



# Interferon- $\gamma$ induces a tryptophan-selective amino acid transporter in human colonic epithelial cells and mouse dendritic cells



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## ABSTRACT

*IDO1*, which encodes the immunosuppressive and tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase-1 (IDO1), is a target for interferon- $\gamma$  (IFN- $\gamma$ ). IDO1-mediated tryptophan catabolism in dendritic cells and macrophages arrests T cell proliferation, thereby providing a molecular basis for the immunosuppressive function of IDO1. Whether the entry of tryptophan into IDO1-expressing cells is also regulated by IFN- $\gamma$  is not known. Here we used a human colonic epithelial cell line (CCD841) and a mouse dendritic cell line (DC2.4) to test the hypothesis that IFN- $\gamma$ , which induces IDO1, also induces a tryptophan transporter to promote substrate availability to IDO1. Upon treatment with IFN- $\gamma$ , there was a marked increase in IDO1 mRNA and a concomitant increase in tryptophan uptake in both cell lines. The induced uptake system was selective for tryptophan and saturable with a Michaelis constant of  $36 \pm 3 \mu\text{M}$  in CCD841 cells and  $0.5 \pm 0.1 \mu\text{M}$  in DC2.4 cells. The induction by IFN- $\gamma$  and the tryptophan-selectivity of the induced transport system were demonstrable even in the presence of physiologic concentrations of all other amino acids. Since kynurenine, the catabolic end product of IDO1, is a signaling molecule as an agonist for the aryl hydrocarbon receptor (AhR), we examined if AhR signaling induces the tryptophan-selective transporter. Treatment of the cells with kynurenine and other AhR agonists increased tryptophan uptake. The present studies demonstrate that IFN- $\gamma$  coordinately induces IDO1 and a tryptophan-selective transporter to maximize tryptophan depletion in IDO1-expressing cells and that the process involves a positive feedback mechanism via kynurenine-AhR signaling.

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## 1. Introduction

Tryptophan is an essential amino acid and the least abundant of all dietary amino acids, required by all forms of life for protein synthesis. It is also the precursor for the synthesis of biologically important signaling molecules serotonin and melatonin. Recent studies have shown that this amino acid plays an obligatory role in the immune system through the enzyme indoleamine 2,3-dioxygenase (IDO) [1,2]. IDO is a tryptophan-catabolizing enzyme and produces kynurenine as one of the catabolic

end products. There are three enzymes that participate in tryptophan catabolism: IDO1, IDO2, and biologically related tryptophan dioxygenase (TDO) [3]. Of these, IDO1 has been investigated the most for its role in immune function. It is considered as an immunosuppressive molecule because its expression in certain immune cells such as the dendritic cells and macrophages is induced by interferon- $\gamma$  (IFN- $\gamma$ ) and the resultant increased catabolism of tryptophan in IDO1-expressing cells arrests T cell proliferation and causes T cell anergy [1,2]. The precise mechanisms by which tryptophan degradation in dendritic cells/macrophages is linked to T cell function are poorly understood. Potential mechanisms include depletion of tryptophan in the T cell microenvironment, thus causing the deficiency of an essential amino acid in T cells and consequent cessation of protein synthesis and initiation of signaling pathways involving the stress kinase GCN2 and suppression of the nutrient-sensing receptor mTOR [4,5]. Since kynurenine, generated by IDO1-mediated degradation of tryptophan, is an important signaling molecule by serving as an agonist for the aryl hydrocarbon receptor AhR, the signaling cascade initiated in T cells by this metabolite may also contribute to the mechanism [6–8]. Yet another potential mechanism is the involvement of the G-protein-coupled receptor GPR35, which is activated by kynurenine acid that is generated from kynurenine [9]. Irrespective of the actual mechanism

**Abbreviations:** IFN, interferon; IDO, indoleamine 2,3-dioxygenase; TDO, tryptophan dioxygenase; HPRT, hypoxanthine/guanine phosphoribosyl transferase; SLC, solute-linked carrier; LAT, L-amino acid transporter; PAT, proton-coupled amino acid transporter; MCT, monocarboxylate transporter; TAT, system T amino acid transporter; 4F2hc, heavy chain of the cell-surface antigen 4F2; BAT, system b<sup>0</sup>+ amino acid transporter; CAT, cationic amino acid transporter; ASCT, system ASC amino acid transporter; ATB<sup>0</sup>+, amino acid transporter B<sup>0</sup>+, B<sup>0</sup>AT, system B<sup>0</sup> amino acid transporter; xCT, system x<sub>c</sub><sup>-</sup> amino acid transporter; SNAT, system N amino acid transporter; EEG, embryonic epithelia gene; ACE, angiotensin converting enzyme; 1-MT, 1-methyl tryptophan; TCDD, 2,3,7,8-tetrachloro dibenzo-p-dioxin; BP, benzo[a]pyrene; 3-MCA, 3-methyl cholanthrene; I3C, indole-3-carbinol; AhR, aryl hydrocarbon receptor; GAS, interferon- $\gamma$  activation sequence

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that is responsible for T cell anergy, the role of IDO1-mediated tryptophan catabolism in dendritic cells and macrophages in immune suppression remains an established phenomenon.

IDO1 is a heme-containing enzyme that is expressed in various organs, particularly the placenta, intestinal tract, and immune cells. IDO1-mediated immunosuppression at the maternal–fetal interface of the placenta might play an important role in the maternal tolerance of the allogeneic fetus [10]. The intestinal tract, specifically the colon, is in contact with a wide variety of microorganisms as part of the gut microbiome, and the IDO1-mediated immunosuppression may be essential to maintain the “truce” between these microorganisms and the host and promote a symbiotic relationship [11]. Many immunogenic and tolerogenic signals regulate IDO1 induction; most importantly, it is induced in response to the inflammatory cytokine IFN- $\gamma$  [12].

The role of IDO1 in colon cancer is not clearly understood since it has potential to act as a double-edged sword [13–15]. Basal expression is seen in immune cells present in the lamina propria of the colon and also in colonocytes to maintain the gut homeostasis but the expression is increased under conditions such as inflammatory bowel disease and inflammatory colitis that facilitate colon cancer. In a mild inflammatory state, IDO1 acts as a natural brake in response to the inflammatory situation but as the inflammation increases in severity, IDO1 expression persists which can ultimately lead to tumorigenesis. The fundamental role of IDO1 being in immune suppression and reduced inflammation, it is perplexing that its expression can also promote colon cancer. It is important however to recognize that IDO1 impacts on multiple signaling pathways, which include not only tryptophan depletion but also kynurenine-dependent AhR signaling and kynurenic acid-dependent GPR35 signaling, and that these pathways may have different effects on inflammation, immune tolerance, tumor progression, and tumor-induced immune escape. The involvement of a plethora of signaling events with different biological effects as a consequence of IDO1 activity potentially underlies the seemingly contradictory reports in the literature on the biological outcome of IDO1 induction under various physiological and pathological conditions.

Tryptophan is the substrate for IDO1; it is the degradation of this amino acid inside the cells via IDO1 that initiates the biological effects irrespective of whether such effects depend on tryptophan depletion or on tryptophan catabolic end products. IDO1, being an intracellular enzyme, cannot elicit its effects unless this amino acid enters the cells from the extracellular milieu. Tryptophan however does not diffuse into cells; the entry is certainly mediated by specific transport systems. Therefore, the biological function of IDO1 must depend on the expression and activity of the transport system that is responsible for tryptophan entry into IDO1-expressing cells. In spite of this obligatory relationship between IDO1 and tryptophan transport, surprisingly the primary focus in IDO1 research has been on IDO1 or its catabolic end products with no or little attention on the tryptophan entry mechanism. To date, there have been only two reports in the literature studying tryptophan transport in the context of IDO1 [16,17]. Both studies have identified a novel transport system in IDO1-expressing cells that shows preferential affinity for tryptophan. However, neither of these studies addressed the question as to whether there is a coordinated induction of IDO1 and the tryptophan transport system. The purpose of the present study was therefore to determine the influence of IFN- $\gamma$ , the cytokine most studied for its ability to induce IDO1, on the transport system responsible for tryptophan delivery into IDO1-expressing cells. Since the enzyme is expressed in colonic epithelial cells as well as immune cells, we conducted the present study with a human colonic epithelial cell line (CCD841) and a mouse dendritic cell line (DC2.4). The results of this study demonstrate that IFN- $\gamma$  induces IDO1 in both of these cell lines and at the same time coordinately induces a tryptophan-selective transport system to optimize the biological effects of the induced enzyme.

