Novel Tryptophan Catabolic Enzyme IDO2 Is the Preferred Biochemical Target of the Antitumor Indoleamine 2,3-Dioxygenase Inhibitory Compound D-1-Methyl-Tryptophan

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Abstract

Small-molecule inhibitors of indoleamine 2,3-dioxygenase (IDO) are currently being translated to clinic for evaluation as cancer therapeutics. One issue related to trials of the clinical lead inhibitor, D-1-methyl-tryptophan (D-1MT), concerns the extent of its biochemical specificity for IDO. Here, we report the discovery of a novel IDO-related tryptophan catabolic enzyme termed IDO2 that is preferentially inhibited by D-1MT. IDO2 is not as widely expressed as IDO but like its relative is also expressed in antigen-presenting dendritic cells where tryptophan catabolism drives immune tolerance. We identified two common genetic polymorphisms in the human gene encoding IDO2 that ablate its enzymatic activity. Like IDO, IDO2 catabolizes tryptophan, triggers phosphorylation of the translation initiation factor eIF2α, and (reported here for the first time) mobilizes translation of LIP, an inhibitory isoform of the immune regulatory transcription factor NF-IL6. Tryptophan restoration switches off this signaling pathway when activated by IDO, but not IDO2, arguing that IDO2 has a distinct signaling role. Our findings have implications for understanding the evolution of tumoral immune tolerance and for interpreting preclinical and clinical responses to D-1MT or other IDO inhibitors being developed to treat cancer, chronic infection, and other diseases. [Cancer Res 2007;67(15):7082–7]

Introduction

Tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO) mediates a protolerogenic mechanism that suppresses T cells, providing balance or feedback control in immune reactions (1, 2). This role for IDO was first established with the demonstration that the specific bioactive IDO inhibitor 1-methyl-tryptophan (1MT; 3) can trigger T cell–mediated rejection of allogeneic mouse concepti (4, 5). More recently, IDO has become recognized as a central mediator of immune tolerance in many settings. In cancer, IDO expression in tumor cells and antigen-presenting cells present in tumor-draining lymph nodes mediates an important mechanism of immune escape (6). IDO inhibitors trigger antitumor immunity (7, 8) and act synergistically with conventional or experimental chemotherapies (9, 10). Based on preclinical efficacy studies, the D stereoisomer of 1MT has emerged as a clinical lead inhibitor that is entering human trials. D-1MT has superior antitumor activity relative to the L stereoisomer in most preclinical models, and IDO is genetically required for the activity of D-1MT (11). However, at the level of biochemical specificity, the distinction between the two isomers is complicated, with the D isomer exhibiting little biochemical activity as an IDO inhibitor relative to L isomer (11). In dendritic cells, both isomers block tryptophan catabolism comparably but the D isomer is again relatively more active biologically (11). Two possible resolutions to this disparity in results are that D-1MT targets either an undefined cellular isoform of IDO, for example, an alternate spliced or modified isoform, or a different target. Here, we corroborate the latter possibility with the discovery of a novel IDO-related enzyme that is a preferential target for biochemical inhibition by D-1MT.

Materials and Methods

All materials and methods are included as online Supplementary Material.

Results

IDO2 is a novel tryptophan-catabolizing enzyme that is preferentially inhibited by D-1MT. We discovered IDO2 by Basic Local Alignment Search Tool searches of the human genome using IDO sequences as probes, identifying a new gene on chromosome 8p12 just downstream of the IDO gene *INDO*. At the time of discovery, genome annotation in this region referred to an anonymous gene termed LOC169355 that was changed later to a misannotated partial gene termed *INDOL1* (IDO like-1; Hs.122077). By trial and error, we identified exons permitting assembly of a full-length IDO-related gene termed *IDO2*. This nomenclature was chosen to distinguish it from *INDOL1*, which remains misannotated as incomplete gene in the database. By homology searching, we also identified the mouse orthologue *Ido2*.

