO in the system to induce GCN2 kinase signalling. This would also have implicated GCN2 kinase as one of the factors behind IDO mediated suppression of immunodominant CTLs in WT mice. However, following completion of two experiments (i.p injection of C57SV cells and marker staining on Day 9 post injection), IDO KO and WT animals exhibited no differences in the frequency of activated site IV or site I CTLs that were also CHOP positive (Figure 14). These results indicate that GCN2 kinase signalling is not responsible for the differences in CTL frequency between IDO KO and WT mice in the TAg system.

This modified ICS approach also provided insight into whether there was any proliferative difference between immunodominant and subdominant CTLs in WT mice, and if the non-activated $CD8^+IFN\text{-}\gamma^-$ cells also varied in proliferative capacity between IDO KO and WT animals. It was found that WT $IFN\text{-}\gamma^+$ site IV specific cells exhibited increased Ki67 expression than their site I counterparts, adding credence to the assertion that immunodominant cells proliferate more robustly than subdominant clones (data not shown). In addition, there was no difference in the $IFN\text{-}\gamma^-$ $Ki67^+$ CTL frequency in IDO KO vs WT mice, suggesting that IDO only affects the proliferation of activated cells (Figure 15).

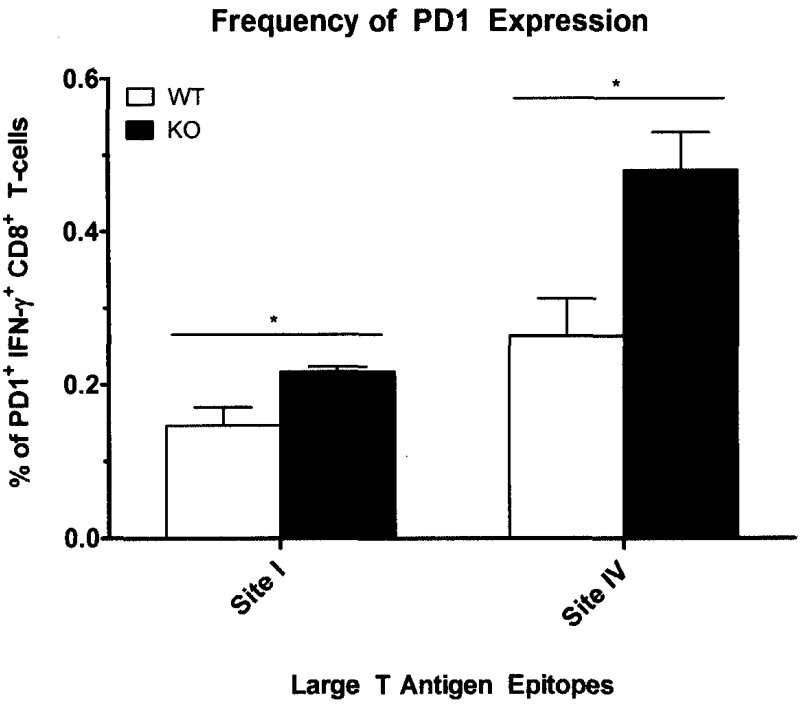


Figure 13 – Percentage of $PD1^{+}IFN\text{-}\gamma^{+}CD8^{+}$ T-cells specific for Site I and Site IV in IDO KO vs WT mice

Mice were injected with C57SV cells i.p on Day 0 and splenocytes were extracted on Day 9 post injection for marker staining. Data representative of two experiments: N= 7 WT & 7 IDO KO

