Chapter 11
IDO in Inflammatory Programming and Immune Suppression in Cancer

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Abstract Tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO) contributes to immune tolerance and inflammatory programming in a variety of tissue microenvironments. In cancer, IDO is overexpressed in both tumor cells and stromal cells where it promotes malignant development and progression by sustaining supportive inflammatory processes and engendering tolerance to tumor antigens. Genetic and pharmacological studies in mice indicate that IDO activity is crucial for cancer development and progression, particularly in settings where inflammatory drivers are essential. IDO is critical for myeloid suppressor functions that contribute to angiogenesis and metastasis. Mechanistic investigations have defined the aryl hydrocarbon receptor (AhR), the master metabolic regulator mammalian target of rapamycin complex 1 (mTORC1), and the stress kinase general control non-repressed 2 (GCN2) as key effector signaling targets of IDO, which also displays a non-catalytic function in transforming growth factor beta (TGF-β) signaling. Small-molecule inhibitors of IDO exhibit anticancer activity and cooperate with radiotherapy, immunotherapy, or chemotherapy to trigger regression of aggressive tumors otherwise largely resistant to treatment. IDO inhibitors that block catalytic activity or selectively reverse IDO-mediated suppression of mTORC1 are being evaluated now in clinical trials. Interestingly, the dramatic antitumor activity of certain targeted therapeutics such as imatinib can be traced, in part, to IDO downregulation. After presenting a historical background on its discovery and early study, this chapter focuses on work that defines IDO as an important mediator of pathogenic inflammation in cancer and summarizes the development of IDO inhibitors as potential anticancer modalities.

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1 Introduction

Advanced metastatic cancer remains a challenge to chemotherapy and other systemic modalities, which provide only limited benefit to ∼50 % of cancer patients who present with advanced disease at diagnosis in developed countries. Similarly, current regimens ultimately fail patients that relapse with disseminated disease following the initial treatment of primary tumors. Tumors display immunogenic antigens but escape immune rejection, somehow evading, subverting, or perhaps reprogramming the immune system for their own benefit. While it has become clear that immune escape is central to the development of a clinically relevant cancer, the basis of this phenomenon remains relatively poorly understood, in part because its role as a critical trait of cancer was not fully appreciated by cancer geneticists until recently [1]–[3].

While an appropriately activated immune system can eradicate cancer, even when it is aggressive and disseminated, spontaneous occurrences of such events in humans are rare. Cancer immunology is one of the oldest parts of the field of cancer research, and during the past century numerous kinds of molecule and cell-based immunotherapy strategies aimed at stimulating an antitumor immune response have been explored. In recent decades, investigators focused on active immune therapies tested many cytokines, tumor-associated antigen peptide vaccines, dendritic cell (DC) vaccines, or adoptive transfers of tumor antigen-specific effector T cells expanded ex vivo from cancer patients [4]–[10]. In contrast to passive immunotherapies, which mainly involve the administration of targeted antibodies, these active immunotherapies are based conceptually on stimulating components of host immunity to elicit an effective response against cancer cells. Having mainly failed historically to generate broadly effective responses, it has become clear that this type of approach is insufficient to overcome tumoral immune suppression and escape mechanisms, which are based upon the dominance of pathological immune tolerance in cancer patients as proposed [12]. While not all escape mechanisms involve an active principle of immune tolerance, as such mechanisms have been defined, it has become increasingly clear that their disruption is important to license the efficacy of active immunotherapies which have failed over the years. In short, to "get on the gas" of immune activation against tumors, it is clear that it is necessary to "get off the brakes" of tumor-associated immune suppression.

Since 2000, there have been rapid advances in understanding how tumors escape the immune system [11], [13]. Intriguingly, it appears that many immune escape mechanisms are configured as active immune suppression by the tumor or stromal cells under the influence of the tumor, implying that continuous activity from the escape mechanism is required. Further, it has been clear that disrupting these active

mechanisms of immune suppression can de-repress (activate) the immune system, enabling it to attack the tumor. Such mechanisms may offer particularly attractive targets for therapeutic intervention with small-molecule drugs [14], which have distinct advantages over biological agents that are currently the norm for immunotherapeutic strategies. Of the mechanisms which have been described to date, one with considerable practical appeal involves the tryptophan-catabolizing enzyme, indoleamine 2,3-dioxygenase (IDO) [15].

2 Background on IDO and Its Recently Discovered Relative IDO2

2.1 Historical Perspective

While most early studies of IDO did not relate to cancer, the discovery of this enzyme was rooted in initial observations made in the 1950s in cancer patients where tryptophan catabolism was found to be elevated [16]. Later studies extended these findings with observations that tryptophan catabolites are elevated in the urine of patients with a variety of malignancies including leukemia, Hodgkin's disease, prostate cancer, and breast cancer [17]–[22]. The hepatic enzyme tryptophan dioxygenase (TDO2), which was the first inducible mammalian enzyme ever to be isolated, had been known since the 1930s to initiate the metabolism of dietary tryptophan [23], [24]. However, no increase in TDO2 activity was detected in cancer patients who presented with elevated tryptophan catabolites [25], implying the existence of a second enzyme.

The extrahepatic tryptophan-catabolizing enzyme termed IDO (aka IDO1; originally D-tryptophan pyrrolase) was first isolated in 1963 [26], [27]. Notably, while IDO catalyzes the same reaction as its hepatic relative TDO2—the conversion of tryptophan to N-formyl-kynurenine—these two enzymes are otherwise remarkably dissimilar [28]. Whereas active TDO2 is a homotetramer of 320 kD, IDO is a monomeric enzyme of 41 kD that is antigenically distinct from TDO2 [29] and lacking in amino acid sequence similarity. Additionally, IDO has less stringent substrate specificity, cleaving a number of indole-containing compounds not recognized by the hepatic enzyme. Lastly, while both enzymes contain heme, IDO utilizes superoxide anion for activity whereas TDO2 does not use superoxide to donate oxygen in the tryptophan catabolic reaction.

Structural and enzymological studies have revealed several interesting features about IDO. Enzymological studies indicate that an electron donor such as methylene blue is critical to achieve full activity in vitro, a role that in vivo is thought to be assumed by tetrahydrobiopterin or flavin cofactors. The binding site on the enzyme for the putative cofactor is distinct from the substrate-binding site [30], implying the potential for allosteric regulation and possibly opportunities for developing non-competitive enzymatic inhibitors (in addition to the more classical substrate-competitive inhibitors). Crystallographic studies of human IDO reveal a two-domain structure of alpha-helical domains with the heme group located in

between [31]. Notably, these findings suggest that strict shape requirements in the catalytic site are required, not for substrate binding but instead for abstraction of a proton from the substrate by iron-bound dioxygen in the first step of the reaction [31]. This detail of the reaction mechanism is important because it is distinct from that used by other monooxygenases (e.g., cytochrome P450), filling a gap in understanding of heme chemistry. In terms of small-molecule inhibitor development, the biochemical differences that distinguish IDO from TDO2 and other monooxygenases are useful because they increase the likelihood of identifying IDO-specific inhibitors.

2.2 IDO: Function in Immune Modulation

In contrast to the biochemical and genetic knowledge about IDO that accumulated relatively quickly in the years since its discovery, a precise understanding of its physiological function remained obscure due to the fact that mammals mostly salvage rather than synthesize nicotinamide adenine dinucleotide (NAD) to meet their metabolic needs. Why then was IDO evolutionarily conserved in mammals? Initial clues as to its function were suggested in the late 1970s by findings from Hayaishi and his colleagues that IDO expression was strongly stimulated in the lungs of mice by viral infection, or exposure to bacterial lipopolysaccharide (LPS) or interferon-γ (IFN-γ) [32]. These findings prompted the interpretation that elevated tryptophan catabolism by IDO at sites of inflammation might provide an antimicrobial benefit. Given the antitumor properties of IFN-γ, this concept was extended to encompass the notion that IDO acted functionally in the manner of a tumor suppressor, contributing to the antitumor effects of IFN-γ activity by starving growing tumor cells of tryptophan [33].

It was not until the late 1990s that a conceptual breakthrough emerged from work by Munn, Mellor, and their colleagues, establishing the possibility that IDO might mediate an immunosuppressive function based on the preferential sensitivity of T cells to tryptophan deprivation. In this radical reconceptualization of the biological role of IDO-based metabolic activity, impaired antigen-dependent T-cell activation occurs in microenvironments where IDO activation results in reduced tryptophan levels [34], [35]. The ability of IDO to promote immune tolerance to "foreign" antigens was supported by the evidence that the specific bioactive IDO inhibitor 1-methyl-tryptophan (1MT) [36] could elicit major histocompatibility complex (MHC)-restricted T-cell-mediated rejection of allogeneic mouse concepti [37], [38]. In cancer, these findings implied that IDO could be prooncogenic by limiting the eradication of tumor cells that occurs through immune-based recognition of "foreign" tumor antigens.