## 2. Materials and methods

### 2.1. Materials

[ $^3\text{H}$ ]-tryptophan (specific radioactivity, 20 Ci/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO, USA). All amino acids including tryptophan, L-1-methyl tryptophan (L-1-MT), D-1-methyl tryptophan (D-1-MT), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), benzo[*a*]pyrene (B[a]P), 3-methylcholanthrene (3MC), and indole-3-carbinol (I3C) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Human and mouse interferon- $\gamma$  (IFN- $\gamma$ ) were purchased from Bio AbChem (Ladson, SC, USA). Fetal bovine serum was from Atlanta Biologicals (Atlanta, GA, USA) and plasticware for cell culture were obtained from Corning (New York, NY, USA). TRIzol and the power SYBR green were procured from Invitrogen Life Technologies (Grand Island, NY, USA).

### 2.2. Cell culture

The human colonic epithelial cell line (CCD841) and the mouse dendritic cell line (DC2.4) were procured from the American Type Culture Collection (Manassas, VA, USA). They were both cultured in RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 units/mL penicillin and 2 mg/mL streptomycin (Sigma-Aldrich). The cells were incubated at 37 °C in a 5% CO $_2$  environment and subcultured every 48–96 h at a 1:5 ratio. The cell lines were propagated, expanded, and frozen immediately after arrival. The cells revived from the frozen stock were used within 10–20 passages, not exceeding a period of 2–3 months. The media were purchased from MediaTech (Atlanta, GA, USA).

### 2.3. Uptake measurements

Uptake experiments were carried out as described previously [18]. Briefly, CCD841 cells were seeded in 24-well culture plates at an initial density of  $8 \times 10^4$  cells/assay. The DC2.4 cell line was cultured in suspension; the approximate number of cells required for the uptake experiment was calculated and allowed to grow in T75 tissue culture flask. The following day, CCD841 and DC2.4 cells were treated with 10 ng/mL of human IFN- $\gamma$  and 20 ng/mL mouse IFN- $\gamma$ , respectively, for 24 h. Following treatment, DC2.4 cells were counted and used at  $2 \times 10^6$  cells/assay whereas CCD841 were seeded in 24-well plate and used as such. Cells cultured under identical conditions but in the absence of IFN- $\gamma$  served as controls in each experiment. To initiate the uptake, the culture medium was first aspirated out and the cells were washed twice with N-methyl-D-glucamine (NMDG)-chloride (uptake buffer). This was followed by the addition of 250  $\mu\text{L}$  uptake buffer containing [ $^3\text{H}$ ]-tryptophan (0.5  $\mu\text{Ci}$ /assay; 0.1  $\mu\text{M}$ ) and 5 mM leucine (to inhibit the activity of the polyspecific amino acid transporter LAT1) and incubated for 2 min at 37 °C. At the end of the uptake reaction, the substrate was aspirated (filtered in case of DC2.4) and the cells were washed with ice-cold uptake buffer twice, and then lysed with 500  $\mu\text{L}$  of lysis buffer (1% sodium dodecyl sulfate/0.2 N NaOH). The cell lysates (CCD841) and membrane filters (DC2.4) were collected and added into a scintillation vial containing the scintillation cocktail. Using the liquid scintillation counter, the radioactivity in the resultant cell lysate was measured. All assays were performed in triplicate. The uptake rate was normalized to cell number and expressed as pmoles/10 $^6$  cells/2 min. The uptake buffer consisted of 25 mM Hepes/Tris (pH 7.5), containing 140 mM NMDG chloride, 5.4 mM KCl, 1.8 mM CaCl $_2$ , 0.8 mM MgSO $_4$ , and 5 mM glucose. Under these experimental conditions, the value for tryptophan uptake in control CCD841 cells (i.e., without treatment with IFN- $\gamma$ ) was  $0.10 \pm 0.01$  pmoles/10 $^6$  cells/2 min; the corresponding value for control DC2.4 cells was  $0.32 \pm 0.04$  pmoles/10 $^6$  cells/2 min. These values were taken as 100% in respective cells to compare the induction of tryptophan uptake in

IFN- $\gamma$ -treated cells. The kinetic parameters (Michaelis constant  $K_t$  and maximal velocity  $V_{max}$ ) were determined using the computer program Sigma Plot, version 10.0 (SPSS, Inc., Chicago, IL, USA). These determinations were made by non-linear regression analysis and the values confirmed by linear regression analysis according to the Eadie–Hofstee transformation of the Michaelis–Menten equation. Statistical analysis was done using the paired Student's  $t$  test. A  $P < 0.05$  was taken as statistically significant. Experiments were repeated at least three times and measurements were made in triplicate for each experimental condition. Data are presented as means  $\pm$  S.E.M.

#### 2.4. Real-time PCR analysis

Real-time PCR was carried out in an Applied Biosystems Step One Plus instrument using the power SYBR green PCR master mix according to the manufacturer's instructions. Total RNA was isolated from CCD841 cells and DC2.4 cells treated with and without human and mouse IFN- $\gamma$ , using Trizol. The RNA was reverse transcribed using high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). The primers used for the real-time PCR analysis are listed in Table 1 (human) and Table 2 (mouse). In each case, the experiment was repeated with biological triplicates.

#### 2.5. Microarray analysis

Total RNA was extracted from CCD841 cells treated with and without human IFN- $\gamma$  (10 ng/mL for 24 h) using Trizol and the quality of the extracted RNA was analyzed using 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). The labeled single-stranded cDNA was prepared using the total RNA (250 ng) according to the Ambion WT Expression Kit (Life Technologies, Carlsbad, CA, USA) and GeneChip WT Terminal Labeling kit (Affymetrix, Santa Clara, CA, USA), and then hybridized to an Affymetrix GeneChip Human Gene 1.0 ST Array. The washing, staining and scanning of the arrays were conducted using a Fluidics 450 station and GeneChip Scanner 3000 (Affymetrix). The analysis was done with biological triplicates for control and treated cells.

### 3. Results

#### 3.1. IFN- $\gamma$ induces IDO in CCD841 and DC2.4 cells

In normal colon, IDO1 is expressed primarily in immune cells present in the lamina propria and, to a lesser extent, in epithelial cells; but the expression in both cell types increase under inflammatory conditions [19,20]. This suggests that pro-inflammatory cytokines might induce the expression of IDO1 not only in immune cells but also in epithelial cells in colon. Several studies have demonstrated the ability of IFN- $\gamma$  to induce IDO1 in immune cells but this phenomenon has not yet been demonstrated directly in colonic epithelial cells. Therefore, we first wanted to investigate the influence of IFN- $\gamma$  on IDO1 expression in the human colonic epithelial cell line CCD841 and compare the findings with the mouse dendritic cell line DC2.4. We treated CCD841 cells with human IFN- $\gamma$  (10 ng/mL) and DC2.4 cells with mouse IFN- $\gamma$  (20 ng/mL) for 24 h and then assessed the expression levels of IDO1 mRNA. Cells cultured in the absence of IFN- $\gamma$  served as controls. We found the expression of the enzyme to be induced markedly by IFN- $\gamma$  in both cell types: ~300-fold in CCD841 cells (Fig. 1A) and ~70-fold in DC2.4 cells (Fig. 1B). These data show that IDO1 is induced by IFN- $\gamma$  in immune cells as well as in epithelial cells.

