IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF GENETIC VARIANTS IN THE HUMAN INDOLEAMINE 2,3-DIOXYGENASE (*INDO*) GENE

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ABSTRACT

IDENTIFICATION AND FUNCTIONAL CHARACTERIZATION OF GENETIC VARIANTS IN THE HUMAN INDOLEAMINE 2,3-DIOXYGENASE (*INDO*) GENE

Million Arefayene

Indoleamine 2,3-dioxygenase (IDO) is a rate limiting enzyme in tryptophan catabolism that has been implicated in the pathogenesis of a number of diseases. Large interindividual variability in IDO activity in the absence of stimuli and as the result of therapy induced changes has been reported. This variability has the potential to contribute to susceptibility to disease and to interindividual variability in therapeutic response.

To identify genetic variations that might contribute to interindividual variability in IDO activity, we resequenced the exons, intron/exon borders and 1.3 kb of the 5'-flanking region of the *INDO* gene in 48 African-American (AA) and 48 Caucasian-American (CA) subjects from the Coriell DNA Repository. A total of 24 *INDO* variants were identified. Seventeen of these were in exons, introns, or exon/intron boundries, while seven were within 1.3 kb upstream of the translation start site. Seventeen are novel and 7 were previously identified.

When transiently expressed in COS-7 or HEK293 cells the amino acid sequence change in $Arg^{77}His$ resulted in significant decrease in activity, and it reduced the V_{max} of IDO. The $Arg^{77}His$ variant and the 9 bp deletion resulted in nearly complete loss of enzyme activity and a lack of detectable protein expression.

The function of the $Arg^{77}His$ variant IDO was restored in a dose dependent manner by the heme analog hemin; but there was no associated increase in IDO protein. Cellular heme concentration was higher in cells transfected with the wild type and $Ala^{4}Thr$ variant constructs, but not in cells transfected with the $Arg^{77}His$ variant. The heme synthesis inhibitor, succinylacetone (SA), blocked IDO activity in cells transfected with $Arg^{77}His$.

We identified 22 putative transcription binding sites within the 1.3 kb upstream of the translation start site. Two of the SNPs were located in GATA3 and FOXC1 sites. A specific 3-SNP haplotype reduced promoter activity when transiently transfected into 2 different cell lines.

We conclude that there are naturally occurring genetic variants in the *INDO* gene which affect both expression and activity. These results make clear that interindividual variability in IDO activity at baseline or in response to therapy may be in part due to inherited genetic variability.

Chairman, David Flockhart, M.D., Ph.D.

TABLES OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

APCs	antigen presenting cells
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DCs	dendritic cells
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
ECL	enhanced chemiluminescence
E.coli	*Escherichia coli*
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HO-1	hemeoxygenase-1
HPLC	High-performance liquid chromatography
hr	hour
IDO	indoleamine 2,3-dioxygenase
*INDOL*1	indoleamine 2,3-dioxygenase - like protein 1
IFN-γ	interferon -γ
Kyn	kynurenine
LNAA	large neutral amino acids
MCS	multiple cloning site
NAD+	nicotinamide adenine dinucleotide
ng	nano gram
PBS	phosphate buffered saline
p-DMAB	paradimethylaminobenzaldehyde
QUIN	quinolinic acid
RNA	ribonucleic acid
RT	room temperature
SA	succinylacetone
SNP	single nucleotide polymorphism
TCA	trichloroacetic acid
TDO	tryptophan 2, 3 dioyxgenase
TNT-RRL	transcription and translation-rabbit reticulocyte lysate
Trp	tryptophan
TBST	tris buffered saline with tween 20
w/v	weight per volume
v/v	volume per volume
1-MT	1-methyl DL tryptophan
3-HAA	3-hydroxy anthranilic acid
5-HT	5-hydroxytryptophan
3-NT	3-nitro tyrosine
ºC	degree Celsius

CHAPTER 1

GENERAL INTRODUCTION

Indoleamine 2,3-dioxygenase (IDO) is a cytosolic enzyme that catalyzes the oxidation of tryptophan into kynurenine. Its expression and activity have demonstrated a large interindividual variability in several clinical studies. This variability includes baseline activity, as well as therapy induced changes in IDO activity. This variability might contribute to differences in the susceptibility to diseases that are influenced by the immune system, such as cancer and autoimmune diseases. The responses to several therapies may also be influenced by the level of IDO. The source of variability could be a number of possible environmental and intrinsic mechanisms. Forexample the mechanism might include exposure to naturally occurring compounds, drugs, and carcinogens; however, it is also possible that genetic variation may play an important role. The contribution of genetic variation in the *INDO* gene to phenotypic variability is the focus of these studies. A comprehensive understanding of the variability in tryptophan metabolism is important because it will likely help to predict therapeutic responses, to develop improved therapies for the subgroups of patients, and to better understand the role of IDO in disease pathogenesis.

The goals of this dissertation are to identify the genetic variants in the *INDO* gene in human and to determine their functional significance.

1.1 Background

Tryptophan (trp) is the least abundant of all essential amino acids. The major metabolic route of tryptophan in mammals is the kynurenine (kyn) pathway. A limited amount of trp is used for protein and serotonin synthesis, but in humans, over 90% of tryptophan catabolism is through the kynurenine pathway [1-3]. This pathway includes a number of other enzymes that further metabolize kynurenine (Figure 1.1). This pathway ultimately leads to the biosynthesis of nicotinamide adenine dinucleotide (NAD) (Figure 1.1).

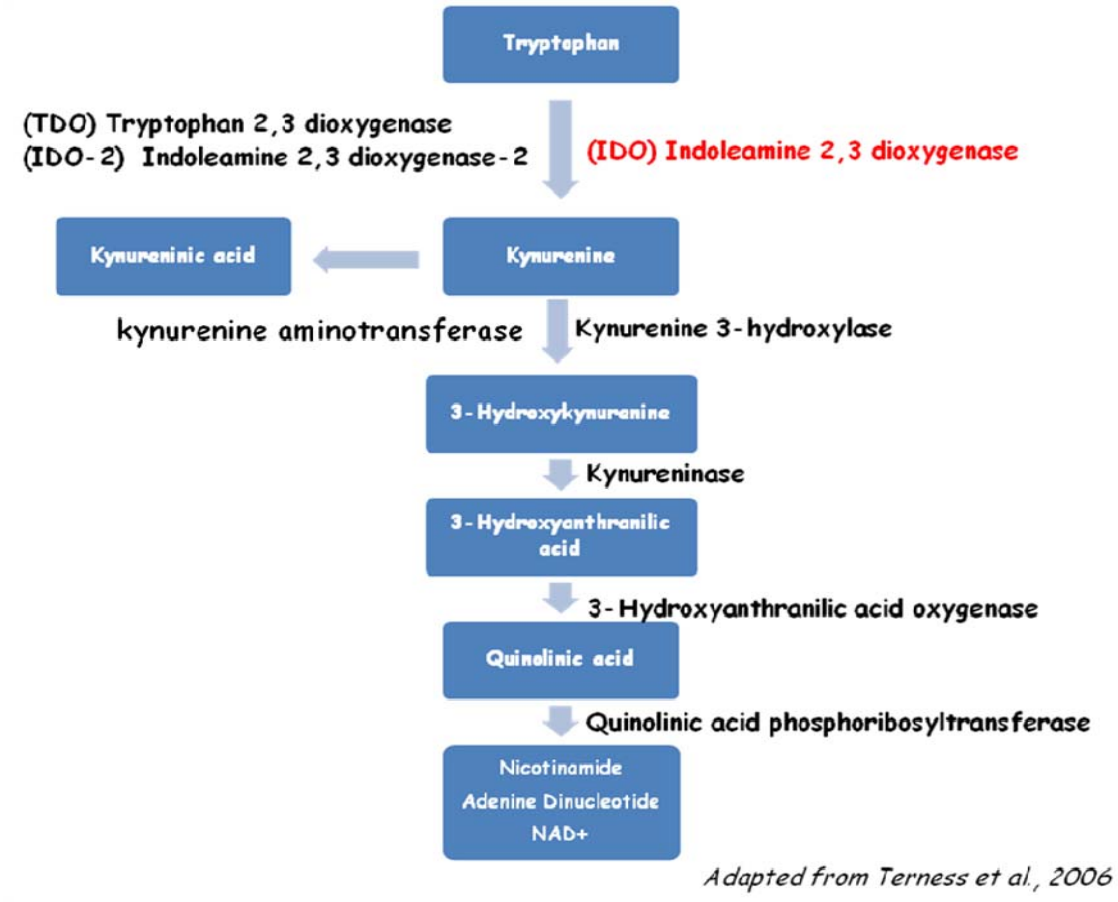

Figure 1.1 Schematic pathway of tryptophan metabolism.

There are three different enzymes that metabolize tryptophan into kynurenine:

- Tryptophan 2,3 dioxygenase (TDO)
- Indoleamine 2,3 dioxygenase (IDO)
- Indoleamine 2,3 dioxygenase like protein (*INDOL-1*) or IDO-2

Tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11) was first discovered in rat liver extracts [4, 5]. The TDO gene is located on the long arm of the human chromosome 4q31-q23 [6]. The gene has 12 exons and spans about 17.5 kb of the human genome. TDO is a glucocorticoid responsive gene [7]. There is one study which showed the presence of a glucocorticoid response element in the human TDO promoter starting at -1174[7]. While it is well known that TDO mRNA is highly expressed in the liver, its expression has also been found in pituitary gland, mammary gland, bladder, colon, lung, testis, pancreas and skin (NCBI Unigene Hs.183671).

Following the identification of TDO, in 1963 a second enzyme was also found that catalyzes the oxidative cleavage of tryptophan [8]. This non-hepatic tryptophan metabolizing enzyme was named D-tryptophan pyrrolase; later, the name was changed to indoleamine 2,3-dioxygenase (IDO). The IDO protein is encoded by the gene designated as *INDO* [9]. The *INDO* gene shares no sequence homology with TDO [10 11]; however, like TDO, IDO is also a heme-containing enzyme.

In humans, *INDO* is a single copy gene comprised of 10 exons spanning ~15 kb. It maps to chromosome 8p12-p11 [6, 9]. Indoleamine 2,3-dioxgyenase (IDO, EC 1.13.11.42) is a cytosolic heme protein which, together with the hepatic enzyme tryptophan 2, 3 dioxygenase (TDO), catalyzes the conversion of tryptophan and other

indole derivatives to kynurenines . IDO is first translated as a 45 kDa apoenzyme; subsequently, the heme prosthetic group is added to make the holoenzyme (Figure 1.2).

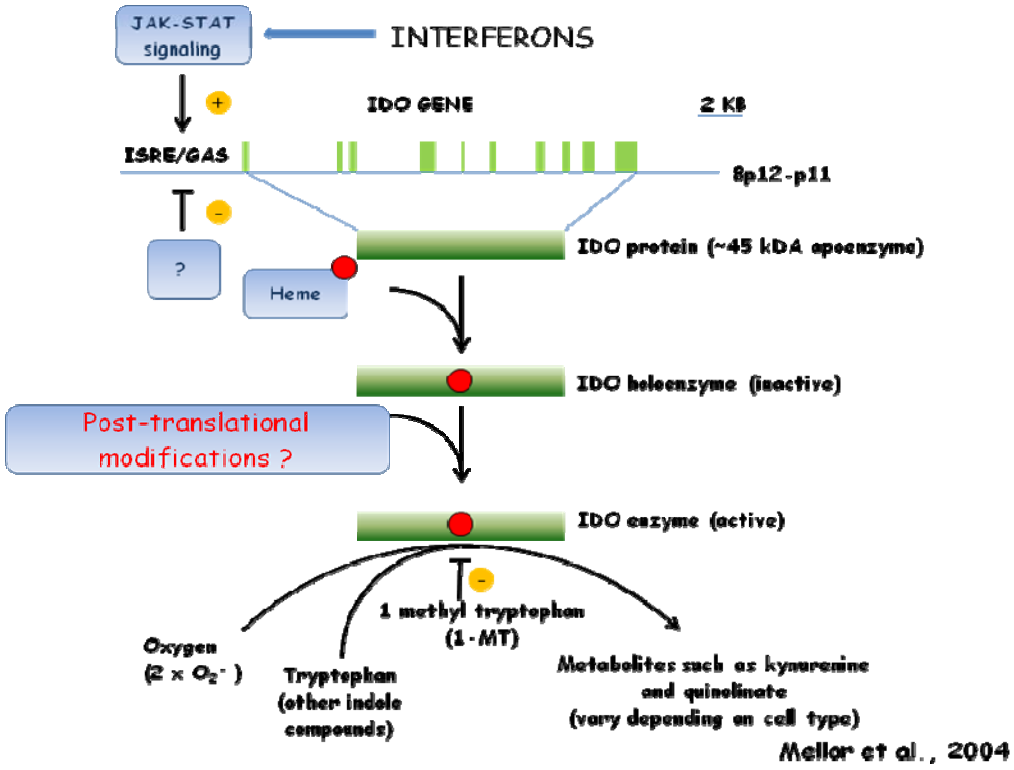

Figure 1.2 Indoleamine 2,3-dioxygenase gene structure.

IDO oxidizes the pyrrole moiety of tryptophan and other indoleamines. In some tissues, it is the initial and rate-limiting enzyme of the kynurenine pathway. It is a unique enzyme in that it can utilize superoxide anion radical ($O_2^{\bullet}$) as both a substrate and a co-factor. The latter role results from the ability of $O_2^{\bullet-}$ to reduce inactive ferric-IDO to the active ferrous form [11, 12]. Antioxidant activity may arise from IDO scavenging $O_2^{\bullet-}$, formation of the potent radical scavengers and kynurenine pathway metabolites, 3-hydroxyanthranilic acid and 3-hydroxykynurenine [13].

IDO is expressed in a variety of cell types; of particular importance are those involved in mediating key steps of an immune response . In many of these cells, IDO is known to be induced mainly by interferon-γ (IFN-γ). This induction is usually a result of infectious agents or chronic inflammatory disorders [12, 14]. Normal induction of IDO, and consequently the metabolism of tryptophan along the kynurenine pathway, is implicated in a variety of physiological processes. Abnormal induction of IDO is implicated in several pathophysiological processes including anti-microbial, anti-tumor defense, neuropathology, immunoregulation and antioxidant activity [14]. IDO is constitutively expressed in healthy tissues with large areas of mucosal surfaces (e.g. lungs, gut, and fetal-maternal unit during pregnancy), in the epididymis, and in the thymus [15]. In healthy tissues that are continuously exposed to antigens, such as mucosal surfaces of the gut, lung, and uterus during pregnancy, IDO expression is normally high [16].

The first suggestion that IDO expression regulates T cell responses came from studies on human macrophages that suppressed T cell proliferation [17]. Two mechanisms have been proposed to explain the inhibitory effect of IDO on T-cell function. First, IDO activity creates a tryptophan depleted microenvironment limiting the proliferation of T-cells[17]. This study prompted further work that revealed a critical role for IDO in preventing fetal rejection by maternal T-cells during pregnancy [18]. Second, downstream tryptophan metabolites have inhibitory effects on T-cells proliferation and function[19]. Today, several studies support the hypothesis that cells expressing IDO regulate T cell-mediated immune responses in inflammatory diseases, transplant rejection, pregnancy, and cancer [17, 20-22].

Recent studies have revealed a critical role of IDO in the regulation of normal immune function; specifically IDO appears to have a role in the local control of tolerance vs. rejection of tissues with foreign antigens [14]. When T-cells migrate into tissues with low tryptophan concentrations and high concentrations of tryptophan degradation products which is caused by IDO, the T-cells undergo growth arrest and/or apoptosis [23]. This mechanism results in the localized suppression of T-cell responses and consequently leads to immune tolerance [24-26].

A third enzyme, which also metabolizes tryptophan has recently been discovered [27, 28]. Because of the similarity of the gene products and their activities, the gene has been named indoleamine 2,3-dioxygenase -like protein (*INDOL1).* The *INDOL1*gene maps to chromosome 8p11.21. It has 11 exons. When the predicted protein is aligned with *INDO,* it has 43% amino acid identity [28]. The *INDOL1* gene is located 3' to the *INDO* gene and separated by 6.5 kb [27]. *INDO* and *INDOL1*to have arisen by gene duplication before the origin of the tetrapods. In the mice, the expression of *INDOL1* is highest in the kidney, followed by epididymis and liver [27, 29]. The identification of *INDOL1* may help to explain the regulation of the diversity of physiological and pathophysiological processes in which the kynurenine pathway is involved [28].

1.1.2 Indoleamine 2,3-dioxygenase and disease

Degradation of the essential amino acid tryptophan is now recognized to play an important role in immunity and in a growing list of pathophysiological conditions.

This rate limiting enzyme in tryptophan catabolism appears to have an active role in the pathogenesis of a number of diseases [30-43]. Several of them are described in the following sections.

1.1.2.1 Immune tolerance and cancer

The first clue to a role for IDO in immune regulation was the discovery that expression of the *INDO* gene is strongly induced at the transcription level by the macrophage activating cytokine interferon-γ [44-47]. The connection between elevated urinary tryptophan metabolites and bladder cancer was first reported in the 1950's [48]. This discovery prompted an interpretation of long-standing clinical observations indicating that tryptophan metabolites are elevated in the urine of cancer patients [49-52]. Consistent with this idea, it was reported later that IDO activity was elevated in lung tumors [53]. Since then, elevated levels of IDO generated tryptophan metabolites have been associated with a number of malignancies [41, 54]. This phenomenon was initially thought to be a tumoricidal consequence of IFN-γ, which stimulates expression of IDO in cells [55].

Several mechanisms of tumor-induced immunosuppression have been proposed, and each is likely to contribute to the overall suppression [56]. Using IDO protection mechanism during normal pregnancy as a starting point, a number of researchers hypothesized that cancer cells may use the same mechanisms, to evade immunosuppression. The mechanisms include impaired antigen presentation [57, 58], expression of death ligands [59] and secretion of soluble inhibitory factors by the tumors [60]. This suppression appears to be mediated through effects on both T-cells

and antigen presenting cells [61]. The first evidence was offered in 2002 that the specific IDO inhibitor 1-methyl tryptophan (1-MT) could partly retard the growth of mouse melanoma cells engrafted onto a syngenic host [62]. It is increasingly clear that tumors induce tolerance to their own antigens. Tumors can also evade local immune destruction despite the systemic presence of tumor reactive T-cells [63].

IDO mediated tryptophan catabolism may participate in immune tolerance in two ways. First, it may induce tolerance by affecting the ability of host APCs to present tumor antigens. Second, it may directly suppress immune effector cells in the tumor microenvironment [64-66]. This strategy appear to utilize a mechanism that normally exists to prevent maternal immune response to paternal fetal antigens [67].

The interaction between developing tumors and the immune system is complex and dynamic. *In vitro*, T-cells and antigen presenting cells are capable of mounting tumor-specific responses if primed with tumor lysates [68, 69]. *In vivo*, patients often have tumor-reactive T-cells. Cancer vaccines using tumor specific antigens are also capable of generating specific responses [70]. However, despite this competency of the immune system and the existence of antigens on tumor cells, there is still a lack of effective immune response in untreated patients and even in many receiving cancer vaccines [71].

The recruitment of IDO expressing cells to tumors and draining lymph nodes may also be a mechanism of immunosuppression by tumors. In one study, 25 of 36 lymph nodes draining human melanoma tumors contained elevated numbers of IDO expressing cells [72]. In another study, an abnormal accumulation of IDO expressing

cells was observed in sentinel lymph nodes of melanoma patients, even in the absence of detectable tumor cells [73].

Macrophages frequently express high levels of IDO and have the ability to suppress T-cell proliferation by the rapid degradation of trp [72, 74]. In human breast cancers, high levels of tumor-associated macrophages in the tumors correlate with a poor prognosis [75]. Part of this effect is thought to be caused by the secretion of various tumor stimulating growth factors [76, 77]. However, the expression of IDO and metabolism of tryptophan to suppress the T-cell response may be another mechanism by which macrophages contribute to breast cancer progression and a poor prognosis. The cancer cells themselves may also be a source of IDO expression. IDO expression was detected in 24 of 25 types of cancers cells [78]. IDO expression has been shown to be induced by IFN-γ in several types of cancer cells, such as breast, ascites tumors, colon cancer and melanoma cancer cells [79]. In inflammation, IFN-γ is secreted at the site of inflammation to help induce a T-cell response against the infected and/or foreign cells. However, in tumors, the IFN-γ induced IDO expression in cancer cells appears to provide a mechanism for the cancer cells to escape the T-cell mediated elimination. The failure of the immune system to respond protectively to the tumor antigens contributes to the progression of cancer to a life-threatening disease [80].

1.1.2.2 Organ transplant

IDO has been shown to play an important role in transplantation immunology [81]. One of the main goals in transplantation immunology is to achieve immune toleration against allo-antigens; however immunity against pathogens needs to be

preserved. IDO mediated tryptophan metabolism appears to be a induce tolerance in organ transplantation [82]. Potent IDO mediated immunoregulation acts locally in the site of antigens. Therefore, locally manipulating IDO mediated immune regulation appears to bepotentailly important therapeutic tool. While experimental systems including *in vitro* and animal studies support the role for IDO mediating transplantation tolerance, its relevance to human transplantation immunology remain to be defined [83]. To date, evidence to support a therapeutic role of IDO for tolerance induction in clinical organ transplantation is limited [83]. *In vivo* studies showed that systemic blockade of IDO with a competitive IDO inhibitor 1-MT after lung transplantation resulted in spontaneous proliferation of T-cells in lung and caused pulmonary inflammation. It has been shown that, over expression of IDO in lung transplants abrogates acute allograft rejection [84]. Survival of transplanted pancreatic islet cells [85] and cardiac allograft [86] is also prolonged by overexpression of IDO.

In conclusion, a better understanding of the implications of IDO activity on different cell types will allow a more focused approach to determine if IDO has a role in generating transplantation tolerance.

1.1.2.3 Depression

Over the last several decades, recombinant preparations of the interferon-α (IFN-α) have played an increasingly important role in the treatment of a number of medical conditions. Two of the most significant are chronic viral hepatitis and several malignancies. IFN-α binds to specific membrane bound receptors and stimulates the expression of several genes. The products of these genes, either individually or

coordinately, mediate IFN-α immunoregulatory activity. In addition to their therapeutic effects, IFN-α also frequently causes dose-dependent neuropsychiatric adverse effects. For example, IFN-α treatment results in major depressive disorders in 30–50% of patients [87, 88]. In addition to their effects on quality of life, these adverse effects also increase the risk of treatment discontinuation; consequently, they can increase the risk of poor treatment outcome [89-92]. Therefore, it is of paramount importance to better understand why some patients are at increased risk for depression during treatment with IFN-α.

IDO overexpression would be expected to reduce the synthesis of brain serotonin (5-HT), which may play a role in the development of depressive symptoms. In fact, IFN-α administration has been shown to suppress 5-HT concentration in brain and serum of patients [92-94]. The link between increased IDO activity and tryptophan depletion has been well established [95]. The results from these studies suggested that reduced availability of tryptophan in patients undergoing interferon therapy could be related to the depression [87, 96]. It is also known that IFN-α enhances IDO gene expression and activity, presumably indirectly through a 15 kD protein which is a product of IFN-α stimulated monocytes and lymphocytes [97]. It is therefore possible that the neuropsychiatric symptoms observed in some patients could be because of several pathophysiological factors. Indeed, many studies have documented different rates of depression in patients with a wide range of medical disorders. They include the conditions against which IFN-α is most typically directed, such as cancer and viral infections [93]. The different rates of depression observed after the IFN-α

treatment could be the reflection of the variability in the IFN-α mediated activation of IDO [98].

In summary, to investigate the therapeutic use of IDO inhibitors would be of great value, given that IDO appearse intimately involved in immuneregulation in transplantation. Genetic variations in one or more of the genes involved in IDO regulation, or other genes that might affect tryptophan metabolism pathway, could account for variable susceptibility to different diseases. These variations could contribute to the interindividual difference in tumor metastasis, immune tolerance development and variable interferon responses.

CHAPTER 2

GENETIC VARIABILITY

2.1 What is a genetic polymorphism?

Genetic polymorphisms occur in multiple forms; they include single nucleotide polymorphisms (SNPs), complete gene deletions, gene duplications, and genetic translocations where portions of similar genes are combined creating a new gene hybrid. Polymorphisms are responsible for the many phenotypic differences between people. Some genetic changes are very rare while others are common in populations. By far the most frequent genetic variations in the human population are SNPs. SNPs involve a single nucleotide change at one specific position. Each of these changes in the gene sequence introduces a variant form of the gene into the population gene pool. Each different sequence at a locus is designated as an allele. Some standards suggest that a gene variant can be classified as an allele when the frequency of the variation is $\geq 1\%$ [99]. Such changes are common enough to be considered as normal variations in the DNA.

SNPs can result in a variety of changes in the expressed protein function. The specific effect depends upon where the polymorphism occurs in the overall gene structure. Single base substitutions are classified into silent substitutions (synonymous; sSNPs) and missense (nonsynonymous; nsSNPs). Synonymus variations did not change the coding sequence for example, AG<u>A</u> →AG<u>G</u>, both encode the amino acid arginine. Nonsynonymous is a base substitution that alters the

codon specifying a different amino acid, e.g. AGA→GGA, would change an amino acid from an arginine to a glycine.

A major interest in human genetics is to determine whether a genetic polymorphism in a gene affects its protein product and consequently, impacts the carrier's health. Amino acid substitutions currently account for approximately half of the known gene lesions responsible for human inherited disease [100]. Therefore, it is important to determine whether genetic variants that affect the amino acid sequence of a gene product can alter protein function and contribute to disease. Genetic variations in the coding and regulatory regions are believed to have the highest impact on phenotype. Variations within the intron regions are typically silent; however, if the variant is in nucleotides that are critical for RNA splicing such variants can also lead to altered protein function.

2.2 The potential effect of genetic variation in protein quality control, degradation and aggressome formation.

Protein quality control is a basic cellular phenomenon through which abnormal proteins become eliminated. All cells are equipped with a proteolytic apparatus that eliminates damaged, misfolded and incorrectly assembled proteins [101-103]. Abnormal proteins can occur during cellular stress, or can be due to a variant that could lead to a none functional protein [102-105]. Several mechanisms could potentially explain decreased protein levels as a result of inherited alterations in encoded amino acid sequence. For example, some SNPs cause altered amino acid sequence which resulted accelerated degradation of the variant protein [106, 107]. Aggressomes are

manifestations of reduced cellular aggregate degradation in the endoplasmic reticulum and cytoplasmic compartments, respectively, and are often associated with disease [108, 109] (Figure 2.1). When the production of aggregation-prone proteins exceeds the cell's (or organelle's) capacity to eliminate them cellular aggregates form [102]. Cellular pathways that resolve these aggregates exist, but appear to have limited capacities. The main engine of cytoplasmic proteolysis, the 26S proteasome, requires that substrates be unfolded to gain access to the active site; consequently, it is relatively ineffective at degrading aggregated proteins[102].

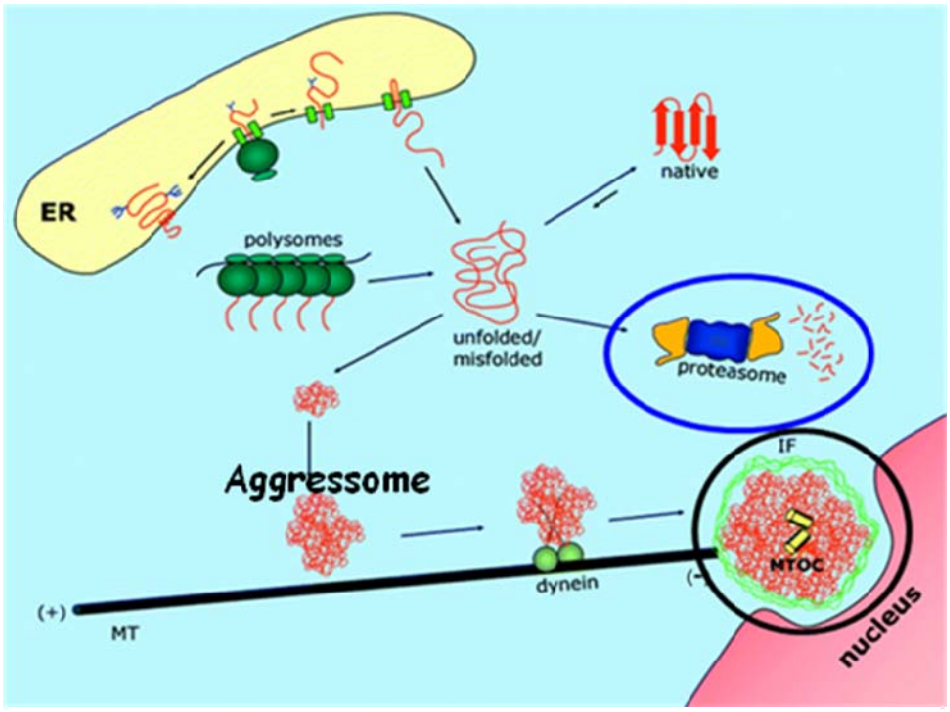

Figure 2.1 Protein degradation and aggressome formation.

2.3 Indoleamine 2,3-dioxygenase and pharmacogenetics

The field of pharmacogenetics is concerned with the ability of genetic variations (polymorphisms) to alter drug concentrations and responses. Genetic differences in the drug metabolizing enzymes cause the same dose of drug to yield varying systemic concentrations in different individuals. Variations in systemic drug concentrations help to explain why some patients respond well to drugs, but some do not. A drug might be toxic to one individual and not another. The medical community has been aware for 50 years of these different responses and of the activities of a limited number of enzymes that are involved in the metabolism of endogenous and exogenous compounds. Examples include the butyrylcholinesterase genetic polymorphism that was initially recognized as a result of decreased metabolism and the prolonged effect of the muscle relaxant succinylcholine [110]. Another early example was inherited variation in *N*-acetylation catalyzed by *N*-acetyltransferase 2 (NAT2), a phase II drug-metabolizing enzyme that catalyzes the biotransformation of isoniazid (the antituberculosis agent) [111] and hydralazine (the antihypertensive agent) [112]. Included among pharmacogenetically relevant proteins that have been studied with regard to the functional effects of nsSNPs is another phase II drug-metabolizing enzyme thiopurine *S*-methyltransferase (TPMT)[113].

Genetic polymorphisms in a number of genes have been studied in an effort to clarify the disparity in drug response among individuals. The body of literature addressing polymorphisms that influences IDO expression and activity is also increased in the past years.

2.4 Genetic polymorphism and tryptophan metabolism

Tryptophan metabolism occurs via both hepatic (TDO) and majority extra-hepatic (IDO and IDO2 or *INDOL1*) pathways. Although these pathways share many enzymes, the first and rate-limiting step in each pathway is carried out by three different enzymes: tryptophan 2,3-dioxygenase (TDO), indoleamine 2,3-dioxygenase (*INDO*) and indoleamine 2,3-dioxygenase like protein IDO2 (*INDOL1*). Over the course of many years, extensive research has led to better understanding of the important biological functions of these enzymes. One of the tasks that now lie ahead is linking the genetic variations in these enzymes with variable human responses in different disease states.

2.4.1 Polymorphisms in TDO

There is a single report of a TDO genetic polymorphism in the coding region. This nsSNP, (A238T; rs11541501, NCBI dbSNP leads to an amino acid change $Gln^{58}His$. As a result of a nucleotide change is on exon 3 (NM_005651.1). Although this genetic polymorphism in TDO was submitted to the NCBI database in 2003, there is still no functional or population frequency data available. There are four TDO promoter variants reported. One of them (rs3755910) has been linked with autism [114].

Association studies of TDO variants also revealed a significant association of two SNPs in intron 6 with Tourette syndrome, attention deficit hyperactivity disorder, and drug dependence [115, 116].

2.4.2 Indoleamine 2,3-dioxygenase genetic polymorphism

A total of 43 *INDO* genetic variants have been reported in the NCBI SNP database. Six of them are coding variants and are listed in Table 2.1. There are 26 intronic and eleven 5'flanking region SNPs.

Table 2.1 Coding variants of *INDO* gene currently described in the SNP data base.

SNP ref ID	Position	Nucleotide change	@Alteration#
rs35059413	Exon 1	G > A	$Ala^{4}Thr$
rs35099072	Exon 3	G > A	$Arg^{77}His$
rs1245877	Exon 4	G > A	$Arg^{105}Lys$
rs4463407	Exon 4	T > G	$Ile^{107}Ser$
rs12545878	Exon 4	G > A	$Lys^{116}Lys$
rs34155785	Exon 6	G insertion	Codon shift

@ The SNP ref ID are assigned by NCBI SNP database at: http://www.ncbi.nlm.nih.gov/sites/entrez , # the amino acid position is relative to the 403 amino acids in the NCBI gene bank protein reference: Version NP_002155

2.4.3 Indoleamine 2, 3 dioxygenase-like protein (*INDOL1)* genetic polymorphism

Based on the Ensemble Human Gene variation Report for *INDOL1* released December 2006, (ENSG00000188676) there are 7 coding variations. These variations include 3 nonsynonymous SNPs. Two of the nonsynonumus SNPs reduced enzymatic activity ~90% in T-REX cells. The third variant T-A SNP affecting Y^{359} generated a premature stop codon ($Y^{359}X$), which completely abolished IDO2 activity [29].

Interestingly, these SNPs were commonly found in human genomic DNAs in public databases. The C-T SNP has the highest prevalence in individuals of European descent. The T-A SNP is the highest in individuals of Asian descent. Neither SNP was as frequent in individuals of African descent. About 50% of individuals of European or Asian descent and 25% of individuals of African descent may lack functional IDO2 alleles.

Chapter 3

hypothesis and specific aims

Central hypothesis

Genetic polymorphisms in the coding and promoter region of the *INDO* gene cause alterations in IDO expression and/or activity.

Specific Aims

Aim 1: To identify IDO genetic polymorphisms in the coding region that affect enzyme expression and/or activity and to evaluate the mechanisms by which these variants influence activity.

The *working hypothesis* for this aim is that the genetic variations in the IDO gene cause functional changes in IDO expression and/or activity. Since IDO plays a key role in the development of tolerance, we have hypothesized that genetic variation in the IDO gene may be at least partly responsible for the variable IDO expression.

The *Rationale* for this research is that a better understanding of the interindividual variability in IDO activity would likely help understand tumor development and variability in responses to cytokine therapy, partially with interferon gamma. Once this information is available, it will be possible to rationally design more effective treatment strategies that incur a minimum of adverse drug effects and allow better compliance to cytokine therapy. Genetic variation of *INDO* gene may also be valuable in interperating the clinical responses to IDO inhibiting drugs.

