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Blocking IDO activity to enhance anti-tumor immunity

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1. ABSTRACT

Tumors express potentially immunogenic antigens, yet the immune response to these antigens is typically profoundly suppressed. Patients with established tumors behave as if they were functionally tolerant to any antigens associated with the tumor. This tolerance reflects a process of active immune suppression elicited by the tumor, and represents a critical barrier to successful anti-tumor immunotherapy. Indoleamine 2,3-dioxygenase (IDO) is a natural immunoregulatory mechanism contributes to immune suppression and tolerance in a variety of settings. In tumor-bearing hosts, animal models suggest that tumor-induced IDO helps create a tolerogenic milieu within the tumor and the associated tumor-draining lymph nodes. IDO directly suppresses the proliferation and differentiation of effector T cells, and markedly enhances the suppressor activity of regulatory T cells (Tregs). Together, these effects contribute to the inability of the immune system to respond effectively to tumor antigens. Treatment of tumor-bearing animals with IDO-inhibitor drugs enhances anti-tumor immune responses, and IDO-inhibitors are synergistic with a variety of chemotherapeutic drugs, anti-tumor vaccines and other immunotherapy. Strategies to pharmacologically inhibit IDO may thus enhance immune-mediated responses following conventional chemotherapy, and may be synergistic with other forms of immunotherapy.

2. TUMOR-INDUCED TOLERANCE

Tumors express a variety of potentially immunogenic antigens (1), and there are many T cells in tumor-bearing hosts that are specific for tumor-associated antigens (2, 3). Despite this antigenicity, once tumors become established they are not spontaneously rejected by the immune system. Functionally, the immune system behaves as if it were tolerant to all antigens associated with an established tumor.

This state of functional tolerance applies not only to self antigens shared by the tumor, but also to authentically foreign antigens (tumor-specific neo-antigens) as well. In some cases the immune system seems unaware of antigens on tumors (immunologic "ignorance") (4, 5); but in many cases the tumor-associated antigens are clearly detected by the immune system, yet there is no effective immune response (tolerance). In the case of autochthonous tumors, this state of acquired tolerance has been shown to be created very early during tumor development (6). During development, the immune system may transiently retard the growth of the developing tumor for a time (7), but all clinically-apparent tumors have evolved mechanisms to escape this immune surveillance.

The molecular mechanisms by which tumors create functional tolerance to themselves are not yet fully defined, but they clearly represent an active process. This

is readily seen in the case of transplantable tumors engineered to express a known, immunogenic foreign antigen: when they are implanted, these tumors rapidly create tolerance to the new antigen (8-10). Of critical importance for understanding this process, tolerization appears driven by the host immune system, involving the active participation of host antigen-presenting cells (8), Tregs (11) and other endogenous suppressive mechanisms. The tumor releases factors that recruit the initial tolerizing milieu (12), but the actual mechanisms of suppression that function in this milieu are generated by the immune system itself.

This represents both a challenge and an opportunity for anti-tumor immunotherapy. The challenge is that the immune system in a tumor-bearing host will actively suppress any attempt to generate a therapeutic immune response against the tumor. But the opportunity is that the molecular mechanisms underlying this pathologic suppression are likely to be the same endogenous tolerogenic mechanisms employed by the immune system in other contexts. Thus, the goal for immunotherapy is to identify these endogenous immunosuppressive mechanisms that have been "hijacked" by the tumor, and develop strategies to circumvent them.

