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IDO inhibits a tryptophan sufficiency signal that stimulates mTOR

A novel IDO effector pathway targeted by *D*-1-methyl-tryptophan

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Tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO) alters inflammation and favors T-cell tolerance in cancer, but the underlying molecular mechanisms remain poorly understood. The integrated stress response kinase GCN2, a sensor of uncharged tRNA that is activated by amino acid deprivation, is recognized as an important effector of the IDO pathway. However, in a mouse model of inflammatory carcinogenesis, ablation of *Gcn2* did not promote resistance against tumor development like the absence of IDO does, implying the existence of additional cancer-relevant pathways that operate downstream of IDO. Addressing this gap in knowledge, we report that the IDO-mediated catabolism of tryptophan also inhibits the immunoregulatory kinases mTOR and PKC-Θ, along with the induction of autophagy. These effects were relieved specifically by tryptophan but also by the experimental agent 1-methyl-*D*-tryptophan (*D*-1MT, also known as NLG8189), the latter of which reversed the inhibitory signals generated by IDO with higher potency. Taken together, our results implicate mTOR and PKC-Θ in IDO-mediated immunosuppressive signaling, and they provide timely insights into the unique mechanism of action of *D*-1MT as compared with traditional biochemical inhibitors of IDO. These findings are important translationally, because they suggest broader clinical uses for *D*-1MT against cancers that overexpress any tryptophan catabolic enzyme (IDO, IDO2 or TDO). Moreover, they define mTOR and PKC-Θ as candidate pharmacodynamic markers for *D*-1MT responses in patients recruited to ongoing phase IB/II cancer trials, addressing a current clinical need.

Introduction

Indoleamine 2,3-dioxygenase (IDO) catalyzes the first rate-limiting step in the breakdown of the essential amino acid tryptophan (Trp). Trp depletion from tissues, as it results from IDO activation, alters inflammation as well as T cell-mediated immune responses implicated in a variety of pathological settings including cancer.[1,2] Indeed, recent genetic studies in murine models of cancer have revealed the nodal importance of IDO in supporting immunoescape, angiogenesis and metastasis.[3] One important aspect of immune regulation by IDO is the programming and expansion of regulatory T cells (Tregs, which essential to generate immune tolerance) from naïve $CD4^+$ T cells and existing Tregs, respectively.[4] IDO has a central role in cancer, especially when chronic inflammation and immunoescape are pivotal to the development or progression of the disease.[3,5] However, neither the molecular basis for the biological effects of IDO nor the mechanism of action of drug-like IDO inhibitors that are now in clinical development for cancer immunochemotherapy are well understood. In particular, greater insights into the mechanism of action of 1-methyl-*D*-tryptophan (*D*-1MT), a lead compound that does not biochemically inhibit IDO but interferes with its function at another level,[6] are needed to fully understand and assess its pharmacodynamics and therapeutic responses in the clinic.

Trp depletion as caused by IDO overactivation leads to an accumulation of uncharged Trp-tRNA in cells. This activates the integrated stress response kinase GCN2, which then phosphorylates and inhibits the translation initiation factor 2α (eIF2α), blocking protein synthesis and arresting cell growth.[7] Notably, the genetic or pharmacological manipulation of GCN2 in T cells phenocopies the effect of IDO-targeted manipulation, thereby modulating T-cell responses to tolerizing signals.[7,8] However, while the crucial effects of IDO on inflammatory carcinogenesis have been demonstrated, the effects of GCN2 deficiency in the same setting have not been investigated.

Recent findings indicate that cells employ different signaling pathways to monitor the depletion or sufficiency of essential amino

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acids such as Trp, integrating this information into cell growth vs. autophagy decisions, which are mainly controlled by the master metabolic regulator mTOR.[9] The signaling mechanisms of amino acid sufficiency are also connected to protein kinase C (PKC-Θ), an isoform of PKC that modulates the ability of the TCR to contribute to activating vs. tolerizing responses to antigens.[10]

In this study, we investigated whether GCN2 is essential for inflammatory carcinogenesis like IDO,[5,11] and we explored the ability of IDO to influence Trp sufficiency signaling by regulating mTOR or PKC-Θ. As part of these studies, we discovered that *D*-1MT acts as a potent Trp mimetic at the level of IDO effectors, offering important insights into the mechanism of action of this experimental clinical agent. Importantly, these findings imply that *D*-1MT may be useful not only to treat cancers that overexpress IDO, but also against cancers that overexpress other enzymes that catabolyze Trp, such as IDO2 or TDO.[12,13]

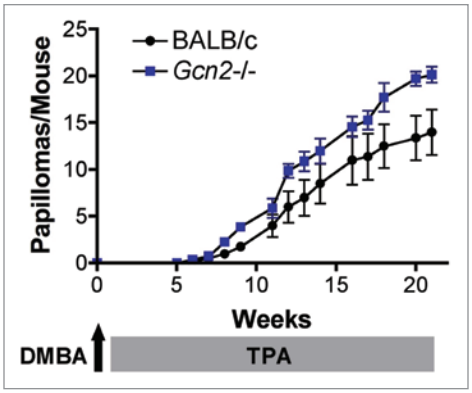

Figure 1. *Gcn2* deficiency in mice does not impede skin carcinogenesis. Wild-type and *Gcn2*$^{-/-}$ BALB/c mice were enrolled to a classical protocol of two-stage skin carcinogenesis and tumor formation was monitored as described.[5,11]