In the past few years, the concept that tryptophan catabolism regulates T-cell immunity has now been corroborated in many laboratories, with regulatory functions identified for both tryptophan depletion and the production of downstream catabolites. In particular, there has been a keen focus on the immune regulatory role of IDO expressed in DCs, an important class of "professional" antigen-presenting cells

(APCs). IDO expression in a small minority population of DCs enables them to dominantly suppress the activation of T cells that occurs through antigen presentation [39], [40]. Tryptophan depletion has been shown to promote T-cell anergy by signaling through the integrated stress-response kinase general control non-repressed 2 (GCN2), which is also required for IDO-induced differentiation of $CD4^+$ T cells into T regulatory (Treg) cells [41]. Likewise, tryptophan catabolites can block T-cell activation and trigger T-cell apoptosis while also promoting the emergence of Treg cells through a transforming growth factor beta (TGF-β)-dependent mechanism, and evidence of synergistic consequences of both depleting tryptophan and elevating tryptophan catabolites have been described [42]. IDO has been implicated widely in cancer, chronic viral infections, allergies, and various autoimmune and inflammatory disorders where immune control is disordered [43].

2.3 IDO2: Discovery and Distinctions from IDO

Mammalian genomes include not only the IDO-encoding gene *IDO1* but also a more recently identified relative termed *IDO2* [44], [45]. Human *IDO1*, located at 8p12–11, comprises 10 exons spanning ~15 kb that encode a 403-amino-acid polypeptide of ~41 kD [46], [47]. Mouse *Ido1* is syntenic and similar in its genomic organization; however, the gene diverges somewhat at the primary sequence level from human *IDO1*, sharing only 63 % identity. The likely existence of a related *IDO2* gene became apparent to us while inspecting sequences immediately downstream of *IDO1* in the human genome [45]. At the time, the genome database in that region was erroneously annotated, referring to a set of partial *IDO1*-related sequences by the anonymous nomenclature *LOC169355*. Correction of the erroneous annotation by trial-and-error exon searches revealed the presence of a 420-amino-acid open-reading frame (ORF) that is 44 % identical to IDO at the primary sequence level. The protein encoded by the *IDO2* ORF conserves all the residues in IDO that have been defined as critical for tryptophan binding and catabolism [31]. The IDO2 proteins in mouse and human are more closely conserved than the mouse and human IDO proteins, displaying 73 % identity at the primary sequence level. The presence of the two IDO-related proteins in such close proximity is likely the result of a gene duplication event, and phylogenetic analysis has been interpreted to indicate that *IDO2* may actually be the ancestral gene [48]. As in the human genome, the mouse *Ido2* gene is located immediately downstream of *Ido1*. Expression of *IDO2* message was detected in a more limited range of tissues than *IDO1* [45]. At the cellular level, evaluation of the National Center for Biotechnology Information (NCBI) SAGEmap database identified the top hits for *IDO2* expression to be bone marrow-derived DCs [45], which is intriguing given the evidence that IDO-based activity profoundly influences the immunogenic nature of DCs.

Most of the signaling and mechanistic data surrounding the IDO proteins have come from studies of IDO and not the more recently identified IDO2. Due to the more restricted localization of IDO2 compared to IDO, it is conjectured that these two molecules do not serve a redundant function. This view is supported by a divergence

in signaling between these two molecules through the integrated stress-response pathway. Local tryptophan depletion due to IDO activity engages this pathway resulting in the elevated expression of liver inhibitory protein (LIP) [45], a truncated isoform of the transcription factor nuclear factor interleukin 6/CCAAT-enhancer-binding protein β (NF-IL6/CEBPβ), which alters the expression of key immune modulatory factors including IL-6, TGF-β, and IL-10. Supplementing with additional tryptophan after depletion quickly abolished the LIP response induced by IDO but not by IDO2 [45]. Thus, after IDO2 induction, LIP expression is maintained in a tryptophan-independent manner, indicating a stable effect of tryptophan catabolic signaling unique to IDO2. While the significance of this distinction has yet to be evaluated in vivo, one implication is that IDO2 might differ from IDO in its ability to transmit a stable immune regulatory signal. LIP-mediated signaling initiated by IDO2 could alter distal immunity, since the signal could persist in microenvironments where tryptophan levels are normal. Alternately, IDO2 might produce a stable differentiation signal. Intriguingly, the IDO2 gene is regulated in DCs by activation of the aryl hydrocarbon receptor (AhR) [49], which as discussed further below has been identified recently as a receptor for kynurenine [50], the product of tryptophan catabolism by IDO or IDO2. Along with other evidence linking AhR and kynurenine in immune control [51], these connections hint at a dynamic signaling node that may act to modulate inflammation as well as adaptive immunity.

Another unique aspect of IDO2 is the considerable genetic variability that exists among different individuals for expressing the active enzyme. This variability is due to the presence of two commonly occurring, non-synonymous single-nucleotide polymorphisms in the *IDO2* gene that ablate its enzymatic activity [45]. Indeed, as many as 50 % of individuals of European or Asian descent and 25 % of individuals of African descent appear to lack functional *IDO2* alleles [45]. The frequent occurrence of inactive genetic variants in human populations suggests that there may be some evolutionary benefit to attenuating IDO2 activity, perhaps reflecting competing selective pressures to establish an optimal degree of immunological responsiveness under differing conditions of infection, autoimmunity, and malignancy. In this vein, one clinical study suggests that active IDO2 alleles may be disproportionally represented among younger individuals with aggressive pancreatic cancer [52]. While the relevance of IDO2 function to immune regulation has yet to be directly corroborated, one recent study offers some support for this expectation based on evidence that IDO2 activity can inhibit the proliferation of human $CD4^+$ and $CD8^+$ T cells in vitro, albeit in a manner insensitive to 1MT treatment [53].

2.4 Immune Suppression by Other Tryptophan Catabolic Enzymes TDO2 and TPH

The fundamental role of IDO in immune tolerance was recognized several years before its connections to cancer were discovered, and it is by far the most broadly expressed and studied of the tryptophan-metabolizing enzymes. IDO action leads to

both tryptophan deprivation and Kyn generation, both of which cooperate to inhibit the activation of immune cells known as T cells, through various mechanisms that also affect the activities of other classes of immune cells. In mammals, there are two other enzymes that catabolize tryptophan, but only one of which also generates kynurenine.

TDO2 is a multimeric enzyme that is structurally distinct from IDO and IDO2, probably an example of convergent evolution. Until recently, TDO2 was widely considered to serve in degrading excess tryptophan in the liver, where it is mainly normally expressed, but it has also been implicated now in cancer and immune modulation. In particular, TDO2 is frequently activated in brain cancers and other cancers where IDO is not activated [54]. In initial studies, one unique aspect of TDO2 in cancer appears to be that its activation promotes cancer cell migration, which has not been reported for IDO, suggesting some divergence in function despite the enzymes' shared ability to generate kynurenine. One possibility is that the preference of TDO2 for substrates may differ, perhaps varying the biological functions of TDO2 from IDO or IDO2 to some extent. Whatever the case, TDO2 appears to be upregulated in a variety of cancers [54], [55], and small-molecule inhibitors of TDO2 may be useful to treat these IDO-independent cancers or cancers that might become resistant to IDO inhibitors through TDO2 activation. In support of this likelihood, in a preclinical model TDO2 expression by tumors was found to prevent their rejection by immunized mice, and systemic treatment with a novel TDO2 inhibitor restored the ability of mice to reject TDO2-expressing tumors [55].

Tryptophan hydroxylase 1 (TPH1), the enzyme responsible for seratonin production from tryptophan, also has been implicated recently in immune control, including in cancer [56]. TPH1 does not generate kynurenine, so it is clear that its immunoregulatory role relates solely to tryptophan deprivation. Studies in mice that are genetically deficient in TPH1 have illustrated its role in skin allograft tolerance, tumor growth, and experimental autoimmune encephalomyelitis, where loss of enzymatic activity can mediate allograft tolerance, induce tumor remission, and intensify neuroinflammation, respectively [56]. These effects were all found to be independent of serotonin. Mast cells are a major source of TPH1 expression, and restoring TPH1 in these cells in vivo was sufficient to correct defects in the genetically deficient mice [56]. Thus, these findings introduced an important and previously unappreciated new role for mast cells in inflammatory programming and immune regulation, through their ability to modulate tryptophan degradation.