3. IDO IS AN ENDOGENOUS MECHANISM OF IMMUNE TOLERANCE

3.1. Natural biologic role of IDO

Indoleamine 2,3-dioxygenase (IDO) is an evolutionarily ancient enzyme that degrades the amino acid tryptophan. In mammals, it is highly inducible by inflammatory stimuli (13). Initially, the role of IDO was assumed to lie in host defense against infection (by starving the microbes of tryptophan), and in certain types of infection this may be true (14, 15). However, IDO is also an endogenous mechanism of immunoregulation and tolerance in the immune system, and it is in this role that it appears to have been co-opted by tumors. In the immune system, the normal role of IDO is to promote certain specific forms of acquired peripheral tolerance, and to control excessive inflammation (reviewed in (16) and (17)). In tissues, local expression of IDO controls innate immunity and excessive inflammation from chronic infection (18, 19), including direct suppression of pro-inflammatory TH17 cytokines IL-6 and IL-17 (20). IDO also can induce antigen-specific tolerance in T cells. IDO is not required for constitutive tolerance to self, as shown by the fact that mice lacking IDO, or treated with IDO inhibitors, do not develop spontaneous autoimmune disorders. However, in the case of acquired peripheral tolerance (i.e., tolerance to new antigens encountered in the periphery), the effects of IDO can be dramatic. IDO is expressed in the placenta, and mice treated with IDO-inhibitor drug during pregnancy spontaneously reject their allogeneic fetuses (21, 22). IDO is also expressed in antigen-presenting cells of the gut, and IDO activity is required for acquired mucosal tolerance (23, 24). In other models, inhibition of IDO markedly exacerbates graft-versus-host disease (25) and autoimmune disorders (26). In tissue-transplantation models, blocking IDO renders the host refractory to a variety of strategies to induce tolerance toward the new graft (27-29). Conversely, tissue allografts that are engineered to overexpress IDO can spontaneously create tolerance to themselves, and are not rejected even across a fully mismatched MHC barrier (29-31). Thus, in certain settings, IDO functions as a potent natural mechanism for creating acquired tolerance and suppressing T cell responses. This natural role can become pathologic when abnormal IDO expression is driven by tumors.

3.2. Biochemical characteristics of IDO

The system historically referred to as "IDO" comprises two related genes, IDO1 and IDO2. These share sequence homology but differ in their regulation and pattern of expression (32, 33). IDO2 was only recently cloned (32, 34), and IDO1 is the more extensively studied of the two. Genetic polymorphisms exist in both genes, and these polymorphisms can affect functional activity (35). The regulation of IDO gene expression is complex. Both IDO1 and IDO2 mRNAs show multiple splice isoforms, and gene expression and enzyme activity are regulated by SOCS3, NF-κB, DAP12 and IRF8 (36-38). The protein is also post-translationally regulated by ubiquitination and protein nitration via iNOS (37, 39).

Given this complexity, it is not surprising that IDO regulation can differ markedly between different cell types. Even in the same cell type (dendritic cells, for example) IDO may be regulated differently depending on the maturation or activation state of the cell. *In vivo*, IDO is inducible in macrophages and dendritic cells by interferons and other pro-inflammatory signals, and can also be expressed by endothelial cells and other cell types under certain conditions (reviewed in ref. (16)).

IDO1 is a monomeric enzyme with a heme prosthetic group that catalyzes the oxidative cleavage of tryptophan to *N*-formylkynurenine. (An analogous reaction is catalyzed by the housekeeping enzyme tryptophan oxygenase in liver, but TDO is not known to have any immunoregulatory role.) For its oxidase function, IDO1 can utilize either molecular oxygen or reactive oxygen species (e.g., as supplied by cytochrome P450 or other sources) (40). Historically, enzymology studies of IDO have relied on *in vitro* systems that use ascorbate and methylene blue as reducing agents to maintain the heme center in the active redox state. This catalase/methylene blue system is not physiologic, but the real reducing system (or systems) that support IDO *in vivo* are at present unknown. As a caveat, however, it has recently been shown that the conditions of the *in vitro* assay system can markedly affect the kinetics and substrate specificity of the IDO enzyme (40). Thus, extrapolation from *in vitro* systems to *in vivo* enzyme characteristics should be undertaken with a certain caution, since IDO expressed under physiologic conditions may be subject to different reducing systems, cofactors, splice variants and post-translational modifications, depending on the cell type.