Aim 2: To identify genetic variants in the IDO promoter and to assess their functional significance.

Working hypothesis: Genetic variations in the IDO promoter cause functional changes in IDO expression.

RATIONALE: The evidence vailable shows a large variability in IDO expression and activity in tumors and therapy interferon –gamma induced IDO expression. Since this variability may be due to *INDO* promoter variants, we assessed genetic variants in the 5'-flanking region of IDO promoter using a luciferase reporter gene. This research will provide the foundation for future clinical studies to determine the role of *INDO* promoter variants with responses to cytokine therapy. The result from this study could help to explain interindividual variation of the therapeutic effectiveness and toxicity of drugs due to altered tryptophan metabolism.

Chapter 4

Present study Methods

4.1 General study plan

Although some genetic variations in the indoleamine 2,3-dioxygenase (*INDO*) gene have been identified, no systematic study that identifies and describes the functional significance of naturally occurring variants has been performed.

In the present study, we have applied a resequencing strategy to identify common sequence variations in the indoleamine 2,3-dioxygenase gene that might cause alterations in IDO enzyme expression and/or activity. This work was followed by characterization of the functional significance of the variants *in vitro*. A schematic "road map" of our IDO pharmacogenetic studies is illustrated in (Figure 4.1).

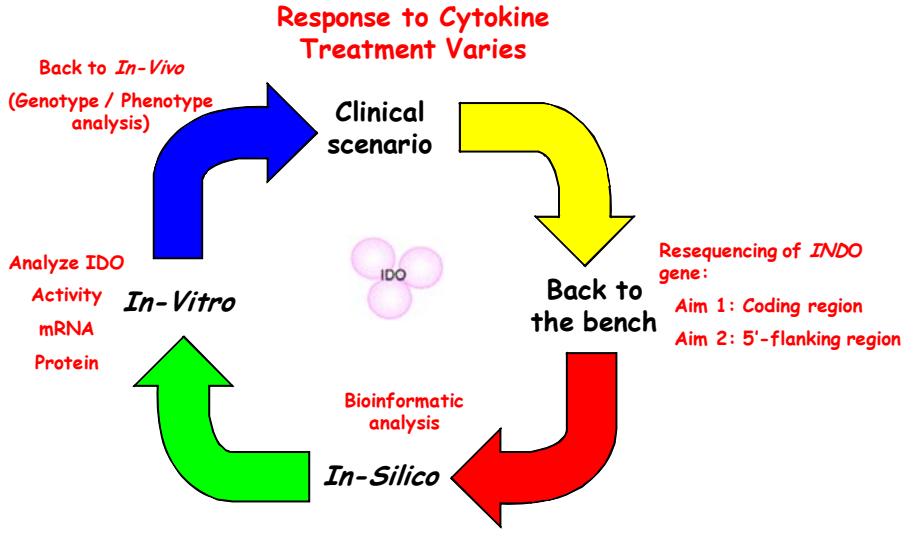

Figure 4.1 The roadmap for the study of indoleamine 2,3-dioxygenase (*INDO*) gene pharmacogentics. The schematic diagram of the experimental plan consists of resequencing of coding and 5'-flnking region, *in-silico* and *in-vitro* analysis of *INDO* gentic variants.

4.2 Specific Aim 1: To identify indoleamine 2,3-dioxygenase genetic polymorphisms in the coding region that affect enzyme expression and/or activity and to evaluate the mechanisms by which these variants influence activity.

Several studies have observed variable tryptophan concentrations in cancer survivors and found an association with patient's quality of life [117]. A number of recent studies showed the correlation between high IDO expression and poor prognosis in ovarian, lung and colorectal cancers [34, 118-120]. Other studies have also reported variable IDO expression in various human cancer tissues [78, 119, 121]. Serum tryptophan concentration was lower and variable in patients with gynecological cancer [120] , suggesting the presence of variable tumormediated IDO activity in these patients. Despite a number of studies indicating variable expression of IDO, the causes are not fully understood. The main goal of *specific aim 1* is to identify and evaluate the mechanism by which genetic polymorphisms in the coding region affect IDO activity.

The complete IDO genetic polymorphism study consists of resequencing of the coding region, sequence data analysis, *in vitro* transient expression, IDO activity assessment and understand the mechanism of loss of IDO activity. A flow chart illustrating the experimental approach is shown in (Figure 4.2). The components of the work flow are discussed in detail in each section below.

4.2.1 Experimental design

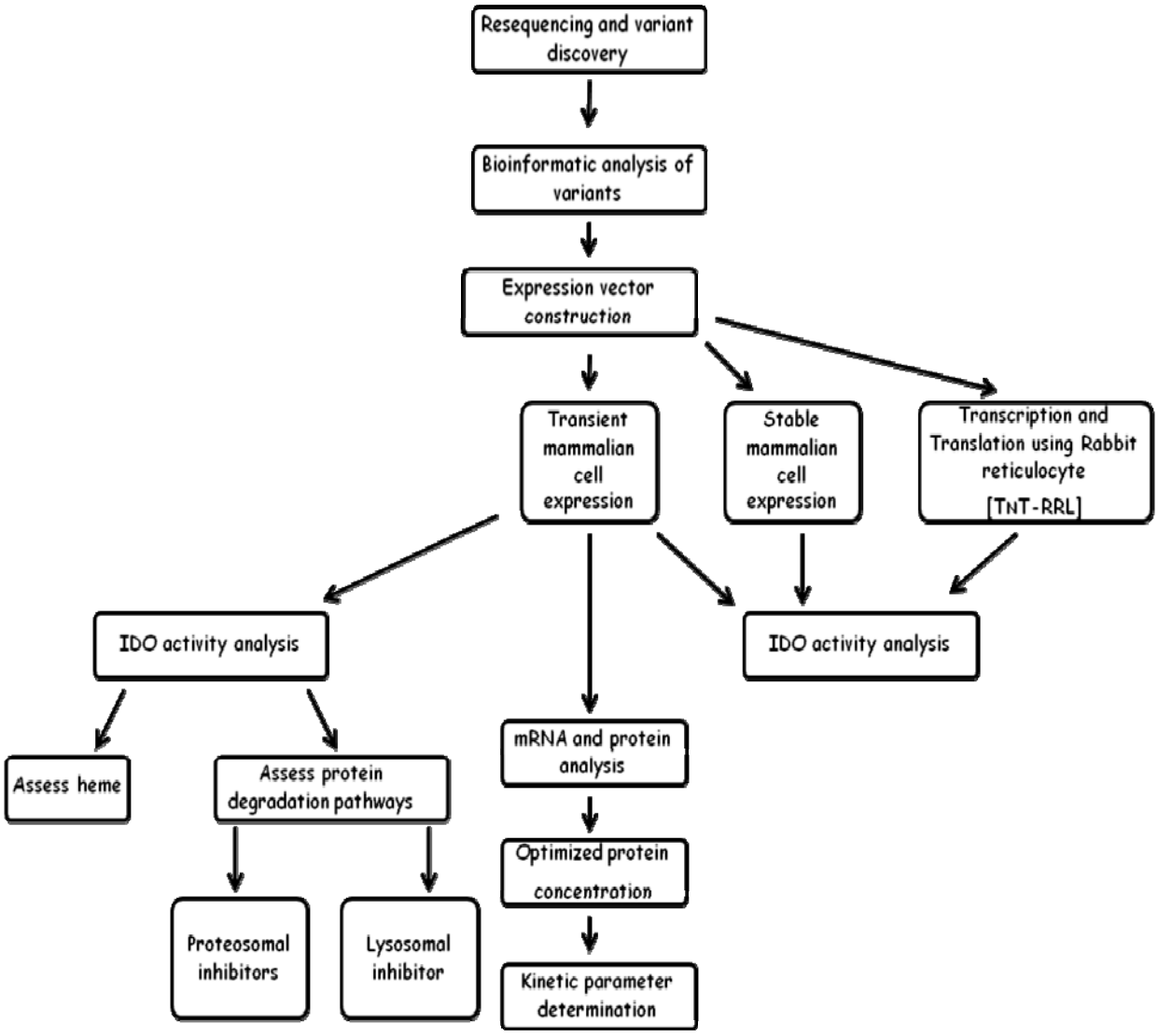

Figure 4.2 Flow chart used in the study of IDO genetic polymorphisms in the *INDO* coding region.

4.2.2 Materials and Methods

Table 4.1 Cell lines used in this study.

Cell Lines	Origin and Characteristics	Base line IDO Activity#
COS-7	Green monkey kidney cell	Undetectable
Ls174T	Human colorectal adenocarcinoma	High
A549	Human lung cancer cell	High
HEK293	Human embryonic kidney cell	Undetectable
SkBr3	Breast cancer	High

IDO activity is measured based on the formation of kynurenine in the culture media after growing cells for 24 hr (if not mentioned).

Table 4.2 Chemical and drugs used in the study.

Drug name	Manufacturer	Catalog #
Hemin	Fluka	WA11440
MG132	Sigma	C2211
Lactosyctin	Sigma	L7035
Trypan Blue Stain	Lonza	17-642E
G418	Sigma	A1720
Chloroquine	Sigma	C6628
Succinylacetone	Sigma	D1415
Interferon-γ	Roche	11 040596001
L-tryptophan	Sigma	T8941
3-nitro tyrosine	Sigma	N7389
4-Dimethylamine-benzaldehyde	ACROS	100-10-7
Kynurenine	Sigma	K8625
D/L-Tryptophan	Sigma	T7425
DMSO	Sigma	D2438
Ethanol	Sigma	E7023

4.2.3 Resequencing/Identification of variants in *INDO* gene coding region

To achieve this aim, we resequenced the exons and intron/exon boarders of the *INDO* gene using DNA samples from 48 African American and 48 Caucasian subjects obtained from the Coriell DNA Repository (Camden, NJ). These samples (Sample sets HD100 AA and HD100 CA) were diversity panels deposited by the NIGMS, National Institute of Health. The samples had been anonymized before deposit, and all subjects had provided written informed consent for the use of their DNA for research purposes.

We used the human *INDO* gene consensus sequence designated as *Homo sapiens* chromosome 8 genomic contig accession number X_17668. Resequencing was completed using the high throughput sequencing services of Polymorphic DNA Technologies, Inc (www.polymorphicdna.com). Primers used for the sequencing of *INDO* coding region are listed in Table 4.3.

Table 4.3 Primers used to resequence coding region. BST- Boost, NST-Nested

Sequence description	Sequence	Amplicon size (bp)
Exon 1_1		
BST5'-	CGGCCACCTGTTTTCATAGT	385
BST3'-	TTGGCCAGGTAAGGAATGAG	
NST5'-	AGGCCTTTCTGGCTTCCTAT	
NST3'-	TTACCTGTGGATTTGGCAGA	
Exon 1_2		
BST5'	TGAAACCATTCCAAAAGTGG	399
BST3'-	CACCACTCTCCGTATCCATGT	
NST5'-	AATTTCTCACTGCCCCTGTGAGATGA	
NST3'-	CTCCATTTCTAGGGAGA	
Exon 2-3_1		
BST5'-	GGTGGAACACAGAGAGGGTCT	434
BST3'-	CCACACATATGCCATGGTGA	
NST5'-	TGACAAGACAGAGGCTGGTG	
NST3'-	TCCCAGAACTAGACGTGCAA	

Exon 2-3_2		
BST5'-	CATTGCTAAACATCTGCCTG	416
BST3'-	GGAGGCCCATGTGTGTTTTA	
NST5'-	GCCAGCTTCGAGAAAGAGTT	
NST3'-	TCAAGCCAGGGCATATTTTC	
Exon 4		
BST5'-	AAGGCCGGAGGATCAC	458
BST3'-	GCAGTCCAAGAAACCGTACC	
NST5'-	TGGTGGCAGGTGCCTATAAT	
NST3'-	CGAGGAACTGAGAGGAACCA	
Exon 5		
BST5'-	CCGGCCCTAGTTCTGAGTT	499
BST3'-	TCATAGTTCCAAAGCCAGATTTT	
NST5'-	CTTGGACAATGGGCCTAAAA	
NST3'-	TACAGAGGTTTCAAGTTGGATTT	
Exon 6		
BST5'	CTGAAGTGGGCGGATCAC	472
BST3'-	GCTTGGAACTCTGGCTTCAC	
NST5'-	GTGGTGGGCACCTGTAGTC	
NST3'-	CCACATAGAAAGTGGAATGGTTT	
Exon 7		
BST5'-	AACAGTTTACTTGTTACGTCAGTAAGC	410
BST3'-	TCCTGAGCAGATTTGGCATA	
NST5'-	TTCCTGGAAGAGGGTAGGAAA	
NST3'-	TCCCCCACTCCAGTACATTC	
Exon 8		
BST5'-	TTATGCCAAATCTGCTCAGG	426
BST3'-	CTGGTTCACAAGGAACCTGTC	
NST5'-	GGCAAGGGGGTATGAAAAA	
NST3'-	CCAAATCCCTACTTGCCTGA	
Exon 9		
BST5'-	TGAGTTTAGCTCATTCAGTTTTCA	441
BST3'-	CCTTCCTTCAAAAGGGATTTC	
NST5'-	GCCAAACATTAGCCTTCAAA	
NST3'-	TCAGTGCCTCCAGTTCCTTT	
Exon 9_2		
BST5'-	TGCCTTTCTCTCCTGGATTG	458
BST3'-	TTACCTTTTGTTGTTTTCCTACAA	
NST5'-	CCACCAGCTCACAGGAACTT	
NST3'-	TGCACAGGTATTTTGAGGTCTTT	
Exon 9_3		
BST5'-	GACCCTTCAAAACTGGAAGC	450
BST3'-	CCAGGTTCACGCCATTCT	
NST5'-	AAAGGAACTGGAGGCACTGA	
NST3'-	CCGTGTTAGCCAAGATGGTC	

For both Boost and Nested PCR the following cycle conditions were used : 94ºC for 4 min, 94ºC for 0:20 min, 55ºC for 0:25 min, 72ºC for 1:00 min (39 cycles) and final 72ºC elongation for 7 min and hold at 6ºC.

4.2.4 Identification of variants in the *INDO* gene and bioinformatics analysis

4.2.4.1 *Vector NTI Advance*

One of the most useful programs for basic DNA sequence analysis and for laboratory maintenance of plasmid constructs is Vector NTI™. Vector NTI Advance is the most highly integrated, multifunctional desktop sequence analysis. Vector NTI Advance contains a comprehensive set of data analysis and management tools implemented across five application modules. All modules share an information-rich graphical user interface that makes sequence analysis both simple and intuitive. The five modules are listed below:

Vector NTI™: mapping, analysis, annotation, illustration and management

ContigExpress™: DNA sequence assembly

GenomBench™: analysis and annotation of reference genomic DNA sequences

BioAnnotator™: functional annotation of proteins and DNAs

AlignX™: multiple sequence alignment of proteins and DNAs

The sequencing was performed in both directions (using the primers in Table 4.3). The sequence information that was collected from the 48 African Americans and 48 Caucasians was available in a chromatogram format *i.e.* .ab1 files and .seq files.

Contig Express module of Vector NTI Advance was used for variant identification. Contig Express is a DNA assembly application for building contigs from individual overlapping sequence runs. Once assembled, a contig can be edited, with linked chromatograms in full view, and an edit history is maintained allowing the option to undo any change made to the contig in the assembly process at any time. The input *INDO* data from the commercial sequencing service was the sequencing chromatograms and the summaries excel files.

Using the *Contig Express*™ module, a new assembly project was created for the combination of DNA sequences of samples of the African American or Caucasian population. This was done by adding fragments in the form of .ab1 files for the above mentioned populations. The wild type sequence of each exon is used as a reference X_17668; this sequence was obtained from ENSEMBL in the form of a text file. After selecting sequence files in both directions, their fragmented ends were trimmed as it includes noisy data with no accurate peaks. Then all these fragments were assembled into one contig to identify variants. The software shows the sequence variations in the form of '+' or 'N' sign. Some variations may be due to artifacts or missing nucleotides. So, all variation marked chromatograms were manually checked to verify the true variations. If they are real variations, they are further analyzed to determine whether they fall in the intron or exon part of the sequence. To exclude PCR induced artifacts, independent amplifications were performed for variants that were observed only once or for any amplicon that had an ambiguous chromatogram.

Once the variant identification process was completed, a subsequent bioinformatics assessment was done for further analysis of the *INDO* variants. One goal of functional genomics is to enhance our ability to predict the functional consequence of naturally occurring polymorphisms. It was shown in a recent study that the impact of amino acid allelic variants on protein structure/function can be reliably predicted via analysis of multiple sequence alignments and protein 3D-structures [122]. The need to obtain functional information for the increasing number of new sequences and structures pushed the development of several methods for the prediction of functional consequences of variants. Depending on the information available for the target protein (example sequence alone, 3D structure, sequence homologous and crystal structure), different sets of methods can be used. Although these methods are not perfect, they are helpful since it is time consuming and difficult to experimentally obtain functional data for the overwhelming stream of new SNPs.

Bioinformatic methods can help prioritize a SNP for *wet* lab functional studies; for example, it can prioritize a set of candidate SNPs/variants to study, rather than blindly trying all SNPs found in the sequence. A combination of both experimental and *in-silico*, approaches can help interpret the functional impact of a set of SNPs. To achieve of this goal for the *INDO* SNPs, we used three different complementary bioinfromatic approaches. They are position specific phyelogenetic information for multiple sequence alignments CLUSTAL W (1.83), from prediction software's *PolyPhen* (*Poly*morphism *Phen*otyping) and *SIFT* (*S*orting *I*ntolerant *F*rom *T*olerant). The properties for each of the 3 are computed based on amino acid characteristics.

4.2.4.2 CLUSTAL W (1.83) Multiple Sequence Alignments

The simultaneous multiple sequence alignments of many nucleotide or amino acid sequence is a key tool in sequence comparison. Multiple alignments are used to find patterns to characterize homology between sequences. It is now one of the most widely used bioinformatics analyses. Conservation was assessed by multiple sequence analysis using CLUSTAL W. The input was the *INDO* sequences from multiple species found by FASTA search in the uniport database. These are Dog (XP_532793), Mouse (NP_032350), Rat (NP_076463) and Cow (XP_582707).

4.2.4.3 *PolyPhen* (*Poly*morphism *Phen*otyping)

PolyPhen (*Poly*morphism *Phen*otyping) is an automatic tool for prediction of the possible impact of an amino acid substitution on the structural and functional properties of a protein. PolyPhen (http://www.bork.embl-heidelberg.de/polyphen) combines a conservation score with additional properties (example physicochemical differences and structural features of the polymorphic variants) in order to predict the functional importance of an amino acid alteration [123]. This prediction uses empirical rules, which are applied to the sequence, phylogenetic, and structural information characterizing the substitution**.**

4.2.4.4 *SIFT* (Sorting *I*ntolerant *F*rom *T*olerant)

SIFT (Sorting *I*ntolerant *F*rom *T*olerant) is based on the premise that important amino acids will be conserved among sequences in a protein family, so changes at amino acids conserved in the family should affect protein function. Given

a protein sequence, *SIFT* chooses related proteins, obtains an alignment of these proteins with the query, and based on the amino acids appearing at each position in the alignment, makes a prediction as to whether a substitution will affect protein function. A position in the protein query that is conserved in the alignment will be scored by *SIFT* as intolerant to most changes; a position that is poorly conserved will be scored by *SIFT* as tolerant to most changes [100, 124, 125]. Unlike *PolyPhen*, SIFT does not require structural information of the protein.It follow that it can be applied to a much larger number of proteins. (http://blocks.fhcrc.org/sift/SIFT.html)

Even when the structural information is available, we still may be unable to accurately predict the functional implication of all alterations in amino acid sequence when placed in the complex context of intracellular environment [106].

The planned approaches for this problem use a range of prediction tools, mainly physical and chemical properties of the amino acids, structural properties of the encoded protein and evolutionary properties derived from sequence alignments of homologous proteins [126].

4.2.5 Reconstruction of vectors by site-directed mutagenesis

To test the functional significance of the two nonsynonomous SNPs (exon 1-Ala^{4}Thr and exon 3-Arg^{77}His) and a 9 bp deletion on exon 7, mutations were introduced in the pCMV-Sport6 vector (Figure 4.3), which contained the full length IDO cDNA (NM 002164.2) in the (clone ID 5208340 purchased from Invitrogen, Carlsbad, CA).

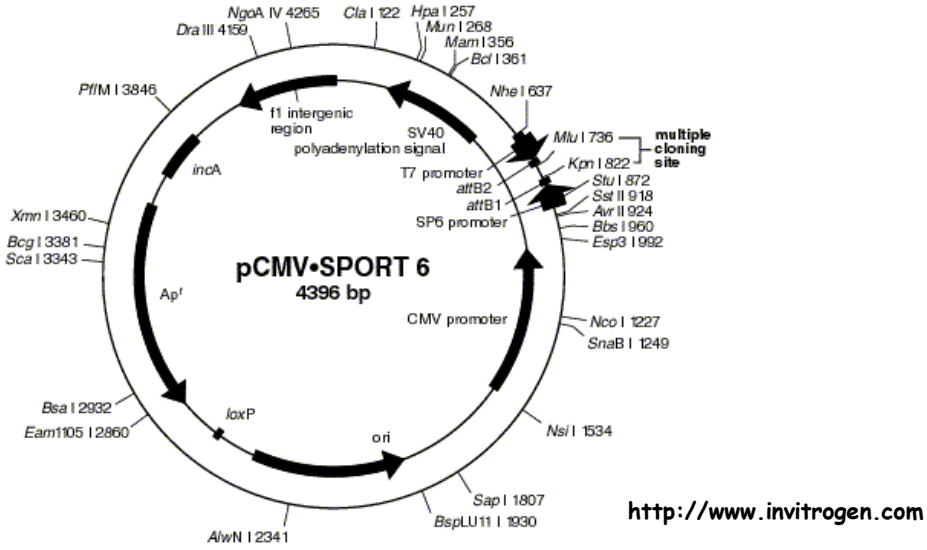

Figure 4.3 Plasmid map of pCMV-Sport6.

Polymerase chain reaction (PCR) based site directed mutagenesis QuickChange® II Site-Directed Mutagenesis kit (Stratagene® La Jolla, CA) was used for the introduction of *INDO* variants. This Strategene QuickChange site directed mutagenesis kit utilizes a simplified way of introducing variations directly into the pCMV-Sport6 expression vector. Variations were introduced separately, using two primers containing the desired mutations (Table 4.4). All primers used for the site-directed mutagenesis were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The primers, each complimentary to opposite strands of pCMV-Sport6-IDO, were extended during temperature cycling using high fidelity DNA polymerase. Incorporation of the primers generated variant vectors containing staggered nicks. Following temperature cycling, the products were treated with a restriction enzyme specific for methylated DNA, and parental DNA templates were digested causing the selection of variant-containing synthesized DNA (Figure 4.4).

The nicked pCMV-Sport6-IDO vectors containing variant IDO were then transformed into XL-blue super competent cells by heat shocking. Clones with variant plasmids were selectively grown in ampicillin-containing medium, during which time the nicks of the variant plasmids were repaired.

Subsequent subcloning of the variant IDO cDNAs into the eukaryotic expression vector pcDNA3 Figure 4.5; Invitrogen (Carlsbad, CA) was performed to exclude the risk of having any additional mutation in the vector generated during mutagenesis. The variant pCMV-Sport6-IDO construct and the native pcDNA3 vector were digested at *Not*I and *EcoR*I sites. The fragments were separated by size on 1% agarose gel and the DNA fragments were purified using a QIAquick Gel Extraction Kit Qiagen (Hilden, Germany). Restriction fragments containing IDO constructs were subsequently ligated into pcDNA3 using a rapid ligation kit New England BioLabs (Ipswich, MA). Transformation into MAX Efficiency®DH5α™ competent cells from Invitrogen (Carlsbad, CA).was achieved by heat shock. Finally, purification of amplified pcDNA3-IDO constructs and verification of variations were completed by sequencing the insert (IDO cDNA) in both directions. The schematic diagram in (Figure 4.4) illustrates the QuickChange® II Site-Directed Mutagenesis system based on polymerase chain reaction.

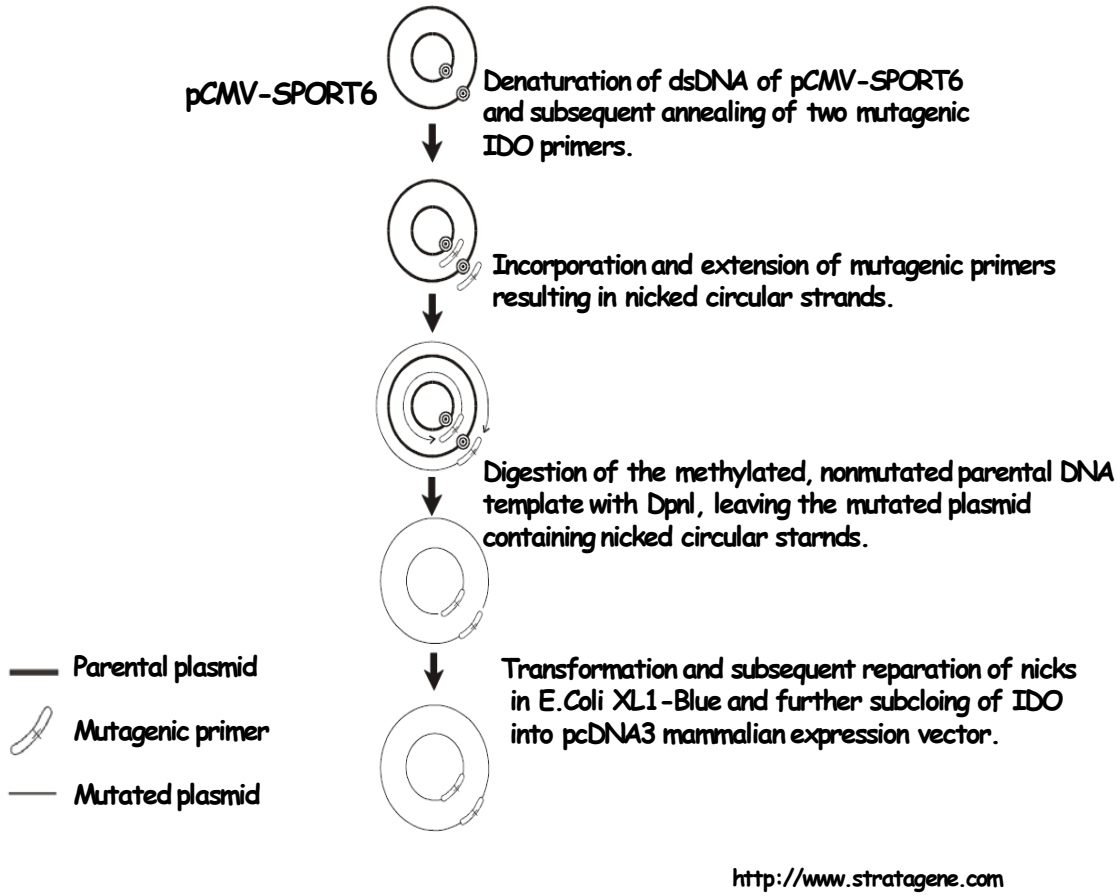

Figure 4.4 Overview of the QuickChange® site-directed mutagenesis method modified from technical manual provided by Stratagene.

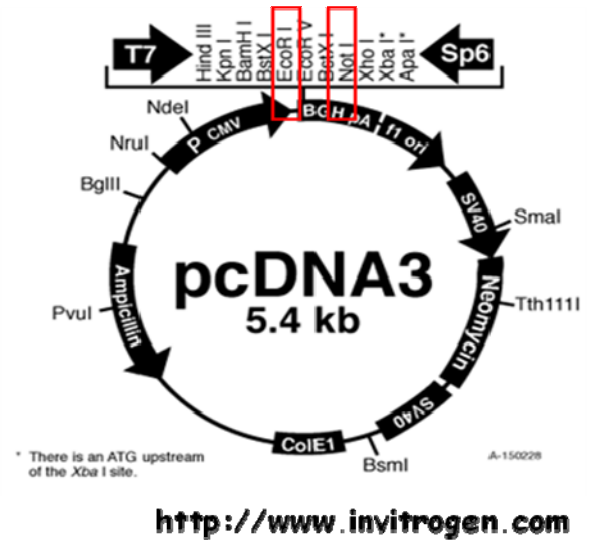

Figure 4.5 A plasmid map of pcDNA3.

Table 4.4 Primers used for the site-directed mutagenesis.

Sequence description	Sequence
Ala^{4} to Thr	
Forward:	GCAGACTACAAGAATGGCACACACTATGGAAAACTCCTGG
Reverse:	CCAGGAGTTTTCCATAGTGTGTGCCATTCTTGTAGTCTGC
Arg^{77} to His	
Forward:	CTCACAGACCACAAGTCACAGCACCTTGCACGTCTAGTTCTG
Reverse:	CAGAACTAGACGTGCAAGGTGCTGTGACTTGTGGTCTGTGAG
#Arg^{77} to Lys	
Forward:	CTCACAGACCACAAGTCACAGAAGCCTTGCACGTCTAGTTCTG
Reverse:	CAGAACTAGACGTGCAAGGTGCTGTGACTTGTGGTCTGTGAG
Ala Leu Leu Glu to Asp	
Forward:	GGACACTTTGCTAAAGGCAATAGCTTCTTG
Reverse:	CCAAGCAAGAAGCTATTGCCTTTAGCAAAGT

The Arg^{77}Lys variant was not identified in the samples we sequenced. We used it as a control for the Arg^{77}His variant.

C-terminal hemagglutinin (HA)-tagged wild-type and variant IDO constructs were also created using the pcDNA3 construct. The wild-type and variant cDNAs were PCR amplified from the plasmids that are described above. To facilitate cloning, *Not*I and *Eco*RI restriction sites were incorporated into the N- and C-terminal ends of the cDNAs. The HA-tag was also incorporated into the C-terminal end, immediately before the stop codon. The primers used for HA-tag subcloning are listed in Table 4.5. All mutations were verified by DNA sequencing after subcloning.

Table 4.5 Primers used to create the HA-tag construct.

Sequence Description	Sequence
Forward:	CCTTTCGAATTCTTAAGCGTAGTCTGGGACGTCGTATG GGTAACCTTCCTTCAAAA
Reverse:	CCTTTCTTCGGCCGCAGACACTGAGG

The amplicon size is 1.5Kb

4.2.6 Plasmid DNA quantification with the Agilent 2100 Bioanalyzer

Several techniques have been used in the quantitive analysis of DNA/RNA. The most common technique is to determine the absorbance at 260 nm (A260) with a UV-spectrophotometer. This detection method has long been the gold standard in nucleic acid quantification, largely due to the ease of sample preparation, requiring no additional mixing of reagents. One major disadvantage to using UV analysis is the impact of sample contaminants such as bacterial genomic DNA during plasmid DNA extraction, which also absorb at 260 nm, thereby giving potentially false quantification readings. An important advantage of the Agilent 2100 Bioanalyzer

DNA 7500 LabChip® kit (Agilent Technologies, Palo Alto, CA) is the ability to accurately determine sample concentrations while checking integrity and purity of the sample. For analysis with the Agilent 2100 Bioanalyzer, one µl of each sample was loaded into a well of a chip prepared according to protocol supplied with the DNA 7500 LabChip kit. Plasmids were digested with restriction enzyme before loading into Agilent. This is because Agilent did not quantify circular plasmid DNA. Briefly, 9 µl gel-dye matrix was loaded into the chip in one well and the chips were pressurized for 30 seconds. Two additional wells were filled with geldye matrix and the remaining wells were loaded with 5 µl each of molecular weight marker. One microliter of DNA ladder was loaded into a ladder well and 1 µl of sample was loaded into each sample well. The chip was vortexed and placed into the Agilent 2100 Bioanalyzer. DNA 7500 assay was run, which applies a current sequentially to each sample to separate products. DNA is detected by fluorescence of the intercalating dye in the gel-dye matrix. The concentration of DNA in each sample was calculated from the area under the curve for each sample product and a size correction was made since an intercalating dye was used to detect DNA (Figure 5.9).

4.2.7 *In vitro* expression of IDO

4.2.7.1 Mammalian cell line expression (COS-7 and HEK293)

A number of mammalian cells were screened for transient expression of IDO (Table 4.1). Most of the cell lines tested had high IDO baseline expression and activity, which makes them unsuitable to use for transient expression. In order to study the effects of variants on the enzymatic function of IDO, Green monkey kidney cells (COS-7) and Human embryonic kidney cells (HEK293) were used for transient

expression analysis. These cell lines were chosen for two main reasons. Primarily, neither cell line has detectable base line IDO activity. The second reason was that they are mammalian cell lines that have the post-translational machinery that is important for process and folding of newly translated proteins. This could be important to assume that the protein is processed correctly and thereby makes it biologically active.

COS-7 cells were purchased from American Type Culture Collection ATCC (Rockville, MD), and Human embryonic kidney (HEK293) cells were obtained from Dr. Theodore Cummin's lab, Department of Pharmacology and Toxicology (IUSOM). All cell lines were cultured according to the ATCC's guidelines and all cell lines tested negative for mycoplasma.

Cells were grown in a humidified atmosphere containing 5% CO_2 at 37°C in DMEM (Dulbecco's modified Eagle's medium BioFluids International, (Rockville, MD) containing a high glucose concentration (4.5 g/l at 25 mM) media was supplemented with 50 units/ml penicillin, 50 μg/ml streptomycin Sigma-Aldrich (St. Louis, MO) and 10% (v/v) fetal bovine serum. All cells were maintained in T-75 flasks and passed every 3-4 days when reached in 80-90% monolayer confluence. Cells from the same passage used in respective experiment. Harvesting was achieved by trypsinization, and subsequently cells were washed re-suspended in medium depending on the use. For expression purpose, cells were seeded in different sized

cell culture plates and flasks based on the experiment (Table 4.6). Immediately before transfection, the medium was removed and replaced with fresh medium without serum.

Table 4.6 Transfection protocol.

#Number of cells	Culture Plate/Flask	@Plasmid DNA	*Lipofectamine™ LTX	*PLUS™ Reagent
1 x 10^5	12-well	0.5	1	1
2 x 10^5	6-well	1	2	2
2.5 x 10^6	T25	10	40	40
7.5 x 10^6	T75	30	120	120

Different tissue culture format, vary the amount of reagent, DNA, cells used in proportion to the relative surface area of the culture plate or flask, as shown in the table (amounts given for the plate is per well).
Number of cells plated 24 hr before transfection unless it is mentioned.
@Plasmid DNA is in µg, all plasmids are diluted to 0.5µg/µl. The amount of plasmid used for transfection is based on the table unless it is mentioned.
*Transfection reagent both Lipofectamine™ and PLUS™ reagent are in µl.