#### 3.2. IFN- $\gamma$ -induced IDO1 expression is associated with a concomitant induction of a tryptophan-selective transporter in colonic epithelial cells and immune cells

Since IDO1-mediated tryptophan catabolism is obligatorily dependent on the availability of tryptophan within the cells, we asked

**Table 1**  
Human primer sequences used for real-time quantitative RT-PCR.

Protein name	Gene name	Orientation	Sequence
ASCT1	SLC1A4	Forward	GGC CTT GGC GTT CAT CAT CA
ASCT1	SLC1A4	Reverse	GTT GCA TAC GTA CGG AAA GCT G
ASCT2, AAAT	SLC1A5	Forward	GCC ATC AAC GCC TCC GTG GGA
ASCT2, AAAT	SLC1A5	Reverse	ACG GGC ACC TTC ACC CTG GTT C
rBAT	SLC3A1	Forward	TCC ACC CAA CAA CTG GTT AAG
rBAT	SLC3A1	Reverse	ACC CTT TGT GAG CCA GAA CC
4F2hc	SLC3A2	Forward	CTC GTG GTT CTC CAC TCA GG
4F2hc	SLC3A2	Reverse	CCG CAA TCA AGA GCC TGT CT
ATB <sup>0,+</sup>	SLC6A14	Forward	GGG TCT CGA TTC TCA GTT TGC T
ATB <sup>0,+</sup>	SLC6A14	Reverse	CCC AGT AAA TTC CAG CCT GAG T
B <sup>0</sup> AT1	SLC6A19	Forward	TCC ATC CAC CCG GCC CTG AAG
B <sup>0</sup> AT1	SLC6A19	Reverse	ACT CGT CCA CAT ACC CTG TCT GGT
CAT-1	SLC7A1	Forward	CTC GGG TGC CGT TGC TGC TGT
CAT-1	SLC7A1	Reverse	CAG GTT AGG CTG CTC TGG CTG G
CAT-2	SLC7A2	Forward	TGT CAA CAA GTC TTC TGG GCT
CAT-2	SLC7A2	Reverse	CAA GCG CCA TGA TTG CAA AC
LAT1	SLC7A5	Forward	CCG TGC CGT CCC TCG TGT TC
LAT1	SLC7A5	Reverse	GGT TCA CCT TGA TGG GCC GCT
LAT2	SLC7A8	Forward	TCG GCT CCT GGC TGC CAT CT
LAT2	SLC7A8	Reverse	CCA GCC AGA AGT ACT CTC CTT TGC
b <sup>0,+</sup>	SLC7A9	Forward	CCT GGC CCA AGG AAA CAC AA
b <sup>0,+</sup>	SLC7A9	Reverse	ATG GCC AAA GGC AGG TTT CT
xCT	SLC7A11	Forward	TGG ACG GTG TGT GGG GTC CT
xCT	SLC7A11	Reverse	CAG CAG TAG CTG CAG GGC GTA
MCT5	SLC16A4	Forward	GGT TGG ATT GGA TCC ATC ATG T
MCT5	SLC16A4	Reverse	CAG AAC CCA AAC CGG GTA GA
MCT6	SLC16A5	Forward	GCC AGC TCT ACT TCA CAG CA
MCT6	SLC16A5	Reverse	GCG GAC AAA GTA GAA GCC CA
MCT7	SLC16A6	Forward	TCA TAT GTA CGT CGC CAT CGG
MCT7	SLC16A6	Reverse	TCA GAG CCA TGA TTG CTG GT
MCT9	SLC16A9	Forward	GAT CCC TGC CAA GTG GAG TT
MCT9	SLC16A9	Reverse	ACC ACA TCC AAG ACC TAC AAC A
TAT1, MCT10	SLC16A10	Forward	TCG GCT CCA AAG ACG ATG AC
TAT1, MCT10	SLC16A10	Reverse	CAG AGG CTC GAT GGA ACT TAC
MCT11	SLC16A11	Forward	CCG CTG GTT TTC GGT GTA CT
MCT11	SLC16A11	Reverse	TTA GGA AGC CTG ACA GGG GA
MCT12	SLC16A12	Forward	ATT GTG GCT GGT GGT TTC CT
MCT12	SLC16A12	Reverse	CTC CCA AGT GGA GCA CAG AG
MCT13	SLC16A13	Forward	TTT GGG AGC CCG GTA GGC
MCT13	SLC16A13	Reverse	GGT CGG AGC GAA GGT CAA A
MCT14	SLC16A14	Forward	AAG AAT TCC ACC AGA GCC GC
MCT14	SLC16A14	Reverse	CGG CAC CCA CAG GTG TTA AT
PAT4	SLC36A4	Forward	ATG TTG TCA GGA ACA TGC CAG
PAT4	SLC36A4	Reverse	TTC AAC GCT TGA GGG AAA CG
SNAT1, ATA1	SLC38A1	Forward	GCC ATT ATG GGC AGT GGG AT
SNAT1, ATA1	SLC38A1	Reverse	ACA CCA TGC AGC CTG TTT CT
SNAT2, ATA2	SLC38A2	Forward	TGG GCA GTG GAA TCC TTG GGC
SNAT2, ATA2	SLC38A2	Reverse	AAA GAC CCT CCT TCA TTG GCA GTC T
SNAT3, SN1	SLC38A3	Forward	ATC GGA GCC ATG TCC AGC TA
SNAT3, SN1	SLC38A3	Reverse	AGG GGC AGA ATG ATG GTG AC
EEG1	SLC43A3	Forward	ACT ACA GCT ATG GCT TGT GC
EEG1	SLC43A3	Reverse	CAG AAG GAG CGG AGT TCC TG
CD147	EMMPRIN	Forward	CAG TCG TGC TAG TCC TTG AA
CD147	EMMPRIN	Reverse	TCG TCG GAG TCC ACC TTG A
ACE2		Forward	GTG GGA GAT GAA GCG AGA GAT
ACE2		Reverse	AGC TGC TTG ACA AAG TGC TTC
IDO1		Forward	CAG GCA GAT GTT TAG CAA TGA
IDO1		Reverse	GAT GAA GAA GTG GGC TTT GC
IDO2		Forward	GGC TCT TGG GAA ACT CCT TC
IDO2		Reverse	TCA GGA CAT CAC CAA AAC CTT
TDO		Forward	TGG GAA CTA CCT GCA TTT GGA
TDO		Reverse	CCA ACT CCC AGA GGA TTT GCT
HPRT		Forward	GCG TCG TGA TTA GCG ATG ATG AAC
HPRT		Reverse	CCT CCC ATC TCC TTC ATG ACA TCT

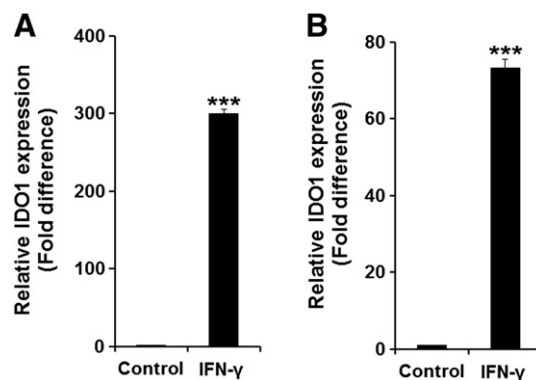
whether IFN- $\gamma$  that induces IDO1 also facilitates the entry of tryptophan into cells to optimize the substrate availability to the enzyme. A Na<sup>+</sup>-independent, tryptophan-selective uptake system has been shown to be present in IDO1-expressing cells [16,17], but the influence of IFN- $\gamma$  on this transport system has never been investigated. To address this issue, we studied tryptophan uptake in control and IFN- $\gamma$ -treated CCD841 cells and DC2.4 cells. Initially we compared tryptophan uptake in these cells in the presence and absence of Na<sup>+</sup> and found that the



