INDOLEAMINE 2, 3-DIOXYGENASE: POTENTIAL IN CANCER IMMUNOTHERAPY

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ABSTRACT

Indoleamine 2, 3-dioxygenase (IDO) is a potent immunosuppressive enzyme that has a significant role in different types of cancers. There is evidence that shows its involvement in a number of infectious diseases and auto-immune disorders. In vitro and in vivo studies indicate that 1-methyl tryptophan, being a competitive inhibitor, has shown to actively control the conditions in which IDO is over-expressed. Dendritic cells are the natural site of secretion of IDO in the host immune system. However, the expression takes place only in the presence of tolerogenic signals that lead to suppression of T-cell mediated immunogenic responses. Different therapies are being designed by employing the role of IDO in conditions such as stress, depression, cancer, pregnancy, and organ transplant, which reflect the promising role of this new target in cancer immunotherapy.

1. IDO AN IMMUNOSUPPRESSIVE MOLECULE

Indoleamine 2, 3-dioxygenase (IDO) is a single chain, heme-containing oxidoreductase enzyme that is involved in rate-limiting step of tryptophan catabolism. It has an imperative role in conditions like pregnancy, autoimmunity, infection, neoplasia and other malignancies (Curti, et al., 2009). The first and rate-limiting step in tryptophan catabolism is the oxidative cleavage of 2, 3 double bond in the indole ring (Stone & Darlington, 2002) [Figure-1] and also being the first step in biosynthesis of the central metabolic regulator, nicotinamide adenine dinucleotide (NAD). This reaction is catalyzed by two heme-containing enzymes: indoleamine 2, 3-dioxygenase (IDO) and tryptophan 2, 3-dioxygenase (TDO). The expression of TDO is localized to liver whereas the expression of IDO is found in many tissues of the human body (Grohmann, Fallarino & Puccetti, 2003). This expression is further regulated by an intricate display of signals from the immune system, which will be discussed later in the review. It is found that inhibition of IDO in pregnant mice leads to lethal immune rejection of allogenic fetus. In order to suppress the T-cell responses from mother, IDO is continuously secreted at the fetal maternal interface throughout the pregnancy (Kudo & Boyd, 2000).

IDO protein is an apo-enzyme encoded by Indo-gene. The size of this gene is about 15 kbp and it consists of 10 exons. This is a well conserved gene and is located on human chromosome 8. The promoters of IDO gene contain multiple sequence elements that respond to different types of interferon, especially interferon-gamma, which is the main inducer of IDO expression (Suzuki, et al., 2003).

IDO is an intra-cellular enzyme and it does not exist in extra-cellular form. It is commonly found at maternal-fetal interface of placenta, gut, lymph nodes, spleen, epididymis, thymus and lungs. Normally the areas with large lymphoid compartments and widespread mucosal surfaces are found to have the highest degree of IDO expression (Takikawa, et al., 1986).

In mammals, the excess dietary tryptophan is not catabolized by IDO, but by a liver enzyme, tryptophan dioxygenase, and levels of nicotinamide adenine dinucleotide are maintained by conserving it and not by synthesis. Seminal work done by Munn and Mellor and their colleagues, revealed that IDO suppresses T-cell activation, thus modulates immunity (Mellor and Munn, 2004), this was initially demonstrated in the allogenic pregnancy setting (Munn, et al., 1998). T-cells were shown to be preferentially sensitive to activation of IDO, such that tryptophan depletion suppressed T-cell division and resultantly were rendered unable to become activated by Antigen Presenting Cells (APC). The T-cells are also sensitive to tryptophan metabolites, such as kynurenines accumulated by IDO pathway (Fallarino, et al., 2002), which have been shown to be important for T_{regs} induction and immune suppression (Fallarino, et al., 2006; Munn and Mellor, 2007).

2. ISOFORMS

IDO enzymes have been reported to catabolize L-tryptophan into kynurenine, which is biologically an active metabolite. Two isoforms of IDO include IDO1 and IDO2. In 2002, it was revealed that heme region of IDO1 was characterized as having histidine-iron bond, strong steric interactions and hydrogen bonding imposed by L-tryptophan on di-oxygen, which were reported to be crucial in governing the catalytic activity (Terentis, et al., 2002). The IDO1 crystal structure was revealed in 2006 and the complete chemistry of IDO1 reaction was more clearly determined (Sugimoto, et al., 2006).