COS-7 cells were transfected with the wild type and variant IDO-pcDNA3 expression constructs using COSFectin (BioRad). Briefly, 2 x 10^5 cells were plated in 6-well plates for 24 hr before transfection and 0.5 µg of IDO expressing constructs were mixed with FBS free DMEM to the final volume to 1ml and mixed with 6 µl of COSfectin and incubate at RT for 20 min. The entire amount was added into the well drop wise. At 24 hr post transfection, media and cells were harvested and IDO activity analyzed using HPLC.

Lipofectamine™ LTX, Plus™ reagent (Invitrogen Carlsbad, CA) was used for the transfection of HEK293 cells according to Table 4.6. An 'empty' pcDNA3 vector

(i.e. one that does not contain cDNA) was used as a control to correct for possible endogenous IDO enzyme activity in the transfected cells. The plasmid DNA concentration was determined by Agilent 2100 Bioanalyzer lab-on-a-chip (Agilent Technologies, Palo Alto, CA). All transfections were performed in triplicate on three different days and a vector which expresses *Renilla* luciferase gene was used as a transfection control. This was done to confirm equal transfection efficiency allowing comparison between trasnfected cells.

Samples of culture medium (~1ml) were collected after 48 hr of transfection in 1.5 ml micro centrifuge tubes. For plasmid dose dependent experiments, 0.1, 0.5 and 1.5 µg of plasmid DNA from respective constructs used. For the time course experiment culture medium was collected at different time points (12, 24, 48 and 72 hr) from cells transfected with 0.5 µg respective plasmids. Cells were harvested for RNA and protein analysis and were kept at -80ºC; culture media was kept frozen at -20ºC for measurement of tryptophan and kynurenine concentrations using HPLC in most of the studies unless specified otherwise.

4.2.7.2 *In vitro* transcription and translation of IDO using rabbit reticulocyte lysate (TNT-RRL).

Transcription and translation of wild type and variant ($Ala^{4}Thr$, $Arg^{77}His$, 9bp deletion and $Arg^{77}Lys$) IDO constructs were performed using TNT® couple RRL system Promega (Madison, WI) with constructs that had been subcloned into pcDNA3. Specifically, 25 µl of T7 buffer, 1 µl of T7 polymerase and, 1 µl of RNasin,

reagents were included in the Promega (Madison, WI) kit. One µg of pcDNA3 expression construct DNA was added to the mixture; the reaction volume was increased to 50 µl with nuclease-free water and the mixture was incubated at 30ºC for 90 min.

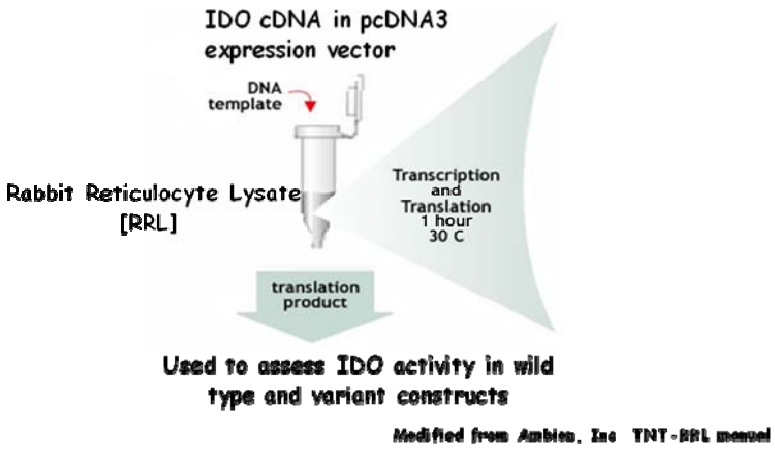

Figure 4.6 A schematic diagram of the Transcription and Translation linked in Rabbit Reticulocyte Lysate system [TNT-RRL] assay.

4.2.7.3 Assay of IDO activity in transient expression systems.

To functionally characterize variant IDO enzymes, the degree of impaired enzyme activity was assayed after transiently expressing it in COS-7 and HEK293 cells. The activity was correlated with the catalytic activity of the wild type IDO. Conversion of the substrate (tryptophan) to metabolite (kynurenine) was achieved by incubation of the substrate for 48 hr. The degree of substrate conversion of the variant was compared with the wild type using substrate concentration of 80 µM in the culture medium. Background activity, i.e. activity found after transfection with empty vector, was below the detection limit. The natural diffusion/transport of substrate and metabolites from the cells to the surrounding medium allowed measurement of both substrate and metabolites in the culture medium.

4.2.7.4 IDO activity measurement in the culture media using HPLC

Using a published HPLC assay for IDO enzymatic activity measurement [127] as a starting point, we optimized and validated a sensitive HPLC assay with UV and florescence detection that allowed effective chromatographic separation and quantification of tryptophan and its metabolite kynurenine in culture medium. Briefly, at the time of analysis, samples were thawed on ice, vortexed, and 200 µl of the conditioned media were transferred to a new micro centrifuge tube containing 50 µl of 3-nitro-tyrosine (3-NT) as the internal standard. Fifty µl of 30% trichloroacetic acid (TCA) was then added to each tube to precipitate out the proteins and the tubes were vortexed immediately. The samples were spun in a microcentrifuge at 12,000g for 5 min and 200 µl of supernatant was transferred to 8×40 mm amber glass tubes for HPLC analysis. All samples were run in triplicate. The two chromatographs (Figure 4.7) show the typical peaks of tryptophan and kynurenine.

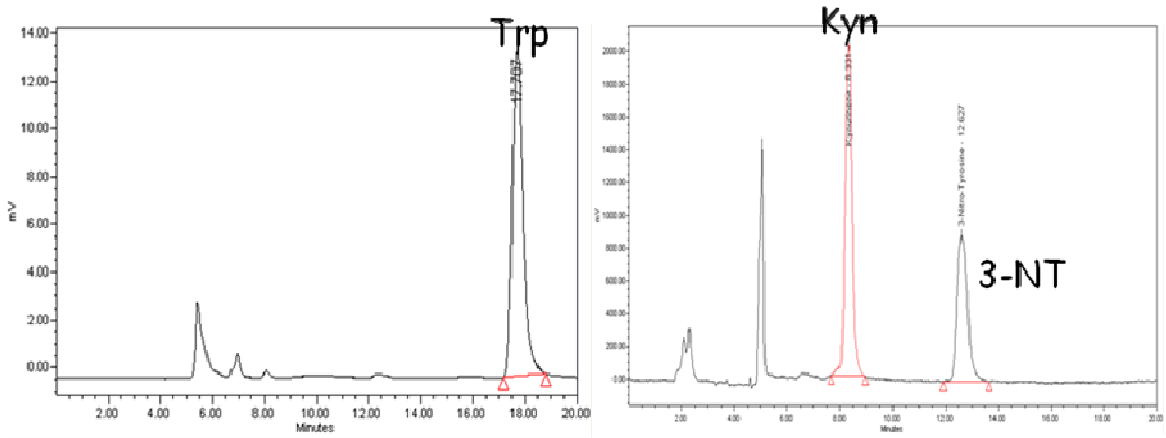

Figure 4.7 Representative chromatograms showing the separation of tryptophan and kynurenine by HPLC**.** Cultured medium from the COS-7 cell line transiently transfected with wild type IDO cDNA was collected after 48 hr, extracted with trichloroacetic acid and the supernatant was loaded on an HPLC. X axis is time in minutes; Y axis is millivolt signal from the detectors, ultraviolet (right) 360nm, fluorescence (left) excitation =285nm, emmission=365nm. Int Std = internal standard (3-nitro-tyrosine).

An HPLC with UV and fluorescence detectors connected in tandem was developed for the quantification of tryptophan and kynurenine. The HPLC system consisted of a Waters (Milford, MA) model 515 pump, model 717 auto sampler, model 490 programmable absorbance UV detector and Hewlett Packard 1046A programmable fluorescence detector. The separation system consisted of a Zorbax SB-C_{18} column (150 x 4.6 mm, 3.5 µm particle size; Phenomenex, Torrance, CA), a Luna C_{18} Guard column (30 x 4.6 mm, 5 µm; Phenomenex), with a mobile phase composed of 97%, 15 mM KH_2PO_4 (pH=5.5) and 2.7% acetonitrile (flow rate, 0.8 ml/min). The column elute was monitored by fluorescence detection at 360 nm for tryptophan and by UV detection for kynurenine at 285 nm. Chromatograms look similar for analyses from cultured media as well as from serum. The values were referred to a standard curve with defined kynurenine (0, 0.16, 0.312, 0.625, 1.25, 2.5, 5, 10, 20 µM) and tryptophan (0, 1.6, 3.12, 6.25, 12.5, 25, 50,100, 200 µM)

concentrations. The limits of quantification for tryptophan and kynurenine are approximately 1.6 μM and 0.16 μM, respectively. Decreasing the mobile phase pH from 6.5 to 5.5 accelerates the elution and sharpens the tryptophan and kynurenine peaks. The retention times of tryptophan and kynurenine were stable over the course of time on a single column.

4.2.7.5 IDO activity measurement using a plate reader

The second method used for the measurement of IDO activity is a colorimetric based assay. This procedure was modified from previous report by [128, 129]. In the 100 μl of conditioned medium, 25 μl of 30% (w/v) trichloroacetic acid (TCA) was added to precipitate residual cells and proteins in the medium. Then 100 μl Ehrlich reagent (*p*-dimethylaminobenzaldehyde in acetic acid) was add into the TCA treated conditioned medium and incubated for 10 minutes at room temperature in a microtiter plate. The absorbance at 480 nm for the yellow color derived from kynurenine was determined using Synergy2 micro plate reader BioTek (Winooski, VT). The limit of quantification for kynurenine is approximately 3.1 μM and limit of detection is approximately 1.6 μM. The values were referred to a standard curve with defined kynurenine (0, 1.6, 3.12, 6.25, 12.5, 25, 50 100 μM) concentrations. All assays were conducted in triplicate. The Ehrlich method used to measure kynurenine is simple, robust and it does not interfere with tryptophan. For these reasons, many groups use this procedure to quantify kynurenine [129, 130]. However, a limitation of the Ehrlich method is that it can interact with downstream kynurenine metabolites and

overestimate kynurenine formation [131]. To avoid this overestimation, we have double tested samples with unexpected high kynurenine formation using HPLC.

4.2.7.6 RNA extraction and real time PCR assay

Total RNA was isolated from the cell cultures after treatment using RNeasy mini-kits, according to the manufacturer's protocol (Qiagen, Inc., Santa Clarita, CA). Initially, endpoint RT-PCR analysis was performed to determine if the wild type and the variant cDNAs were expressed in both COS-7 and HEK293 cells. In later experiments, the assay was converted to a real-time quantification PCR assay as described below. Cells were harvested after 48 hr and total RNA was extracted using RNeasy kit (Qiagen, Inc., Santa Clarita, CA), following the manufacturer's protocol. Following the extraction of total RNA, the samples were treated with DNase I (DNA-free™; Ambion, Austin, TX). Total RNA concentrations were measured using RNA6000 Nano LabChip kit from Agilent Bioanalyzer lab-on-a-chip (Agilent Technologies, Palo Alto, CA). The RNA quality was checked based using the ratio of 28S/18S ribosomal RNA. Reverse transcription (RT) was performed on 1 μg of RNA using Reverse Transcription kit (Promega, Madison, WI).

Table 4.7 Primers used for the RT-PCR.

Sequence description	Sequence	Amplicon size (bp)
IDO-Forward:	ACTACAAGAATGGCACACGC	406
IDO-Reverse:	TTGCCAAGACACAGTCTAC	
GAPDH-Forward:	GAA GGT GAA GGT CGG AGT	227
GAPDH-Reverse:	GAA GAT GGT GAT GGG ATT TC	

All primers were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). The specific sequences of primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi)

Quantitative real time RT-PCR analysis using SYBR Green was performed to assess the mRNA level in HEK293 cells transiently transfected with the wild type and variant IDO constructs. Briefly, the reaction volume was set at 25 µl with the following composition. Primers were diluted to a 20 µM stock solution and used 0.25 µl each (to a final concentration 200 nM). SYBR green was used to quantify the PCR products. Fluorescein was included for the iCycler well-factor correction. The samples were amplified using the Platinum Supermix UDG (2X) (Invitrogen, Carlsbad, CA). Two µl of cDNA were used in the reaction and the volume was made up by water to a final volume of 25 µl. Samples were amplified using the following conditions. Samples were incubated at 50ºC for 2 minutes followed by 30 cycles of amplification using the following program: denaturation at 95ºC for 30 sec, annealing at 54ºC for 1 minute and extension of PCR product at 72ºC for 1 minute and final extension at 72ºC for 10 minutes. The $2^{-\Delta\Delta Ct}$ method was used to calculate

relative changes in gene expression determined from real-time quantitative PCR experiments [132]. GAPDH was selected as the housekeeping gene for normalization. Basically, each gene was running along with GAPDH and difference between threshold cycle (C_T) was designated as ΔC_T. Then the difference between their respective controls was calculated and designated as $\Delta\Delta C_T$.

4.2.7.7 Determination of total protein content

HEK293 cells were harvested and washed twice with PBS and subsequently mixed with cell lysis buffer and cell disruption was performed by sonication. Briefly, pellets from HEK293 cells trasfected with respective IDO plasmids were washed and resuspended in 500 µl lysis buffer. The suspension material was sonicated by a multi-step scheme. Each step consisted of three cycles of disruption using a microtip with 40W Ultrasonic homogenizer model 150VT from Biologics, Inc (Manassas, VA) for 30 sec on ice bath and incubation on ice for 5 min, followed by centrifugation at 12 000 *g* and at 4ºC for 5 min to separate the soluble fraction from the pellet.

Protein concentration was measured using a protein assay based on the BCA method (Pierce, Rockford, IL). Supernatants from sonicated cells were incubated with protein assay buffer using a range of concentrations of bovine serum albumin as a protein standard. Samples were measured thereafter on a plate reader and the protein concentration was estimated using the reference values from the protein standards.

4.2.7.8 Western blot analysis

The indoleamine 2,3-dioxygenase protein concentrations in the transfected HEK293 cells were determined by Western blot. Total protein concentrations were determined using the BCA method (Pierce, Rockford, IL). Briefly, 25-100 µg of total protein from each samples were loaded and separated on a 4-20% SDS-polyacrylamide gel Invitrogen, (Carlsbad, CA). Electrophoresis was performed for 1 hr and 30 min at 125 V, and proteins were transferred to Polyvinylidene fluoride (PVDF) membranes overnight at 4ºC at 35 V. The membranes were then blocked overnight with 5% milk powder in TBST (Tris-HCl (25 mM; pH 7.5), KCl (3 mM), NaCl (140 mM) Tween-20 (0.05%). The following day, the membrane was incubated with an anti-IDO primary antibody (final concentration 1 µg/ml) in 5% milk powder in TBST. We used a mouse anti-human monoclonal antibody directed against human IDO (hIDO) amino acids 78-184 US Biological, (Swampscott, MA; Catalog # I7545-01). Following the primary incubation, the membranes were washed three times. The secondary antibody was a goat anti-mouse horseradish peroxidase (Pierce, Rockford, IL) diluted 1:10,000 in 5% milk powder in TBST. The secondary antibody was incubated for 2 hr and then washed three times in TBST. The bound antibody was detected by enhanced chemiluminescence performed with the ECL™ Western Blotting Analysis Kit Pierce, (Rockford, IL). For the HA-tag western blots, the same procedure was used with the following modifications: The primary antibody (anti-HA, Upstate, Temecula, CA; catalog #05-904; final concentration 2 µg/ml) was incubated in phosphate buffered saline Tween-20 (0.05%) in 5% milk powder. β-actin was used as a loading control. Membrane was stripped using

Restore™ Western Blot Stripping Solution Pierce, (Rockford, IL) according to manufacturer's specifications. Membranes were blocked and probed with anti-beta actin antibody (1:1000, A5441; Sigma) for 3 hours in blocking solution, and then membranes were washed three times. The secondary antibody was a goat anti-mouse horseradish peroxidase (Pierce, Rockford, IL) diluted 1:10,000 in 5% milk powder in TBST. The bound antibody was detected as described above.

4.2.7.9 Quantitative Western blotting and IDO activity

To evaluate whether variation in enzymatic activity resulted from differences in enzyme expression, rather than reduced function of the variants, wild type and variant proteins were analyzed by Western blotting. Since enzyme activities were expressed as pmol/mg/hr, it was crucial to accurately assess the total amount IDO protein expressed. A comparison of enzyme activities could be done only after correction for protein content was completed.

HEK293 cells were seeded in a T225 flask and transfected with wild type, $Ala^{4}Thr$ and $Arg^{77}His$ variants for 48 hr using the pcDNA3-HA IDO constructs according to the transfection protocol in Table 4.6. Cell lysates from several T225 flasks for each construct were pooled together. Because cell harvesting by ordinary trypsinization may lead to degradation of proteins, mammalian protein extraction reagent (Pierce, Rockford, IL; Catalog # 78501) was directly applied on the cell culture plate. Total protein concentration was measured as described above using BCA. To obtain the equivalent amounts of immunoreactive IDO protein, we

gradually reduced the amount of total protein loaded on the gel for the wild type and $Ala^{4}Thr$ variant, but at the same time by increasing for the $Arg^{77}His$ and $Arg^{77}Lys$ until we got comparable bands for all IDO constructs. The same western blot procedure was used as described above paragraph.

4.2.7.10 Kinetic parameter determination

Prior to enzymatic assays, IDO proteins were expressed in HEK293 cell systems and cell lysates were prepared as described above. For a more accurate assessment of the quantitative differences in IDO activity for variant proteins, HEK293 cells transiently expressing different IDO constructs were characterized for IDO protein expression by Western blot analysis. To establish the functional effect of nonsynonymous polymorphisms of $Ala^{4}Thr$, $Arg^{77}His$ and $Arg^{77}Lys$ on tryptophan metabolism, we conducted enzymatic assays with IDO proteins obtained from HEK 293 cells over expressing each variant protein. First the enzymatic assay was done with the wild type IDO containing 0, 50, 100 and 200 mg total proteins in 200 µL. The incubation medium contained the following: 0.8 mmol/l L-tryptophan, 40 mmol/l ascorbic acid, 20 µmol/l methylene blue, 200 U/ml catalase, and 100 mmol/l potassium phosphate buffer (pH 6.5) in 200 µL. Both the cell lysate and the reaction buffer were pre-incubated at 37ºC for 5 min. Reactions were started by adding 200 µL of reaction buffer into the tube with the cell lysates and incubated at 37ºC for 1hr. The reaction was terminated by adding 100 µL of 30% (w/v) trichloroacetic acid and incubated at 50ºC for 30 min to hydrolyse *N*-formylkynurenineurenine produced by

indoleamine 2,3-dioxygenase to kynurenine. The reaction mixture was then centrifuged for 10 min at 12000 *g* to remove sediment. Formation of kynurenine was measured as described above using the plate reader assay. Relative IDO activities are expressed as formation of kynurenine in pmol/mg/hr.

For kinetic analysis of the different IDO constructs, data derived from determination of enzyme activity at each of the six substrate concentrations were used (6.25, 12.5, 25, 50,100 and 200 μM). Determination of the kinetic variables was done using simple ligand binding one site saturation Sigmaplot 8.0 (Systat Software, Point Richmond, CA). Kinetic constants (K_m and V_{max}) were estimated by nonlinear regression analysis using Sigma Plot. Formation rates (V) of metabolites versus substrate concentrations (S) were fit to a simple Michaelis - Menten Equation ($V = V_{max} \cdot S/ (K_m + S)$). Based on this equation, the intrinsic clearance $CL_{int} = V_{max}/K_m$ estimation values were calculated.

4.2.7.11 Protein degradation inhibitors and IDO activity

HEK293 cells were seeded in a 6-well plate and transfected with wild type or variant IDO for 48 hr using the pcDNA3 IDO constructs as described above in Table 3.6. To assess the effect of proteasomal inhibition on IDO activity cells were treated with hemin, MG132, or lactocystin for 24 hr after transfection. Each drug was used at 2.5, 5 and 15μM. Media was harvested after 24 hr drug exposure and formation of kynurenine measured using HPLC.

The effect of the lysosmal pathway inhibitor, chloroquine, was tested in HEK293 cells transiently transfected with $Arg^{77}His$ variant. Cells were treated with 5,

10 and 20 μM chloroquine for 24 hr and the formation of kynurenine analyzed in the culture media. Cells harvested from each well and IDO expression assessed by Western blot.

4.2.7.12 Alteration in cellular heme concentrations

IDO must have heme incorporated into it to function. Heme is one important member of the porphyrin family. It is synthesized in both mitochondria and cytoplasm, and is a key prosthetic group for various essential proteins such as cytochromes, catalases and indoleamine 2,3-dioxygenase. Since alteration in the heme component of IDO could be a mechanism by which the genetic variants affect the enzymes function, we evaluated heme concentrations and manipulated them in a transient expression system.

HEK293 cells were treated with succinylacetone for 12 hr before transfection with wild type and variant IDO plasmids. Heme concentrations in the cell lysate were measured using QuantiChrom™ Heme Assay kit (Bioassay systems, Hayward, CA). This assay is based on an aqueous alkaline solution method, in which the heme is converted into a uniform colored form. The intensity of color, measured at 400 nm, is directly proportional to the heme concentration in the sample. The linear detection range of this assay is 0.6-125 μM heme. Fifty μl of sample mixed with 200 μl of reagent and incubated at room temperature for 5 minutes. After 5 minutes incubation optical density was read using Synergy2 microplate reader (BioTek, Winooski, VT).

Heme concentrations were calculated based on the formula below.

$$= OD_{sample} - OD_{blank} / OD_{calibrator} - OD_{blank} \text{ X } 62.5 \text{ X n } (\mu M)$$

Water used as a OD_{Blank} , $OD_{Sample,}$ $OD_{Calibrator,}$ *n* is a dilution factor.

Cell viability was assayed using CellTiter 96® AQ_{ueous} One Solution Reagent (Promega, Madison, WI). Twenty µl of one solution reagent were added to each well of the 96-well plate containing HEK293 cells transfected with wildtype and variant IDO construct and subsequently treated with hemin in 100 µl of culture medium. Plates were incubated for 2 hr at 37ºC in a humidified, 5% CO_2 atmosphere and absorbance was recorded at 490 nm using a 96-well plate reader (BioTek, Winooski, VT). The absorbance values at 490 nm were corrected by subtracting the average absorbance from the vehicle treated wells.

The proteasomal, lysosome and heme synthesis inhibitors effect on cell viability were tested using Trypan Blue Stain (Lonza Inc., Allendale, NJ). Viable cells, excluding trypan blue, were counted in a hemocytometer at the indicated drug concentrations and compared with the vehicle treated cells. Cells were harvested after 24 hr drug exposure and 100 µL cell suspension was mixed with 100 µL of 0.4% trypan blue and incubated for 5 minutes at room temperature. Ten µl of stained cells placed in a hemocytometer and count the number of viable (unstained) and dead (stained) cells.

4.2.8 Statistical analysis

Data are expressed as mean ± SD, and statistical analyses were performed with the SPSS statistical program. One-way analysis of variance with the Dunett *t*-test was used to evaluate the difference between wild type and variants. A p-value less than 0.05 was considered significant.

4.3 Specific Aim 2: To identify genetic variants in the IDO promoter and to assess their functional significance.

We hypothesize that genetic variations in the IDO promoter cause functional changes in IDO expression and activity.

The *long term goal* for this research is to have a better understanding of potentially damaging variants; this would enable physicians to make reliable assessments of individuals' risks of particular disease susceptibility, and help explain interindividual variation of the therapeutic effectiveness and toxicity of drugs caused by altered tryptophan metabolism.

Endogenous and exogenous cytokines play an important role in regulating IDO expression. The molecular mechanisms responsible for IFN-γ mediated IDO inductions have been characterized in human fibroblasts [133]. The region upstream of the IDO transcriptional start site contains numerous regulatory elements, several of which are characterized as being important in IFN-γ responsiveness [134, 135]. There are two distantly separated IFN-stimulated response elements (ISRE-1 and ISRE-2). There are three gamma activation sequences (GAS-1, GAS-2 and GAS-3) with in the 1.3 kb region upstream of the transcription start site [136-138]. Studies using a reporter

construct have been effective in characterizing the regulatory region important for the response of IFN-γ. These response elements have shown to be essential to the transcription of IDO in response to IFN-γ [138]. Although IDO is primarily an IFN-γ induced gene, other inflammatory mediators also mediate the induction of IDO [137, 138]. There is variability in IFN-γ mediated expression of IDO in different tumor cell lines [Cao unpublished data]. However, the molecular mechanisms contributing to this variability are unknown. Little is known about the genetic variants of the IDO promoter and the functional significance of these variants. It is likely that genetic polymorphisms in the IDO promoter region alter IDO expression.

4.3.1 Experimental design

Our studies focused on genetic polymorphisms in the *INDO* 5'-flanking region consisted of SNP identification and functional studies. The SNP identification studies involved gene resequencing and bioinformatic analysis of the sequence data. The functional studies involved *in vitro*, transient transfections; these were used to evaluate the promoter activity. A flow chart describing the experimental approach is shown in Figure 4.8.

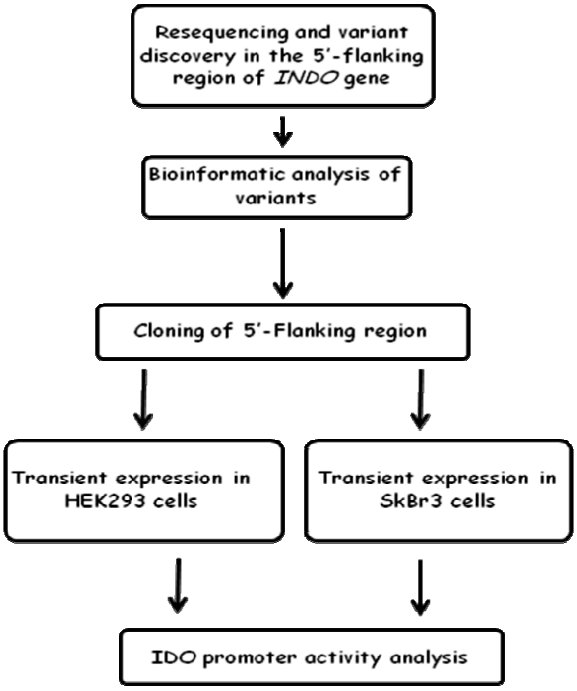

Figure 4.8 Experiment flow chart used in the study of IDO genetic polymorphism in the 5'-flanking region.

4.3.2 Resequencing/Identification of variants in the 5'-flanking region of *INDO* gene

In addition to the core promoter sequence, the *INDO* 5'-flanking region contains several other functional transcription factor binding sites. In fact, functional elements -1246/-1018 and -150/-10 control activity of the human IDO promoter; this is particularly important for the interferon gamma regulation [139, 140]. Therefore, we engaged in a systematic analysis of these two particular portions of the *INDO* gene promoter.

Two PCR primer pairs, encompassing 400 bp were designed to resequence a portion of the 5'-flanking region. These were used to assess the nature and extent of nucleotide variation in what is believed to be the functionally important parts of thehuman *INDO* gene promoter. The primer sets used for each target were designed to include sequences corresponding to the known functional transcription factor binding sites (Table 4.7). The portion of the 5'-flanking region covered by the sequencing primers are highlighted in Figure 5.22.

We used the same DNA sample set as used in the specific aim 1. This consisted of 48 African-American and 48 Caucasian subjects from the Coriell DNA Repository (Camden, NJ.). Sample Sets HD100CAU and HD100AA were used. The resequencing and SNP identification was done using commercial services of Polymorphic DNA, Inc. DNA was sequenced in both the forward and reverse directions. Each polymorphic site was verified by examining the chromatograms using the Vector NTI Advance^{TM} software and the excel spread sheet from received from polymorphic DNA.

Table 4.8 Primers used to sequence the 5'-flanking region of *INDO* gene.

Sequence description	Sequence	Amplicon size (bp)
Ref. sequence 1		
#BST5'-	TTTCTCCTTTTGATCATCTAGAGGA	461
BST3'-	ACACAACCTGTGTTATACTCAACTA	
*NST5'-	GCAACTTGGTTTCTTCTTTAGC	
NST3'-	TCAATGGCTCTGAAGTAGGAAA	
Ref. sequence 2		
BST5'-	TAAAACGTTGTTCCATGTCTTCACT	484
BST3'-	TTCCTCTAGATGATCAAAAGGAGA	
NST5'-	TGACTCACCCATAAAAACTTCAAGT	
NST3'-	AGAGAAATAGTTCGTGTGAGTAAAC	

For both #Boost and *Nested PCR the following cycle conditions were used : 94ºC for 4 min, 94ºC for 0:20 min, 54ºC for 0:25 min, 72ºC for 1:00 min (39 cycles) and final 72ºC elongation for 7 min and hold at 6ºC.

The analysis of regulatory regions in the genome sequence is mainly based on the identification of potential transcription binding sites (TFBS). *Vector NTI Advance* was used to analyze the SNPs identified by the resequencing chromatograms.

The genomic sequence of 1.3 kb upstream of the IDO translation site was analyzed for putative transcription factor binding site using TRANSFAC and JASPAR. The Sequence was screened at high levels of stringency for human transcription factors with a transcription factor score of 95% (the default is 80%). For the analyses based upon cross-species conservation, we aligned each human promoter sequence with 10 kb of mouse sequence that is immediately 5' to the relevant orthologous reference mRNA sequence in GenBank. We used the 'all vertebrate' transcription factor setting combined with the default settings for conservation and transcription factor scores.

4.3.3.1 TRANSFAC

Computational analysis of regulatory properties of DNA is most often based on the use of matrix models describing binding preferences of transcription factors, or other DNA patterns [141].

In order to elucidate transcription factor binding sites (TFBS) within the *INDO* promoter, we used TRANSFAC. This is a database on eukaryotic *cis*-acting regulatory DNA elements and trans-acting factors. TRANSFAC employs a simple correlation calculation with binding site profile matrices to derive results. Data within TRANSFAC have been generally extracted from the original literature. We submitted the 1.3 kb IDO promoter sequence and selected a high-scoring threshold of 95.0 [142].

4.3.3.2 JASPAR

In addition to TRANSFAC, we also queried TFBSs using JASPAR. It is an open-access annotated database of experimentally derived TFBS profiles. We used the same promoter sequence and a threshold value of 95.0. This database (JASPAR) is the most comprehensive open-access database holding such models [143, 144].

4.3.4 Isolation of 1.3 kb of 5'-flanking region of *INDO* gene

Primers were designed using Primer 3 (http://frodo.wi.mit.edu). The 3' primer for target promoter was designed to include sequence corresponding to the transcription start site in the amplicon. It did not include any coding sequence in order to avoid changing the open reading frame of the reporter gene or making a target-reporter fusion protein. We included no more than 100 bp of 5'-fanking region and avoided inclusion of any untranslated ATG sequences. DNA from individuals with

specific promoter variants has amplified using Expand High Fidelity DNA polymerase (Roche, Indianapolis, IN) to minimize mis-incorporation of nucleotides. According to the following PCR condition 1.3 kb of 5'-flanking region of *INDO* gene amplified: denaturation at 95ºC for 30 sec, annealing at 54ºC for 1 minute and extension of PCR product at 72ºC for 1 minute and final extension at 72ºC for 10 minutes.

To clone the insert into a luciferase reporter plasmid, the PCR products were digested at *Nhe*I and *Xho*I sites (Table 4.8) and the fragments were separated by size on 1% agarose gel, DNA fragment were purified using a QIAquick Gel Extraction Kit (Qiagen; Hilden, Germany). Restriction fragments containing 1.3 kb of 5'-flanking region of IDO promoter were subsequently ligated into pGL4.10 (Figure 4.9) using a rapid ligation kit (New England BioLabs; Ipswich, MA). Transformation into MAX Efficiency®DH5α™ competent cells (Invitrogen; Carlsbad, CA) was achieved by heat shock. Finally, purification of amplified constructs and verification of variations was completed by sequencing the insert (*INDO* 5'-flanking region in pGL4.10 vectors) in both directions.

Table 4.9 Primers used to clone 1.3kb of IDO promoter region.

Sequence description	**Sequence**
Forward:	GGGTTTGCTAGCTCAATATTTTATTTGTAGTG *Nhe*I
Reverse:	TTTTTTCTCGAGTCTTGTAGTCTGCTCCTCTG *Xho*I

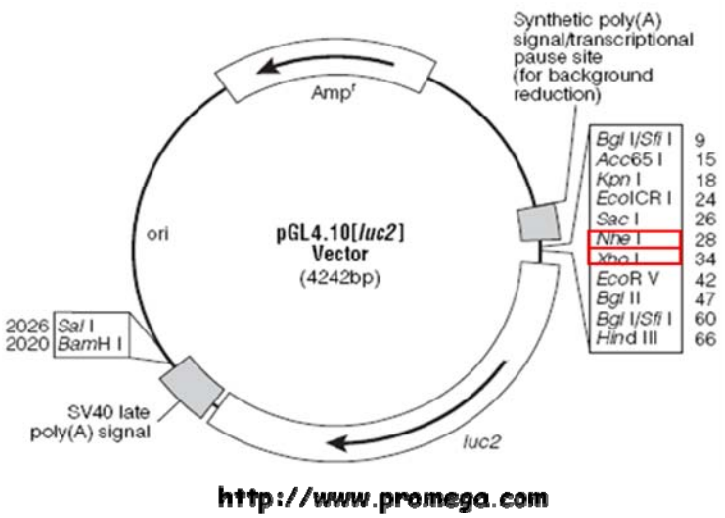

Figure 4.9 Plasmid map of pGL4. 10[*luc2*] vector used to clone 5'-flanking region of *INDO* gene.

4.3.5 *In vitro* luciferase reporter experiment

After the insert sequence was verified by independent sequencing and restriction enzyme digestion, its activity was tested by luciferase reporter gene assay HEK293 and SkBr3 cell lines. To control for transfection efficacy, we used the *Renilla* luciferase reporter plasmid (pRL-SV40) was used. HEK293 and SkBr3 cells were plated in 12-well plates 1 day before transfection. On the day of transfection, cells were washed twice with Phosphate Buffered Saline (PBS) solution and replaced with serum-free DMEM media. Cells were transfected using Lipofectamine™ LTX, Plus™ reagent (Invitrogen; Carlsbad, CA) with 250 ng of respective constructs and 5 ng of *Renilla* Luciferase were co-transfected (a plasmid containing the cytomegalovirus promoter upstream of the *Renilla* Luciferase gene to normalize for transfection efficiencies). After 4 hr of incubation at 37ºC, a complete medium described above added, and cultured for an additional period of 24 hr at 37ºC.

