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IDO Induces Expression of a Novel Tryptophan Transporter in Mouse and Human Tumor Cells

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IDO is the rate-limiting enzyme in the kynurenine pathway, catalyzing tryptophan to kynurenine. Tryptophan depletion by IDO-expressing tumors is a common mechanism of immune evasion inducing regulatory T cells and inhibiting effector T cells. Because mammalian cells cannot synthesize tryptophan, it remains unclear how IDO+ tumor cells overcome the detrimental effects of local tryptophan depletion. We demonstrate that IDO+ tumor cells express a novel amino acid transporter, which accounts for ~50% of the tryptophan uptake. The induced transporter is biochemically distinguished from the constitutively expressed tryptophan transporter System L by increased resistance to inhibitors of System L, resistance to inhibition by high concentrations of most amino acids tested, and high substrate specificity for tryptophan. Under conditions of low extracellular tryptophan, expression of this novel transporter significantly increases tryptophan entry into IDO+ tumors relative to tryptophan uptake through the low-affinity System L alone, and further decreases tryptophan levels in the microenvironment. Targeting this additional transporter could be a way of pharmacological inhibition of IDO-mediated tumor escape. These findings highlight the ability of IDO-expressing tumor cells to thrive in a tryptophan-depleted microenvironment by expressing a novel, highly tryptophan-specific transporter, which is resistant to inhibition by most other amino acids. The additional transporter allows tumor cells to strike the ideal balance between supply of tryptophan essential for their own proliferation and survival, and depleting the extracellular milieu of tryptophan to inhibit T cell proliferation. The Journal of Immunology, 2011, 187: 1617–1625.

Metabolism of tryptophan, like that of other essential amino acids, is tightly regulated. Its degradation through the kynurenine pathway is mediated by two functionally similar, but structurally unrelated, enzymes: IDO and tryptophan 2,3-dioxygenase (TDO) (1, 2). TDO is liver specific and is expressed constitutively (3), whereas IDO is induced by IFN-γ in many cell types, including those of the epithelial and monocytic lineage (4, 5).

The IDO-dependent kynurenine pathway is an important component of antibacterial innate immune responses, because depletion of intracellular tryptophan hinders replication of invading microorganisms (6). In recent years, the kynurenine pathway has been implicated in regulation of a number of immune processes, as a consequence of tryptophan depletion or metabolite toxicity, or both (7–9). Placental expression of IDO prevents fetal rejection by suppressing the maternal immune response to fetal Ags (10). In keeping with this immunosuppressive function, loss of IDO expression or activity has been shown to enhance several autoimmune diseases, including experimental autoimmune encephalomyelitis (11, 12) and insulin-dependent diabetes mellitus (13), and to shorten graft survival (14, 15). The steroid dexamethasone was found to inhibit onset of allergic inflammation by inducing reverse signaling into plasmacytoid dendritic cells (pDC) and inducing expression of IDO (16). Induction of IDO in the context of experimental autoimmunity including graft-versus-host disease and diabetes can lead to alleviation of the disease (17, 18).

Van den Eynde and colleagues (19) showed that many human tumors constitutively express IDO, and that expression of IDO by immunogenic mouse tumor cells prevents their rejection by preimmunized mice. IDO can be upregulated by human and murine DCs on maturation (20–22), and IDO-expressing regulatory DCs were found to suppress T cell proliferation in MLRs in a manner that is reversed by the addition of tryptophan (23, 24). In mice, these cells were defined as a subset of pDC, found in abundance in tumor-draining lymph nodes (25). A functionally similar population of “IDO+” pDC was found in human tumor-draining lymph nodes and was shown to negatively correlate with survival of melanoma patients (26). IDO expression by DC indicates that IDO activity, immunosuppression, and tolerance could be linked (27). A population of DC constitutively expressing IDO has recently been described in the mesenteric lymph nodes of mice (28), and they may play a role in maintenance of oral tolerance.

Although it is well established that IDO expression by APCs or tumors can inhibit immune responses, it is unclear whether IDO-dependent immunosuppression is mediated by tryptophan de-
Tryptophan depletion is known to halt cell cycle progression by triggering the anti-proliferative GCN2 pathway in lymphocytes (31–33). However, there is currently little understanding of why some cells (such as T lymphocytes) are sensitive to tryptophan depletion, whereas others such as tumor cells are resistant to conditions of low tryptophan concentration. It is possible that regulation of tryptophan transport accounts for the differential susceptibility to tryptophan depletion between different cell types.