Results

GN2 deficiency does not phenocopy IDO deficiency in suppressing carcinogenesis. Trp depletion by IDO causes an increase of uncharged Trp-tRNA, activating the integrated stress response (ISR) kinase GCN2. GCN2 then phosphorylates eIF2-α and blocks protein synthesis, hence arresting cell growth. This pathway is critical for T-cell suppression by IDO, as the genetic deletion of *Gcn2* abolishes this response.[7] However, the role of GCN2 has not been examined in models in which IDO is crucial for tumor development.[3,5,14] In a classical two-stage mouse model of inflammatory skin carcinogenesis, we found that absence of *Gcn2* does not phenocopy the tumor suppressive effects of the *Ido*$^{-/-}$ genotype[5,11] (**Fig. 1**). This was not a strain-specific effect, because the same effects were observed in C57BL/6J mice when the *Gcn2* null allele was backcrossed from BALB/c mice (data not shown). In this cancer model, results from bone marrow transplantations indicate that the contribution of IDO expressed by hematopoietic cells is less crucial than that of IDO expressed by tumor cells or non-hematopoietic stromal cells.[11] Our results therefore imply that localized Trp deprivation as caused by IDO activation[5] must exert effector mechanisms beyond those transduced by GCN2 that are also essential to support the development of inflammatory cancer.

Trp deprivation by IDO inhibits mTOR and *D*-1MT relieves this event. In exploring other cell signaling pathways that IDO might affect, we considered the mTOR pathway because of its key role as a nutrient sensor that is influenced by amino acid sufficiency.[9] When amino acids are sufficient and the AKT pathway is active, the mTOR complex 1 (mTORC1) becomes active and phosphorylates the translational regulators S6K1 and 4EBP1, stimulating their activity. Thus, in cells experiencing Trp limitation due to the activation of IDO, both GCN2 and mTOR should be affected. However, the impact of IDO activation on mTOR activity has not been examined previously. In addressing this question, we found that the potent IDO inducer interferon-γ (IFNγ) depleted Trp and repressed mTOR activity in human HeLa cells, as monitored by the levels of S6K phosphorylated on T359 (**Fig. 2A**). These changes relied upon Trp depletion, as determined by the ability of Trp-deficient culture media to phenocopy the effects of IDO activation, as well as by the capacity of Trp replenishment to reverse S6K phosphorylation. In a variant of this experiment, we obtained similar results using cell culture media that were deprived of Trp upon the inducible activation of IDO,[15] excluding the possibility that mTOR would be directly affected by IFNγ (data not shown).

Notably, we discovered that *D*-1MT acts as a potent Trp mimetic in relieving such an mTOR-dependent effect (**Fig. 2A**). Indeed, both the *D* and *L* racemic isomers of 1MT could restore mTOR activity after Trp deprivation (**Fig. 2B**). Rapamycin abolished the ability of Trp or *D*-1MT to resuscitate mTOR activity after Trp deprivation, confirming S6K phosphorylation as a reliable readout for mTOR activity (**Fig. 2C**). Pharmacological inhibition of the phosphoinositide-3-kinase (PI3K), which acts upstream of mTOR to license its activity, also abolished the effects of Trp and *D*-1MT on mTOR activity (**Fig. S1**). Titration of *D*-1MT revealed an estimated EC_{50} of ~70 nM (**Fig. 2D**), which is well within the relevant pharmacokinetic range of clinical responses reported for *D*-1MT in Phase I clinical trials.[16] To confirm our results in a primary cell setting, we examined the ability of *D*-1MT to reactivate mTOR after Trp deprivation in primary mouse splenic B cells that were stimulated in vitro with interleukin-4 (IL-4) and lipopolysaccharide (LPS), a treatment that induces both IDO and IDO2 in this setting (data not shown). Confirming our previous observations, upon IDO-mediated Trp deprivation mTOR activity could be restored by either Trp or *D*-1MT (data not shown). Rapamycin abolished these effects, consistent with a specific targeting of mTOR-transduced signals by Trp and *D*-1M. Thus, we concluded that *D*-1MT can serve as a potent mimetic of Trp in its ability to relieve the inhibitory effects of IDO-mediated Trp deprivation on mTOR activity.

***D*-1MT activates a Trp sufficiency signal that stimulates mTOR but does not affect GCN2.** We reasoned that if *D*-1MT stimulated mTOR activity by mimicking a Trp sufficiency signal, then *D*-1MT should be able to restore the activity of mTOR following the deprivation of Trp but not other essential amino acids. This prediction was confirmed with the observation that *D*-1MT

