Indoleamine 2,3-Dioxygenase in Immune Suppression and Cancer

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Abstract: The extrahepatic enzyme indoleamine 2,3-dioxygenase (IDO) catalyzes tryptophan degradation in the first and rate-limiting step towards biosynthesis of the central metabolic co-factor nicotinamide adenine dinucleotide (NAD). While this pathway has been known for decades, the actual physiological role for IDO in mammals remained obscure, because (i.) most cell types do not express the downstream enzymes in the NAD biosynthesis pathway and (ii.) mammals salvage rather than synthesize NAD to meet their metabolic needs. An immunological role for IDO was hinted at with the observation that IDO expression is stimulated by interferon-γ and subsequently confirmed by the discovery of its physiological importance in protecting the fetus from maternal immunity. Similarly, elevations in tryptophan catabolism in cancer patients were known since the 1950s, but the basis and meaning of this phenomenon were uncertain until it was shown that IDO, which is commonly elevated in tumors and draining lymph nodes, suppresses T cell immunity in the tumor microenvironment. Indeed, by creating peripheral tolerance to tumor antigens, IDO can undermine immune responses that thwart tumor cell survival in the context of an underlying inflammatory environment that facilitates tumor outgrowth. In preclinical studies, small molecule inhibitors of IDO compromise this mechanism of immunosuppression and strongly leverage the efficacy of a variety of classical chemotherapeutic agents, supporting the clinical development of IDO inhibitors as a therapeutic goal. This essay summarizes key findings that implicate IDO as an important mediator of peripheral tolerance and discusses the development of anti-cancer modalities that incorporate the use of IDO inhibitors.

Keywords: Tumor immunology, immune suppression, tolerance, T cells, IDO.

INTRODUCTION

The traditional goal of cancer treatment, exemplified by radiotherapy and chemotherapy, has been to kill any residual tumor that cannot be surgically removed. However, the effectiveness of such approaches is limited by the inherent nature of the cancer cell. Being of host origin, cancer cells are particularly difficult targets for the development of cytotoxic agents that are sufficiently selective to avoid severe side effects in patients, and the therapeutic window for such agents is usually narrow. Due to their plastic nature, tumors are also remarkably resilient in their ability to rebound from such treatments. Even when the vast majority of cancer cells are killed by a cytotoxic agent, a small number of residual cells resistant to the agent can be sufficient to seed the regrowth of a tumor. Making matters worse, the regrown tumor may no longer respond to the previously successful therapy, due to the capacity of tumor cells to evolve resistance under selective pressures applied by cytotoxic agents. This feature of tumors is a unique consequence of the genetic plasticity that is characteristic of all cancer cells. As demonstrated for infectious agents with high rates of mutation, such as HIV, successful targeting of tumor cells may require the application of multiple agents that target different survival mechanisms. However, compared to HIV, the genetic space available for the evolution of a cancer cell is far larger, due to the far greater size of the cancer cell genome. Thus, effective eradication of tumors may be difficult to realize, even using multiple agents, because of the ability of the cancer genome to evolve mechanisms of survival in response to the multiple selection pressures that such agents apply. One solution to this conundrum is suggested by the fact that tumors depend on interactions with the host for their growth and survival. Exploiting these constraints imposed by the host/tumor interface might offer attractive points of vulnerability to manipulate therapeutically. The recent successes illustrated by angiogenesis inhibitors for cancer therapy offers a tangible cornerstone to this conceptual construct.

IDO FUNCTION: FROM AMINO ACID CATABOLISM TO IMMUNE REGULATION

The IDO enzyme (EC 1.13.11.42) is a single-chain polypeptide that catalyzes cleavage of the pyrrole ring of L-tryptophan, incorporating both atoms of molecular oxygen in the reaction. The catalytic activity of IDO requires binding of a heme prosthetic group that is critical for the reaction mechanism, which differs from other monooxygenases (e.g. peroxidases, cytochrome P450s). X-ray crystallographic analysis of IDO structure reveals two α-helical domains with the heme group and catalytic site positioned between the domains. Unlike other heme-containing monooxygenases, the IDO amino acid residues do not contribute to oxygen activation or proton abstraction. Instead, it appears that the heme iron-bound oxygen species abstracts a proton from the substrate [1]. Thus, shape complementarities between the substrate indole ring and the IDO protein side chains are required, not for binding, but to permit an interaction between the substrate and iron-bound oxygen in the first step of the reaction. From a biochemical standpoint, the structural analysis of IDO has addressed a

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gap in understanding of heme chemistry as it pertains to dioxygenase catalytic mechanisms, possibly contributing to the design of potent and specific small molecule inhibitors.