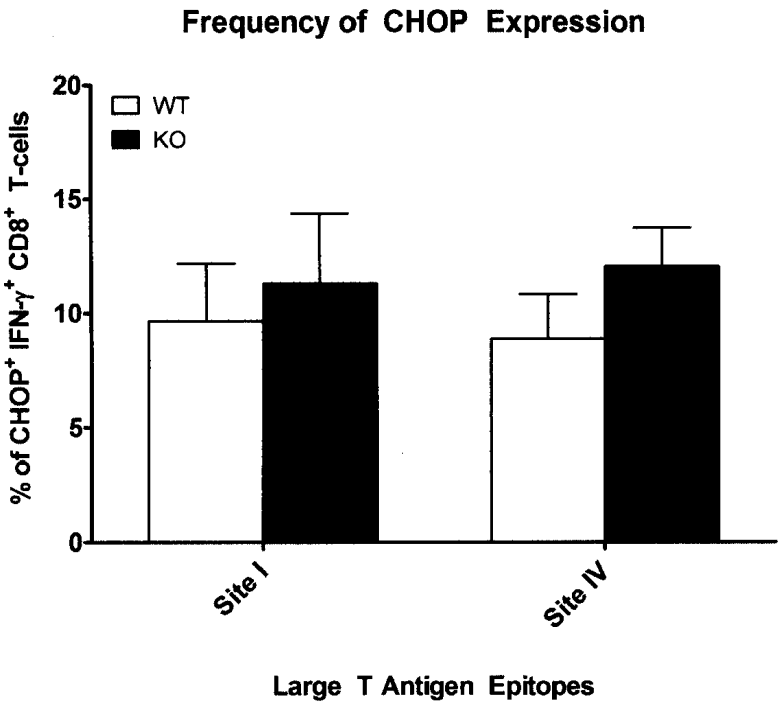


Figure 14 – Percentage of CHOP$^+$IFN-γ$^+$CD8$^+$ T-cells specific for Site I and Site IV in IDO KO vs WT mice

Mice were injected with C57SV cells i.p on Day 0 and splenocytes were extracted on Day 9 post injection for marker staining. Data representative of two experiments: N= 7 WT & 7 IDO KO

Based on these results, it was logical to conclude that IDO suppresses the proliferation of activated immunodominant CTLs in an L-kynurenine and CHOP independent manner, thus modulating the frequency of these cells *in vivo*. However, in order to further this assertion it became necessary to determine whether IDO also affects the frequency of cells with the specific site IV or site I TCR, regardless of activation state.

IDO does not affect the frequency of T cell clones with the site IV or site I specific TCR

The experiments up to this point have focussed on $CD8^+$ T-cells that produce IFN-γ when re-stimulated by synthetic peptides that equate with specific epitopes of TAg or IAV. This cell population corresponds to activated CTLs that recognize the antigenic determinant in question (e.g., site IV), but does not include all of the clones with the appropriate epitope specific TCR. There are also cells with the correct TCR that were not activated *in vivo* and thus do not respond to peptide re-stimulation. In order to adequately evaluate whether IDO only affects the frequency of activated CTLs, it was thus necessary to determine its effects on the entire pool of clones, regardless of their activation state.

Through the use of tetramer staining on splenocytes extracted on Day 9 post C57SV injection, it was determined that IDO does not affect the frequency of CTLs with either the site IV or site I TCR (Figure 16). There was no significant difference in the percentage of site IV $tetramer^+CD8^+$ cells between IDO KO and WT mice. This was consistent with the hypothesis that IDO only suppresses clones that are activated *in vivo*, which was suggested by the ICS and proliferation marker data.

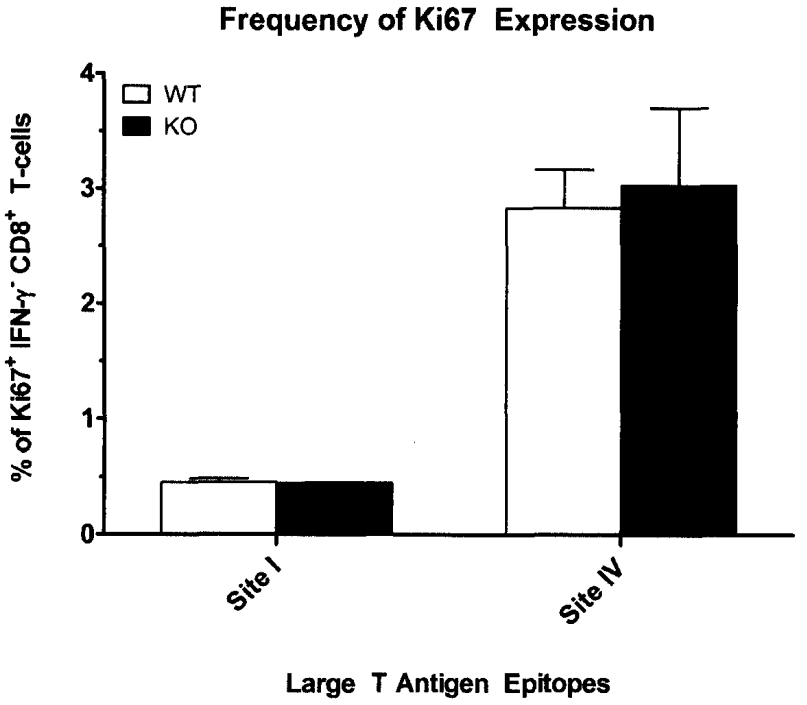


Figure 15 – Percentage of Ki67$^+$IFN-γ$^-$CD8$^+$ T-cells specific for Site I and Site IV in IDO KO vs WT mice

