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Indoleamine 2,3-dioxygenase in malaria immunity and pathology

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Abstract

Indolamine 2,3-dioxygenase (IDO) is known as the first and rate limiting enzyme in the kynurenin pathway degrading tryptophan. As well as its known inducer IFNγ, IDO is increased in malaria infections but its physiological role is not yet well established. The discovery of a shorter variant of IDO has further complicated the picture and there are now both a longer and a shorter, truncated IDO isoform to characterise and put in the context of malaria. This study, with the aim to further typify the two isoforms of IDO, showed that the longer IDO isoform, which has tryptophan degrading activity, was strongly induced during malaria infection. The truncated isoform is constitutively expressed in the tissues examined and less induced in malaria-infected tissues than the longer IDO isoform. The data confirm the role of IFNγ as being the key inducer of both isoforms. This work gives a wider insight into the role of IDO to further elucidate the reasons behind the pathogenesis of malaria.

Keywords

Indoleamine 2,3-dioxygenase, kynurenin pathway, cerebral malaria

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INDOLEAMINE 2,3-DIOXYGENASE IN MALARIA IMMUNITY AND PATHOLOGY

CHRISTINA JOHANSSON

Sammanfattning

Malaria är en av vår tids största sjukdomar som dödar mer än en miljon människor årligen. Sjukdomen, som bärs vidare genom myggor, kommer av en blodburen protozo infektion av *Plasmodium* släktet.
Enzymet Indoleamine 2,3-dioxygenase (IDO), har visats vara starkt uttryckt under malariainfektion men dess roll och funktion i sjukdomen är oklar. Nyligen upptäcktes en kortare variant av IDO, den så kallade trunkerade IDO. Detta projekts mål har varit att vidare karaktärisera de två isoformerna av IDO för att få insikt om deras respektive roll i malaria samt att finna likheter och olikheter emellan dem.
Projektet har varit indelat i tre områden. För det första undersöktes nivån av den ena isoformen jämfört med den andra i nio olika vävnader från malariainfekterade och oinfekterade möss. Närvaron av cytokinen IFNγ, som är den enda kända positiva induktionsfaktorn för IDO uttryck, visade sig ha stor betydelse för de förhöjda nivåer av IDO som man ser i malaria. Ett annat fokus var att bestämma aktiviteten på promotorn, startsekvensen, av IDO generna. Olika längder av isoformernas promotorer infördes i en välkänd cellinje (HEK293). Cellerna fick sedan en tillsats av IFNγ, och resultatet jämfördes mot kontroll celler. Till sist undersöktes även enzymernas funktion med bland annat HPLC teknik.

Examensarbete 20p inom Molekylär bioteknikprogrammet

Uppsala Universitet, september 2005

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Abbreviations

ABTS	2,2´-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid)
CM	Cerebral malaria
FRET	Fluorescence resonance energy transfer
HPLC	High pressure liquid chromatography
HPRT	Hypoxanthine Phosphoribosyltransferase
IDO	Indoleamine 2,3-dioxygenase

IFNγ	Interferon gamma
iNOS)	Inducible nitric oxide synthase
KA	Kynurenic acid
KO+	IFNγ knockout infected mouse
KO-	Uninfected IFNγ knockout mice
Lf-IDO	Long form of IDO
PbA	*Plasmodium berghei* ANKA
PbK	*Plasmodium berghei* K173
p.i	Post infection
Tr-IDO	Truncated IDO
QA	Quinolinic acid
WT+	Infected wild type mouse
WT-	Uninfected wild type mouse (control)

1 Background

1.1 Malaria and the murine models

Malaria, the largest parasitic disease in the world, is due to a blood-borne protozoan infection caused by *Plasmodium* species. More than 500 million people are affected yearly with over one million cases of deaths. Africa (south of Sahara), large parts of Asia and also

South America are areas where malaria is common. Cerebral malaria (CM), the major life-threatening complication, is caused by an infection of the protozoe *Plasmodium falciparum*. The nature of the process, leading to the cerebral complications, is poorly understood and as a help in the search of insight into the pathogenesis several rodent models exists as tools.

Plasmodium berghei ANKA (PbA) causes neurological symptoms similar to those in human CM such as ataxia, convulsions and coma followed by death. *Plasmodium berghei K173* (PbK) is another strain that causes severe malaria, without any neurological symptoms. Instead, the PbK infected mice become sick and die with severe anemia between days 15 and 22 post infection. These models are useful for identifying those changes in gene expression which are specific to CM alone.

1.2 The Kynurenine pathway and Indoleamine 2,3-dioxygenase

Being a neurological complication, CM share features with disorders like AIDS dementia and other inflammatory neurological diseases. A particular example is elevated levels of a potent neuro-excitotoxin, quinolinic acid [1]. It has been suggested that a change in the ratio of quinolinic acid (QA) to kynurenic acid (KA), which antagonizes the neuro-excitotoxic effects, could contribute to the symptoms of cerebral malaria [2]. QA and KA are two of several neuroactive metabolites being products from the kynurenine pathway with altered levels in CM [3] (Fig. 1).

Indoleamine 2,3-dioxygenase (IDO) is the first and rate limiting enzyme of the kynurenine pathway and catalyses the degradation of tryptophan. As well as for its known inducer Interferon gamma (IFNγ), the concentration of IDO is increased in malaria infections. Its physiological role, however, is not yet well established. Since being discovered in the 1960s [4] it has been found in many tissues in the mammalian body and it is strongly induced in rodents and humans following immune activation by infectious pathogens [5], [6] and cancer.

The cellular source of expression was initially thought to be cells in the macrophage lineage but later studies have shown that IDO may be induced in fibroblasts as well as epithelial cells followed upon stimulation with IFNγ [7], [8], [9]. Hansen *et al.* (2000) found that vascular endothelial cells was a big source of expression during malaria infection [10], which indicated that IDO may be a protective response against the intravascular parasite. As several cells are expressing IDO, many other theories about its activity and function, apart from catabolising tryptophan, have been considered.

Another anatomic region of interest in IDO research is the female sex organ. In reports where IDO has been suggested to defend the female reproductive tract against ascending bacterial and parasitic infections [11], [12], again, a protective mechanism is assumed. Other studies have focused of the role of IDO in the regulation of fetomaternal tolerance in the mouse pregnancy. Here an immunosuppression function for IDO is suggested, as

inhibition of IDO resulted in T-cell-mediated rejection of allogeneic conceptuses [13]. High levels of expression in the placenta and also in the lung have been reported [14].

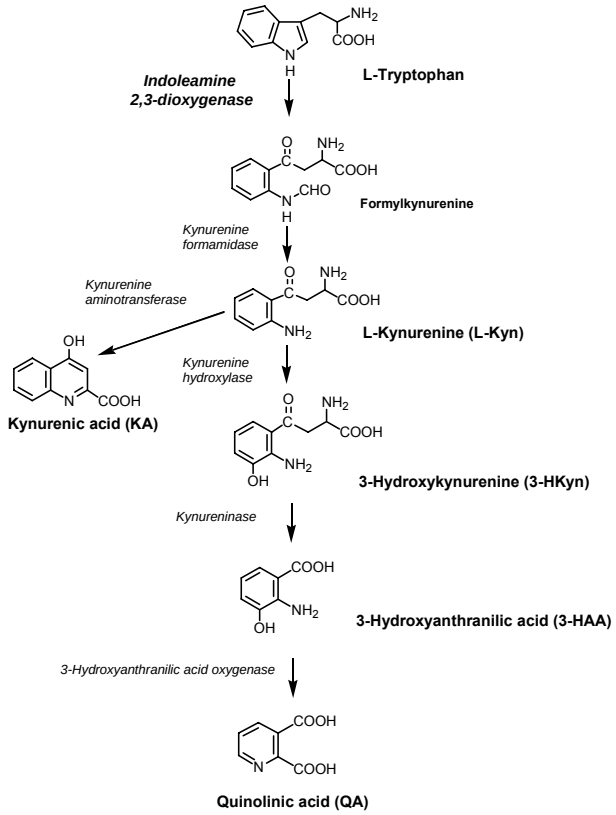


Fig. 1. Indoleamine 2,3-dioxygenase (IDO) is the first and rate limiting enzyme in the kynurenine pathway degrading tryptophan. Quinolinic acid and kynurenic acid are two of several neuroactive metabolites being products of the kynurenine pathway with altered levels in CM. Figure adapted from ref. 1.

The discovery of a shorter transcript of IDO by the Hunt laboratory (unpublished result) raised the interest for the enzyme further. The sequence of this truncated form was already deposited in the GenBank database but without any associated publication (accession number: AK033783). Being regulated by different promoters and with the promoter and transcription start of the truncated IDO (Tr-IDO) within the gene of the longer form of IDO (Lf-IDO, accession number: BC049931), the two transcripts vary in their 5´end resulting in two isoforms. Tr-IDO is predicted to be 90 amino acids shorter than the Lf-IDO based on the presence of a conserved methionine residue and a Kozac consensus site, a common motif in promoters, but the native protein has not been isolated from tissue for determination of size or sequence. It was not clear whether the truncated form of the protein had the same function as the longer. Another difference between the two isoforms was that the Tr-IDO seemed to be constitutively expressed at all times while the Lf-IDO was highly induced in malaria infection.

1.3 The aim of this project

This study, with the aim to further characterise the two isoforms of IDO, was divided into three parts. First of all, nine tissues of PbK infected mice were examined for the presence of IDO. The abundance of Tr-IDO and Lf-IDO was measured with quantitative real time PCR using primers specific for each isoform, to see if and in which tissue there was a difference in IDO expression as compared with non-infected control. The second part involved the promoter regions and their response to IFNγ. Human embryonic kidney cells, HEK239, were transfected with vector constructs that included different lengths of the promoters. The vector included a reporter gene (β-lactamase) after the inserted promoter region. Thereby, an active promoter resulted in transcription of β-lactamase. Samples which got an addition of IFNγ was compared to those who did not (control samples), by measuring the fluorescence from intact and degraded CCF2-FA (a β-lactamase substrate) with FRET (Fluorescence Resonance Energy Transfer). A third approach was to investigate if the two forms of IDO had the same activity of converting tryptophan into kynurenine, for this HPLC analysis was applied.

2 Materials and methods

2.1 The abundance of IDO isoforms in mouse tissues

2.1.1 Inoculation of mice and collection of tissues

16 C57B16 mice were used in this study with uninfected and infected wild type and IFNγ gene knockout mice in groups of four. The mice were inoculated with 10^6 parasitised red blood cells obtained from the blood of infected animals and suspended in 200µl of phosphate buffered saline (PBS). The IFNγ knock out mice were from Grenentech south (San Fransisco, California) and wild types were obtained from the Blackburn Animal House, University of Sydney being 6-8 weeks old at the time of the study. The parasite used were *Plasmodium berghei K173* (PbK) from Dr Ian Clark, Australian National University, Canberra, Australia.

On day 8 after inoculation, infected mice and controls were euthanized. Tissues (brain, lung, spleen, heart, liver, kidney, muscle, epididymis– the testicular appendages and aorta) were immediately collected and transferred to tubes containing 1ml TRIzol® reagent (Sigma) and Zirconica beads, 1mm (Biospec Products Inc). The tubes were immediately put on dry ice and stored in -80˚C freezer.

2.1.2 RNA extraction

The samples were thawed and tissues homogenized by using a FastPrep homogenizer (BIO 101, Savant). Chloroform (0.2 ml) was added, the lysate was mixed well and samples were centrifuged at 14,000 rpm for 15 minutes. The resulting aqueous layer was transferred to a new tube. To precipitate the RNA, 500µl of isopropanol was added. In the samples with

lower mRNA yields (aorta and epididymis) 3µl glycogen (25µg/µl) was added to visualize the pellet. Samples were microfuged at 14,000 rpm for 15 minutes and the resulting pellet was washed with 70% (volume/volume) ethanol, air dried and resuspended in 200µl RNAse free water.

2.1.3 cDNA synthesis

To remove any contaminating genomic DNA all samples were DNase treated by use of the DNA-free™ kit (Ambion). The concentration of each sample was determined with spectrophotometry. mRNA was synthesised to cDNA by using the Sensiscript reverse transcriptase kit (Qiagen). 1µl oligo dT (1µg/µl) was mixed with 1µg mRNA. Water was added to make up a total volume of 11µl. After 10 minutes of incubation at 70˚C the samples were immediately transferred to ice. 9µl of master mix, was added to each tube to contain 1X RT buffer, 10mM DTT, dNTP, RNAse out and reverse transcriptase, all included in the kit. The samples were incubated at 37˚C for one hour and then for 2 further minutes at 95˚C, before cooling them down on ice. Finally the cDNA were diluted up to 300µl in RNAse free water and stored in the -20˚C freezer.

2.1.4 Standard curve set up

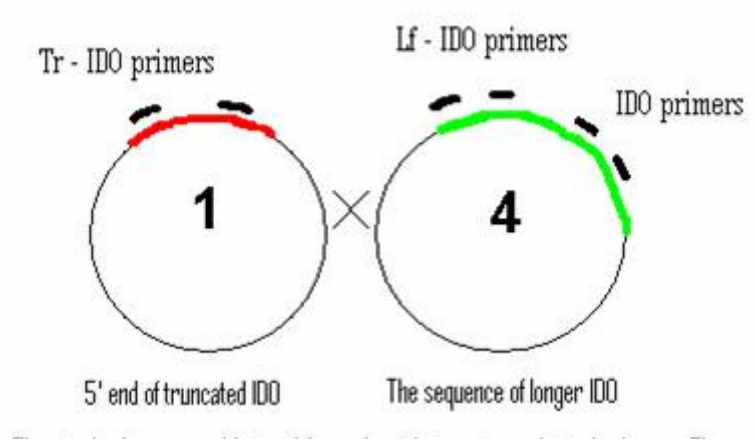


Fig. 2. The standard curve used to give a relative quantification of the isoforms was set up from a mix of two plasmids, where one contained the sequence on the longer IDO and the other only the 5´end of the truncated IDO. With different primer pairs, both isoforms and the total IDO could be measured with aliquots from the same standard stock. Because of primer efficiency problems, a better standard curves were given when the plasmids was mixed 1:4 (Tr-IDO:Lf-IDO).

The set of standards were based on plasmids with the sequences of IDO isoforms inserted. By using a plasmid with only the 5´end of the truncated form and primers for this part the level of the Tr-IDO would be revealed. Another plasmid with the sequence of the longer IDO together with primers attaching to its 5´end would give the level of the other isoform in each tissue. The latter plasmid standard could also be used when measuring the

total amount of IDO in the tissues by using a set of primers that were complementary to the 3´ end, which is identical for both isoforms.

The concentrations of the two plasmid standard stocks were measured spectrophotometrically, which did not allow for accurate concentration comparisons between the truncated and the longer IDO isoform. More accurately we could compare the amount of the long form versus the total amount of IDO since these data originating from the same plasmid. Then it could also be assumed that what was not the long form of the total amount of IDO must be the truncated isoform. By doing this assumption a good picture of the expression of the two isoforms was given.