IDO is encoded by the *INDO* gene, which in humans is comprised of 10 exons spanning approximately 15 kb at chromosome site 8p11-p12 [2, 3]. IDO catalyzes the initial and rate limiting step in degradation of tryptophan which leads to biosynthesis of nicotinamide adenine dinucleotide (NAD) [4, 5]. IDO does not handle dietary catabolism of tryptophan in the liver, which is the role of the structurally distinct liver-specific enzyme tryptophan dioxygenase (TDO2). Additionally, IDO is not involved in maintaining NAD levels, which in mammalian cells is handled predominantly if not exclusively by salvage pathways. Thus, the precise physiological role for IDO and the basis for its evolutionary conservation in mammals was unclear for decades following its initial description.

The first clue to a role for IDO in immune regulation was the discovery that expression of the *INDO* gene is strongly induced at the transcriptional level by the macrophage-activating cytokine interferon-γ (IFN-γ). This discovery prompted an interpretation of long-standing clinical observations indicating that tryptophan catabolites are elevated significantly in the urine of cancer patients [6]. Briefly, the idea was that IDO levels driven by IFN-γ activity in cancer patients was responsible for their elevated levels of tryptophan catabolites. Consistent with this idea, it was reported later that IDO activity was elevated in lung tumors [7]. Given the antitumor properties of IFN-γ, a consensus emerged around the interpretation that IDO performed a tumor suppressor function that contributed to the antitumor effects of IFN-γ activity by starving growing tumor cells of tryptophan [8]. Subsequently, seminal work by Munn and Mellor and their colleagues introduced the revolutionary concept that IDO functions to suppress immunity based on experimental evidence that T cells are selectively affected by tryptophan starvation leading to an impairment of antigen-dependent T cell activation in microenvironments in which tryptophan levels are reduced by IDO activation [9, 10]. In cancer, this interpretation implied that IDO could perform an oncogenic function to limit immune eradication of tumor cells based on recognition of 'foreign' tumor antigens. The ability of IDO to promote immune tolerance to 'foreign' antigens was most dramatically illustrated by the ability of the specific bioactive IDO inhibitor 1-methyl-tryptophan (1MT) [11] to elicit MHC-restricted T cell-mediated rejection of allogeneic mouse concepti [12, 13], a result that has been corroborated by our group.

Subsequent studies have furthur substantiated the concept that tryptophan catabolism can regulate T cell immunity. In particular, it has been found that antigen-presenting cells (APCs) can upregulate IDO in response to IFN-γ that is produced by activated T cells, suggesting that IDO participates in a negative feedback loop that regulates T cell activation. With regard to the role of IDO in cancer, recent findings have produced a radical rethinking of what elevated tryptophan catabolism means to the developing tumor, by introducing the concept that IDO activity may be a way for tumors to promote pathological tolerization, defeating anti-tumor immunity and facilitating immune escape.

COMPLEX CONTROL OF IDO BY IMMUNE REGULATORY FACTORS

IDO is integrated within a complex milieu of factors that promote immune tolerance. The nature of the integration of IDO appears to be complex and may be context-dependent. One important regulator of IDO expression levels may be the proinflammatory prostaglandin PGE-2, which is elevated frequently during cancer progression as a result of activation of cyclooxygenase-2 (COX-2). IDO is induced by PGE-2, consistent with its respective role in promoting immune suppression. Interestingly, while PGE-2 is employed widely as an *in vitro* maturation factor for dendritic cells, treatment of these cells with PGE-2 has been reported to elevate IDO expression ~100-fold [14]. Whether such preparations may compromise the desired immune stimulatory activity of DCs used in the setting of cancer vaccines is unclear: PGE-2 treatment is sufficient to induce IDO message, but the induction of IDO enzymatic activity appears to require an additional signal(s) that can be triggered by exposure to tumor necrosis factor (TNF) or agonists of Toll-like receptors (TLRs). In support of the concept that IDO mediates some of the immune inhibitory effects of COX-2 and PGE-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 [15]. The relationship between PGE-2 and IDO is clearly complex, insofar IDO can provide a negative feedback on prostaglandin synthesis [16].