Oligonucleotide primers specific to murine and human coding regions were used to amplify cDNAs by reverse transcription-PCR (RT-PCR) from total RNA isolated from various tissues (Supplementary Figs. S1 and S2). In this manner, we obtained full-length cDNAs with complete coding regions including four alternatively spliced variants of each gene. The primary human transcript is derived from 11 exons encompassing a 74 kb region of chromosome 8p12 (Fig. 1*A* and Supplementary Fig. S3). In three of the five splice isoforms of *IDO2* mRNA we identified, introduction of an out-of-frame stop codon causes a premature truncation of IDO2 protein. Transcripts are initiated only 5 to 7 kb

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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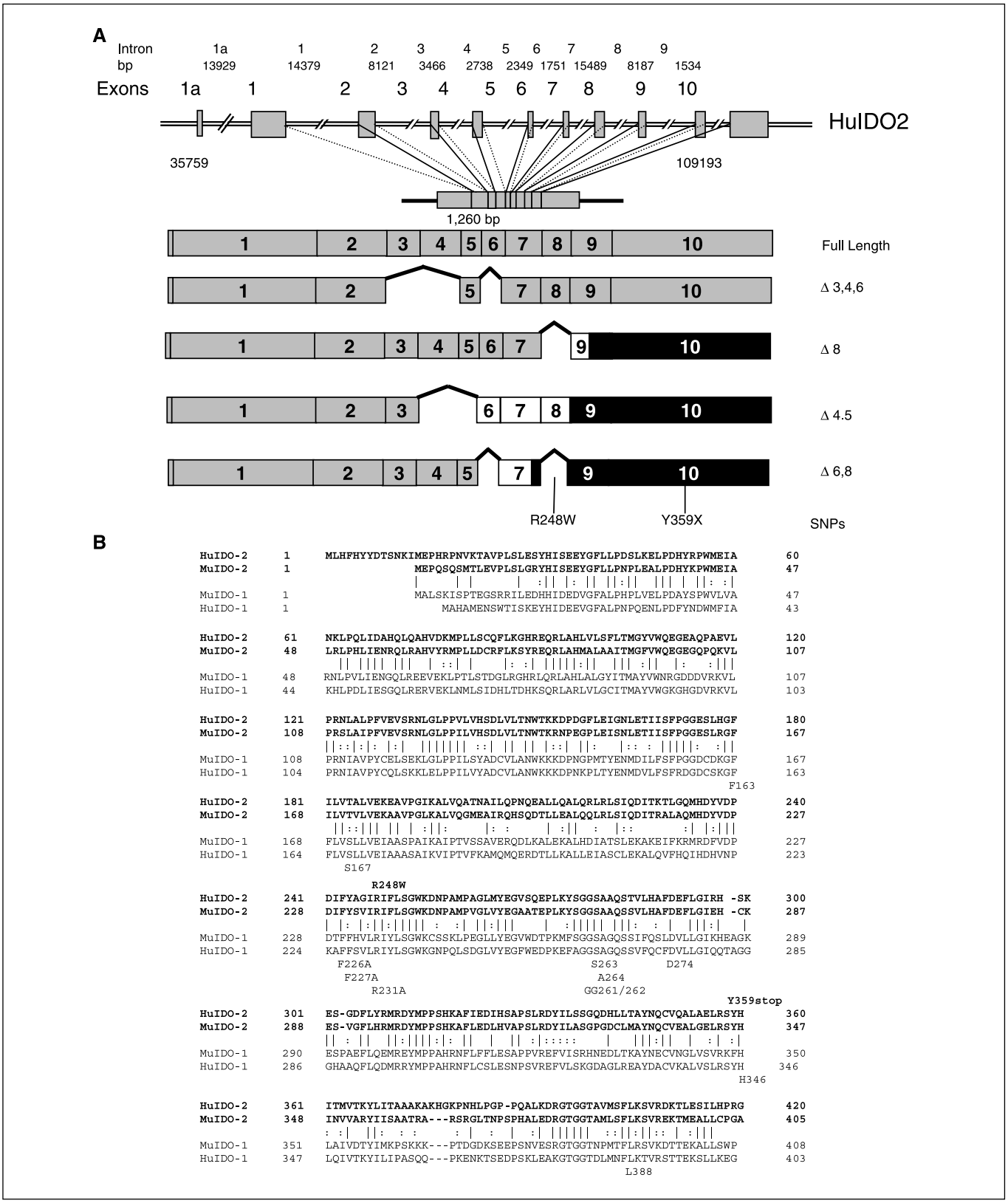