Amino acids are taken up by substrate-specific transmembrane transporters. In mammalian cells, transporter-mediated tryptophan uptake occurs mainly via the ubiquitously expressed, neutral amino acid transporter System L, which transports large hydrophobic amino acids in a sodium-independent manner (34, 35). System L is heterodimeric, composed of a heavy glycoprotein chain (CD98, encoded by SLC3A2), and one of two catalytic L chains LAT1 or LAT2, encoded by SLC7A5 and SLC7A8 genes, respectively (36, 37). System L is inhibited by 2-aminoisobicyclo-(2,2,1)-heptane-2-carboxylic acid (BCH) under sodium-free conditions (34, 38–40). Altered tryptophan uptake into IDO-expressing cells could account for the ability of tumors to overcome the effects of tryptophan shortage. Consistent with this hypothesis, monocytic differentiation into macrophages, which is accompanied by induction of IDO, is associated with the upregulation of a high-affinity tryptophan transporter, in addition to System L (41).

In this article, we demonstrate a link between IDO expression and transporter-mediated tryptophan uptake. Our results indicate that IDO expression, both in mouse and in human tumor cells, results in modified tryptophan uptake through a novel transport system that has functional properties different from those of System L. These findings highlight a mechanism by which IDO+ tumors can survive under conditions of low tryptophan concentrations and may, indeed, contribute to IDO-mediated depletion of tryptophan from the tumor microenvironment.

**Materials and Methods**

**Reagents**

All chemicals including tryptophan, 1-methyl L-tryptophan, and BCH were purchased from Sigma-Aldrich unless otherwise stated. Compounds sparingly soluble in aqueous solution (such as tryptophan) were dissolved in dimethyl sulfoxide before further dilution. [3H]L-tryptophan, [14C]L-histidine, and [14C]L-lysine were obtained from Amersham Biosciences in dimethyl sulfoxide before further dilution. [3H]L-tryptophan, [14C]L-sparingly soluble in aqueous solution (such as tryptophan) were dissolved were purchased from Sigma-Aldrich unless otherwise stated. Compounds including tryptophan, 1-methyl DL-tryptophan, and BCH under sodium-free conditions (34, 38–40).

**Measurement of amino acid uptake**

Cells were cultured for 48 h, then washed and incubated in PBS or choline buffer (pH 7.4, choline chloride, CaCl2, MgCl2, KC1, KH2PO4 [monobasic and dibasic]) for 20–30 min. [3H]L-tryptophan or [14C]L-histidine was added at 50 mmol/ml. After a fixed time at 37°C, uptake was terminated by layering the EG7 cells onto an oil layer before spinning in a microfuge to separate cells from the [3H]-tryptophan-containing solution. Tryptophan uptake by EG7 cells was performed with duplicate samples.

For HeLa cells, assays were performed in 6- or 12-well plates at 90–100% confluence. The uptake at 37°C was stopped by removing the supernatant and adding 20 mmol/l -lysine in ice-cold PBS. The cells were lysed with lysis buffer (1% NaOH, 0.1% SDS). [3H]-tryptophan uptake was determined by liquid scintillation. A time course of [3H]-tryptophan uptake was performed to determine the period for which uptake was linear (initial rate); it showed that uptake increased in a linear fashion over the initial rate. For intracellular detection of IDO protein in transduced EG7 clones, cells were fixed with 2% paraformaldehyde, permeabilized with saponin buffer (10 mmol Hepes, 5% FCS, and 5 mmol/ml saponin), and stained with a rabbit polyclonal anti-mouse IDO Ab (Santa Cruz) and PE-conjugated goat anti-rabbit IgG as a secondary Ab.

**Measurement of IDO enzymatic activity**

Cells were washed twice and resuspended in ice-cold PBS with pepstatin (1 mg/ml) and leupeptin (1 mg/ml). Cells were lysed by repeated freeze/thawing and centrifuged at 4°C for 5 min at 14,000 × g. Lysate was stored at −20°C for use in enzymatic assays. The enzyme assays were initiated at 37°C by mixing equal volumes of lysate and incubation medium (100 mmol potassium phosphate buffer, pH 6.5, 40 mmol ascorbic acid, 20 mmol/methylene blue, 200 U/ml catalase, and different concentrations of 1-tryptophan). The reaction was terminated after 1 h by addition of 30% (v/w) Trichloroacetic acid and the mixture were incubated for a further 30 min at 50°C. After centrifugation, the clear supernatant was injected onto a Spherisorb S5-ODS1 column, 4.6 × 150 mm (Waters, Milford, MA) using an HPLC system consisting of a Krueger pump and variable wavelength detector and a BioTek S650 autosampler. The mobile phase consisted of 40 mmol sodium citrate buffer (pH 2.25), 50% methanol, and 0.4 mmol SDS. Kynurenine and tryptophan were detected at 265- and 280-nm wavelengths, respectively. Concentrations were normalized to the amount of protein in the cell lysate.