Other important immune regulatory agents that can influence IDO activity are nitric oxide (NO) and transforming growth factor-ß (TGF-ß). IDO and inducible nitric oxide synthase (iNOS) appear to be mutually antagonistic in DC-based studies [17-19]. In the 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 PTEN activity relieves the negative regulation that phosphorylated Akt imposes on FOXO3a-mediated transcription of superoxide dismutase (SOD2), which degrades peroxynitrate [20]. Through this complex route, activation of IDO gene expression is blocked by NO + superoxide. Conversely, this blockade can be reversed by limiting NO + superoxide production or by CTLA-4-mediated activation of SOD2 *via* PTEN/Akt/FOXO-3a signaling. 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 would be useful. Second, small molecule inhibitors of Akt that are being developed as anticancer therapeutics will tend to heighten immunosuppression by phenocopying the effects of CTLA-4-Ig on IDO expression. Recent findings suggest that Akt inhibition may also heighten the invasive capability of cancer cells [21]. Thus, for cancer treatment, the desirable proapoptotic quality of Akt inhibitors may be balanced by their undesirable proinvasive and immunosuppressive properties. TGF-β has also been reported to antagonize IFNγ-mediated induction of IDO expression, in this case in fibroblasts [22]. This appears to run counter to immunosuppressive activity ascribed to TGFβ

but is consistent with its ability to antagonize positively regulated targets of IFNγ. The balance between the effects of TGFß and IFNγ signaling on IDO expression in different cells of tumor and peripheral microenvironments provides a complex mechanism for local control of IDO activity.

IMMUNE TOLERANCE *VIA* IDO IN DENDRITIC CELLS

Although the default mode for immature DCs appears to be toleragenic, even more effective suppression may be achieved with appropriate maturation [23]. A number of DC subgroups in mice have been ascribed toleragenic properties [24-28]. Whether any of these represent distinct lineages or alternative differentiation outcomes for a common precursor has yet to be determined, however, the tumor itself plays a key role in determining the type of response that tumor-associated DCs will elicit [29, 30]. In particular, a novel subset of potently toleragenic IDO-expressing plasmacytoid DCs coexpressing the B-cell lineage marker CD19 has been identified that specifically accumulates in mouse tumor draining lymph nodes [31]. These IDO-positive pDCs have been implicated in stifling antitumor T cell responses through cross-tolerization as well as other inhibitory mechanisms [23].

Local catabolism of tryptophan through induction of the enzyme indoleamine 2,3-dioxygenase (IDO) in DCs has been proposed as a mechanism for inducing tolerance [32]. In addition to being directly toleragenic, mature DCs have the capacity to expand T_{regs} [33]. B7 costimulatory signals from DCs appear to play a critical role in T_{reg} development as both CD28-null and B7-1/B7-2-double null mice were found to exhibit markedly reduced T_{reg} cell populations [34], and constitutively expressed B7 costimulatory molecules maintain self tolerance through suppression of T cell activation by sustaining a T_{reg} population [35]. $CD4^+CD25^-$ T cells transferred into congenic animals can be converted into T_{reg}-like cells *in vivo*, but this does not occur if the recipient mice lack expression of B7 costimulatory molecules [36]. Reciprocally, CTLA-4 binding of B7 molecules on DCs induces IDO [37-39].

Accumulating evidence indicates that $CD4^+CD25^+$ T_{regs} play an indispensable role in the maintenance of negative control over pathological as well as physiological immune responses [40-42] and that removal of T_{regs} not only elicits autoimmune diseases but also enhances responses to non-self antigens including xenogenic proteins and allografts [43]. In mice, the absence of T_{regs} has been reported to lead to gestational failure due to immunological rejection [44] and adoptive transfer of pregnancy-induced T_{regs} can protect against fetal rejection in abortion prone mice [45]. CTLA-4 is a major signaling molecule for T_{regs} and IDO has been implicated to be an important downstream effector for CTLA-4-mediated immune tolerance. The first *in vivo* evidence for this was the observation that, in a diabetic mouse model, the ability of administered CTLA-4-Ig to effectively suppress immune rejection of pancreatic islet allografts was defeated by concurrent treatment with the IDO inhibitor 1MT [46]. This study further suggested that CTLA-4-Ig-mediated tolerance occurs through a heterodox mechanism of 'reverse' signaling through B7 molecules on APCs, which promotes IFN-γ to induce IDO. $CD4^+CD25^+$ T_{regs} that constitutively express CTLA-4 on their surface could likewise promote IDO activity in DCs through a CTLA-4 dependent mechanism [38] and defects in CTLA-4 signaling that promote IDO activity have been implicated in the failure of IFN-γ to activate the toleragenic properties of DCs derived from female non-obese diabetic (NOD) mice early in prediabetes [20]. As mentioned above, *in vivo* evidence from this model indicates that IFN-γ signaling in DCs is impaired as the result of NO-dependent nitration of STAT1 that can be overcome by CTLA-4-Ig treatment *via* Akt/FOXO3a-mediated activation of SOD2 [20].