Figure 1. IDO2 structure and similarities to IDO. *A,* structure of human *IDO2* gene and transcripts. Complete coding region is 1,260 bp encoding a 420-amino-acid polypeptide. Alternate splice isoforms lacking the exons indicated are noted. *White boxes,* a frameshift in the coding region to an alternate reading frame leading to termination. *Black boxes, 3′* untranslated regions. Nucleotide numbers, intron sizes, and positioning are based on IDO sequence files NW_923907.1 and GI:89028628 in the Genbank database. *B,* amino acid alignment of IDO and IDO2. Amino acids determined by mutagenesis and the crystal structure of IDO that are critical for catalytic activity are positioned below the human IDO sequence. Two commonly occurring SNPs identified in the coding region of human IDO2 are shown above the sequence that alter a critical amino acid (R248W) or introduce a premature termination codon (Y359stop).

downstream of the *INDO* gene. The mouse gene seems to differ in its lack of the alternate exon 1a found in the human gene; otherwise, exon positions are conserved, indicating gene duplication during evolution of this region of the genome. Human and mouse IDO2 proteins are 420 and 405 amino acids, respectively, and are more conserved (72% identical, 84% similar) than IDO proteins (62% identical, 77% similar). Alignments between IDO and IDO2 sequence reveal highly conserved features that mediate heme and substrate binding (Fig. 1*B*), although the overall level of sequence conservation is not particularly high (43% identical, 63% similar for human). Significantly, residues determined by IDO mutagenesis and crystallographic analysis to be critically important for catalytic activity are highly conserved in IDO2 (Fig. 1*B*).

To confirm the expectation that IDO2 catabolizes tryptophan, we expressed it in a doxycycline-regulated T-REX cell system where formation of the enzymatic product *N*-formyl-kynurenine (Kyn) was monitored. Stable cell lines expressing V5 epitope–tagged or untagged proteins with similar levels of doxycycline-induced expression were used for analysis (Fig. 2*A*; Supplementary Fig. S4). As expected, both human and murine IDO2 catabolized tryptophan effectively as measured by Kyn production (Fig. 2*B*). Based on IDO-IDO2 similarity, we compared the ability of known IDO inhibitors to block the activity of IDO2 in T-REX cells. For reasons mentioned above, the IDO inhibitor D-1MT was of particular interest based on uncertainties about its biochemical target (9, 11). Therefore, we evaluated how IDO1 or IDO2 activity was affected by the D or L stereoisomers of 1MT, or by a third inhibitor MTH-trp (9). Consistent with previous observations (11), we found that IDO activity was modestly inhibited by L-1MT but not D-1MT. In contrast, IDO2 activity was inhibited by D-1MT but not L-1MT. This pattern of inhibition was specific to these 1MT isomers insofar as MTH-trp inhibited the activity of both enzymes (Fig. 2*C*). These results identify IDO2 as a relevant target for biochemical inhibition by D-1MT, which may explain its well-documented antitumor effects.