Mice were injected with C57SV cells i.p on Day 0 and splenocytes were extracted on Day 9 post injection for marker staining. Figure shows the percentage of Ki67$^+$ non-activated (IFN-γ$^-$) CTLs in IDO KO vs WT mice. Data representative of two experiments: N= 7 WT & 7 IDO KO

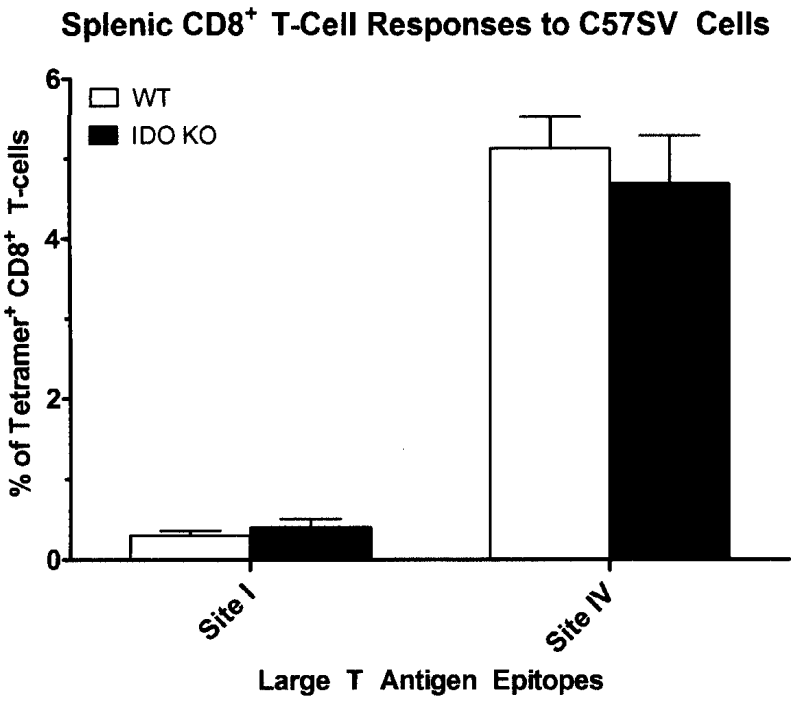


Figure 16 – Percentage of Tetramer$^+$CD8$^+$ T-cells specific for Site I and Site IV in IDO KO vs WT mice
Mice were injected with C57SV cells i.p on Day 0 and splenocytes were extracted on Day 9 post injection for tetramer staining. Data from pooled experiments: N = 5 WT & 5 IDO KO

Taking all of the experimental results into account, it is possible to conclude that IDO decreases the frequency of activated immunodominant CTLs, by reducing their proliferative capacity *in vivo*, and that it does not simultaneously affect non-activated T-cell clones. This makes inherent sense because non-activated T-cell clones would not necessarily be proliferating above homeostatic levels (66), and thus the presence or absence of the IDO enzyme should not affect the frequency of these cells.

IDO moderately suppresses epitope specific CTL cytotoxicity

Though ICS for IFN-γ is a CTL functional assay, it does not allow for the enumeration of actual CTL killing capacity. The ability of CTLs to kill virally infected cells is particularly important to ameliorate infection and limit morbidity in a variety of different contexts (67,68). Thus, it was crucial to determine whether the IDO KO mediated increase in immunodominant CTL frequency, actually has salient clinical implications, by measuring its affect on CTL cytotoxicity.

After the completion of three *in vivo* killing assays, during which IDO KO and WT mice were injected with C57SV cells and then re-challenged nine days later with syngeneic, site IV or control peptide pulsed splenocytes, it was determined that the absence of IDO induces a moderate, yet statistically significant increase, in the cytotoxicity of site IV specific CTLs (Figure 17). Though the increase in killing was relatively small, it can be argued that the induction of a 5% higher rate of cytotoxicity by knocking out one enzyme is still important. In a therapeutic context, an IDO inhibitor could be used in conjunction with other pharmaceutical approaches to further augment CTL responses.