Other work has confirmed that systemic administration of CTLA-4-Ig results in IDO upregulation in DC subsets, including $CD8\alpha^+$ DCs. Systemic CTLA-4-Ig can also block clonal expansion and CTL activity of H-2Kb-specific T cells from TCR transgenic mice adoptively transferred into H-2Kb hosts. This effect of CTLA-4-Ig was abrogated by treatment with 1MT or by using IDO-null knockout mice as hosts [47]. This latter control is especially informative because it argues against effects of 1MT on targets other than IDO, thereby offering direct evidence that 1MT is a specific inhibitor of IDO and not other tryptophan- or indoleamine-binding proteins [48]. Further studies showed that administering either CTLA-4-Ig or a clonal $CTLA\text{-}4^+$ Treg cell line to mice *in vivo* resulted in elevated IDO activity in specific subsets of splenic DCs that correlated with increased T cell inhibitory activity in a mixed lymphocyte reaction (MLR) but was independent of IFN-γ expression [49]. In particular, following CTLA-4-Ig administration, the $CD11c^+$ $CD19^+$ splenic subpopulation of pDCs, akin to the IDO-expressing suppressor population identified in tumor draining lymph nodes, was shown to induce IDO though a STAT1 dependent mechanism elicited by type I interferon signaling and not IFN-γ [28]. The $CD19^+$ pDC population was found to also respond to CpG- oligodeoxynucleotide (ODN) signaling through the TLR9 receptor to induce IDO, again through type I IFNs [50]. However, the role of interferon signaling in IDO induction has not been fully resolved. The group that first identified IFN-γ as important for IDO induction in DCs has found that type I interferons can induce IDO activity in a splenic pDC population, but that this occurs in response to engagement of CD200 on the pDCs while CTLA-4-Ig engagement of B7 was found to signal through IFN-γ [51, 52]. Another study has reported that systemic administration of CpG-ODN elicits toleragenic induction of IDO through TLR9 except that the induction was independent of both type I and II interferons [53]. Clear resolution of the precise role of interferons in the induction of IDO in DCs may be obfuscated by the complexities of the experimental systems used to make these determinations, such as genetic variability between models, methods of cell isolation, and culture conditions.

The finding that IDO mediates the effect of CTLA-4 runs counter to the idea that CTLA-4 is directly antagonistic to CD28 on T cells, either through out-competing CD28 for access to B7 ligand, inducing immunosuppressive cytokines, or directly interfering with CD28-mediated and/or TCR-mediated signaling [54]. The different mechanisms need not be mutually exclusive, however, especially considering that loss of IDO activity, either pharmacologically or genetically, does not completely

phenocopy genetic loss of CTLA-4. Thus, IDO does not account for all of the immunological activity of CTLA-4. Notably, IDO-null knockout mice have not been observed to exhibit spontaneous autoimmune disease. This observation suggests that IDO is not required for homeostatic maintenance of central or peripheral tolerance to self antigens. In contrast, acquired tolerance is defective in IDO-null mice suggesting that induction of IDO activity might play a role in the acquisition of tolerance to neoantigens [47]. Becoming tolerized to neoantigens is critical to maintaining pregnancy and may be similarly important to permit progression of early neoplastic lesions, which present mutated oncoproteins to the immune system.