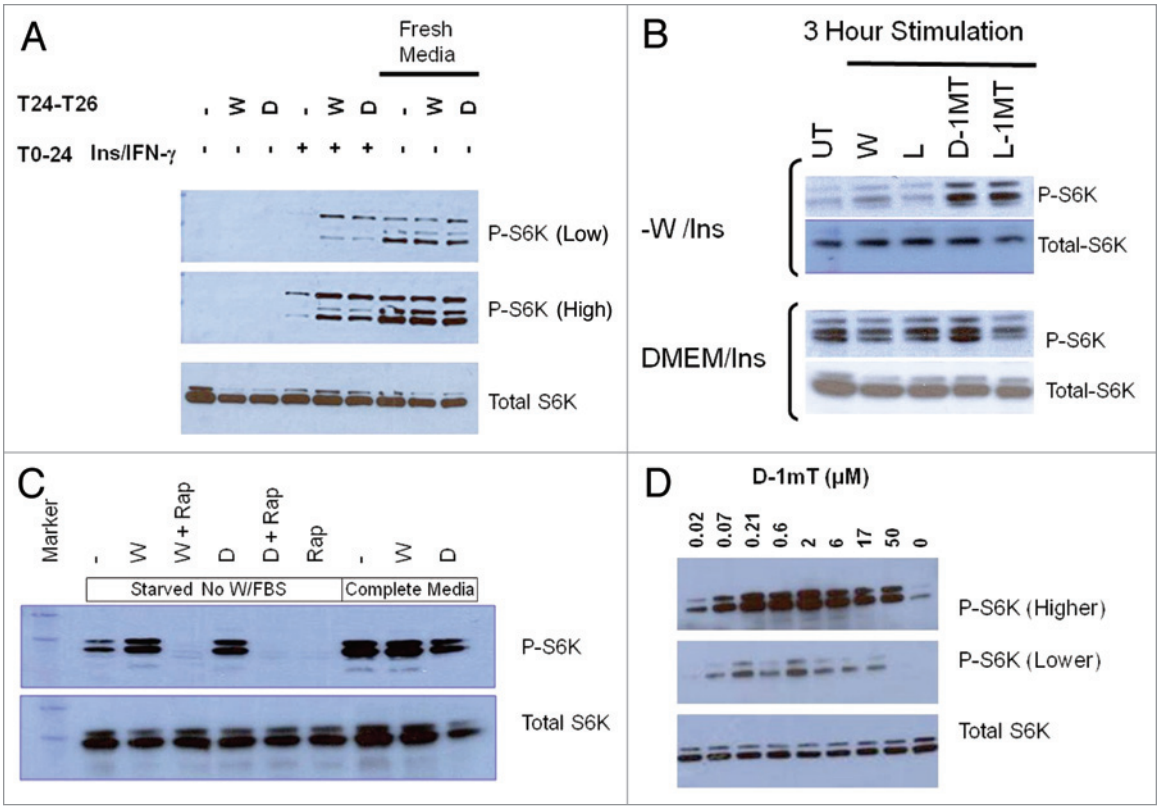

Figure 2. mTOR activity is inhibited by IDO and relieved by either *D*-1MT or L-1MT. mTORC1 activity was monitored by immunoblotting analysis of the steady-state levels of T359-phosphorylated S6K in cells using total S6K levels as a loading control. (**A**) mTORC1 activity inhibited by IDO-mediated Trp deprivation is relieved by Trp or *D*-1MT. HeLa cells were treated 24 h with insulin and interferon (IFN) before the addition of 60 M Trp (W) or *D*-1MT (D), either with or without replenishment of fresh media containing 10% FBS before immunoblotting analysis. (**B**) Both the *D* and *L* isomers of 1MT relieve IDO-mediated inhibition of mTORC1 activity. HeLa cells were cultured 24 h in serum-free medium plus insulin (DMEM/Ins) with or without Trp (-W/Ins), after which cells were left untreated (UT) or treated for 3 h with Trp (W), leucine (L), *D*-1MT or *L*-1MT before immunoblotting analysis. (**C**) Rapamycin abolishes the ability of Trp or *D*-1MT to restore mTOR kinase activity in Trp-starved cells. MCF-7 cells were cultured 24 h in serum-free medium lacking Trp or in medium supplemented with FBS (complete media) and then left untreated (-) or treated with Trp (W) or *D*-1MT in the presence or absence of 1 μM rapamycin (Rap) before immunoblotting analysis. (**D**) *D*-1MT potency in restoring mTOR activity. Trp-starved MCF-7 cells were treated 3 h with *D*-1MT (0–50 μM) prior to immunoblotting analysis. Two different radiographic exposures of phospho-S6K on the same blots are shown (low, high).

could not relieve the effects of leucine, glutamine or arginine deprivation (**Fig. 3A**). The fact that *D*-1MT did not stimulate mTOR activity in leucine-deprived cells is important, because it rules out possible effects related to activation of permeases that are known to transport Trp, leucine and other essential amino acids. Moreover, it directly establishes that the biochemical target of *D*-1MT can specifically bind or sense the levels of Trp and signal them to mTOR.

Further considering the specificity of *D*-1MT in restoring a Trp sufficiency signal that can activate mTOR, we also compared the ability of Trp and the *D* and *L* isomers of 1MT to relieve GCN2 activation in Trp-starved cells, as assessed by monitoring the levels of phosphorylated eIF2α. Strikingly, while Trp itself relieved eIF2α phosphorylation upon Trp deprivation, *L*-1MT had a weak intermediate effect and *D*-1MT had none (**Fig. 3B**). To strengthen the argument that *D*-1MT specifically stimulates mTOR after Trp deprivation, we confirmed that GCN2 is critical for Trp to relieve S6K phosphorylation by repeating the experiment in cells transfected with GCN2-specific siRNA, a condition in which the effects of Trp deprivation on GCN2 were ablated (**Fig. 3C**). Under these conditions, *D*-1MT enhanced mTOR activity, regardless of whether cells expressed normal or reduced levels of GCN2. Similarly, mTOR activity was little affected by Trp deprivation in primary B cells derived from *Gcn2*$^{-/-}$ animals (data not shown). Altogether, these observations indicate that *D*-1MT can mimic the effects of Trp in restoring a Trp sufficiency signal to mTOR, but not the effects of Trp in inhibiting GCN2 as activated by Trp deprivation. In summary, *D*-1MT phenocopies Trp restoration in the mTOR but not in the GCN2 pathway, implying that *D*-1MT counteracts IDO-mediated Trp deprivation by de-repressing the mTOR pathway in a GCN2-independent manner.