**Measurement of tryptophan transport in tumors**

To generate lentiviral vectors encoding murine IDO, we reverse transcribed mouse intestinal mRNA, and we used the cDNA as a template for the amplification using the following forward and reverse primers: 5′-CCTGTACACCCCATGGCACCAGATGAAATTAC-3′ and 5′-AACTC-GAGCTAGGGCAGCTGAGACG-3′.

The PCR product was ligated into the lentivector pH-SIN-BXRES-EM, also containing the cDNA for GFP (42). 293T cells were used to produce viral particles. Control viral particles were also generated using a vector encoding GFP alone. Transduced EG7 cells were sorted on the basis of GFP expression and cloned by limiting dilution. IDO expression in EG7 clones was verified by RT-PCR and flow cytometry.

HeLa cells expressing human IDO were made in a similar manner. Human IDO1 cDNA was amplified from mRNA of IFN-γ-treated TH-1 cells using the following forward and reverse primers: 5′-AATGATCACAGCGGACAGCTGATG-3′ and 5′-GCCGTGACTTAACTCTTCCATCCAAAAGGGATTCC-3′.

IDO1 expression in sorted, GFP+, transduced HeLa cells was verified by RT-PCR. Concentrations of tryptophan and kynurenine were measured in the culture supernatants by HPLC.

**Detection of IDO protein by flow cytometry**

For intracellular detection of IDO protein in transduced EG7 clones, cells were fixed with 2% paraformaldehyde, permeabilized with saponin buffer (10 mmol Hepes, 5% FCS, and 5 mmol/ml saponin), and stained with a rabbit polyclonal anti-mouse IDO Ab (Santa Cruz) and PE-conjugated goat anti-rabbit IgG as a secondary Ab.

**CFSE T cell proliferation**

HeLa cells were cultured for 72 h, and the supernatants were harvested and filtered through a 0.2-μm filter. Flat-bottom plates (96-well) were coated with 5 μg/ml anti-human CD3 and CD28 Abs. PBL were isolated from the blood of healthy volunteers and labeled with 0.5 μM CFSE, resuspended in preconcentrated supernatant, and 1–2 × 10⁵ cells were added to each well. In some wells, the preconditioned supernatant had 25 μmol/l -tryptophan added. After 3–4 d, cells were harvested and stained with an anti-human CD3-allophycocyanin Ab. The number of T cell divisions was determined by flow cytometry, gating on propidium iodide-negative (live) cells. The analyses were performed on duplicate wells.
Results
IDO-dependent modulation of tryptophan uptake by tumor cells

To study the relationship between IDO expression and tryptophan uptake, we first set up an in vitro cellular model for constitutive IDO expression. EG7 tumor cells were transduced with a lentiviral vector encoding mouse IDO with GFP. Wild type (WT) EG7 cells did not express detectable Idol, whereas Idol-transduced EG7 cells expressed Idol at levels comparable with IFN-γ-treated WT EG7 cells (Supplemental Fig. 1A). After cloning Idol-lentivirus–transduced EG7 cells, five clones (clones 7, 12, 23, 25, and 26) were selected, based both on GFP expression (data not shown) and the level of intracellular IDO protein (Supplemental Fig. 1B). IDO protein expression was highest in clone 7 (C7), whereas clones 25 and 26 expressed the lowest amount of IDO. In these clones, the level of IDO expression correlates with that of GFP expression.

Consistent with the level of IDO expression, clone 7 displayed the highest rate of kynurenine production as determined by HPLC, whereas clones 25 and 26 showed the lowest protein (Supplemental Fig. 1C) at different tryptophan concentrations. As a control, EG7 cells were transduced with a lentivirus encoding GFP alone. These cells neither produce Idol transcript nor have IDO activity (data not shown).

Uptake of [3H]tryptophan in sodium-containing and sodium-free buffer showed that tryptophan uptake in EG7 cells was sodium independent (data not shown). Therefore, subsequent experiments were conducted under sodium-free conditions. Initial time-course experiments indicated that the absolute rate of tryptophan uptake was similar in all of the cell types tested and was not influenced by expression of IDO (data not shown).

IDO-dependent tryptophan uptake is not mediated solely by System L

We next compared tryptophan uptake in WT, GFP controls, and IDO+ C7 cells. The effect of unlabeled tryptophan as an inhibitor was compared between the different cell types, and there was no apparent difference between IDO+ and IDO− cells (Fig. 1A). To determine whether tryptophan transport in IDO+, EG7-C7 tumor cells was mediated solely through the ubiquitous System L transporter or whether other transporters were induced, we tested the potent and selective inhibitor of System L, BCH, under in vitro conditions (34, 38–40) (Fig. 1B). High concentrations of BCH completely abolished transporter-mediated [3H]tryptophan uptake in EG7-WT+, GFP+, and IDO+ cells. In contrast, inhibition of tryptophan uptake at lower BCH concentrations was significantly greater in WT and GFP cells than in the IDO+ C7 cells. Using these data, we calculated that the inhibition constants (Ki) for the EG7-GFP and WT cells for BCH were 12 μM, whereas for the IDO-expressing EG7 cells, the Ki was 80 μM, indicating the presence of a BCH-resistant component to tryptophan uptake in IDO+ cells.