Transfections were performed in duplicate, and repeated on at least 3 different days. For the dose dependent IFN-γ treatment of cells, four different doses used (0, 50, 250 and 500 IU/ml). Measurement of luciferase activity was performed 24 hr after IFN-γ exposure using the Luciferase Assay Kit (Promega; Madison, WI) according to the manufacturer's protocol. Briefly, cells were washed with PBS solution, 100 µl of Passive Lysis Buffer (Promega; Madison, WI) was added to each well, and cells were incubated for 10 min at room temperature in an orbital shaker. Luciferase assays were performed using 20 µl aliquots of supernatant in a Sirus Luminometer (Berthhold Detection Systems, Pforzheim, Germany). Each lysate was measured twice. Luciferase activities were normalized for *Renilla* luciaferase activity in each extract to correct for transfection efficiency, and reporter gene expression was expressed in relative light units. The luciferase activity of each construct was compared with that of the wild type promoter at base line and after interferon treatment.

4.3.6 Statistical analysis

Data are expressed as mean ± SD, and statistical analyses were performed with the SPSS statistical program. One-way analysis of variance with the Dunett *t*-test was used to evaluate the difference between wild type and variants. A p-value less than 0.05 was considered significant.

CHAPTER 5

Results

5.1 Specific Aim 1: To identify indoleamine 2,3-dioxygenase genetic polymorphisms in the coding region that affect enzyme expression and/or activity and to evaluate the mechanisms by which these variants influence activity.

The series of experiments described in this study began with the resequencing of the *INDO* gene using 96 samples obtained from the Coriell DNA repository (Camden, NJ). Of these samples 48 were African American (AA) and 48 were Caucasian (CA). These DNA samples were obtained from unrelated individuals without any known hereditary diseases. Since these subjects have consented for their DNA to be used for a variety of discovery purposes, these DNA samples are frequently used for identifying common genetic variants in the human population. All 10 exons were resequenced, including splice junctions, as well as approximately 100 bp of the 5'-untranslated region of the gene.

In these samples, we identified 17 genetic variants in *INDO* gene. The detailed information on these variants is in Table 5.1. Eight of the variants were observed exclusively in the African American samples, 4 exclusively in the Caucasians samples and 5 in both populations. Allelic frequencies of 1 of the 9 newly identified intronic SNPs were greater than 36%; one in putative intron/exon splice junctions, which may alter the *INDO* mRNA processing. We also identified four variants that change the predicted IDO amino acid sequence; three are nonsynonymous SNPs and one is a 9 bp deletion.

In total, 5 of the 48 samples (~10%) of the African American samples contained nonsynonymous variant alleles (Table 5.1). Three nonsynonymous SNPs and one 9 pb deletion were observed only in the African American population; each of which was present in only a single heterozygous sample. These variants resulted in changes in amino acid $Ala^{4}Thr$, $Arg^{77}His$, $Leu^{197}Ile$ and the 9 bp deletion replaced Ala-Leu-Leu-Glu with Asp. The presence of these variants was verified by performing independent PCR amplification followed by sequencing.

The variants observed during this resequencing study were comparable to those present in the publicly accessible databases. A total of 43 human *INDO* variant sequences had been deposited in the SNP database (www.ncbi.nlm.nih.gov/SNP). Of these, 26 variants were located in introns, 2 were in the 3'-flanking region and 9 SNPs near the 5' UTR. Only 7 of the variants were observed during our resequencing studies. The remaining 36 publicly available variants were not observed in our sample cohort. Therefore, of the 17 *INDO* polymorphisms that we observed, 10 of the SNPs are novel. These results now make it possible to test the hypothesis that IDO genetic polymorphisms might contribute to individual variation in IDO expression and tryptophan metabolism.

Table 5.1 Genetic polymorphisms in the *INDO* gene. [1]The nucleotide positions referred to are relative to the translation start site. The first nucleotide of the translation initiation codon assigned the number (+1). The numbering is based on accession # X_17668. Allelic frequencies were calculated as the number of variant alleles divided by the total of 96 alleles (48 subjects) in each group.

Position[1]	Exon /Intron	Base pair change	Location /Alteration	Allelic Frequency	
				AA	CA
-89	Ex 1	gataaa*[C > T]tgtggt	5' UTR	3	0
+10 rs#35059413	Ex 1	gcacac[G > A]ctatgg	$Ala^{4}Thr$	2	0
+4211 rs#35099072	Ex 3	cacagc[G > A]ccttgc	$Arg^{77}His$	1	0
+6180	Int 5	taaggc[C > T]cctgac	Intron	15	20
+8545	Int 5	ttgaaa[C > T]taaaat	Intron	1	0
+8624	Int 5	caattt[C > T]ctcagg	Splice junction	19	27
+8733	Int 6	aaagta*[C > A]gtctat	Intron	0	1
+9598	Ex 7	actttg*[C > A]taaagg	$Leu^{197}Ile$	1	0
+9605	Ex 7	*GCTGTTGGA Deletion	Ala-Leu-Leu-Glu > Asp	1	0
+9674	Int 7	tgcagt*[G > A]caatag	Intron	0	2
+10917	Int 8	gtccct*[A > G]atatcc	Intron	1	0
+10957	Int 8	caaaaa*[T > G]tcacat	Intron	36	2
+11309	Int 8	ctctga[T > C]agctgg	Splice junction	1	0
+13855	Int 9	atgctg[T > C]gacctc	Intron	25	39
+13863	Int 9	acctcc*[G > A]tatttc	Intron	1	1
+13872	Int 9	tttcct*[C > A]tttctc	Splice junction	0	1
+14444	Int 10	cacaaa*[C > A]taatac	Intron	0	1

African American (AA) and Caucasian (CA). *Genetic variants not in public databases.

5.1.1 Bioinformatics analysis

After obtaining reliable sequencing data, we outlined computational approaches for *in silico* analysis of IDO genetic variants. The first step in this series of experiments involved analysis of the sequencing data in order to predict the functional significance of these amino acid variations. To do this we performed a comparative genomic analysis of *INDO* gene.

The first method used was a FASTA algorithm to compare the human *INDO* gene with four other non-human species; we were able to identify evolutionarily conserved residues as highlighted in Figure 5.1 and Table 5.2. The Arg[77] and two amino acids in the 9 bp deletion (Ala-Leu) were conserved among all 4 species.

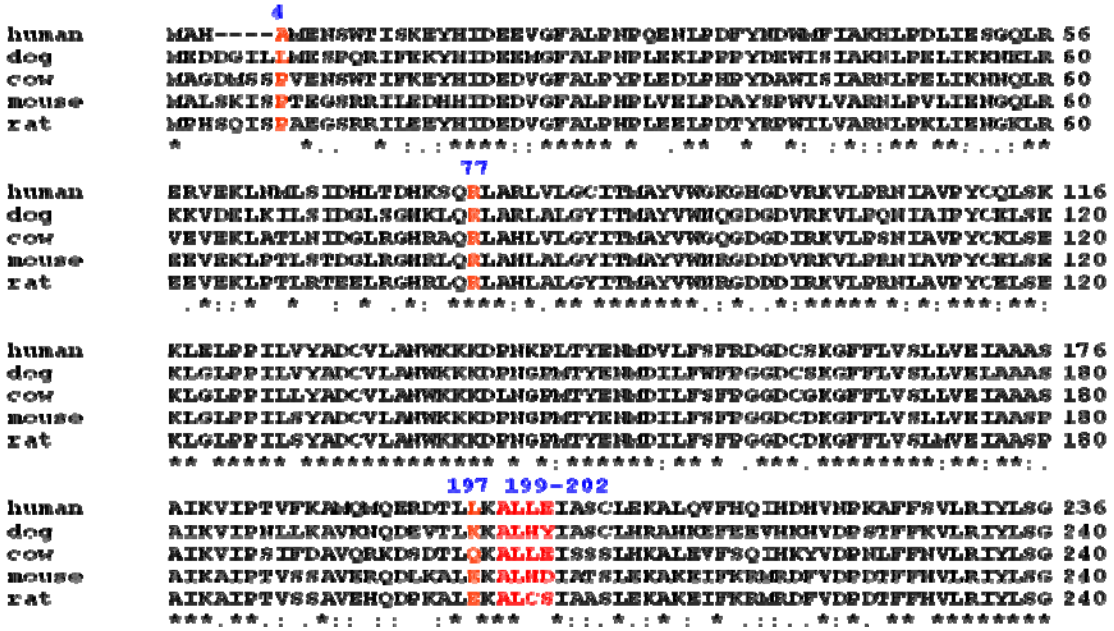

Figure 5.1 Sequence alignments of IDO orthologues. CLUSTALW alignment of amino acid sequences for Human (NP_002155), Dog (XP_532793), Mouse (NP_032350), Rat (NP_076463) and Cow (XP_582707).

Table 5.2 Indoleamine 2,3-dioxygenase comparative genomics: amino acid conservation for human IDO polymorphic residues.

Variant alleles	**Exon 1**	**Exon 3**	**Exon 7**	**Exon 7**
Amino acid	**Ala^{4}Thr**	**Arg^{77}His**	**Leu^{179}Ile**	**(9 base pair deletion) 199-200-201-202 Ala-Leu-Leu-Glu >Asp**
Conservation	**U**	**C**	**U**	**C U$^{@}$**
Human	A	R	L	ALLE
Mouse	P	R	E	ALHD
Rat	P	R	E	ALCS
Dog	L	R	K	ALHY
Cow	P	R	Q	ALLE

Amino acid locations of variant residues in human indoleamine 2,3-dioxygenase are shown. Data for four non-human species are listed. Those amino acids which are conserved among species are highlighted.C-Conserved and U-unconserved. $^{@}$ Partially conserved (Ala-Leu) conserved, but not (Leu-Glu);

The second bioinformatic method used was *P*oly*P*hen (Polymorphism Phenotyping). *P*oly*P*hen predicts the impact of an amino acid substitution on protein structure and function. To make this prediction, it uses both phylogenetic and structural information. *P*oly*P*hen predicted that the Arg^{77}His variation would be potentially damaging (Table 5.3). It also predicted that changing the Arg^{77}Lys residue, which has similar physical and chemical properties would also be damaging. It predicted that the Leu^{179}Ile would be benign. The Ala^{4}Thr could not be analyzed with this software because that region of the molecule was not included in the crystal structure. Likewise, the exon 7 deletion could not be analyzed by this software because it only works for single amino acid changes.

The third method tested was SIFT; it is an algorithm that assigns scores to changes in amino acids on the basis of both residues involved. Using this method, the Ala^{4}Thr and the Arg^{77}His were predicted to be intolerant. The deletion of a 9 bp

fragment in exon 7 replaces four predicted amino acids at position 199-202 (Ala-Leu-Leu-Glu) with an Asp. This minor allele was observed in the 48 African American samples (96 alleles), an allelic frequency of 1%. Using the SIFT prediction tool, the $Leu^{179}Ile$ variant was predicted to be tolerant. These programs are exciting new tools. They may be useful for research investigating a protein by suggesting regions that may be more interesting than others to be studied thoroughly for their function. The few variants that we showed indicate that, in silico investigations should never replace real functional testing of the protein. The main reason that confidence in these programs should be limited is that they can only minimally consider interactions of the particular protein with other molecules, such as chaperones; or part of the protein involved in signaling.

Table 5.3 Human indoleamine 2,3 dioxygenase (IDO) variant amino acid "scoring".

hIDO	**Amino acid changes**	**Scoring system for variant residues**	
		***P*oly*P*hen**	**SIFT**
Wild-type	-	-	-
Exon 1	$Ala^{4}Thr$	NP	IT
Exon 3	$Arg^{77}His$	POS	IT
Exon 3	$Arg^{77}Lys^{\#}$	POS	IT
Exon 7	$Leu^{179}Ile$	BN	T
Exon 7	Ala-Leu-Leu-Glu > Asp	ND	IT/T

The table summarizes the results from *P*oly*P*hen (Polymorphism Phenotyping) SIFT (*S*orting *I*ntolerant *F*rom *T*olerant), # this variant does not occur naturally; (NP) not predicted; (POS) possibly damaging; (BN) benign; (IT) intolerant; (T) tolerant

5.1.2 *In vitro* analysis

We next addressed the possible functional consequences of *INDO* nonsynonymous variants. Based on the bioinformatic analysis above, these appeared to be most likely to have functional effects on IDO enzyme activity. We focused our studies on the nonsynonymous SNPs Ala[4]Thr and Arg[77]His and the 9 bp deletion (Figure 5.2).

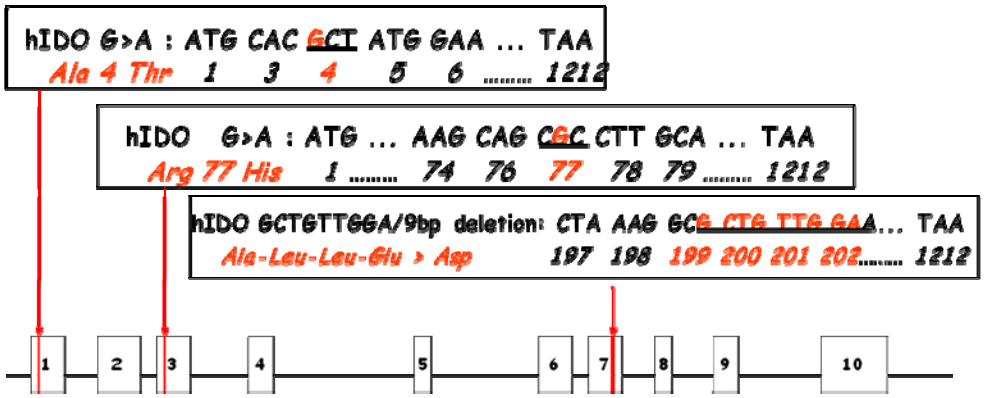

Figure 5.2 Functionally tested indoleamine 2,3-dioxygenase genetic variants. The figure shows a schematic representation of human *INDO* gene with the location of the coding polymorphisms located by arrows. The boxes numbered 1-10 represent the exons. The lines connecting the boxes represent the introns. The arrow in exons 1 and 3 represent the nonsynonymous SNPs. The arrow in exon 7 represents the 9 bp deletion. Two functionally tested nsSNPs in the coding region of IDO, Ala[4] (GCT) replaced with Thr (ACT) and Arg[77] (CGC) replaced with His (CAC). Nucleotide sequences are shown for the start and stop codon of hIDO as well as the respective amino acid changes.

Expression constructs for the three naturally occurring IDO coding variants ($Ala^{4}Thr$, $Arg^{77}His$, 9 bp deletion) and the $Arg^{77}Lys$ as a control construct for $Arg^{77}His$ variant, as well as the wild type were expressed in COS-7 and HEK293 cells. We tested the functional effects of the genetic variants on the IDO enzyme activity.

The wild-type and variant cDNAs were expressed in COS-7 and HEK293 cells using the pcDNA3 vector. These cells were used because they have very low endogenous IDO activity and the transfected wild-type IDO is highly active in these cell lines. Since they are a mammalian cell line, they also contain the post-translational modification machinery and protein degradation systems that may affect the activity of the variant IDO enzymes. To determine the IDO enzyme activity, we analyzed the tryptophan and kynurenine concentrations of the cultured media harvested 48 hr after transfection. Some amount of tryptophan from the culture media is going to be used for protein synthesis by the cells.

Representative chromatograms from wild type and variant IDO proteins are shown in (Figure 5.3). Chromatograms from the wild type and $Ala^{4}Thr$ constructs showed the complete metabolism of tryptophan in the culture media and increased kynurenine peak. In contrast there were no reduction in tryptophan peak in the chromatograms from $Arg^{77}His$ and 9 pb deletion constructs (Figure 5.3).

As expected, the transfection of the wild-type cDNA was highly active. The cultured media contained reduced tryptophan and elevated kynurenine concentrations. The expression of the $Ala^{4}Thr$ resulted in similar tryptophan metabolism. In contrast,

the Arg^{77}His transfected cells had very little metabolic activity. Relative to the wild-type cDNA, the Arg^{77}His kynurenine production was reduced by >90% (Figure 5.4). When the Arg at position 77 was replaced by a Lys, another basic and hydrophilic amino acid, the activity was also reduced, but to a lesser extent. Even though Arg and Lys have similar physicochemical properties replacing of Arg^{77}His with Lys did not restored IDO activity (Figure 5.4). These data suggest that Arg at position 77 play a significant role in IDO activity. This amino acid is also conserved among all the four species analyzed.

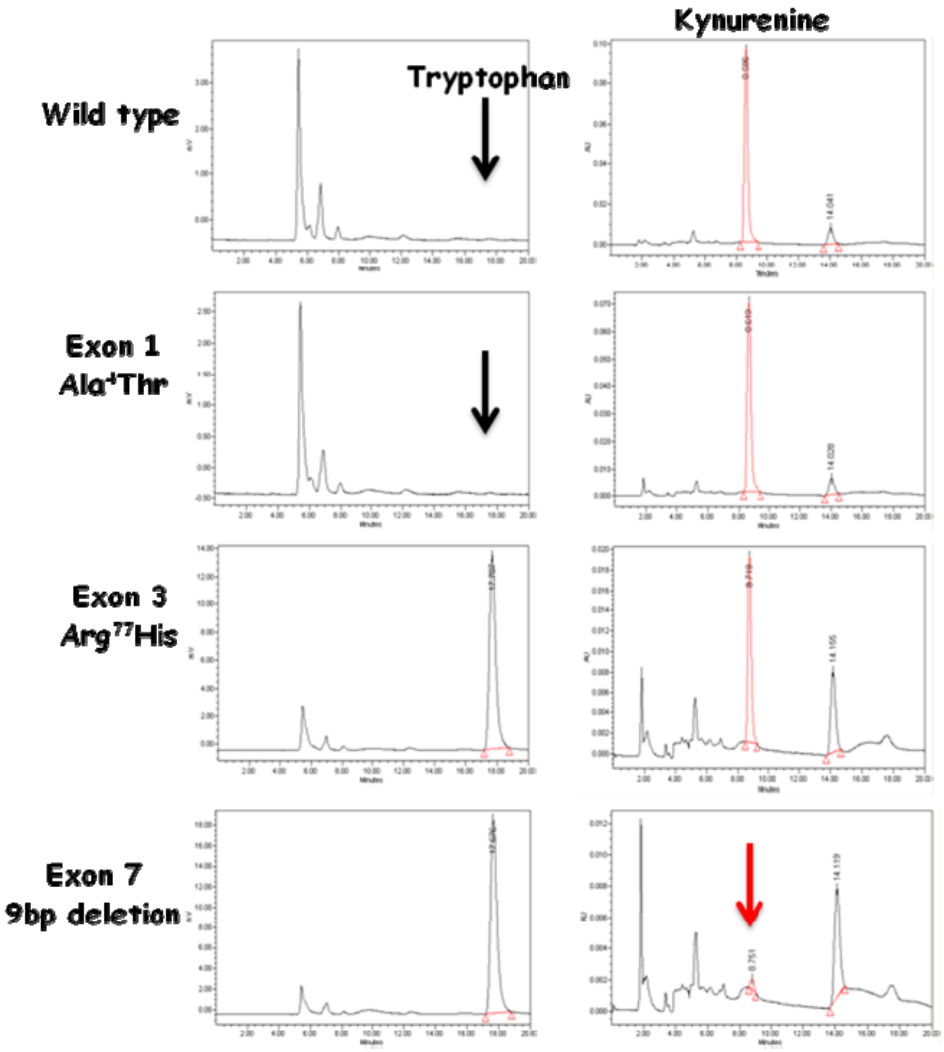

Figure 5.3 Representative chromatograms showing the separation of tryptophan and kynurenine using HPLC from wild type and variant IDO transfected cells**.** Cultured medium from COS-7 cell line transiently transfected with (**from top**) wild type, Ala^{4}Thr, Arg^{77} and exon7-9 bp deletion IDO cDNA was collected after 48 hr, extracted with trichloroacetic acid and the supernatant was loaded on an HPLC. X axis is time in minutes; Y axis is millivolt signal from the detectors, the right panel shows ultraviolet kynurenine detection at 360 nm, the right fluorescence, excitation =285 nm, emmission=365 nm for tryptophan detection.

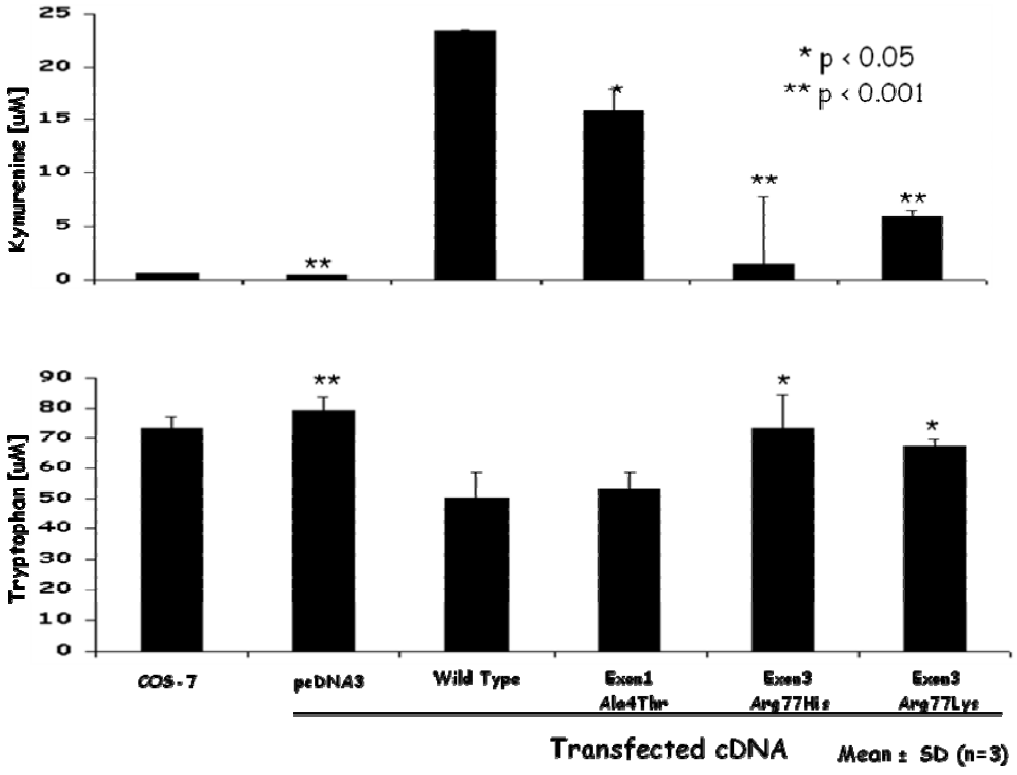

Figure 5.4 The effect of IDO variants on kynurenine formation in COS-7 cells. IDO activity was determined by measuring kynurenine (**top**) and tryptophan (**bottom**) concentrations in COS-7 cells after a 48 hr transient transfection of IDO cDNAs. Tryptophan and kynurenine measured by HPLC. All data are presented as mean ± SD (n=3). *, value ($p<0.05$) significantly different from the value obtained for the wild type compared with $Ala^{4}Thr$. **, value ($p<0.001$) significantly different from the value obtained for the wild type compared with $Arg^{77}His$. $Arg^{77}Lys$ variant, which was not observed in the general population, was a control for the His^{77} variant.

Increasing the amount of plasmid DNA in the transfection resulted in a dose-dependent increase in the kynurenine formation in cells transfected with the wild type and Ala^{4}Thr variant IDO constructs. The dose dependent increment of plasmid DNA did not affect the formation of kynurenine in the Arg^{77}His variant (Figure 5.5). Both wild type and Ala^{4}Thr IDO showed a time dependent increase in the formation of kynurenine, but not the Arg^{77}His variant (Figure 5.6).

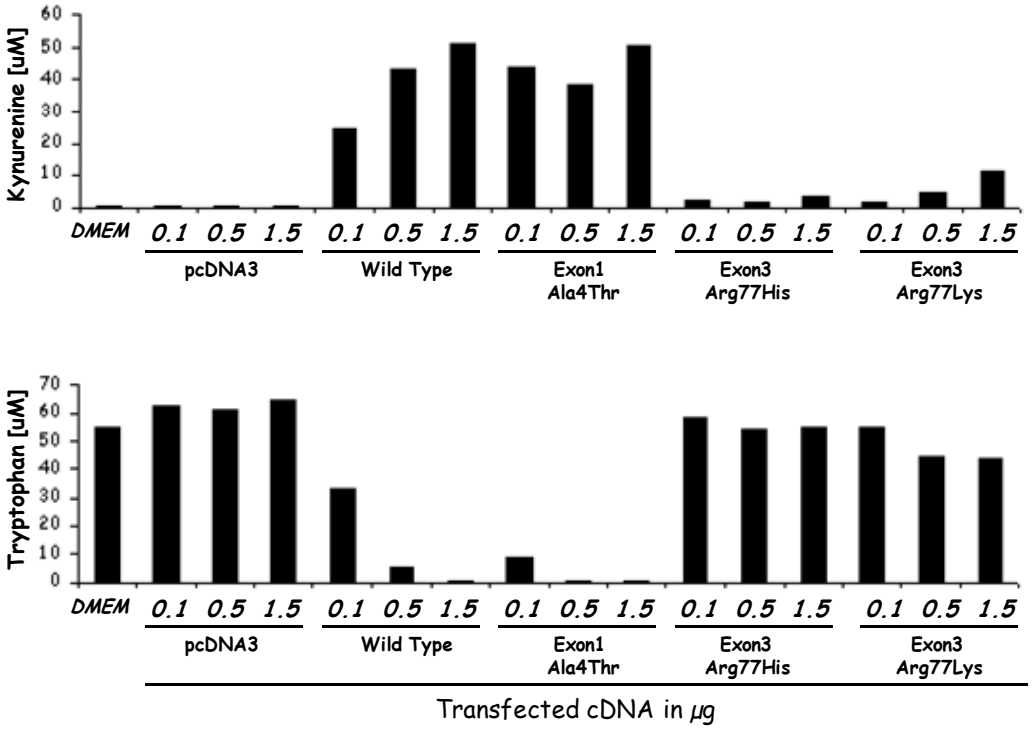

Figure 5.5 The effect of IDO variants on kynurenine formation in HEK293 cells with increasing plasmid concentrations. IDO activity was determined by measuring kynurenine (**top**) and tryptophan (**bottom**) concentrations in HEK293 cells after a 48 hr transient transfection of IDO cDNAs with increasing concentration expression vector. Tryptophan and kynurenine measured by HPLC. All data are presented as mean (n=2). Arg^{77}Lys variant, which was not observed in the general population, was a control for the His^{77} variant.

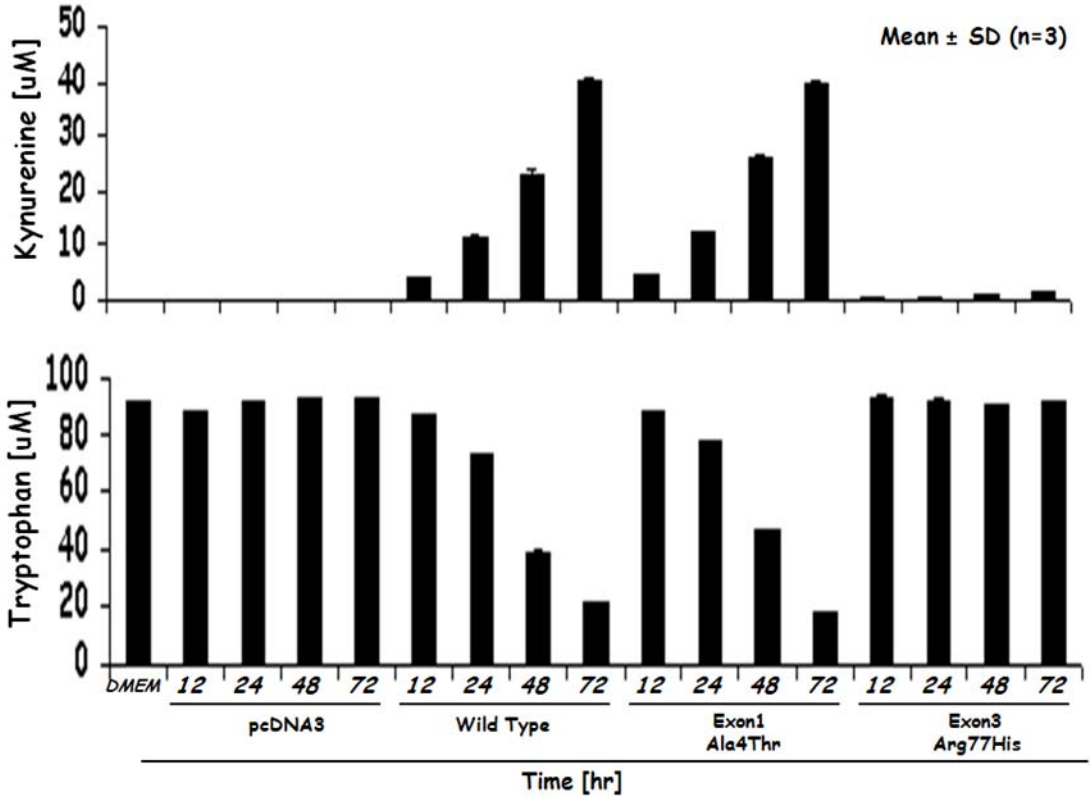

Figure 5.6 The effect of transfection time on kynurenine formation in HEK293 cells in a time dependent manner. IDO activity was determined by measuring kynurenine (**top**) and tryptophan (**bottom**) concentrations in HEK293 cells after 12, 24, 48 and 72 hr transient transfection with 0.5 μg of IDO expression vectors. Tryptophan and kynurenine measured using HPLC. All data are presented as mean ± SD (n=3). $Arg^{77}Lys$ variant, which was not observed in the general population, was a control for the His^{77} variant.

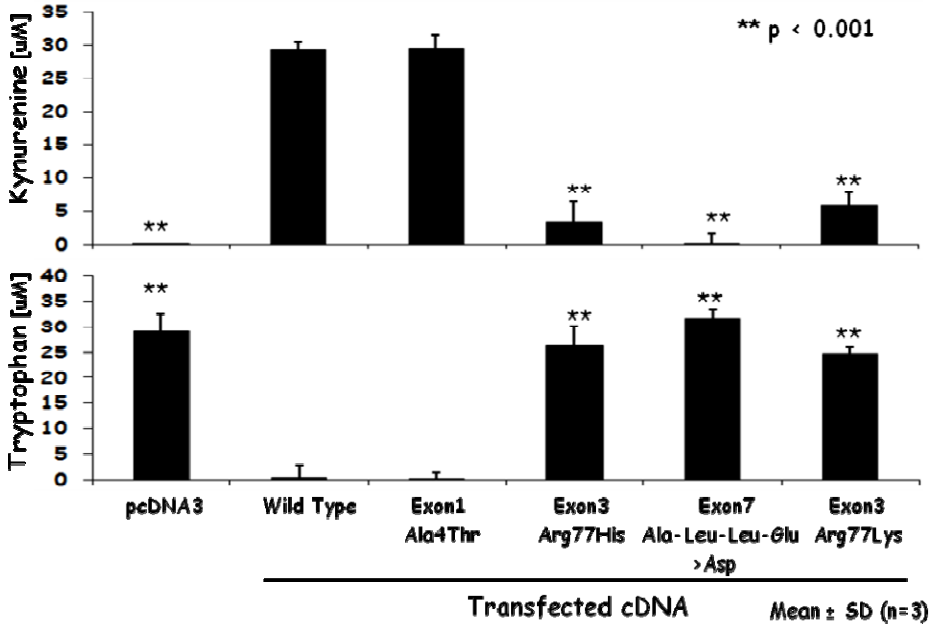

Figure 5.7 The effect of IDO variants on kynurenine formation in HEK293 cells. IDO activity was determined by measuring kynurenine (**top**) and tryptophan (**bottom**) concentrations in HEK293 cell media 48 hr after transient transfection with the IDO cDNAs. Tryptophan and kynurenine measured by HPLC. All data are presented as mean ± SD (n=3), **, value ($p<0.001$) significantly different from the value obtained for the wild type compared with other variants.

A similar reduction in IDO activity was observed (Figure 5.4 and 5.7) in cDNA's expressed in the COS-7 and HEK293 cells using both plasmids (pCMV-SPORT6 and pcDNA3). To verify that the variant IDO mRNAs were being equally expressed from the plasmids in the transient transfected COS-7 and HEK293 cells, we also quantified the IDO mRNA expression. To test the hypothesis that altered IDO activity is because of reduced mRNA expression we used end-point RT-PCR analysis. The data suggest that altered IDO activity in $Arg^{77}His$ and the 9 bp deletion variants were not due to reduced mRNA expression, but it could also potentially be, because of decreased translation or altered post- translational regulation (Figure 5.8).

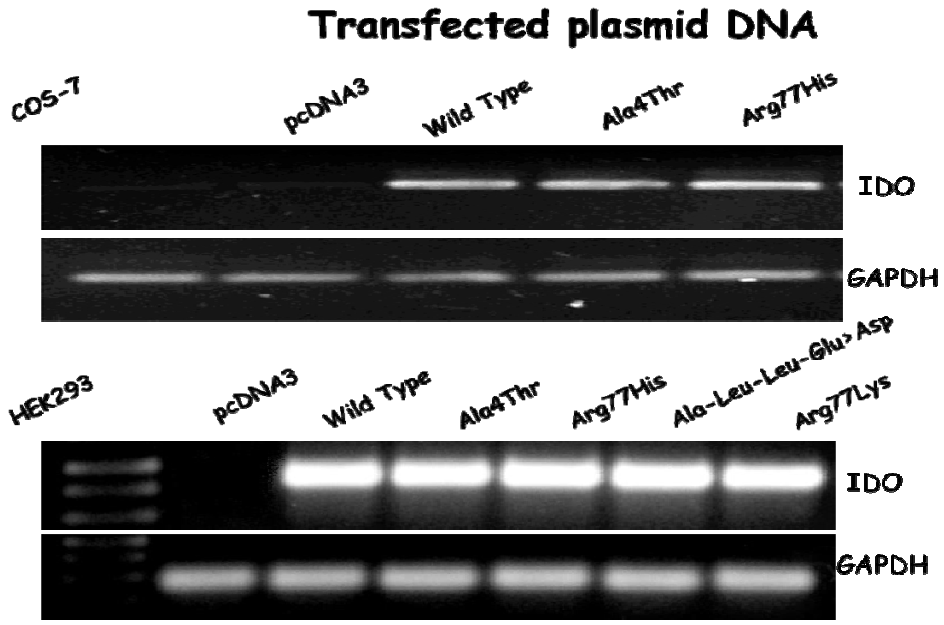

Figure 5.8 IDO mRNA expression in COS-7 and HEK293 cells. The levels of IDO mRNA in COS-7 cells transfected with blank pcDNA3, wild type, exon 1- $Ala^{4}Thr$, exon 3- $Arg^{77}His$ and exon 3 -$Arg^{77}Lys$ IDO cDNA were measured by an end point PCR **(top)**. The levels of IDO mRNA in HEK293 cells transfected with blank pcDNA3, wild type, exon 1- $Ala^{4}Thr$, exon 3- $Arg^{77}His$, exon 7- 9 bp deletion and exon 3 -$Arg^{77}Lys$ IDO cDNA were measured by an end point PCR **(bottom)**. A representative gel of 3 replicates of products. GAPDH was used as the control gene.