**Table 2**  
Mouse primer sequences used for real-time quantitative RT-PCR.

Protein name	Gene name	Orientation	Sequence
ASCT1	SLC1A4	Forward	TGC TCT GGC GTT CAT CAT CA
ASCT1	SLC1A4	Reverse	AGT GAA TGC GGC AAC CAC AA
ASCT2, AAAT	SLC1A5	Forward	TGG CCA GCA AGA TTG TGG AGA T
ASCT2, AAAT	SLC1A5	Reverse	TTT GCG GGT GAA GAG GAA GT
rBAT	SLC3A1	Forward	ATG TCA ACG CCA TGC ACA TGC T
rBAT	SLC3A1	Reverse	AGG TGT GGT TGG CCT CAG TAA A
4F2hc	SLC3A2	Forward	ACC TAC TGA ACA CTC CAC CG
4F2hc	SLC3A2	Reverse	TCA TGT CCA CTT CGG TGT CC
ATB <sup>0,+</sup>	SLC6A14	Forward	CGG CCA GGA CAA CTT CCC AGT
ATB <sup>0,+</sup>	SLC6A14	Reverse	GCC ACT AGG CCA CCC CAA GC
B <sup>0</sup> AT1	SLC6A19	Forward	TTC ACA TCT GTG TAT GCG GCC A
B <sup>0</sup> AT1	SLC6A19	Reverse	AGT GGC ATT GCA CCA CTG TT
CAT-1	SLC7A1	Forward	ATG CCA TGG CTG AAG ATG GAC T
CAT-1	SLC7A1	Reverse	AAA CAC AGG CAG CCA CCA AA
CAT-2	SLC7A2	Forward	ACT TCT TTG CCG TGT GCC TTG T
CAT-2	SLC7A2	Reverse	TTT CAC AAA CCC AGC CAC CA
LAT1	SLC7A5	Forward	ATG GAG TGT GGC ATT GGC TT
LAT1	SLC7A5	Reverse	TGC ATC AAC TTC TGG CAG AGC A
LAT2	SLC7A8	Forward	AGC GAA ACA ACA CCG CGA AGA A
LAT2	SLC7A8	Reverse	TTT CCA GCA CAC CTT TCG GT
b <sup>0,+</sup>	SLC7A9	Forward	TGC AGA GCT TGG CAC AAT GA
b <sup>0,+</sup>	SLC7A9	Reverse	AGG AGG CTT GCA ACC TGA GTA A
xCT	SLC7A11	Forward	TGC AAT CTG CAT CTC CAT GGC T
xCT	SLC7A11	Reverse	AAG CAG GAG AGG GCA ACA AA
MCT5	SLC16A4	Forward	GGT GGC TAC CTG GCA CTA AT
MCT5	SLC16A4	Reverse	ATC CAG CCT GCT ATT GGT GGT
MCT6	SLC16A5	Forward	GGC ATG GTA GTC AGC ACC TT
MCT6	SLC16A5	Reverse	ACA AGC CCA CTA CCG TGA TG
MCT7	SLC16A6	Forward	CCT CCT TCT CCC AAA GGG TC
MCT7	SLC16A6	Reverse	GCT GTG ATA GGT GGT GCG AA
MCT9	SLC16A9	Forward	GAG CAG TCT TGC CCC CAA TA
MCT9	SLC16A9	Reverse	ACC CAC ACT CGA ACC TGT TG
TAT1, MCT10	SLC16A10	Forward	GTG CCT TAC GTT CAC TTG ATG A
TAT1, MCT10	SLC16A10	Reverse	TGG ACA TCA GGC CAA TGA AGA A
MCT11	SLC16A11	Forward	CGG AGC ACT TTG AAC GAA GC
MCT11	SLC16A11	Reverse	AAG CCA AGC GAG GTT AGG AC
MCT12	SLC16A12	Forward	GGC AGT CAC CAG ATG TAT CTC CA
MCT12	SLC16A12	Reverse	ATG ATC CCC GCT TGA CAG GA
MCT13	SLC16A13	Forward	CTT CCT GAA CTG GTG GGG AC
MCT13	SLC16A13	Reverse	ATC CCG GAG GTA GCG CAG TA
MCT14	SLC16A14	Forward	GAT CGT GGG ACC TTT CAT CG
MCT14	SLC16A14	Reverse	CAC TGC AGG TAG GTA AGC CA
PAT4	SLC36A4	Forward	GGA TTT CGT TCC TGC AGA CCT
PAT4	SLC36A4	Reverse	TGT GCA TAC AGT GGA CCG AA
SNAT1, ATA1	SLC38A1	Forward	ACG CGT GCA CAC CAA AGT AT
SNAT1, ATA1	SLC38A1	Reverse	AAA GAT GGC CGT CAG GAA GT
SNAT2, ATA2	SLC38A2	Forward	TTG CAG GCC AGC CTA TTT CA
SNAT2, ATA2	SLC38A2	Reverse	AGC ACA GCC AAT CGG ACA ACA A
SNAT3, SN1	SLC38A3	Forward	TGG CCT GCT TAC GTG CAT CAA T
SNAT3, SN1	SLC38A3	Reverse	AGC GAA ACA AAG GGC CAG GAT T
EEG1	SLC43A3	Forward	GCC CGC CTG ATA GCG ATA TT
EEG1	SLC43A3	Reverse	CCC AAA GAG GTT CCC AAT CTG T
CD147	EMMPRIN	Forward	GCC TGC GCG GCG GCT GGT TT
CD147	EMMPRIN	Reverse	CAC GGT CCA GGT GGA CCG CA
ACE2		Forward	CAG CTG AGG CCG TTG TAT GA
ACE2		Reverse	GGC TTG ATC TCT GCG AAG GT
IDO1		Forward	TGG CAA ACT GGA AGA AAA AG
IDO1		Reverse	AAT GCT TTC AGG TCT TGA CG
IDO2		Forward	TGG CGG TTC TCG ATT AAG TG
IDO2		Reverse	GGC TTT CTC CTT CCA AAT CC
HPRT		Forward	GCG TCG TGA TTA CCG ATG ATG AAC
HPRT		Reverse	CCT CCC ATC TCC TTC ATG ACA TCT

uptake activity is almost equal in both experimental conditions, indicating that the transport system responsible for tryptophan entry in these cells is Na<sup>+</sup>-independent with no or little involvement of any Na<sup>+</sup>-dependent system. We then assessed the contribution of the Na<sup>+</sup>-independent but polyspecific amino acid transporter LAT1 (SLC7A5), a leucine-preferring transporter, to tryptophan uptake in these cells. Most cells express LAT1 and this transporter can mediate the uptake of several amino acids including tryptophan with high affinity [21,22]. The Michaelis constant for the transport of leucine via



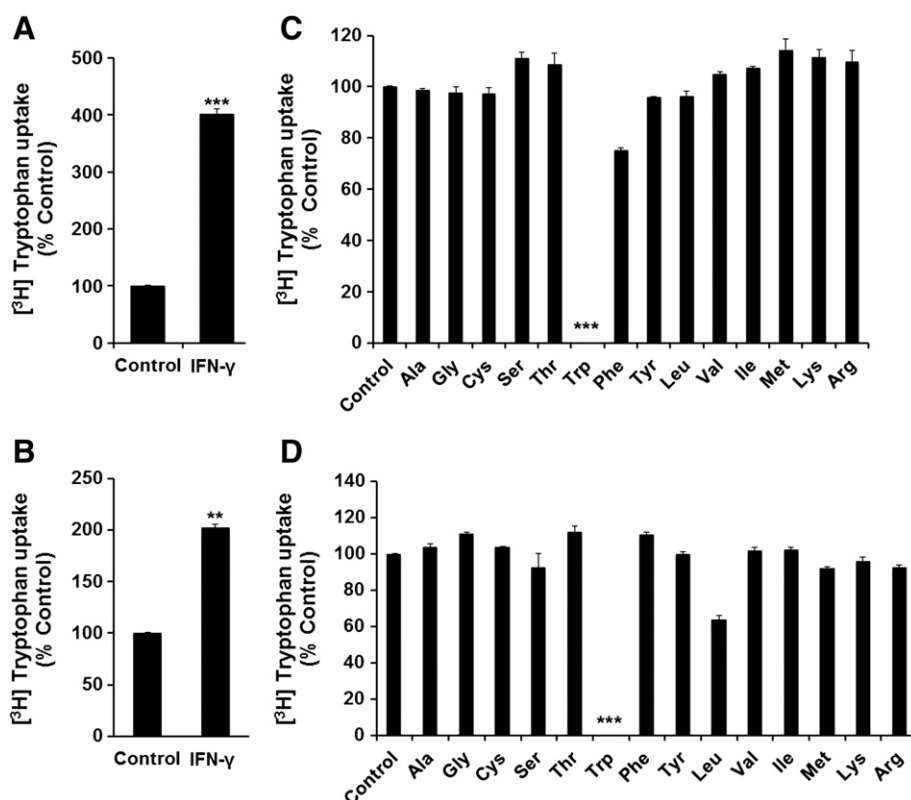
**Fig. 1.** IFN- $\gamma$  treatment induces IDO1 in human CCD841 colonic epithelial cell line and mouse DC2.4 dendritic cell line. (A) Real-time PCR showing the relative IDO1 mRNA expression in IFN- $\gamma$ -treated (10 ng/mL of human IFN- $\gamma$ ; 24 h) CCD841 cells versus untreated CCD841 cells. \*\*\*,  $P < 0.001$ . (B) Real-time PCR showing the relative IDO1 mRNA expression in IFN- $\gamma$ -treated (20 ng/mL of mouse IFN- $\gamma$ ; 24 h) DC2.4 cells versus untreated DC2.4 cells. \*\*\*,  $P < 0.001$ .