Much of the published work on the role of IDO in DCs has come from investigating the toleragenic mechanism elicited by CD11c$^+$CD8α^+ DCs in the context of challenge with self/tumor antigens. One model that has been used extensively for immune tolerance experiments is a delayed type hypersensitivity (DTH) skin test model, in which mice receiving peptide-pulsed DCs were assayed for MHC class I-restricted reactivity by footpad challenge with peptide. Fractionated CD8α^- myeloid DC presented with tumor/self peptide were actively immunogenic but could be effectively inhibited by reintroduction of a minority population of CD8α^+ lymphoid DC. The Th1-asssociated cytokines IL-12 and IFN-γ were found to work at cross purposes in this system, with toleragenic suppression relieved by exposure of the CD8α^- population to IL-12 but reestablished by exposure of the CD8α^+ population to IFN-γ [55, 56]. Addition of the IDO inhibitory compound 1MT suppressed the ability of IFN-γ to overcome the adjuvant effect of IL-12. Induction of IDO activity by IFN-γ in CD8α^+ cells, which exhibit a significant basal level of IDO expression, has been shown to be regulated both at the level of expression as well as post-translationally [57]. Interestingly, the basal and induced levels of IDO expression appear to be comparable in the CD8α^- population but IDO activity remained suppressed in response to IFN-γ. Responses to engagement of cell surface molecules on DCs were found to play a key role in determining whether DC activity would be immunogenic or toleragenic. Engagement of CD40 with agonistic antibody blocked IFN-γ-mediated induction of IDO activity in toleragenic CD8α^+ DCs and induced these cells to actively prime rather than suppress CTL responses to self/tumor peptide in the DTH model [58]. The agonistic CTLA-4-Ig receptor/antibody fusion protein had an opposite effect. Immunogenic CD8α^- DCs treated with CTLA-4-Ig induced IDO activity and were rendered toleragenic [59]. Together, these findings highlight the functional plasticity of these different DC subsets.

CD28-Ig binds B7 molecules, like CTLA-4-Ig, except with the opposite effect of promoting immunogenicity [60]. DCs treated with CD28-Ig exhibited early and sustained production of IL-6 that was not induced by CTLA-4-Ig. IL-6 upregulates SOCS3 which inhibits the STAT-dependent IFN-γ signaling required for IDO induction and, consistent with these signaling connections, silencing of SOCS3 expression in DCs caused CD28-Ig to elicit a CTLA-4-Ig-like toleragenic response [60, 61]. Gene expression profiling in CD8α^+ DCs has revealed that the *Tyrobp*-encoded DAP12 protein, which is controlled by IFN-γ *via* negative transcriptional regulation imposed by IRF-8, is important for post-translational suppression of IDO activity [62]. DAP12 belongs to the family of immunoreceptor tyrosine-based activation motif (ITAM)-bearing membrane adaptor molecules that associate with the transmembrane regions of activating receptors in NK and myeloid cells. CD8α^+ DC from transgenic mice overexpressing the DAP12 protein exhibited impaired toleragenic function, while CD8α^+ DCs lacking DAP12 function exhibited increased IDO-dependent toleragenic activity [62]. These observations argue that DAP12 acts as a negative modifier of IDO activity.

In addition to DCs, other immune cells that may utilize IDO for immunosuppression include macrophages, granulocytes and neutrophils. The first indications of IDO involvement in the suppression of T cell activation were obtained from *in vitro* studies of macrophages exposed to CSF-1, which induced IDO activity in the context of a mixed lymphocyte reaction (MLR). Expression of CSF-1 in tumors has been implicated in polarizing macrophages toward an M2 suppressor phenotype [63]. Studies in the STAT6 knockout mouse support a role for both myeloid suppressor cells (MSCs) and tumor-associated macrophages (TAMs) in tumor metastasis. STAT6 knockout mice are resistant to metastases produced by isogenic tumor grafts of the highly metastatic breast cancer cell line 4T1. The resistance observed in these animals has been linked to diminished MSC induction coupled with a reduction in M2 TAMs. In CD-1 knockout mice, which lack IL-13 producing NKT cells, polarization toward an M2 phenotype is no longer supported after implantation of 4T1 cells and the resulting production of cytotoxic M1 macrophages is associated with rejection of 4T1 tumors [64]. Thus, a permissive environment for NKT cells and activated T cells combined with a reduction in MSCs is sufficient to result in effective immune surveillance and rejection of metastatic 4T1 tumors. Local catabolism of the amino acid arginine through induction of the ARG1 enzyme is one mechanism by which M2 macrophages can promote tumor tolerance through suppression of effector T cell responses [65]. In addition, when cultured in the presence of CSF-1, macrophages display elevated IDO activity [10], which also suppresses effector T cell responses. The production of TGF-β and IL-10 by TAM would also perpetuate polarization toward a pro-toleragenic state [63].