To assure that the differences in the enzyme activity in the variant IDO constructs were not due to artifacts in plasmid production, we replicated these studies in several ways. Constructs expressed in two different cell lines using two different types of plasmids as described above suggest that the enzyme activity were similar in both cell lines tested COS-7 and HEK293 (Figure 5.4 and 5.7).

To be certain that the concentrations of wild-type and variant plasmids used for the transfections were similar, we quantified the working dilutions of the plasmids using DNA 7500 chip in the Agilent Bioanalyzer 2100 (Figure 5.9). In addition we also used multiple preparations of the plasmids in the replicates to assure that the reduced activity was not due to an artifact in any of the preparations.

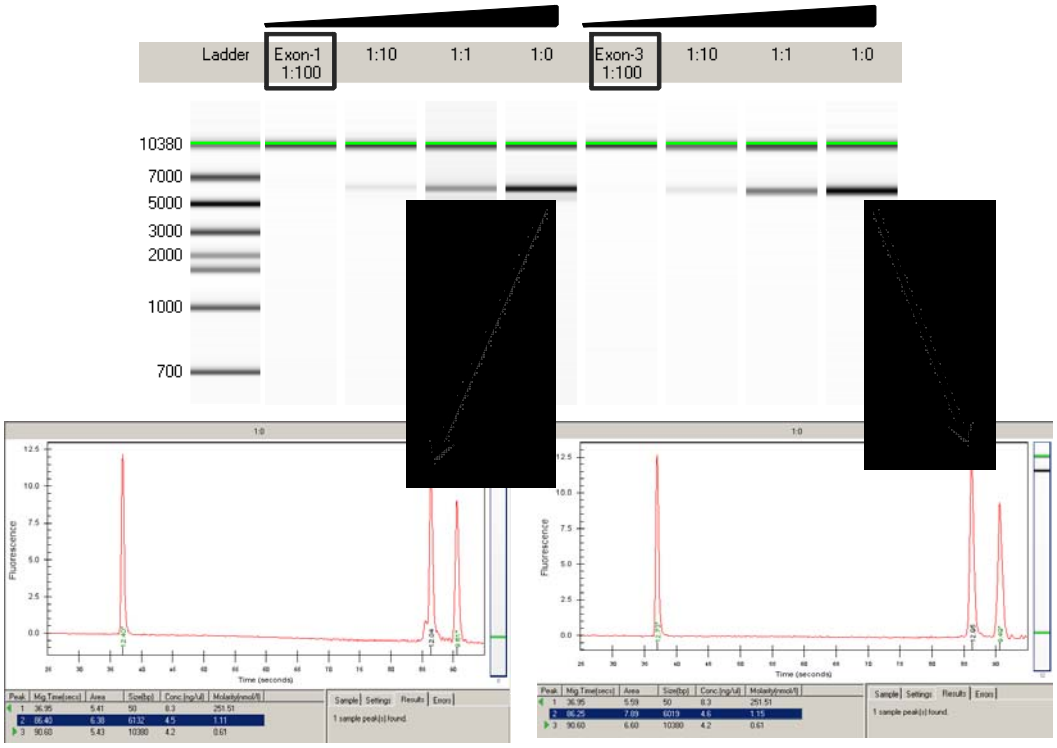

Figure 5.9 Representative pseudo gel from Agilent 2100 Bioanalyzer. From the left a serial dilution of plasmid from exon-1 Ala[4]Thr variant showed the same band intensity as the exon-3 Arg[77]His variant in the right **(top)**. A chromatogram showing a corresponding peak from bands of respective samples (**bottom)**.

Part of this variability could be a result of altered mRNA expression levels transfected cells. To assess this variability, we analyzed our transfected cells using a quantitative RT-PCR assay to determine the effect of the nsSNPs on the IDO RNA concentration. The differences in the mRNA expression levels of the variant IDO mRNAs were not sufficient to account for the large decrease in IDO enzyme activities of the Arg^{77}His and the 9 bp deletion. Similar mRNA expression was seen in the transfected COS-7 and HEK293 cells (Figure 5.10).

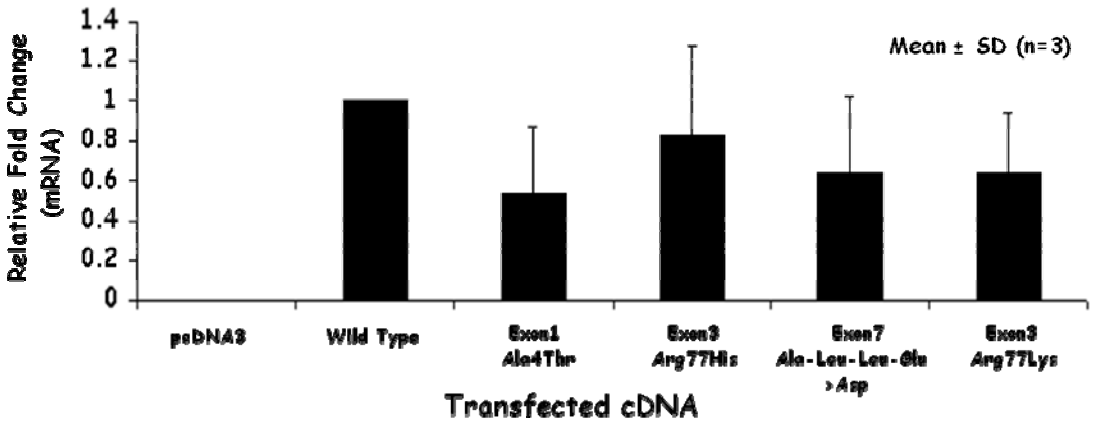

Figure 5.10 IDO mRNA expression in HEK293 cells**.** The levels of IDO mRNA in HEK293 cells transfected with the wild type, exon 1- Ala^{4}Thr, exon 3- Arg^{77}His, exon 7- 9 bp deletion and exon 3 -Arg ^{77}Lys IDO cDNA were measured by real-time SYBR Green RT-PCR. Samples are normalized using GAPDH and the relative fold change was calculated based on the $2^{-\Delta\Delta Ct}$ method. All data are presented as mean ± SD (n=3).

To assess the effects of these genetic variants on the expression of the IDO protein, we harvested HEK293 cells transiently transfected with IDO expression vectors and analyzed them by Western blot. We detected immunoreactive IDO

protein in the cells transfected with the wild-type and the Ala^{4}Thr plasmids; however, the Arg^{77}His and the Arg^{77}Lys showed reduced protein level and no protein was detected for the 9 bp deletion. The negative control, pcDNA3 vector also had no detectable expression (Figure 5.11). These results were consistent with the enzyme activity results, which indicate that the reduced enzyme activity of the variant proteins may be due to altered stability of the variant proteins.

Since the reduced IDO immunoreactivity on the Western blot could be due to reduced antibody binding to the variant IDO protein, we created cDNAs for the wild-type and variant proteins that have an HA-tag incorporated into the C-terminal end of the IDO protein. Similar to the results of the transfections without the HA-tag (Figure 5.11), cells transfected with the HA-tagged cDNAs encoding the Arg^{77}His had reduced tryptophan metabolic activity; likewise, the 9 bp deletion totally abolished tryptophan metabolism (Figure 5.11). Furthermore, similar to the anti-IDO Western blots, when using the anti-HA tag antibody, the cells transfected with the cDNAs encoding for the Arg^{77}His and Arg^{77}Lys variants had reduced immunoreactive HA-tagged IDO protein; and, no protein as detected for the 9 bp deletion. We stripped the blots and probed them using the anti-IDO antibody and obtained similar results to the anti-HA tag Western blots (Figure 5.11).

Levels of IDO protein, as detected by Western blot analysis, did not correlate with the IDO transcript data. For example, no IDO protein was detected for the 9 bp deletion and there was a significant reduction in the IDO protein for the Arg^{77}His variant (Figure 5.9). However, comparable amount of transcript was detected from all constructs. Therefore, the alterations must be at the posttranscriptional level.

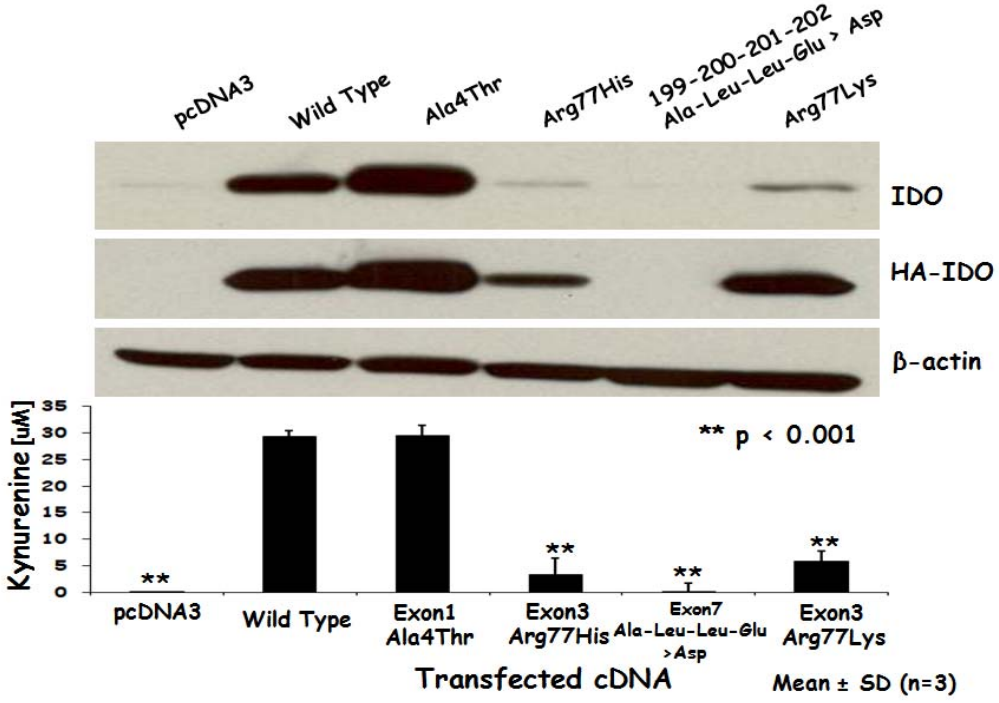

Figure 5.11 IDO expression and activity in HEK293 cells. The HEK293 cells were transiently transfected with plasmids containing cDNAs for HA-tagged wild type, Ala^{4}Thr, Arg^{77}His, 9 bp deletion, and Arg^{77}Lys IDO proteins **(top)**. The amounts of the respective IDO proteins were determined by Western blot using an anti-IDO and anti-HA-tag antibodies. Kynurenine production by the HEK293 cells following transfection with the IDO cDNAs (**bottom**). Values are the concentrations of kynurenine in the media 48 hr after transfection. All data are presented as mean ± SD (n=3), **, value ($p<0.001$) significantly different from the value obtained for the wild type compared with other variants.

One of the limitations of these types of studies is that the activities were evaluated by transient expression systems. To overcome this issue, we established stable cell line expressing IDO wild type and variant constructs and assessed IDO activity using these cell lines. The results from the stable cell lines were also consistent with the transient expression. This provides additional support that the low metabolic activity from the Arg^{77}His was not due to reduced IDO transient expression.

Up to now, we characterized the formation of kynurenine in COS-7 and HEK293 cells transiently expressing different IDO constructs. IDO activity was assessed based on the consumption of tryptophan in the culture media (~80 μM) or by the formation of kynurenine (Figures 5.4. to 5.7). Using a quantitative western blot analysis, corrected for equivalent amount of immunoreactive IDO protein, enzyme kinetic parameters were determined for the wild type and variant proteins. The 9 bp deletion variant was not included in this experiment, since it does not give any detectable IDO activity or protein. Each variant's ability to convert tryptophan into kynurenine was determined and compared to the wild type activity.

In an effort to reveal potential subtle differences in the tryptophan metabolism among the three IDO variants, analysis of tryptophan metabolism was studied using a range of substrate concentrations (Figure 5.12). A representative Western blot for the optimization of equal IDO protein loading is shown in (Figure 5.12 top). These observations represent additional evidence that the alteration of only a single amino acid as a result of genetic polymorphism can significantly alter the level of protein.

Although we observed reduced IDO protein levels due to single amino acid changes, variability could also be a result of altered enzyme kinetic properties. Therefore, we determined the kinetic parameters of wild type, $Ala^{4}Thr$ and $Arg^{77}His$ variants using cell lysate from cells transfected with respective constructs. First, we tested a range of protein concentrations (0, 50, 100 and 200 mg) to choose the optimum protein concentration for the kinetic study (Figure 5.12 bottom). Since IDO can display profound substrate inhibition at high substrate (tryptophan) concentrations, these experiments were conducted in two stages. During the first

stage of experiment, a wide range of substrate concentrations were tested. In the next step, based on the results of the initial studies, the concentration range narrowed to the values appropriate for the determination of K_m and V_{max}. The Arg^{77}His variant showed a significant reduction in V_{max} compared to wild type and Ala^{4}Thr variant (Figure 5.13). The results obtained also showed that both Arg^{77}His variants resulted in a four fold reduction in the intrinsic clearance. The Ala^{4}Thr variant V_{max} and K_m were similar to the wild type (Figure 5.13 and Table 5.4).

The reduction in catalytic activity of Arg^{77}His variant was accompanied by changes in the K_m value which also affected the intrinsic clearance. The Ala^{4}Thr showed the same metabolism profile as the wild type; these data were consistent with the results from cell culture studies (Figure 5.4 and 5.7). Consistent with the transient expression data, the Arg^{77}His variant showed a significant reduction in the metabolism of tryptophan; this suggests that the Arg^{77}His residue is important for IDO activity. Relative to the wild type or the Ala^{4}Thr variant the Arg^{77}His variant had changes in both V_{max} and K_m. The Arg^{77}His variant showed lower apparent substrate affinity as reflected by the higher K_m values compared to the wild-type and the Ala^{4}Thr variant enzyme.

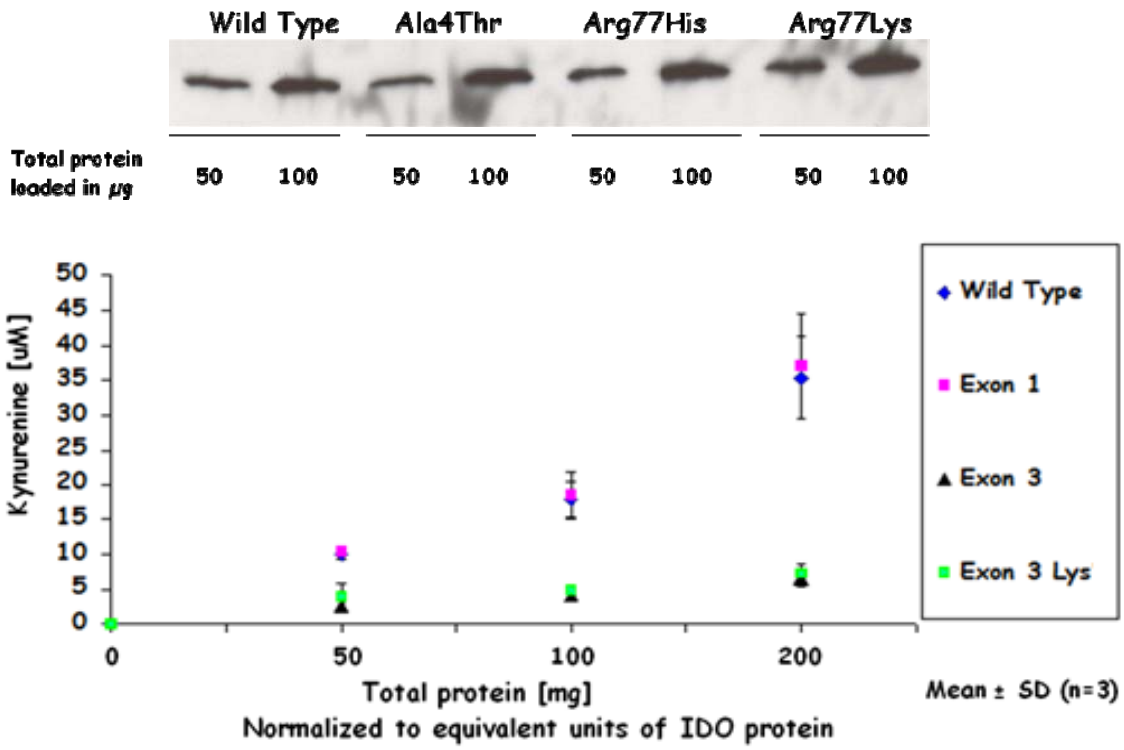

Figure 5.12 Exon 3 Arg^{77}His variant has a reduced activity. Immunoreactive recombinant human IDO expressed in HEK293 cells and enzyme activity. The HEK293 cells were transiently transfected with plasmids containing IDO cDNAs for HA-tagged wild type, Ala^{4}Thr and Arg^{77}Lys respectively (**top**). The amounts of the respective IDO proteins were determined by Western blot using an anti-HA-tag antibody. The formation of kynurnine by respective IDO constructs with increasing normalized units of equivalent IDO protein (**bottom**).

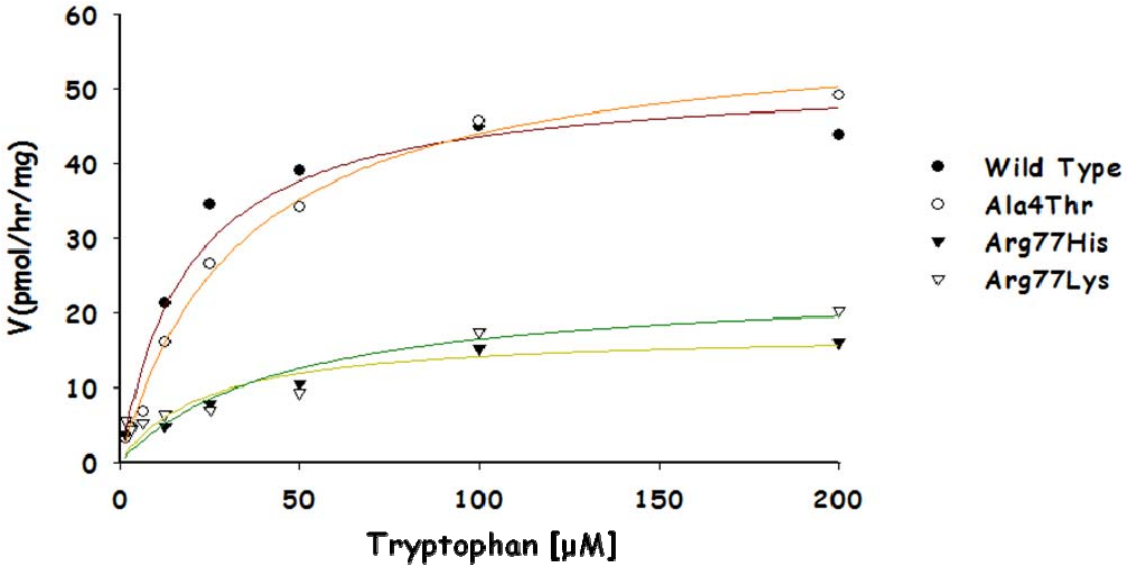

Figure 5.13 Kinetics of tryptophan metabolism. Recombinant wild type and variant IDO proteins (100 mg) were incubated with a range of tryptophan concentrations (0, 3.12, 6.25, 12.5, 25, 50, 100, 200 µM) for 1hr and formation kynurenine measured using a calorimetric assay, as described under material and methods. Representative fitted lines are depicted; parameters estimates were obtained by fitting the kinetics models to actual data points.

Table 5.4 Kinetic parameters estimated for tryptophan metabolism by wild type and variant IDO proteins.

Estimate	Wild type	Ala^{4}Thr	Arg^{77}His	Arg^{77}Lys
V_{max}	52.2 ±1.0	53.0 ± 5.6	19.7 ± 3.1**	22.7 ± 6.9**
K_m	18.0 ± 4.5	23.4 ± 9.1	29.6 ± 11.8	34.2 ± 18.2
V_{max}/K_m	3.0 ± 0.8	2.4 ± 0.7	0.70 ± 0.2*	0.78 ± 0.3*

Data are expressed as estimate ± SD., n=3, estimate unites are as follows: V_{max}, pmol/hr/mg; K_m, micro molar; V_{max}/K_m, ml/min/mg. **, value ($p<0.001$) and * ($p<0.005$) significantly different from the value obtained for the wild type compare with Arg^{77} variant.

The reduction in IDO expression and activity in the Arg^{77}His variant (Figure 5.11 and 5.13) could be due to accelerated degradation. To assess this possibility, we used the TNT-RRL system to transcribe and translate wild type and variant proteins to *in vitro.* IDO activity was determined by using recombinant protein translated in the

TNT-RRL system. In contrast to the other experiments, the Arg^{77}His variant exhibited IDO activity that was similar to the wild type (Figure 5.14). This was a surprising observation, because in the transient expression system the Arg^{77}His variant exhibited a dramatic reduction in IDO activity.

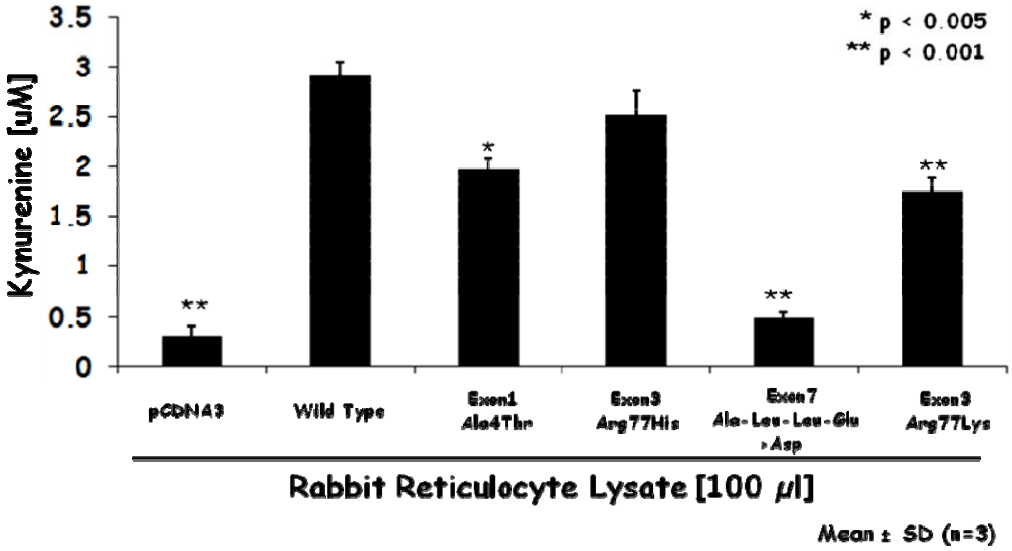

Figure 5.14 IDO enzyme activity assessment using protein translated in the rabbit reticulocyte lysate [TNT-RRL] system. IDO activity was determined by measuring kynurenine concentration using IDO protein translated in the rabbit reticulocyte lysate (RRL) system. Kynurenine measured by HPLC as described in the methods section. IDO activity was determined by measuring kynurenine in the cell lysate, values shown are mean ± SD for three independent experiments **, values ($p<0.001$) and * ($p<0.005$) significantly different from the value obtained for the wild type compared with other variants.

Protein degradation inhibitors and IDO activity

Since the TNT-RRL results differ from the transient transfection data, we explored possible mechanisms. One possibility is that the hemin in the TNT-RRL system might affect IDO activity. This was a likely scenario since the results from our previous studies demonstrated that the Arg^{77}His and 9 bp deletion resulted in the

reduction of both protein expression and enzyme activity. Furthermore, it has been shown in a number of studies that coding variants potentially result in structural disruption and misfolding of newly translated proteins [109]. One of the most common mechanisms by which misfolded proteins are removed from the cell is by accelerated degradation. This degradation is usually through the Ubiquitin Protesome Pathway (UP)[145]. The misfold proteins can also form aggregates [109]. To keep a dynamic balance between protein degradation and aggregation mammalian cells have developed a highly regulated protein quality control mechanism [102, 103, 109] (Figure 2.1).

To further analyze this observation, transiently transfected cells were treated with hemin in a dose dependent manner. The effect of hemin in restoring IDO activity in the Arg^{77}His construct became apparent for the first time during the use of *in vitro* translated proteins using TNT-RRL system (Figure 5.14). This *in vitro* translation kit, rabbit retculocytes are treated with 20 μM hemin (Figure 5.15), because it suppresses an inhibitor of the initiation factor elF2α. In the absence of hemin, protein synthesis in the Reticulocyte Lysate System will cease after a short period of incubation [146].

We tested if hemin has the same effect in transiently transfected HEK293 cells. In cells transfected with Arg^{77}His hemin showed a dose dependent increase in the formation of kynurenine (Figure 5.16 and 5.17). Hemin restored IDO activity in the Arg^{77}His variant without increasing the immunereactive protein (Figure 5.16). This data suggest that hemin might stabilize the Arg^{77}His variant IDO protein and increased formation of kynurenine without affecting protein concentration.

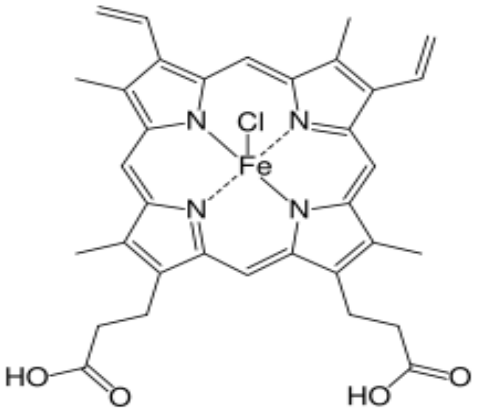

Figure 5.15 Molecular structure of hemin (Adapted from Sigma-Aldrich).

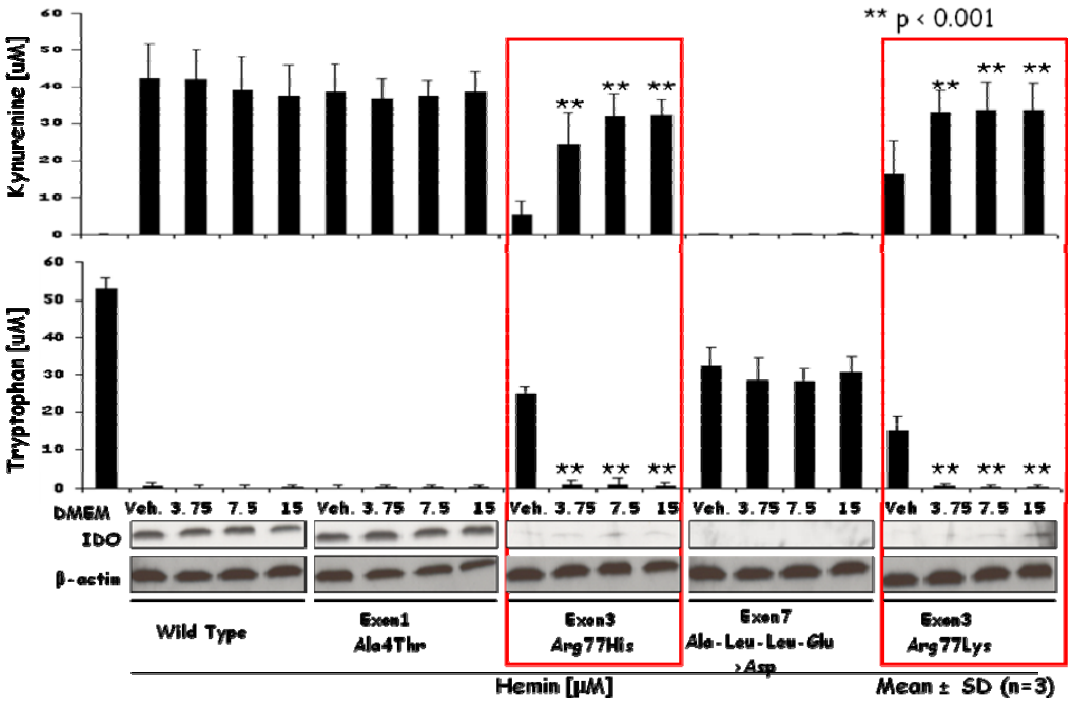

Figure 5.16 The effect of exposure to increasing concentration of hemin on IDO activity. HEK293 cells transfected with a wild type and variant IDO constructs and treated with increasing concentration of hemin. IDO activity was determined by measuring kynurenine (**top**) and tryptophan (**bottom**) concentrations in HEK293 cell media after 24 hr. Tryptophan and kynurenine measured by HPLC. Values shown are mean ± SD for three independent experiments **, value ($p<0.001$) significantly different from the value obtained for the wild type compared with other variants.

Since hemin can affect multiple cellular processes, we also tested additional proteosomal inhibitors to confirm that the effect was through the proteosome inhibition (Figure 5.16). Two specific proteosomal pathway inhibitors were tested. The first one MG132 is an aldehyde -based reversible inhibitor. The second is lactocystin, a lactone- based irreversible inhibitor. Neither inhibitor changed IDO activity in any of expressed IDO proteins (Figure 5.17). We also tested the lysosomal pathway inhibitor, chloroquine. It did not affect tryptophan metabolism in the $Arg^{77}His$ expressing cells (Figure 5.18).

The results presented in this study strongly suggest that the mechanism underlying the altered protein expression in the $Arg^{77}His$ and the 9 bp deletion is a translational or post-translational event. The 9 bp variant yielded virtually no detectable protein when expressed in HEK293 cells (Figure 5.11). Furthermore, data from the $Arg^{77}His$ variant showed a decreased level of protein, which at least in part, could be due to accelerated degradation pathway. Exposure of cells with proteasome pathway inhibitors (MG132 and lactocystin) after transient transfection with $Arg^{77}His$ construct did not increase in IDO activity (Figure 5.17). These proteasome pathway inhibitors bind to the 26S-proteasome and prevent degradation of short –lived proteins [147]. Likewise inhibition of the lysosomal pathways with chloroquine did not affect the $Arg^{77}His$ activity (Figure 5.18).

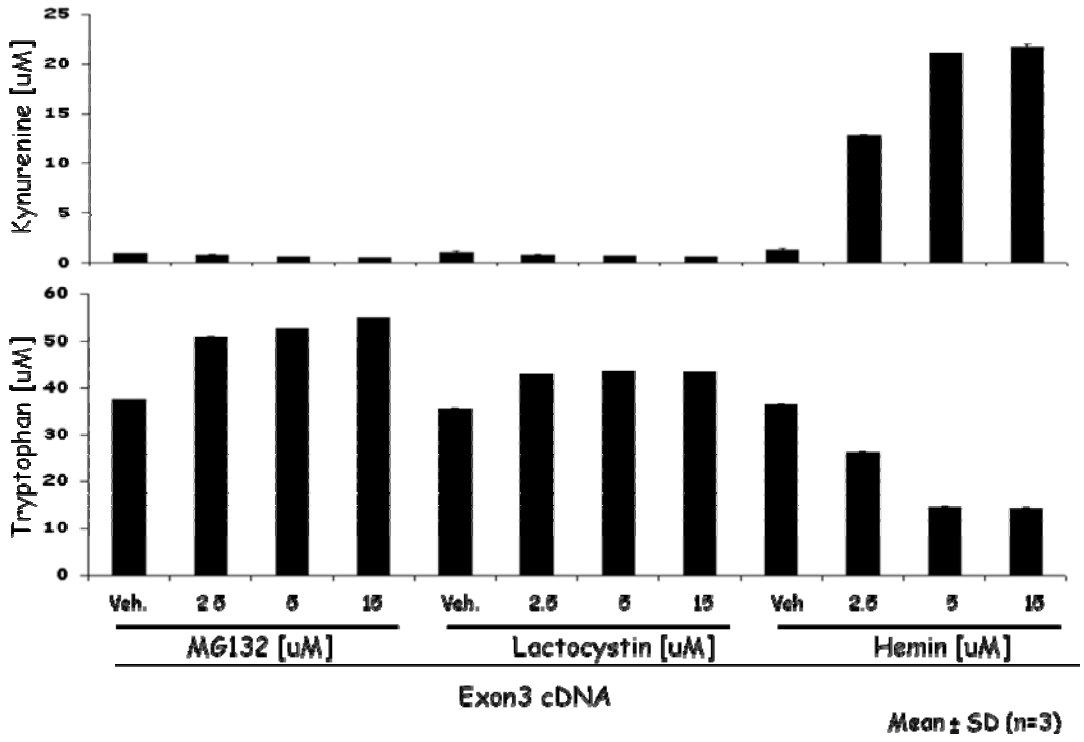

Figure 5.17 The effect of proteasome inhibitors MG132, lactocystin and hemin on $Arg^{77}His$ activity. IDO activity was determined by measuring kynurenine (**top**) and tryptophan (**bottom**) concentrations in HEK293 cells treated with increasing concentration of proteasome inhibitors after a transient transfection with the $Arg^{77}His$ construct. Tryptophan and kynurenine measured by HPLC. Values shown are mean ± SD for three independent experiments.