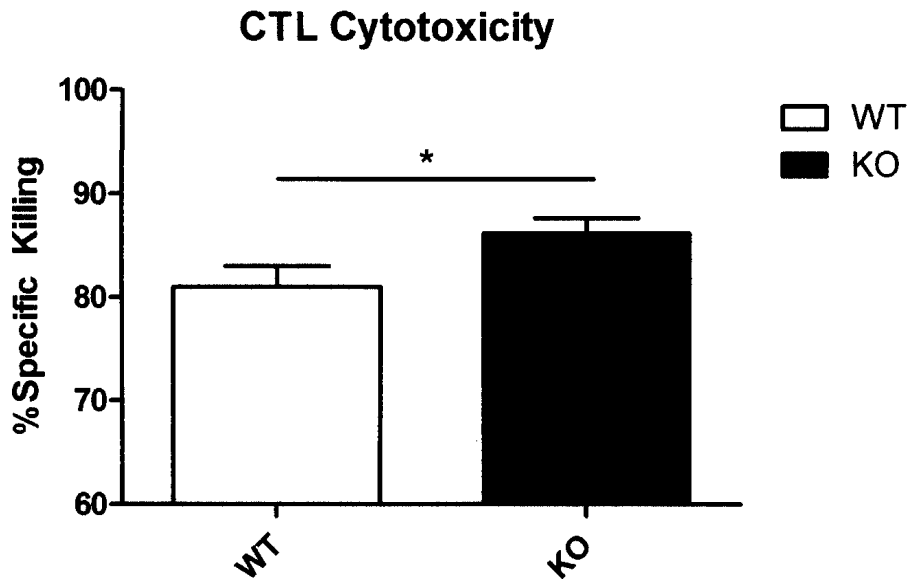


Figure 17 – Cytotoxicity of IDO KO vs WT CTLs specific for Site IV

Mice were injected with C57SV cells i.p on Day 0, and on Day 9 re-challenged i.v. with CFSE labelled syngeneic splenocytes pulsed with either Site IV or control peptide. Killing was allowed to occur for 30 minutes *in vivo* at which point the mice were sacrificed. The figure shows % specific killing of Site IV peptide pulsed C57Bl/6 splenocytes by CTLs in IDO KO vs WT mice

Data from pooled experiments: N = 6 WT and 6 IDO KO

Due to the small but statistically significant increase in CTL killing, it became important to put these findings into a more physiological perspective and determine whether the results were biologically relevant. To accomplish this objective, the role of IDO was elucidated in an actual disease model of IAV.

IDO does not affect morbidity in an intranasal model of IAV infection

Since intraperitoneal IAV injection fails to induce an active disease state in mice, it became necessary to devise a model of infection that produces detectable morbidity. This would serve to elucidate whether the ICS findings in the i.p systems were applicable to other modes of infection, and if the increase in both the frequency and cytotoxicity of immunodominant CTLs translated to better protection against disease.

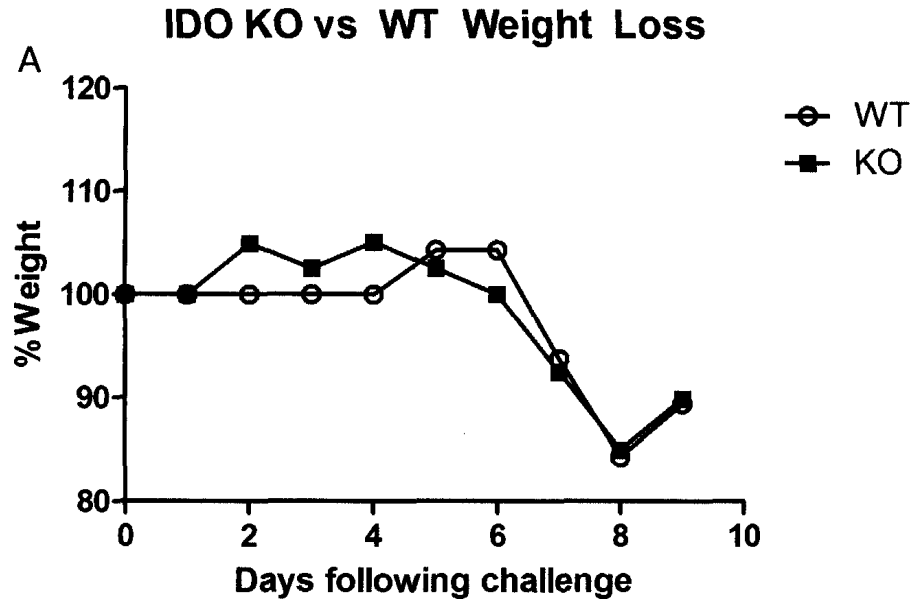
Animals treated with 6-8 pfu of IAV intranasally began losing weight between 5-7 days following inoculation, and the ICS was then conducted on day 9 or when the mice had lost 20% of their body weight. It was hypothesized that mice lacking the IDO enzyme would be better protected against IAV insult, due to the higher frequency of immunodominant CTLs and the slightly improved cytotoxic capacity of these cells.