We then examined [3H]tryptophan uptake in the presence of a panel of inhibitors shown to discriminate between various members of the LAT transporter family (43). Experiments were performed using a combination of BCH together with an excess of the following inhibitors: methyl-aminosobutyric acid (MeAIB) that inhibits System A transporters such as ATA2; N-ethylmaleimide (NEM) that inhibits LAT2, LAT3, and LAT4; and d-methionine (d-Met) that inhibits LAT1 (43). Use of either NEM or MeAIB with BCH at high concentrations completely inhibited tryptophan uptake in WT and GFP-expressing cells, whereas in IDO+ cells, a component of the uptake was resistant to inhibition (data not shown). In the presence of both BCH and d-Met, inhibition of [3H]tryptophan uptake was complete in both the WT and GFP-transduced cells, whereas a significant proportion of [3H]tryptophan uptake was still present in IDO-expressing cells (data not shown).

We titrated the effect of d- or l-Met on tryptophan uptake in the absence of BCH. Both isomers inhibited >80% of the tryptophan uptake both in WT and GFP-expressing cells at 0.05 mM, whereas uptake in EG7-C7 cells was maintained at values >40% (Fig. 1C, 1D). The Ki for d-Met, calculated based on a single tryptophan uptake system, was ~10-fold higher in the IDO+ cells than the IDO− cells (100–300 and 10–30 μM, respectively). When the Ki were calculated using an equation based on two independent systems with different affinities, the data for the WT and GFP+ cells indicated the presence of a single transporter, whereas IDO+ C7 cells appeared to have more than one tryptophan transporter: the first with the same affinity for d- and l-Met as in the WT and GFP cells, and an additional transporter with a 10-fold lower affinity for d- and l-Met (36).

In conclusion, these data indicate that tryptophan transport in IDO+ cells occurs through two separate mechanisms: the BCH and d-/l-Met–sensitive System L (CD98 and LAT1/2) and an additional transporter that is relatively resistant to BCH and d-/l-Met inhibition. NEM or MeAIB alone, known to inhibit LAT2, LAT3, and LAT4 or System A, respectively, had minimal effect on tryptophan uptake in IDO+ cells (data not shown), suggesting the additional tryptophan uptake observed is unlikely to be mediated via one of the other LATs.

Expression of IDO in HeLa cells inhibits T cell proliferation in vitro

To establish whether results obtained using mouse tumors could be extended to human tumor cells, we generated a lentiviral vector encoding human IDO and used this to transduce HeLa cells. Untransduced HeLa cells were also treated with IFN-γ to induce endogenous IDO expression. RT-PCR and quantitative real-time PCR confirmed the expression of IDO1 mRNA (data not shown), whereas analysis of the culture supernatants confirmed conversion of tryptophan to kynurenine in cultures of IDO1-transduced and IFN-γ–treated HeLa cells, but not with untreated or GFP-transduced cells (Supplemental Fig. 1D). To confirm functional differences between IDO+ and IDO− HeLa cells in their ability to reduce tryptophan levels in tissue culture medium, T cell proliferation in the presence of medium from IDO+ and IDO− HeLa cells was compared (Fig. 2). IDO+ or IDO− HeLa cells were cultured for 72 h in complete MEM in the presence or absence of IFN-γ. PBLs purified from healthy donors and labeled with CFSE were cultured in the preconditioned media in 96-well plates precoated with anti-CD3 and anti-CD28 Abs (Fig. 2A, 2B). T cell proliferation as indicated by CFSE dilution was completely inhibited when cells were cultured in supernatant from IDO+ HeLa cells, whereas they proliferated when cultured in supernatant from WT or GFP cells (Fig. 2A). T cell proliferation was restored by diluting the IDO-conditioned supernatant 1:2 with fresh complete MEM or by reconditioning the MEM with IDO+ cells in the presence of the IDO inhibitor 1-methyl dl-tryptophan (data not shown). Finally, we used preconditioned MEM that was reconstituted with 25 μM l-tryptophan (Fig. 2B). T cell proliferation in conditioned MEM from IDO+ cells reconstituted with tryptophan was restored to the same levels as seen in WT cells (Fig. 2B), despite the presence of kynurenine metabolites, suggesting that depletion of tryptophan is likely to be the mechanism of T cell inhibition in this model.
Expression of human IDO by HeLa cells induces expression of a BCH-resistant tryptophan transporter