LAT1 is  $\sim 10 \mu\text{M}$ ; therefore, we used 5 mM leucine in the uptake medium to eliminate any tryptophan uptake that might occur via LAT1. The presence of leucine did inhibit tryptophan uptake by about 50%, indicating that LAT1 does contribute to tryptophan uptake in these cells to a significant extent. However, the remaining 50% of tryptophan uptake activity did not occur via LAT1. It is the presence of a significant tryptophan uptake activity that is insensitive to leucine and not mediated by LAT1 that is relevant to the present study because of the focus on the tryptophan-selective transport activity rather than on the polyspecific LAT1. Based on these initial experiments, subsequent uptake measurements were made in the absence of Na<sup>+</sup> but in the presence of 5 mM leucine to monitor the leucine-insensitive tryptophan uptake system. We compared tryptophan uptake in control and IFN- $\gamma$ -treated CCD841 cells and DC2.4 cells under these uptake conditions. We found a significant increase in tryptophan uptake in both cell types in response to IFN- $\gamma$  treatment (Fig. 2A for CCD841 cells and Fig. 2B for DC2.4 cells). We also analyzed the influence of IFN- $\gamma$  on the leucine-sensitive tryptophan uptake (i.e., LAT1-mediated uptake) and found that IFN- $\gamma$  treatment had no effect on the activity of this transport system (data not shown).

We then sought to determine the substrate specificity of the induced transporter. To address this, we conducted [<sup>3</sup>H]-tryptophan uptake in IFN- $\gamma$ -treated CCD841 and DC2.4 cells in the presence of a panel of unlabeled amino acids (alanine, glycine, cysteine, serine, threonine, tryptophan, phenylalanine, tyrosine, leucine, valine, isoleucine, methionine, lysine and arginine). The concentration of [<sup>3</sup>H]-tryptophan was 0.1  $\mu\text{M}$  and that of unlabeled amino acids was 1 mM. Under these conditions, none of the amino acids tested except tryptophan competed with [<sup>3</sup>H]-tryptophan for uptake. This is evident in CCD841 cells (Fig. 2C) as well as in DC2.4 cells (Fig. 2D). In contrast, unlabeled tryptophan abolished the uptake of [<sup>3</sup>H]-tryptophan almost entirely. We then used a much lower concentration of unlabeled tryptophan (50  $\mu\text{M}$  instead of 1 mM) and found the uptake of [<sup>3</sup>H]-tryptophan to be inhibited markedly even at this low concentration (data not shown), indicating the high-affinity nature of the transport system. These findings lead to the conclusion that IFN- $\gamma$  induces a tryptophan-selective transport system in the colonic epithelial cell line CCD841 as well as in the mouse dendritic cell line DC2.4.

### 3.3. Kinetic features of the IFN- $\gamma$ -inducible tryptophan transport system

We then determined the kinetic parameters (Michaelis constant  $K_m$  and maximal velocity  $V_{\text{max}}$ ) of the tryptophan-selective transport



**Fig. 2.** IDO1 expression is associated with induction of a tryptophan-selective transporter. [<sup>3</sup>H]-tryptophan (0.1 μM) uptake in CCD841 (A) and DC2.4 (B) cells cultured for 24 h in the presence and absence of 10 and 20 ng/mL of human and mouse IFN-γ, respectively. The transport function was monitored by measuring tryptophan uptake for 2 min in NMDG-chloride buffer in the presence of 5 mM leucine. Control uptake measured in CCD841 and DC2.4 cells without IFN-γ treatment was taken as 100%. \*\*\*,  $P < 0.001$ ; \*\*,  $P < 0.01$ . Amino acid selectivity of the IFN-γ-induced tryptophan uptake system in CCD841 (C) and DC2.4 (D) cells. Cells were cultured in the presence of 10 and 20 ng/mL of human and mouse IFN-γ, respectively, for 24 h. [<sup>3</sup>H]-tryptophan (0.1 μM) uptake was measured in NMDG-chloride for 2 min in the presence of 5 mM leucine. When present, the concentration of unlabeled tryptophan and other amino acids was 1 mM. Uptake values measured in the absence of competing amino acids were taken as 100%. \*\*\*,  $P < 0.001$ .

system in IFN-γ-treated CCD841 cells and DC2.4 cells. Again, uptake was monitored in the absence of Na<sup>+</sup> but in the presence of 5 mM leucine. The transport system was saturable in both cell types (Fig. 3A for CCD841 cells; Fig. 3B for DC2.4 cells), and the uptake data fit well to a model describing a single saturable transport system. The values for  $K_t$  and  $V_{max}$  for CCD841 cells were  $36 \pm 6 \mu\text{M}$  and  $62 \pm 8 \text{ pmoles}/10^6 \text{ cells}/2 \text{ min}$ ; the corresponding values for DC2.4 cells were  $0.5 \pm 0.1 \mu\text{M}$  and  $1.3 \pm 0.3 \text{ pmoles}/10^6 \text{ cells}/2 \text{ min}$ . These findings lead to the conclusion that the IFN-γ-inducible tryptophan-selective transport system is of a high-affinity type in both cell types. Interestingly, the data show a significant species-specific difference in the affinity; the affinity of the transport system for tryptophan is almost 70-fold higher in the mouse cell line DC2.4 compared to that in the human cell line CCD841.

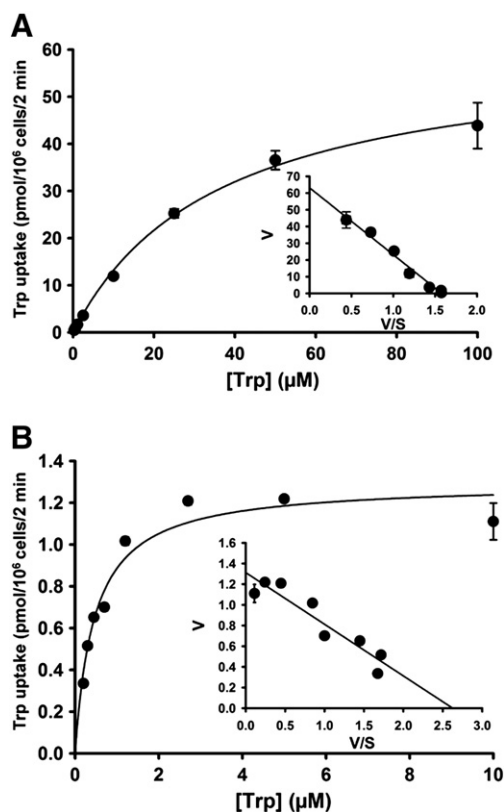
#### 3.4. Tryptophan selectivity of the IFN-γ-inducible transport system in the presence of physiological concentrations of amino acids

We then wanted to confirm the induction of the tryptophan-selective transport system by IFN-γ in both cell types even when assessed in the presence of physiological concentrations of all amino acids. This was done by measuring [<sup>3</sup>H]-tryptophan uptake in IFN-γ-treated CCD841 cells and DC2.4 cells in the presence of an amino acid mixture consisting of 18 different amino acids, each at its respective physiological concentrations found in plasma [18]. Again, cells cultured in the absence of IFN-γ were used as controls. IFN-γ-mediated induction of tryptophan uptake was noticeable even under these

experimental conditions mimicking a physiological situation (Fig. 4A for CCD841 cells; Fig. 4B for DC2.4 cells).

#### 3.5. Evidence for the non-involvement of the Na<sup>+</sup>-independent amino acid transporter PAT4 in IFN-γ-inducible tryptophan uptake

Until recently, among the multitude of amino acid transporters that have been characterized at the molecular level, LAT1 (SLC7A5) was the only transporter that was capable of tryptophan uptake with high affinity. Therefore, previously published reports [16,17] as well as the present study had to address the issue of whether or not the IFN-γ-inducible tryptophan uptake in IDO1-expressing cells occurs via LAT1. These studies concluded that the IFN-γ-inducible tryptophan uptake system is not identical to LAT1 at the molecular level. A more recent study has identified another amino acid transporter, PAT4 (SLC36A4), as capable of transporting tryptophan with high affinity (Michaelis constant,  $\sim 2 \mu\text{M}$ ) [23]. This transporter belongs to the family of proton-coupled amino acid transporters with high affinity for proline [24]. Therefore, we sought to determine the relevance of PAT4 to the IFN-γ-inducible tryptophan uptake. PAT4 is Na<sup>+</sup>-dependent and has high affinity for tryptophan; but it also has high affinity for proline. As such, the relevance of PAT4 to the present investigation could be assessed easily by studying the effect of proline on tryptophan uptake. We used IFN-γ-treated DC2.4 cells for this purpose and compared the efficacy of proline and unlabeled tryptophan to compete with [<sup>3</sup>H]-tryptophan (0.1 μM) for uptake (Fig. 4C). Proline did not inhibit [<sup>3</sup>H]-tryptophan uptake even at a concentration as high as 1 mM whereas unlabeled tryptophan, as expected, showed robust competition with radiolabeled