Because of differences in primer binding efficiencies the standards were mixed in a 4:1 ratio, thus four times as much of the plasmid with the truncated IDO piece as with the longer IDO. This mix resulted in a good standard curve for all cases.

2.1.5 Real-time PCR

Real-time PCR analyses were performed with a Corbett Research Rotor-Gene™ (RG 3000, Applied Biosystems). 9µl of cDNA (~3µg/ml) template was mixed with 10µl of Invitrogen™ PCR mix (Platinum® SYBR® Green qPCR SuperMix-UDG) and 1µl of a 10µM primermix (table 1). The thermal conditions for the PCR started with an incubation at 95˚C for 1 min followed by 50 cycles with 15 seconds at 95˚C, 56˚C for 20 seconds and 72˚C for 20 seconds. After that, a stepwise temperature increase from 60˚C to 95˚C was applied to determine melting curves for the products. These were used to check the quality of the PCR products for correct amplification. All samples were quantified by using a standard curve and normalised to the levels of a reference housekeeping gene, HPRT (Hypoxanthine Phosphoribosyltransferase) which has been shown to be appropriate for this disease model since it is constitutively expressed during infection.

Table 1. Shown here are the primer sequences used for the detection of IDO isoforms in mice tissues. Primers specific for the truncated isoform (Tr-IDO) and the longer isoform (Lf-IDO) recognizes the 5´end of respective enzyme while primers for detection of both isoforms (IDO) attach to the 3´end where the enzymes are identical. HPRT was used as a housekeeping gene in this experiment.

	Primer sequences, 5' - 3'	
Gene	Forward	Reverse

Tr-IDO	TGA CCC CGG ACG GTA AAA TT	GGC AGA TTT CTA GCC ACA AGG A
Lf-IDO	AGA TGA AGA TGT GGG CTT TGC T	GGC AGA TTT CTA GCC ACA AGG A
IDO	CAA AGC AAT CCC CACTGT ATC C	GCC AGC CTC GTG TTT TAT TCC
HPRT	CAT CTA AGA GGT TTT GCT CAG TGG	ACA GCC AAC ACT GCT GAA ACA T

2.1.6 Statistical analysis

Statistical analyses were performed using the Mann Whitney test, (http://www.graphpad.com/articles/interpret/Analyzing_two_groups/mann_whitney.htm. A Kruskal-Wallis test with Dunn´s post test was also performed in addition to that, (http://www.graphpad.com/articles/interpret/ANOVA/kruskal_wallis.htm).

2.2 Induction of IDO promoter region with IFNγ

2.2.1 Amplification of promoter fragments and insertion into TOPO cloning vector

Mouse genomic DNA extracted from tail was used as a template. Primers were designed to amplify promoter fragments of different lengths from a 1500bp region upstream from transcription start of both isoforms. The different lengths were 1500bp, 1200bp (for cIDO only), 1100bp, 700bp and 300bp (table 2).

Table 2. Primers were designed to amplify different lengths of the promoters for each isoform. The 1200bp fragment was only examined for the truncated isoform (Tr-1200).

	Primer 5' - 3'	
Gene	**Forward**	**Reverse**
Tr-1500	GAC GAA GAG AGA TCC TTT GTG G	CAG GAC ACT TGT AGC AAG GAT ATC
Tr-1100	TGTCAAATTCAGAGCCCACTAC	"
Tr-1200	CCA CAT AGA TGA AGA TGT GGG C	"
Tr-700	CCT TGA TTG TGC TTT TGT GC	"
Tr-300	CAG AGT AAG TAG TCA GTC GCA CGT	"
Lf-1500	ACA TAT GCA GCTA AAG TCA AGA GC	AAG GAT CCT TCT AGA ACC TTC TGT AG
Lf-1100	CAT CCT TTT GTC TCA CCT CCA	"
Lf-700	GGT GGA CCA CCT TCC AAG AT	"
Lf-300	TAA CAG GTG GCC ACC CAA AC	"

For each reaction 12.5µl 2XBio-x-act Short Mix (Invitrogen™), 5µl DNA, 1µl primer mix (Table 2, conc. 10µM) and water was mixed to a total volume of 25µl. The PCR reactions were performed with a Mastercycler personal, Eppendorf. The PCR program was initiated with 95˚C for 2 minutes, followed by 35 cycles of 95˚C for 20 seconds, 50˚C for 30

seconds and 72˚C for 90 seconds. A final elongation step at 72˚C for 20 minutes finished the reaction and temperature was set to 4˚C until samples were to be collected. To make sure that the products were of correct length a fraction of each sample (18µl) were run on an agarose gel for 45 minutes at 120V. The gel was then stained with ethidiumbromide and the DNA bands visualized with a UV camera.

The following procedure were performed according to the protocol of TOPO TA Cloning® Kit but described here briefly. 4µl of fresh PCR product were mixed with TOPO vector cloning construct. Electrocompetent *E. coli* cells were heat chocked at 42˚C to promote an uptake of the vectors. The bacteria were grown in S.O.C media (supplied in the kit) at 37 ˚C in a shaking incubator for 1h and then plated out onto LB agar plates selecting for ampicillin resistance. The next day 6 colonies of each vector variant were picked and cultured overnight in 3 ml LB medium with ampicillin (100µg/ml) to give larger amount of DNA. As a control, to make sure that the colonies had the correct insert another PCR reaction was run. 1µl culture was mixed with 22.5µl of PCR Mix High Fidelity (Invitrogen™) and 1.5µl primer mix. Apart from starting the reaction at 95˚C for 10 minutes, the program was set as described above. One successful clone with an insert of each length was grown further o/n.

The plasmid DNA was extracted using the S.N.A.P.™ MiniPrep Kit (Invitrogen) and the quantity determined by absorbance at 260nm (SpectraMax 190 microplate reader, Molecular devices). To make sure that both the sequence and the orientation of the insert were correct, the DNA was sequenced. The successful clones were grown further and scaled up to give a bigger amount of DNA. By using the S.N.A.P.™ MidiPrep Kit (Invitrogen) the plasmid DNA was separated from the bacteria and the resulting concentration was determined by absorbance.

2.2.2 Transfection of HEK293 cells

HEK293 cells- a transformed human embryonic kidney cell line was cultured in DMEM™ (GIBCO) supplied with 10% (v/v) heat inactivated FCS, L-glutamine (2mmol/l), HEPES (10mmol/lit), Benzylpenicillin (100U/L) and streptomycin sulphate (100µg/l) prior to use. $1X10^6$ cells were seeded in 500µl antibiotic free DMEM in the wells of a 24 well culture dish, incubated at 37˚C and 5% CO_2, reaching confluence after 1 day. According to the protocol, 0.8µg of DNA per reaction was mixed with Lipofectamine™ 2000 (Invitrogen) in serum- and antibiotic free DMEM prior addition to cells. The Lipofectamine-DNA mix of each plasmid variant was distributed into the media of four wells each and cells were transfected over night.

2.2.3 Induction of IFNγ

The media from transfected HEK293 cells was removed. Two out of four wells, with cells transfected with a specific vector insert, got an addition of 500µl antibiotic free DMEM including human IFNγ (62.5U/µl). The two remaining got media without IFNγ, to serve as

transfected controls. The plate was incubated o/n. The following day the media was removed and cells were washed in 100µl fridge cold PBS. 100µl of CHAPS (0.5%) was added to each well and the lysate was transferred to a fresh eppendorf tube. The lysates were then centrifuged on maximum speed at 4˚C and the supernatant was collected into new tubes.

GeneBLAzerTM Detection Kit was used when investigating the expression of the β-lactamase reportergene and the exact procedure can be found in the protocol supplied with the kit. 45µl of the supernatant and 5µl of a 100µM CCF2-FA stock solution was transferred to a 96 well reading plate to obtain a final concentration of 10µM of the substrate. A Spectra Max fluorescence reader (Gemini EM, Molecular devises) excitated the samples at 409nm and red the outgoing emission signal for intact substrate at 520nm and cleaved substrate at 447nm. Average fluorescence values and the ratio of cleaved to intact substrate were calculated to reveal the level of expression and the promoters' response to IFNγ.

2.3 Analysis of IDO activity

Another focus of this work was to determine whether both IDO isoforms had the enzymatic capability of converting tryptophan into kynurenine. Expressionvectors (Gateway® pDEST™26 Vector, Invitrogen) containing the DNA sequence for each isoform (Lf-IDO and Tr-IDO) and also one containing the sequence of the related gene, IDO-2, was constructed and transfected into *E. coli* cells. The plasmids of the colonies were extracted and the resulting DNA concentrations were measured as described in previous section.

2.3.1 The first experiment

HEK293 cells were plated out and cultured over night in a 24 well plate as explained previously. The next day, three wells for each isoform were supplied with DNA-Lipofectamine mix with the expression vectors containing DNA inserts of either Tr-IDO or Lf-IDO. Three wells got the same volume of serum- and antibiotic free media to serve as controls. After a night's incubation, the old media was removed and 500µl of fresh antibiotic free DMEM containing tryptophan (final conc. 200µM) was supplied to all nine wells. The following day, 750µl of the supernatant from all nine wells were collected in eppendorf tubes and mixed with 250µl TCA 20%. After vortexing, the samples were frozen down at -20˚C and stored until HPLC analysis. To make sure that the transfection reaction was successful and that IDO was expressed and translated in the cells, the cells were lysed to preserve mRNA as well as protein for control experiments. This experiment was also tried with B-end cells to see if the outcome would be different. Cells were cultured in RPMI supplied with supplied with 10% (v/v) heat inactivated FCS, L-glutamine (2mmol/l), HEPES (10mmol/lit), Sodium pyruvate (1mmol/lit), Benzylpenicillin (100U/L) and streptomycin sulphate (100µg/l) prior to use, otherwise the procedure was the same.

2.3.2 The second experiment

Another method, where co-factors and L-Tryptophan was added to cell lysate rather than to intact cells, was also tried to test if it would have any impact on the results of the protein activity. After being plated out, cultured and transfected as previously described, HEK293 cells were washed three times with 100µl PBS and then lysed by 5 cycles of freezing at -80˚C and thawing at 37˚C in PBS (100µl/well) supplemented with protease inhibitors (Protease Inhibitor Cocktail Tablets, complete, mini, EDTA-free, Roche). The cell lysates were collected in eppendorf tubes and centrifuged at 14 000rpm for 5 minutes at 4˚C. The resulting supernatants were transferred into fresh tubes and used for the IDO assay.The IDO assay reaction buffer contained ascorbic acid (10mM), methylene blue (25µM), L-Trp 200µM and catalase (0.2mg/mL) in PBS (pH 7.4). To start the reactions, 50µl of the lysate was mixed with 50µl reaction buffer (1:1 ratio). After 30 minutes at 37˚C, the reactions were terminated by addition of 25µl TCA. To allow a complete degradation of N-formyl-Kynurenine into kynurenine the samples sat in room temperature for 1 hour. The samples were centrifuged at 14000rpm and 20µl of the resulting supernatants were run on HPLC for the concentrations of kynurenine and tryptophan.

2.3.3 HPLC analysis

Tryptophan (Trp) and kynurenine (Kyn) in the resulting supernatant were separated on a VeloSep RP-18 column (Applied Biosystems, Inc., Foster City, CA; 10 x 0.32 cm with i-cm guard column, 3µm particle size) with 100 mM choloroacetic acid/acetonitrile (pH 2.2) (8:2, vol/vol) and detected photometrically (Kyn, 365 nm; Trp, 280 nm) with a flow of 0.5 ml/minute.

2.3.4 Control experiments

In the first experiment control steps were performed to make sure that the transfection worked properly and to be convinced that IDO isoforms were produced by the cells. The transfection was done in triplicates for each isoform with non-transfected HEK293 cells (mock cells) as control. The cells were lysed with two different solutions (Trizol or STET lysis buffer), after taking off the media for HPLC analysis, two samples for protein extraction and a third for IDO mRNA were prepared.

Confirmation of IDO mRNA in cell lysates by rt-PCR

The cells in one of the three wells for each isoform transfection and mock cells were lysed by the addition of 100µl Trizol (50µl/cm^2). The mRNA was extracted from the lysates, converted to cDNA and analysed with an rt-PCR machine, Rotor-gene 3000 (Corbett

research) using the method previously described. In the step when isopropanol is brought to the tubes, all samples also got an addition of 3µl glycogen before centrifugation. After washing the resulting pellet in EtOH, the samples were resuspended in 30µl RNA-water. After DNAse treating the three samples using a DNAfree kit (Ambion), 1µg mRNA was synthesised to cDNA. The samples were diluted to a final volume of 300µl with RNAse free water and a PCR with IDO primers (table 1) was set up to control that IDO mRNA was present in the samples.

Gel Electrophoresis and His-staining for IDO

For the two remaining wells of each IDO isoform and mock cells, 100µl STET lysis buffer (50µl/cm^2) supplied with protease inhibitors (Protease Inhibitor Cocktail Tablets, complete, mini, EDTA-free, Roche) was added. These lysates were then stored at -20˚C to be examined for IDO protein content.

As 10% SDS polyacrylamid gel was poured to separate and visualize the proteins. To seal the plates, 1ml of 10% resolving gel (with 20µl TEMED added) was polymerized in the bottom before the rest (7ml) of the 10% resolving gel (refer to) was poured. The acryl amide solution was overlaid with water saturated butanole to even out the gel level and to prevent oxygen from inhibit the polymerization. The gel was placed in a vertical position for the resolving gel to fully polymerise.
After the polymerisation was complete (45minutes), the overlay was poured off and the gel surface was washed using milliQ water. A 5 % stacking gel solution was prepared and poured on top of the resolving gel and a comb creating 12 wells was inserted immediately. After 30 minutes, the stacking gel was polymerised. An equal amount of sample buffer was added to the protein cell lysates and samples were heated for 5 minutes at 95˚C. 7µl of BenchMark™ His-tagged Protein Standard, a molecular weight marker (Invitrogen life technologies) and 30µl of the samples were loaded into the corresponding wells. The gel was run at 20mA for approximately 4 hours in running buffer and then left in fixation solution over night. The following day the gel was stained using InVision™ His-tag In-gel Stain (Invitrogen) and the image was immediately visualized with UV camera. After this, the gel could still be used for techniques such as Western blotting.

Membrane transfer, Western blot and detection of IDO

The proteins separated on the SDS gel, was transferred onto a PVDF membrane. The membrane was first wet in methanol before being soaked in transfer buffer along with the gel, Whatman filter papers and the sponges. These were then put together in a determined order to create a “sandwich”. To prevent air bubbles from interfering with the transfer process a pipette was rolled over the sandwich before placing it in the right direction allowing proteins to flow onto the membrane towards the anode. The apparatus was set up with everything submerged in cold transfer buffer. A Magnetic stirring flea and an ice pack ensured that the apparatus did not over-heat. The transfer was run at 10V for 4 hours.