3 Complex Control of IDO by Immune Regulatory Factors

3.1 Transcriptional Control

Initial clues regarding the involvement of IDO in inflammation originated with the finding that its expression and activity in many cell types is stimulated strongly by the cytokine IFN-γ [57]. IFN-γ is now recognized as a major inducer of IDO, especially

in APCs including macrophages and DCs [58]–[61]. Transcriptional induction of the *IDO1* gene through IFN-γ is mediated through the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway, in particular JAK1 and STAT1α [62]. STAT1α appears to act to induce *IDO1* gene expression both directly through binding of gamma-activated sequence (GAS) sites within the *IDO1* promoter as well as indirectly through induction of interferon regulatory factor 1 (IRF-1) which binds the *IDO1* promoter at two interferon-stimulated response element (ISRE) sites [63]–[65], [62], [66]. The upregulation of IDO in APCs that occurs in response to IFN-γ, which is produced by activated T cells, suggests that IDO participates in a negative feedback loop that regulates T-cell activation.

The transcription factor NF-κB, which has a central role in directing inflammatory processes, has also been identified as a key factor controlling the induction of IDO. The precise mechanisms for NF-κB-mediated control of *IDO1* expression are not fully elucidated and may be contextually based in various cells, given that both the canonical and non-canonical pathways have been found to be important under different experimental conditions [67]–[69]. IRF-1 may be a common element through which both STAT1α and NF-κB contribute to the induction of *IDO1*, as both IFN-γ and tumor necrosis factor alpha (TNF-α) (which signals through NF-κB) can synergistically induce expression of IRF-1 through a novel composite binding element for both STAT1α and NF-κB in the IRF-1 promoter (termed a GAS/κB element) that combines a GAS element overlapped by a non-consensus site for NF-κB [70].

A recent analysis of functional polymorphisms in the IDO1 gene promoter was conducted which may explain some interindividual variability in IDO expression that has been documented [71]. This study identified a variable nucleotide repeat (VNTR) polymorphism, the presence of which correlated with serum tryptophan concentration in female but not male subjects. Interestingly, this VNTR did not affect basal or cytokine-induced activity of the IDO1 promoter, but it harbored functional binding sites for the transcription factor lymphoid enhancer-binding factor 1 (LEF-1) which is responsible for changes in gene expression mediated by WNT signaling, which is activated in many epithelial cancers, most notably colon cancers. However, the pathogenic role of this polymorphism in promoting cancer progression, if any, will require further analysis.

3.2 IDO Control in Dendritic Cells

In DCs, interferons (both type 1 and type 2) have been found to act at a central interface between IDO and other components of inflammation and immunity. Toll-like receptor 9 (TLR9) ligands such as CpG were found to induce IDO expression in a subset of DCs through a type 1 interferon-dependent signaling pathway [72]. Interactions with immune cells are also implicated in IDO regulation. The first of these interactions to be characterized was an intriguing reverse-signaling mechanism described for the inhibitory T-cell co-receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is constitutively expressed on Treg cells. By binding to B7 ligands (CD80 and CD86) on DCs, CTLA-4 was shown to elicit the IFN-γ-dependent induction of IDO

[73]. The stimulatory T-cell co-receptor CD28 also binds the same B7 ligands but fails to similarly induce IDO because of the concomitant induction of IL-6 which interferes with IFN-γ elicited STAT signaling through upregulation of suppressor of cytokine signaling 3 (SOCS3) [74]. Other cell surface proteins including CD40, CD200 and glucocorticoid-induced TNF receptor family-related gene (GITR) have since been shown to induce IDO through similar reverse-signaling mechanisms all of which appear to share the non-canonical NF-κB pathway as a common point of convergence [75].

TGF-β was initially reported to antagonize IFN-γ-mediated induction of IDO expression [76]. These experiments, carried out in fibroblasts, appear to run counter to immunosuppressive activity ascribed to TGF-β but are consistent with its ability to antagonize positively regulated targets of IFN-γ. More recently, the opposite relationship between IDO and TGF-β has been reported in experiments carried out in DCs suggesting that the regulatory impact of TGF-β on IDO expression may be complex and contextual. In these experiments, autocrine TGF-β sustained the activation of IDO in a tolerogenic subpopulation of $CD8^+$ DCs while exogenous TGF-β could convert immunogenic $CD8^-$ DCs into tolerogenic cells in conjunction with induction of IDO [77]. In this milieu, it was found that even DCs that lack expression of IDO could be rendered tolerogenic by exposure to tryptophan catabolites produced by IDO-expressing cells [78] as part of a feedforward expansion of IDO-elicited immune suppression described as "infectious tolerance" [79].

3.3 COX2 and Prostaglandins in IDO Control

The proinflammatory prostaglandin E-2 (PGE-2), which is frequently elevated during cancer progression as a result of activation of cyclooxygenase-2 (COX-2), has also been implicated as an important inducer of IDO activity. In support of the concept that IDO acts downstream of COX-2, induction of IDO activity can be blocked in vitro by COX-2 inhibitors such as aspirin, indomethacin, and phenylbutazone but not by anti-inflammatory agents that do not affect prostaglandin production [80]. This signaling mechanism may be relevant to the biological activity of upstream regulators of *COX2* expression as well. For instance, hepatocyte growth factor (HGF), which is known to be able to elevate COX2 activity, has been found to also elevate IDO in monocyte-derived DCs [81]. The relationship between PGE-2 and IDO is complicated insofar as IDO activity can affect the ratio of prostaglandin synthesis [82]. The complex interplay between IDO and COX2 in inflammatory processes in cancer and autoimmune and chronic inflammatory diseases has been reviewed in detail elsewhere recently [83]. Interestingly, while PGE-2 is employed widely as an in vitro maturation factor for DCs, treatment of these cells with PGE-2 has been reported to elevate IDO expression ~ 100-fold [84]. Although the induction of IDO enzymatic activity does appear to require an additional signal(s) (i.e., exposure to TNF or agonists of TLRs), these findings raise the concern that such preparations may inadvertently compromise the desired immune stimulatory activity of the DCs used in the setting of cancer vaccines.

3.4 IDO Control by Aryl Hydrocarbon Receptor Signaling

More recently, interconnections identified between IDO and the xenobiotic AhR have been generating particular interest due to a developing appreciation for the importance of AhR in modulating immune function especially at the level of mucosal immunity [85] where IDO may also be particularly relevant. AhR activation by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCCD) resulted in the induction of both IDO1 and IDO2 in vitro [86]. Furthermore, it was shown that TCDD treatment of mouse splenic T cells resulted in increased levels of FoxP3—an effect that was abrogated in *AhR*-null mice, suggesting that AhR is important for the development of Treg cells possibly through the induction of IDO [86]. Conversely, several tryptophan catabolites have been implicated as physiological ligands for AhR including kynurenine [54], [87], produced by the ubiquitous arylformamidase enzyme following the IDO- or TDO2-initiated catabolism of tryptophan. Further biological ramifications can be inferred from studies by DiNatale et al. [88] showing that kynurenic acid, another downstream tryptophan catabolite, induces AhR-mediated induction of IL-6, an important inflammatory cytokine for promoting tumor progression. Interestingly, *Ido1*-nullizygous mice exhibited a marked reduction in IL-6 levels in primary lung tumors and pulmonary metastases which was functionally linked to increased tumor resistance [89]. As noted earlier in this section, IL-6 has been demonstrated to antagonize IDO expression, suggesting its involvement in an important negative regulatory feedback loop that may go awry during the development of cancer.