IDO2 expression is more restricted than IDO but includes dendritic cells. By RT-PCR analysis, we found *IDO2* is expressed in a subset of tissues expressing IDO. Primers spanning the complete human coding region detected full-length mRNAs only in placenta and brain, whereas primers specific to exon 10 (found to be common to all human *IDO2* cDNAs) detected *IDO2* mRNAs in human liver, small intestine, spleen, placenta, thymus, lung, brain, kidney, and colon (Fig. 3*A* and *B*). Although RT-PCR reactions spanning exons 1 to 8 might not have been sensitive enough to detect low-level transcripts, exon 1a–specific primers gave similar results (data not shown), implying that other transcription start

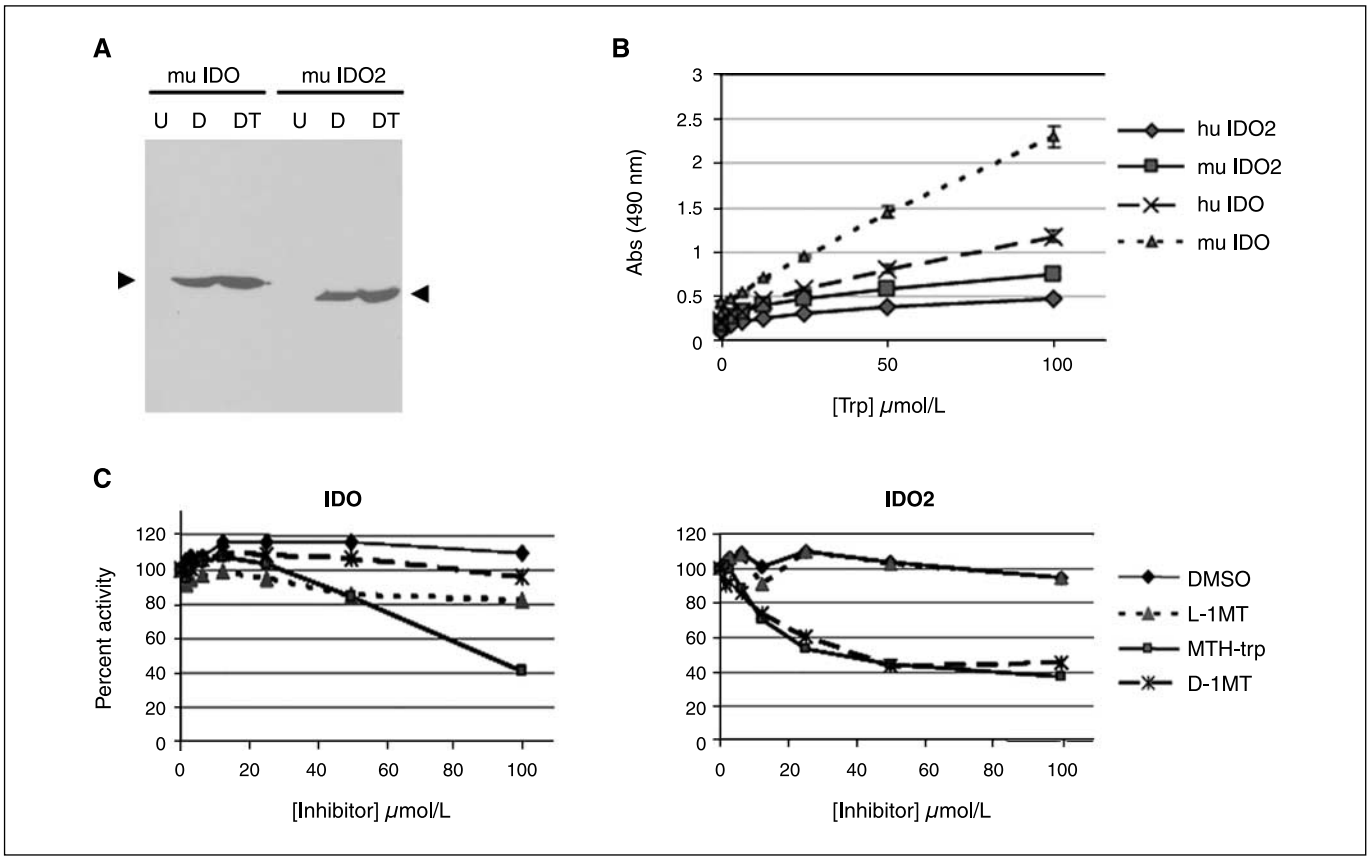

Figure 2. Tryptophan catabolic activity of IDO2 and inhibition by D-1MT. *A,* inducible expression of IDO and IDO2 in representative T-REX cells. Western blot analysis of the V5 epitope–tagged proteins indicated was done with a horseradish peroxidase–conjugated anti-V5 antibody (Invitrogen) in cells that were untreated (*U*), treated with 20 ng/mL doxycycline (*D*), or treated with doxycycline and 100 μmol/L tryptophan (*DT*). *B,* tryptophan catabolism. T-REX cells were seeded at 60% to 70% confluence in 96-well dishes in medium supplemented with 0 to 100 μmol/L tryptophan. Kyn production was determined 48 h later and normalized to protein levels as determined by sulforhodamine B assay. Each enzyme was catalytically active, based on increased Kyn levels with increasing substrate concentrations, although IDO2 seemed to be 2- to 4-fold less active than IDO when normalized to protein levels as determined by sulforhodamine B assay. *Points,* mean of values determined in triplicate and normalized to cellular protein levels. *Abs,* absorbance. *C,* effect of IDO inhibitors on IDO2 catalytic activity. T-REX cells were seeded and processed as above except for the addition to the medium of 0 to 100 μmol/L of the IDO inhibitors MTH-trp, L-1MT, D-1MT, or vehicle control (DMSO). *Points,* mean of values determined in triplicate and normalized to cellular protein levels as before.