After completing the transfer, the membrane was washed in TBS-T (TBS with 0.05% Tween 20) and then blocked for 1h in room temperature using 5% Blotto (5% Skim milk powder in TBS) to prevent non-specific binding of proteins. After washing 3x10 minutes in TBS-T, the membrane was incubated with a primary antibody to IDO [15] diluted 1:2000 in Blotto. That was either done over night at 4˚C or for 1-2h in room temperature on a rocking platform. Another 3x10 minutes washing step in TBS-T followed before the membrane was incubated for one hour with a biotinylated secondary antibody (ELC Anti-rabbit IgG, Amersham Biosciences) diluted 1:5000 in 5% Blotto. The unbound antibodies were cleared of the membrane with a final 3x10 minutes wash in TBS-T. The detection solutions (ECL Plus™ Western Blotting System, Amersham Biosiences) were mixed 1:1 and pipetted onto the membrane to cover the surface. The prepared membrane was used to expose photopaper and the resulting image was developed.

2.3.5 IDO peroxidase activity

HEK293 cells transfected with cIDO, iIDO and IDO-2 was lysed with a freeze-thaw method as described above. The IDO proteins were purified from the lysates with a His-tag kit (Ni-NTA Spin Kit, QIAGEN) by following the protocol included in the kit. Flow through from the first spin was saved as a control step. Purified protein was incubated with heme (prepared fresh in 0.1M NaOH) in room temperature. 2,2´-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) was then dissolved in the heme protein solution. Final concentrations in 200µl were; 0.25µM heme, 1.25mM ABTS, and 2µM protein mixed with H_2O_2 (final concentration: 100µM). The absorbance was measured for all samples at both 414 and 700nm (time=0). Solutions containing heme protein and ABTS were rapidly mixed with H_2O_2 solutions in a 96-well plate, the absorbance was measured by several time points and curves were drawn. See reference 21 (Moffet et al., 2000) for background to this experiment.

3 Results

3.1 Abundance of IDO isoforms in PbK infected tissues

3.1.1 Observations of infected mice

On the day when the mice were put to death, symptoms of malaria were seen among all infected animals. They were slower, had ruffled fur, and hunched up against each other. Some of the organs of infected animals, most obviously the

spleen, were bigger than in uninfected. The brighter color of some of the organs was also an indication on the pathogenesis of malaria, with lysis of red blood cells and anemic tissues. Due to the same reason the blood was noticeably less red, the lung appeared grey and the liver was darker in infected mice.

3.1.2 Diagrams over the expression of IDO isoforms in mice tissues.

The following diagrams are showing the average measured amount of Lf-IDO and Tr-IDO in the nine tissues of PbK infected mice (WT+), IFNγ knockout infected mice (KO+) and uninfected IFNγ knockout mice (KO-), compared to that of uninfected wild type mice (WT-, control).

Figure 3a are showing values of the long isoform in comparison with the total amount of IDO. What is left between these two columns should correspond to the truncated IDO isoform. These numbers are based on the same standard plasmid and are therefore directly comparable. The truncated isoform was also detected by its own set of primers attaching to the other standard plasmid included in the standard mix. The separate diagrams of the truncated IDO isoform (Fig. 3b), the total amount of IDO (Fig. 3c) and the longer isoform (Fig. 3d) are showing levels, for infected wild type as well as infected and uninfected knockouts, relative to an uninfected wild type control. The numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT), which is often used as a housekeeping gene.

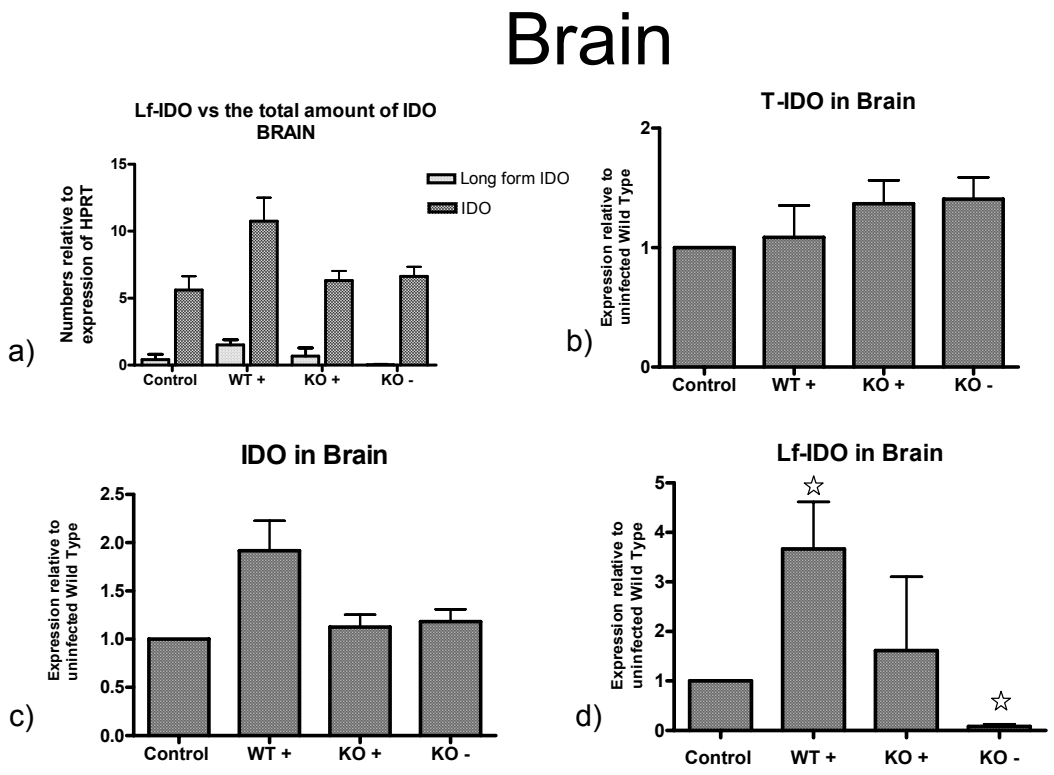


Fig. 3. Brain expression data. **Indoleamine 2,3-dioxygenase mRNA levels in brain of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. Mice were killed on day 8 p.i. and RNA was extracted from the tissue. After reverse transcribing mRNA the levels of IDO isoforms were measured by quantitative real time PCR as described in the method section. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-).**
In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). There was an increase of the total amount of IDO in brain in malaria infected mice which the longer IDO seems to be responsible for. A significant increase in the expression of the longer but not of the truncated IDO can be seen when comparing diagrams b) and d). Columns and vertical bars represent mean ± SEM (n=4). Significantly different from control: ☆ P<0.05.

In brain of the controls, Tr-IDO was the more abundant isoform (Fig. 3a). Malaria made the total amount of IDO increase mildly (Fig. 3a, 3c) and the longer isoform gave the biggest contribution to this increase. A significant increase in the expression of the longer but not of the truncated IDO can be seen when comparing diagrams in Figure 3 (b, d). The brain contained more of the shorter IDO both in non-infected and infected mice, but after infection the concentration of the longer form increased more. IFNγ is needed to trigger this increase in expression since infected IFNγ KO mice had IDO levels close to control. Levels of the longer isoform in uninfected KO mice were significantly lower than control, indicating that IFNγ is important not only for an increase but for normal levels to be produced. There might also be another substance involved in the increased expression of the longer form in brain as infected KO mice have a higher level of IDO than uninfected KOs.

Lung

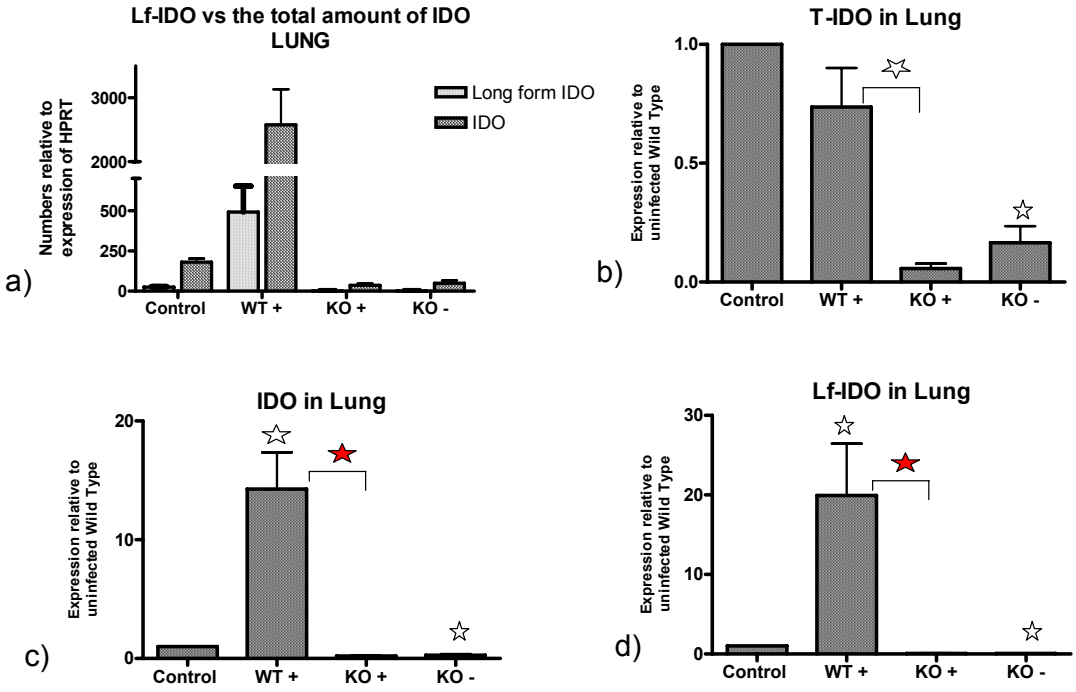


Fig. 4. Lung expression data. **Indoleamine 2,3-dioxygenase mRNA levels in lung of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-).**
In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). There was a high increase of the total amount of IDO in lung in malaria infected wild type mice. According to diagram a) the increase is caused by both isoforms. A higher expression of the longer isoform was a fact with reference to diagram d) but the same can not be seen for the truncated form. According to the result for Tr-IDO the expression is rather lower in infected lung than in control. The increase was significantly IFN dependent with levels of IDO in lung from IFN knockout mice being close to that of control. Columns and vertical bars represent mean +/- SEM (n=4). Significantly different from control: ☆ P<0.05. Filled stars indicate a significant difference presented by both statistical tests (Mann Whitney and Kruskal-Wallis test).

A very large increase in IDO expression for WT+ mice was demonstrated in the tissue from the lung. Since the expression of IDO in IFNγ KO mice were close to that in the non-infected control, this showed that the expression of IDO in lung was dependent on IFNγ. The absence of IFNγ appeared to affect the normal expression of Tr-IDO, since the IFNγ KO mice had a Tr-IDO level lower than that in the control (Fig. 4b). In uninfected lung tissue, Tr-IDO is more abundant than Lf-IDO and that seems to be the case in malaria infected animals as well (Fig. 4a). With malaria, IDO increased a lot in lung (Fig. 4a, 4c) however which isoform that contributes more to this increase is uncertain. Assuming that what is left between the columns of total IDO and the longer form (Fig. 4a) is Tr-IDO one would say that levels of both isoforms increased but the longer increased

more. However, when inspecting the data generated with the shorter isoform it appears as if the level rather decrease after infection (Fig. 4b, 4c). What is correct here is difficult to say. Lung was the tissue that displayed the highest IDO levels in infected as well as non-infected tissue of all tissues examined.

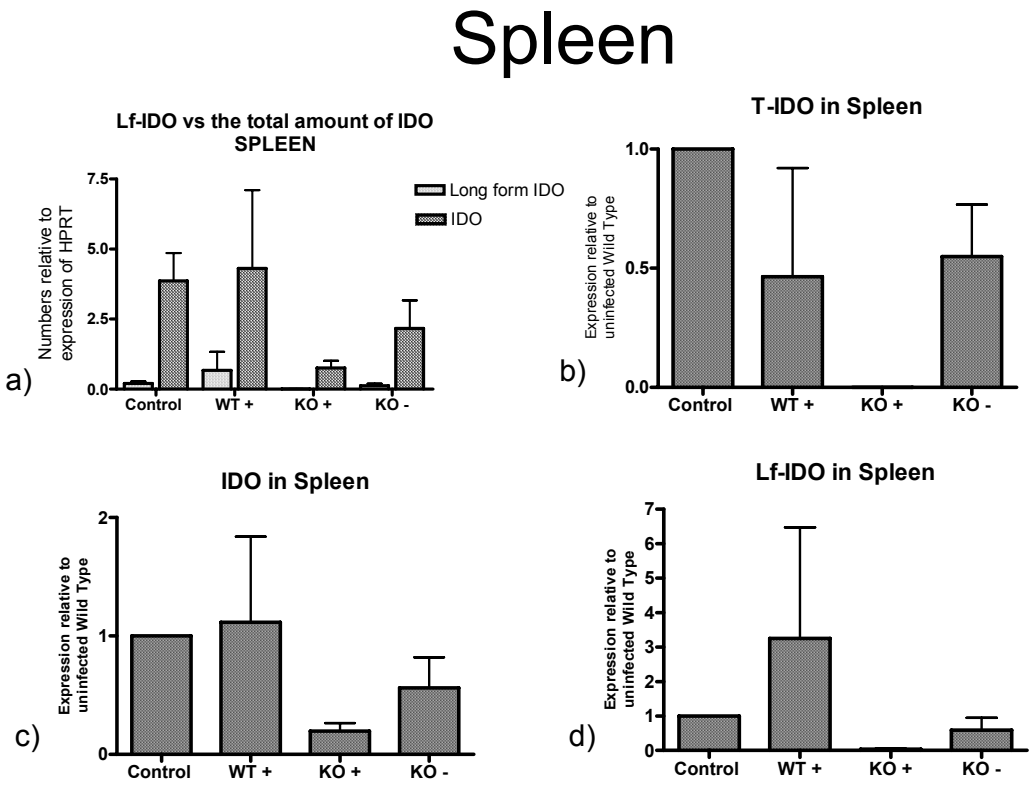


Fig. 5. Spleen expression data. **Indoleamine 2,3-dioxygenase mRNA levels in spleen of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-). In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). The total expression of IDO in spleen is not increased in malaria (a, d). The basal transcription of IDO is lowered when IFNγ is knocked out.**

The total IDO expression in spleen was not increased after infection by the Plasmodium parasite. Looking at the specific diagrams the truncated isoform was actually decreased in infected animals (Fig. 5b) whereas the level of the longer IDO was only slightly elevated (Fig. 5d). Comparing the abundance, there is a lot more of Tr-IDO in healthy than in infected spleen (Fig. 5a). As seen in the diagrams in figure 5, IFNγ is playing a role in triggering the expression of IDO in spleen. For both isoforms the basal level of IDO transcription was lower in the absence of IFNγ, indicating that IFNγ is not only the trigger but essential for the base level of IDO in normal case. Another reflection is that when IFNγ KO mice get infected with malaria, the levels of both isoforms are further decreased pointing to some repression in the regulation of IDO (Fig. 5b, 5d).