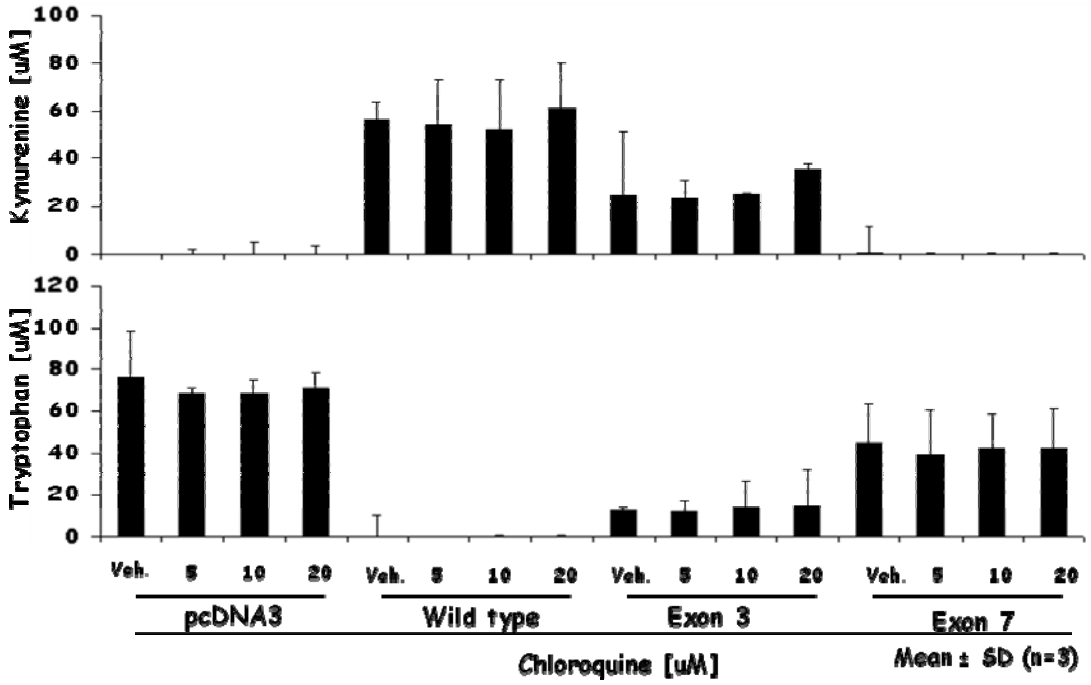

Figure 5.18 The effect of lysosomal pathway inhibitor on IDO activity in HEK293 cells. IDO activity was determined by measuring kynurenine (**top**) and tryptophan (**bottom**) concentrations in HEK293 cells transiently transfected with wild type exon3 ($Arg^{77}His$) and exon7 (9 bp deletion) IDO constructs and treated with increasing concentration of lysosomal pathway inhibitor. Tryptophan and kynurenine measured by HPLC. Values shown are mean ± SD for three independent experiments.

Hemin increased IDO activity in HEK293 cells transiently transfected with the $Arg^{77}His$ IDO variant in a dose dependent manner (Figures 5.16, 17 and 21). The increase in IDO activity occurred without increasing detectable immuno reactive IDO protein as measured by Western blot (Figure 5.16). This suggests that hemin might be

working through another non proteosomal mechanism. One such mechanism may be through restoring the cellular heme concentration. Since heme is required for IDO activity incorporation of heme into the IDO protein could be the rate limiting factor in IDO activity in the $Arg^{77}His$ variant.

To address this possibility we assessed heme concentrations in HEK293 cells transfected with wild type and variant constructs. Heme concentrations were significantly lower in HEK293 cells transfected with the variant constructs compare with wild type and $Ala^{4}Thr$ variant. Heme concentrations for the variants were in the 2-5 μM range, but for the wild type and $Ala^{4}Thr$ were up in the 30-35 μM (Figure 5.19). The interesting finding in this study was that the heme concentration correlated with the kynurenine production. Furthermore, the addition of hemin, which can substitute for heme, restored the IDO activity. This provided strong evidence that heme incorporation into the IDO may be a limiting factor. This promoted further investigation into the role of endogenous heme in the IDO activity (Figures 5.19 bottom).

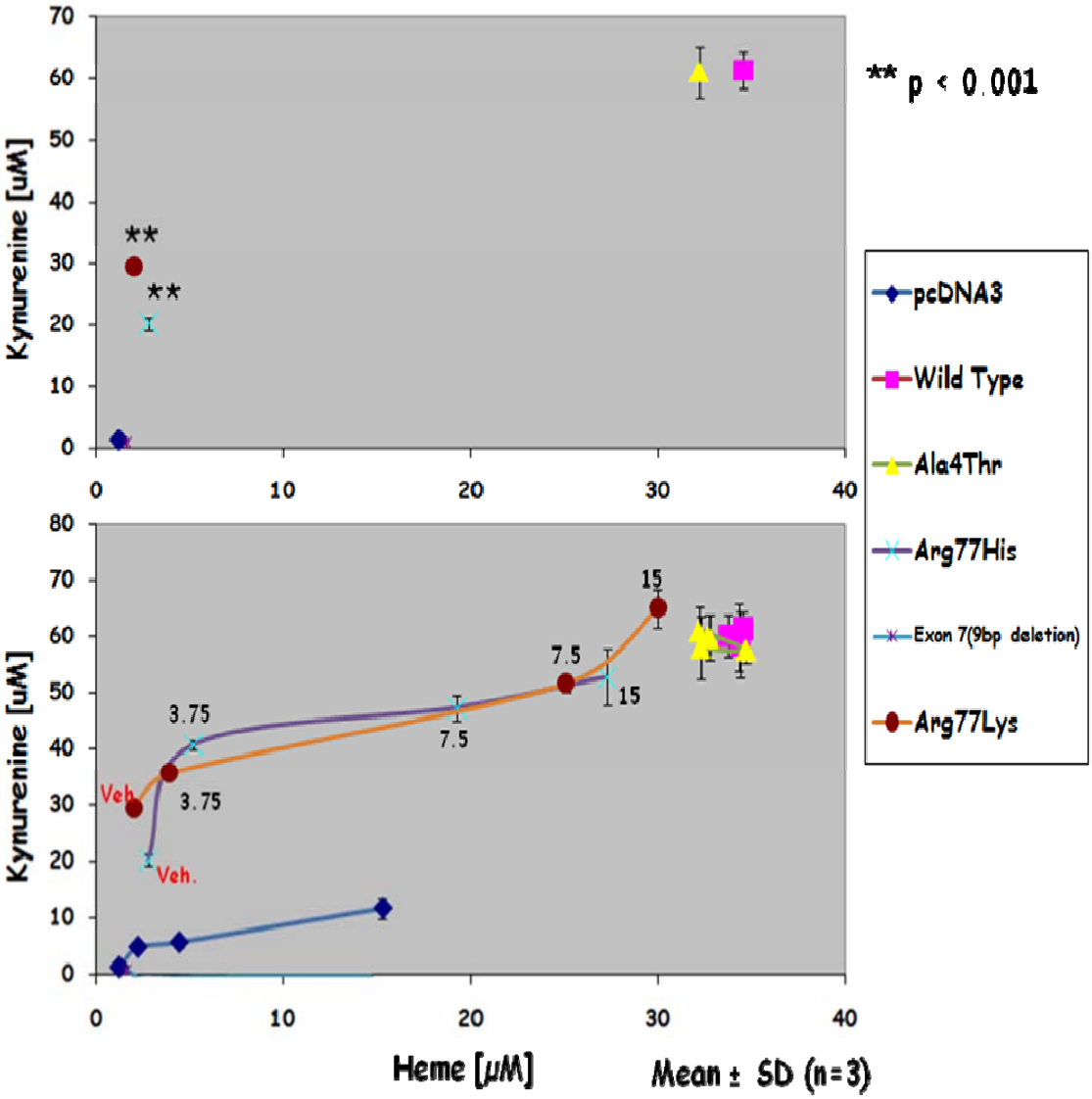

Figure 5.19 The effect of exposure to increasing concentration of hemin on IDO activity and heme concentration. IDO activity and heme concentrations were assessed after the transfection of HEK293 cells with a wild type and variant IDO constructs. IDO activity was determined by measuring kynurenine (y-axis) and heme (x-axis) concentrations in HEK293 cell media 24 hr after hemin exposure (**top**) only vehicle and (**bottom**) vehicle, 3.75, 7.5 and 15µM. Kynurenine was measured in the media and heme concentration measured in the cell lysate from respective constructs. Kynurenine and heme were measured using a plate reader. IDO activity determined values are shown as means ± SD for three independent experiments. **, value ($p<0.001$) significantly different from the value obtained for the wild type compared with $Arg^{77}His$ variant.

Since *de novo* synthesis of heme is important for IDO activity, we further evaluated the impact of the Succinyl acetone is a potent heme synthesis inhibitor succinylacetone (SA). Succinyl acetone is an inhibitor of 5-aminolevulinic acid dehydratase, the second enzyme involved in heme synthesis [148]. To examine the role of SA, HEK293 cells were pretreated with 500 μM SA for 12 hr; cells were then transiently transfected with wild type or $Arg^{77}His$ variant IDO. IDO activity was assessed by measuring kynurenine formation in the culture media (Figure 5.20).

In the wild type cells, the inhibition of heme synthesis substantially reduced IDO activity. In the $Arg^{77}His$ cells, the SA completely abolished the formation of kynurenine (Figure 5.20). This experiment showed that heme synthesis is necessary for optimal IDO activity. The effect of SA on cell viability has tested using Trypan Blue Stain and there was no difference between the SA and vehicle treated cells. Since we did not analyz IDO mRNA after SA treatment it is possible that SA could affect the mRNA concentration. Next we measure heme concentration in the cell lysates after SA treatment and correlated it with IDO activity (Figure 5.21). The inhibition of IDO activity in the $Arg^{77}His$ transfected cells were reversed by addition of hemin (Figure 5.19). The hemin effect was dose dependent (Figures 5.16, 5.19 and 5.21). Since, hemin did not increase the protein concentration; it appears not to be through the proteasome pathway (Figure 5.18). Hemin also restored IDO activity in cells transiently transfected with the wild type IDO construct at low dose. SA clearly reduced IDO activity in the wild type, as the $Arg^{77}His$ variant (Figures 5.20 and 21). Together, these results suggest that hemin restores the $Arg^{77}His$ IDO activity by

increasing the incorporated heme into IDO in the cell and increases the modification of the apo to holo enzyme. Hemin did not increase heme concentration in cell that were pre treated with SA (Figure 5.21) compared to cells that were not treated (Figure 5.19) which suggest that SA has inhibited *de novo* synthesis of heme and reduced the heme concentration (Figure 5.21).

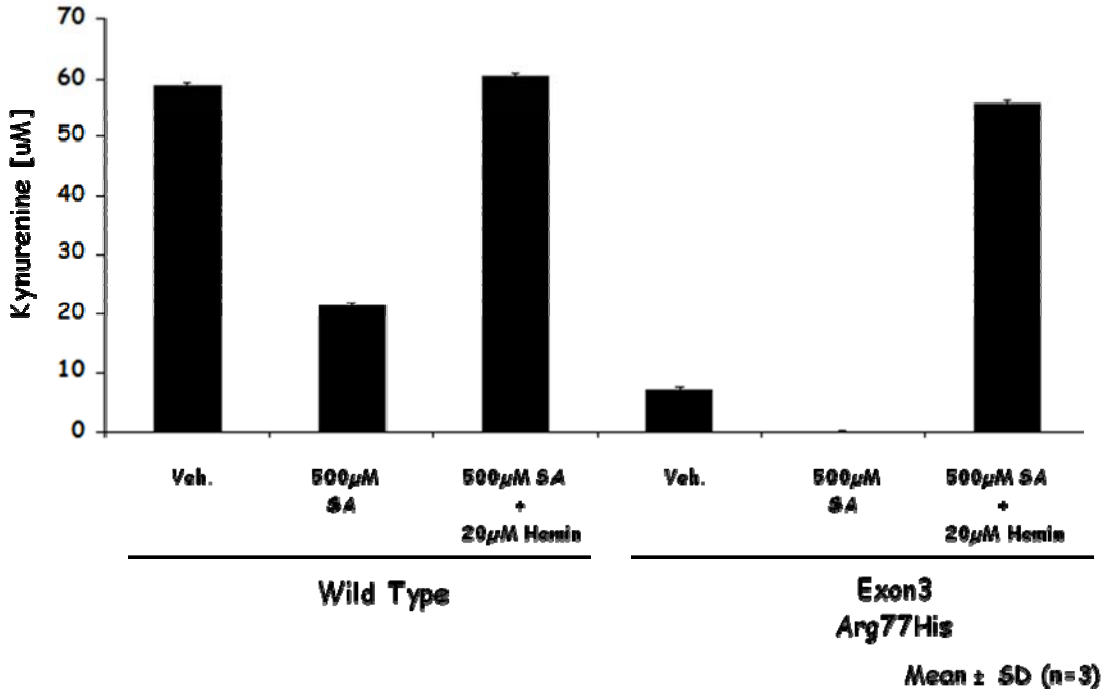

Figure 5.20 The effect of the heme synthesis inhibitor succinylacetone (SA) on IDO activity. IDO activity was determined by measuring kynurenine concentrations in HEK293 cell media 24 hr after SA treatment. HEK293 cells transiently transfeted with wild type and $Arg^{77}His$ constructs after SA treatment. The effect of SA reversed by hemin exposure 12 hr after transient transfection with IDO cDNAs. Values shown are mean ± SD for three independent experiments.

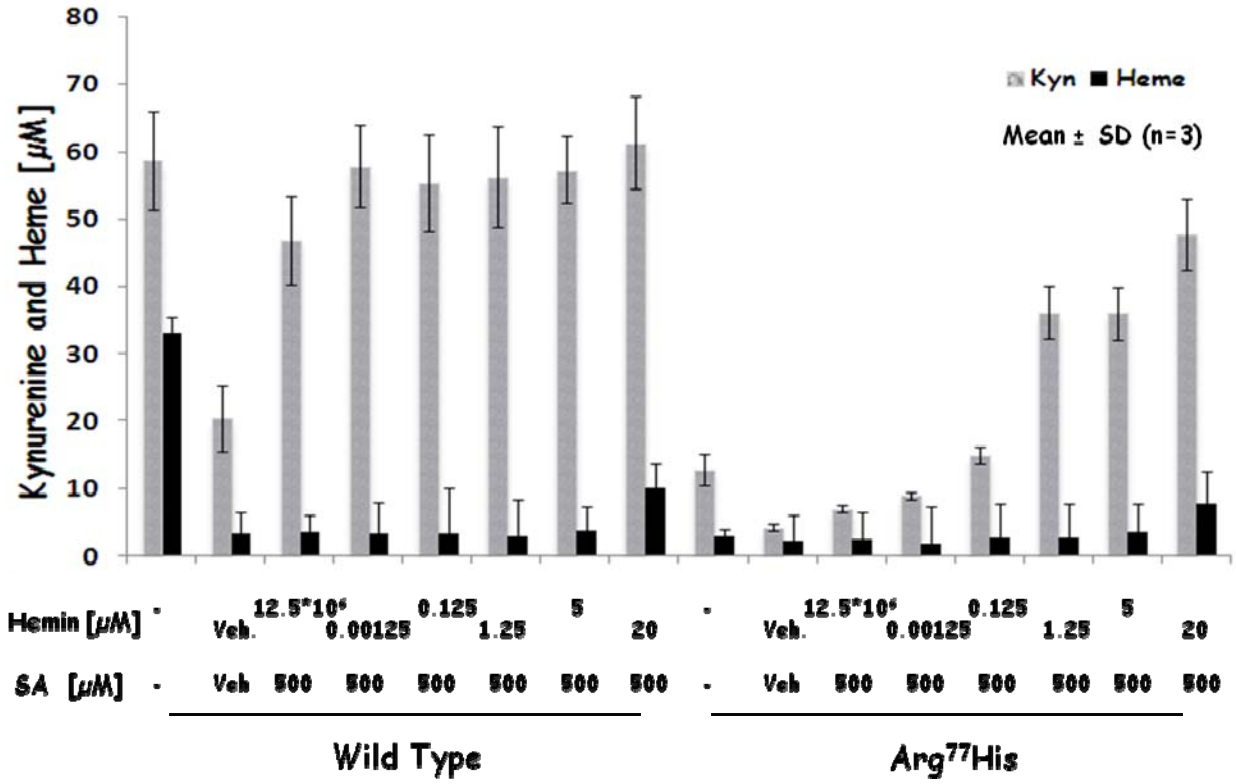

Figure 5.21 The effect of the heme synthesis inhibitor succinylacetone (SA) on IDO activity in HEK293 cells transiently transfeted with wild type and Arg^{77}His construct and its response to hemin. IDO activity was determined by measuring kynurenine concentrations in HEK293 cell media 24 hr after 500 μM SA treatment. The effect of SA dose dependently reversed by hemin exposure 12h after transient transfection with IDO cDNAs. Values shown are mean ± SD for three independent experiments.

Since the X-ray crystal structure of human indoleamine 2,3-dioxygenase has been solved [149], we were able to map the Arg^{77}His and the 9 bp deletion to the 3-D structure of the IDO protein**.** The consequence of genetic variants in the regions that are directly part of a functional domain, such as substrate binding pocket of the enzyme, are likely to cause a more severe phenotype than variation in regions of less

functional importance. In the crystal structure, the first 11 amino acids were not determined; therefore, the Ala^{4}Thr could not be mapped. Neither the Arg^{77}His nor the 9 bp deletion appeared to be part of the substrate recognition or heme binding site (Figure 5.22). Both variants are upstream from the highly conserved substrate recognition site Phe^{226}, Phe^{227} and Arg^{231} identified by site directed mutagenesis experiments [149]. However, it is possible that the changes indirectly alter those binding sites through changes in the protein folding or structure.

The Arg^{77}His amino acid and the first two amino acids affected by the 9 bp deletions were strictly conserved among vertebrate IDO orthologs. The variant affecting residue Ala^{4}Thr did not show any functional impairment which is also not conserved among the vertebrate orthologs compared (Figure 5.1 and Table 5.2). This observation suggest that residues Arg^{77}His and (199-202) Ala-Leu-Leu-Glu replaced by Asp due to the 9 bp deletion are important in maintaining normal IDO function. In our experimental system, 9 bp deletion variant abolishes enzyme function completely. This supports the hypothesis that conserve residues are of important for maintaining normal enzyme function.

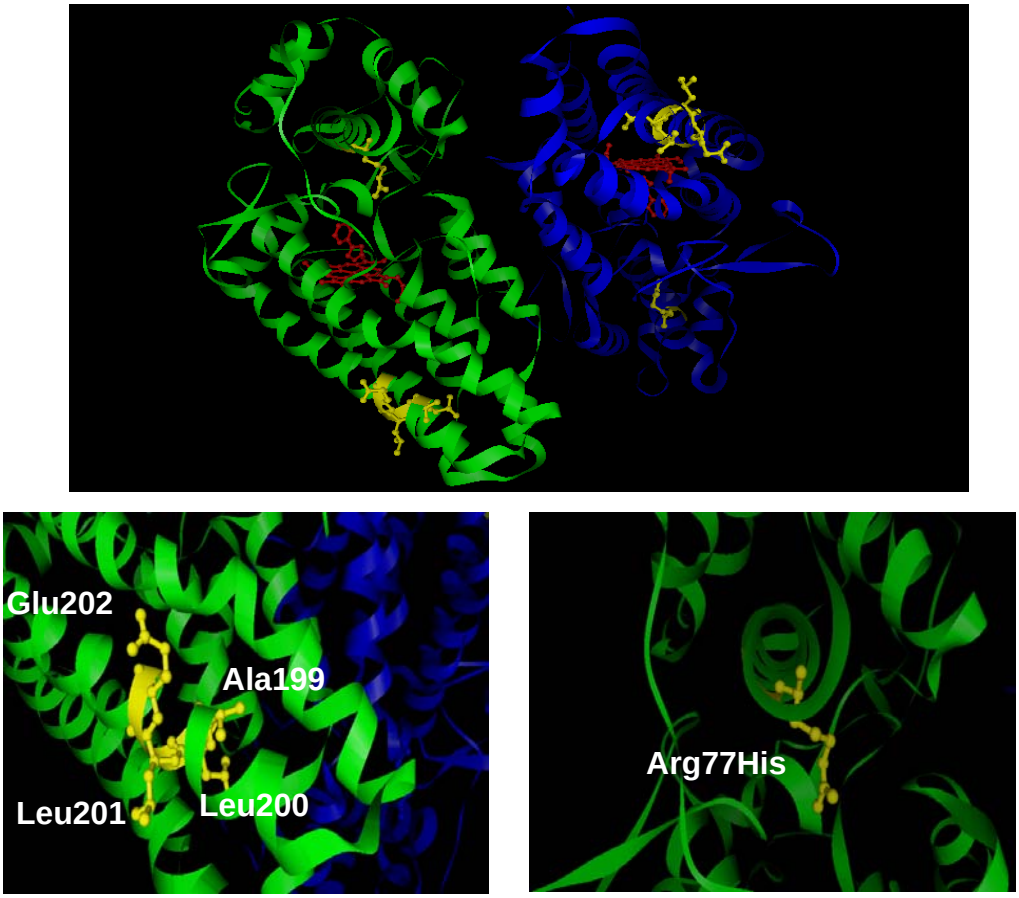

Figure 5.22 Location of the variant positions in the human indoleamine 2,3-dioxygenase crystal structure. **Top,** The ribbon representation of the overall structure of human Indoleamine 2,3-dioxygenase (PDB entry code 2D0T). Each monomer has small and large domains which create a cavity for the heme where the substrate binding site is located. **Bottom left,** Close up of the structure around the four amino acids (Ala-Leu-Leu-Glu>Asp) at a position 199-202. **Bottom right,** The single nucleotide polymorphism on exon 3 causes the change of $Arg^{77}His$. The red and green are separate monomers as taken from the PDB. The yellow are the amino acids that are changed by the genetic variants. The red is the heme and the substrate in the binding site.

5.2 Specific Aim 2: To identify genetic variants in the IDO promoter and to assess their functional significance.

We resequenced portions of IDO promoter to identify and functionally characterize genetic variants which could affect IDO expression.

In the present study, we identified seven novel genetic variants in the 5'-flanking region of *INDO* gene (Table 5.5).

Table 5.5 Genetic polymorphisms in the 5'-flanking region of *INDO* gene. [1]The nucleotide positions referred to are relative to the translation start site. The numbering is based on accession NM_002164. Allelic frequencies are the number of variant alleles out of the 96 alleles (48 subjects) in each group.

Position	Base pair change	CA (n=96) #	AA (n=96) #
-1144	cctatt (C > G)aaggaa	0	1
-1135	agggaa(C > T)attttcc	0	3
-1113	atgaca(G > T)gttgaa	0	3
-1104	gttgaa(G > A)aaaaca	0	3
-1081	ttagta(G > C)agaaata	0	4
-742 [FOXC1]	tattac(A > G)ttattag	1	0
- 624 [GATA3]	aatgct(A > G)tcattgg	1	0
[@]-89 5'UTR	gataaa(C>T)tgtggt	3	0

African American (AA) and Caucasian (CA). # number of variant alleles identified in a total of 96 alleles (48 samples).[@] This SNP discovered during the resequencing of the coding region (aim 1), and has functionally tested in aim 2.

5.2.1 Bioinformatics analysis

The mechanisms by which IDO expression is regulated differently in different people remains unknown. This makes it difficult to ascertain whether variable IDO expression is a cause or an effect of the variability in therapy outcomes. Thus, knowledge concerning the molecular mechanisms involved in IDO gene expression is of significant importance.

Gene expression, and in particular transcription, in eukaryotic cells is an important process that is under complex regulation. The complex interaction of transcription factors with the transcription binding motifs could be affected by genetic variants in the promoter of the gene.

To identify the presence of important transcription-factor-binding sites, we used JASPAR and TRANSFAC software's, we focused our search on the 1.3 kb of the 5'-flanking region. This 5'-flanking region revealed a variety of putative transcription factor binding sites (Figure 5.23 and Table 5.6). As shown in Figure 5.23, two of the novel SNPs are located within the putative transcription factor binding sites FOXC1 and GATA3. When we analyze the variant sequence with these transcription factor binding sites the prediction were below the threshold.

IDO PROMOTER SEQUENCE

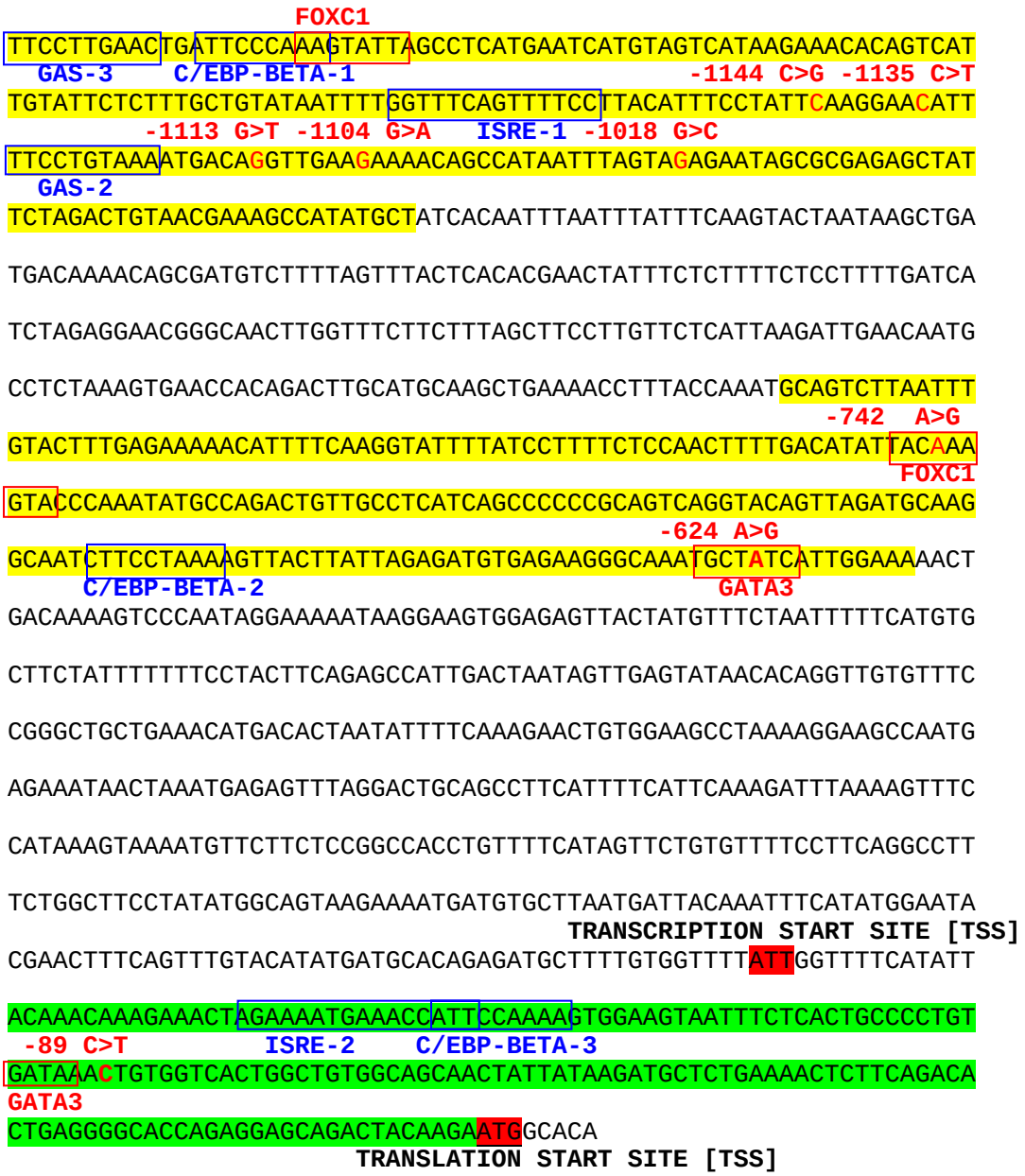

Figure 5.23 DNA sequence of the 5'-flanking region of the *INDO* gene. The SNPs that we functionally tested are highlighted in red. The two newly identified transcription binding sites -742 FOXC1 and -624 GATA3 are also highlighted in red boxes. Transcription factor binding sites that have been characterized before (ISRE-Interferon Stimulating Response Element, C/EBP-Beta -CCAAT/Enhancer binding protein-beta, GAS-Gamma activating sequence are labeled in (Blue boxes). The sequences covered during the 5'-flanking region resequencing are highlighted in yellow. The 5'-UTR region highlighted in green and it was covered during the coding region resequencing.

Table 5.6 Twenty-two putative TFBS were predicted using JASPAR (95% relative score threshold). Two SNPs were discovered within TFBSs: FOXC1 and GATA3.

Start from upstream	Model Name	Relative Score	Predicted site sequence	Actual sequence
1	FOXC1	0.958	CCAAAGTA	CCAAAGTA
2	IRF1	0.951	AAAACTGAAACC	GGTTTCAGTTTT@
3	SPIB	0.967	ACAGGAA	TTCCTGT@
4	GATA3	0.988	TGATAG	CTATCA@
5	FOXC1	0.955	TGTGAGTA	TACTCACA@
6	SPIB	1.000	AGAGGAA	AGAGGAA
7	SPI1	0.978	AGGAAG	CTTCCT@
8	GATA3	0.959	AGATTG	AGATTG
9	RUNX1	0.964	TCTGTGGTT	AACCACAGA@
10	GATA2	1.000	GGATA	TATCC@
11	FOXL1	0.964	TTGACATA	TTGACATA
12	§FOXC1*	0.958	ACAAAGTA	ACAAAGTA
13	HAND1	0.968	AGTCTGGCAT	ATGCCAGACT@
14	GATA3	0.959	AGATTG	CAATCT@
15	SPI1	0.978	AGGAAG	CTTCCT@
16	¢GATA3*	0.988	TGATAG	CTATCA@
17	SPI1	0.978	AGGAAG	AGGAAG
18	SPI1	0.978	AGGAAG	AGGAAG
19	SPI1	0.978	AGGAAG	CTTCCT@
20	RUNX1	1.000	TTTGTGGTT	TTTGTGGTT
21	IRF1	0.978	GAAAATGAAACC	GAAAATGAAACC
22	RUNX1	0.954	ACTGTGGTC	ACTGTGGTC

*TFBS (Transcription Factor Binding Site with SNP), @Reverse complement sequence. §-742, ¢-624, these positions are the same in (Figures 5.22-24).

5.2.2 *In vitro* analysis

In the present study, we elucidate for the first time, the existence of IDO promoter variants that may play a significant role in the therapy induced side effect. Since our aim was to identify polymorphisms transcriptional activities of the IDO promoter and contribute to the reduced or increased IDO expression, the variants investigation was limited to the 1.3 kb of the IDO promoter, which has been shown to be active in reporter-gene assays.

A 1.3 kb fragment of human genomic DNA was obtained by PCR and subsequently cloned into a pGL4.10 basic vector. Plasmids containing the 8 genetic variants were created to study their effects on the promoter activity. The cell lines used for these studies were the human HEK293 and SkBr3 cells. The SkBr3 cells were chosen because they consistently express high levels of IDO [Cao unpublished data]. In contrast, the HEK293 cells express very little IDO. The use of these two different cell lines may provide important transcription factors patterns that may reveal different effects of the genetic variants.

Using the 1.3 kb IDO promoter, we have provided evidence that this promoter fragment drives expression of luciferase reporter constructs. This is shown in two human cell lines and at base line, as well as after interferon exposure. Differential regulation of IDO promoter in SkBr3 and HEK293 cells were assessed. IDO promoter activity was tested by luciferase reporter gene assay. Reporter gene transfection efficiencies were corrected by dividing firefly luciferase activity with *Renilla* luciferase activity.

Sequence analysis in the JASPAR database has shown that the -742 A/G interrupts FOXC1 site and -624 A/G interrupts one of the putative GATA3 box sites. These variants did not affect the luciferase activity compare to the wild type in both cells types tested (Figures 5.24 and 5.25). Three of the newly identified variants (-1113 G/T, -1135 C/T, -1144 C/G) were in linkage disequilibrium and formed a single haplotype containing the rare alleles. We only identified these three variants in the African American population (Table 5.5). The 3 SNP and -1144 constructs reduced luciferase activity dramatically in HEK293 cells after interferon gamma exposure (Figure 5.24). On the other hand, in SkBr3 cells there was a decrease in luciferase activity with constructs -1081 and 3SNP compared to the wild type (Figure 5.25). These data suggest the presence of important element located in around -1080 to -1144. The 3 SNP is the only construct that reduced promoter activity in both HEK293 and SkBr3 cells by more than 50% compare to the wild type. This indicates that these SNPs lie in a portion of the promoter that is important for promoter activity. All constructs show a dose dependent increase in promoter activity with increase interferon gamma dose (Figures 5.24 and 5.25). No difference in promoter activity detected using the constructs with SNPs at position -1081, -724, -624, -89 at base and after interferon gamma treatment compared to the wild type in the HEK293 cells.

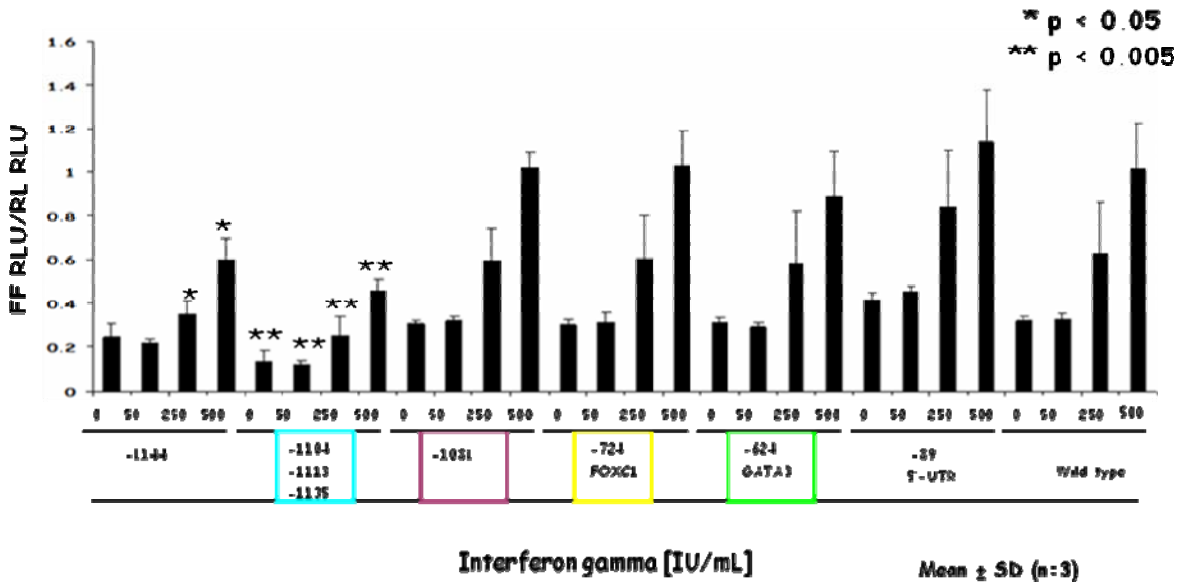

Figure 5.24 Functional analyses of SNPs in the IDO 5'-flanking region of IDO using luciferase reporter assay in HEK293 cells. Average levels of luciferase activity for IDO 5'-flanking region reporter constructs transfected into HEK293 cells. The constructs have been labeled with respective SNPs to indicate the polymorphic nucleotide position numbered with respect to the translation start site. Bars represent the average of at least three independent transfection each done in triplicate (mean ± SD). *($p < 0.05$) and ** ($p < 0.05$), represents data that are significantly different from the value obtained for the wild type compared with other variants.