3.5 Negative Control of IDO by Nitric Oxide

In addition to IL-6, other important negative regulators of IDO have been identified. Inducible nitric oxide synthase (iNOS) and IDO appear to be mutually antagonistic in DC-based studies [90]–[92]. The production of NO by iNOS prevents the IFN-γ-induced expression of IDO [93], interferes directly with its enzymatic activity [93]–[95], and promotes its proteolytic degradation [96]. NO can directly inactivate IDO by binding to the heme iron, which under lowered pH conditions induces iron–His bond rupture and the formation of a 5C NO-bound derivative that is associated with protein conformational changes that may be sufficient to target the protein for ubiquitination and proteosomal degradation [97]. In the non-obese diabetic (NOD) mouse model of diabetes, in vivo evidence suggests that IFN-γ signaling is impaired as the result of nitration of the downstream STAT1 transcription factor by peroxynitrate, which is derived from NO and superoxide. This impairment can be overcome by CTLA-4-Ig treatment, which, by promoting phosphatase and tensin homolog (PTEN) activity, relieves the negative regulation that phosphorylated Akt imposes on FOXO3a-mediated transcription of superoxide dismutase (SOD2) which degrades peroxynitrate [98]. Through this complex route, the blockade to activation of IDO gene expression, to which iNOS contributes through peroxynitrate-mediated nitration of STAT1, is relieved. Two implications of the configuration of this mechanism

are the following. First, NO agonists will tend to reverse immunosuppression at the level of DCs in cancer, which should benefit treatment. Second, small-molecule inhibitors of Akt that are being developed as anticancer therapeutics will tend to heighten immunosuppression by phenocopying this effect of CTLA-4-Ig on IDO expression. Other findings suggest that Akt inhibition may also heighten the invasive capability of cancer cells [99]. Thus, for cancer treatment, the desirable proapoptotic quality of Akt inhibitors may be balanced by their undesirable proinvasive and immunosuppressive properties.

4 IDO Dysregulation in Cancer Pathogenesis

4.1 IDO Upregulation in Cancer Cells Through Attenuation of Tumor Suppressor Gene Bin1

IDO overexpression is associated with poor prognosis in many different cancers [100]. Tumor transplant studies in mice have likewise linked IDO expression with enhanced tumor outgrowth in the context of an active immune system [68], [101], [102]. Upregulated IDO expression occurs commonly in human cancer cells [102]. While the basis for this upregulated expression is not fully understood, studies of the tumor suppressor gene *Bin1* have identified it as a central regulatory event in this process. *Bin1* is among the most frequently attenuated genes in human cancer, due to aberrant RNA splicing patterns that eliminate tumor suppressor function [103]–[107], or due to altered gene methylation patterns that extinguish expression [108]–[112]. Loss of *Bin1* function affects cancer cell proliferation, motility, survival, and immune escape [113]. However, in vivo studies clearly suggest that the most pathogenically significant effect of *Bin1* loss in promoting cancer is through IDO activation and IDO and IDO-mediated immune suppression [113], [114].

Genetic studies in the mouse have established an antagonistic relationship between *Bin1* and *Ido1*, such that functional ablation of *Bin1* causes transcriptional upregulation of *Ido1* with increased responsiveness to IFN-γ [68]. Oncogenic transformation of murine embryo fibroblasts or skin keratinocytes with *c-Myc* + *Ras* indicated that while *Bin1* loss affected cell growth, invasion, and survival [115], more dramatic differences were revealed in the growth of tumors in immunocompetent hosts, where *Bin1*-null cells formed large tumors in contrast to *Bin1*-expressing cells which formed only indolent nodules [114]. This dichotomy reflected different immune responses to the cells, as *Bin1*-expressing cells produced rapidly growing tumors when introduced into T cell-deficient mice. IDO was identified as a key target of *Bin1*-dependent transcriptional repression that was activated in *Bin1*-deficient cells [114], although other genes implicated in immune suppression such as *CD39* and *Arginase-1* were also identified (unpublished observations). Notably, treatment of *Bin1*-deficient cells with small-molecule inhibitors of IDO suppressed the outgrowth of *Bin1*-null tumors in syngeneic mice but not immunocompromised nude mice or

mice that were immunologically deprived of $CD4^+$ T cells [114]. Taken together, these findings established that *Bin1* loss led to IDO upregulation and tumor promotion by enabling IDO-mediated escape from T-cell immunity. Given the relationship between *Bin1* and *IDO* established by genetic studies in the mouse, which causally link Bin1 attenuation to IDO overexpression, it will be important to further evaluate their mechanistic relationship and integrate it with immunometabolic regulatory processes that may be affected by *Bin1*, such as adenosine or arginase signaling.

4.2 IDO is a Crucial Contributor to the Inflammatory Tumor Microenvironment

The tissue microenvironment where a tumor arises poses a huge barrier to its development and progression. In particular, it is clear that the interplay of cancer cells with immune cells is one of the most important determinants for whether an early cancer is destroyed by the immune system, persists in a dormant or slowly growing state (which often makes the tumor localized and treatable), or progresses to an invasive or metastatic state that becomes clinically challenging.

Unlike a conventional proto-oncogene, the role of IDO in cancer is predominantly to create a more hospitable environment for the tumor rather than enhancing malignant properties intrinsic to the tumor cells. Therefore, it is not surprising that normal cells outside the tumor have also been found to be a relevant source of IDO expression. In particular, a subset of DCs, with characteristics indicative of the B-cell lineage [116], expresses high levels of IDO in the proximal lymph nodes of mice with subcutaneous melanoma tumor grafts that exhibited no expression of IDO in the tumor cells themselves [117]. Elevated levels of IDO have also been reported in the tumor-draining lymph nodes (TDLNs) of human cancer patients [117]. Preclinical studies using a classical two-stage model of inflammatory skin carcinogenesis demonstrated that mice lacking the *Ido1* gene encoding IDO were quite resistant to the development of tumors [118]. In this model, tumors are initiated with a single exposure of the *ras*-activating carcinogen 7,12-dimethylbenz [*a*]anthracene (DMBA) followed by multiple exposures to the proinflammatory phorbol ester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) (also known as phorbol 12-myristate 13-acetate or PMA), the latter of which drives a state of chronic inflammation that promotes tumor outgrowth. These studies provided the first direct genetic evidence that IDO is crucial for de novo tumorigenesis. In this model of inflammatory carcinogenesis, but also within a T cell-suppressive population of DCs localized at TDLNs, which had the same characteristics as those observed previously in a melanoma tumor graft model [117], [118]. Notably, in TDLNs in the skin model, TPA strongly upregulated IDO expression [119] and subsequent findings confirmed this proinflammatory stimulus acting through protein kinase C (PKC) stimulates a unique pathway of IDO activation [120]. Together, these findings provided a glimpse of the complexity of interpreting IDO effects in biological systems, given that its expression can be either intrinsic or extrinsic to cancer cells.

Subsequent genetic studies in different mouse models of carcinogenesis have established that IDO contributes a crucial function to the inflammatory tumor microenvironment. Further, they argue that the initial characterization of IDO solely as a modifier of adaptive immune tolerance may oversimplify its role in cancer pathogenesis. Studies in the skin model showed that IDO loss did not exacerbate classical inflammatory responses to TPA but that its induction was integral to the inflammatory tissue microenvironment even in the absence of cancer [120]. In connecting IDO to inflammatory stimuli, it was found that IDO loss had little impact on tumor outgrowth if the carcinogenesis model employed lacked an explicit inflammatory promoter. For example, IDO ablation did not affect induction of skin tumors elicited by multiple topical exposures to DMBA that are sufficient for carcinogenesis in the absence of TPA, nor did IDO ablation affect induction of breast tumors where progesterone was used instead of TPA as a non-inflammatory promoter after a single intraperitoneal exposure to DMBA [120]. Moreover, it is clear that IDO deficiency does not influence the engraftment of established tumor cell lines that have previously developed an effective immunoediting route, unlike transgenic models where the route must be developed and will therefore vary between individual mice. In the context of TPA-driven skin carcinogenesis, where IDO was critical, bone marrow transplant experiments revealed that the most important source of IDO function were radiation-resistant, non-hematopoietic cells in the model, supporting evidence that *Bin1* deficiency in *myc* + *ras*-transformed skin cells was sufficient to facilitate IDO-mediated immune escape by a cell autonomous mechanism [120]. Together, these findings argued that IDO was a key element of "cancer-associated" inflammation that tilts the immune system toward tumor support. More broadly, they prompted the concept that mediators of immune escape and cancer-associated inflammation may be genetically synonymous.

Other observations from *Ido1*-deficient mice strengthen the concept that IDO exerts a proximal influence on inflammation that is too subtle to understand as simply immunosuppressive. If IDO were a solely immunosuppressive enzyme, inflammation might be expected to run rampant in *Ido1*-deficient mice where this presumptive check is no longer in place. However, *Ido1* deficiency does not produce such effects, in contrast to deficiency of an immunosuppressive function like *CTLA-4*. Moreover, the inflammation that develops in *Ido1*-deficient mice treated with TPA is not discernibly different than in wild-type control animals receiving the same treatment [121]. So, rather than IDO simply being an immunosuppressive counterbalance in inflammatory reactions, a more nuanced interpretation for the role of IDO is required in which IDO shapes the pathogenicity of the tissue microenvironment.