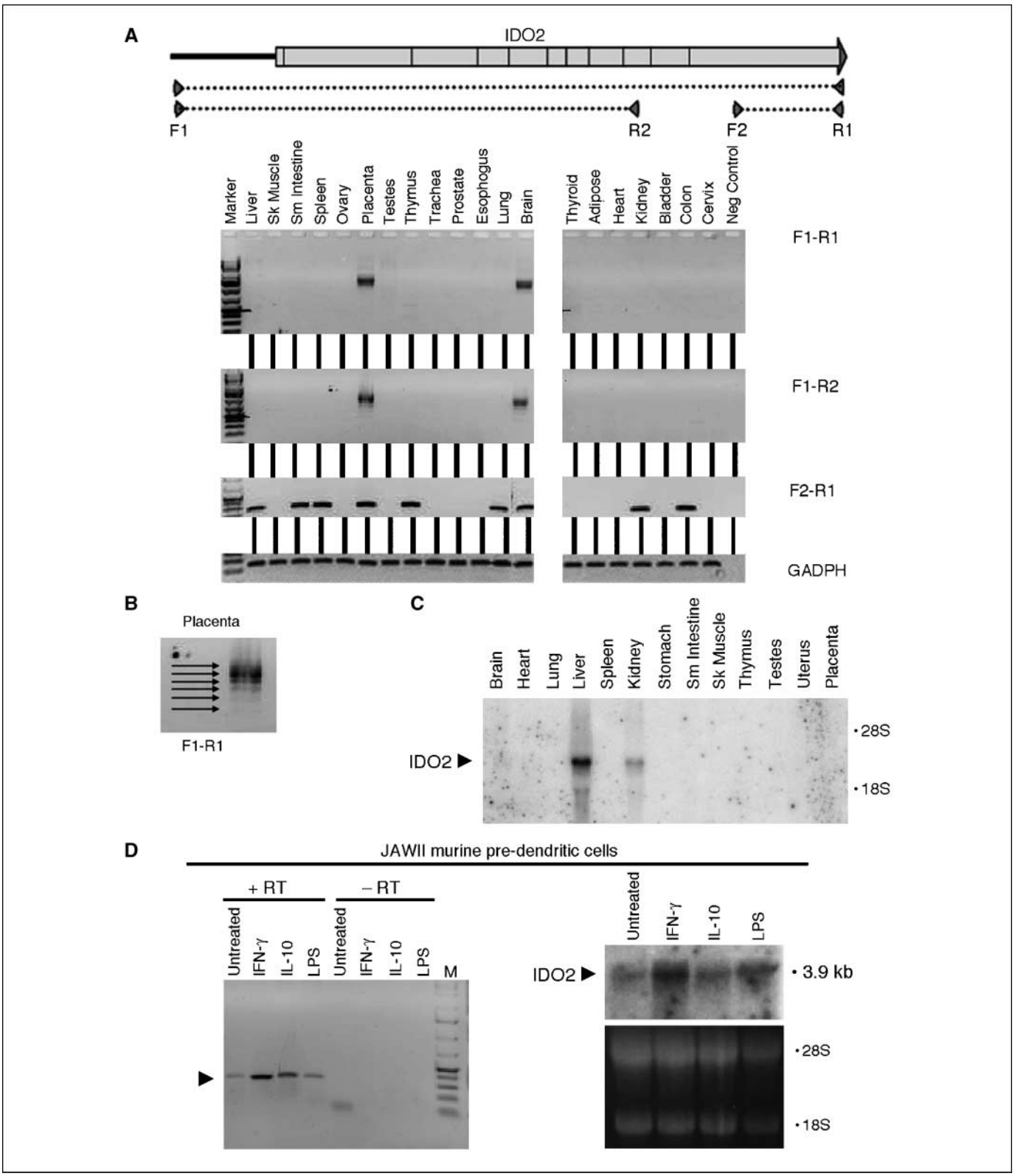

Figure 3. Tissue-specific and dendritic cell expression of IDO2. *A,* human tissues. A panel of total RNAs (Ambion) was analyzed by RT-PCR and agarose gel electrophoresis. *Cartoon above the figure,* location of primer pairs used for RT-PCR as indicated next to the gel photos. Glyceraldehyde-3-phosphate dehydrogenase was used as a positive control. *B,* human placenta. Expression of splice variants characterized as detected by the F1-R1 primer pair spanning the full-length cDNA. A similar pattern of expression was observed with primers extending through exons 8 to 10 as F2-R1. *C,* murine tissues. A commercial Northern blot (Seegene) was hybridized to murine IDO2 cDNA probe before washing and autoradiography using standard methods. *D,* murine JAWII predendritic cells. *Top,* RT-PCR analysis. Total RNA isolated from cells that were unstimulated or stimulated 24 h with IFN-γ, IL-10, or lipopolysaccharide was analyzed using primers F6 and R5 for murine IDO-2 (Supplementary Fig. S2). *Bottom,* Northern analysis. RNAs were fractionated on an agarose gel, blotted to nitrocellulose, and hybridized with a murine IDO2 cDNA probe. *Top,* ethidium-stained gel photograph showing intact 28S and 18S rRNAs. *LPS,* lipopolysaccharide.