Initially it was believed that the kynurenine pathway involving tryptophan degradation in extra-hepatic tissues is governed by IDO1, and similarly only IDO1 was thought to perform immunosuppressive effect

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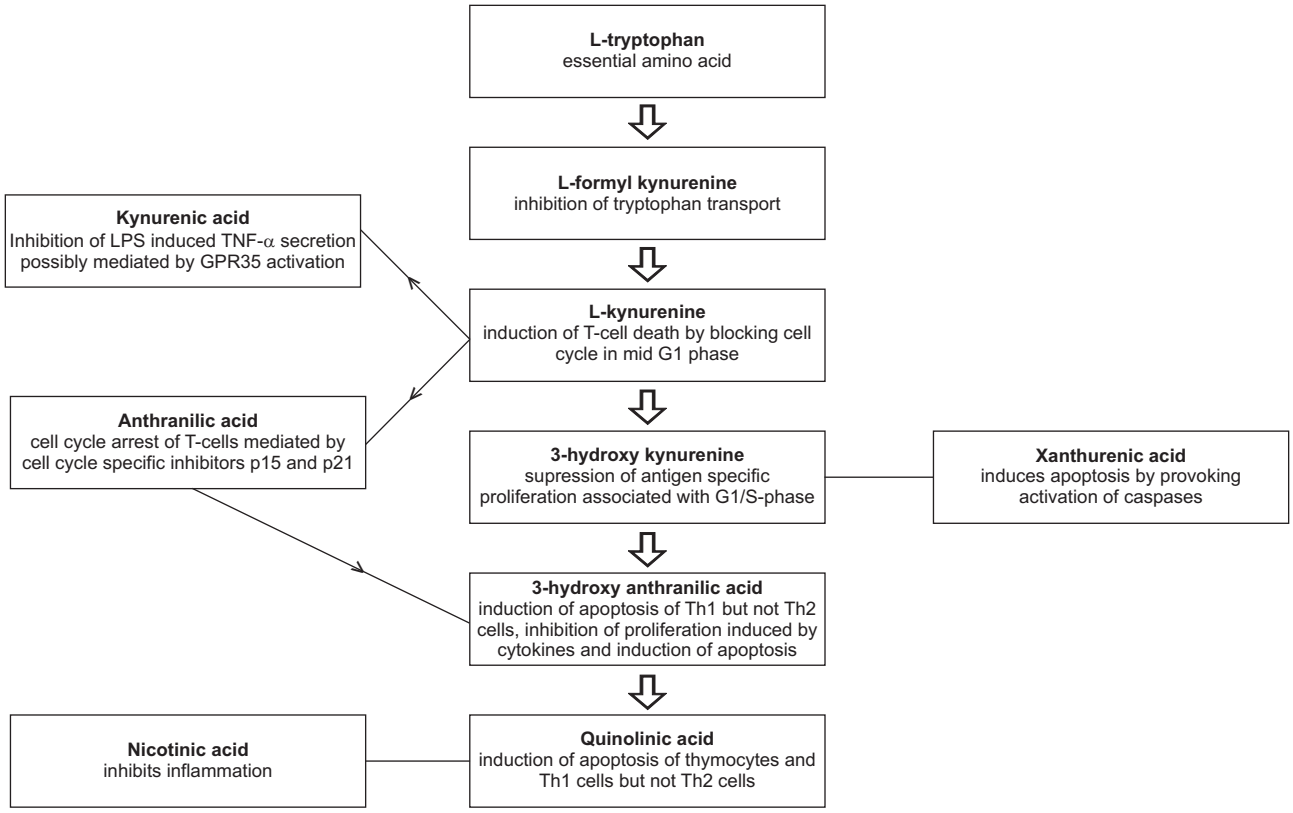

Figure-1: Tryptophan Catabolism Pathway

until recently; however, IDO2, a new isomer and variant was described in 2007 (Ball, et al., 2007; Metz, et al., 2007). The gene responsible for encoding IDO2 is reported to be adjacent to IDO1, and has similar structure as well. These two genes are located at chromosome 8, next to each other in mice as well as humans (Metz, et al., 2007). Both isomers catabolize the same substrate but with different efficiency rate, and show distinct responses while interacting with inhibitors (Lob, 2008; Ball, et al., 2009). Also several reports have shown that the levo isoform of 1MT plays a role in inhibiting IDO1, while D-1MT has shown inhibiting potential against IDO2 (Lob, et al., 2008; Lob, et al., 2009; Ball, et al., 2009).