Liver

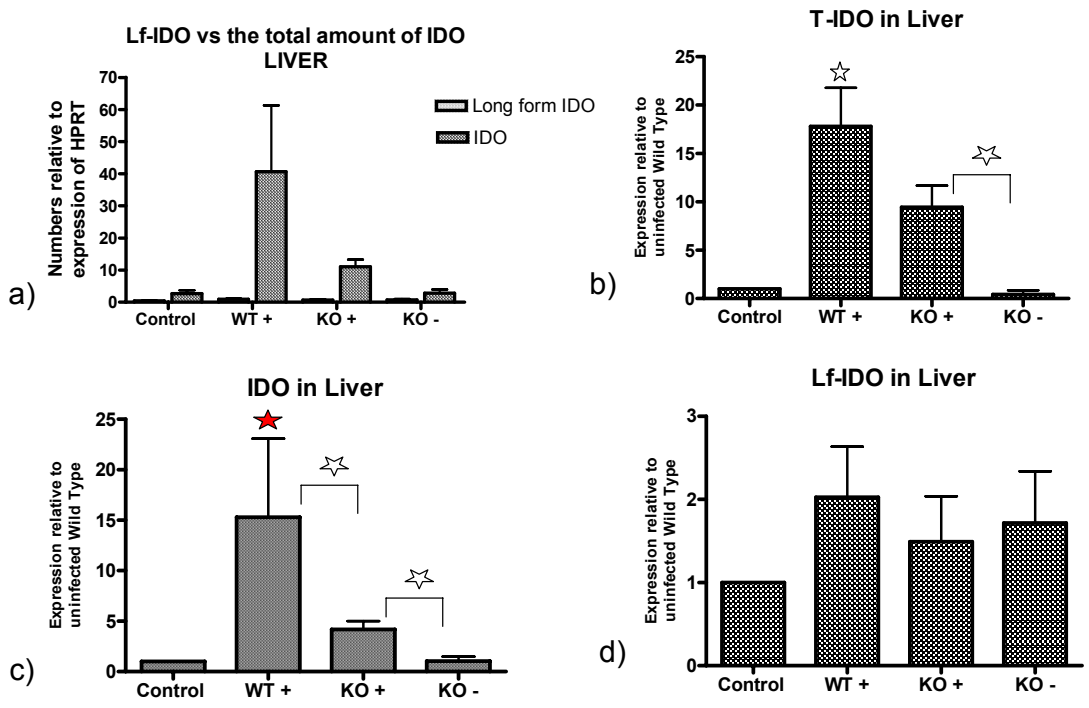


Fig. 6. Liver expression data. **Indoleamine 2,3-dioxygenase mRNA levels in liver of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-).**
In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). Compared to control, the level of IDO was 15 times higher in malaria infected mice. The truncated IDO was the source to that big increase and also the more abundant isoform both in uninfected and infected liver. This increase was IFNγ dependent. Columns and vertical bars represent mean +/- SEM (n=4). Significantly different from control: ☆P<0.05. Filled stars indicate a significant difference presented by both statistical tests (Mann Whitney and Kruskal-Wallis test).

There were almost no IDO in the liver of the uninfected mice and just slightly more of the truncated than the longer isoform. Malaria infection causes Tr-IDO to rise to more than 15 times the level of control while the longer, not presenting the same rise, increases 2 fold. This upregulation is partly IFNγ dependent since liver from the infected IFNγ KO mice contained higher levels of both isoforms than the control, but less than in the infected wild type mice. The truncated IDO form was more abundant in infected IFNγ KO mice than in non-infected IFNγ KO mice (Fig. 6b). Regarding that, one can assume that the additional factor needed to boost the expression of this isoform is probably itself induced in liver in malaria infection.

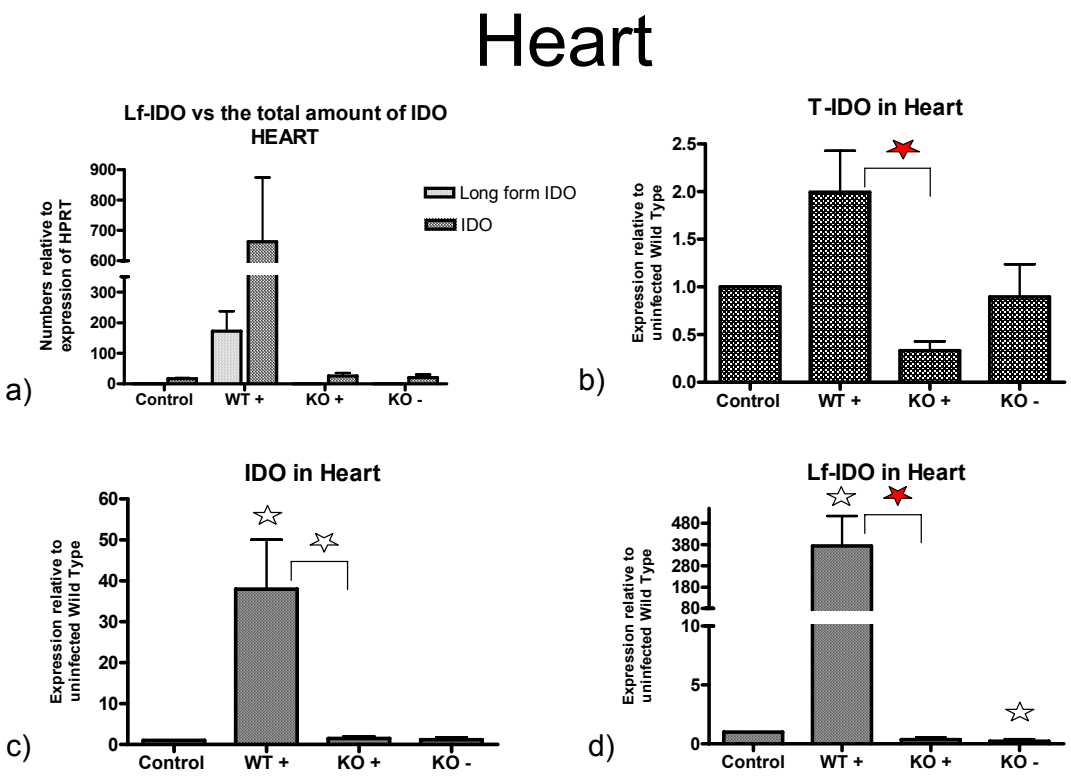


Fig. 7. Heart expression data. **Indoleamine 2,3-dioxygenase mRNA levels in heart of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-).**
In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). The longer IDO was strongly induced in the hearts of malaria infected mice showing an evident IFNγ dependency (a, d). The truncated isoform was also increased compared to control but not as obvious (b). In the heart of controls as well as infected mice the truncated IDO is more abundant than the longer (a). Columns and vertical bars represent mean +/- SEM (n=4). Significantly different from control: ☆ P<0.05. Filled stars indicate a significant difference presented by both statistical tests (Mann Whitney and Kruskal-Wallis test).

Under non-infected conditions, there are low levels of both IDO isoforms in the heart. As in most other tissues mentioned earlier, malaria tends to increase the expression of the IDO isoforms. The level of the short form was very much increased when looking at figure 7a but this induction is not quite equivalent with that of its individual diagram (Fig. 7b). The level of the longer isoform was significantly increased in infected wild type mice, to over 300 times the level of control (Fig. 7d). However, the truncated isoform is the one more abundant in hearts of controls and WT+ (Fig. 7a). Similarly as in the other tissues mentioned, IFNγ triggered the expression of IDO isoforms in heart as well. There is a significant difference between infected wild type and infected IFNγ KO mice (Fig. 7b, 7c, 7d).

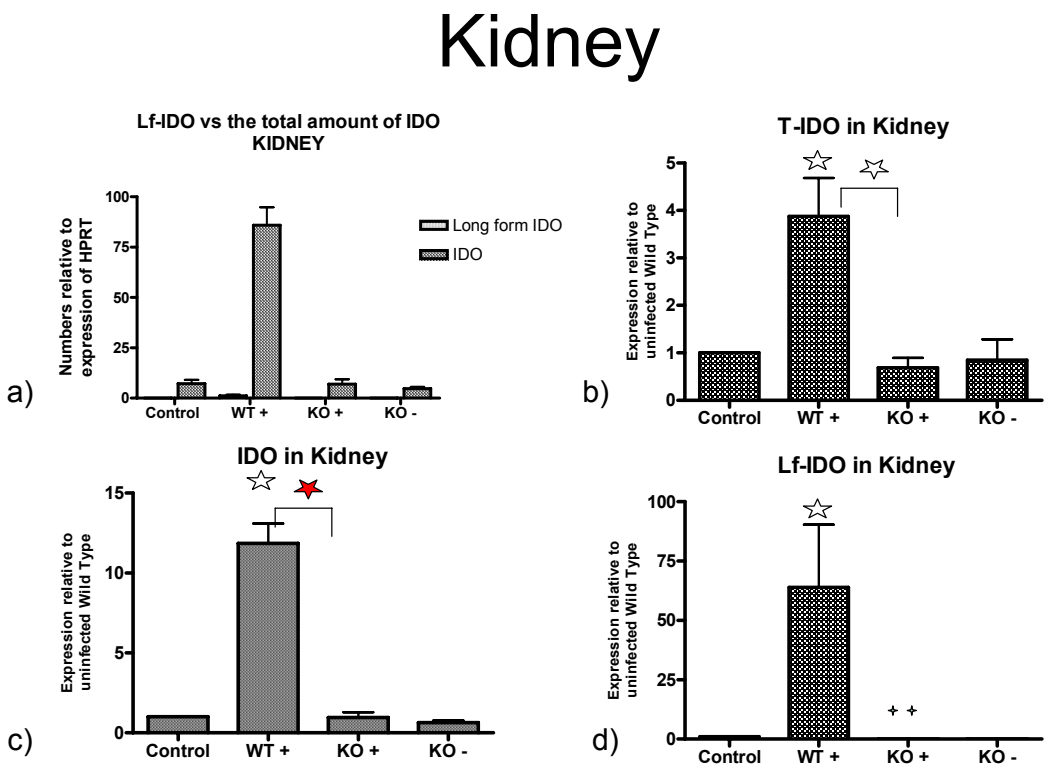


Fig. 8. Kidney expression data. Indoleamine 2,3-dioxygenase mRNA levels in kidney of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-). In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). A high and IFNγ dependent induction of IDO in malaria infected kidney compared to control (a,c). Both isoforms are increasing in kidney during malaria with the truncated having the biggest increase according to diagram a). Furthermore Tr-IDO also seems to be the more abundant in uninfected controls as well as infected mice (a). However when comparing diagram (b) and (d) the longer is the isoform more increased compared to control. Columns and vertical bars represent mean +/- SEM (n=4). Significantly different from control: ☆ P<0.05. Filled stars indicate a significant difference presented by both statistical tests (Mann Whitney and Kruskal-Wallis test).

Almost no Lf-IDO was detected in the kidney which makes the Tr-IDO the more abundant variant in that particular tissue. In fact, the longer form was not detected in any other group than the infected wild types and in one of the control animals. Again, there was a significant difference between the results for Tr-IDO. The fold induction, comparing WT+ to control, that can be seen in figure 5a is a lot higher than the 4 fold increase visualised in the individual diagram of Tr-IDO (Fig. 5b). The elevated level of IDO isoforms in kidney during malaria infection was clearly IFNγ dependent.

Muscle

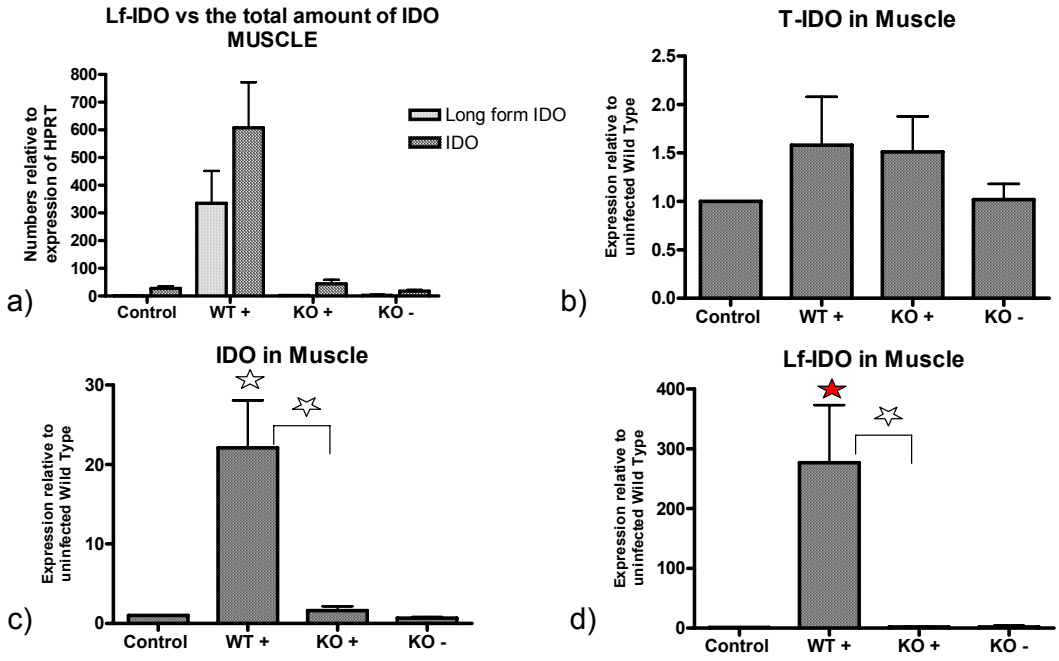


Fig. 9. Muscle expression data. Indoleamine 2,3-dioxygenase mRNA levels in muscle of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-). In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). In muscle of controls, Tr-IDO was detected at a higher level than Lf-IDO (a). When malaria is present, the level of the longer IDO is 250 fold increased (a, d) while Tr-IDO only increases slightly (a, d). With this big raise the longer IDO is more abundant in malaria infected tissue. Columns and vertical bars represent mean +/- SEM (n=4). Significantly different from control: $P<0.05$. Filled stars indicate a significant difference presented by both statistical tests (Mann Whitney and Kruskal-Wallis test).

The muscles of non-infected mice contain more of the truncated isoform. Both IDO isoforms were induced in malaria and the longer form is expressed at a level around 250 times the normal value (Fig. 9d). This large increase was clearly IFNγ dependent. Tr-IDO being the isoform more constitutively expressed does not show the same elevated level in malaria infected muscle (Fig. 9b), which turns the long IDO to the more abundant form in malaria infected muscle.