sites may exist in human *IDO2*. Northern analysis of mouse tissue RNAs confirmed a more narrow range of expression, revealing detectable *IDO2* transcripts only in liver and kidney (Fig. 3*C*). In a query of the NCBI SAGEmap database with a sequence tag to *IDO2*, the top four hits in terms of tag count prevalence were all identified as bone marrow–derived dendritic cell libraries. Because D-1MT inhibits kynurenine production in dendritic cells and block their ability to activate T cells (11), we examined *IDO2* expression in an established predendritic mouse cell line (JAWII) that matures to dendritic cells after treatment with IFN-γ. *IDO2* mRNA was expressed in unstimulated JAWII cells, and IFN-γ treatment and, to a lesser extent, IL-10 or lipopolysaccharide treatment increased levels modestly (Fig. 3*D*). Using an IDO2-specific monoclonal antibody, we confirmed expression of IDO2 protein in JAWII cells by Western blotting and indirect immunofluorescence microscopy, the latter of which revealed a generally cytoplasmic pattern of expression like IDO (data not shown). Although we could not detect Kyn production in JAWII cells, we confirmed that full-length cDNAs cloned from these cells encoded a fully active enzyme in T-REX cells (data not shown). Together, these observations defined a pattern of expression for IDO2 that includes dendritic cells.