The degradation of normal cellular physiology leading to malignancy involves acquisition of the cell-intrinsic traits of immortalization, growth sufficiency, insensitivity to growth inhibitory signals, and resistance to apoptosis, along with the cell-extrinsic traits of angiogenesis, invasive capability, metastatic capacity, and immune escape. In this context, immune escape mechanisms utilized by tumors, such as IDO induction, have been postulated to be a terminal feature of the immunoediting process, which comprises the three distinct phases of elimination, equilibrium, and escape [122]. However, a contrarian argument has also been made that tumoral immune escape is not a late event driven by selective pressure, but instead develops

as an early, integral component of the tumorigenic process [123]. The multistage aspect of the DMBA/TPA carcinogenesis protocol described above provided us with a unique opportunity to investigate this question with regard to the role of IDO induction in the contextual setting of de novo tumor development. The immunoediting postulate would require that there be at least some nascent tumor present for IDO to be induced. Instead, however, TPA treatment alone was sufficient to induce IDO in the proximal lymph nodes [118]. Because these mice were never exposed to DMBA-based tumor initiation, this elevation of IDO occurred in the absence of cancer, as TPA alone is not able to drive the development of neoplasia in the absence of an initiating agent. This outcome, therefore, was more in line with IDO elevation being an early event driven by TPA-elicited inflammation, rather than a late event driven by immune selection.

Other studies extend the notion that IDO acts in a proximal manner to program pathogenic inflammatory processes which then go on to direct antigenic tolerization in the adaptive immune system at a more distal level. In one study, ectopic modulation of IDO in murine breast cancer cells not only influenced T-cell responses in immunocompetent mice but also affected primary tumor growth and metastasis in immunodeficient severe combined immunodeficiency *(scid)/beige* mice which lack T, B, and natural killer (NK) cells [124]. Thus, these pathogenic effects of IDO overexpression could not be readily interpreted as mediated solely by adaptive immunological mechanisms. The conceptual realization that IDO acts as an integral component of the inflammatory milieu is supported additionally by evidence of a role in supporting other pathogenicities associated with chronic inflammation. For example, IDO-mediated tryptophan degradation is elevated in rheumatoid arthritis and systemic lupus erythematosus patients, suggesting a role for increased IDO activity in promoting autoimmune disease [125], [126] that has some direct corroborative support from studies in the K/BxN spontaneous mouse model of arthritis [127]. In the KxB/N model, IDO activity is elevated at disease onset, and administration of the IDO inhibitor 1MT resulted in alleviation of joint inflammation, with 1MT-treated animals exhibiting minimal synovial expansion and fewer infiltrating inflammatory cells [128]. In this setting, 1MT treatment did not affect levels of Treg cells or T helper type 1 (Th1)/Th2/Th17 cytokines, but it did greatly diminish the autoreactive B-cell response, indicative of a role for IDO upregulation in supporting the development of autoimmune disease by supporting the activation of autoreactive B cells. In conjunction with results from cancer models, these results argue strongly that IDO contributes to pathogenic forms of chronic inflammation in a manner that is more complex than simply acting as an immunosuppressive brake.

4.3 IDO Activation Is a Critical Contributor to Tumor Angiogenesis and Metastasis

Our most recent findings further elucidate how IDO contributes to cancer development by altering the inflammatory milieu. *Ido1*-deficient mice exhibit a reduced tumor burden in a K-RasV12-induced model of lung adenocarcinoma and a reduced

susceptibility to development of pulmonary metastases in the 4T1 model of breast cancer, in both settings displaying improved survival [129]. Notably, IL-6 levels were attenuated by *Ido1* deficiency in each model, leading to an impairment of myeloid-derived suppressor cell (MDSC)-mediated suppression of T cells. The importance of these findings to pulmonary tumor development was demonstrated in the metastatic model where restoration of IL-6 overcame the MDSC impairment and allowed metastatic disease to progress at the rate observed in *Ido1*-competent mice [129]. The implication that IL-6 serves as a key regulator of tumor growth downstream of IDO has therapeutic value as increased IL-6 levels are associated with recurring tumors in patients [130]. In yet another clue to the role of IDO beyond adaptive immune control, *Ido1*-deficient mice were found to display an angiogenic defect in lungs even in the absence of tumors. Together, these studies highlight a more complex and nuanced interpretation of what tryptophan catabolism means to a developing tumor, extending beyond adaptive immunoregulation to inflammatory programming, metastasis, and angiogenesis.

5 IDO Effector Pathways in Cancer Pathogenesis

5.1 Kynurenine Activates the Aryl Hydrocarbon Receptor to Modulate Inflammation

Kynurenine production resulting from IDO-mediated tryptophan catabolism is widely recognized as one of the elements which mediate the immunosuppressive effects of IDO [131], [132]. How kynurenine may contribute to inflammatory programming by IDO has been less clear, but an important perspective has opened up on this aspect with the identification of AhR as the physiological receptor for kynurenine [54]. This connection links the fields of toxicology, immunology, and cancer biology, and it may help explain why tryptophan consumption assists pathogenic inflammatory programming and drives malignant progression. In activating AhR, kynurenine not only mediates an effector signaling pathway from IDO but also TDO2 in driving cancer growth [54]. Kynurenine binding to AhR is essential to generate Treg cells that suppress adaptive immunity [133]. In binding AhR, kynurenine triggers nuclear translocation of this receptor, licensing activation of its target genes. A broad literature implicates AhR in immune regulation, inflammation, and carcinogenesis [134] in the same vein that IDO has been implicated [135]. Elevated levels of AhR correspond with poor prognosis in cancer patients [54]. The discovery that kynurenine is an endogenous ligand for AhR helps explain why there is a selection for tryptophan consumption mediated by IDO or TDO2 during tumor development, because the kynurenine that is produced binds AhR to help tumors program a pathogenic inflammation in their microenvironment that can tilt it from an antagonist to a facilitator role (i.e., from immunosurveillance toward immune escape). By connecting tryptophan consumption to AhR activation, this discovery also helps explain why immune escape and tryptophan consumption are so integrally connected in cancer [135].

5.2 IDO Activation Stimulates Stress Kinase GCN2 and Elevates IL-6 Synthesis

In a nutrient-deprived tissue microenvironment, such as that which occurs within tumors, tryptophan degradation by IDO (or IDO2 or TDO2) may cause a local tryptophan deficiency that leads to the accumulation of uncharged tryptophan-transfer RNA (tryptophan-tRNA). In this way, IDO activity may lead to activation of GCN2, a stress-response kinase that is simulated by elevations in uncharged tRNA and that limits or alters protein translation in response to this condition. Notably, T cells where GCN2 is genetically disrupted are not susceptible to IDO-mediated suppression of proliferation in vitro or in vivo, and these T cells cannot be anergized by IDO-expressing DCs [136]. Further, IDO-expressing DCs can induce the production of immunosuppressive Treg cells, but this effect is abolished by genetic disruption of GCN2. Thus, one critical downstream effector pathway for IDO to blunt T cell-mediated tumor immunity appears to involve GCN2 activation in T cells, which allows them to respond to IDO activity manifested in a local tissue microenvironment.

GCN2 functions to blunt protein translation by phosphorylating the initiation factor eukaryotic initiation factor 2 alpha (eIF-2α) which blunts its activity and prevents the readout of most RNA transcripts. However, under such conditions, some RNA transcripts actually become preferentially translated, including LIP, an isoform of the immunoregulatory transcription factor NF-IL6 (also known as CEBP-β), which then goes on to activate the expression of certain immunoregulatory cytokines such as IL-6 [137]. The relevance of this pathway is documented in vivo in tumor-bearing animals, where IDO genetic deficiency leads to reduced IL-6 production, a factor that is causally related to tumor outgrowth and metastasis [129]. The consequences of GCN2 activation by IDO in this regard may differ between cell types, since the effect of IDO on IL-6 production through this pathway can be repressive or inductive [129], [136]. Nevertheless, in experiments conducted in at least two mouse models of cancer, it appears that IDO supports IL-6 production and that this production is critical for MDSC function and malignant progression [129].