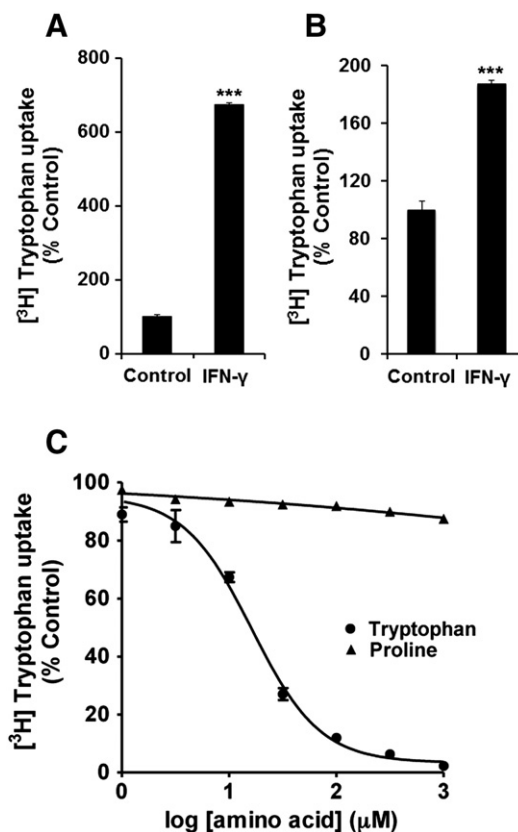


**Fig. 3.** Substrate saturation kinetics of the IFN- $\gamma$ -induced tryptophan-selective transport system in CCD841 cells (A) and in DC2.4 cells (B). Cells were treated with human and mouse IFN- $\gamma$ , respectively, for 24 h. Tryptophan uptake was measured at increasing concentrations of tryptophan (0.3–100  $\mu$ M) in CCD841 cells and (0.2–10  $\mu$ M) in DC2.4 cells. [ $^3$ H]-tryptophan (0.1  $\mu$ M) was present as the tracer during uptake measurements. The uptake buffer contained NMDG-chloride (in place of NaCl) and 5 mM leucine. Values are the means  $\pm$  S.E.M. (n = 6). Inset, Eadie-Hofstee plot (V versus V/S; V, tryptophan uptake in pmol/10<sup>6</sup> cells per 2 min; S, tryptophan concentration in  $\mu$ M).

tryptophan for uptake. Similar results were obtained with CCD841 cells (data not shown). These data rule out involvement of PAT4 in IFN- $\gamma$ -inducible tryptophan uptake in both cell types.

### 3.6. Efficacy of L-1-methyl tryptophan versus D-1-methyl tryptophan for the inhibition of IFN- $\gamma$ -inducible tryptophan uptake

L-1-Methyl tryptophan (L-1-MT) is an inhibitor of IDO1 whereas D-1-methyl tryptophan (D-1-MT) is not [25–27]. Therefore, the L-isomer is routinely used in vitro and in vivo to determine the consequences of IDO1 inhibition. Here we examined the influence of L-1-MT on the IFN- $\gamma$ -induced tryptophan-selective transport system because the ability of this molecule to interfere with IDO1-induced tryptophan depletion may not be solely due to its inhibitory effect on IDO1 activity but may also involve potential inhibition of tryptophan entry into IDO1-expressing cells. For this, we investigated the influence of both stereoisomers of 1-MT on the tryptophan-selective uptake system in IFN- $\gamma$ -treated CCD841 cells and DC2.4 cells. L-1-MT or D-1-MT was present only in the uptake medium along with tryptophan to see if these tryptophan derivatives interfere with tryptophan uptake in IFN- $\gamma$ -treated cells. We observed that the L-isomer was highly effective in inhibiting tryptophan uptake in both cell types with an IC<sub>50</sub> value of  $54 \pm 4$   $\mu$ M in CCD841 cells (Fig. 5A) and  $5 \pm 1$   $\mu$ M in DC2.4 cells (Fig. 5B). In contrast, the D-isomer had almost no effect. These data suggest that L-1-MT is not only an inhibitor of IDO1 activity but also an inhibitor of tryptophan entry into IDO1-expressing cells. On the other hand, the D-isomer shows no effect on the IFN- $\gamma$ -inducible tryptophan transporter.



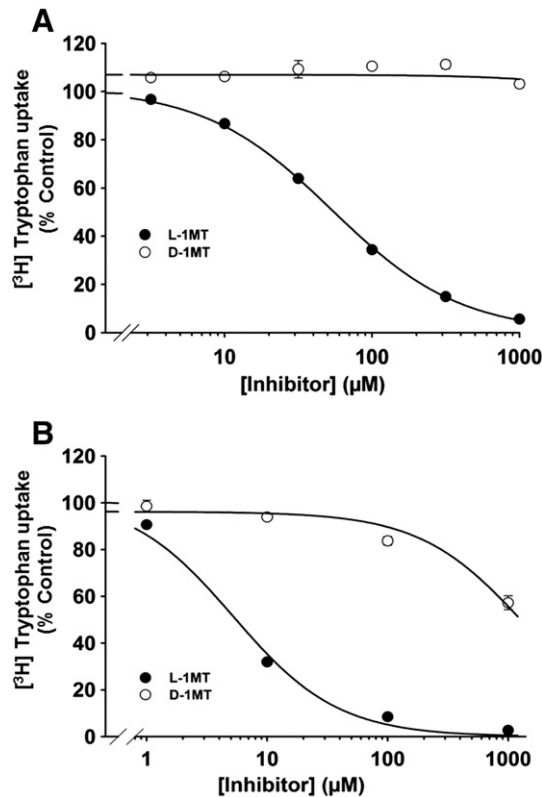
**Fig. 4.** Tryptophan uptake in the presence of physiological concentrations of amino acids reconfirms the specificity of the tryptophan transporter. [ $^3$ H]-tryptophan (0.1  $\mu$ M) uptake in CCD841 cells (A) and DC2.4 cells (B) cultured for 24 h in the presence of human and mouse IFN- $\gamma$ , respectively. Uptake was conducted in NMDG-chloride buffer containing 18 different amino acids (but no tryptophan), all at their normal physiological concentrations in plasma [18]. \*\*\*,  $P < 0.001$ . (C) Dose-response relationship for the inhibition of [ $^3$ H]-tryptophan (0.1  $\mu$ M) uptake by proline and unlabeled tryptophan in DC2.4 cells treated with mouse IFN- $\gamma$  (20 ng/mL; 24 h). The concentrations of the inhibitors ranged from 1 to 1000  $\mu$ M. Values are the means  $\pm$  S.E.M. (n = 6).

### 3.7. Induction of the tryptophan-selective transport system by aryl hydrocarbon receptor

Kynurenine is a catalytic end product of IDO1 and it is a physiological agonist for the aryl hydrocarbon receptor (AhR). Activation of AhR induces IDO1 [28–31]. We therefore asked whether AhR signaling, just like IFN- $\gamma$  signaling, is also involved in coordinated induction of the tryptophan-selective transport system along with the induction of IDO1. To address this question, we examined the effects of kynurenine treatment on the activity of the tryptophan-selective transport system in CCD841 cells. Cells cultured under identical conditions but in the absence of kynurenine were used as controls. We found that kynurenine treatment increased the activity of the tryptophan-selective transporter to a significant extent (Fig. 6A). We then examined the effects of several pharmacological agonists of AhR in the same cell line. With each of the agonist examined, we found a significant increase in the activity of the tryptophan-selective transport system compared to cells cultured in the absence of the agonists (Fig. 6B). These findings lead to the conclusion that AhR signaling induces not only IDO1 expression as evident from published reports but also the tryptophan entry into IDO1-expressing cells.