Precisely how IDO-mediated tryptophan catabolism elicits immune tolerance remains somewhat uncertain given evidence for different but not necessarily mutually exclusive mechanisms of action. Some studies in DCs have indicated that induction of IDO activity, triggered by exposure to either IFNγ or CTLA-4-Ig, may be necessary for acquiring rather than directly eliciting the suppressor phenotype [55, 62]. The former possibility is consistent with the finding that DC maturation in response to TNF or LPS treatment is suppressed by 1MT along with the chemokine receptors CCR5 and CXCR4, which mediate tumor migration and infiltration [66]. Most attention, however, has focused on the direct suppression of T cells by IDO-mediated tryptophan catabolism. In fact, both depletion of tryptophan and accumulation of tryptophan catabolites have been implicated in mediating T cell suppression, and *in vitro* studies meant to demonstrate which of the two is more relevant to immunological suppression have produced contradictory results [67]. Recent *in vivo* data are likewise

incongruent. There is genetic evidence that signaling through the GCN2 kinase pathway, which responds to environmental depletion of tryptophan, elicits the arrest response in T cells: T cells from Gcn2-null mice are no longer responsive to IDO-expressing DCs, arguing that IDO-mediated induction of growth arrest and anergy in responding T cells is signaled by stress signals mediated by GCN2 kinase [68]. On the other hand, systemic treatment of mice with the IDO catabolite mimetic N-(3,4-dimethoxycinnamoyl) anthranilic acid (3,4-DAA) has been reported ameliorate symptoms in a mouse model of multiple sclerosis in conjunction with a reduction in inflammatory foci in the brain and spinal cord [69]. In this study, 3,4-DAA interfered with IFN-γ induced STAT1 signaling in a microglial cell line and suppressed the activation of APCs *in vivo*, but no data on its effects on T cells independent of APCs was presented. One possible explanation consistent with these two *in vivo* reports is that depletion of tryptophan might have a direct effect in suppressing T cell activation while the accumulation of tryptophan catabolites might act indirectly to further impair T cell activation by suppressing immunogenic APCs.

IDO DYSREGULATION IN CANCER CELLS

Malignant development is accompanied by a breakdown normal cellular physiology. During this process, cell-intrinsic traits acquired include immortalization, growth sufficiency, insensitivity to growth inhibitory signals, and apoptosis resistance, whereas cell-extrinsic traits include angiogenesis, invasive capability, metastatic capacity, and immune escape. The latter trait can be viewed as a terminal feature of immunoediting, which is comprised of surveillance, equilibrium, and ultimately escape of tumor cells from effective control by the immune system [70]. Immune escape has only recently gained wide recognition among cancer geneticists as a crucial feature of malignant development: this following a definitive proof in knockout mice (IFN-γ, Stat1, and Rag2 knockouts) that genetic ablation of T cell immunity is sufficient to increase tumor incidence [71]. The dynamic interplay of plastic tumor cells with the innate and adaptive immune systems constitutes thrust and parry in a battle for the upper hand. On one hand, inflammatory properties provided by immune cells can provide a supportive tumor microenvironment [72]. On the other hand, immune surveillance limits malignant growth but also provides a selective pressure for the evolution of mechanisms capable of subverting or evading the immune response that tumor antigens elicit [73].

IDO activation represents one mechanism evolved by tumor cells to escape the immune system. The value of this mechanism to a tumor cell that has evolved it is balanced by the cost of depriving itself of an essential amino acid. Thus, like other pro-oncogenic alterations that accumulate in cancer cells, IDO activation represents a stochastic event whose value is determined by the particular setting of transformation and immunoediting pathways that are relevant to a particular malignancy. As mentioned above, elevated levels of tryptophan catabolites have been documented in the urine of cancer patients and this elevation is reversed upon surgical tumor reductive therapy (e.g. [6]). While it has not been proven that IDO elevation is responsible for this phenomenon, it seems likely given the common elevation of IDO in human tumors [74] as well as the lack of other IDO-related genes reported in the human genome.

How does IDO become deregulated in cancer cells? One mechanism that has been reported involves *Bin1*, a gene that is frequently attenuated in breast cancer, prostate cancer, melanoma, neuroblastoma, and other cancers [75-78]. Bin1 was initially identified in a two-hybrid screen for Myc-interacting proteins [79]. Numerous investigations of *Bin1,* also known as *Amphiphysin II*, have suggested a function for this gene in cancer suppression [75-83]. Recent genetic studies in mice targeted for homologous deletion of *Bin1* indicates that it facilitates apoptosis and limits proliferation and immune escape in oncogenically transformed cells [84, 85]. Along with the Bin3 gene, Bin1 is one of two evolutionarily conserved members of an adapter protein family termed BAR adapters. This family of adapters is named for the presence of a signature fold called the BAR domain, which can mediate interaction with curved vesicular membr**31**ernately spliced to generate at least 10 isoforms that differentially localize to diverse nuclear, cytosolic, and membraneous sites in cells [87-93]. Only two of these splice isoforms are ubiquitously expressed, whereas the remainder are restricted to specific terminally differentiated tissues including neurons and skeletal muscle cells. Additionally, only the ubiquitous and muscle-specific isoforms of Bin1 that can access the nucleus display anticancer properties. BAR proteins make diverse interactions in cells and a simple and readily classifiable function for Bin1 has yet to emerge. However, existing information suggests that the ubiquitous Bin1 isoforms and certain other nucleocytosolic BAR family adapter proteins may act not only in trafficking processes at vesicular membranes but also in transcriptional processes at the nucleus [80, 94]. For example, recent studies offer some genetic support for the notion that *Bin1* may modify the efficiency of nuclear trafficking or function of the NF-kB and STAT transcription factors [84, 85]. These connections are interesting in the present context given their important role in immunity as well as cancer.