Having established that IDO⁺ HeLa cells can deplete tryptophan from tissue culture medium, we sought to determine whether (in line with our previous results with murine cells) in addition to IDO-dependent tryptophan degradation, IDO⁺ HeLa cells could further reduce tryptophan in the medium by upregulating a second tryptophan transporter. To address this possibility, we compared the effects of BCH and d- and l-Met on [³H]tryptophan uptake by HeLa cells (Fig. 3). [³H]tryptophan uptake by WT and GFP-transduced cells was strongly inhibited by BCH (Fig. 3A), d-Met (Fig. 3B), and l-Met (Fig. 3C), but less so by MeAIB or NEM (data not shown). In contrast, each of the three inhibitors was significantly less effective at inhibiting [³H]tryptophan uptake by IDO⁺-transduced cells. Similar effects were seen with cells expressing endogenous IDO in response to IFN-γ treatment. The Km for BCH was calculated to be ~300 and 600 μM for IDO- and IDO⁺-treated cells, respectively, whereas for control cells, it was 12 μM. For d- and l-Met, similar differences in the calculated Km were observed. Similar to murine tumor cells, tryptophan uptake by IDO⁺ HeLa cells and the effects of d-Met were independent of both sodium concentration (Supplemental Fig. 2A–D) and external pH (data not shown).

These data indicate that similar to the results obtained with murine tumor cells, tryptophan uptake by human tumor cells expressing IDO contains two components: one that is probably System L, as defined by its high sensitivity to BCH and d-Met, and a second that is relatively insensitive to either BCH or d-Met. Similar effects were seen when the tumor cells were treated with IFN-γ to induce expression of IDO.

Characterization of the amino acid specificity of the IDO-induced tryptophan transporter

We performed a number of [³H]tryptophan uptake experiments in the presence of competing unlabeled amino acids. Initially, we used an excess concentration of a panel of amino acids including tryptophan, histidine, glycine, and glutamine (Fig. 4A). Unlabeled tryptophan effectively competed for uptake with [³H]tryptophan to a similar extent in all cell types, irrespective of IDO expression. Mediated tryptophan uptake ranged from 80 to >95% in different experiments.

In contrast, 5 mM glycine had no effect on tryptophan uptake in any of the cell types tested. However, with 5 mM histidine or 5 mM glutamine, we noticed a reproducible difference between IDO⁺ and IDO⁻ cells. Tryptophan uptake was completely inhibited in IDO⁺ cells with a high concentration of histidine or glutamine, whereas a proportion of the tryptophan uptake was retained in IDO⁺ HeLa cells (Fig. 4A).

To determine the affinity of the different amino acids for the tryptophan transporters, we used a range of concentrations of tryptophan, glutamine, or histidine. There was little difference between cell types in the inhibition by unlabeled tryptophan at a range of concentrations (Fig. 4B). In contrast, the lower concentrations of glutamine almost completely abrogated tryptophan uptake in WT or GFP-transduced HeLa cells, whereas even at 5 mM, >50% of tryptophan uptake was maintained in IDO⁺ cells (Fig. 4C). A qualitatively similar effect was seen for 5 mM histidine (Fig. 4D). The calculated Km for tryptophan was almost identical in each cell type, whereas for both glutamine and histidine, there was a 10-fold increase in the Km for IDO⁺ cells compared with IDO⁻ controls. These data indicate that tryptophan...
transport occurs through two distinct systems, one of higher and one of lower affinity for histidine, and that there are both glutamine-sensitive and -insensitive components of tryptophan transport. Tryptophan uptake by murine EG7-IDO+ cells was also resistant to inhibition by glutamine, whereas uptake in WT and GFP-transduced cells was inhibited (data not shown).

As a control for nonspecific effects, we measured uptake of other amino acids. We found that tryptophan, histidine, or glutamine inhibited uptake of [14C]histidine, showing that IDO expression did not affect histidine uptake (data not shown).

We also performed experiments using the human prostate adenocarcinoma cell line PC-3 and found that, similar to that seen in HeLa cells, expression of IDO by PC-3 cells either by transfection or indirectly through treatment of the cells with IFN-γ led to the upregulation of a glutamine-resistant tryptophan transport phenotype (Supplemental Fig. 3). These data, together with the mouse EG7 cell data, suggest that induction of such a transporter may be a common response to expression of IDO across different tumor cells, although whether expression is restricted to tumors of certain cellular origins remains to be established.

Using the property of glutamine resistance to functionally characterize the IDO-induced tryptophan transporter in tumor cells demonstrates a high degree of selectivity for aromatic amino acids.

Once we had found that tryptophan uptake by WT and GFP-transduced HeLa cells was almost completely abrogated with
2.5–5 mM unlabeled glutamine, whereas a significant component was resistant to inhibition in IDO+ cells, we screened a panel of representative amino acids, as well as BCH, in the presence of 2.5 mM glutamine for effects on \[^{3}H\]tryptophan (and \[^{14}C\]histidine) uptake (Supplemental Table I).