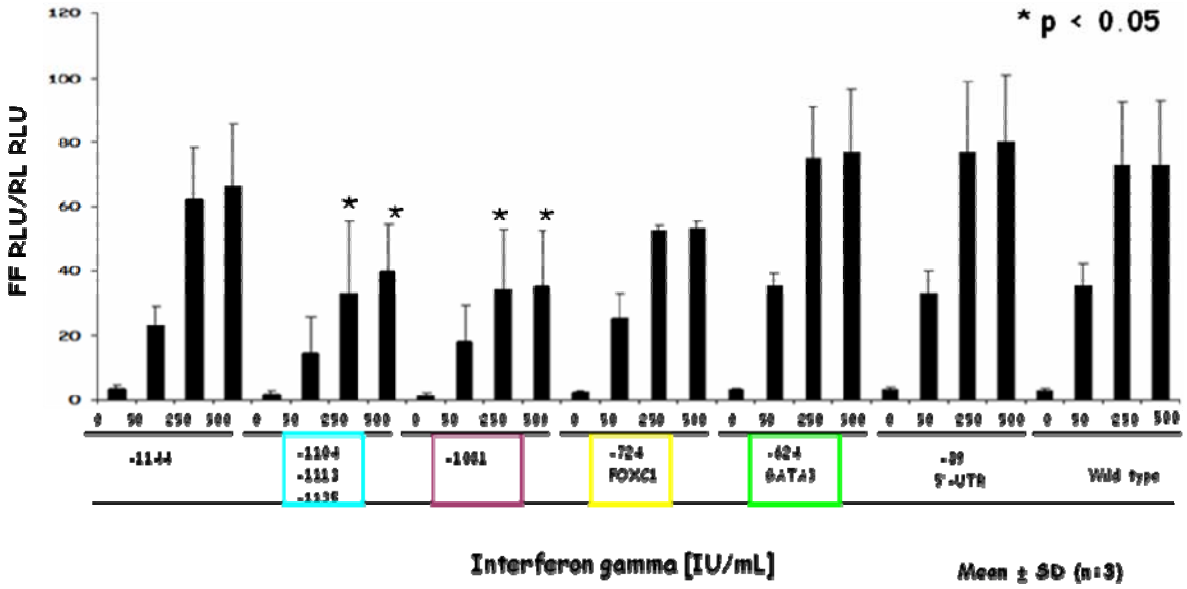

Figure 5.25 Functional analyses of SNPs in the IDO 5'-flanking region of IDO using aluciferase reporter assay in SkBr3 cells. Average levels of luciferase activity for IDO 5'-flanking region reporter constructs transfected into SkBr3 cells are depicted. The constructs have been labeled with respective SNPs to indicate the polymorphic nucleotide position numbered with respect to the translation start site. Bars represent the average of at least three independent transfection each done in triplicate (mean ± SD). * ($p < 0.05$), significantly different from the value obtained for the wild type compared with other variants.

Functional analysis of constructs containing different variants in the 5'-flanking region demonstrated a variable profile of promoter activity; this was evident at base line and after interferon treatment. In fact, there was suggestive evidence for a significant decrease in luciferase activity of the constructs that carry 3-SNP (-1104, -1113 and -1135) compared with the wild type. This suggests that SNPs may play a significant role in IDO expression. On the other hand, in HEK293 cells, when compared to the SkBr3 cells the promoter activity was constant and low from all constructs. Luciferase activity in SkBr3 cell lines is several folds higher than the HEK293 cells.

The data obtained from the promoter construct transfections were consistent in the IDO expression in the two human cell lines used. This difference in IDO expression suggests that cell-specific mechanisms regulate transcription of this gene. Since it is known that IDO is highly expressed in SkBr3 cells, the high luciferase activity in these cells could be due to the expression of transcription factors that are important for IDO expression. In contrast, the HEK293 cells may express relatively low levels of these transcription factors.

CHAPTER 6

DISCUSSION

To our knowledge, no effect of IDO variants on tryptophan metabolism has been reported to date. We have identified naturally occurring genetic variants in the coding and 5'-flanking region of indoleamine 2, 3-dioxygenase (*INDO*) gene that cause impaired functional activity. The existence of these alleles in the Coriell diversity panel subjects indicate that the variants occur in the general population. Although we observed genetic variants in both Caucasian and African American samples, the impaired function alleles in the coding and promoter region were detected exclusively in the African American subjects. Since our sample size was relatively small, it remains possible that these variants also exist in Caucasian populations and in other ethnic groups. Within the African American samples, ~4% of the subjects carried a nonfunctional *INDO* allele.

The reduced function alleles included the $Arg^{77}His$ in exon 3 and the 9 bp deletion in exon 7. A number of the other genetic variants are also in important functional sites, such as those in the exon-intron splice junctions, and may also have important functional effects. These will require additional studies and are the focus of ongoing research. In addition, large number of SNPs that occur in the non-protein coding regions of a gene (e.g. 5'-flanking region), have also been analyzed in this work. Non-protein coding region SNPs (e.g. intronic) are likely to affect the level of gene expression to various extents, but an understanding of their mechanisms of

influence is still very incomplete. *In silico* methods for predicting the functional impact of these types of SNPs are currently under development.

Two SNPs of the SNPs that we identified encode $Ala^{4}Thr$ and $Arg^{77}His$ amino acid changes. Sequence comparison demonstrates that Ala at amino acid position 4 is not conserved in *INDO* among the Dog, Mouse, Rat and Cow (Table 5.2). Since this amino acid resides within the N-terminal sequence of the protein, it might be part of the cytosolic signaling sequence of the protein. Consistent with it being a signal sequence, it is also missing from the crystal structure. As shown in a number of experiments the $Ala^{4}Thr$ variant did not affect IDO expression/activity. In contrast to the $Ala^{4}Thr$, the $Arg^{77}His$ is conserved in IDO among the Dog, Mouse, Rat and Cow. These analyses predicted that the amino acid sequence at position 77 would be important for IDO activity. Our laboratory data confirmed this prediction. The absence of functional changes in $Ala^{4}Thr$ is consistent with findings in both Cos-7 and HEK293 cells transfected with variant IDO cDNA. Therefore, $Ala^{4}Thr$ allele is unlikely to cause significant interindividual differences in tryptophan metabolism. We did not find a noticeable difference in tryptophan metabolism between wild type and $Ala^{4}Thr$ variant allele. Interestingly, we observed a minor increase in activity and expression of $Ala^{4}Thr$ variant protein in HEK293 cells.

In the current work, tryptophan was used as a substrate for IDO kinetics. IDO may have several substrates [150], but tryptophan is the primary one. Therefore, tryptophan is normally used as the substrate in the IDO enzyme assay. We observed differences in the rates of tryptophan metabolism between the wild type and $Arg^{77}His$

variant (Table 5.4). The Arg^{77}His variant displayed a lower level of immunoreactive protein (Figure 5.11). Our data suggested that Arg^{77}His amino acid variant might contribute to both the altered intrinsic property of the enzyme and expression of protein.

Genetic variants that alter the amino acid sequence of a gene product may affect the cellular phenotype at various levels. They may directly alter the stability of the native protein structure and the folding rate, resulting in a reduced concentration of the protein [151]. Polymorphisms residing in the substrate recognition site may further affect protein interactions and other biochemical activities inside the cell [152]. Effects at the level of transcription, translation as well as post-translational modification can also occur. In our studies, we have begun to elucidate these mechanisms for the *INDO* variants. *In vitro* data are of particular importance for rare variations where large groups of people are not available for further investigation. Functional studies are also valuable for studying various mechanisms of altered enzyme function. Furthermore, they can improve our current understanding of the variable IDO expression and activity in the molecular level.

We have yet to identify any subjects that have two nonfunctional alleles. This may be a result of the low frequency of these genetic variants; however, it is also possible that having two nonfunctional alleles may cause sufficiently impaired immune function to result in a high susceptibility to a variety of immunity related diseases. If this is the case, it is unlikely that those people would have been included in the Coriell DNA samples, since these are people who do not have any known

diseases at the time of DNA sample collection. Since IDO activity appears to be involved in the maintenance of pregnancy, it is also possible that homozygosity for the reduced activity alleles is embryonic lethal [153]. However the newly identified *INDOL1* like enzyme may compensate for reduced IDO activity. Although the *INDO* knockout is not lethal in transgenic mice [19, 154], the effect may differ in humans. In humans, which may involve a much greater immune response to the paternal antigens. In addition, compared to mice that are raised in clean animal laboratories, humans are also exposed to a large diversity of environmental pathogens.

The functional effects of the Arg^{77}His and the 9 bp deletion could be due, at least in part, to reduce IDO protein concentrations. For other proteins, the altered folding of variant proteins, and consequently, recognition by the proteasomal degradation pathway has been shown to cause reduced protein levels [145]. This is often the result of rapid variant protein degradation through a proteasome-mediated process[145]. Studies on other genes indicate that this is a common mechanism of reduced enzyme activity caused by genetic variants. Our results showing that immunoreactive IDO protein is reduced in cells transfected with variant *INDO* cDNAs (Arg^{77}His and the 9 bp deletion) are consistent with this mechanism. Since we observed reduced activity in the variants when they were expressed from three different plasmid constructs, this would seem unlikely to be caused by problems with the variant expression vectors. Furthermore, the variability in the IDO mRNA concentrations did not appear to be large enough to cause the large difference in amounts of protein and enzyme activity. Consistent results with both an anti-IDO

antibody, as well as with the anti-HA-tag antibody, indicate that the lack of immunoreactive protein is unlikely to be due to an altered immunogenicity of the variant proteins.

Our results indicate that the Arg^{77}His variant also affects the IDO enzyme kinetics. Compared to the wild type IDO, the V_{max} for the Arg^{77}His variant was reduced by ~60%. The Arg^{77}His K_m appeared to be slightly increased. The K_m of IDO extracted from human placenta has been estimated to be 24.6 μM [20]. This is similar to the wild type IDO transiently expressed in HEK293 cells 18.0 μM. The K_m of the Arg^{77}His variant was estimated to be slightly higher 29.6 μM. When the V_{max} and K_m were combined to calculate the intrinsic clearance, the Arg^{77}His was substantially reduced (Table 5.4).

As shown in (Figure 5.11) the expression level of Arg^{77}His can not account for the difference between the wild type and variant IDO's. One mechanism that may be responsible for the decreased levels of variant IDO enzyme activity is a decrease in the quantity of enzymatically active protein (holoenzyme).

At present, little is known about translational and posttranslational regulation events involved in the IDO protein. It is known that the incorporation of the heme prosthetic group is a required posttranslational regulation step [155, 156]. Our studies now indicate that alternations in the heme incorporation into IDO may be affected by genetic variants in the *INDO* gene. Exposing cells with heme analog, hemin increased IDO activity in the Arg^{77}His DO variant; it is most likely by increasing the amount of holoenzyme (Figures 5.16, 5.18-21). The effect of hemin in IDO activity was first

obsereved in the cell free expression system in which cells were treated with 20 μM hemin (Figure 5.14). The effect of hemin was negligible in the wild type and Ala^4 variant (Figure 5.14). It is possible that the exogenous hemin added into this system might increase the proportion of holo to apo $Arg^{77}His$ variant protein ratio in the system and resulted in increase kynurenine formation. A previous study has shown that addition of hemin to IFN-γ activated human monocyte derived macrophages (hMDM) culture and cell lysate prepared from this activated cells increased formation of kynurenine [157]. These data suggested that cellular heme may be the limiting for IDO activity. As a consequence, a significant amount of IDO expressed in the cells may be present as apoenzyme [157]. The concentration of heme in HEK293 cells was 5-fold higher than in cells that do not transfected with the neuronal nitric oxide synthase (nNOS) cDNA [158]. These data are consistent with our findings which showed several fold higher heme concentration in cells transefcted with wild type IDO compared to pcDNA3 transfected cells (Figure 5.19). Although the major portion of cellular heme is probably associated with heme containing proteins, it is generally assumed that cells contain low levels of free heme (unbound) [158, 159]. It should be pointed out that heme homeostasis is tightly regulated in a cell and heme oxygenase (HO) is the only physiological mechanism of heme degradation [159, 160].

The inhibition of heme synthesis by SA was shown to reduce IDO activity in the wild type and profoundly blocked in the $Arg^{77}His$ variant (Figure 5.19). This indicates that the availability of heme is important for IDO activity. The reversing of the SA effect by hemin indicates that hemin can apparently substitute for the

endogenous heme (Figures 5.19 and 5.21). Furthermore, our studies with other proteasome inhibitors and lysosomal inhibitors did not restore enzyme activity (Figure 5.17 and 5.18). This suggests that the $Arg^{77}His$ may have a defect in incorporating heme and it may exist as an apoenzyme; however, the 9 bp deletion may be degraded through other mechanisms. In the light of this tight heme regulation, and the fact that heme concentrations in cells transfected with variant IDO constructs were low, we hypothesized that the heme degradation is accelerated or heme synthesis is slowed in this cells due to less incorporation into IDO. Future studies assessing the level of heme oxygenase (HO) expression in cells transfected with $Arg^{77}His$ will help to distinguish between these possibilities.

Some insight into the effects of the variants on the IDO protein may be provided by the examination of the 3D structure of the IDO protein. First, based on the crystal structure of the IDO protein, these amino acids affected by the genetic variants do not appear to directly contact the substrate or the heme moiety; however, they may indirectly affect the structure of one of these domains. Secondly, since the positions of the mutations appear to be on the surface of the enzyme, the variant proteins may be easily identified by the cellular degradation machinery. Furthermore, since the variant amino acids are on the surface of the protein, it is possible that they interfere with protein-protein interactions. Although these amino acids could be involved in protein-protein interactions, little is known about IDO's binding partners, making it difficult to speculate further on this potential mechanism.

Specific Aim 2:

This thesis provides a new promoter polymorphisms of the *INDO* gene for which its functionality was demonstrated *in vitro.* The variable induction by interferon gamma due to variants in the 5'-flanking region is also assessed. In order to understand the effect of SNPs in *INDO* gene promoter activity, we have cloned 1.3 kb of *INDO* promoter covering a number of important transcription factor binding sites. In this thesis, we have identified and described the allele frequencies for eight *INDO* promoter variant by resequncing the 5'-flanking region of *INDO* promoter. The distributions of these alleles were described in the African American and Caucasian population. Eight of the SNPs at positions (-1144, -1135, -1113, -1104, -1081, -742, -624 and -89) relative to the translation start site have never been reported before. 5'-flanking region of *INDO* gene contain several interesting structural and potential regulatory elements. For example, with in the 1.3 kb region using JASPAR 22 putative transcription binding consensus sequences were predicted at 95% threshold. Two of the newly identified SNPs are located in (FOXC1 at -742 and GATA3 at -624) TFBS. The functionality of all variants were tested *in vitro* in two different cell lines, and only one of them (-1144, -1135, -1113) was shown to influence the transcription activity of the reporter gene in both SkBr3 and HEK293 cells. Based on the *in vitro* functional reporter assay, results the 3-SNP variant (designated as haplotype in this study) leads to a considerable reduction in the transcriptional activity of the reporter gene in both cell lines tested after interferon gamma exposure (Figures 5.24 and 5.25). The -1081 variant showed reduction of promoter activity only in SkBr3 cells at high dose of interferon gamma (Figure 5.25). Most of factors that bind to these regions

remain to be identified and characterized. Two of the SNPs described in this study are localized within the FOXC1, -742 and GATA3,-624. These sites have been shown to play an important role in the transcription of a wide variety of eukaryotic genes [161, 162]. *In vitro* functional analysis using a luciferase reporter construct carrying a variant in FOXC1and GATA3 sites reveled no reduction in the transcription level of luciferase reporter gene at base line and after interferon exposure (Figures 5.24 and 5.25). Furthermore, during TFBS analysis using a sequence with these two SNPs at (-742 and -624) the outcome did not include FOXC1and GATA3. One possibility is the prediction was wrong or the model system we used is not ideal to test the effect of these variants.

We have identified and characterized IDO promoter variants that played a significant role in regulating the expression of human IDO. IDO gene have been cloned and sequenced revealing transcriptional promoter elements (GAS and ISRE motifs) that confer responsiveness to interferons via the JAK-STAT signaling pathway [163]. Mutagenesis experiments revealed the importance of these motifs for the interferon mediated IDO induction and it was also recognized by a putative cellular specific regulatory transcriptional factor(s) ISRE and GAS [164].

IDO is one of the enzymes that is able oxidize tryptophan into kynurenine, which may be an important defense mechanism against pathogens. In addition, the transcriptional regulation of IDO might have potential significance in therapy induced increased IDO expression. It has been reported that levels of IDO transcript, protein and its primary metabolite increased in patients treated with interferon [81, 165]. The mechanisms underlying the interindividual variations in IDO expression is remain

unknown. Future studies will be required to determine whether the variable IDO expression is a cause or an effect of the variable outcomes.

The variants we have identified may have phenotypic effects in the human population. Since many preclinical studies indicate that IDO frequently induces immune tolerance [166-168], people with genetically reduced IDO activity may be at an elevated risk for inflammatory and autoimmune type diseases. As described above, maternal tolerance of paternal antigens may also be affected. Natural killer (NK) cells play a significant role in immune responses to pathogenic agents as well as in the defense against cancer cells [169]. The mechanisms underlying NK cell recognition of target cells have been studied in great detail. In contrast to the potential effects on tolerance, other preclinical studies indicate that NK cell activity is partially dependent on IDO activity [169, 170]. Consequently, people with reduced IDO activity may have impaired immune function and have an increased susceptibility to infectious diseases. Since there does not appear to be redundancy of tryptophan metabolizing enzymes that are induced in peripheral tissues by inflammation, it is likely that people with no functional *INDO* alleles would have phenotypic consequences. These phenotypes would likely be revealed to a greater extent in human populations that are exposed to many pathogenic antigens than in *INDO* knockout mice that are housed in relatively clean environments. These genetic variants may also contribute to inter-individual variability in IDO expression/activity

We hypothesized that patients who are treated with interferon based therapy and have the 3-SNPs, would have reduced IDO induction compared with wild type alleles. Although, these variants are not widely distributed in the population and

occurs in approximately 3% (allelic frequency) of the African American population, it is important to note who is benefiting from this therapy and minimize therapy induced side effects. In addition, the interindividual variability of therapy outcomes and IDO expression is undoubtedly multi factorial. Therefore a large study would be needed to confirm possible relationship between the 3-SNPs and interferon therapy outcome. An understanding of the impact of these 3-SNPs and interferon therapy will be valuable information.

An interesting finding from our study is the higher prevalence in African Americans. These data raise the question of their potential contribution to clinical disease in this population. Since a key function of IDO is to down regulate T-cell immunity, it is interesting to speculate that these variants may have a role in diseases highly prevalent in African Americans, such as asthma or type I diabetes. Since the sample size was modest and the frequencies of each of these individual variants were low, it will be important to conduct larger studies to accurately determine the allelic frequencies in the different ethnic populations. The work described in this thesis provides a foundation for future studies addressing the association between IDO genotype and clinical response to drugs (interferons) and a firm basis to allow impaired mechanistic understanding of the interaction between tryptophan metabolism and theraputics. These questions will be addressed in future studies.

In summary, we have identified and described the allele frequencies for IDO coding and promoter variants in the African American and Caucasian population. We have assessed the functional significance of three coding and eight promoter variants.

This is the first report on the identification of allelic variants of the *INDO* gene in these two populations combined with functional data. In the African American population a total 18 alleles were identified, including the four functional variants (two in the promoter and two in the coding region). These four functional variants were not identified in the Caucasian population.

Future studies

More research needs to be conducted to clarify the functional consequences of known IDO genetic variant and their impacts on human health. As additional information about the biological and physiological functions of the gene is gathered, un understanding of the role of IDO polymorphisms will also increase. In this context it will be important to consider the possible complexity of interactions between different polymorphisms; especially in genes at different steps and different pathways that are involved in IDO regulation.

In the future, it would be valuable to further evaluate the functional significance of these IDO variants *in vivo* in studies such as the following:

1. Determine whether IDO activity in human tissues or cells associated with the genetic variants in the *INDO* gene.

2. Determine the association of *INDO* genetic variants in disease susceptibility; populations to focus on would be those with disease such as autoimmune and cancer?

3. Determine if interferon responses are different between the groups of people with different *INDO* geneotypes. Afirst step would be to conduct haplotype analysis using all known *INDO* variants. Subsequently, it would be appropriet to test haplotype-phenotype associations for common IDO haplotypes under baseline conditions and after interferon treatment.

CHAPTER 7

CONCLUSIONS

The current study has examined genetic factors that have the potential to the variability in IDO enzyme expression and activity. We have investigated genetic factors that may contribute to IDO interindividual variability at three levels.

A. By the resequencing, identification and characterization of *INDO* genetic variants in the coding and promoter regions.
B. By predicting functional significance of *INDO* variants *in silico*.
C. By determining the functional effects of *INDO* variants *in vitro.*

We conclude the following:

- There are naturally occurring genetic variants in the human population that result in altered enzyme function. The $Arg^{77}His$ (exon 3) and the exon 7 (9 bp deletion) variants are examples of reduced functional alleles.
- The Ala4Thr variant exhibited similar metabolism and substrate affinity towards tryptophan (V_{max} and K_m) as the wild type.
- The $Arg^{77}His$ (exon 3) variant protein does not appear to be degraded through the proteasomal and lysosomal pathway.
- Transfection of HEK293 cells with wild type IDO cDNA increases cellular heme concentrations; however, this increase is much less when the cells are transfected with impaired function $Arg^{77}His$ and 9 bp deletion variants.
- In the wild type cells, the inhibition of heme synthesis substantially reduced the IDO activity. In the $Arg^{77}His$ cells, the SA completely abolished the formation of kynurenine.

- We have identified seven novel genetic variants in the 5'-flanking region of *INDO* gene.
- Two of the novel SNPs are located with in the putative transcription factor binding sites FOXC1 and GATA3.
- All constructs show a dose dependent increase in promoter activity with increase interferon gamma dose.
- The 3-SNP haplotype and -1144 constructs reduced luciferase activity in HEK293 cells after interferon gamma exposure.
- Our data suggest the presence of important element around -1080 to -1144 bp of *INDO* promoter.

REFERENCES

1. Berney A, Nishikawa M, Benkelfat C, Debonnel G, Gobbi G, Diksic M. An index of 5-HT synthesis changes during early antidepressant treatment: alpha-[(11)C]methyl-l-tryptophan PET study. *Neurochem Int* 2008; **52**(4-5)**:** 701-708.

2. Praschak-Rieder N, Hussey D, Wilson AA, Carella A, Lee M, Dunn E*, et al*. Tryptophan depletion and serotonin loss in selective serotonin reuptake inhibitor-treated depression: an [(18)F] MPPF positron emission tomography study. *Biol Psychiatry* 2004; **56**(8)**:** 587-591.

3. Young SN, Teff KL. Tryptophan availability, 5HT synthesis and 5HT function. *Prog Neuropsychopharmacol Biol Psychiatry* 1989; **13**(3-4)**:** 373-379.

4. Greengard O, Feigelsonp. The purification and properties of liver tryptophan pyrrolase. *J Biol Chem* 1962; **237:** 1903-1907.

5. Kotake Y. Xanthurenic acid, an abnormal metabolite of tryptophan and the diabetic symptoms caused in albino rats by its production. *J Vitaminol (Kyoto)* 1955; **1**(2)**:** 73-87.

6. Najfeld V, Menninger J, Muhleman D, Comings DE, Gupta SL. Localization of indoleamine 2,3-dioxygenase gene (INDO) to chromosome 8p12-->p11 by fluorescent in situ hybridization. *Cytogenet Cell Genet* 1993; **64**(3-4)**:** 231-232.

7. Altman K, Greengard O. Tryptophan pyrrolase induced in human liver by hydrocortisone: effect on excretion of kynurenine. *Science* 1966; **151**(708)**:** 332-333.

8. Higuchi K HS, Hayaishi O: Enzymatic formation of D-kynurenine. *Federation Proc* 1963**:** 22**:**243.

9. Kadoya A TS, Maeda H:. Gene structure of human Indoleamine2, 3-dioxyegenase. *Biochem Biophys Res Commun* 1992;(189)**:** 530-536.

10. Yamamoto S, Hayaishi O. Tryptophan pyrrolase of rabbit intestine. D- and L-tryptophan-cleaving enzyme or enzymes. *J Biol Chem* 1967; **242**(22)**:** 5260-5266.

11. Forouhar F, Anderson JL, Mowat CG, Vorobiev SM, Hussain A, Abashidze M*, et al*. Molecular insights into substrate recognition and catalysis by tryptophan 2,3-dioxygenase. *Proc Natl Acad Sci U S A* 2007; **104**(2)**:** 473-478.

12. Thomas SR, Stocker R. Redox reactions related to indoleamine 2,3-dioxygenase and tryptophan metabolism along the kynurenine pathway. *Redox Rep* 1999; **4**(5)**:** 199-220.

13. Thomas SR. Antioxidant activities and redox regulation of interferon-gamma-induced tryptophan metabolism in human monocytes and macrophages *Adv Exp Med Biol*. 1999;467**:** 541-52.

14. Grohmann U, Fallarino, F., Puccetti, P. Trends Immunol.,Tolerance, DCs and tryptophan: much ado about IDO. 2003; (24)**:** 242-248.

15. Taylor MW, Feng GS. Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. *FASEB J* 1991; **5**(11)**:** 2516-2522.

16. Pfaff AW, Mousli M, Senegas A, Marcellin L, Takikawa O, Klein JP*, et al*. Impact of foetus and mother on IFN-gamma-induced indoleamine 2,3-dioxygenase and inducible nitric oxide synthase expression in murine placenta following Toxoplasma gondii infection. *Int J Parasitol* 2008; **38**(2)**:** 249-258.

17. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B*, et al*. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science* 1998; **281**(5380)**:** 1191-1193.

18. Mellor AL, Sivakumar J, Chandler P, Smith K, Molina H, Mao D*, et al*. Prevention of T cell-driven complement activation and inflammation by tryptophan catabolism during pregnancy. *Nat Immunol* 2001; **2**(1)**:** 64-68.

19. Mellor AL, Keskin DB, Johnson T, Chandler P, Munn DH. Cells expressing indoleamine 2,3-dioxygenase inhibit T cell responses. *J Immunol* 2002; **168**(8)**:** 3771-3776.

20. Kudo Y, Boyd CA. Human placental indoleamine 2,3-dioxygenase: cellular localization and characterization of an enzyme preventing fetal rejection. *Biochim Biophys Acta* 2000; **1500**(1)**:** 119-124.

21. Mellor AL, Munn DH. Tryptophan catabolism prevents maternal T cells from activating lethal anti-fetal immune responses. *J Reprod Immunol* 2001; **52**(1-2)**:** 5-13.

22. Mellor AL, Chandler P, Lee GK, Johnson T, Keskin DB, Lee J*, et al*. Indoleamine 2,3-dioxygenase, immunosuppression and pregnancy. *J Reprod Immunol* 2002; **57**(1-2)**:** 143-150.

23. Mellor AL, Munn DH. Tryptophan catabolism and T-cell tolerance: immunosuppression by starvation? *Immunology today* 1999; **20**(10)**:** 469-473.

24. Moffett JR, Namboodiri MA. Tryptophan and the immune response. *Immunol Cell Biol* 2003; **81**(4)**:** 247-265.

25. Le Rond S, Gonzalez A, Gonzalez AS, Carosella ED, Rouas-Freiss N. Indoleamine 2,3 dioxygenase and human leucocyte antigen-G inhibit the T-cell alloproliferative response through two independent pathways. *Immunology* 2005; **116**(3)**:** 297-307.

26. Chen W. Dendritic cells and (CD4+)CD25+ T regulatory cells: crosstalk between two professionals in immunity versus tolerance. *Front Biosci* 2006; **11:** 1360-1370.

27. Ball HJ, Sanchez-Perez A, Weiser S, Austin CJ, Astelbauer F, Miu J*, et al*. Characterization of an indoleamine 2,3-dioxygenase-like protein found in humans and mice. *Gene* 2007; **396**(1)**:** 203-213.

28. Murray MF. The human indoleamine 2,3-dioxygenase gene and related human genes. *Curr Drug Metab* 2007; **8**(3)**:** 197-200.

29. Metz R, Duhadaway JB, Kamasani U, Laury-Kleintop L, Muller AJ, Prendergast GC. Novel tryptophan catabolic enzyme IDO2 is the preferred biochemical target of the antitumor indoleamine 2,3-dioxygenase inhibitory compound D-1-methyl-tryptophan. *Cancer Res* 2007; **67**(15)**:** 7082-7087.

30. Oertelt-Prigione S, Mao TK, Selmi C, Tsuneyama K, Ansari AA, Coppel RL*, et al*. Impaired indoleamine 2,3-dioxygenase production contributes to the development of autoimmunity in primary biliary cirrhosis. *Autoimmunity* 2008; **41**(1)**:** 92-99.

31. Prendergast GC. Immune escape as a fundamental trait of cancer: focus on IDO. *Oncogene* 2008; **27**(28)**:**3889-3899.

32. Puccetti P, Grohmann U. IDO and regulatory T cells: a role for reverse signalling and non-canonical NF-kappaB activation. *Nat Rev Immunol* 2007; **7**(10)**:** 817-823.

33. Platten M, Ho PP, Youssef S, Fontoura P, Garren H, Hur EM*, et al*. Treatment of autoimmune neuroinflammation with a synthetic tryptophan metabolite. *Science* 2005; **310**(5749)**:** 850-855.

34. Brandacher G, Perathoner A, Ladurner R, Schneeberger S, Obrist P, Winkler C*, et al*. Prognostic value of indoleamine 2,3-dioxygenase expression in colorectal cancer: effect on tumor-infiltrating T cells. *Clin Cancer Res* 2006; **12**(4)**:** 1144-1151.

35. Burudi EM, Marcondes MC, Watry DD, Zandonatti M, Taffe MA, Fox HS. Regulation of indoleamine 2,3-dioxygenase expression in simian immunodeficiency virus-infected monkey brains. *J Virol* 2002; **76**(23)**:** 12233-12241.

36. Guillemin GJ, Brew BJ, Noonan CE, Takikawa O, Cullen KM. Indoleamine 2,3 dioxygenase and quinolinic acid immunoreactivity in Alzheimer's disease hippocampus. *Neuropathol Appl Neurobiol* 2005; **31**(4)**:** 395-404.

37. Hansen AM, Driussi C, Turner V, Takikawa O, Hunt NH. Tissue distribution of indoleamine 2,3-dioxygenase in normal and malaria-infected tissue. *Redox Rep* 2000; **5**(2-3)**:** 112-115.

38. Ino K, Yoshida N, Kajiyama H, Shibata K, Yamamoto E, Kidokoro K*, et al*. Indoleamine 2,3-dioxygenase is a novel prognostic indicator for endometrial cancer. *Br J Cancer* 2006; **95**(11)**:** 1555-1561.

39. Kohl C, Sperner-Unterweger B. IDO and clinical conditions associated with depressive symptoms. *Curr Drug Metab* 2007; **8**(3)**:** 283-287.

40. Le AV, Broide DH. Indoleamine-2,3-dioxygenase modulation of allergic immune responses. *Curr Allergy Asthma Rep* 2006; **6**(1)**:** 27-31.

41. Mazzolini G, Murillo O, Atorrasagasti C, Dubrot J, Tirapu I, Rizzo M*, et al*. Immunotherapy and immunoescape in colorectal cancer. *World J Gastroenterol* 2007; **13**(44)**:** 5822-5831.

42. Melchior D, Meziere N, Seve B, Le Floc'h N. Is tryptophan catabolism increased under indoleamine 2,3 dioxygenase activity during chronic lung inflammation in pigs? *Reprod Nutr Dev* 2005; **45**(2)**:** 175-183.

43. Wolf AM, Wolf D, Rumpold H, Moschen AR, Kaser A, Obrist P*, et al*. Overexpression of indoleamine 2,3-dioxygenase in human inflammatory bowel disease. *Clin Immunol* 2004; **113**(1)**:** 47-55.

44. Hissong BD, Byrne GI, Padilla ML, Carlin JM. Upregulation of interferon-induced indoleamine 2,3-dioxygenase in human macrophage cultures by lipopolysaccharide, muramyl tripeptide, and interleukin-1. *Cell Immunol* 1995; **160**(2)**:** 264-269.

45. Sekkai D, Guittet O, Lemaire G, Tenu JP, Lepoivre M. Inhibition of nitric oxide synthase expression and activity in macrophages by 3-hydroxyanthranilic acid, a tryptophan metabolite. *Arch Biochem Biophys* 1997; **340**(1)**:** 117-123.

46. MacKenzie CR, Langen R, Takikawa O, Daubener W. Inhibition of indoleamine 2,3-dioxygenase in human macrophages inhibits interferon-gamma-induced bacteriostasis but does not abrogate toxoplasmastasis. *Eur J Immunol* 1999; **29**(10)**:** 3254-3261.

47. Grant RS, Naif H, Thuruthyil SJ, Nasr N, Littlejohn T, Takikawa O*, et al*. Induction of indolamine 2,3-dioxygenase in primary human macrophages by human immunodeficiency virus type 1 is strain dependent. *J Virol* 2000; **74**(9)**:** 4110-4115.

48. Boyland E , Williams DC. The metabolism of tryptophan. 2. The metabolism of tryptophan in patients suffering from cancer of the bladder. *Biochem J.*1956; (64)**:** 578-582.

49. Friedlander E, Morrison AS. Urinary tryptophan metabolites and cancer of the bladder in humans. *J Natl Cancer Inst* 1981; **67**(2)**:** 347-351.

50. Perissinotto B, Benassi CA, Allegri G. Urinary Excretion of Tryptophan Metabolites in Patients with Renal Pelvis and Parenchyma Tumours. *Urol Int* 1964; **17:** 175-182.