Epididymis

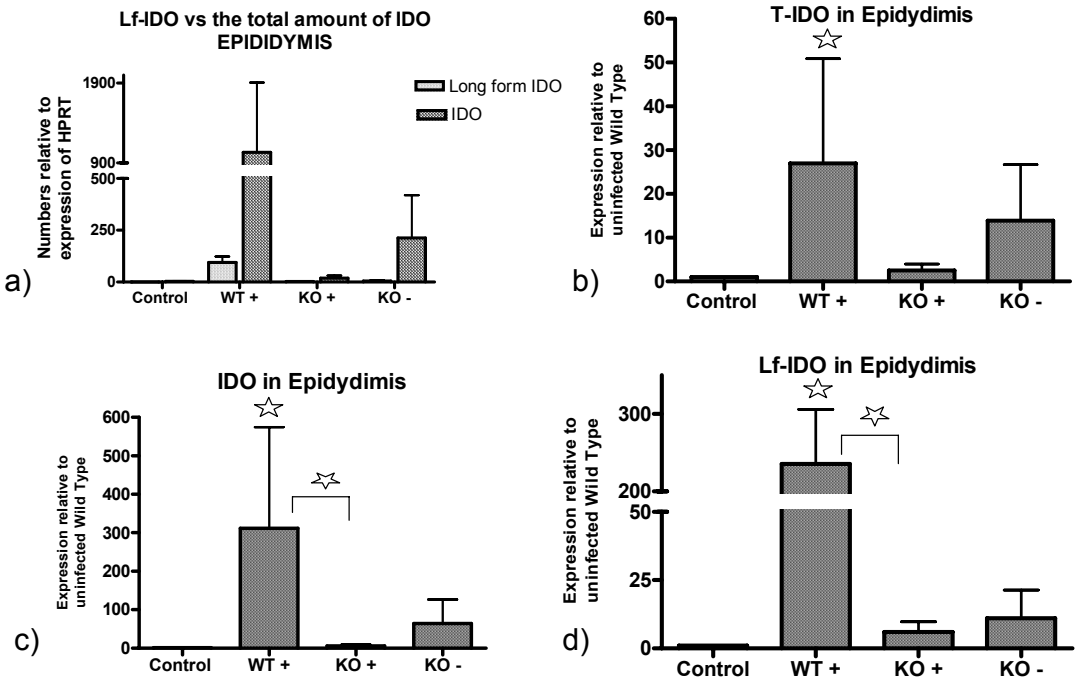


Fig. 10. Epididymis expression data. **Indoleamine 2,3-dioxygenase mRNA levels in epididymis of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-). In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). Malaria causes both isoforms of IDO to rise in epididymis with expression of the longer isoform more elevated than as of the truncated (a). Lacking IFNγ, the KO+ mice does not have the same increase. The longer IDO has the bigger increase (d) but the truncated is more abundant in the epididymis of normal and infected mice (a). Columns and vertical bars represent mean +/- SEM (n=4). Significantly different from control: ☆P<0.05.**

For the results of the epididymis it must be taken into account that this small part was hard to find and to remove without including anything from the surrounding tissue into the sample and that there were some animals that gave really high numbers included in the analysis. One of the infected wild type mice presented a very high number for the total IDO which brought up the average. Only a small amount of IDO was found in controls. There was very little of the long form and just slightly more of the truncated form (Fig. 10a). In WT+, apparently the longer form had a relatively higher increase than the truncated (Fig. 10b, 10d). Still the results indicated that the truncated was more abundant in the tissue (Fig. 10a). Without IFNγ, as in the IFNγ KO mice, these big increases were absent. Again the dependence of IFNγ was a fact.

Aorta

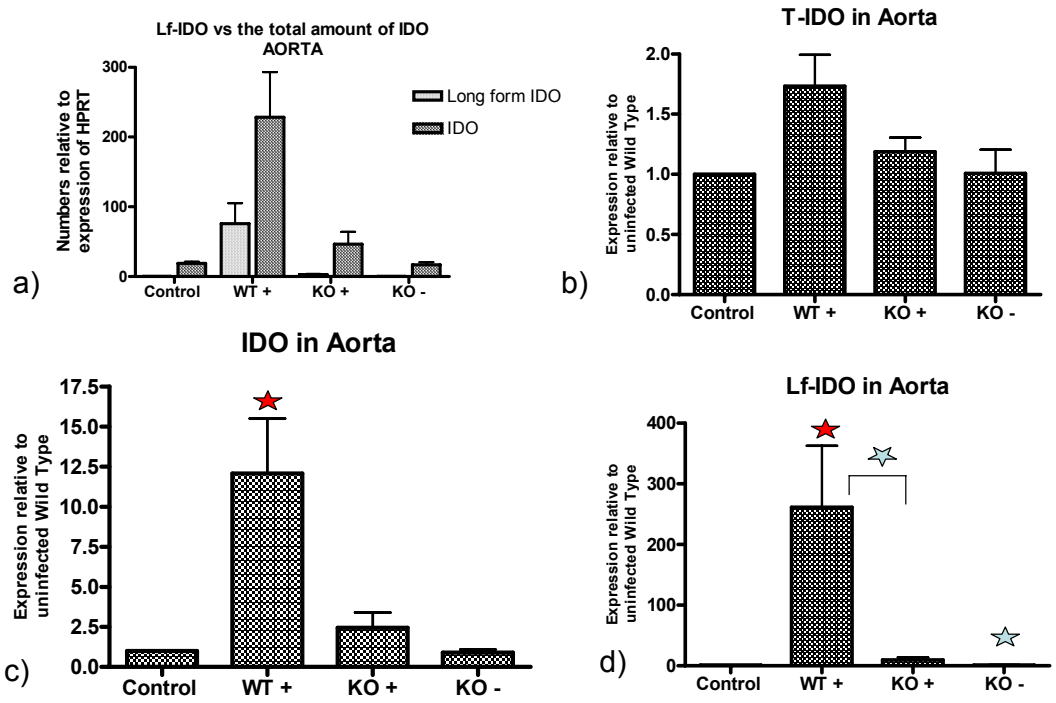
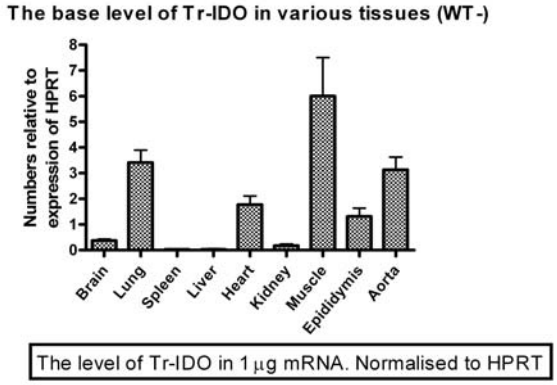
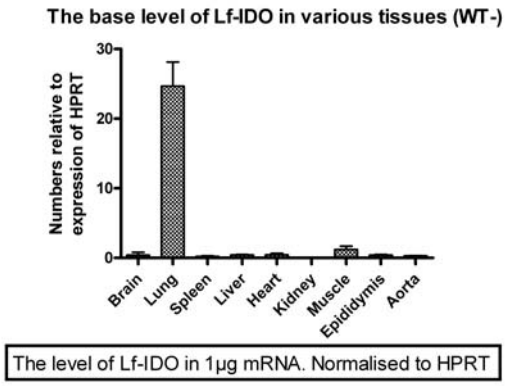


Fig. 11. Aorta expression data. Indoleamine 2,3-dioxygenase mRNA levels in aorta of *P. berghei* K173 infected wild type and infected as well as uninfected IFNγ gene knockout mice. The groups shown are uninfected wild type mice (control), infected wild type (WT+), infected IFNγ knockout mice (KO+) and uninfected IFNγ knockout mice (KO-). In diagram (a), the total amount of IDO is compared to the long IDO isoform. In the individual diagrams of the truncated IDO (b), the total amount of IDO (c) and the longer IDO (d), the levels are relative to that of control. All numbers are normalised to the expression of Hypoxanthine Phosphoribosyltransferase (HPRT). With malaria, the level of total IDO is significantly increased (c). The longer IDO showed elevated levels to approximately 250 times its normal expression (d). The amount of Tr-IDO is also higher in infected aorta but the relative increase is not even close to that of the longer IDO. However Tr-IDO is more abundant than Lf-IDO in healthy and infected aorta. The increase in malaria is IFNγ dependent, at least partly. Columns and vertical bars represent mean +/- SEM (n=4). Significantly different from control: $P<0.05$. Filled stars indicate a significant difference presented by both statistical tests (Mann Whitney and Kruskal-Wallis test).

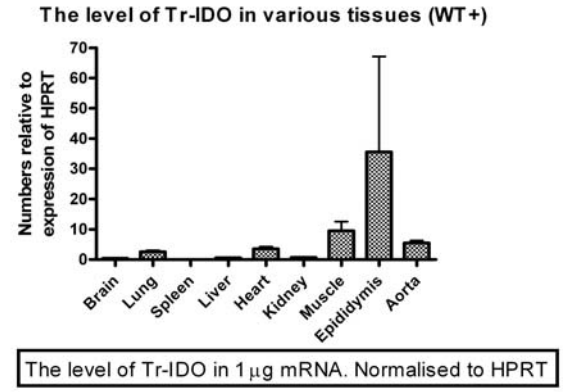
In aorta the truncated IDO was more abundant within uninfected controls and according to figure 11a, this was also true for WT+. Malaria upregulated the level of both isoforms but the relative increase was higher for the longer isoform. This increase of IDO isoforms in aorta of infected mice was IFNγ dependent.



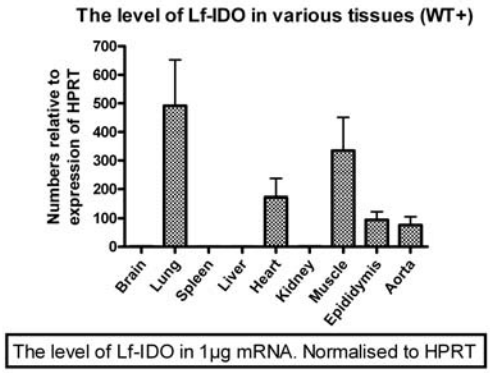
a)



b)



c)



d)

Fig. 12. Assuming that the level of expression of the housekeeping gene, HPRT, is the same in all tissues, the level of each isoform in different tissues can be compared as amount of IDO expressed per 1µg RNA. In controls, the muscle had the highest level of truncated IDO compared to the other tissues examined (a) and lung, a very high expression of the longer IDO (b). The pattern changed when mice became infected with malaria. The Tr-IDO was most common in the tissues of epididymis after malaria infection (c). At a higher level than before, lung still had the highest amount of the longer IDO when tissue was infected (d).

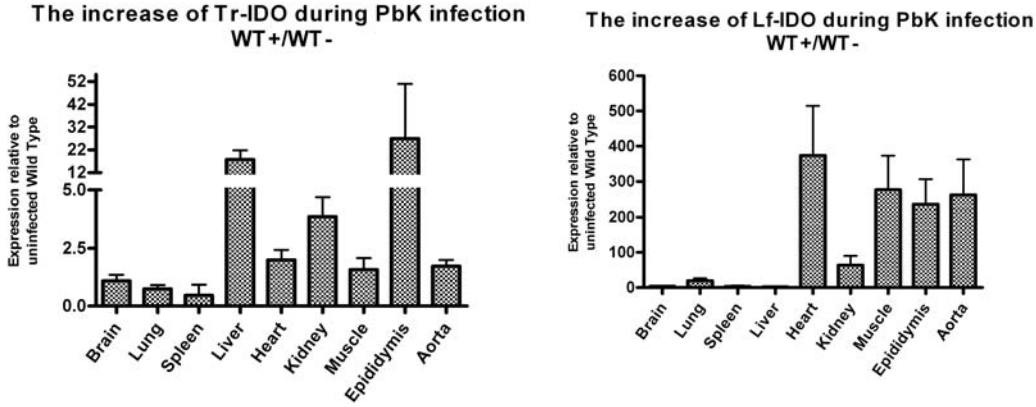


Fig 13. The relative increase of the longer IDO isoform is bigger than the increase of the truncated IDO. Heart, muscle and aorta were the tissues that had the biggest raise in expression of the longer IDO (right diagram). The truncated IDO level were elevated the strongest in epididymis, liver, kidney and heart

Table 3. Ranked from 1-9, the tissue having the highest expression of any of the two isoforms during healthy conditions or infected are listed with the highest expression higher up in the table.

	Control			WT+			Largest increase		
	Truncated	**Longer**	**Total**	**Truncated**	**Longer**	**Total**	**Truncated**	**Longer**	**Total**
1	Muscle	Lung	Lung	Epididymis	Lung	Lung	Epididymis	Heart	Epididymis
2	Lung	Muscle	Muscle	Muscle	Muscle	Epidydimis	Liver	Muscle	Liver
3	Aorta	Heart	Spleen	Aorta	Heart	Muscle	Kidney	Aorta	Heart
4	Heart	Epidydimis	Aorta	Heart	Epidydimis	Heart	Heart	Epidydimis	Muscle
5	Epidydimis	Brain	Heart	Lung	Aorta	Aorta	Muscle	Kidney	Kidney
6	Brain	Aorta	Kidney	Kidney	Liver	Kidney	Aorta	Liver	Aorta
7	Kidney	Spleen	Brain	Liver	Brain	Liver	Brain	Lung	Lung
8	Spleen	Liver	Epidydimis	Brain	Kidney	Brain	Lung	Brain	Brain
9	Liver	Kidney	Liver	Spleen	Spleen	Spleen	Spleen	Spleen	Spleen

To summarise the results of the quantification experiments with the nine different tissue types, the Tr-IDO was the more abundant isoform in both non-infected as well as infected tissue. However, the expression of the Lf-IDO increased much more after malaria infection. The only exception was in the liver, where the Tr-IDO increased more after infection as compared with the longer isoform.

3.2 The promoter activity and response to IFNγ

IFNγ is well known to enhance the expression of IDO but the way this cytokine is affecting the promoter to become more active is not known. By amplifying smaller fragments of the promoters (the region 1500bp upstream from transcription start) and then inserting these in TOPO vector to transfect HEK239 cells, the effect of IFNγ could be studied.

A transcription factor search (http://www.cbrc.jp/research/db/TFsearch.html) of the promoter regions revealed positions of potential binding sites for IFNγ (Fig. 14).

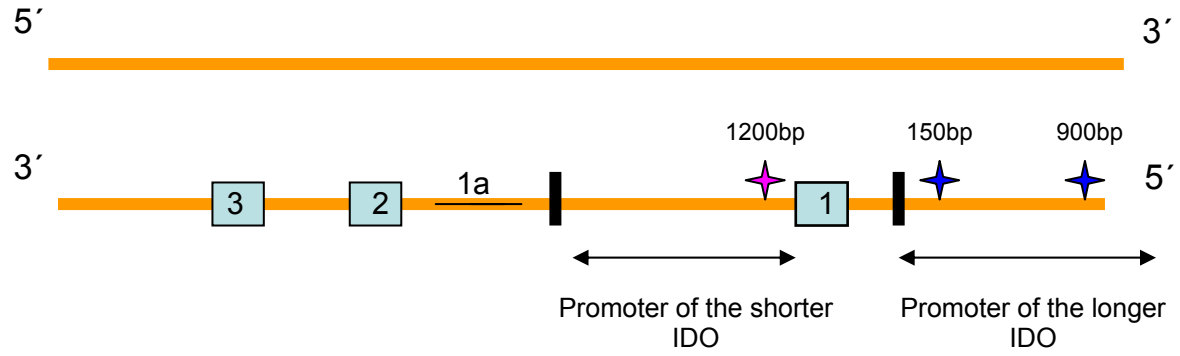


Fig. 14. This schematic picture shows the location of the truncated IDO as a gene within the longer IDO gene. When the transcript of the longer IDO includes exon 1, instead the truncated IDO contains a non-translated region (1a) and consequently the isoforms differ in their 5´end. The promoter regions of the two isoforms are marked with arrows. Black vertical bars represent transcription start of the truncated (the left bar) and the longer IDO (the right bar). The stars reveal the location of potential IFNγ transcription factor (TRF) sites in base pairs from transcription start. The TRF sites given by the blue stars correspond to the promoter of the longer IDO and the purple star to the promoter of the truncated IDO.