3. IMMNOSUPRESSION INDUCED BY TRYPTOPHAN DEPLETION

Dendritic cells (DCs) act as the main information management system of the immune system because of their ability to receive a wide array of signals and then determining the type of response needed. When the incoming signals are immunogenic in nature, dendritic cells function to activate the immune system. However, if the signals are tolerogenic in nature like transforming growth factor-β (TGF-β), Interleukin-10 (IL-10) or T-regulatory cells (T_{regs}), the result is modulation of host response against tumor (Andrew & David, 2004). As IDO is found to up-regulate the differentiation of T_{regs}, it is considered to be an operative target for promoting an effective immune response in the state of cancer.

IFN-γ released in response to the Treg induced CTLA-4/B7-dependent T-cell cell-signalling, up-regulates IDO in DCs. Tryptophan degradation caused as a result in the local environment limits the survival and proliferation of T-cells, which otherwise are activated by APCs presenting tumour antigens to them. This process occurs preferentially in tumour draining lymph nodes. In tumour cells IDO expression is super activated by IFN-γ due to BIN1 attenuation (Figure-2), and results in direct suppression of T-cells in local tumour environment.

Research has shown that superoxide ions are a pre-requisite for IDO-mediated tryptophan depletion. This is because the area of infection where leukocytes

actively produce superoxide ions is the prime site for IDO-mediated tryptophan depletion (Hirata & Hayaishi, 1975). In 1984, Pfefferkorn and his coworkers performed some experiments to establish the fact that the growth of intra-cellular parasites can be inhibited by promoting interferon-gamma induced IDO expression. Further, in vitro studies showed that if tryptophan was replenished in the culture media, the conditions would once again become favorable for the growth of infectious micro-organisms. By that time, the role of IDO in limiting the growth of micro-organisms was well established. Later, a new concept appeared on the horizon. According to this, IDO-mediated tryptophan depletion was responsible for regulating the host immune cells. In tryptophan free cultures, T-cells enter the cell cycle but their growth is halted in the mid of G1 phase and they are subjected to apoptotic signals. IDO also has a significant role in suppressing the active immune responses against the allogenic graft. Transfection of IDO to the allograft can prevent or at least delay the immune rejection by sending tolerogenic signals to APCs (Adrian & Angus, 2007).

4. EXPRESSION IN CANCER

Tumor development requires cancer cells to obtain certain characteristics of intrinsic nature, such as resistance to apoptosis, immortalization and self-sufficiency, as well as some cellular extrinsic properties. The latter includes the interaction of tumor cells with immune system that is the most crucial step in determining the transformation of malignant-cells (Curti, et al., 2009). Tumor progression and growth essentially require immune escape (Whiteside, et al., 2006; Gajewski, 2006). Recent studies have shown that IDO is one of the key factors contributing to immunosuppressive strategies induced by tumors (Munn & Mellor, 2007). Various tumor-cell types over-express IDO (Uyttenhove, et al., 2003). IDO protein has been revealed to be involved at both the sites of tumor and draining lymph nodes. At tumor site, the tryptophan depletion, and rise & accumulation of tryptophan metabolites cause loss of effector function and reduce clonal expansion, which result in reduced survival of T-cells. While in draining lymph nodes, plasmacytoid DCs express IDO, thus generating tolerogenic T-cells. In addition, IDO producing DCs have been detected in sentinel lymph nodes of melanoma patients and are associated with poor clinical outcome (Munn, et al., 2004). In humans, correlation of poor prognosis and over-expression of IDO has been reported in endometrial, ovarian and colon carcinoma (Okamoto, et al., 2005; Brandacher, et al., 2006; Ino, et al., 2006). Moreover, in colon cancer, tumoral IDO expression causes reduced tumor infiltration in lymphocytes (Brandacher, et al.,