### 3.8. Attempts to identify the IFN- $\gamma$ -inducible tryptophan-selective transporter

To establish the molecular identity of the tryptophan-selective transporter that is induced by IFN- $\gamma$  in IDO1-expressing cells, we took



**Fig. 5.** Interaction of the IDO1 inhibitor 1-methyl tryptophan (1MT) with the tryptophan-selective transporter. Dose–response relationship for inhibition of tryptophan (0.1  $\mu$ M) uptake (NMDG-chloride buffer; 5 mM leucine) by D-1-MT and L-1-MT examined in IFN- $\gamma$ -treated CCD841 (A) and DC2.4 (B) cells. The concentrations of the inhibitors ranged from 1 to 1000  $\mu$ M. Values are the means  $\pm$  S.E.M. ( $n = 6$ ).

two different approaches. The first approach involved analysis of ~30 transporters that included those that have already been established as amino acid transporters, others that currently remain as orphan transporters with no known transportable substrates, and transporter chaperones that do not function by themselves as transporters but facilitate the stability and plasma membrane recruitment of certain amino acid transporters. We compared the expression levels of these ~30 genes by qPCR between control and IFN- $\gamma$ -treated CCD841 cells and DC2.4 cells. The rationale for this approach was that IFN- $\gamma$  treatment induces a tryptophan-selective transport system in these cells and therefore

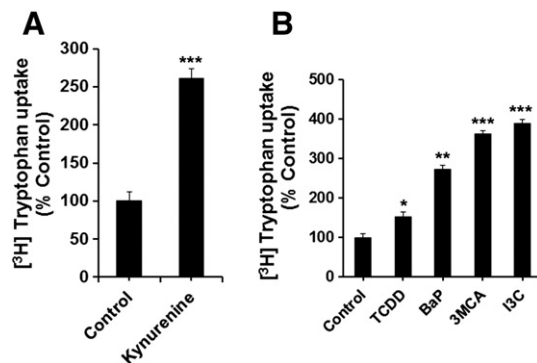
must be associated with an increase in the steady-state levels of the mRNA for the transporter in both cell types. Comparison of the mRNA levels for the potential transporter genes between control and IFN- $\gamma$ -treated cells might lead to identification of the transporter. The second was a non-biased approach that involved analysis of transcriptome by microarray in control and IFN- $\gamma$ -treated CCD841 cells with the goal to identify the transporter genes that are induced by IFN- $\gamma$  as the potential candidates for the tryptophan-selective transporter.

The real-time PCR data for CCD841 cells and DC2.4 cells are given in Tables 3 and 4, respectively. In CCD841 cells, we identified six transporters that were induced significantly by IFN- $\gamma$ ; these are SLC6A14 (ATB<sup>0,+</sup>), SLC6A19 (B<sup>0</sup>AT1), SLC7A2 (CAT2), SLC7A8 (LAT2), SLC7A9 (b<sup>0,+</sup>) and SLC16A11 (MCT11). The same six transporters were also induced in DC2.4 cells. Among these transporters, five of them have already been characterized at the functional level, and the functional features of these five transporters did not match those of the IFN- $\gamma$ -induced tryptophan-selective transporter, thus ruling them out as the potential candidates for the transport system. SLC16A11 seemed a promising candidate because it has no known transport function and it is induced by IFN- $\gamma$  in both cell types. Therefore, we cloned this transporter, functionally expressed in mammalian cells, and examined its ability to transport tryptophan. These studies failed to show tryptophan uptake activity for the transporter (data not shown). There were four additional transporter genes that were induced by IFN- $\gamma$  in DC2.4 cells (SLC7A11, SLC16A5, SLC16A9, and SLC43A3), but these genes were not induced in CCD841 cells. Since IFN- $\gamma$  induced the tryptophan-selective transport activity in both cell types, induction in one cell type but not in the other made it very unlikely that any of these four transporters represents the tryptophan-selective transporter.

We then examined the transcriptome profiles in control and IFN- $\gamma$ -treated CCD841 cells for the genes belonging to the known SLC gene families. The microarray data contained expression information for a total of 430 transporter genes, of which ~90% of the genes remained largely unaffected by IFN- $\gamma$  treatment. Among the genes that were upregulated at least by 50% were SLC2A3 (glucose transporter 3), SLC6A6 (taurine transporter), SLC6A12 (betaine transporter), SLC9A7

**Table 3**  
Real-time quantitative RT-PCR with CCD841 cells.

Sr. No.	Protein name	Gene name	+ IFN- $\gamma$ treated	SE
	Control		1.00	0.03
1	ASCT1	SLC1A4	0.76	0.08
2	ASCT2	SLC1A5	1.07	0.02
3	rBAT	SLC3A1	1.06	0.25
4	4F2hc	SLC3A2	0.75	0.04
5	ATB <sup>0,+</sup>	SLC6A14	4.08	0.26
6	B <sup>0</sup> AT1	SLC6A19	2.01	0.22
7	CAT-1	SLC7A1	0.91	0.01
8	CAT2	SLC7A2	1.86	0.13
9	LAT1	SLC7A5	0.82	0.03
10	LAT2	SLC7A8	6.46	1.04
11	b <sup>0,+</sup>	SLC7A9	2.46	0.51
12	xCT	SLC7A11	0.85	0.13
13	MCT5	SLC16A4	1.03	0.08
14	MCT6	SLC16A5	1.07	0.02
15	MCT7	SLC16A6	0.96	0.06
16	MCT9	SLC16A9	1.00	0.31
17	TAT1, MCT10	SLC16A10	0.67	0.02
18	MCT11	SLC16A11	3.19	0.30
19	MCT12	SLC16A12	1.67	0.15
20	MCT13	SLC16A13	0.72	0.03
21	MCT14	SLC16A14	0.50	0.03
22	PAT4	SLC36A4	0.98	0.03
23	SNAT1, ATA1	SLC38A1	0.83	0.01
24	SNAT2, ATA2	SLC38A2	0.95	0.02
25	SNAT3, SN1	SLC38A3	0.95	0.04
26	EEG1	SLC43A3	0.95	0.02
27	CD147	EMMPRIN	1.09	0.16
28	ACE2		0.91	0.31
29	IDO1		21466.20	1249.55



**Fig. 6.** Activation of aryl hydrocarbon receptor (AhR) induces the tryptophan-selective transporter. (A) [ $^3$ H]-tryptophan (0.1  $\mu$ M) uptake measured for 2 min in CCD841 cells (NMDG-chloride buffer; 5 mM leucine) that had been cultured in the presence and absence of 1 mM kynurenine for 24 h. \*\*\*,  $P < 0.001$ . (B) [ $^3$ H]-tryptophan (0.1  $\mu$ M) uptake measured for 2 min in CCD841 cells (NMDG-chloride; 5 mM leucine) that had been cultured in the presence of various AhR ligands for 24 h: TCDD (10 nM), BaP (5  $\mu$ M), 3MCA (5  $\mu$ M) and I3C (5  $\mu$ M). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ .



**Table 4**  
Real-time quantitative RT-PCR in DC2.4 cells.

Sr. No.	Protein name	Gene name	+ IFN- $\gamma$ treated	SE
1	Control		1.00	0.03
2	ASCT1	SLC1A4	0.59	0.02
3	ASCT2	SLC1A5	0.83	0.01
4	rBAT	SLC3A1	0.35	0.04
5	4F2hc	SLC3A2	0.71	0.07
6	ATB <sup>0,+</sup>	SLC6A14	2.62	0.12
7	B <sup>0</sup> AT1	SLC6A19	34.21	13.02
8	CAT-1	SLC7A1	0.93	0.02
9	CAT-2	SLC7A2	3.78	0.17
10	LAT1	SLC7A5	0.85	0.03
11	LAT2	SLC7A8	2.70	0.09
12	b <sup>0,+</sup>	SLC7A9	455.16	102.02
13	xCT	SLC7A11	1.90	0.04
14	MCT5	SLC16A4	1.83	0.19
15	MCT6	SLC16A5	1.22	0.24
16	MCT7	SLC16A6	1.31	0.02
17	MCT9	SLC16A9	1.83	0.32
18	TAT1, MCT10	SLC16A10	0.96	0.04
19	MCT11	SLC16A11	7.03	0.60
20	MCT12	SLC16A12	0.60	0.04
21	MCT13	SLC16A13	0.90	0.04
22	MCT14	SLC16A14	0.19	0.01
23	PAT4	SLC36A4	0.84	0.01
24	SNAT1, ATA1	SLC38A1	0.72	0.00
25	SNAT2, ATA2	SLC38A2	0.63	0.02
26	SNAT3, SN1	SLC38A3	2.16	1.02
27	EEG1	SLC43A3	4.46	0.18
28	CD147	EMMPRIN	1.66	0.13
29	ACE2		1.46	0.38
30	IDO1		73.20	2.18

(a Na<sup>+</sup>/H<sup>+</sup> exchanger), SLC9B1 (a K<sup>+</sup>/H<sup>+</sup> exchanger), SLC25A22 (a mitochondrial glutamate transporter), SLC22A28 (a member of the anion/cation transporter gene family), SLC37A1 (glucose-6-phosphate transporter), and SLC37A3 (a lysosomal sugar phosphate exchanger) (Supplemental Table 1). The functional identities of all of these transporters are already known, and it is unlikely that any of them has the ability to transport tryptophan, thus ruling them out as potential candidate genes for the tryptophan-selective transporter. Among the transporter genes that were downregulated, only 6 genes showed quantitatively significant reduction in expression (>30%) (Supplemental Table 2). These genes are not likely to do anything with the tryptophan-selective transporter because of the suppressing effect instead of the inductive effect of IFN- $\gamma$  on their expression.