5.3 IDO Activation Inhibits mTORC1 and Stimulates Autophagy

While GCN2 is recognized as an important effector of the IDO pathway, studies in our laboratory suggested that its role in detecting tryptophan deprivation and regulating IL-6 may be insufficient for the manifestation of inflammation-driven cancers. In particular, in the mouse model of DMBA + TPA-induced inflammatory skin carcinogenesis, we found that genetic ablation of *GCN2* did not promote resistance to papilloma tumor development in the same manner that IDO did [119], [138]. This difference implied the existence of additional cancer-relevant pathways that operate downstream of IDO. In considering effector mechanisms beyond GCN2 activation, we hypothesized that IDO may suppress the master metabolic regulator mammalian

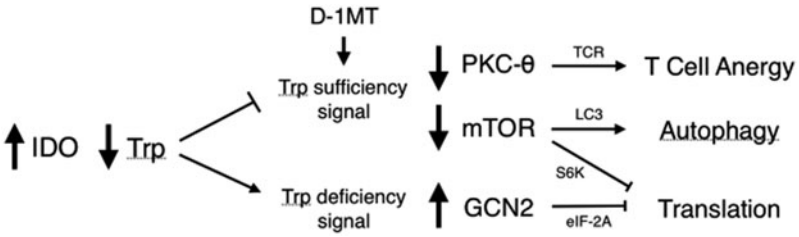

Fig. 11.1 Trp deprivation caused by IDO generates signals sensed by distinct amino acid sufficiency and deficiency pathways. Trp deficiency is sensed by the integrated stress kinase GCN2 that inhibits eIF-2α and blocks translation. Through a distinct pathway, the lack of Trp sufficiency causes mTOR to be inactivated, leading to autophagy via LC3 de-repression and translational blockade via S6 kinase inactivation. D-1MT acts as a mimetic of Trp in the sufficiency pathway, thereby functionally reversing the effects of IDO on mTOR. (The figure and legend are taken from [138])

target of rapamycin complex 1 (mTORC1), which is known to monitor not only energy status through adenosine monophosphate-activated protein kinase (AMPK) but also essential amino acid status [139], [140]. Indeed, in a set of experiments employing cells harboring an inducible IDO gene, we demonstrated [138] that IDO-mediated catabolism of tryptophan inhibits mTORC1 as well as the T-cell receptor (TCR) regulatory kinase PKC-θ, both of which are regulatory targets of a master amino acid-sensing kinase glucokinase 1 (GLK1) acting upstream of mTORC1 (also known as mitogen-activated protein kinase kinase kinase kinase 3, MAP4K3) [141]. Our findings suggest that tryptophan deprival resulting from IDO activation is read out in two distinct effector pathways, one of which is activated by tryptophan insufficiency (GCN2) and the other suppressed by tryptophan insufficiency (mTOR/PKC-θ via presumptive GLK1 blockade). As expected, mTORC1 suppression by IDO triggered autophagy, as measured by light chain 3 (LC3) processing and relocalization in cells, and this effect could be reversed by tryptophan restoration which relieved mTOR blockade [138]. The finding that IDO can regulate mTORC1 and autophagy distinct from GCN2 control may advance understanding of IDO function in the many settings where mTOR acts as a pivotal immune regulator. Further, this work offers a novel conceptual perspective on IDO by suggesting its analogy to the mTOR inhibitor rapamycin and by revealing how IDO can trigger autophagy to anergize T cells in the tumor microenvironment.

5.4 An Integrated Model for Effector Signaling by IDO-Mediated Tryptophan Deprival

Integrating our findings with existing knowledge of IDO signaling, our work supports a model in which IDO coordinately affects pathways of essential amino deficiency and sufficiency via GCN2 and mTOR, respectively, in controlling inflammatory responses and immune tolerance (see Fig. 11.1). Nutrient-sensing processes in

mammalian cells involve a set of master regulatory kinases, including AMPK, which monitors levels of ATP (energy), GCN2, which monitors levels of uncharged tRNA (amino acids), and mTOR, which integrates all nutrient information to control cell growth and autophagy. Studies in yeast [142] and hepatocytes [143] suggest that the GCN2 and mTOR pathways function in concert, for example, by demonstrations that deprivation of an essential amino acid can elevate insulin sensitivity through coordinate GCN2 activation and mTOR repression in settings where AMPK is active (i.e., energy is sufficient) [144], [145]. 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 tuberous sclerosis 2 (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 guanosine triphosphatase (GTPase) complex and other signals needed to recruit mTOR to late-stage autophagosomes where it blocks autophagy (a starvation-induced process for generating amino acids). In this way, mTORC1 licenses protein synthesis if AMPK, PI3K/AKT, and Ragulator signals are all positive.

While it is not yet clear how the mTORC1 complex receives amino acid sufficiency signals, recent work [146] suggests a pivotal role for MAP4K3/GLK1, a kinase that is stimulated by undefined amino acid-binding molecules acting further upstream. MAP4K3/GLK1 would seem to offer a logical effector molecule for IDO acting upstream of mTOR and PKC-θ, based on present evidence of its role in regulating amino acid sufficiency signaling [141], [147]. In considering direct sensors of tryptophan that act further upstream of MAP4K3/GLK, the most logical candidates are the tryptophan-tRNA synthetases WARS1 and WARS2. The candidacy of a WARS molecule or variant as a proximal effector molecule for IDO is based not only upon existing evidence of WARS multifunctionality [148] but also on the recent striking discovery that the leucine-tRNA synthetase LARS senses branched chain amino acid to control mTOR activation status [149]. In future work, it will be important to establish whether WARS and MAP4K3/GLK will complete the connections of IDO to mTOR and PKC-θ to fully define this new IDO effector pathway which influences amino acid sufficiency signaling.

IDO-mediated tryptophan deprival may provide an integrated molecular switch to establish an immunosuppressive environment by amplifying tolerogenic APCs, expanding Treg cells, downregulating cytotoxic T-cell activity, and sustaining other cells that provide critical support to inflammatory carcinogenesis [120], [129]. By analogy to the mTOR inhibitory agent rapamycin, IDO may blunt immune activation and D-1MT may reorient this process by controlling tryptophan sufficiency signals needed to license mTOR activation, relieve immunosuppression, and reestablish proinflammatory states that together limit the progression of cancer or other diseases characterized by disordered inflammation and immunity. Given the implications of all mammalian tryptophan-catabolizing enzymes IDO, IDO2, and TDO in cancer progression [54], [102], [129], [135], [150], [151], further investigation is needed to understand how tryptophan depletion promotes immune escape by supporting the

development of Treg cells and MDSCs that are important to IDO-mediated cancer progression [129]. On the other hand, deprivation of any essential amino acid may be sufficient to reorient naïve $CD4^+$ T helper cells to support Treg generation [140], as the rarest amino acid Trp may assume a special position in modulating local GCN2 and mTOR status in tissue microenvironments. In future work, it will be important to explore in more detail the crosstalk between IDO and the Ragulator, MAP4K3/GLK1, and PKC-θ signals which all exert major physiological and pathophysiological effects on inflammatory programming and immune control. PKC-θ is a notable connection given its predominant function in TCR signaling which has been elucidated most fully only recently [152]. PKC-θ is dispensable for general T-cell development but critical for Treg development [153]. Its activation relies upon T538 phosphorylation [154], 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 [141], which is essential for signaling and differentiation of Th2 cells and IL-17-producing helper cells (Th17 cells), but not for 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 tryptophan catabolism.

6 IDO Inhibitors in Cancer Therapy

6.1 Therapeutic Prototype 1MT

Many studies have now provided evidence that IDO inhibition with 1MT or other small-molecule inhibitors of the IDO pathway can exert anticancer effects. Initial evidence was offered in 2002 that the IDO inhibitor 1MT could partly retard the growth of mouse lung carcinoma cells engrafted onto a syngeneic host [155]. Similar results were obtained as part of an investigation to assess the ramifications of IDO overexpression, which was detected in a wide range of human tumors [102]. In this study, ectopic overexpression of IDO in an established tumor cell line was shown to be sufficient to enable tumor formation in animals pre-immunized against a specific tumor antigen, and 1MT partially suppressed tumor outgrowth in this context as well. Against established, autochthonous (spontaneously arising) mammary tumors in the mouse mammary tumor virus-neu/human epidermal growth factor receptor 2 (MMTV-neu/HER2) transgenic mouse model of breast cancer, we found that 1MT could likewise retard tumor outgrowth [68]. By itself, however, 1MT was unable to elicit tumor regression in this model, as shown previously in the tumor cell graft models, suggesting that IDO inhibition may produce limited antitumor efficacy when applied as a monotherapy.