The first notable observation is that the induced transporter is relatively tryptophan and isomer specific. Tryptophan uptake was reduced to baseline in IDO+ cells (in the presence of 2.5 mM glutamine) by 2 mM unlabeled L-tryptophan, whereas there was minimal inhibition by D-tryptophan. In IFN-\(\gamma\)-treated cells, we observed additional effects on tryptophan uptake in the presence of D-tryptophan (Supplemental Table I), possibly resulting from trans stimulation, the mechanism by which transport of one amino acid stimulates transport of another amino acid, sometimes through secondary or tertiary transporters, as previously described for the cationic CAT family of heterodimeric amino acid transporters (36). It is highly likely that the profile of amino acid transporters present in IFN-\(\gamma\)-treated cells is significantly more complex than those in IDO1-transfected cells, and it is perhaps not surprising that the degree and pattern of trans stimulation by different amino acids is different between the two cell types.

There was no additional inhibition of tryptophan uptake by BCH in IDO+ HeLa cells in the presence of glutamine. Together with the almost complete inhibition in IDO- cells, this indicates that glutamine is likely to inhibit tryptophan uptake via System L. It also confirms that the additional transporter in IDO+ cells has properties distinct from those of System L. Other than L-tryptophan, the only amino acid that had any detectable effect on tryptophan uptake in both IFN-\(\gamma\)-treated and IDO1-transduced HeLa cells was phenylalanine, which inhibited tryptophan uptake by >50%.

We then measured the affinity for tryptophan of the IDO-induced transporter in tumor cells. Tryptophan uptake by WT and GFP-transduced cells was completely abolished in the presence of glutamine alone and was not reduced further by the addition of unlabeled tryptophan. In contrast, in IDO+ tumor cells in the presence of glutamine, a clear reduction in tryptophan uptake was observed as the concentration of unlabeled tryptophan increased (Fig. 5A). The \(K_i\) of the induced glutamine-resistant \[^{3}H\]tryptophan transport in IDO+ tumors was 2.3–4.2 \(\mu M\).

We measured \[^{3}H\]tryptophan uptake in the presence of glutamine with a titration of tyrosine (Fig. 5B), phenylalanine (Fig. 5C), or L-3,4-dihydroxyphenylalanine (L-dopa) (Fig. 5D). Both phenylalanine and L-dopa inhibited the glutamine-insensitive tryptophan flux in IDO+ cells, whereas tyrosine had little additional effect, suggesting that the induced tryptophan transporter has specificity for more hydrophobic, aromatic amino acids, although qualitatively the effect of tryptophan was of significantly higher affinity.

Together, these data indicate that tumor cells can adapt to the relative scarcity of tryptophan in their microenvironment by modifying their capacity for tryptophan uptake by upregulating a second tryptophan transporter. This mechanism ensures sufficient tryptophan is taken up by tumor cells for optimal protein synthesis,
whereas further depleting the tumor microenvironment of tryptophan and thus inhibiting T cell proliferation.

**Discussion**

In this article, we have examined the link between IDO activity and transporter-mediated tryptophan uptake. Our experiments have established that transporter-mediated tryptophan uptake is regulated by the expression of IDO in tumor cells. Our central finding is that IDO activity alters tryptophan uptake in human and mouse tumor cells by inducing a second sodium-independent, glutamine-resistant tryptophan transporter that is distinct from the constitutively expressed System L.

We have shown that the IDO-induced tryptophan transporter in IDO+ EG7 and HeLa cells is tryptophan specific, as the uptake of other amino acids, including lysine and histidine, was similar between IDO+ and IDO− cells. The observation that excess glutamine effectively inhibited tryptophan uptake by IDO+ tumor cells, although only partially inhibiting uptake in their IDO+ counterparts, provided us with a useful tool to dissect the specificity of the induced transporter. These data indicated that the IDO-induced transporter has a high degree of selectivity and a higher affinity for tryptophan than System L, as defined by the observation that [3H]tryptophan uptake in the presence of glutamine is efficiently inhibited by unlabeled tryptophan, and with lower affinity by phenylalanine or L-dopa, but by none of the other amino acids tested.

Upregulation of a highly tryptophan-specific, IDO-induced transporter, as compared with the broader amino acid uptake mediated by System L (44), has two main advantages for IDO+ tumor cells. First, it ensures that tryptophan uptake in an environment depleted of tryptophan is not impaired by the competitive transport of other, more abundant amino acids. Second, it further contributes to the IDO-dependent tryptophan depletion from the tumor microenvironment, hence further impairing T cell proliferation.