#### 4. Discussion

Here we report for the first time that IFN- $\gamma$ , which is known to induce the tryptophan-catabolizing enzyme indoleamine 2,3-dioxygenase-1 (IDO1), also induces a tryptophan-selective transporter in colonic epithelial cells and in dendritic cells. This is a very important finding because the primary purpose for the induction of IDO1 is to promote intracellular degradation of tryptophan with consequent depletion of this essential amino acid in the extracellular milieu. It is this decreased availability of tryptophan in the extracellular medium that suppresses T cell proliferation and causes immune tolerance. This whole rationale however relies on effective transfer of tryptophan from the extracellular medium into IDO1-expressing cells. Therefore, a concomitant induction of a tryptophan transport system along with IDO1 is necessary for this process to occur in an effective manner. Our data showing that IFN- $\gamma$  coordinately induces IDO1 as well as a tryptophan-selective transporter provide a molecular basis for the ability of the enzyme to deplete this essential amino acid in the extracellular medium. The existence of a tryptophan-selective transporter in IDO1-expressing cells has been demonstrated previously in two different laboratories [16,17] but this is the first time that the influence of IFN- $\gamma$  on the transporter is reported.

The selectivity of the transporter to tryptophan is unique among the known amino acid transport systems, but since the function of the transporter is coupled to IDO1, an enzyme that degrades tryptophan, biologically this selectivity does make sense. Another interesting aspect of the present study is the demonstration that L-1-methyl tryptophan (L-1-MT) interferes with the transfer of tryptophan into cells via the transporter. This tryptophan derivative is widely used to inhibit the activity of IDO1 and it does prevent the enzyme-induced tryptophan depletion and its consequences [25–27]. But until now it has been assumed that IDO1 is the sole target for this compound. Our studies show that L-1-MT not only inhibits IDO1 but also blocks tryptophan entry into cells. The D-isomer (D-1-MT) exhibits a much lower efficacy in blocking tryptophan transport via the transporter. It is interesting to note that this isomer does not inhibit IDO1 also. Even though it is the L-isomer that is capable of preventing IDO1-induced tryptophan depletion, the D-isomer has been found to be effective in vivo in blocking the IDO1-dependent immunotolerance in some mouse models [5]. The molecular basis of the efficacy of the D-isomer in vivo is not well understood. It is possible that the D-isomer has a much greater half-life than the L-isomer due to the lack of robust enzyme activities to metabolize the D-isomer; therefore, even though D-1-MT is less effective than L-1-MT in inhibiting IDO1 and the tryptophan transporter, the pharmacokinetics of D-1-MT may be more favorable than L-1-MT in vivo to prevent IDO1-induced tryptophan depletion. Alternatively, the ability of the D-isomer to block the IDO1-induced immunotolerance may not have anything to do with IDO1 and tryptophan entry; the compound might have some other, not yet identified, biological targets in cells.

The third observation in the present study that has functional and biological significance to IDO1-induced tryptophan depletion and immunotolerance is the ability of kynurenine to induce the tryptophan-selective transporter via the aryl hydrocarbon receptor (AhR). It is already known that AhR is capable of inducing IDO1 [28–31], but the present study demonstrates that AhR is also capable of inducing the transporter responsible for the entry of tryptophan in IDO1-expressing cells. The coordinated induction of IDO1 and the tryptophan transporter by kynurenine suggests the existence of a positive feedback loop in the process. When tryptophan is degraded by IDO1, it generates kynurenine, which then induces the expression of the tryptophan transporter and IDO1 by serving as an agonist for AhR, thus amplifying the signal to elicit further depletion of tryptophan. A previous study has shown that exogenous expression of IDO1 in mammalian cells leads to induction of the tryptophan-selective transporter [7], but the molecular mechanism underlying the phenomenon has not been determined. Our findings that kynurenine induces the transporter via AhR indicate that the IDO1-dependent induction of the transporter occurs most likely via the kynurenine-AhR signaling pathway.

Though it is unequivocal from the present study as well as from the other two previous studies that IDO1-expressing cells have a functionally active tryptophan-selective transporter, the molecular identity of the transporter remains unknown. The transport activity does not seem to represent or resemble the function of any of the already characterized amino acid transporters. The present studies represent the first comprehensive attempt to identify the transporter at the molecular level. Unfortunately, these studies also failed to establish the molecular identity of the transporter. Since the list of the transporter genes that we examined in the present study for tryptophan-selective transport function included several orphan transporters, it is possible that the transporter represents the product of a novel transporter gene not yet identified. It is also possible that, like many of the known amino acid transporters, the tryptophan-selective transporter requires a chaperone for its function and that the induction of this chaperone is responsible for the IFN- $\gamma$ -inducible tryptophan uptake activity. The list of genes that we examined in the present studies did include all of the known chaperones associated with amino acid transporters, but it is possible that the chaperone associated with the tryptophan-selective transporter is different from the ones that have been already identified. Thus, the



molecular nature of the IDO1-associated tryptophan transporter still remains elusive.

Despite the failure of the present study to successfully establish the molecular identity of the tryptophan-selective transporter in IDO1-expressing cells, there is no doubt about the biological and therapeutic importance of the transporter. Without the function of the transporter, IDO1 will not be effective in depleting tryptophan in the extracellular environment to elicit T cell anergy. The transporter and the enzyme have to be functionally coupled for this purpose. The coordinated induction of both genes by IFN- $\gamma$  underscores the obligatory nature of this functional coupling. The positive feedback loop in the control of the expression of the transporter and the enzyme through kynurenine-AhR signaling also highlights the significance of the functional cooperation between the two proteins. The therapeutic relevance of the transporter is readily apparent. Currently, only IDO1 is being investigated as a drug target for the modulation of immune function via tryptophan catabolism. The fact that IDO1 cannot bring about tryptophan catabolism without partnering with the tryptophan transporter indicates that the transporter is also a potential drug target to modulate immune function. Ideally, an inhibitor that blocks the function of the transporter as well as that of the enzyme would be desirable for this purpose because such an inhibitor would be more effective in preventing tryptophan catabolism than the inhibitors that target either the transporter or the enzyme individually.

There is a growing interest in the potential use of IDO1 inhibitors as anticancer drugs [13–15]. The enzyme is induced in immune cells present in tumor-draining lymph nodes where it is thought to play a role in suppressing T cell proliferation and consequently promote the escape of tumor cells from attack by cytotoxic T cells. Based on this proposed mechanism, pharmacological inhibition of IDO1-mediated tryptophan catabolism is expected to enhance immune surveillance and suppress tumor growth via T cell-mediated antitumor immunity. Several studies with preclinical animal models of breast cancer, ovarian cancer and melanoma have provided evidence in support of antitumor activity of IDO1 inhibitors, effective either alone or in combination with chemotherapy [32–35]. There are reasons to believe that the biological function of IDO1, tryptophan-selective amino acid transporter, and tryptophan catabolism and their relevance to inflammation and carcinogenesis might be different in colon versus other organs. IDO1 is expressed in normal colon and in immune cells located in lamina propria where the enzyme-mediated tryptophan depletion and consequent immune tolerance might be essential to facilitate the existence of normal microbiota in the host colon. In the present study we have shown that IDO1 and the tryptophan-selective amino acid transporter are expressed not only in immune cells but also in colonic epithelial cells. It is likely that IDO1-mediated tryptophan depletion occurs in both cell types in normal colon contributing to the maintenance of immune tolerance. Pharmacological blockade of IDO1 and tryptophan-selective amino acid transporter in colon would interfere with this phenomenon and promote inflammation, particularly in view of the fact that the colonic epithelial cells and immune cells are constantly exposed to bacterial antigens. It is well known that inflammation is a potent driver of colon carcinogenesis, suggesting that inhibition of IDO1-mediated tryptophan depletion may actually promote colon cancer. The findings from published reports on the consequences of IDO1 loss or inhibition in colon are conflicting, some studies showing suppression of colon cancer [36,37] while others showing enhancement of colon cancer [38]. Further studies are needed in this area to evaluate the role of IDO1-mediated tryptophan depletion in colonic epithelial cells and immune cells under normal and pathological conditions.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.bbamm.2014.10.021>.

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