The thought was to clone promoter fragments of different lengths; 300bp, 700bp, 1100bp and 1500bp. This was done for the truncated isoform but there were some problems with the longer isoform where, most likely, a repetitive sequence complicated the amplification of the two longer fragments so these could not be included in the study. Since the promoter of the truncated isoform is inside the 5´end of the longer isoform, it reaches the beginning of the promoter for the longer isoform. This was not ideal, so this fragment was not used either. To begin with only the 300bp and the 700bp fragments were used. The fragments were inserted into TOPO vector that includes a reporter gene for β–lactamase. An activated promoter will thereby be identified by the production of β–lactamase. By transfecting HEK293 cells with these vectors and then stimulate them with an addition of human IFNγ a difference compared to control was expected to be found.

The first results pointed at a high base activity for the truncated isoform. The calculated ratio was a lot higher than that of the longer isoform for both lengths and no difference was seen comparing the sample in which IFNγ had been added with controls. This meant that there was a lot of cleaved substrate in the samples compared to intact, with other words; loads of β–lactamase and thus a high activity of the promoter fragments even in the absence of IFNγ.

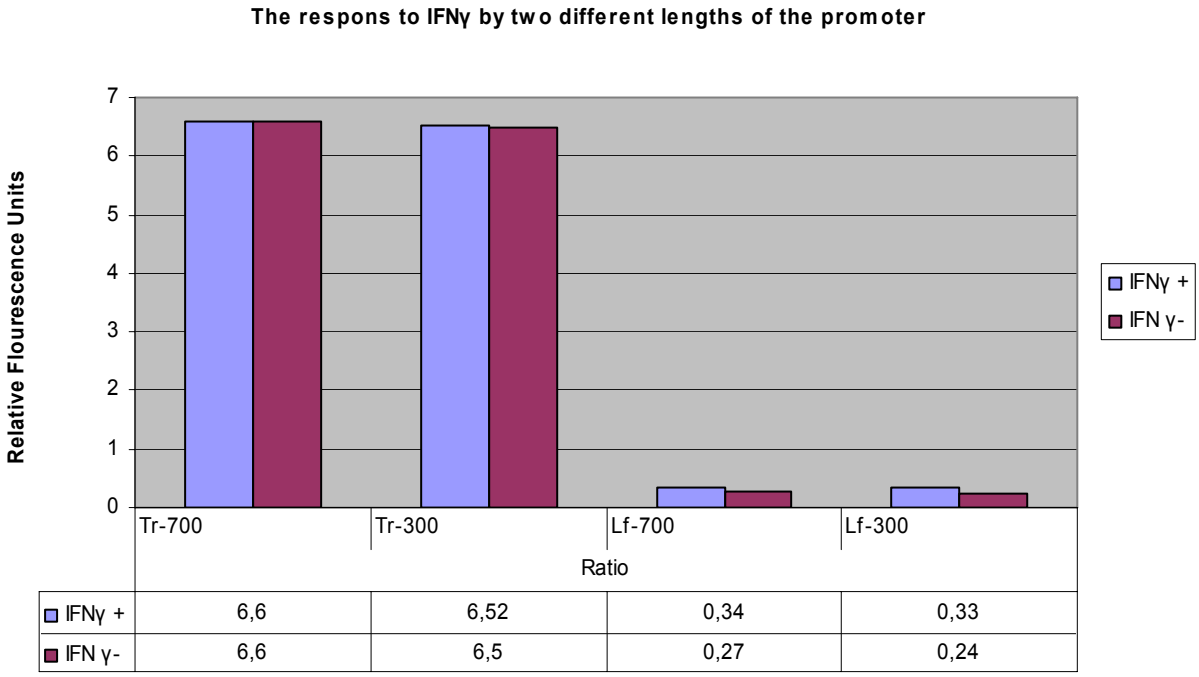
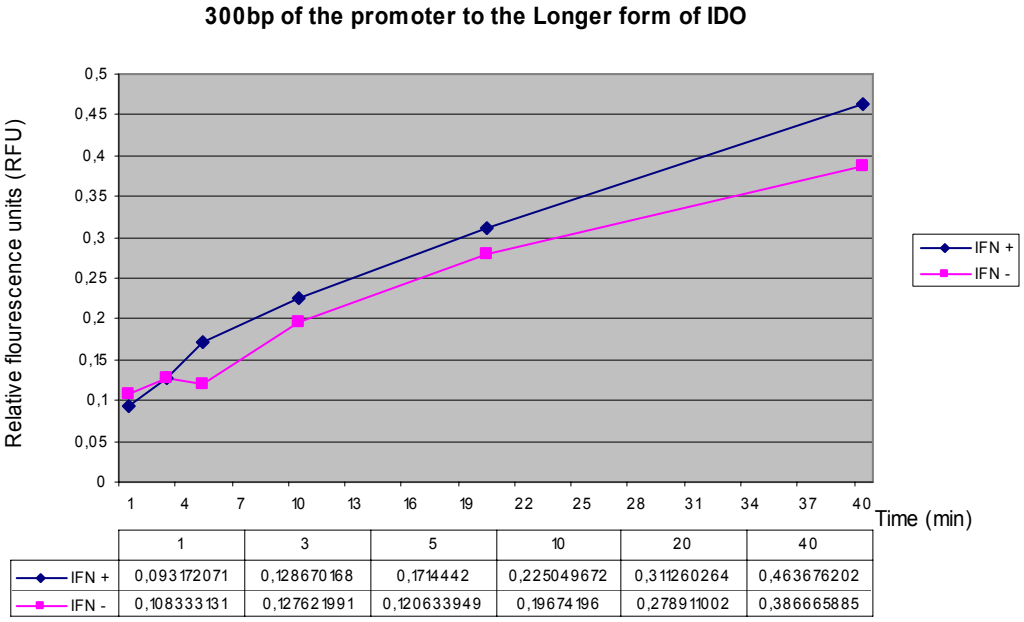


Fig. 15. Result from the first experiment investigating the promoter response to IFNγ of the IDO isoforms. There was a big difference in base activity set by the promoters. The promoter of the truncated IDO showed a very large base activity while the longer IDO seems to have a rather low level of expression. A small difference was observed between the IFNγ induced samples and control for the longer IDO. No difference could be seen between the different lengths of the promoters 300 and 700bp for any isoform. (Tr-700; 700bp of the promoter of Tr-IDO, Tr-300; 300bp of the promoter of Tr-IDO, Lf-700; 700bp of the promoter of Lf-IDO, Lf-300; 300bp of the promoter of Lf-IDO)

Samples containing fragments from the promoter of the longer IDO isoform showed a lot lower activity (Fig. 15. Lf-700, Lf-300). The IFNγ induced samples from both lengths (700bp and 300bp) got a slightly higher ratio compared to control suggesting that IFNγ had effect by binding to that region. Since the numbers were low some further work was done trying to enhance this effect. Increasing the concentration of IFNγ was one approach. The dose response curve drawn did not show any significant increase in the numbers (result not shown here). A try to transfect another cell line, B-end cells, did not succeed either.

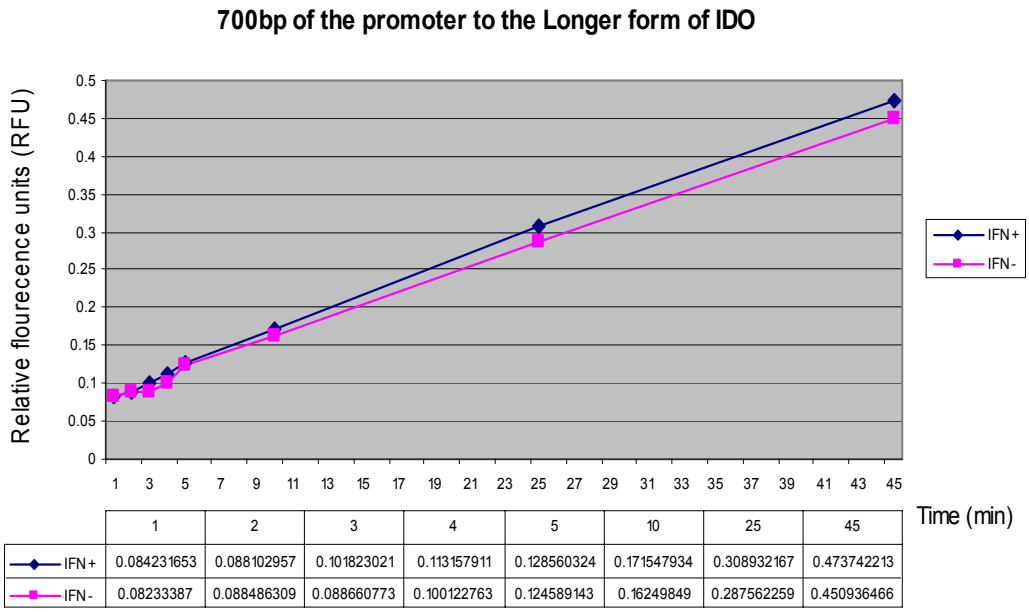
Concerning the very high ratio of cleaved to uncleaved substrate, given by the samples with the Tr-IDO constructs; the excess of β–lactamase probably cleaved the substrate to give an outgoing emission signal over the capacity range of the machine. To reduce the amount of substrate being cleaved the amount of β–lactamase, and consequently the promoter, had to be reduced. To keep the ratios of Lipofectamine to DNA constant "filler-DNA", plasmids without any promoter insert or reporter gene, substituted a part of the plasmids with a promoter insert. Using the same amount of DNA in the transfection but in a 25:75 mixture of filler-DNA:Lf-IDO promoter plasmids. After loading the samples with substrate the fluorescence reading had to be initiated very quickly since β–lactamase cleaves the substrate at a high rate and quickly brings up the

ratio. Along with this, the fluorescense was read at several time points to follow the reaction.



	1	3	5	10	20	40
IFN +	0,093172071	0,128670168	0,1714442	0,225049672	0,311260264	0,463676202
IFN -	0,108333131	0,127621991	0,120633949	0,19674196	0,278911002	0,386665885

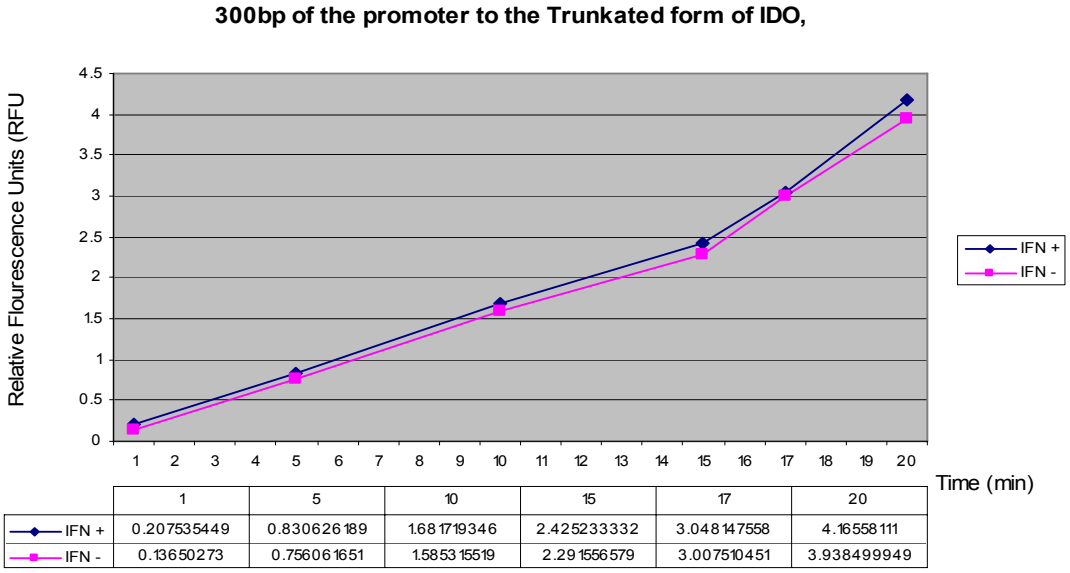
Fig 16. The curves are showing the ratio of cleaved to intact CCF2-FA substrate over time. HEK293 cells were transfected with a vector with 300bp of the promoter for the longer IDO isoform, inserted in front of a reporter gene. With an active promoter the product of the reporter gene, β–lactamase, accumulated. After lysing the cells the amount of β–lactamase and thereby the activity of the promoter was measured by adding a substrate, CCFA-FA. Converted by β–lactamase, the ratio of the amounts of cleaved and intact substrate was calculated. The blue line corresponds to samples in which the transfected cells were induced with IFNγ (IFNγ +). The purple line is the control sample without addition of IFNγ (IFNγ -).



	1	2	3	4	5	10	25	45
IFN+	0.084231653	0.088102957	0.101823021	0.113157911	0.128560324	0.171547934	0.308932167	0.473742213
IFN-	0.08233387	0.088486309	0.088660773	0.100122763	0.124589143	0.16249849	0.287562259	0.450936466

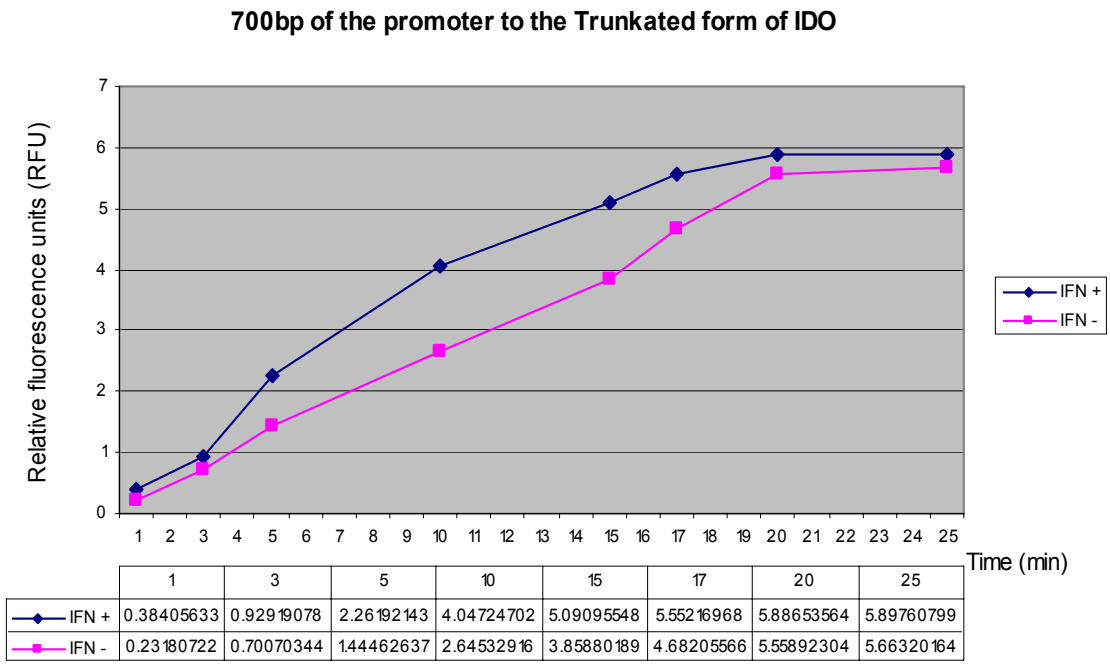
Fig 17. The curves are showing the ratio of cleaved to intact CCF2-FA over time.
HEK293 cells were transfected with a vector containing 700bp of the promoter of the longer IDO isoform, inserted in front of a reporter gene. With an active promoter the product of the reporter gene, β–lactamase, accumulated. After lysing the cells the amount of β–lactamase and thereby the activity of the promoter was measured by the addition of the substrate, CCFA-FA. Converted by β–lactamase, the ratio of the amount cleaved over non-cleaved substrate was calculated. Blue line corresponds to sample where transfected cells were induced with IFNγ (IFNγ +). The purple line is the control sample without addition of IFNγ (IFNγ -).