Trp deprivation by IDO induces an autophagic response that is relieved by *D*-1MT. Deprivation of an essential amino acid triggers autophagy in an mTOR-dependent manner, and induction of autophagy is reversed by restoring that essential amino acid. Thus, we predicted that the IDO-mediated Trp deprivation would trigger autophagy and that either Trp or *D*-1MT would reverse such an autophagic response, based on their common ability to restore Trp sufficiency signaling in the mTOR pathway. After

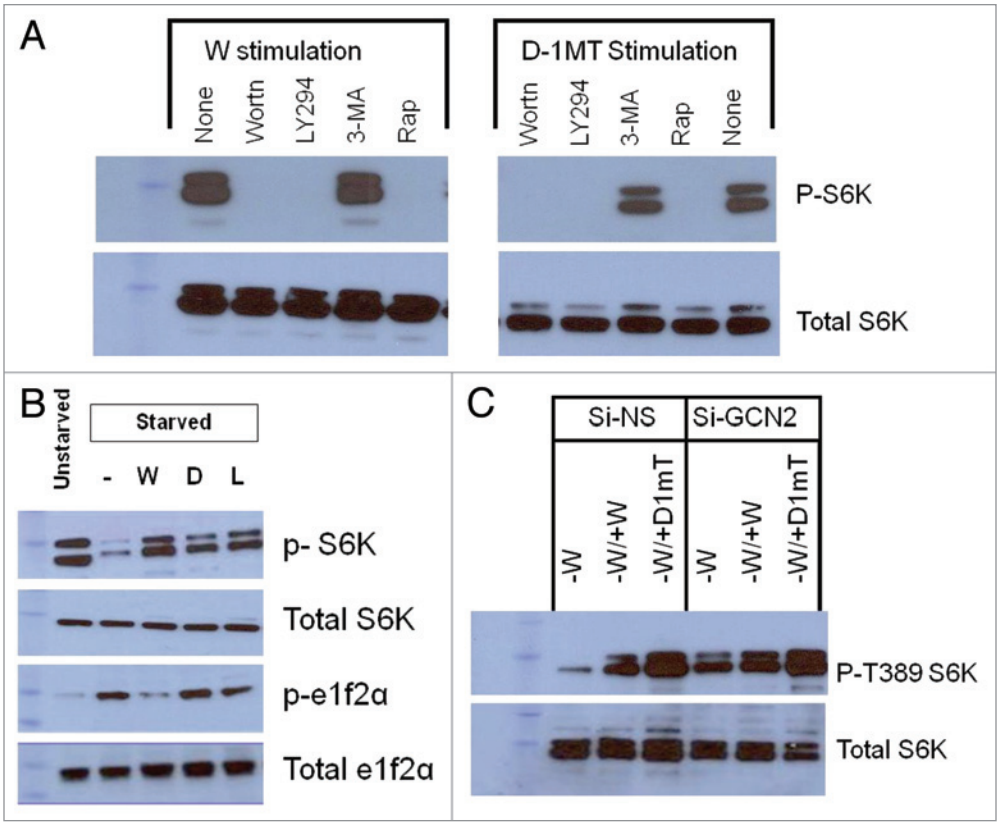

Figure 3. mTOR stimulation by *D*-1MT relies specifically on Trp depletion and not GCN2. (**A**) *D*-1MT specificity. MCF-7 cells were cultured for 24 h in insulin-containing medium deprived of leucine (L), glutamine (Q), arginine (R) or Trp (W) and then left untreated (-) or treated with *D*-1MT or the corresponding deficient amino acid at standard concentrations before harvesting and immunoblotting analysis. (**B**) *D*-1MT does not relieve GCN2 activation after Trp starvation like Trp or *L*-1MT. MCF-7 cells cultured for 24s hr in insulin-containing medium without Trp (Starved) or in complete medium supplemeted with % FBS (Unstarved) were left untreated (-) or treated with 50 μM Trp (W), *D*-1MT (D) or *L*-1MT (L) before harvesting and immunoblotting analysis. Phospho-eIF2α was monitored as a reporter of GCN2 activity after amino acid starvation. *L*-1MT produces an intermediate effect between Trp and *D*-1MT. (**C**) *D*-1MT restores mTOR activation after Trp starvation in a GCN2-independent manner. HeLa cells were transfected with non-specific (Si-NS) or GCN2-specific (Si-GCN2) siRNAs and cells were deprived of Trp for 24 h after, which they were treated 3 h with Trp (-W/W) or *D*-1MT (-W/D1MT), respectively. RNAi-mediated attenuation of GCN2 expression was confirmed by immunoblotting analysis.

IFNγ treatment, HeLa cells expressing the immunofluorescent autophagic marker LC3-GFP exhibited a punctate staining that is indicative of autophagy (**Fig. 4A**). This phenotype was relieved to the same degree by supplementing the cell culture media with either Trp or *D*-1MT (**Fig. 4A**). These results were confirmed by immunoblotting analysis of endogenous LC3, which is lipidated thus acquiring a fast electrophoretic mobility, along with the induction of autophagy. Appearance of the lipidated LC3 isoform LC3-II that was induced directly by Trp starvation was relieved to the same extent by the *L* and *D* isomers of either Trp or 1MT (**Fig. 4B**). Taken together, these results offer a cell biological confirmation of the molecular signaling events described above.

Downregulation of PKC-Θ activity by Trp deprivation and restoration by *D*-1MT. The kinase pathways that signal amino acid sufficiency to mTOR also branch to PKC-θ, a regulator of TCR function.[10] Therefore, we examined whether Trp deprivation and *D*-1MT may also reciprocally influence the phosphorylation status of PKC-Θ, which determines its immunoregulatory role. In the experimental design employed, we transiently expressed a glutathione-S-transferase (GST)-PKC-Θ chimera in human MCF-7 cells, and compared the GST-PKC-Θ phosphorylation status, as monitored by immunoblotting analysis after 24 h of Trp deprivation and 1 h of stimulation with vehicle only, 50 μM Trp or 10 μM *D*-1MT. Antibodies specifically recognizing phosphorylated T538 in PKC-Θ were used to determine the PKC-Θ activation status. We found that the activation level of ectopically expressed GST-PKC-Θ was low in Trp-deprived cells, but both Trp and *D*-1MT could restore activation, with *D*-1MT exhibiting greater potency than Trp (**Fig. 5**), as seen above for mTOR. These results confirmed the expectation that Trp deprivation as triggered by IDO not only affects amino acid insufficiency signals activating GCN2, but also amino acid sufficiency signals needed to sustain the activation of mTOR and PKC-Θ. Further, these results corroborate the concept that *D*-1MT acts as a more potent mimetic of Trp with regard to its ability to restore signaling to mTOR and PKC-Θ in the amino acid sufficiency pathway. In summary, our studies of the effects of Trp catabolism on mTOR and PKC-Θ activation status frame a novel IDO effector pathway that is well suited to help explain the ability of IDO to modulate inflammatory responses and immune tolerance.