Common genetic polymorphisms in human IDO2 compromise or abolish enzymatic activity. During characterization of *IDO2* cDNAs, we identified two single nucleotide polymorphisms (SNP) that abolished enzymatic activity. One C-T SNP affecting R248 in human IDO2 was structurally analogous to R231 in human IDO, which makes a critical contact with the indole ring of tryptophan (12). The nonsynonymous substitution (R248W) reduced catalytic activity ~90% in T-REX cells (Supplementary Fig. S5). A second T-A SNP affecting Y359 generated a premature stop codon (Y359X), which completely abolished activity (Supplementary Fig. S5). Strikingly, both SNPs were commonly found in human genomic DNAs in public databases, with the C-T SNP being highly represented in individuals of European descent, the T-A SNP being highly represented individuals of Asian descent, and neither SNP being as prevalent in individuals of African descent (Supplementary Fig. S6). Thus, as many as 50% of individuals of European or Asian descent and 25% of individuals of African descent may lack functional IDO2 alleles. This analysis implicates these SNPs as having a broad effect on IDO2 activity in human populations, which may have a significant bearing on the interpretation of clinical responses to drug-like inhibitors of IDO2 like D-1MT.

IDO2 and IDO each activate LIP, an inhibitory isoform of immune regulatory transcription factor NF-IL6, but IDO2 produces a tryptophan-independent signal. Tryptophan catabolism by IDO triggers GCN2-dependent phosphorylation of the translation initiation factor eIF-2α (13). Activation of this pathway inhibits translation of most messages with the exception of certain messages essential for stress-related functions. Additional contributions of IDO to tolerogenesis are imparted by Kyn and other downstream catabolites (14–16). We evaluated the ability of IDO2 to activate this pathway in T-REX cells. In IDO-expressing cells, Kyn production was constant for 4 days postinduction after which cell growth rate slowed appreciably. This effect related to tryptophan depletion rather than Kyn elevation, because supplementing the culture medium with tryptophan rescued the effect (Supplementary Fig. S7). In IDO2-expressing cells, tryptophan consumption was slower such that cell growth was not affected (Supplementary Fig. S7). Nevertheless, induction of IDO2 caused GCN2-dependent phosphorylation of eIF-2α like IDO (data not shown). To compare downstream effects, we examined how IDO or IDO2 activation affected translation of LIP, an inhibitory isoform of the transcription factor NF-IL6/CEPBβ that is up-regulated by amino acid deprivation by a switch to an alternate translational start site (17). Both enzymes up-regulated LIP strongly, however, restoring tryptophan to culture medium reversed LIP induction only when stimulated by IDO (Fig. 4). Thus, IDO2 produced a distinct signal for LIP activation that was independent of tryptophan availability. This signal required catalytic activity because it was inhibited by D-1MT (Fig. 4). These findings implied that IDO2 has a distinct signaling role in cells compared with IDO.

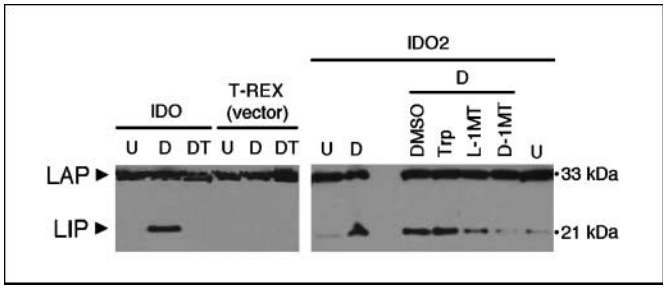

Figure 4. Distinct role of IDO2 in tryptophan catabolic signaling to transcription factor LIP. Western analysis of LIP and LAP isoforms of NF-IL6/CEBPβ was done using lysates isolated from T-REX cells seeded into 12-well dishes that were uninduced (*UI*), treated with 20 ng/mL doxycycline (*Dox*), or treated with doxycycline and 100 μmol/L tryptophan (*Tryp*). In the lanes indicated, cells were also treated with 100 μmol/L L-1MT or D-1MT.