As previous result had shown (Fig. 15), there was only a small difference between the samples that got an addition with IFNγ and control. Initially, the ratio was higher for the control than that for the induced sample for both the 300bp and 700bp fragments. After some readings that shift occurred pointing to more β–lactamase in those samples induced. This was better seen for the shorter 300bp promoter (Fig. 16) of longer IDO than for the 700bp (Fig. 17), where the difference increased more and more with time. But, again the ratio was low for Lf-IDO (Fig. 16, Fig. 17).



	1	5	10	15	17	20
IFN +	0.207535449	0.830626189	1.681719346	2.425233332	3.048147558	4.16558111
IFN -	0.13650273	0.756061651	1.585315519	2.291556579	3.007510451	3.938499949

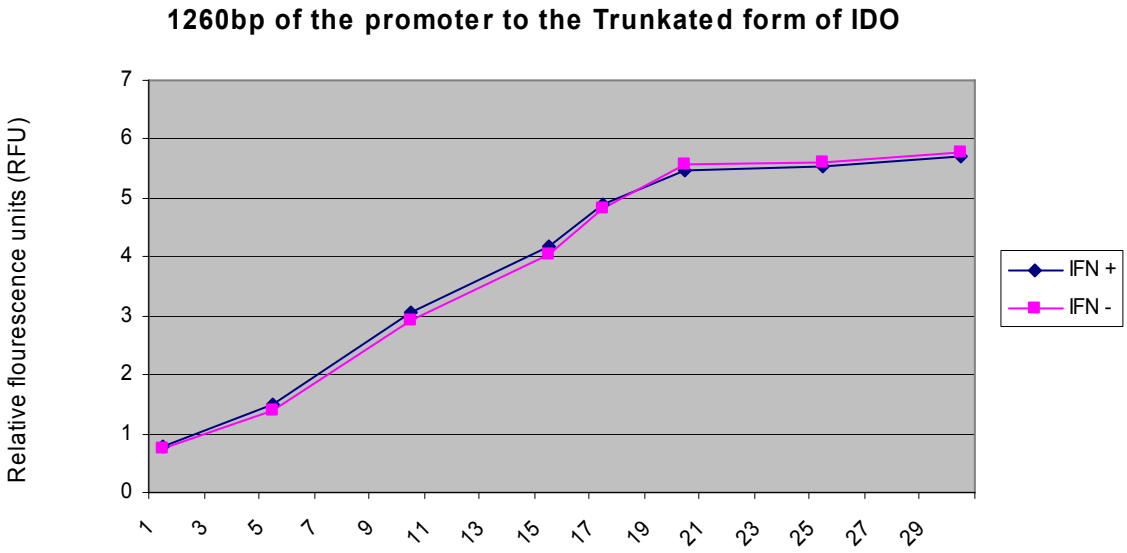
Fig. 18. Investigating the effects of IFNγ as the key inducer of IDO. The curves are showing the ratio of cleaved to intact CCF2-FA over time. HEK293 cells were transfected with a vector with 300bp of the promoter for the truncated IDO isoform, inserted in front of a reporter gene. With an active promoter the product of the reporter gene, β–lactamase, accumulated. The promoter of Tr-IDO was more active than Lf-IDO even when IFNγ was not added (Fig. 15). Using 75% filler DNA in the transfection that did not carry any promoter region or a reporter gene, the result was easier to interpret. After lysing the cells the amount of β–lactamase and thereby the activity of the promoter was measured by the addition of the substrate, CCFA-FA. Converted by β–lactamase, the ratio of the amount cleaved over non-cleaved substrate was calculated. Blue line corresponds to sample where transfected cells were induced with IFNγ (IFNγ +). The purple line is the control sample without addition of IFNγ (IFNγ -).



	1	3	5	10	15	17	20	25
IFN +	0.38405633	0.92919078	2.26192143	4.04724702	5.09095548	5.55216968	5.88653564	5.89760799
IFN -	0.23180722	0.70070344	1.44462637	2.64532916	3.85880189	4.68205566	5.55892304	5.66320164

Fig. 19. Investigating the effects of IFNγ as the key inducer of IDO. The curves are showing the ratio of cleaved to intact CCF2-FA over time. HEK293 cells were transfected with a vector with 700bp of the promoter for the truncated IDO isoform, inserted in front of a reporter gene. With an active promoter the product of the reporter gene, β–lactamase, accumulated. The promoter of Tr-IDO was more active than Lf-IDO even when IFNγ was not added (Fig. 15). Using 75% filler DNA in the transfection that did not carry any promoter region nor a reporter gene, the result was easier to interpret. After lysing the cells the amount of β–lactamase and thereby the activity of the promoter was measured by the addition of the substrate, CCFA-FA. Converted by β–lactamase, the ratio of the amount cleaved over non-cleaved substrate was calculated. Blue line corresponds to sample where transfected cells were induced with IFNγ (IFNγ +). The purple line is the control sample without addition of IFNγ (IFNγ -).

The aim of lowering the amount of β–lactamase using non-coding DNA, in the samples of the truncated isoform, gave better results. The calculated ratio from the first of the kinetic readings was essentially lower than previously and the reaction was possible to follow. Nothing from the 300bp fragment pointed to a difference between IFNγ induced sample and control (Fig. 18). However, a very interesting result was given by the 700bp promoter fragment. Starting from the same ratio, the IFNγ affected sample converted the substrate faster, resulting in a higher ratio compared to control (Fig. 19). The reason that the curves later merged could be that the limit of the machine was reached or that most of the substrate was cleaved. This suggested that the promoter of the truncated IDO has an IFNγ response element located within the region of 300bp to 700bp upstream from transcription start.



	1	5	10	15	17	20	25	30
IFN +	0,792688	1,479338	3,071397	4,174082	4,90812	5,470577	5,547992	5,707332
IFN -	0,744299	1,383956	2,92852	4,034719	4,814579	5,566166	5,589921	5,78941

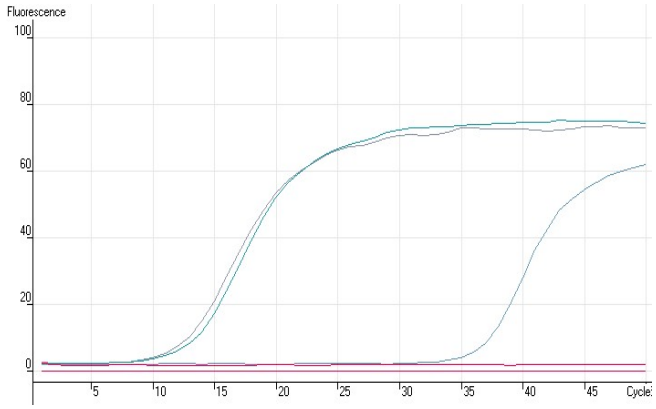
Time (min)

Fig. 20. Investigating the effects of IFNγ as the key inducer of IDO. The curves are showing the ratio of cleaved to intact CCF2-FA over time. HEK293 cells were transfected with a vector with 1260bp of the promoter for the truncated IDO isoform, inserted in front of a reporter gene. With an active promoter the product of the reporter gene, β–lactamase, accumulated. The promoter of Tr-IDO was more active than Lf-IDO even when IFNγ was not added. Using 75% filler DNA in the transfection that did not carry any promoter region nor a reporter gene, the result was easier to interpret. After lysing the cells the amount of β–lactamase and thereby the activity of the promoter was measured by the addition of the substrate, CCFA-FA. Converted by β–lactamase, the ratio of the amount cleaved over non-cleaved substrate was calculated. Blue line corresponds to sample where transfected cells were induced with IFNγ (IFNγ +). The purple line is the control sample without addition of IFNγ (IFNγ -).

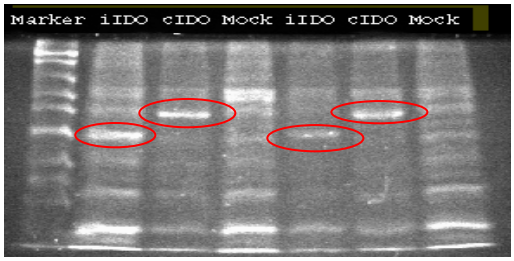
According to the TRF search another response element was located about 1200bp away from transcription start. With the result of the 700bp fragment it was exciting to investigate any changes with that element included. A new primer for the longer isoform was designed. That resulted in a 1260bp fragment of the promoter that included another IFNγ binding site but excluded as much as possible of the 5´end of the longer isoform not to interfere with its promoter. The difference between the samples with and without IFNγ seen previously (700bp fragment; Fig. 19) could not be seen with the longer fragment (1260bp; Fig. 20). The reason for this is not clear.

3.3 The activity of IDO

With two isoforms of IDO, it was not clear if both had the same enzyme activity. It was suggested that the longer isoform were the one to convert tryptophan into kynurenine but whether the truncated IDO had the same role was not clear. To explore the activity of the isoforms, HEK293 cells were transfected with expression vectors, to produce either the longer or the truncated IDO (see methods section). Transfections had been successful since control experiments revealed both mRNA (Fig. 21a) and protein of the IDO isoforms in the particular samples (Fig. 21b). Two samples from each isoform transfection and mock cells were loaded onto the polyacrylamide gel to look for IDO-protein in supernatant of the lysates. His-staining of the gel, demonstrated expression of both protein isoforms (Fig. 21b). However, in the result from a western blot of the gel the Tr-IDO was not detected (Fig. 23). The conclusion was that the IDO-antibody apparently only recognises the longer IDO.



a)



b)

Fig. 21. Cells were transfected with expression vectors for IDO isoforms, to produce either the long form or the truncated IDO. As controls for the transfection, cells were lysed with Trizol to preserve RNA or STET lysis buffer in the samples for protein analysis. Of the extracted RNA, 1µg was translated into cDNA for analysis. With primers to detect IDO (table 1), quantitative rt-PCR technique found each isoform in its corresponding sample at approximately the same concentration (a). Mock cells produced a curve coming up later at a much lower concentration, probably resulting from the natural production of IDO in the cells. Two controls per isoform were checked for the presence of IDO protein. The samples were separated on a 10% SDS polyacrylamid gel (b). Making use of the designed His-tag that flank the 5´end of the IDO isoforms, the gel was stained using a His-tag In-gel kit and the bands were visualized with a UV camera. Also here, both isoforms were found in the samples at their predicted sizes (marked with red circles). The gel was loaded as following; Lane 1: ladder, 2: longer IDO, 3: truncated IDO, 4: mock cells, 5: sample two of longer IDO, 6: sample two of truncated IDO, 7: sample two of mock cells.

Cells were also prepared in another way which involved a gentle freeze thawing method to lyse the cells. Co-factors and tryptophan were then added to the samples prior to HPLC

analysis. However both experiments generated the same results. The longer isoform effectively converted all the added tryptophan into kynurenine (Fig. 22a). The truncated isoform did not show the same enzymatic activity as all of the tryptophan remained and no kynurenine was detected (Fig. 22b).

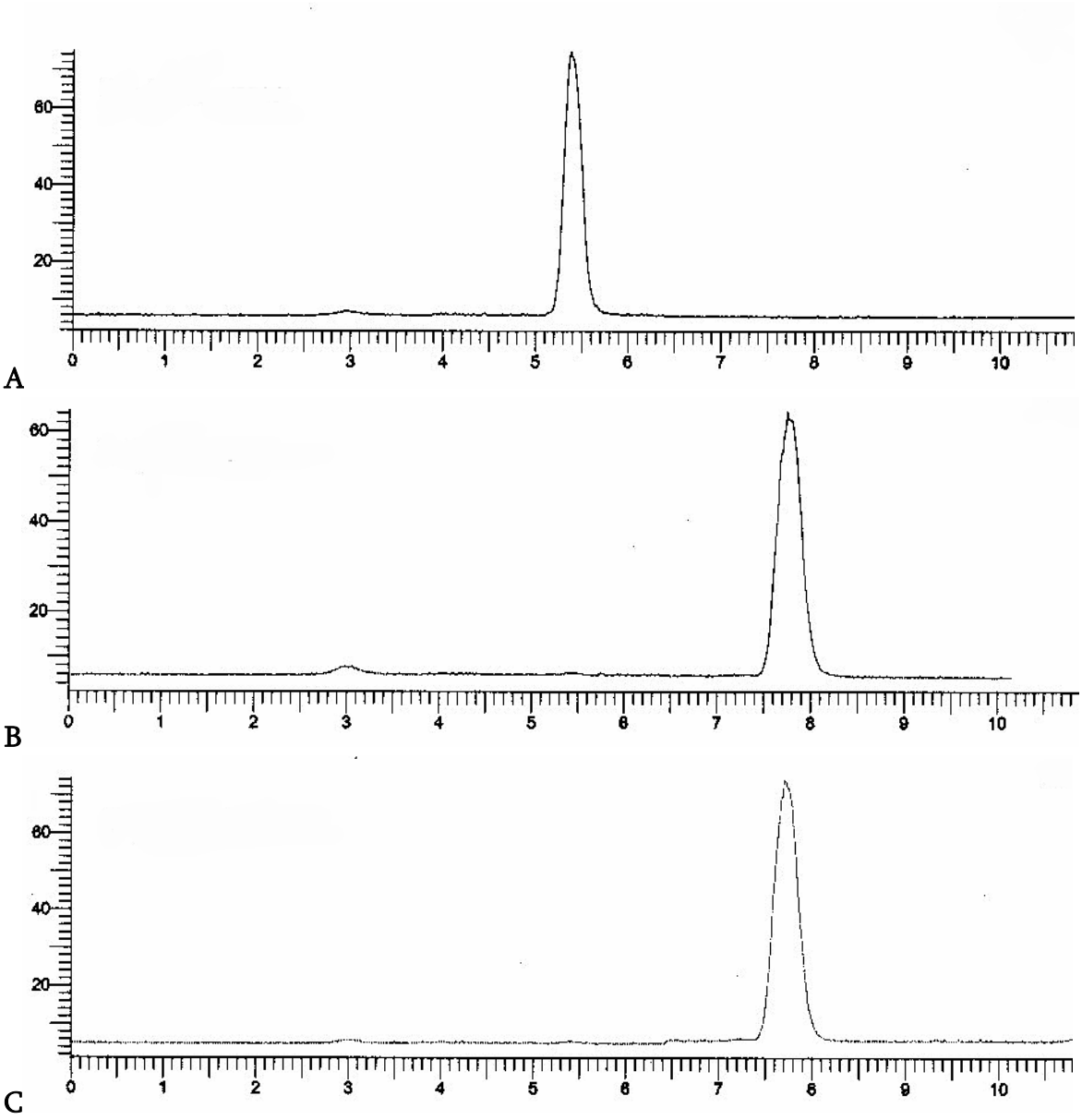


Fig. 22. Investigating the enzymatic activity of the IDO isoforms, HEK293 cells were transfected with expressionvectors to produce IDO isoforms. Tryptophan was supplied to the enzymes either before or after the cells were lysed. Tryptophan (Trp) and kynurenine (Kyn) in the resulting supernatant were separated on a VeloSep RP-18 column, with a flow of 0.5 ml/minute and detected photometrically (Kyn, 365 nm; Trp, 280 nm). The earlier peak corresponds to kynurenine (5.5 min) and the later tryptophan (~7.5 min). All tryptophan was converted to kynurenine by the longer IDO isoform (A). With no sign of tryptophan degradation, there was no peak for kynurenine in the samples with the truncated IDO protein (B), resulting in a diagram identical to the one of mock cells (C).