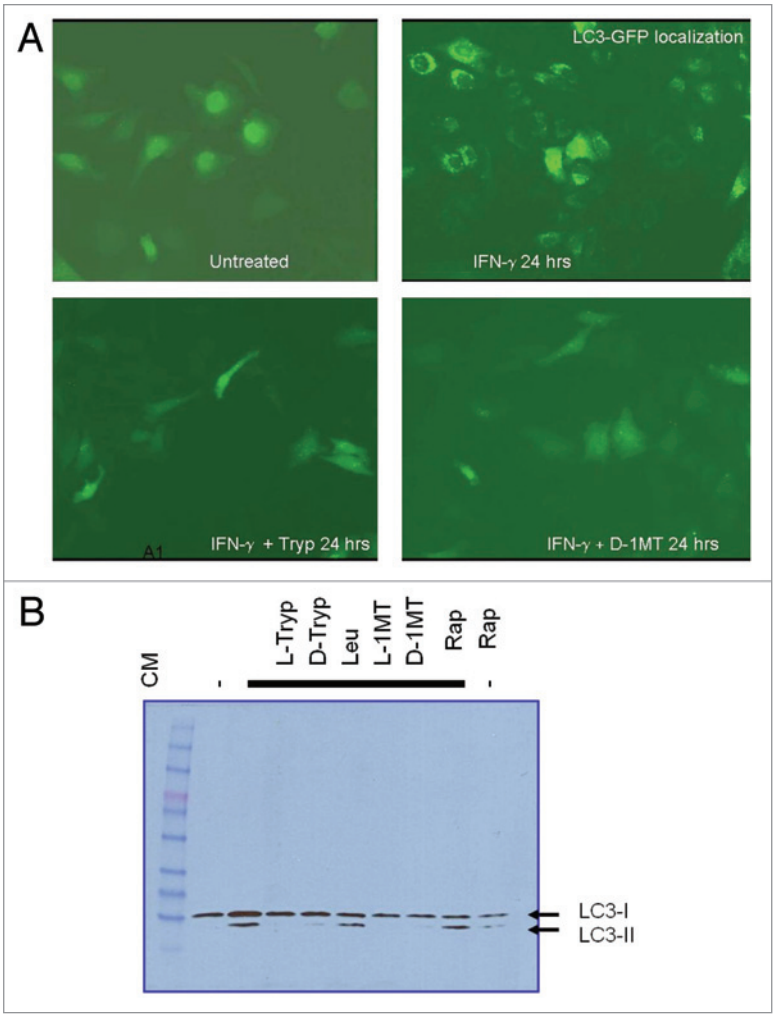

Figure 4. IDO-mediated deprivation of Trp triggers autophagy that is relieved by *D*-1MT. (**A**) Cellular assays. HeLa cells were transfected with a LC3-GFP expression vector, treated as in **Figure 2A** and then subjected to immunofluorescence analysis. The punctate green fluorescence pattern is indicative of autophagic induction (top right panel). IDO stimulated under the experimental conditions is responsible for this induction as established by its abrogation by Trp supplementation (lower left panel). (**B**) Molecular assays. HeLa cells were treated as above and then the cleavage of endogenous LC3 was monitored in whole cell lysates by immunoblotting analysis.

Discussion

Our study identifies an IDO effector pathway that involves a blockade of Trp sufficiency signaling leading to the metabolic regulator mTOR and the TCR regulator PKC-Θ. The finding that IDO can regulate mTOR and autophagy independently from GCN2 control may advance our understanding of IDO function in distinct settings where mTOR acts as a pivotal immunomodulator. By suggesting an analogy between IDO and the mTOR inhibitor rapamycin and revealing how IDO can trigger autophagy to limit T-cell function in the tumor microenvironment, our work offers a novel conceptual perspective on IDO. Moreover, we showed that the IDO pathway inhibitor *D*-1MT acts as a Trp mimetic in regulating mTOR. This finding offers a seminal advance in the understanding of the mechanism of action of this experimental agent, which is currently being investigated in Phase IB/II clinical trials. Additionally, it illustrates the mechanistic distinctions between *D*-1MT and direct enzymatic inhibitors of IDO, the clinical development of which may pose greater safety risks,[17] particularly in the setting of combinatorial therapies, given their general rather than subtle disruption of IDO function. In defining mTOR and PKC-Θ as IDO effector signaling elements, our work also highlights these kinases as candidate pharmacodynamic markers to monitor clinical responses to *D*-1MT or other IDO inhibitors. In this regard, we note that the concentrations at which *D*-1MT affects these key immunoregulatory molecules are consistent with clinical pharmacokinetics data as previously obtained for *D*-1MT in clinical trials.[16]