Under normal plasma concentrations of tryptophan (∼60 μM) (45), expression of an additional high-affinity transporter would have little effect on the total tryptophan uptake by cells. However, its effect becomes increasingly more important for tryptophan uptake as the local extracellular tryptophan concentration decreases. Previous work has shown that tryptophan depletion to concentrations <10 μM reduces T cell proliferation, whereas at <1 μM, T cell proliferation is completely inhibited (46). We confirmed using an in vitro model that depletion of tryptophan by IDO+ tumor cells inhibited T cell proliferation, and that replacement of fresh tryptophan or inhibition of IDO was sufficient to restore proliferation to normal levels. In the presence of an additional transporter, the permeability for tryptophan entry at low tryptophan concentrations would be substantially greater. It follows that the additional transporter would increase both depletion of local extracellular tryptophan by IDO+ cells and availability of tryptophan for metabolism by IDO+ tumors. Notably, the affinity of tryptophan for the novel transporter (3 μM) falls precisely into the critical concentration range for the inhibition of T cell proliferation (1–10 μM). Interestingly, preliminary data suggest that a glutamine-resistant tryptophan transporter is unlikely to be

**FIGURE 5.** [3H]Tryptophan uptake by IDO+ cells in the presence of unlabeled glutamine is inhibited by a range of aromatic amino acids. A. Unlabeled tryptophan in the presence of 2.5 mM glutamine inhibits tryptophan uptake by IDO+ cells with a Ki of 2.3–3.2 μM. Curve fitting in the presence of glutamine and tryptophan was performed using SigmaPlot. Individual data points are indicated by open (HeLa WT) or filled (GFP) triangles or by open (WT + IFN-γ) or filled circles (IDO). The IDO-induced tryptophan transporter is inhibited by some of the aromatic amino acids and their derivatives. (B) Tyrosine, (C) phenylalanine, and (D) L-Dopa were tested in the presence of 2.5 mM glutamine at a range of concentrations. Plotted data are normalized, and the means of triplicate measurements ± SE are representative of at least two independent experiments. Asterisks indicate statistical significance p < 0.05 as assessed using Student t test. Experiments were performed in 12-well plates.
expressed by activated human T cells because tryptophan uptake in such cells is highly sensitive to inhibition by glutamine (data not shown). It is also known that activation of T cells leads to rapid upregulation of System L (CD98 and LAT1) (47). Indeed, CD98 is widely recognized as a marker of activated T cells.

Our findings and the previously published results indicating the induction of a high-affinity tryptophan transporter in IDO+ macrophages (41) highlight the presence of different IDO-inducible tryptophan transporters in nonmalignant and rapidly proliferating malignant cells, which may be necessary for the different physiological requirements for tryptophan by different cell types. Importantly, we also showed that physiological stimuli capable of inducing IDO expression, such as IFN-γ, also led to a similar modification in the tryptophan transport phenotype. It is interesting to note that in microarray experiments, IFN-γ–treated HeLa cells appear to be highly metabolically active and upregulated, at least at the mRNA level, expression of a large number of transporters for a range of different nutrients (datasets: GSE11299; location available online at: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE11299) (48).

System L comprises an H chain (CD98/4F2hc) paired with one of two catalytic L chains, either LAT1 or LAT2, that define the transport specificity. We demonstrated that none of the components of System L is likely to contribute to formation of the novel IDO-induced transporter because we found no evidence for significantly increased expression of CD98, LAT1, or LAT2 either at RNA or protein levels (data not shown), in addition to the decreased sensitivity to inhibition by BCH or d-Met. We also consider that it is protein levels (data not shown), in addition to the decreased sensitivity to inhibition by BCH or d-Met. We also consider that it is

It is as yet unclear whether additional amino acid transporters are expressed in other IDO+ cells such as matured DC, DC resident in the mesenteric lymph nodes known to constitutively express IDO (28), or TDO+ cells such as those in the liver or in tumor cells expressing IDO2 (57, 58). It is distinctly possible that the activity of other catabolic enzymes such as TDO or DIO2, possibly through localized tryptophan depletion or accumulation of metabolites, may indeed drive modifications in the repertoire of amino acid transporters expressed by certain cell types. The mechanisms of induction of the novel tryptophan transporter and candidate molecules are currently under investigation. However, tryptophan uptake in human placenta (a site of high IDO activity) is mediated by a number of different transporters at different poles of the cell (34). It is also likely that other cell types, such as myeloid-derived suppressor cells, that are expanded during infectious diseases and cancer growth and that suppress T cell proliferation by a combination of arginase and inducible NO synthase expression (59), may require an altered amino acid uptake capacity. Indeed, it has been demonstrated that myeloid-derived suppressor cells can regulate T cell function by depletion of cystine through differential expression of transporters, thereby acting as a sink for cystine in the microenvironment (60).