Investigation of how *Bin1* loss facilitates the outgrowth of oncogenically transformed cells has identified immune tolerance *via* IDO activation in the transformed cells as a mechanistic explanation [85]. Targeted deletion of *Bin1* in mouse cells resulted in superinduction of IDO gene expression by IFN-γ. Transformation of *Bin1*-null and *Bin1*-expressing mouse embryo keratinocytes with *c-myc+ras* oncogenes produced cell lines with similar *in vitro* growth properties. However, when these cells were introduced into syngeneic animals, the *Bin1*-null cells formed large tumors whereas the *Bin1*-expressing cells formed only indolent nodules. This dichotomy reflected a difference in immune response to the cells, as *Bin1*-expressing cells produced rapidly growing tumors when introduced into either athymic nude mice or syngeneic mice depleted of $CD4^+$/$CD8^+$ T cells. Treatment with the IDO inhibitor 1MT suppressed the outgrowth of *Bin1*-null tumors in syngeneic mice but this effect was absent in both nude mice and immunodepleted syngeneic animals. Taken together, the findings showed how the deregulation of IDO by *Bin1* loss promoted tumorigenicity by enabling immune escape. Given the frequent attenuation of *Bin1* expression and the frequent overexpression of

IDO in human cancers, it will important to further evaluate the relationship between these two events.

Several recent studies suggest that IDO overexpression is associated with poor prognosis in cancer. In a small study of ovarian cancers, immunohistochemical overexpression of IDO in tumor sections correlated inversely with patient survival, such that higher immunohistochemical levels of IDO staining in tumors was associated with poor survival [95]. In contrast, all patients with tumors classified as negative for IDO staining in this study survived at least 5 years after surgery. For sporadic, focal, and diffusely staining tumors, the 50% survival of patients was 41, 17, and 11 months, respectively [95]. A similar trend was found in a larger study of IDO in colorectal cancer, where levels and activity of the enzyme were examined by RT-PCR, immunohistochemistry, and HPLC analysis [96]. IDO expression and enzyme activity in colon cancer cells was strictly dependent on IFN-γ stimulation. High IDO expression was associated with increased incidence of liver metastases, as compared to tumors expressing low IDO, and with reduced infiltration of tumors by $CD3^+$ T cells. High IDO was, likewise, significantly associated with reduced survival and comparative analyses assigned it as an independent prognostic variable [96]. Interestingly, the same group has observed elevated IDO in inflammatory bowel disease, a condition associated with a significant increase in the risk of colon cancer [97]. Additional studies also capture the possibility that IDO expression in stromal cells may have prognostic value. In a retrospective pilot study of patients with malignant melanoma, abnormal accumulation of IDO-positive cells in the tumor draining lymph node was significantly associated with poor survival outcome [31]. These infiltrating IDO-expressing cells have been further characterized as expressing markers that are consistent with mature myeloid DCs but they do not appear to be conventional interdigitating DCs or macrophages, but rather a distinct population [98]. Another small study of non-small cell lung cancer patients revealed IDO overexpression in tumor infiltrating eosinophil granulocytes, rather than in DCs or tumor cells, and associated the level of IDO-positive eosinophil infiltrate with poor survival [99]. While it will be important to extend these early observations, they exhibit a similar trend that is consistent with the expectation that by facilitating tumoral immune escape IDO activation may provide a powerful driver of malignant progression.