3.3. Mechanism of action of IDO

Cells that express IDO create two effects in the milieu around them: depletion of the essential amino acid

tryptophan, and production of a series of kynurenine-pathway metabolites. Both of these effects have immunoregulatory properties. Several kynurenine metabolites have been shown to have immunomodulatory effects (41), in particular 3-hydroxyanthranylic acid (42). In certain models, local production of these kynurenine metabolites by IDO is required to control excessive inflammation in tissues (18). The molecular mechanism of action of kynurenines has not been fully elucidated, but may involve inhibition of PDK1→NFkB signaling (43) and/or signaling through the aryl-hydrocarbon receptor (44).

In addition, IDO reduces the local concentration of tryptophan. Tryptophan is an essential amino acid, and low levels have the dual effect of inhibiting the mTOR kinase pathway (an effect analogous to rapamycin), and activating the amino-acid sensitive GCN2 kinase pathway (45). mTOR kinase is a known regulator of immune responses (46). The GCN2 pathway responds to cellular deficiency of one or more amino acids (47), and cells of the immune system appear highly sensitive to regulation by GCN2 (48, 49). GCN2 activation by IDO affects gene expression in both the cell that expresses IDO (50), and in adjacent T cells to which the IDO^+ cell presents antigen (45, 51, 52). The effects of local amino-acid depletion on mTOR and GCN2 are not confined only to tryptophan; immunoregulatory enzymes such as arginase can produce analogous affects in other contexts (53). Thus, it has been proposed that amino-acid based regulation may represent a generalized mechanism of control in the immune system (54).

3.4. IDO and Tregs

As described above, kynurenine production and tryptophan depletion can directly inhibit the activation, proliferation and survival of effector T cells. In addition, IDO can create potent indirect suppression by activating the regulatory T cell (Treg) system. Naive $CD4^+$ T cells exposed to IDO during activation are biased to become $Foxp3^+$ inducible Tregs (55-58). IDO can also directly activate mature, pre-existing Tregs for markedly enhanced suppressor function (51). In addition, IDO stabilizes the suppressive phenotype of Tregs under inflammatory conditions, and prevents inflammation-induced reprogramming of Tregs into T-helper-like cells (52, 59, 60). IDO-activated Tregs are found in tumor-draining lymph nodes in mice, where they may contribute to tumor-induced immunosuppression and tolerance (51).

3.5. IDO expression by human cell types

Most mechanistic studies of IDO have been performed in mouse models, but IDO can also be expressed by a variety of human cell types. Cultured human monocyte-derived (myeloid) dendritic cells can express high levels of IDO under certain conditions, although (like mouse dendritic cells) the lineage, maturation state and activating stimulus determines whether IDO is expressed or not. In the case of cultured human dendritic cells, a variety of stimuli and conditions have been reported to lead to IDO expression (36, 58, 61-68). Under other conditions, IDO may be expressed at the protein level but without enzymatic activity (62, 69); while under other conditions dendritic cells may not express IDO at all (70). Human macrophages and plasmacytoid dendritic cells can also express IDO (56-58, 71-73). Functionally, IDO expression by dendritic cells or macrophages can inhibit proliferation of human T cells *in vitro* (62, 63, 65, 71-73), and can promote the differentiation of $Foxp3^+$ regulatory T cells from human $CD4^+$ T cells (56-58, 74).

4. IDO AFTER HEMATOPOIETIC STEM CELL TRANSPLANTATION

In mouse models of acute graft-versus-host disease, IDO plays a protective role against lethal inflammation and end-organ damage. One key target appears to be the gastrointestinal tract, since mice lacking IDO suffer severe gut damage in graft-versus-host disease (25, 75). This is reasonable, since IDO is naturally expressed in gut, and has been implicated in mucosal tolerance (24) and control of inflammation during colitis (26). In other models, histone deacetylase inhibitor drugs are immunosuppressive and can ameliorate experimental graft-versus-host disease; it has recently been shown that this effect requires induction of host IDO (76). In humans, monocytes from patients following hematopoietic stem cell transplantation showed elevated levels of IDO, and increased IDO-mediated suppression of T cells *in vitro* (77). The degree to which IDO participates in controlling graft-versus-host disease in humans (and perhaps also contributing to post-transplant immunosuppression (78)) remains to be elucidated; however, based on the results in mouse models, IDO seems likely to play a significant role.