Discussion

The findings of this study are significant and timely regarding how tryptophan catabolism suppresses T-cell immunity, how immune escape evolves during cancer progression, and how the D stereoisomer of the widely studied IDO inhibitor 1MT, presently entering phase I clinical trials, acts to elicit antitumor responses in animals. Given the striking therapeutic effects of D-1MT in preclinical models of cancer and other diseases (6), our findings point to IDO2 as an important therapeutic target and genetic modifier for understanding disease susceptibility. The existence of widely dispersed genetic polymorphisms in human populations that ablate catalytic activity argues that knowing the genetic status of IDO2 of individuals enrolled in D-1MT trials may be important for understanding clinical responses. Given the likelihood that IDO2 may contribute to immune tolerance, two implications are that individuals heterozygous or homozygous for catalytically inactive alleles may be (*a*) less susceptible to developing diseases driven by immune suppression, and (*b*) less susceptible to manifesting clinical responses to D-1MT or other IDO2 inhibitory compounds. Due to deficiencies in IDO2 activity, such individuals may be relatively less prone to immune escape and malignant progression of oncogenically initiated lesions, but relatively more prone to autoimmune disorders. Given differences in the antitumor responses seen in various preclinical cancer models to L-1MT versus D-1MT (11), it may also be interesting to evaluate the murine *IDO2* gene for related polymorphisms.

In LIP, we have defined a novel component of the tryptophan catabolism signaling pathway triggered by IDO or IDO2, using it here to reveal a mechanistic difference in how translational control by these enzymes may modulate immune tolerance. As a downstream reporter, LIP could provide a useful biomarker for genetic and biochemical pathways activated by IDO1 or IDO2 in cells that express

NF-IL6 (also known as CEBPβ). In essence, LIP is a dominant inhibitory isoform composed of only the DNA binding region of NF-IL6 (17). By interfering with target genes that control stress signaling, cell growth, and immune modulation, LIP is well positioned to mediate stable effects of IDO or IDO2 on immune tolerance generated by antigen-presenting cells or other cell types. Using LIP, we found that transient activation of IDO2 generates a stable signal that persists independently of tryptophan availability. The potential significance of this mechanism is that it could be used to propagate tolerance from a local to a peripheral immune environment, away from an initial site of tryptophan catabolism (18), for example, to support cancer metastasis. Differences in LIP response argue that the functions of IDO and IDO2 may be distinct, even if outcomes for eliciting immune tolerance are similar.

IDO2 may address key questions about how 1MT manifests its antitumor activity. Previous studies indicated that D-1MT can inhibit tryptophan catabolism in human dendritic cells and that the *IDO* gene is needed for antitumor activity, implicating IDO in the D-1MT mechanism at some level (11). Our findings do not rule out the possibility that 1MT may target an endogenous IDO protein differing at some level, for example, due to posttranslational modification (11); however, identifying IDO2 addresses a key gap in knowledge concerning the biochemical target of D-1MT. In most models, D-1MT displays much better antitumor activity than L-1MT prompting the choice made for clinical development. One implication is that compounds with dual specificity for IDO and IDO2 may exert more potent antitumor efficacy, and MTH-trp fulfills this expectation (9). Based on genetic knockout studies supporting a role for IDO in the response to D-1MT at some level (11), our findings strongly suggest cross-talk or cooperation between the functions of IDO and IDO2 in immune regulation. Consistent with this idea, IDO activity may be supported by other elements involved in tryptophan catabolism (16, 19). In future work, it will be important to examine IDO-IDO2 cooperation as well as how catabolites of tryptophan catabolism may figure into IDO2 action.

Addendum

Recently we became aware of another group reporting the identification of this gene (20).

Acknowledgments

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We apologize to investigators whose work was not cited due to size restrictions for publication.

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