After the conclusion of two experiments, it became apparent that IDO KO mice lost the same amount of weight as WT mice, at about the same time (Figure 18). IDO KO mice did, however, exhibit a trend toward increased frequency of immunodominant CTLs in the spleen, which was consistent with the results from the i.p systems (Figure 19). Thus, even though IDO suppresses the frequency of epitope specific CTLs as well as their killing capacity, it does not seem to affect an animal's susceptibility to disease at least in an acute context. However, it is possible that the IDO KO mice would have

recovered more quickly than WT mice from IAV infection, had the experimental time course been adjusted to study this phenomenon. In addition, IDO KO mice may have been better protected against a higher dose of IAV following an initial vaccination with the virus.

It was also interesting to conduct ICS on the cells collected via bronchoalveolar lavage (BAL), since these were the in situ responding cells in the lungs and would be directly responsible for killing virally infected host cells. IDO KO and WT mice exhibited a similar frequency of epitope specific CTLs in the BAL fluid, and this might speak to why IDO KO mice were not better protected from acute IAV infection (Figure 20). It is possible that the presence or absence of IDO has differential effects on CTL responses, depending on whether the response is local (BAL) or systemic (spleen).

In addition, there appeared to be a modified immunodominance hierarchy in the lung. The subdominant epitopes induced a much higher CTL response than in the spleen, and the levels were on par with the immunodominant peptides. This was a side observation but none the less a potentially important one, because such a phenomenon has never been reported before.



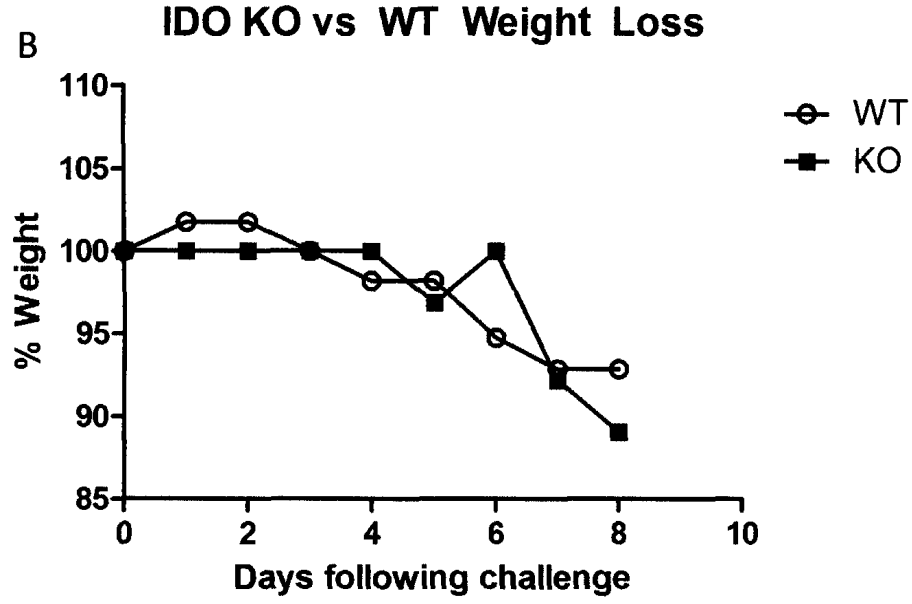
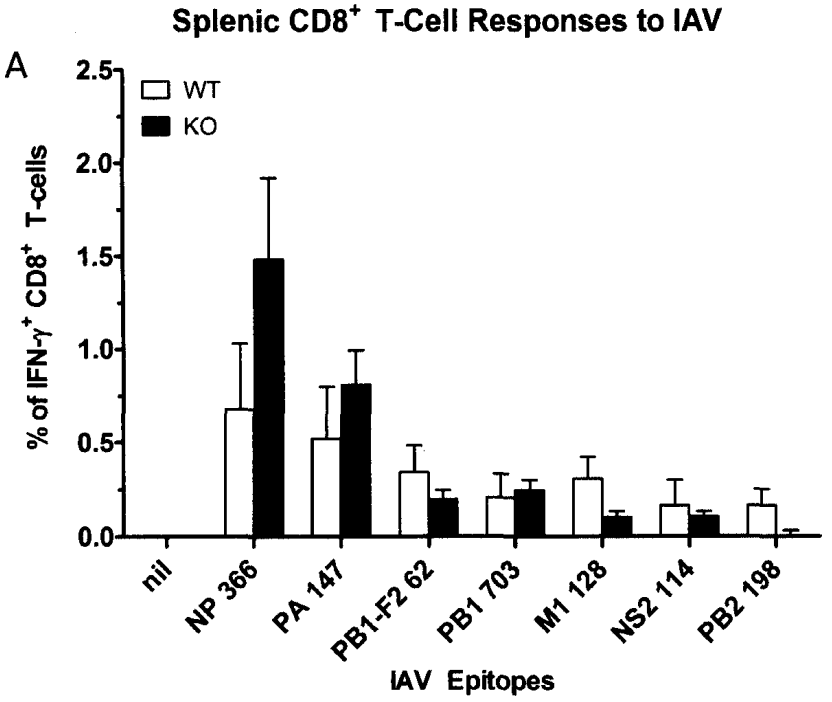