Finally, our data have potential clinical implications, as identification of small molecule inhibitors of the IDO-dependent tryptophan transporter expressed by tumor cells would be of therapeutic importance, not only to relieve IDO-dependent immunosuppression, but also to enhance the sensitivity of IDO+ tumor cells to tryptophan shortage, slowing down tumor growth. Further experiments are warranted to identify this novel IDO-induced tryptophan transporter, to characterize its expression and assess its potential as a therapeutic target.

Disclosures

The authors have no financial conflicts of interest.

References


SUPPLEMENTARY INFORMATION

Supplementary Figure 1: Isolation of IDO-lentivirus transduced EG7 clones expressing different levels of IDO protein. A. Reverse transcription (RT)-PCR for Ido transcript from WT and IDO lentivirus transduced cells, before limiting dilution (20% GFP positive) and after (100% GFP positive). IFN-γ-treated EG7 cells were used as a positive control. Amplification of β-actin transcript was also used as an internal control. B. Intracellular staining of individual sorted IDO positive clones with an anti-mouse IDO antibody. Flow cytometric analysis showed a hierarchy of the level of IDO protein expression between the individual clones. C. Rate of IDO enzymatic activity in lysates of WT and IDO-lentivirus transduced EG7 clones as measured in cell-lysate based assay. Kynurenine levels were measured in duplicate using an HPLC based detection method. D. WT Hela cells with or without 1000U/ml IFN-γ, GFP-transduced or IDO transduced Hela cells were cultured for 48h. The concentrations of tryptophan and kynurenine (μM) in the supernatant were determined by HPLC, measuring the absorbance at 280nm and 365nm respectively and comparing to standard curves.

Supplementary Figure 2: Tryptophan uptake in Hela cells is sodium-independent while thyroid hormones have little or no effect on 3H-Tryptophan uptake by Hela cells in the presence of 2.5mM glutamine. A. WT, B. WT + IFN-γ, C. Hela-GFP or D. Hela-IDO cells were cultured for 48h before washing with either PBS, sodium-free choline buffer or sodium and chloride-free buffer. 3H-tryptophan uptake was measured over 3 minutes in the indicated buffers either in the absence of any other amino acids (untreated) or in the presence of 2mM unlabelled tryptophan or
1.5mM D-Met to inhibit uptake by System L. $^3$H-tryptophan uptake is sodium independent and is similar across cell types. The difference between IDO positive and negative cells in the presence of D-Met is also observed in the presence or absence of sodium. Plotted data are the means of triplicate measurements ± SE and experiments were performed in 12 well plates. E. $^3$H-tryptophan uptake was measured in IDO, WT + IFN-γ, GFP or Hela WT cells in the presence or absence of 2.5mM unlabeled glutamine and/or 10μM the individual thyroid hormones 3,5,3'-triiodo-L-thyronine (T3), 3,3',5'-triiodo-L-thyronine (rT3), thyroxine (T4) or a thyroid hormone analog triiodothyroacetic acid (TRIAC). Data are shown as the normalized mediated uptake (subtracting the uptake in the presence of 2mM unlabeled tryptophan and are the means of triplicate measurements ± SE. Experiments were performed in 12 well plates.

Supplementary Figure 3: PC-3 prostate cancer cells upregulate a glutamine-resistant tryptophan transporter in response to IDO activity. $^3$H-tryptophan uptake was measured over 3 minutes in IDO, WT + IFN-γ, GFP, GFP +IFN-γ or PC-3 WT cells in the presence or absence of 2.5mM unlabeled glutamine or 2mM unlabeled tryptophan and are the means of triplicate measurements ± SE. Experiments were performed in 12 well plates.

Supplementary Table I. Inhibition of $^3$H-tryptophan uptake by a panel of amino acids and BCH (all at 2mM) in the presence of 2.5mM L-glutamine. Data are shown as the mean of triplicate wells ± SE as normalized (percentage uptake compared with untreated controls) and mediated (subtracting $^3$H-tryptophan uptake in the presence of
2mM unlabelled tryptophan) \( ^3 \)H-tryptophan uptake in the presence of each amino acid or inhibitor indicated.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Hela WT</th>
<th>Hela WT + IFN-( \gamma )</th>
<th>Hela GFP</th>
<th>Hela IDO</th>
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<tr>
<td>Untreated control</td>
<td>100±5</td>
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</tbody>
</table>
Supplementary Figure 1.

A

Ido-lentivirus transduced

WT 20% 100% IFN-γ treated

β-Actin

B

Counts

IDO

C

Kynurenine production

WT (Clone 7)

IDO+ (Clone 12)

IDO+ (Clone 23)

IDO+ (Clone 25)

IDO+ (Clone 26)

Tryptophan concentration µM

D

Tryptophan or Kynurenine (µM)

WT WT + IFN-γ GFP IDO ND
Supplementary Figure 2.

A.

B.

C.

D.

E.
Supplementary Figure 3.