51. Ivanova VD. Studies on tryptophan metabolites in the blood and urine of patients with leukemia. *Probl Gematol Pereliv Krovi* 1959; **4:** 18-21.

52. Boyland E, Williams DC. The estimation of tryptophan metabolites in the urine of patients with cancer of the bladder. *Biochem J* 1955; **60**(Annual General Meeting)**:** v.

53. Yasui H, Takai K, Yoshida R, Hayaishi O. Interferon enhances tryptophan metabolism by inducing pulmonary indoleamine 2,3-dioxygenase: its possible occurrence in cancer patients. *Proc Natl Acad Sci U S A* 1986; **83**(17)**:** 6622-6626.

54. Liakou CI, Narayanan S, Ng Tang D, Logothetis CJ, Sharma P. Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human bladder cancer. *Cancer Immun* 2007; **7:** 10.

55. Ozaki Y EM, Duch DS. Induction of indoleamine 2,3-dioxygenase: a mechanism of the antitumor activity of interferon γ. *Proc Natl Acad Sci* 1988; **85**(4)**:** 1242-1246.

56. Katz JB, Muller AJ, Prendergast GC. Indoleamine 2,3-dioxygenase in T-cell tolerance and tumoral immune escape. *Immunol Rev* 2008; **222:** 206-221.

57. Enk AH, Jonuleit H, Saloga J, Knop J. Dendritic cells as mediators of tumor-induced tolerance in metastatic melanoma. *International journal of cancer* 1997; **73**(3)**:** 309-316.

58. Gabrilovich DI, Corak J, Ciernik IF, Kavanaugh D, Carbone DP. Decreased antigen presentation by dendritic cells in patients with breast cancer. *Clin Cancer Res* 1997; **3**(3)**:** 483-490.

59. Restifo NP. Not so Fas: Re-evaluating the mechanisms of immune privilege and tumor escape. *NatMed,* 2000; **6**(5)**:** 493-495.

60. Kobie JJ, Wu, R. S, Kurt, R. A, Lou, S, Adelman, M. K., Whitesell, L. J, Ramanathapuram, L. V., Arteaga, C. L., and Akporiaye, E. T. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. *Cancer Res,* 2003; **63**(8)**:** 1860-1864.

61. Gabrilovich DI, Corak, J, Ciernik, I. F, Kavanaugh, D, and Carbone, D. P. Cli. Decreased antigen presentation by dendritic cells in patients with breast cancer.*Cancer Res.*,1997; **3**(3)**:** 483-490.

62. Friberg M, Jennings R, Alsarraj M, Dessureault S, Cantor A, Extermann M*, et al*. Indoleamine 2,3-dioxygenase contributes to tumor cell evasion of T cell-mediated rejection. *Int J Cancer* 2002; **101**(2)**:** 151-155.

63. Pardoll D. Does the immune system see tumors as foreign or self? *Annu Rev Immunol* 2003; **21:** 807-839.

64. Munn DH, Sharma MD, Mellor AL. Ligation of B7-1/B7-2 by human CD4+ T cells triggers indoleamine 2,3-dioxygenase activity in dendritic cells. *J Immunol* 2004; **172**(7)**:** 4100-4110.

65. Belladonna ML, Grohmann U, Guidetti P, Volpi C, Bianchi R, Fioretti MC*, et al*. Kynurenine pathway enzymes in dendritic cells initiate tolerogenesis in the absence of functional IDO. *J Immunol* 2006; **177**(1)**:** 130-137.

66. Fallarino F, Grohmann U, You S, McGrath BC, Cavener DR, Vacca C*, et al*. The combined effects of tryptophan starvation and tryptophan catabolites down-regulate T cell receptor zeta-chain and induce a regulatory phenotype in naive T cells. *J Immunol* 2006; **176**(11)**:** 6752-6761.

67. Munn DHZ, M, Attwod, J.T; Bndarev, I; Conway, S.J.; Marshall, B.; Brown, C. and Mellor, L. Prevention of allogeneic fetal rejection by tryptophan catabolism. *Science*,1998; **281**(5380)**:** 1191-1193.

68. DeMatos P, Abdel-Wahab, Z, Vervaert, C, Hester, D, Seigler, H, Pulsing of dendritic cells with cell lysates from either B16 melanoma or MCA-106 fibrosarcoma yields equally effective vaccines against B16 tumors in mice. *J. Surg. Oncol* 1998; **68**(2)**:** 79-91.

69. Fields RC, Shimizu, K, Mule, J. Murine dendritic cells pulsed with whole tumor lysates mediate potent antitumor immune responses in vitro and in vivo. *Proc.Natl.Acad.Sci.U.S A*,1998; **95**(16)**:** 9482-9487.

70. Romero P, Dunbar, P. R., Valmori, D., Pittet, M., Ogg, G. S., Rimoldi, D., Chen, J. L., Lienard, D., Cerottini, J. C., and Cerundolo, V. Ex vivo staining of metastatic lymph nodes by class I major histocompatibility complex tetramers reveals high numbers of antigen-experienced tumor-specific cytolytic T lymphocytes. *J Exp Med,* 1998; **188**(9)**:** 1641-1650.

71. Jager E, Jager, D, Knuth, A. Antigen-specific immunotherapy and cancer vaccines. *Int J Cancer,* 2003; **106**(6)**:** 817-820.

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

73. Lee JR, Dalton, R., Messina, J. L., Sharma, M., Smith, D., Burgess, R, Mazzella, F., Antonia, S, Mellor, A, and Munn, D. H. Pattern of recruitment of immunoregulatory antigen-presenting cells in malignant melanoma. *Lab Invest,* 2003; **83**(10)**:** 1457-66.

74. Munn DH, Shafizadeh, E., Attwood, J. T., Bondarev, I., Pashine, A., and Mellor, A. L. Inhibition of T cell proliferation by macrophage tryptophan catabolism. *JExpMed,* 1999; **189**(9)**:** 1363-72.

75. Leek RD, Lewis, C, Whitehouse, R., Greenall, M., Clarke, J., and Harris, A. L. Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Res,* 1996; **56**(20)**:**4625-9.

76. Lewis JS, Landers, R. J., Underwood, J. C., Harris, A, Lewis, C. E. J.Pathol., Expression of vascular endothelial growth factor by macrophages is up-regulated in poorly vascularized areas of breast carcinomas. *JPathol,* 2000**:** **192**(2)**:**150-8.

77. Leek RD, Hunt, N. C., Landers, R. J., Lewis, C. E., Royds, J. A., and Harris, A. L. Macrophage infiltration is associated with VEGF and EGFR expression in breast cancer. *J Pathol,* 2000; **190**(4)**:**430-6.

78. Uyttenhove C, Pilotte L, Theate I, Stroobant V, Colau D, Parmentier N*, et al*. Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. *Nat Med* 2003; **9**(10)**:** 1269-1274.

79. Liebau C, Baltzer AW, Schmidt S, Roesel C, Karreman C, Prisack JB*, et al*. Interleukin-12 and interleukin-18 induce indoleamine 2,3-dioxygenase (IDO) activity in human osteosarcoma cell lines independently from interferon-gamma. *Anticancer Res* 2002; **22**(2A)**:** 931-936.

80. Staveley-O'Carroll K SE, Montgomery J, Borrello I, Hwang L, Fein S, Pardoll D, Levitsky H. . Induction of antigen-specific T cell anergy: An early event in the course of tumor progression. *Proc Natl Acad Sci U S A* 1998; **3**(95)**:** 1178-1183.

81. Brandacher G, Margreiter R, Fuchs D. Implications of IFN-gamma-mediated tryptophan catabolism on solid organ transplantation. *Curr Drug Metab* 2007; **8**(3)**:** 273-282.

82. Bauer TM, Jiga LP, Chuang JJ, Randazzo M, Opelz G, Terness P. Studying the immunosuppressive role of indoleamine 2,3-dioxygenase: tryptophan metabolites suppress rat allogeneic T-cell responses in vitro and in vivo. *Transpl Int* 2005; **18**(1)**:** 95-100.

83. Hainz U, Jurgens B, Heitger A. The role of indoleamine 2,3-dioxygenase in transplantation. *Transpl Int* 2007; **20**(2)**:** 118-127.

84. Swanson KA, Zheng, Y., Heidler, K, Mizobuchi, T, Wilkes, D. S. CDllc+ cells modulate pulmonary immune responses by production of indoleamine 2,3-dioxygenase. 2004; **30**(3)**:** 311-8.

85. Alexander AM, Crawford, M., Bertera, S., Rudert, W, Takikawa, O., Robbins, P. D., and Trucco, M. Indoleamine 2,3-dioxygenase expression in transplanted NOD Islets prolongs graft survival after adoptive transfer of diabetogenic splenocytes. *Diabetes* 2002; **51**(2)**:**356-65.

86. Li J, Meinhardt A, Roehrich ME, Golshayan D, Dudler J, Pagnotta M*, et al*. Indoleamine 2,3-dioxygenase gene transfer prolongs cardiac allograft survival. *Am J Physiol Heart Circ Physiol* 2007; **293**(6)**:** 3415-3423.

87. Capuron L RA, Neveu PJ, Miller AH, Maes M, Dantzer R. Association between decreased serum tryptophan concentrations and depressive symptoms in cancer patients undergoing cytokine therapy. *Mol Psychiatry;* 2002; **7**(5)**:** 468-73.

88. Musselman D, Paroxetine for the prevention of depression induced by high-dose interferon alfa. *N Engl J Med* 2001; **344**(13)**:** 961-6.

89. Schaefer M EM, Gut O. Interferon α (IFNa) and psychiatric syndromes: a review. *Prog Neuropsychopharmacol Biol Psychiatry;* 2002; **4**(26)**:** 731-746.

90. Dieperink E WM, Ho SB. A review Neuropsychiatric symptoms associated with hepatitis C and interferon α: *Am J Psychiatry* 2000; **6**(157)**:** 867-876.

91. Trask P EP, Riba M,.Psychiatric side effects of interferon therapy: prevalence, proposed mechanisms, and future directions. *J Clin Oncol* 2000; **11**(18)**:** 2316-2326.

92. Owens MJ NC. Role of serotonin in the pathophysiology of depression: focus on the serotonin transporter. *Clin Che,* 1994; **2**(40)**:** 288-295.

93. Bonaccorso S PA, Marino V, Pasquini M, Biondi M, Artini M, Almerighi C, Levrero M, Egyed B, Bosmans E, Meltzer HY, Maes M. Immunotherapy with interferon-alpha in patients affected by chronic hepatitis C induces an intercorrelated stimulation of the cytokine network and an increase in depressive and anxiety symptoms. *Psychiatry Res* 2001**: 105**(1-2)**:** 45-55.

94. Capuron L RA, Gualde N, Bosmans E, Dantzer R, Maes M, Neveu P Association between immune activation and early depressive symptoms in cancer patients treated with interleukin-2-based therapy. *J Psycho neuroendocrinology* 2001; **8**(26)**:** 797-808.

95. Taher YA, Piavaux BJ, Gras R, van Esch BC, Hofman GA, Bloksma N*, et al*. Indoleamine 2,3-dioxygenase-dependent tryptophan metabolites contribute to tolerance induction during allergen immunotherapy in a mouse model. *J Allergy Clin Immunol* 2008. **121**(4):983-91

96. Capuron L HP, Hinze-Selch D, Miller AH, Neveu PJ. Treatment of cytokine-induced depression. *BrainBehav Immun,* 2002; **5**(6)**:** 575-580.

97. Recht M BE, Knight E Jr. A human 15-kDa IFN-induced protein induces the secretion of IFN-gamma. *J Immunol;* 1991; **8**(147)**:** 2617-2623.

98. Capuron L, Neurauter G, Musselman DL, Lawson DH, Nemeroff CB, Fuchs D*, et al*. Interferon-alpha-induced changes in tryptophan metabolism. relationship to depression and paroxetine treatment. *Biol Psychiatry* 2003; **54**(9)**:** 906-914.

99. Brookes AJ, Lehvaslaiho H, Siegfried M, Boehm JG, Yuan YP, Sarkar CM*, et al*. HGBASE: a database of SNPs and other variations in and around human genes. *Nucleic Acids Res* 2000; **28**(1)**:** 356-360.

100. Ng PC, Henikoff S. Accounting for human polymorphisms predicted to affect protein function. *Genome Res* 2002; **12**(3)**:** 436-446.

101. Gregersen N, Bross P, Vang S, Christensen JH. Protein misfolding and human disease. *Annu Rev Genomics Hum Genet* 2006; **7:** 103-124.

102. Kopito RR. Aggresomes, inclusion bodies and protein aggregation. *Trends Cell Biol* 2000; **10**(12)**:** 524-530.

103. Kopito RR, Sitia R. Aggresomes and Russell bodies. Symptoms of cellular indigestion? *EMBO Rep* 2000; **1**(3)**:** 225-231.

104. Varshavsky A. Recent studies of the ubiquitin system and the N-end rule pathway. *Harvey Lect* 2000; **96:** 93-116.

105. Aridor M, Hannan LA. Traffic jams II: an update of diseases of intracellular transport. *Traffic* 2002; **3**(11)**:** 781-790.

106. Salavaggione OE, Wang L, Wiepert M, Yee VC, Weinshilboum RM. Thiopurine S-methyltransferase pharmacogenetics: variant allele functional and comparative genomics. *Pharmacogenet Genomics* 2005; **15**(11)**:** 801-815.

107. Wang L, Nguyen TV, McLaughlin RW, Sikkink LA, Ramirez-Alvarado M, Weinshilboum RM. Human thiopurine S-methyltransferase pharmacogenetics: variant allozyme misfolding and aggresome formation. *Proc Natl Acad Sci U S A* 2005; **102**(26)**:** 9394-9399.

108. Roth J, Yam GH, Fan J, Hirano K, Gaplovska-Kysela K, Le Fourn V*, et al*. Protein quality control: the who's who, the where's and therapeutic escapes. *Histochem Cell Biol* 2008; **129**(2)**:** 163-177.

109. Garcia-Mata R, Gao YS, Sztul E. Hassles with taking out the garbage: aggravating aggresomes. *Traffic* 2002; **3**(6)**:** 388-396.

110. Kalow W. The Pennsylvania State University College of Medicine 1990 Bernard B. Brodie Lecture. Pharmacogenetics: past and future. *Life Sci* 1990; **47**(16)**:** 1385-1397.

111. Evans DA, Manley KA, Mc KV. Genetic control of isoniazid metabolism in man. *Br Med J* 1960; **2**(5197)**:** 485-491.

112. Timbrell JA, Harland SJ, Facchini V. Polymorphic acetylation of hydralazine. *Clin Pharmacol Ther* 1980; **28**(3)**:** 350-355.

113. Weinshilboum RM, Otterness DM, Szumlanski CL. Methylation pharmacogenetics: catechol O-methyltransferase, thiopurine methyltransferase, and histamine N-methyltransferase. *Annu Rev Pharmacol Toxicol* 1999; **39:** 19-52.

114. Nabi R, Serajee FJ, Chugani DC, Zhong H, Huq AH. Association of tryptophan 2,3 dioxygenase gene polymorphism with autism. *Am J Med Genet B Neuropsychiatr Genet* 2004; **125**(1)**:** 63-68.

115. Comings DE, Gade R, Muhleman D, Chiu C, Wu S, To M, *et al*. Exon and intron variants in the human tryptophan 2,3-dioxygenase gene: potential association with Tourette syndrome, substance abuse and other disorders. *Pharmacogenetics* 1996; **6**(4)**:** 307-318.

116. Rickards H, Dursun SM, Farrar G, Betts T, Corbett JA, Handley SL. Increased plasma kynurenine and its relationship to neopterin and tryptophan in Tourette's syndrome. *Psychol Med* 1996; **26**(4)**:** 857-862.

117. Schroecksnadel K, Fiegl M, Prassl K, Winkler C, Denz HA, Fuchs D. Diminished quality of life in patients with cancer correlates with tryptophan degradation. *J Cancer Res Clin Oncol* 2007; **133**(7)**:** 477-485.

118. Brandacher G, Winkler C, Schroecksnadel K, Margreiter R, Fuchs D. Antitumoral activity of interferon-gamma involved in impaired immune function in cancer patients. *Curr Drug Metab* 2006; **7**(6)**:** 599-612.

119. Okamoto A, Nikaido T, Ochiai K, Takakura S, Saito M, Aoki Y, *et al*. Indoleamine 2,3-dioxygenase serves as a marker of poor prognosis in gene expression profiles of serous ovarian cancer cells. *Clin Cancer Res* 2005; **11**(16)**:** 6030-6039.

120. Schroecksnadel K, Winkler C, Fuith LC, Fuchs D. Tryptophan degradation in patients with gynecological cancer correlates with immune activation. *Cancer Lett* 2005; **223**(2)**:** 323-329.

121. Sedlmayr P, Semlitsch M, Gebru G, Karpf E, Reich O, Tang T, *et al*. Expression of indoleamine 2,3-dioxygenase in carcinoma of human endometrium and uterine cervix. *Adv Exp Med Biol* 2003; **527:** 91-95.

122. Pei J, Kim BH, Grishin NV. PROMALS3D: a tool for multiple protein sequence and structure alignments. *Nucleic Acids Res* 2008. **36**(7):2295-300.

123. Sunyaev SR, Lathe WC, 3rd, Ramensky VE, Bork P. SNP frequencies in human genes an excess of rare alleles and differing modes of selection. *Trends Genet* 2000; **16**(8)**:** 335-337.

124. Ng PC, Henikoff S. SIFT: Predicting amino acid changes that affect protein function. *Nucleic Acids Res* 2003; **31**(13)**:** 3812-3814.

125. Ng PC, Henikoff S. Predicting deleterious amino acid substitutions. *Genome Res* 2001; **11**(5)**:** 863-874.

126. Mooney S. Bioinformatics approaches and resources for single nucleotide polymorphism functional analysis. *Brief Bioinform* 2005; **6**(1)**:** 44-56.

127. Widner B, Werner ER, Schennach H, Fuchs D. An HPLC method to determine tryptophan and kynurenine in serum simultaneously. *Adv Exp Med Biol* 1999; **467:** 827-832.

128. Hara T, Yamakura F, Takikawa O, Hiramatsu R, Kawabe T, Isobe K, *et al*. Diazotization of kynurenine by acidified nitrite secreted from indoleamine 2,3-dioxygenase-expressing myeloid dendritic cells. *J Immunol Methods* 2008; **332**(1-2)**:** 162-169.

129. Travers MT, Gow IF, Barber MC, Thomson J, Shennan DB. Indoleamine 2,3-dioxygenase activity and L-tryptophan transport in human breast cancer cells. *Biochim Biophys Acta* 2004; **1661**(1)**:** 106-112.

130. Grant RS, Naif H, Espinosa M, Kapoor V. IDO induction in IFN-gamma activated astroglia: a role in improving cell viability during oxidative stress. *Redox Rep* 2000; **5**(2-3)**:** 101-104.

131. Alegre E, Lopez AS, Gonzalez A. Tryptophan metabolites interfere with the Ehrlich reaction used for the measurement of kynurenine. *Anal Biochem* 2005; **339**(1)**:** 188-189.

132. Kreuzer KA, Lass U, Bohn A, Landt O, Schmidt CA. LightCycler technology for the quantitation of bcr/abl fusion transcripts. *Cancer Res* 1999; **59**(13)**:** 3171-3174.

133. Sarkhosh K, Tredget EE, Uludag H, Kilani RT, Karami A, Li Y, *et al*. Temperature-sensitive polymer-conjugated IFN-gamma induces the expression of IDO mRNA and activity by fibroblasts populated in collagen gel (FPCG). *J Cell Physiol* 2004; **201**(1)**:** 146-154.

134. Babcock TA, Carlin JM. Transcriptional activation of indoleamine dioxygenase by interleukin 1 and tumor necrosis factor alpha in interferon-treated epithelial cells. *Cytokine* 2000; **12**(6)**:** 588-594.

135. Du MX, Sotero-Esteva WD, Taylor MW. Analysis of transcription factors regulating induction of indoleamine 2,3-dioxygenase by IFN-gamma. *J Interferon Cytokine Res* 2000; **20**(2)**:** 133-142.

136. Shirey KA, Jung JY, Maeder GS, Carlin JM. Upregulation of IFN-gamma receptor expression by proinflammatory cytokines influences IDO activation in epithelial cells. *J Interferon Cytokine Res* 2006; **26**(1)**:** 53-62.

137. Robinson CM, Hale PT, Carlin JM. The role of IFN-gamma and TNF-alpha-responsive regulatory elements in the synergistic induction of indoleamine dioxygenase. *J Interferon Cytokine Res* 2005; **25**(1)**:** 20-30.

138. Robinson CM, Shirey KA, Carlin JM. Synergistic transcriptional activation of indoleamine dioxygenase by IFN-gamma and tumor necrosis factor-alpha. *J Interferon Cytokine Res* 2003; **23**(8)**:** 413-421.

139. Chon SY, Hassanain HH, Pine R, Gupta SL. Involvement of two regulatory elements in interferon-gamma-regulated expression of human indoleamine 2,3-dioxygenase gene. *J Interferon Cytokine Res* 1995; **15**(6)**:** 517-526.

140. Dai W, Gupta SL. Regulation of indoleamine 2,3-dioxygenase gene expression in human fibroblasts by interferon-gamma. Upstream control region discriminates between interferon-gamma and interferon-alpha. *J Biol Chem* 1990; **265**(32)**:** 19871-19877.

141. Stormo GD. DNA binding sites: representation and discovery. *Bioinformatics* 2000; **16**(1)**:** 16-23.

142. Heinemeyer T, Chen X, Karas H, Kel AE, Kel OV, Liebich I*, et al*. Expanding the TRANSFAC database towards an expert system of regulatory molecular mechanisms. *Nucleic Acids Res* 1999; **27**(1)**:** 318-322.

143. Bryne JC, Valen E, Tang MH, Marstrand T, Winther O, da Piedade I*, et al*. JASPAR, the open access database of transcription factor-binding profiles: new content and tools in the 2008 update. *Nucleic Acids Res* 2008; **36**(Database issue)**:** D102-106.

144. Sandelin A, Alkema W, Engstrom P, Wasserman WW, Lenhard B. JASPAR: an open-access database for eukaryotic transcription factor binding profiles. *Nucleic Acids Res* 2004; **32**(Database issue)**:** D91-94.

145. Kahana C. Ubiquitin dependent and independent protein degradation in the regulation of cellular polyamines. *Amino Acids* 2007; **33**(2)**:** 225-230.

146. Jackson RJ, Campbell EA, Herbert P, Hunt T. The preparation and properties of gel-filtered rabbit-reticulocyte lysate protein-synthesis systems. *Eur J Biochem* 1983; **131**(2)**:** 289-301.

147. Benaroudj N, Goldberg AL. PAN, the proteasome-activating nucleotidase from archaebacteria, is a protein-unfolding molecular chaperone. *Nat Cell Biol* 2000; **2**(11)**:** 833-839.

148. De Matteis F, Marks GS. The effect of N-methylprotoporphyrin and succinyl-acetone on the regulation of heme biosynthesis in chicken hepatocytes in culture. *FEBS Lett* 1983; **159**(1-2)**:** 127-131.

149. Sugimoto H, Oda S, Otsuki T, Hino T, Yoshida T, Shiro Y. Crystal structure of human indoleamine 2,3-dioxygenase: catalytic mechanism of O2 incorporation by a heme-containing dioxygenase. *Proc Natl Acad Sci U S A* 2006; **103**(8)**:** 2611-2616.

150. Sun Y. Indoleamine 2,3-dioxygenase--a new antioxidant enzyme. *Mater Med Pol* 1989; **21**(3)**:** 244-250.

151. Karplus K, Karchin R, Draper J, Casper J, Mandel-Gutfreund Y, Diekhans M, *et al*. Combining local-structure, fold-recognition, and new fold methods for protein structure prediction. *Proteins* 2003; **53 Suppl 6:** 491-496.

152. Sunyaev S, Lathe W, 3rd, Bork P. Integration of genome data and protein structures: prediction of protein folds, protein interactions and "molecular phenotypes" of single nucleotide polymorphisms. *Curr Opin Struct Biol* 2001; **11**(1)**:** 125-130.

153. Suzuki S, Tone S, Takikawa O, Kubo T, Kohno I, Minatogawa Y. Expression of indoleamine 2,3-dioxygenase and tryptophan 2,3-dioxygenase in early concepti. *Biochem J* 2001; **355**(Pt 2)**:** 425-429.

154. Mellor AL, Baban B, Chandler P, Marshall B, Jhaver K, Hansen A, *et al*. Cutting edge: induced indoleamine 2,3 dioxygenase expression in dendritic cell subsets suppresses T cell clonal expansion. *J Immunol* 2003; **171**(4)**:** 1652-1655.

155. Aitken JB, Thomas SE, Stocker R, Thomas SR, Takikawa O, Armstrong RS, *et al*. Determination of the nature of the heme environment in nitrosyl indoleamine 2,3-dioxygenase using Multiple-scattering analyses of X-ray absorption fine structure. *Biochemistry* 2004; **43**(17)**:** 4892-4898.

156. Terentis AC, Thomas SR, Takikawa O, Littlejohn TK, Truscott RJ, Armstrong RS, *et al*. The heme environment of recombinant human indoleamine 2,3-dioxygenase. Structural properties and substrate-ligand interactions. *J Biol Chem* 2002; **277**(18)**:** 15788-15794.

157. Thomas SR, Salahifar H, Mashima R, Hunt NH, Richardson DR, Stocker R. Antioxidants inhibit indoleamine 2,3-dioxygenase in IFN-gamma-activated human macrophages: posttranslational regulation by pyrrolidine dithiocarbamate. *J Immunol* 2001; **166**(10)**:** 6332-6340.

158. Kamada Y, Jenkins GJ, Lau M, Dunbar AY, Lowe ER, Osawa Y. Tetrahydrobiopterin depletion and ubiquitylation of neuronal nitric oxide synthase. *Brain Res Mol Brain Res* 2005; **142**(1)**:** 19-27.

159. Kitamuro T, Takahashi K, Ogawa K, Udono-Fujimori R, Takeda K, Furuyama K*, et al*. Bach1 functions as a hypoxia-inducible repressor for the heme oxygenase-1 gene in human cells. *J Biol Chem* 2003; **278**(11)**:** 9125-9133.

160. Shibahara S. The heme oxygenase dilemma in cellular homeostasis: new insights for the feedback regulation of heme catabolism. *Tohoku J Exp Med* 2003; **200**(4)**:** 167-186.

161. Berry FB, Skarie JM, Mirzayans F, Fortin Y, Hudson TJ, Raymond V*, et al*. FOXC1 is required for cell viability and resistance to oxidative stress in the eye through the transcriptional regulation of FOXO1A. *Hum Mol Genet* 2008; **17**(4)**:** 490-505.

162. Zhao X, Zheng B, Huang Y, Yang D, Katzman S, Chang C*, et al*. Interaction between GATA-3 and the transcriptional coregulator Pias1 is important for the regulation of Th2 immune responses. *J Immunol* 2007; **179**(12)**:** 8297-8304.

163. Feng GS, Dai W, Gupta SL, Werner-Felmayer G, Wachter H, Takikawa O*, et al*. Analysis of interferon-gamma resistant mutants that are possibly defective in their signaling mechanism. *Mol Gen Genet* 1991; **230**(1-2)**:** 91-96.

164. Chon SY, Hassanain HH, Gupta SL. Cooperative role of interferon regulatory factor 1 and p91 (STAT1) response elements in interferon-gamma-inducible expression of human indoleamine 2,3-dioxygenase gene. *J Biol Chem* 1996; **271**(29)**:** 17247-17252.

165. Carlin JM, Borden EC, Byrne GI. Enhancement of indoleamine 2,3-dioxygenase activity in cancer patients receiving interferon-beta Ser. *J Interferon Res* 1989; **9**(2)**:** 167-173.

166. Guillonneau C, Hill M, Hubert FX, Chiffoleau E, Herve C, Li XL*, et al*. CD40Ig treatment results in allograft acceptance mediated by CD8CD45RC T cells, IFN-gamma, and indoleamine 2,3-dioxygenase. *J Clin Invest* 2007; **117**(4)**:** 1096-1106.

167. Tan PH, Yates JB, Xue SA, Chan C, Jordan WJ, Harper JE*, et al*. Creation of tolerogenic human dendritic cells via intracellular CTLA4: a novel strategy with potential in clinical immunosuppression. *Blood* 2005; **106**(9)**:** 2936-2943.

168. von Bubnoff D, Hanau D, Wenzel J, Takikawa O, Hall B, Koch S*, et al*. Indoleamine 2,3-dioxygenase-expressing antigen-presenting cells and peripheral T-cell tolerance: another piece to the atopic puzzle? *J Allergy Clin Immunol* 2003; **112**(5)**:** 854-860.

169. Kai S, Goto S, Tahara K, Sasaki A, Kawano K, Kitano S. Inhibition of indoleamine 2,3-dioxygenase suppresses NK cell activity and accelerates tumor growth. *J Exp Ther Oncol* 2003; **3**(6)**:** 336-345.

170. Kai S, Goto S, Tahara K, Sasaki A, Tone S, Kitano S. Indoleamine 2,3-dioxygenase is necessary for cytolytic activity of natural killer cells. *Scand J Immunol* 2004; **59**(2)**:** 177-182.

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Honors and Awards:

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Professional Memberships:

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Peer-reviewed Publications:

Sequence diversity and functional characterization of the 5'-regulatory region of human CYP2C19. **Arefayene, M**., T. Skaar, X. Zhao, J.E. Tanus-Santos, U. Brinkmann, I. Brehm, A. Nguyen, Z. Desta, and D. A. Flockhart. *Pharmacogenetics*. 2003; **13**(4):199-206.

Pharmacokinetics and QT interval pharmacodynamics of oral haloperidol in poor and extensive metabolizers of CYP2D6. M. Desai, J.E. Tanus-Santos, Z. Desta, L. Li, K. Lee, **Arefayene, M** and D. A. Flockhart**.** *Pharmacogenomics J*. 2003; **3**(2):105-13

Cytochrome P450 pharmacogenetics as a predictor of toxicity and clinical response to pulse cyclophosphamide in lupus nephritis. Takada K*****, **Arefayene, M**.***,** Desta Z, Yaboro CH, Boumpas DT, Balow JE, Flockhart DA Illei GG. *Arthritis Rheum.* 2004 ; **50**(7):2202-10.***contributed equally to this work**

Inhibition of human intestinal wall metabolism by macrolide antibiotics: effect of clarithromycin on cytochrome P450 3A4/5 activity and expression. Pinto AG, Wang YH, Chalasani N, Skaar T, Kolwankar D, Gorski JC, Liangpunsakul S, Hamman MA, **Arefayene, M**., Hall SD. *Clin Pharmacol Ther*. 2005 ; **77** (3):178-88.

Possible interethnic differences in quinidine-induced QT prolongation between healthy Caucasian and Korean subjects. Shin JG, Kang WK, Shon JH, **Arefayene, M.,** Yoon YR, Kim KA, Kim DI, Kim DS, Cho KH, Woosley RL, Flockhart DA. *Br J Clin Pharmacol*. 2007 ; **63**(2):206-15.

Selected Presentations:

Comparison of DNA from Blood and Buccal Swabs for genotyping of different CYP450 isoforms using Affymetrix P450 Gene Chip and Conventional Method. **Arefayene, M**., M. Alexander and D. A. Flockhart. Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics, 2000, Poster Presentation.

Frequencies of CYP2B6 allelic variants differ between Caucasian, African American and Korean population. **Arefayene, M.,** Z. Desta and D. A Flockhart. Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics, 2002, Poster Presentation.

Low doses of Haloperidol do not cause QT prolongation in poor Metabolizers of CYP2D6. M. Desai, J.E Tanus-Santos, Z. Desta, L. Li, K. Lee, **Arefayene, M** and D. A.Flockhart. Annual Meeting of the International Society for the Study of Xenobiotics, 2002.

Cytochrome P450 genetic polymorphism as predictor of response to cyclophosphamide in lupus nephritis. **Arefayene, M.,** Z. Desta, K. Takada, C.Yarboro, H. Carrero, G. Illei, D.A. Flockhart. Annual Meeting of the International Society for the Study of Xenobiotics, 2002, Poster Presentation.

Cytochrome P450 2B6 is the major catalyst of oxidative metabolism of the HIV-1 reverse transcriptase inhibitor efavirenz. Z. Desta, B. A Ward, M. A Hamman, **Arefayene, M.,** D.R Jones, S.D. Hall and D.A. Flockhart. Annual Meeting of the International Society for the Study of Xenobiotics, 2002.

Functional characterization of the 5'regulatory region of human CYP2C19. **Arefayene, M.,** T. Skaar, J.E Tanus-Santos, X. Zhao, U. Brinkmann, I. Brehm, A. Nguyen, Z. Desta and D.A Flockhart. Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics, 2003, Poster Presentation.

Pharmacogenetics determinant of cyclophosphamide therapy in patients with lupus nephritis. **Arefayene, M**., K. Takada, C. Yarboro, Z. Desta, G. G. Illei and David A Flockhart. Annual Meeting of the American Society for Clinical Pharmacology and Therapeutics, 2003, Poster Presentation.

Identification of indoleamine 2,3 dioxygenase gene variants. **Arefayene, M.,** Mamidipalli S, Philips S, A Flockhart, Wilkes D, and Skaar T. Gordon Research Conference 2005, Minisymposium and Poster Presentation.

Identification of functional genetic variants of the indoleamine 2,3 dioxygenase gene. **Arefayene, M.,** Mamidipalli S, Philips S, Cao D, D. A Flockhart, Wilkes D, and Skaar T. Annual Meeting of the American Association of Cancer Research 2006, Poster Presentation.

Functional Analysis of the Indoleamine 2, 3 Dioxygenase Genetic Variants. **Arefayene, M.,** Mamidipalli S, Philips S, Cao D, D. A Flockhart, Wilkes D, and Skaar T. Annual Meeting of the American Association of Cancer Research 2007, Poster Presentation

MUC1 contributes to the immunosuppressive microenvironment in pancreatic tumors. Mukherjee P, Tinder T, Bradley J, Gendler S, Basu G, Subramani D, **Arefayene, M.**, Skaar T. Annual Meeting of the American Association of Cancer Research 2007 (Minisymposium).

The Role of Indoleamine 2, 3 Dioxygenase in Sarcoid Immunoregulation. Rose AS*, **Arefayene, M*.,** Skaar TC, Kohli LL, Wilkes DS, Knox KS. Annual Meeting of the American Society of Transplant 2007 *contributed equally to this work

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Additional:

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