Figure 18 – Percent weight loss in i.n IAV challenge IDO KO and WT mice
Mice were challenged with 6-8 pfu of IAV i.n on Day 0 and health status (weight loss) was monitored on a daily basis with a digital scale. The figure shows A) percent weight loss in Experiment 1: N = 3 WT & 3 IDO KO, and B) percent weight loss in Experiment 2: N = 3 WT & 3 IDO KO



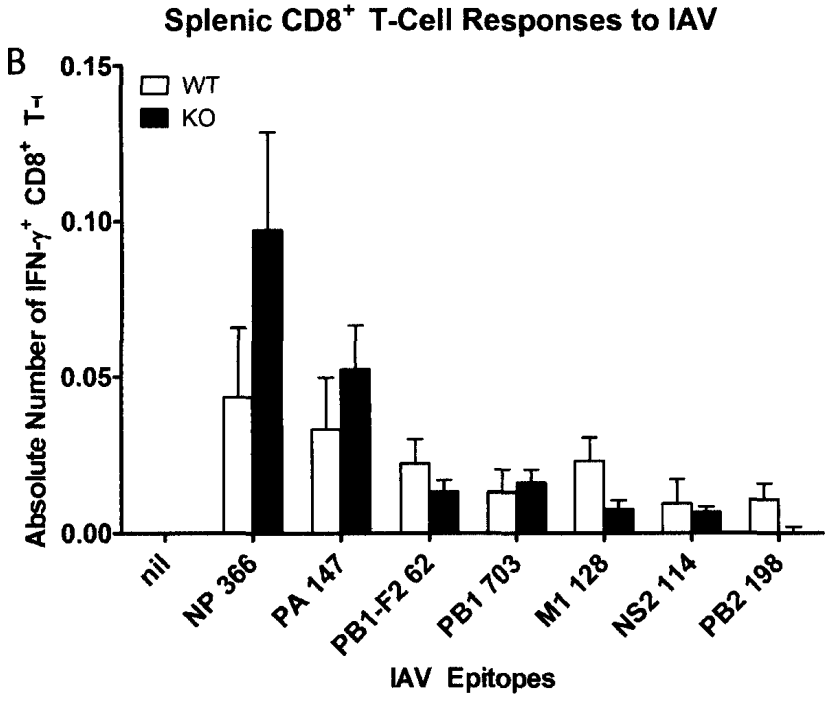


Figure 19 – Splenic epitope specific T-cell responses to i.n IAV in IDO KO vs WT mice
Mice were challenged with 6-8 pfu of IAV i.n on Day 0 and ICS was conducted on splenocytes on Day 9 post injection. The figure shows A) the percentage and B) the absolute number of IFN-γ$^+$CD8$^+$ T-cells specific for IAV epitopes. Data representative of two experiments: N = 6 WT & 6 IDO KO