5. IDO AND TUMORS

5.1. IDO and tolerance to tumors

Studies suggest that resting T cells initially become aware of tumor antigens primarily through cross-presentation on host antigen-presenting cells (8, 79, 80). The tumor actively modifies the local milieu (i.e., the tumor microenvironment and draining lymph nodes) so that antigen presentation that occurs in this milieu become anergizing and and tolerogenic (12, 16, 81). Analysis of lymph nodes draining sites of established tumors often show abnormal over-expression of IDO. This can be seen in sentinel lymph nodes of human cancers (62, 82-84), and in experimental tumors in mice (85-87). IDO has also been demonstrated in host stromal cells that are actively recruited by growing mouse tumors, and which appear important for tolerance induction (12) (although the mechanistic contribution of IDO these cells is not yet known).

In mice, IDO-expressing dendritic cells isolated from tumor-draining lymph nodes actively suppress T cell proliferation and create antigen-specific anergy *in vitro* and *in vivo* (45, 85). These IDO^+ dendritic cells also activate $Foxp3^+$ Tregs *in vitro* to become markedly more suppressive (51). The phenotype of IDO^+ cells in mouse tumor-draining lymph nodes is consistent with plasmacytoid dendritic cells, but with additional co-expression of the B cell-lineage marker CD19 (85, 88).

Similar $CD19^+$ plasmacytoid dendritic cells expressing IDO are found in other, non-tumor models of inflammation and tolerance (89, 90). One important point to note is that, in these mouse models, the tumor cells themselves often do not express IDO. The relevant site of IDO expression is thus the host immune cells, and these are often cryptically located in the tumor-draining lymph nodes or in the surrounding stroma at the margins of the tumor (12, 85, 87). This has relevant implications for human studies, because simply biopsying the tumor itself would not have revealed the presence and biological importance of IDO.

5.2. IDO expression in patients with malignancy

In humans, the IDO^+ cells found in tumor-draining lymph nodes are less well characterized, but they often display a "plasmacytoid" morphology (82). Studies of melanoma sentinel lymph nodes showed co-expression of the plasmacytoid-dendritic cell marker BDCA2 on IDO^+ cells (91), and a study of ovarian cancer suggested that the IDO^+ cells co-express CD123 (92). These markers would be consistent with plasmacytoid dendritic cells, although other cell types such as macrophages and endothelial cells in tumors may also express IDO in tumors (72, 93).

IDO has been demonstrated by immunohistochemistry in a wide spectrum of human cancers (94). IDO has been studied in patients with malignant melanoma (85, 95, 96); pancreatic cancer (97, 98); colorectal cancer (99, 100); prostate cancer (93); ovarian cancer (92); acute myelogenous leukemia (74, 101, 102); endometrial cancer (103, 104); and ovarian cancer (105). In several malignancies, the presence of IDO was an independent predictor of a worse clinical outcome (sometimes dramatically worse) (85, 99, 101, 102, 104, 105). In many of these studies, IDO was expressed by the tumor cells themselves (although in most cases the tumor-draining lymph nodes were not studied).

6. CHEMO-IMMUNOTHERAPY WITH IDO-INHIBITOR DRUGS

6.1. Immunologic effects of chemotherapy

An important recent advance in cancer immunotherapy has been the realization that standard chemotherapy drugs can in some cases be combined with anti-tumor immunotherapy to produce synergistic effects (106). Mechanistically, chemotherapy induces a combination of antigen release from dying tumor cells, depletion of suppressor cells, and an immune-activating period of lymphopenia; which together can generate a beneficial (though transient) anti-tumor immune response (107). Indeed, the effects of even standard chemotherapy may be more dependent on this transient immune activation than previously appreciated (108, 109). Unfortunately, the immune response is limited (the tumor invariably re-establishes tolerance); nevertheless, conventional chemotherapy may create a useful window of opportunity in which active immunotherapy can be added to enhance and sustain the immune activation. Clinically, this synergy between chemotherapy and immunotherapy could have profound implications, because patients would no longer need to fail all conventional therapy before becoming candidates for immunotherapy.