Integrating our findings with the existing knowledge on IDO signaling, our work supports a model in which IDO coordinately affects pathways of essential amino acid deficiency and sufficiency via GCN2 and mTOR, respectively, in controlling inflammatory responses and immune tolerance (**Fig. 6**). Nutrient sensing processes in mammalian cells involve a set of master regulatory kinases, including the AMP-dependent protein kinase (AMPK), which monitors the levels of ATP (energy), GCN2, which monitors the levels of uncharged tRNA (amino acids), and mTOR, which integrates multiple thread of information to control cell growth and autophagy. Studies in yeast[18] and hepatocytes[19] suggest that the GCN2 and mTOR pathways function in concert. Thus, it has been shown that the deprivation of an essential amino acid can elevate insulin sensitivity through a coordinate activation of GCN2 and repression of mTOR in settings in which AMPK is inactive (i.e., energy is sufficient).[20,21] mTOR receives insulin- or other growth factor-derived signaling information via the PI3K/AKT pathway, with AKT directly phosphorylating and activating mTOR in the rapamycin-sensitive mTORC1 complex and directly phosphorylating and inactivating TSC2 in the mTORC1 repressor complex RHEB/TSC1–2. When activated, mTOR licenses protein synthesis by phosphorylating S6K and other translational regulators, but only if amino acid sufficiency is established by the Ragulator small GTPase complex and other signals needed to recruit mTOR to late stage autophagosomes, where it blocks autophagy. In this way, mTORC1 licenses protein synthesis if AMPK, PI3K/AKT and Ragulator all convey permissive signals.

While it is not yet clear how the mTORC1 complex receives amino acid sufficiency signals, recent work[22] suggests a pivotal role for MAP4K3/GLK1, a kinase that is stimulated by upstream undefined amino acid-binding molecules. MAP4K3/GLK1 would seem to offer a logical effector molecule for IDO, acting upstream of mTOR and PKC-Θ, based on present evidence on its role in regulating amino acid sufficiency signaling.[10,23] In considering direct sensors of Trp that act further upstream of

MAP4K3/GLK, the most logical candidates are the Trp-tRNA synthetases WARS1 and WARS2. This is based not only on the fact that WARSs exert multiple functions[24] but also on the recent striking discovery that the leucine-tRNA synthetase LARS senses branched chain amino acids to control mTOR activation status.[25] In future work, it will be important to establish whether WARS and MAP4K3/GLK are connected to IDO, mTOR and PKC-Θ to fully define this new IDO effector pathway that influences amino acid sufficiency signaling.

IDO-mediated Trp depletion provides an integrated molecular switch to establish an immunosuppressive environment by amplifying tolerogenic antigen-presenting cells (APCs), expanding Treg, downregulating cytotoxic T-cell activity, and sustaining other cells that provide critical support to inflammatory carcinogenesis.[3,11] By analogy to the mTOR inhibitory agent rapamycin, IDO may blunt immune activation and *D*-1MT may re-orient this process by controlling Trp sufficiency signals needed to license mTOR activation, relieve immunosuppression and re-establish pro-inflammatory states. Altogether, these phenomena would limit the progression of cancer or other diseases characterized by disordered inflammation and immunity. Given the implication of all mammalian Trp-catabolizing enzymes IDO, IDO2 and TDO in cancer progression,[1,3,6,12,13,26] our findings will undoubtedly stimulate further investigations into how Trp depletion promotes the immune escape by supporting the development of Treg and myeloid-derived suppressor cells (MDSC) that are important to IDO-mediated cancer progression.[3] While deprivation of any essential amino acid may be sufficient to reorient naïve $CD4^+$ T helper cells to support Treg generation,[27] Trp (as the rarest amino acid) may assume a special position in modulating the GCN2 and mTOR status in the tumor microenvironment. In future work, it will be important to explore in more detail the crosstalk between IDO, the Ragulator complex, MAP4K3/GLK1 and PKC-θ, which all exert major physiological and pathophysiological effects on inflammatory programming and immune control. Given its predominant function in TCR signaling (which has been elucidated only recently), PKC-θ may constitute a notable connection.[28] PKC-θ is dispensable for general T-cell development but critical for Treg development.[29] Its activation relies upon T538 phosphorylation,[30] which occurs only upon stimulation of the TCR along with co-activator signals such as those provided by CD28 ligation. Notably, the kinase responsible for PKC-θ activation is MAP4K3/GLK,[10] which is essential for the differentiation and function of Type 2 T helper (Th2) cells and interleukin-17-secreting T helper (Th17) cells, but not for Type 1 T helper (Th1) cells. In summary, our work supports a role for PKC-Θ function in IDO effector signaling, perhaps through MAP4K/GLK, as a novel potential mechanism for Treg control by IDO-mediated Trp catabolism.