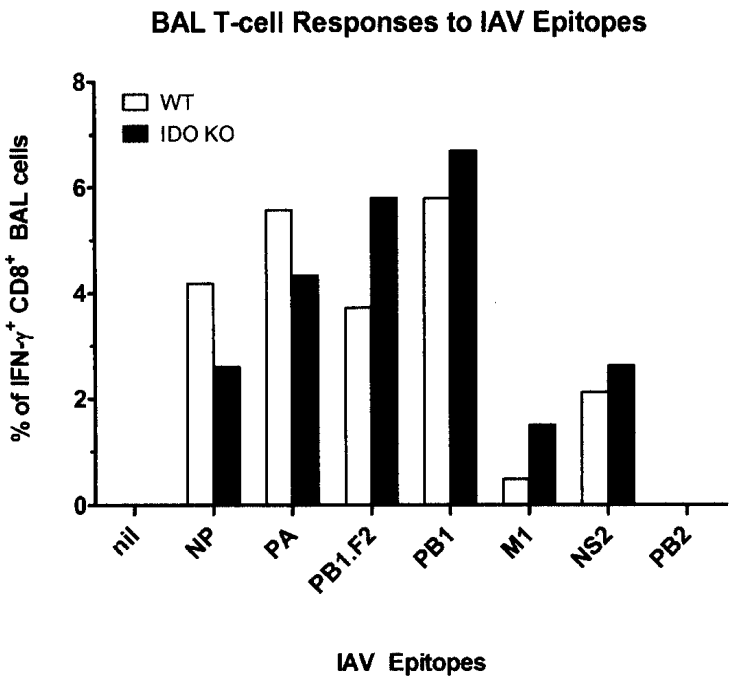


Figure 20 – Bronchoalveolar lavage (BAL) T-cell responses to i.n IAV in IDO KO vs WT mice
Mice were challenged with 6-8 pfu of IAV i.n on Day 0 and ICS was conducted on BAL lymphocytes on Day 9 post injection. Figure shows the percentage of IFN-γ^+CD8^+ T-cells specific for IAV epitopes Data representative of two experiments. BAL cells pooled from 3 WT and 3 IDO KO mice

Discussion

The data detailed in this thesis clearly show that IDO plays a role in the negative regulation of epitope specific T-cell responses to viral antigens. This effect is mediated by the decreased proliferative capacity of activated immunodominant CTLs when IDO is present, and also translates to less efficacious cytotoxic functions of these cells. This phenomenon occurs in an L-kynurenine independent manner, and therefore must be primarily mediated by the absence of tryptophan in the cellular microenvironment. Though IDO does not affect morbidity or survival in mice infected with i.n IAV in an acute context, it is still likely that it would modulate disease progression and/or recovery from illness in a vaccination challenge model, since IDO also suppresses the generation of memory recall responses *in vivo*.

The finding that IDO primarily affects the most immunodominant CTLs is an important one, because it highlights a previously unappreciated mechanism of IDO mediated immune regulation. Given the increased propensity of immunodominant CTLs to proliferate, it makes sense that these cells would be most sensitive to changes in microenvironment tryptophan levels. In fact, this was one of my earliest hypotheses, and it was derived from the published observation that CTLs and certain pathogens require different levels of tryptophan to function; the data was used to explain why IDO is considered to be immuno-suppressive, but also paradoxically linked to microbial defence (69,70). Thus, though inhibiting or knocking out IDO does not alter the immunodominance hierarchy (i.e., increasing Site II/III responses) it still provides an avenue for the control and/or enhancement of immunodominant CTL function. This

provides some rationale to investigate the use of IDO inhibitors to help combat viral infection and disease progression in hosts of varied immuno-competence status.

In addition, given the published contradictory role that IDO plays in viral infection (i.e., positive in some contexts and negative in others) it was important to utilize a variety of model systems to ensure that the results were applicable in more than one context. The above study employed several different approaches (e.g., i.p TAg, i.p IAV acute and memory, i.n IAV) to arrive at the same conclusion, which adds credence to the findings amid the often paradoxical results in different publications. Importantly, the i.n IAV system was an actual disease model, which allows for the comparison of these results with published literature in other viral infections such as dengue and HCV. In the case of dengue, although IDO was actually shown to be beneficial in combating morbidity in one study, the particular nature of dengue virus infection (e.g., the fact that it infects DCs) may perhaps explain why IDO is beneficial in this case (55).