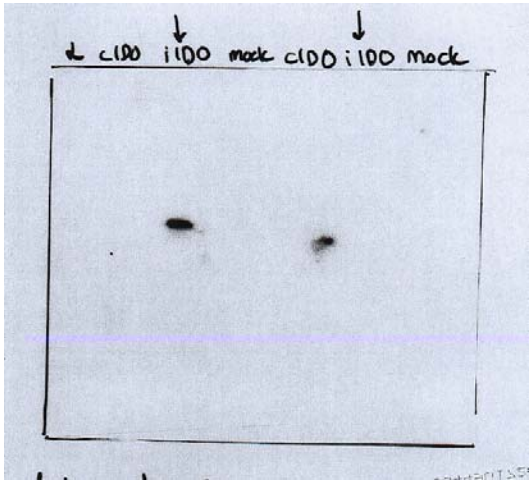


Fig. 23. The proteins separated on the SDS gel, was western blotted onto a PVDF membrane. The membrane was blocked in TBS with 4% milk and then incubated o/n with a primary IDO antibody (diluted 1:2000). After washing the membrane 3x10min in TBS-T a secondary antibody (ELC Anti-rabbit IgG, diluted 1:5000) was introduced. After a second washing step the membrane was covered with detection solution, photo paper was exposed by the signal and the image was developed. The longer IDO was the only one detected so the antibody is specific for that isoform only.

3.3.1 Truncated IDO, a peroxidase?

An IDO-like myoglobin, found in gastropod molluscs, have rather high homology (35%) with mammalian IDO [16]. So in molluscs the gene could have evolved to have a different function - acting as a myoglobin [17]. Myoglobin is a monomeric, heme containing enzyme that functions as an oxygen storage unit providing oxygen to working muscles, but myoglobin also has the function of a peroxidase when the concentration of hydrogen peroxide (H_2O_2) is high. H_2O_2 is a by product of fatty acid oxidation and are also produced by white blood cells to kill bacteria but in large amounts it can damage the cell itself. This is prevented by catalases. The long form of IDO in mammals carries a heme group and binds oxygen but the interaction is not stable. With this and a known homology to mammalian myoglobin, another role for IDO, such as that for myoglobin has been suggested. In addition, this work showed that the truncated IDO was highly expressed in a lot of vascular organs, where the supply of oxygen is of importance.

To further elucidate the role of the truncated IDO one more experiment was performed, investigating whether the truncated IDO isoform had any peroxidase activity. The protein extracts were prepared as described in methods section. The experiment involved, mixing the purified protein with heme and addition of a substrate: ABTS. Addition of H_2O_2 initiated the reaction. The ABTS radical produced is absorbing light around 700nm wavelength (Fig. 24) and the reaction was followed in time by spectrophotometry. Using pure myoglobin (working as a control) to different concentrations of hydrogen peroxide

(protein:H_2O_2; 1:5, 1:10, 1:20, 1:30, 1:40 and 1:50) a response curve was made. The reason for the inclusion of this control was to see if a higher concentration of H_2O_2 would affect the heme binding pocket or alter the reaction. It did not have any effect other than speeding up the reaction.

The purified IDO samples were checked at the same concentration as the myoglobin control, with the highest concentration of H_2O_2 (1:50) but neither one did show a raise in absorption that would indicating a proceeding reaction.

Enzyme (Mb/IDO) + H_2O_2	→	Compound 1 + H_2O	(1)
Compound 1 + ABTS	→	Compound 2 + ABTS*	(2)
Compound 2 + ABTS	→	Enzyme (Mb/IDO) + ABTS*	(3)

Fig. 24. Natural peroxidases catalyse the reduction of hydrogen peroxide to water as described by the reaction mechanism shown above. Compound 1 is an intermediate two oxidation states above the resting state and compound 2 is one oxidizing state above the resting state. 2,2´-azino-di(3-ethyl-benzthiazoline-6-sulfonic acid) (ABTS) is a commonly used reducing agent. ABTS become colored upon oxidation, allowing peroxidase activity to be monitored spectrophotometrically. By measuring the absorbance at 700nm the oxidation of ABTS could be monitored to detect the occuring reaction.

The concentrations of the samples, purified with a His-tag kit, were rather low, similar to the mock sample. Flow through from the first spin was saved. As a control after that non-succeeding peroxidase reaction, a spectroscan of what was left of the samples was performed. Heme proteins are absorbing light around 400nm. With the same concentration as for the IDO samples, Hemoglobin, myoglobin and pure heme were scanned to get an image of where the characteristic heme peak would show up and at what height. According to the IDO spectrogram, a lot of proteins (280nm) were in the flow through and just a tiny fraction in the volume purified. Neither flowthrough nor purified IDO samples had a peak that represented heme.

4 Discussion

To be able to compare gene expression data from different tissues a reference gene (housekeeping gene) with proven invariable expression between cells must be used for normalization. No such housekeeping gene has yet been identified but HPRT has been identified as a good alternative in this matter [18]. Therefore it might not be accurate to compare the levels of IDO isoforms between different tissues as in figure 12 and table 3. The numbers can, however, indicate tissues in which there are much lower or higher expression levels than others.
The results obtained in this study were in agreement with previously reported data. Lung and epididymis have a high activity of IDO expression. In both control and infected mice the lung was the tissue where the total expression of IDO was highest. The longer isoform was the more abundant, giving the highest contribution to that high level. Epididymis had the biggest increase of the truncated isoform when comparing infected to control and topped the list of tissues containing most of the tr-IDO in infected animals.

With constant inhalation of air, the lung is exposed to a large number of airborne pathogens. The observation that respiratory infections are nevertheless rare demonstrates the presence of an efficient host defence system at the mucosal surface of the lung. As seen in this work and previously reported the lung is a tissue with high expression of IDO [14] and the assumption of IDO as having a role in the defence against pathogens is again being strengthened. Which of the cells of the airway epithelium that are expressing IDO and thus play a key role in this host defence system remains to be solved.

The IDO activity in spleen was unchanged during malaria infection. The level of truncated IDO is actually lowered in malaria infected spleen and the level of longer IDO, that normally increases quite a lot, is just slightly elevated. The reason for this result is probably Inducible nitric oxide synthase (iNOS). As an important molecule involved in the host defense against infectious agents, iNOS is induced by IFNγ and generates the product of its action Nitric oxide (NO). There have been reports about nitric oxide (NO) inhibiting IDO activity in macrophages [19], which there are a lot of in spleen.
When comparing the uninfected and infected IFNγ KO groups the level of IDO is lower than control as expected when the IFNγ is not up regulating the gene. For some reason, the infected IFNγ KO group has an even lower level than the uninfected. This suggests that there still is something that inhibits IDO during an infection even when IFNγ is not present to up regulate iNOS, perhaps the other forms of IFN have the same role. In that case, the lack of IFNγ, together with some kind of inhibition in IDO expression in infected IFNγ KO mice, is lowering the level of the truncated IDO further. In fact no truncated IDO was detected in spleen for KO+ at all. The effect was the same with the longer IDO.

A protein similar to IDO, called Tryptophan 2,3-dioxygenase (TDO), has previously been found in liver [4]. TDO has the same tryptophan degrading activity as the longer isoform of IDO and could be the reason why there is no need for longer IDO to be highly induced and expressed in liver, as presented by the diagrams in figure 6.

The role of IDO was previously determined to be the key enzyme in the kynurenine pathway, catabolising tryptophan. Now, having two isoforms of IDO, the question was if they both had the same activity. The truncated IDO did not show the capability to degrade tryptophan while the longer isoform clearly has this function. Since tryptophan is an essential amino acid necessary for a variety of metabolic processes, depletion of available tryptophan may be an important mechanism for control of rapidly dividing microbial pathogens and tumors. This explanation goes hand in hand with the expression pattern of the longer isoform as being highly induced during malaria infection. So the truncated did not show the same activity but expression of enzymes *in vitro* are not often without trouble. Factors like optimal temperature and pH or essential substances not included might give an incorrect folding of the enzyme and an inactive mode. Expressing the enzyme with a histidine tail is also something out of its nature that might cause changes. However, since Tr-IDO is expressed at a rather high level in uninfected state, a tryptophan degrading function would not be easy to explain. Healthy cells expressing Tr-IDO would then be depleted of tryptophan, if the efficiency in the enzymatic activity were the same as for the longer IDO (Fig. 22a).

The level of the truncated IDO isoform increased a lot in the vascular tissues after malaria infection. This data, together with the homology of IDO to myoglobin, is perhaps clues to its function. Sequestration of peripheral red blood cells is a typical feature in malaria as the production of parasites forces the cells to burst, with anaemia and hypoxia as a consequence. Perhaps the expression of truncated IDO is increased as a defence mechanism to help supplying the tissues with oxygen. The oxygen binding capability of truncated IDO remains to be examined.

It was examined if IDO isoforms have the same peroxidase activity as myoglobin but no signs of enzymatic activity could be seen. The protein concentration of the purified samples was low and the sample from mock cells was showing the same quantity as the ones from cells expressing IDO. The reason for that might be that other proteins, containing histidine, attach to the column in lack of histidine tagged IDO. Spectroscanning the samples and the saved flow through fraction, from the purification of the proteins, did not reveal any heme protein. However, if the truncated isoform has got a heme group is not known. Because of time limitations, the experiment was only tried once. In case the purification was not successful this should be repeated and the peroxidase experiment performed again.

As reported previously, IFNγ is the key inducer of IDO and as seen in this work, an inducer of both isoforms of the enzyme. Examining the expression of IDO in mice tissues revealed a clear IFNγ dependent induction of IDO in malaria, in all tissues examined.

However, the results from the experiments, to localise the essential parts of the promoter important for this IFNγ dependent induction, was not straightforward to interpret but still informative. To only study smaller parts of the promoter, might imply that parts of the sequence, essential for IFNγ induction, have been cut out. Binding sites for transcription factors can be located within the gene as well as further away than 1500bp from transcription start. Repressor sites included in the examined promoter sequence could affect the activity of the promoter and is also important to consider when results are analysed. The 700bp fragment of the isoform did display a difference between the IFNγ induced sample as compared to the control (Fig. 19) while a longer fragment, including these 700bp was not affected at all (Fig. 20). That loss in induction could be caused by a repressor fragment, included in this longer piece. The hypothesis that IFNγ is the only inducer for IDO expression might not be correct. TNFα , IL-1 and lipopolysaccharide (LPS) of bacteria have been mentioned as possible inducers of IDO in macrophages [20]. In a study where *Toxoplasma gondii* were used, co-stimulation of human brain microvascular endothelial cells with IFNγ and TNFα, induced IDO activity and increased the capacity of cells to restrict bacterial growth [6] more than a stimulation of IFNγ alone.

Combining the results of the IDO mRNA in mice tissues and those from the promoter investigation, this indicated that the promoter of the longer isoform is normally rather silent. Being strongly induced by IFNγ the levels increased a lot in malaria infected animals. The promoter of the truncated IDO is always switched on producing this shorter variant with or without IFNγ. Malaria and IFNγ effects the expression of both IDO isoforms but the longer IDO is the isoform induced the most.

Doing a western blot of IDO isoforms as a control when studying the activity, the IDO antibody did not detect the truncated IDO. When the gel was His stained in an earlier experiment, a protein of the expected size was clearly seen. This antibody has previously been used in immunohistostaining for IDO. Performing this western blot as a second test was also meant to serve as a control whether the antibody actually could distinguish the two isoforms from each other. Three immunizing peptides were used to produce antibodies in rabbit. However, the truncated isoform only contains two of these while the longer has all three of them. Since the antibody did not recognize the truncated IDO protein, the results from previous immunohistostaining using this antibody must be the result of the longer isoform only.

One development for the future that will help a lot when studying the activity would be a method for growing and purifying the IDO isoforms in a bigger scale. For instance, cloning the expression vector and express the enzyme using bacteria and then purify and separate the isoforms with different kinds of chromatography.

Since the IDO antibody used here were only recognising the longer isoform, an antibody specific for the shorter isoform should be prepared. With this antibody the abundance of IDO isoforms in tissues, studied in this work, could be confirmed by immunohistostaining, the cellular source and perhaps the location in the cell for each of

these isoforms could be found which in turn could tell about the optimal conditions for IDO activity.

To broaden the experiment with the abundance of IDO in tissues the level of the two isoforms could be measured for the same organs during PbA infection.

For characterisation of promoter activity, other transcription factors could be tested, separate and mixed together. As mentioned earlier, TNFα, IL1 and LPS have all been referred to as IDO inducers. By trying other cell lines for the transfection, to make the environment as close to the *in vivo* environment as possible, could make a difference and give a different result than seen in this work.

Worth mention is that within 20kb of the mouse IDO gene there is another gene that encodes a cDNA with high homology to IDO. This gene (IDO-2) probably arose through gene duplication and based on the predicted protein sequence the protein from this gene would be likely to have IDO activity. Interestingly the analysis of transcripts from this gene by our lab indicates that this gene also uses two promoters generating transcripts with alternate 5´ end. The primers used in this work should not interfere with this IDO-2 (non-published).

Structural analysis and bioinformatics will create more ideas and theories can grow to be tested in the lab to find answers to the many questions asked. There are many experiments that remains to be done with Indoleamine 2,3-dioxygenase in the future.

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