IDO AS A TARGET FOR THERAPEUTIC INTERVENTION

A small number of studies have offered evidence that IDO inhibition with 1MT or other small molecule inhibitors can exert antitumor effects. Initial evidence was offered in 2002 that the IDO inhibitor 1MT could partly retard the growth of mouse melanoma cells engrafted onto an syngeneic host [100]. Similar results were obtained as part of an investigation to assess the consequences of IDO overexpression in human tumors that had been documented widely [74]. In this study, ectopic overexpression of IDO in an established tumor cell line was shown to be sufficient to promote tumor formation in pre-immunized animals which could be at least partially reversed by treatment with IMT. However, pharmacokinetic analysis was not performed in these studies, so it was unclear whether 1MT dosing may have been insufficient to achieve more pronounced efficacy, or whether it might be possible to regress the growth or survival of an established tumors (as compared to inhibiting the outgrowth of subcutaneously seeded tumor cells). Subsequent studies performed in our laboratory determined that 1MT was pharmacologically stable in serum, and that under dosing conditions that were effective in eliciting allogeneic conceptus rejection it could accumulate to levels consistent with *in vivo* inhibition of IDO. Under such conditions, we confirmed that 1MT could retard tumor outgrowth, in this case in established autochthonous (spontaneously arising) mammary tumors in the MMTV-neu/HER2 transgenic mouse model of breast cancer [85]. By itself, 1MT was unable to elicit tumor regression in the model, as shown previously in the 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 [85]. Immunodepletion of $CD4^+$ or $CD8^+$ T cells from the mice before treatment abolished the combinatorial efficacy observed in this model, confirming the expectation that 1MT acted indirectly through activation of T cell-mediated antitumor immunity. We extended these observations using novel small molecule inhibitors that we have identified, including several thiohydantoin derivatives of tryptophan. For example, continuous administration of methylthiohydantoin-tryptophan was found to retard the growth of MMTV-neu/HER2 tumors and to elicit regressions in combination with paclitaxel, in the absence of increased side-effects, displaying the same pattern of antitumor properties as 1MT [85]. In each case, regressions could not be explained by 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). Recently, we have observed that efficacy in achieving 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. Strikingly, as little as 4-5 days of 1MT administration on a 2 week trial is sufficient to produce regressions in combination with chemotherapy in the model (A.J.M, J.B. DuHadaway, and G.C.P., unpublished observations). In future work, to validate IDO as the target of putative inhibitors such as 1MT, it will be important to show that additional bioactive inhibitors have similar antitumor properties, that antitumor and pharmacodynamic responses can be correlated appropriately, and that genetic manipulations of IDO can alter the response to putative inhibitors. However, taken together, the existing results offer an initial step in validating IDO for drug development in the context of a cytotoxic combination treatment modality.

DISCOVERY AND DEVELOPMENT OF IDO INHIBITORS

IDO has a number of appealing features as a target for drug development. First, as a single-chain catalytic enzyme

with a well-defined biochemistry, IDO is very tractable for discovery and development of small molecule inhibitors. By comparison, many other proposed therapeutic targets in cancer are more challenging in terms of tractability. Second, no genomic relatives of IDO have been reported. The other enzyme that catalyzes tryptophan degradation, TDO2, is structurally distinct and has a much more restricted pattern of expression and substrate specificity. This quality of IDO 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, an *Indo* gene ''knockout'' mouse that has been constructed is reported to be viable and healthy [101]. While further analysis is necessary, this observation encourages the notion that IDO inhibitors will not produce unmanageable mechanism-based toxicities. Fifth, pharmacodynamic evaluation of the IDO inhibitors can be performed easily 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 therapies which can modulate T cell immunity.

The rational design and development of new inhibitory compounds requires understanding 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 [102]. The recent publication of an X-ray crystal structure for IDO complexed with a simple inhibitor will greatly facilitate this work [1]. Alternately, screening for novel inhibitors is likely to identify novel structural series to evaluate. Through this route, our group has identified the natural product brassinin as an IDO inhibitor and evaluated brassinin derivatives for *in vitro* potency and cell-based activity [103]. Brassinin is a phytoalexin compound found in cruciferous vegetables that has potent chemopreventative activity against breast and skin cancer in rodent models [104, 105]. In order to probe the relationship between inhibitors and the active site, that is, perform a structure-activity relationship (SAR) analysis, we synthesized a series of derivatives from the core brassinin structure [103]. 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 [11], 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 ~1 μM 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 number of IDO inhibitory compounds representing diverse structural classes has recently been reported in a unique yeast screen [106]. Consistent with SAR-based evidence that the indole core is not critical for enzyme inhibition, at least one of the non-indole compounds identified in the yeast screen exhibited sub-micromolar potency. Given the intriguing features of IDO as an immune modulator in cancer and other diseases, future efforts to identify IDO inhibitors may broaden quickly.

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