6.2. 1MT plus chemotherapy in mouse models

Preclinical studies in mouse tumor models have demonstrated that the IDO inhibitor 1-methyl-tryptophan displays synergy when combined with a variety of chemotherapeutic drugs (22, 110). Active agents included cyclophosphamide, doxorubicin, paclitaxel, cisplatin and gemcitabine. The synergy between 1MT and chemotherapy was immune-mediated, since the effect required an intact immune system. Importantly, this synergy was also demonstrated a model of autochthonous breast tumors, in which each tumor arises through its own unique series of mutations following the initial oncogenic transformation (111). Even though each tumor thus had to develop its own strategy to evade the immune system, all of the tumors responded to the combination of 1MT plus chemotherapy (22). Thus, the fact that 1MT was synergistic with multiple classes of chemotherapy, in multiple different tumor types and genetic mutations, suggests that IDO plays a fundamental and broadly-applicable biologic role in helping suppress the host anti-tumor immune response following chemotherapy.

7. IDO-INHIBITOR DRUGS COMBINED WITH VACCINES AND IMMUNOTHERAPY

Hosts with established tumors respond poorly to therapeutic anti-tumor vaccines (112). In the mouse B16 melanoma model, response to therapeutic vaccination could be significantly enhanced by administration of D-1MT at the time of immunization (52, 60). In part, this appeared to result from the fact that blocking IDO allowed some of the host Tregs to be converted ("re-programmed") into a polyfunctional TH17-like helper phenotype, resulting in enhanced vaccine-induced $CD8^+$ T cell responses (60).

In other studies, administration of D-1MT enhanced the anti-tumor effects of immunotherapy with IL-12 in a 4T1 breast-cancer model (86). Of note, in this latter study the high levels of immunosuppressive IDO were actually created as an unwanted byproduct of the therapy itself (via IFNγ secreted in response to IL-12). This brings up the important point that IDO is frequently induced as a physiologic counter-regulatory mechanism in response to a variety of intense inflammatory stimuli. Thus, for example, IDO is induced by acute graft-versus-host disease (25), excessive lung inflammation (18), high-dose systemic CpG (90) or mycobacterial infection (20, 113). In physiologic settings this counter-regulatory IDO is beneficial, since it prevents mice from dying of uncontrolled inflammation (18, 25). However, in the case of cancer immunotherapy, collateral IDO induction – whether by adjuvants, immune stimulants or activated T cells – would be highly undesirable. Unfortunately, tumor-bearing hosts may be particularly prone to unwanted IDO induction in response to inflammation, especially in the tumor microenvironment and tumor-draining lymph nodes (where IDO is already increased). This could explain why a variety of different mouse models of vaccination and immunotherapy, with diverse mechanisms of action, all benefit from combination with an IDO-inhibitor drug. The common factor may be

that any successful anti-tumor inflammatory response, regardless of how it is generated, is liable to trigger unwanted collateral induction of IDO.

8. PRECLINICAL AND CLINICAL STUDIES OF IDO-INHIBITOR DRUGS

8.1. 1-methyl-tryptophan: D and L isomers

The first pharmacologic inhibitor of IDO to be described *in vivo* was 1-methyl-tryptophan (1MT) (21). A number of additional inhibitors of IDO have been reported in the literature (see refs. (22, 114, 115) and further reviewed in ref. (116)). The most extensively studied inhibitor is 1MT, and this is the first compound to reach clinical trials. 1MT exists as D and L stereoisomers, each with somewhat different properties. L-1MT is far more potent at inhibiting purified IDO in cell-free systems (with the caveat mentioned above that stereoselectivity of the enzyme may be influenced by the choice of *in vitro* assay conditions (40)). L-1MT is also superior at inhibiting IDO expressed in tumor cell lines (110). However, using assays based on primary human or mouse dendritic cells, D-1MT appears somewhat better at reversing inhibition of T cell proliferation by IDO (110). *In vivo*, murine studies directly comparing D-1MT vs. L-1MT showed the D isomer to be superior in combination chemo-immunotherapy (86, 110).