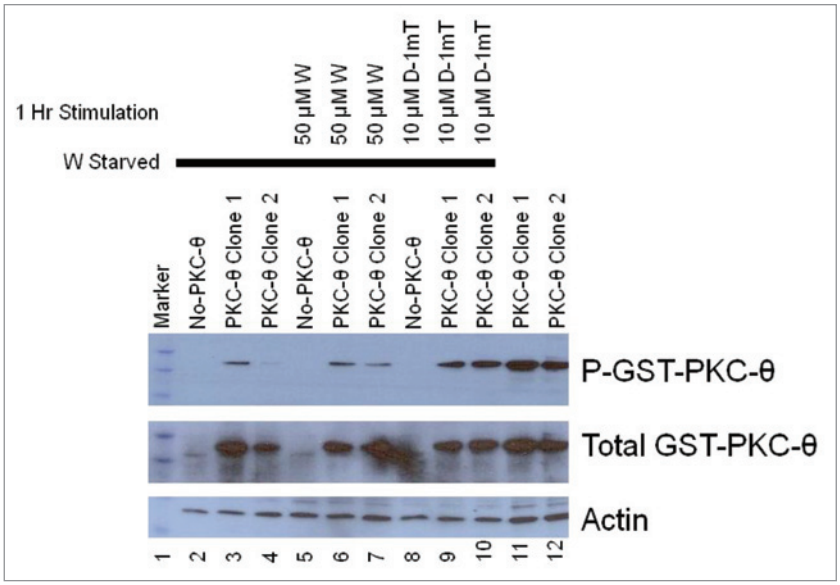

Figure 5. Impact of Trp deprivation and *D*-1MT addition on PKC-Θ activation. MCF-7 cells were transfected with expression vectors for a chimeric GST-PKC-Θ protein or a control GST-encoding vector. One GST and two GST-PKC-Θ transfected cell populations were subjected to analysis. Twenty-four hours after transfection, cells were incubated for additional 24 h in serum-free insulin-containing medium lacking Trp and then left untreated or treated for 1 h with 50 μM Trp (W) or 10 μM *D*-1MT (D) before harvesting for immunoblotting analysis with a antibodies recognizing phospho-T538 PKC- (top panel), total PKC-Θ (middle panel) or actin (bottom panel), the latter as a lane loading control.

Our study also addressed the mechanism of action of *D*-1MT, which so far has been enigmatic. In cells *D*-1MT can selectively blunt IDO2 activity,[15] but this effect may be indirect and cell-type specific.[31] *D*-1MT has been reported to upregulate IDO in cells, but seemingly only at very high concentrations that are irrelevant in vivo.[32] In standard in vitro assays that assess the enzymatic activity of IDO, the 1MT stereoisomer *L*-1MT turned out to be more potent than *D*-1MT, the latter of which exhibits little, if any, inhibitory activity in this setting. In contrast, *D*-1MT clearly relieves IDO-mediated suppression of T-cell proliferation in MLR assays involving IDO^+ plasmacytoid DCs, and it elicits potent biological responses in vivo including superior antitumor effects.[6] Moreover, IDO has been validated genetically as a target of *D*-1MT, based on the loss of *D*-1MT antitumor activity in $Ido^{-/-}$ mice.[6] Questions concerning *D*-1MT as a direct inhibitor of IDO[33] must be tempered by evidence that the in vitro reactions routinely employed to monitor the enzymatic activity of IDO invariably employ non-physiological reductants. This issue is critical, because these reductants exert differential effects on inhibitor binding and activity when compared with physiological reductants (Metz R, DuHadaway JB and Prendergast GC, unpublished observations).

Nonetheless, while it is clear that the physiological relevance of IDO enzymology must investigated further if any of the biochemical inhibitors now entering clinical trials are to be understood at a mechanistic level, our findings may at least offer some insight into why *D*-1MT is superior to *L*-1MT in breaking IDO-dependent immune tolerance in preclinical mouse models of cancer.[6] *D*-1MT had indeed no effects on the activation status of GCN2 in Trp-deprived cells, contrarily to Trp and *L*-1MT,

arguing that *D*-1MT may act exclusively by restoring the mTOR pathway, unlike Trp or *L*-1MT. Mechanistically, we also have preliminary evidence indicating that *L*-1MT but not *D*-1MT can inhibit WARS1A-mediated tRNA aminoacylation (R.M., unpublished observations), explaining why *D*-1MT would not alter the levels of uncharged Trp-tRNA that would be needed to reverse the activation of GCN2 as triggered by IDO-mediated Trp deficiency. The possibility that *L*-1MT inhibits WARS1A activity might also explain why *L*-1MT is inferior to *D*-1MT as an anticancer compound, because WARS1A inhibition would be expected to counteract IDO inhibition by limiting the changes in uncharged Trp-tRNA levels that activate GCN2. Moreover, it should also be taken into account the fact that *L*-1MT can serve as a substrate of IDO, unlike *D*-1MT (M.R.M., unpublished observations). The catabolism of *L*-1MT leads to the production of *N*-methyl-kynurenine, which—by activating the aryl hydrocarbon receptor (AhR) pathway like kynurenine[34]—may actually limit the immunostimulatory effects of *L*-1MT as an IDO inhibitor. Taken together, this information provides a more complete understanding of why *L*-1MT functions as a poor physiological inhibitor of IDO function compared with *D*-1MT and therefore a weaker candidate for clinical exploration.

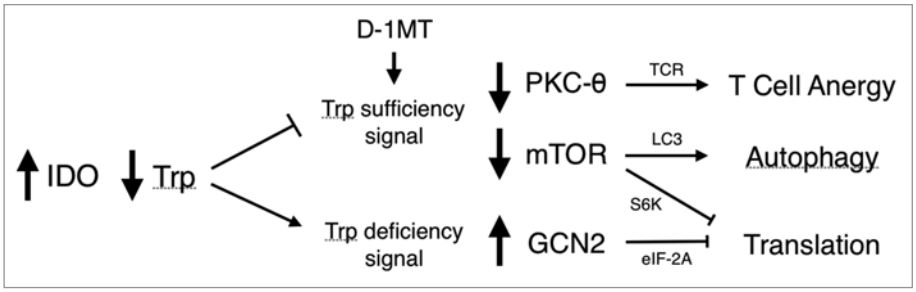

Figure 6. Mechanistic model. Trp deprivation caused by IDO generates signals sensed by distinct amino acid sufficiency and deficiency pathways. Trp deficiency is sensed by the integrated stress kinase GCN2 that inhibits eIF-2α and blocks translation. Through a distinct pathway, the lack of Trp sufficiency causes mTOR to be inactivated, leading to autophagy via LC3 de-repression and translational blockade via S6K inactivation. *D*-1MT acts as a mimetic of Trp in the sufficiency pathway, thereby functionally reversing the effects of IDO on mTOR and autophagy and potentially Treg formation controlled by PKC-Θ.