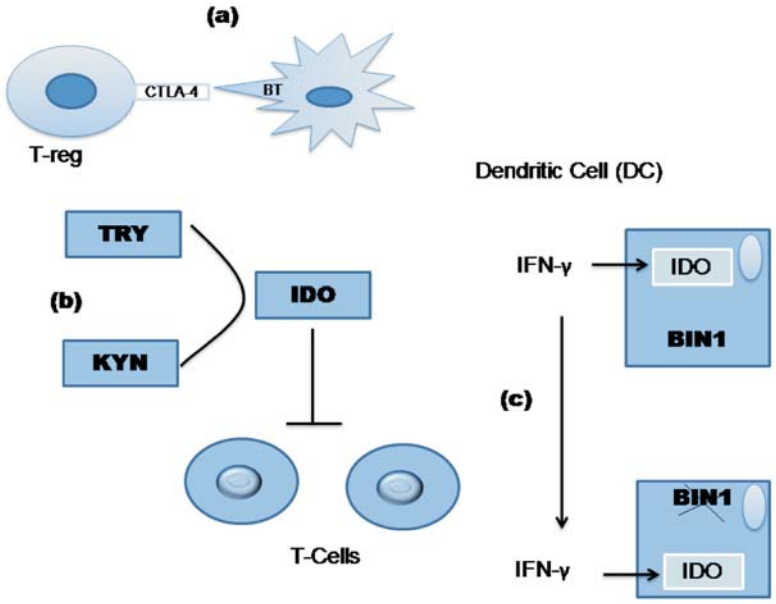

Figure-2: Mechanisms of IDO-Induced Tumor Immune Escape

(a) Treg cells presents tolerogenic signals to the IDO+ cells resulting in release of IDO, which in turn suppresses the T-cell activity; (b) IDO breaks down the essential amino acid tryptophan into kyneurine and inhibits the immunogenic activity if T-cells; (c) IFN-gamma induced IDO expression can be blocked in presence of BIN1

2006). Recently, it has been demonstrated that BIN1, the tumor suppressor gene, has genetic control of IDO expression and is reported to be down regulated in various human cancers. BIN1 loss, might account for pathological condition of IDO deregulation through super-induction of IFN-gamma mediated Indo-gene expression (Muller, et al., 2005).

Studies have shown high expression of IDO in advanced ovarian cancer, and poor patient survival has been associated with it. In addition, it has been demonstrated that overexpression of IDO increases peritoneal tumor dissemination in vivo (Inaba, et al., 2009).

A recent study has revealed that high IDO expression correlates with clinical stage of breast cancer, and therefore plays a very important role in suppressing the immune system in such patients (Sakurai, et al., 2005). The proposed mechanism of immunoregulation that facilitates metastasis is achieved by elevated IDO levels, which results in local T-cell immune suppression in the tumor microenvironment (Brandacher, et al., 2006). The infiltration and amplification of CD4+CD25+T_{regs} promotes and facilitates metastasis and can be related to poor prognosis of cancer (Jinpu, et al., 2011).

Higher cases of lung cancer have reported expression of IDO1, but no significantly determined correlation has been found between expression of IDO1 and clinico-pathological parameters. Immunohistochemical analysis of non-small-cell lung cancer has shown that IDO is not expressed by tumor cells but are instead expressed by eosinophilic granulocytes. There is significant correlation found between IDO expressing infiltrate and overall survival rate determined by analysis of follow-up of lung cancer patients. IDO has been reported as a prototype that bridges vascularization, inflammation and immune escape in order to promote primary, as well as metastatic growth of tumor (Smith, et al., 2012).

Similarly, the BAR-adapter encoding gene BIN1 has a very important role in cancer suppression (Sakamuro, et al., 1996; Elliott, et al., 1999; Ge, et al., 1999; Ge, et al., 2000; Tajiri, et al., 2003). BIN1 gene controls the sub-cellular movement of STAT and NF-κB, which in turn monitors the expression of Indo-gene. The reason behind it is that the afore-mentioned transcription factors are required for the activation of the promoters of Indo-gene. If BIN1 is deleted, the expression of Indo-gene remains unchecked resulting in a high and persistent expression of interferon-gamma induced IDO expression.

5. INHIBITORS

A wide range of bioactive inhibitors has been screened for their activity against IDO due to its crystal structure. There are two major classes of IDO inhibitors, competitive inhibitors and non-competitive inhibitors. 1-methyl tryptophan (1MT) is the key competitive inhibitor of IDO. Since its discovery in 1991, 1MT has been the most studied inhibitor of IDO because of its favorable pharmacokinetic activity like low protein binding, oral availability and low clearance. It works tremendously in retarding the growth of tumor cells when given in combination with potent chemotherapeutic agents, such as cyclophosphamide, cisplatin, doxorubicin or paclitaxel. To date, there have been no cases of toxicity reported against 1-methyl tryptophan, except dehydration in a study performed on experimental mouse model. 1-methyl tryptophan was administered to mice through drinking water (Uyttenhove, et al., 2003).