In contrast, the delivery of 1MT in combination with a variety of classical cytotoxic chemotherapeutic agents elicited regression of established MMTV-neu/HER2 tumors which responded poorly to any single-agent therapy [68]. In each case, the observed regressions were unlikely to result from a drug–drug interaction, that is,

by 1MT acting to raise the effective dose of the cytotoxic agent, because efficacy was increased in the absence of increased side effects (e.g., neuropathy produced by paclitaxel, which is displayed by hind leg dragging in affected mice). Immunodepletion of $CD4^+$ or $CD8^+$ T cells from the mice before treatment abolished the combinatorial antitumor effect, confirming the expectation that 1MT acted indirectly through activation of T cell-mediated antitumor immunity. We have observed that combinatorial efficacy in achieving tumor regressions can be replicated by oral dosing of 1MT at 400 mg/kg on a b.i.d. schedule, again in the absence of any detectable side effects [156]. These striking findings were one harbinger in emerging concepts of immunochemotherapy, which is the combination of conventional chemotherapy with modalities that interfere with tumoral immune escape as a strategy to improve therapeutic outcomes [157], [158]. Our results demonstrating the powerful combinatorial effects of 1MT with conventional chemotherapy helped propel this compound onto a select list of immunotherapeutic agents identified by a National Cancer Institute (NCI) workshop panel in 2008 as having high potential for use in cancer therapy [159]. First-in-man trials were initiated that year with the D isoform of 1MT, a racemic agent. Phase IA single-agent evaluation and phase IB combinatorial evaluations (with taxotere or an adenoviral p53 vaccine) are now reported to be complete (H. Soliman, N. Vahanian, pers. comm.). Some preclinical results obtained during these trials have encouraged IDO inhibitor applications in immunochemotherapy, for example, with illustrations that the powerful efficacy of the anticancer drug imatinib (Gleevec®) in the treatment of solid gastrointestinal stromal tumors relies on a blockade in the IDO expression driven by activated KIT oncogenes in this disease [160].

6.2 Mechanisms of Action of D-1MT (Indoximod): Relief of mTORC1 Suppression by IDO

Since 1MT is a racemic compound, it was necessary to choose a single molecular species for clinical testing. For a variety of reasons, D-1MT was selected instead of L-1MT, but this choice has drawn some controversy because the mechanism of action of these isoforms, particularly D-1MT, has been somewhat enigmatic. Using classical in vitro assays that employ recombinant IDO1 enzyme and the non-physiological reductant methylene blue, L-1MT acts as a weak catalytic inhibitor. In contrast, under the same conditions, D-1MT exerts little, if any, effect as a catalytic inhibitor [150]. Our laboratory has observed that in cell-based assays D-1MT can inhibit IDO2 activity [137]. Further, efficacious effects of D-1MT which have been observed in the K/BxN mouse model of rheumatoid arthritis indicate a genetic requirement for *Ido2* but not *Ido1* in the drug-targeting mechanism (L. Merlo, E. Pigott, J. DuHadaway, S. Grabler, R.M., G.C.P., and L. M-N, unpublished observations). However, in studies conducted in different cell systems, other groups have not extended the evidence that D-1MT can inhibit IDO2 activity [53], [161], [162], finding instead that D-1MT was inactive and that L-1MT was either effective [53] or ineffective [53] in blocking

IDO2 catalytic activity (including in a human T-cell assay where IDO2 function was biologically relevant [53]).

Overall, it is clear that L-1MT is more potent than D-1MT as a biochemical inhibitor of IDO, but under traditional non-physiological assay conditions that may impact catalysis by IDO and possibly even more by IDO2 (which, in such assays, displays weaker catalytic activity). As a further complexity, D-1MT can also upregulate IDO expression in cells [50] albeit only at relatively high concentrations that may be irrelevant in vivo. Factors in choosing to clinically translate D-1MT instead of L-1MT included evidence that D-1MT is more potent at relieving IDO-mediated suppression of T-cell proliferation in mixed lymphocyte reactions involving human IDO^+ plasmacytoid DCs; displays superior anticancer activity relative to L-1MT in preclincal models, both as a single agent and in combination with chemotherapy; and has been genetically validated in terms of IDO targeting based on the loss of anticancer activity in *Ido1*-nullizygous mice [150]. As alluded to above, questions concerning D-1MT as a direct inhibitor of IDO enzymes [163] must be tempered by concerns about the use of non-physiological reductants in enzyme assays. This issue is critical to appreciate, because of emerging evidence that these reductants can exert differential effects on inhibitor binding and activity when compared to physiological reductants used in the reactions (R.M., J.D., and G.P., unpublished observations).

Our recent work in identifying mTORC1 suppression as an effector mechanism in IDO-mediated tryptophan catabolism also revealed a likely mechanism of action for D-1MT (which having entered clinical trials is now also known as NLG8189 or indoximod). Specifically, we found unexpectedly that D-1MT acts as a high-potency tryptophan mimetic in reversing mTORC1 inhibition and autophagic induction by IDO, even though D-1MT is insufficient to charge tryptophan-tRNA and therefore to rescue protein translation or return GCN2 to a quiescent state. Strikingly, D-1MT relieved mTOR suppression by IDO at even higher potency than L-tryptophan itself (i.e., at lower concentrations), within a nanomolar range concentration consistent with clinical pharmacodynamics associated with patient responses in phase I trials (H. Soliman, pers. comm.). The implications of this discovery are discussed in more detail below and elsewhere [138], [164], but they provide timely insight into the unique mechanism of action of D-1MT relative to indisputable catalytic inhibitors of IDO.

The findings suggest why D-1MT is generally superior to L-1MT at breaking IDO-dependent immune tolerance in preclinical mouse models of cancer [150]. D-1MT did not affect GCN2 activation status in Trp-deprived cells, like Trp or L-1MT, arguing that D-1MT may act exclusively by restoring the mTOR pathway, unlike Trp or L-1MT which would also be expected to restore GCN2 quiescence and block kynurenine production. Mechanistically, preliminary work suggests that L-1MT but not D-1MT can inhibit WARS1A-mediated tryptophan-tRNA amino acylation (R.M., unpublished observations), thereby explaining why D-1MT could not alter levels of uncharged Trp-tRNA that would be needed to reverse activation of GCN2 triggered by IDO-mediated Trp deficiency. In contrast, an inhibition of WARS1A activity by L-1MT could explain why L-1MT is inferior to D-1MT as an anticancer compound, because WARS1A inhibition would counteract IDO inhibition

by blocking elevation of uncharged Trp-tRNA levels that are needed to activate GCN2. Moreover, given evidence [138] that L-1MT may serve as a weak substrate of IDO, unlike D-1MT, it is conceivable that catabolism of L-1MT leads to production of the product N-methyl-kynurenine, which by activating the AhR pathway like kynurenine [165] may actually limit the immunostimulatory effects of L-1MT as an IDO enzyme inhibitor. Developing these concepts may yield a more complete understanding of why L-1MT serves as a poor physiological inhibitor of IDO function compared to D-1MT and therefore a weaker candidate for clinical exploration.

The discovery that D-1MT can reverse the suppression of mTORC1 by IDO is important in translational terms, because it suggests that D-1MT may have broader clinical uses against cancers that overexpress any tryptophan catabolic enzyme (IDO, IDO2, or TDO), perhaps even being suitable to combine with specific biochemical inhibitors of these enzymes. The definition of mTORC1 and PKC-θ as candidate pharmacodynamic markers for D-1MT responses may be useful in studying the response of patients recruited to ongoing phase IB/II cancer trials, addressing a current clinical need. In this regard, we note that the concentrations at which D-1MT affects these key immunoregulatory molecules are consistent with the clinical pharmacokinetics documented in human trials [166].

6.3 Discovery and Development of Novel Enzymatic Inhibitors of IDO

IDO has a number of appealing features as a target for small-molecule drug development. First, IDO is a single-chain catalytic enzyme with a well-defined biochemistry. Unlike many proposed therapeutic targets in cancer, this means that IDO is very tractable for discovery and development of small-molecule inhibitors. Second, the other known tryptophan-catabolizing enzyme on the kynurenine pathway, TDO2, is structurally distinct from IDO and has a much more restricted pattern of expression and substrate specificity, which mitigates "off-target" issues usually posed by novel agents. Third, bioactive and orally bioavailable "lead" inhibitors exist which can serve as useful tools for preclinical validation studies. Fourth, the *Ido1*-deficient mouse that has been constructed is viable and healthy [167], and further analysis encourages the notion that IDO inhibitors will not produce unmanageable, mechanism-based toxicities [168]. Fifth, pharmacodynamic evaluation of IDO inhibitors can be performed by examining blood serum levels of tryptophan and kynurenine, the chief substrate and downstream product of the IDO reaction, respectively. Lastly, small-molecule inhibitors of IDO offer logistical and cost advantages compared to biological or cell-based therapeutic alternatives to modulating T-cell immunity.