In elucidating mTOR restoration as a unique aspect of *D*-1MT action, it is intriguing to consider that mammalian cells can sense nanomolar concentrations of a *D* amino acid derivative. *D*-amino acids are irrelevant to mammalian physiology but bacteria use them to dissociate and invade biofilms,[35] a function potentially affecting the physiology of the mammalian immune system and suggesting that *D*-1MT might function as a generalized immune adjuvant.[36] In future work, it will be important to further evaluate whether WARS, like LARS,[25] may not only participate in amino acid sufficiency signaling but also function as an immunomodulator, akin to Toll-like receptors.

Materials and Methods

Reagents. LY294002, wortmannin, Trp, arginine and leucine were purchased from Sigma. Glutamine was obtained from Mediatech. Rapamycin, blasticidin, zeomycin were purchased from Invitrogen. Ampillicin sodium salt (Acros 61177–0250), doxycycline hyclate (Sigma D9891) and methyl-thiohydantoin tryptophan (MTH-Trp, also known as necrostatin 1) were obtained from Biomol. siRNAs were purchased from IDT. Anti-p70S6K, anti-Beclin-1, anti-phospho-eIF2α (Ser51), anti-eIF2α, anti-GCN2, anti-phospho-4E-BP1 (Thr37/46), anti-mTOR, anti-PKC-, anti-phospho-PKC-, anti-phospho-mTOR (Ser2448 or Ser2481), anti-phospho-Raptor (Ser 792), anti-RagC, anti-PRAS40, anti-phospho-PRAS40 (Thr246), anti-IgG HRP and anti-mouse IgG HRP were purchased from Cell Signaling. Anti-PI3K p100 (H-300) and goat anti-VPS39 were purchased from Santa Cruz. Anti-LC3B was obtained from Sigma-Aldrich. PremoAutophaGY and Sensor LC3-GFP wERE purchased from Invitrogen (P36235). An expression vector for the GST-PKC-chimeric protein described[10] was generated by PCR and subcloning into pcDNA4TP (Invitrogen).

Cell culture. MCF-7, HeLa, 293 and 3T3 cells were maintained in DMEM plus 10% fetal bovine serum (FBS) and pen/strep (50 U/mL penicillin, 50 μg/mL streptomycin). For Trp starvation cells were passaged to 60% confluence in wells of a 12-well plate in 1 mL media per well, allowed to attach and recover 24 h, washed three times with PBS and then replenished with 1 mL DMEM lacking arginine, glutamine, leucine and/or Trp supplemented with arginine, glutamine and leucine to normal concentrations such that only Trp was absent. No FBS was added because dialyzed serum contains 1–12 uM Trp bound to serum proteins. To activate the canonical AKT/mTOR pathway media, were supplemented with 1 μM human insulin. Where indicated, IFN was added to HeLa cells to induce IDO or doxycyclin (dox) was added in TRex-293 cells to stimulate a dox-responsive IDO1-pCDNA4TO transgene, as described.[15] Cells under these conditions were typically starved for 18–24 h before stimulation for 3 h with Trp, arginine, glutamine, or leucine as indicated or with complete DMEM including human insulin ± IFN (R&D Systems). Cell proliferation was monitored by sulforhodamine B assays performed in 96-well plates with absorbance (510 nm) read after cell staining on a plate reader and data collected and analyzed using Excel software (Microsoft). Mature naïve B cells isolated by magnetic depletion of mouse total splenocytes with anti-CD43 (Miltenyl Biotech) were seeded into multiwell dishes at 1×10^6 cells/well in RPMI 1640 medium (Gibco) with or without Trp supplemented with 10% dialyzed FBS (Hyclone), 2 mM glutamine, 50 U/mL penicillin, 50 μg/mL streptomycin, 50 μM mercaptoethanol, 25 mM 1× non-essential amino acids and 1 mM sodium pyruvate. After overnight incubation cells were stimulated where indicated with lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 (Sigma), mIL-4 (R&D Systems) or Fab_2 goat anti-mouse IgM (2 μg/mL) and maintained 2–3 d prior to harvesting. Blast formation induced under these conditions was observed

microscopically. Transfections of HeLa or MCF7 cells with siRNA or plasmids were performed by mixing with Lipofectamine 2000 (Invitrogen) for 20 min at room temperature before addition to cell culture media 24–48 h, following the vendor's instructions.

Immunoblotting studies. Cells were washed 2× with ice-cold PBS before whole cell lysates were prepared by the addition of lysis buffer [1× PBS, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 1 mM PMSF, 1 mM Na_3VO_4, 10 mM E-64 (Sigma), and protease and phosphatase inhibitor cocktails (Calbiochem)]. Total protein concentrations were determined using the BCA Protein Assay Reagent Kit (Pierce). SDS-PAGE proteins were electroblotted to nitrocellulose or PVDF membranes and stained with Ponceau S to assure equal protein loading and transfer of each gel lane. Membranes were blocked in Tris-buffered saline containing 0.1% Tween 20 (TBST), 5% BSA or powdered milk and probed with specific antibodies 1 h at RT or overnight at 4°C followed by secondary horseradish peroxidase-conjugated antibodies under the same conditions. ECL reagents (Pierce) were used for developing blots by chemiluminescence.

Disclosure of Potential Conflicts of Interest

A conflict of interest for several authors is stated based on their relationship with New Link Genetics Corporation, reflecting inventorship in IDO technology licensed for clinical development by the company from the authors' institutions, including as compensated scientific advisors and grant recipients (D.H.M., G.C.P.) and equity shareholders (J.B.D., D.H.M., G.C.P.).

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Supplemental Material

Supplemental material may be found here:
www.landesbioscience.com/journals/oncoimmunology/article/21716

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