There are two stereo-isomers of 1-methyl tryptophan: D-isomer and L-isomer. It was found that the L-isomer has highest degree of efficacy when tested *in vitro* on HeLa cell lines and other cell lines or cell free assays. On the contrary, the efficacy of D-isomer was found to be almost equal to that of L-isomer when tested *in vivo* in mouse model and humans. The main reason for the superior activity of D-isomer *in vivo* is that L-isomer did not promote a high level of T-cell proliferation as activated by D-isomer. 1MT has emerged as a promising small molecule inhibitor of IDO over several clinical trials, data suggests that 1MT is synergistic with chemotherapy (Muller, et al., 2005) and as IDO regulates the reprogramming of T_{regs} into TH-$_{17}$-like T-helper cells in tumour draining lymph nodes in part, via CGN2-mediated suppression of IL-6 expression, therefore, is also synergistic with vaccines against established tumors.

Non-competitive inhibitors of IDO are mainly beta-carboline derivatives, which bind to the heme portion of IDO and compete with oxygen to bind to the active site iron. A large number of beta-carboline derived inhibitors has been developed but the fact that these inhibitors behave as benzodiazepine ligands; plays a limiting role in their development. This is because the binding of beta-carboline to the benzodiazepine receptors increase the chance of occurrence of effects caused by Central Nervous System (CNS) permeation

in cancer patients (Muller, 2005). In case of competitive or non-competitive inhibition of IDO, the basic mechanism of inhibition is independent of indole ring substitution. Lately research is being carried out on the development of such potent IDO inhibitors that work by substituting the indole ring of IDO.

6. COMPLIMENTING CANCER THERAPY

Tumor development and immune system involve dynamic and complex interactions. On one side, inflammation presents a pro-tumorigenic microenvironment and immune suppression can sometimes actually result in tumor regression in some models (Erdman, et al., 2004), while on the other side, cancer cells are subjected to evade immune response elicited by tumor antigens (Dunn, et al., 2004). Hence the development of immunotherapeutic strategies have predominately focused on supplementing and stimulating the effector cells of immune system. It is becoming apparent that in cancer patients, immune tolerance is the dominant factor and it will be essential to first breach the immunosuppressive processes in order to make immunotherapy effective (Zou, 2005).

Tryptophan depletion is being recognized as an essential factor in establishing tumor microenvironment that down regulates immune response. The proposed process of immune suppression is tryptophan catabolism in tumor tissues governed by IDO (Platten, et al., 2012).

Immune escape provides an essential gateway to malignancy. The emergence of this attribute of cancer shows the collapse of immune surveillance, an essential, multi-armed and potent way of cancer suppression that critically influences the ultimate clinical outcome of an early tumor stage. Immune escape may act as central modifier of clinical consequences by affecting progression versus dormancy, promoting invasion mechanisms, metastasis, and regulating therapeutic response. Although limited studies have been done until now, suppression of immune system and its escape presents potential areas of research and therapy.

It has emerged through recent evidence that activation of IDO pathway occurs during cancer progression and that may act as a modifier nodal pathway for cancer immune escape. IDO inhibitors have been reported to enhance the efficacy of chemotherapy in mouse model and a lead compound has already entered the phase-1 of clinical trials as an IDO inhibitor. New perspectives in this area of research offer promising ways to target advanced cancers, where pivotal support is provided by immune escape (Prendergast, 2008).

A recent development in mechanistic aspect of immune escape has been the finding of an interface with metabolic alterations that is another attribute of cancer. Furthermore, important mechanisms contributing immune tolerance to cancer antigens have been identified, which involve degradation of arginine and tryptophan by arginase-I and IDO, respectively (Bronte and Zanovello, 2005; Muller, et al., 2005b; Muller and Prendergast, 2007; Munn and Mellor, 2007; Popovic, et al., 2007).