Another interesting result was that L-kynurenine and its metabolites did not affect CTL responses to viral antigens. Most literature on the subject states that tryptophan catabolites exude immunosuppressive functions *in vivo* (32,38,42,46,47), but in the systems studied here they had no effect. In fact, the data seems to suggest that treating mice with L-kynurenine actually improves the CTL response, although given the controversial nature of this conclusion, more experimentation is needed to solidify the results beyond any reasonable doubt. In any event, it is possible that high concentrations of tryptophan metabolites actually inhibit IDO in a negative feedback loop, although no studies have ever identified such a mechanism. There is, however, precedent for such a phenomenon with TDO, which is the housekeeping enzyme in the liver, because its

function is inhibited by nicotinic acid derivatives, which are one of the downstream metabolites of tryptophan (62,63).

The fact that CHOP did not seem to play a role in the responsiveness of IDO KO T-cells and their WT counterparts is also surprising, given that this protein was used as part of a reporter system to study IDO expression in another study (39). Since CHOP expression is linked to GCN2 kinase activation, which in itself senses amino acid stress inside a cell (34), it is logical that IDO KO CTLs would exhibit lower levels of CHOP because of the absence of tryptophan degradation through IDO. However, it is possible that the level of amino acid stress in WT CTLs caused by physiological IDO expression was not enough to induce elevated CHOP levels in this system, which is why there was no difference between IDO KO and WT mice in the context of CHOP expression.

One important limitation to consider is that all experiments here were conducted in a mouse model, and more research is required in order to elucidate whether the findings are applicable to humans. In fact, a vast proportion of published literature on the subject of IDO mediated immune modulation is based on the mouse system (31,34,35,38,53,69,71). Therefore, it is crucial to consider that murine IDO differs from its human ortholog in a variety of different aspects, not the least of which is their genetic sequence. Mouse IDO (407 amino acids) and human IDO (403 amino acids) only share 62% sequence identity, which is comparably low amongst other enzymes in the L-kynurenine pathway. In addition, recombinant mouse IDO remains significantly more active at alkaline pH when compared with human IDO, and also exhibits a three-fold higher substrate efficiency ratio for L-tryptophan (72). Therefore future work will have to take these differences into account and ensure that findings using the mouse system are

also relevant to humans. This is particularly important because a Phase 1 clinical trial of the IDO inhibitor 1-MT is already underway (H.Lee Moffat Cancer Center and Research Institute: NCT00617422) in a subset of cancer patients, but the basic science behind the trial may not be perfectly sound yet.

It is also important to note that a paralog of the IDO was recently discovered and termed IDO-2 (73). The capacity of this new enzyme to degrade tryptophan differs from IDO-1, as does its tissue and cell distribution. IDO-2 is primarily expressed in the reproductive tract, liver, and kidneys, and the two IDO paralogs exhibit 42% similarity in amino acid sequence (74). In addition, IDO-2 is able to function at more alkaline pH than IDO-1 (global maximum pH 7.5 for IDO-2 and 6-6.5 for IDO-1) which is consistent with the reported pH in the tissues in which IDO-2 is expressed (i.e., seminiferous tubules pH 7.2-8.0). Furthermore, the reduced substrate activity of IDO-2 when compared with IDO-1 may be due to an amino acid mutation in the catalytic pocket of the enzyme (F227 to Y231) which makes it prone to phosphorylation or sulfation. In both humans and mice, the two IDO genes are located adjacent to each other on chromosome 8, suggesting that a gene duplication event may be responsible for the presence of either gene (29).

Importantly, the functional significance of IDO-2, especially in the realm of immune regulation, is an active field of investigation still in its infancy. The IDO KO mice used in this study were missing the IDO-1 enzyme and not its paralog, which calls into question the relevance of this newly discovered IDO-2 molecule. It is still possible however, that in the complete absence of both IDO paralogs CTL responses would be even more enhanced than in animals without IDO-1 only. For instance, it would be interesting to determine the effect of this double knockout on susceptibility to intranasal

IAV, and investigate whether it is protective, unlike IDO-1 KO. However, given the unique tissue expression profile of IDO-2, it is less likely to play a role in global immune regulation than IDO-1 and may be more important in maternal fetal tolerance instead.

Though the role of IDO mediated tryptophan metabolism in CTL immunobiology is of clinical interest and import, much work remains to be done to generate a unified model of T-cell regulation by this enzyme, especially in the realm of viral infection. Taking the differences between humans and mice into account, and also further investigating the function of IDO-2, may illuminate a previously unappreciated facet of CTL regulation.

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