While early-phase clinical trials with D-1MT are currently ongoing, concerns regarding its inhibitory effects on IDO catalytic activity prompt the development of pharmacologically superior IDO inhibitory compounds. The rational design and development of new inhibitory compounds requires understanding of the IDO active

site and catalytic mechanism. Proposed models for the processes at work in the active site have been developed based on mechanistic studies [169]. The publication of an X-ray crystal structure for IDO complexed with a simple inhibitor [31] has greatly facilitated this work. Alternately, screening for novel inhibitors is likely to identify novel structural series to evaluate. Through this route, our group initially identified the natural product brassinin as an IDO inhibitor and evaluated brassinin derivatives for in vitro potency and cell-based activity [170]. Brassinin is a phytoalexin-type compound found in cruciferous vegetables that has potent chemopreventive activity against breast and colon cancer in rodent models [171], [172]. In order to probe the relationship between inhibitors and the active site, that is, to perform a structure–activity relationship (SAR) analysis, we synthesized a series of derivatives from the core brassinin structure [170]. Among the conclusions drawn, we determined that the indole core is not essential for enzyme inhibitory activity, consistent with the known promiscuity of the active site in IDO [36], thus broadening the spectrum of potential inhibitory compounds. In addition, we found that the dithiocarbamate segment of brassinin is an optimized moiety for inhibition, probably on the basis of chelation of the heme iron at the active site. Of the large number of derivatives evaluated, the most potent were only $\sim 1\ \mu M$ suggesting that it may be difficult to achieve significant improvements in potency within this simple structural class.

High-throughput screening of comprehensive compound libraries remains the most effective way to identify new structural series. A unique yeast screen has been used to identify IDO inhibitory compounds representing diverse structural classes [173] including several complex natural products with potent IDO inhibitory activity [174], [175]. The insight that a naphthoquinone pharmacophore might be at the core of several of the most potent IDO inhibitory compounds led us to conduct an SAR-driven study that yielded a promising series of pyranonapthoquinone-based IDO inhibitory compounds, some with inhibition constants of less than 100 nM [176]. Similar studies based on the phenylamidazole pharmacophore have likewise yielded a series of IDO inhibitory compounds, though not achieving the degree of inhibitory potency seen with pyranonapthoquinones [177].

Recently, starting from the IDO inhibitory compound 4-phenyl-1,2,3-triazole [178], Rohrig et al. employed computational structure-based methods to design a set of more potent bioactive inhibitors that lacked cellular toxicity and exhibited high selectivity for IDO over TDO2. Explanative power in understanding the activities of this set of compounds was gained through a quantitative SAR based on electrostatic ligand–protein interactions in the docked binding modes and on quantum chemically derived charges of the triazole ring.

Starting from another structural class, a new IDO inhibitor termed INCB024360 entered phase I trials for advanced malignancies in 2010. INCB024360 is a hydroxyamidine that competitively blocks the degradation of tryptophan to kynurenine by IDO with an IC_{50} of approximately 72 nM [179]. Oral administration of this compound in mice and dogs reduced kynurenine levels in the plasma as well as in tumors and TDLNs [180]. Using several mouse models, INCB024360 delayed tumor growth in wild-type mice but not in nude mice or *Ido1*−/− mice indicating not only that this drug targets IDO1 but also that it mediates its antitumor effects through the immune

system [179], [180]. The in vivo data complement in vitro experiments showing that INCB024360 does not inhibit IDO2 or TDO2 activity [179]. An important mechanistic observation is the ability of INCB024360 to increase the survival and decrease the apoptosis of DCs suggesting that this drug may improve the number of functional DCs, thereby allowing T cells to be more effectively primed against tumor cell antigens [179].

6.4 Inhibitors of IDO Expression: Gleevec and Beyond

Possible alternative targeting strategies include inhibiting IDO expression (upstream) or inhibiting the signaling pathway through which IDO acts (downstream). As mentioned earlier, some non-steroidal anti-inflammatory drugs (NSAIDs) have been shown to indirectly block IDO activity by inhibiting COX2 [80], and the anti-inflammatory compound, ethyl pyruvate, previously found to inhibit NF-κB activity, has been shown in mouse models to be an effective inhibitor of IDO expression and to produce robust antitumor responses that were both T cell and IDO dependent [69]. Inadvertent targeting of IDO expression may already be providing a clinical benefit, as recent investigations into the therapeutic role of Gleevec in treating gastrointestinal stromal tumor (GIST) found that the inhibition of oncogenic Kit signaling could potentiate antitumor T-cell responses by interfering with the induction of IDO [181]. Downstream of IDO, the integrated stress-response kinase GCN2 has been identified as responding to tryptophan depletion to limit T-cell responses [136] and thus might represent an alternative target. Recent attention has also focused on AhR in mediating the downstream response to tryptophan catabolites, including kynurenine and kynurenic acid [87], [88]. The liver enzyme TDO2 catalyzes the same reaction as IDO, and recent reports indicate that TDO2 elevation in some cancers may serve as an alternate mechanism for eliciting the same immune escape mechanism [54], [182]. Thus, while targeting the IDO pathway has clearly been established as an attractive approach for leveraging cancer treatment, it remains to be determined how this will be translated to provide the greatest benefit to patients.

6.5 Potential Safety Risks of IDO Inhibitors Suggested by Studies of Ido1-Deficient Mice

As summarized above, small-molecule inhibitors of IDO are being developed to treat cancer, chronic infections, and other diseases, so the systemic effects of IDO disruption on inflammatory phenomena may influence the design and conduct of early-phase clinical investigations of this new class of therapeutic agents. In assessing potential safety risks that might be monitored clinically in patients during trials of IDO inhibitors, phenotypes revealed in *Ido1*-deficient mice may be useful to consider. In a recent report, our group summarized a set of cardiac and gastrointestinal

phenotypes we have observed during several years of study of *Ido1*-deficient mice in various contexts that may warrant consideration in planned assessments of the safety risks of IDO inhibitors [183]. The most striking phenotype observed to date was calcification of the cardiac endometrium proximal to the right ventricle. This phenotype was 30 % penetrant, specific to *Ido1* deficiency on the BALB/c strain background and sexually dimorphic in nature [183]. Additionally, we observed that administration of complete Freund's adjuvant containing Toll-like receptor ligands known to induce IDO caused acute pancreatitis in *Ido1*-deficient mice [183], with implications for the design of planned combination studies of IDO inhibitors with cancer vaccines. Further, in an established model of hyperlipidemia, caused by homozygous deletion of the murine low-density lipoprotein (LDL) receptor, we found that IDO deficiency caused a dramatic elevation in levels of serum triglycerides [183]. This risk factor may be relevant in cancer patients receiving IDO inhibitors who may also have occult unstable cardiovascular plaques. Lastly, we observed an increased sensitivity of *Ido1*-deficient mice to induction of acute colitis, with a marked elevation in tumor incidence, multiplicity, and staging in animals subjected to regimens of inflammatory colon carcinogenesis [183]. These findings suggested risks of colitis in the short term and colon carcinoma in the longer term in patients who may receive IDO inhibitors as part of their therapy. Here, we note that administration of D-1MT has never been observed to produce any of these phenotypes, but the benign nature of this compound may relate to its limited activity in blocking the IDO pathway at the level of mTORC1 restoration (or other targets), relative to more potent biochemical inhibitors in development. Together, while the phenotypes in *Ido1*-deficient mice characterized to date have been observed only under certain stress conditions, they suggest potential cardiac and gastrointestinal risks of IDO inhibitors, in particular as they will be tested in combination with other therapeutic modalities, that should be monitored in patients as this class of drugs proceeds through clinical development.

7 Conclusions

In a relatively short period, IDO has become recognized as a major regulator of the immune system. Pathophysiologically, IDO has been strongly implicated in tumoral immune tolerance and immune escape and appears to be widely overexpressed in cancer at the level of tumor cells and/or tumor-associated immune regulatory cells. IDO has a variety of characteristics that make it an appealing target for cancer drug development. To date, preclinical validation of IDO inhibitors suggests they may offer the greatest promise in combination with classical cytotoxic drugs, but their potential to heighten the response to active immunotherapeutic agents such as TLR ligands or tumor vaccines is also important to consider. Given the provocative preclinical findings that have emerged from studies of agents targeting IDO and the IDO pathway, one would expect therapeutic interest in this pathway to continue to grow.

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