The molecular basis for the difference in biological properties of D-1MT and L-1MT is not yet fully elucidated. It has been suggested that the IDO1 and IDO2 enzymes might be differentially sensitive to D-1MT vs. L-1MT (32), although this has not yet been tested *in vivo*. Perhaps more importantly, as mentioned above the cofactors and reducing system that interact with IDO appear to exert a significant influence on the stereo-selectivity of the enzyme (40). Hence, the relative potency of the D and L isomers of 1MT may differ between different cell types depending on which cofactors, redox systems, and other intracellular conditions predominate in that cell. IDO is also clearly subject to regulation by allosteric effects (40, 117), as well as to transcriptional and post-translational regulation, so some of the inhibitory effects of 1MT may occur in a non-competitive fashion. (For all of these reasons, it may therefore be more accurate to refer to "inhibitors of the IDO pathway", rather than assuming that all inhibitors are competitive.) Finally, it is relevant to consider the possibility of off-target effects, since the L isomer of 1MT is accepted by IDO as a substrate and metabolized (albeit more slowly than authentic tryptophan) into methylated downstream products (117, 118). All of these possibilities will need to be clarified by additional investigation.

That said, both D and L isomers of 1MT have been shown to inhibit functional IDO enzymatic activity (kynurenine production) *in vitro*, when assays are based on physiologically-relevant IDO-expressing cells. The D isomer of 1MT, which is the isomer in clinical trials, has good biological activity in mouse studies, and inhibits functional IDO activity in human cells in multiple models (56, 62, 68, 72, 73, 110, 119). In all of these models, IDO was physiologically expressed by dendritic cells or macrophages. It is possible that the forms of IDO aberrantly expressed by tumor cells may behave differently (92). However, the preponderance of mouse preclinical studies suggest that the relevant IDO-expressing cells are usually those of the host immune system. Thus, while the topic remains the subject of some debate (120), the on-going clinical trials of D-1MT should soon help clarify the issue of biologic activity.

8.2. Phase I clinical trials of D-1MT

The first IDO-inhibitor drug to reach the clinic, D-1MT, is now in Phase I trials. Preclinical pharmacology/toxicology studies showed D-1MT to have good oral bioavailability and a long half-life (compatible with once- or twice-daily administration), with no dose-limiting toxicity in mice, rats or dogs (121). A first-in-humans clinical trial of D-1MT is ongoing, and only interim results have been reported (122). No efficacy data are yet available, but D-1MT appeared well tolerated. Two patients who had previously received immunotherapy with other agents developed Grade 2 hypophysitis while receiving 1MT. This may represent a recall toxicity, because subsequent patients without prior immunotherapy have not shown hypophysitis. The study is ongoing.

9. SUMMARY AND PERSPECTIVE

In patients with established tumors, the immune system actively suppresses anti-tumor immune responses (tumor-induced tolerance). In order for anti-tumor vaccines and immunomodulators to be optimally effective, strategies must be developed to overcome this tumor-induced suppression. IDO is positioned at the intersection of three key immunosuppressive pathways: inhibition of effector T cells, activation of Tregs, and suppression of inflammation. Abnormal levels of IDO are induced in host cells by the presence of tumor, and IDO can also be expressed by the tumor itself. In addition, IDO can be collaterally induced as a counter-regulatory pathway by a variety of pro-inflammatory stimuli, which may act to antagonize the efficacy of vaccine adjuvants and other immunotherapy approaches. Thus, pharmacologic inhibition of IDO may be synergistic with existing strategies of active immunotherapy, particularly if administered in the window of opportunity following conventional chemotherapy.

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Abbreviations: 1MT: 1-methyl-tryptophan; IDO: indoleamine 2,3-dioxygenase

Key Words: IDO, Indoleamine 2 3-Dioxygenase, Tumors, Tolerance, Immunotherapy, Checkpoint Blockade,

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