BIN1 attenuation occurs in several human cancers, including prostate, breast, colon and lung cancers, melanoma and neuroblastoma (Ge, et al., 1999; Ge, et al., 2000a, 2000b; Tajiri, et al., 2003; Chang, et al., 2007a). By knocking out gene in the mouse, it has been indicated that BIN1 plays an essential role in minimizing inflammation and suppressing cancer. Specifically, BIN1 gene ablation enhances the rate of incidence of liver and lung carcinomas (Chang, et al., 2007a). Loss of BIN1 and activated-ras coordinate and enhance the progression of colon and breast carcinomas, supporting the fact that BIN1 limits the oncogenic potential of c-myc (Chang, et al., 2007b).

7. DISCUSSION

Various studies have shown that the tumors where IDO has been over-expressed, 1MT potentially limits the growth and progression of tumors (Friberg, et al., 2002; Uyttenhove, et al., 2003). However, due to lack of pharmacokinetic aspect of these studies, it is difficult to determine whether dosage has been inadequate to initiate the regression of developed tumors, rather than just limiting the growth. Various laboratories investigated that 1MT is stable in serum and accumulates to sufficient levels to inhibit IDO *in vivo* when dosed under circumstances that could initiate rejection of an allogenic fetus (Muller, et al., 2005a).

It has also been revealed that under same conditions, 1MT only limits the tumor outgrowth in an autochthonous mouse model of breast cancer, the MMTV-neu transgenic mouse (Muller, et al., 2005a). Hence, 1MT alone is unable to initiate tumor regression on its own, thus it shows very limited efficacy in terms of monotherapy. Alternatively by combining 1MT with any cytotoxic agent of chemotherapy such as paclitaxel, resulted in strong regressive response to established tumors in the

mouse model, where single therapy had poorly responded (Muller, et al., 2005a).

Administering subsequent dose, route and schedule of 1MT, it has been shown that a twice oral dose for just 4-5 days was plentiful to produce required tumor regression response in tumor bearing mice on trial (Hou, et al., 2007). It has also been shown that presence and targeting of IDO is essential for the action of 1MT, which was revealed by lack of 1MT antitumor activity demonstrated in IDO deficient mice (Hou, et al., 2007).

The concept that chemotherapy can be made effective by inhibiting IDO has been further supported by efficacy patterns of novel small inhibitor molecules of IDO that have been discovered recently. The continuous delivery to overcome its rapid clearance, the new inhibitor of IDO, methyl-thiohydantoin tryptophan presents greater potency but the same level of efficacy as 1MT (Muller, et al., 2005a). In addition natural inhibitors of IDO, such as brassinin, with already known cancer prevention activity in rodents (Mehta, et al., 1995), present antitumor efficacy on similar lines as 1MT (Gaspari, et al., 2006; Banerjee, et al., 2007).

Preclinical data for a potent IDO inhibitor called NLG919, has been presented at the annual meeting of American Association for Cancer Research (AACR) 2013. This data shows that NLG919 in addition to inhibition of IDO pathway *in vitro* and cell based assays, is bioavailable orally, and presents a favorable pharmacologic and toxicity profile. Indoximod (NLG8189 or D1-MT) is another inhibitor produced by the same entity is already in clinical trials, for the treatment of breast and prostate cancer. Both indoximod and NLG919 demonstrate synergic antitumor activity and T-cell activation.

So far, Indoximod has appeared to be efficient and favorable as a single agent as well as in association with docetaxel or dendritic cell vaccines. Thus, Indoximod serves as an ideal candidate to experiment other IDO novel candidates to improve the response of immune system from several other checkpoint inhibitors including PD-1/PD-L1 and CTLA-4 antibodies. By incorporating IDO inhibitors to protocols of immunotherapy, response rate to several checkpoint inhibitors can be increased. It appears that these inhibitors work best in association with immunomodulatory agents. Further determination of best combinations to use will still need some further clinical trial.

In conclusion, IDO presents a strong candidate for immunotherapy and synthetic approaches should be applied to obtain better candidates for inhibitors with lesser side effects and dose adjustment issues. Further the role of the IDO1/ IDO2 balance in cancer also warrants study to map the precise pathways of immune escape and broadening the understanding of the complex inter cellular interplay in immune evasion. The results for Clinical trials of 1-MT in glioblastoma patients of USA are highly anticipated and will open new avenues of study once data on IDO inhibition in human patients is revealed.

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