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Indoleamine 2,3-Dioxygenase and Its Therapeutic Inhibition in Cancer

George C. Prendergast, William, Malachowski, Arpita Mondal, Peggy Scherle, and Alexander J. Muller

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ABSTRACT

The tryptophan catabolic enzyme indoleamine 2,3-dioxygenase-1 (IDO1) has attracted enormous attention in driving cancer immunosuppression, neovascularization, and metastasis. IDO1 suppresses local CD8+ T effector cells and natural killer cells and induces CD4+ T regulatory cells (iTreg) and myeloid-derived suppressor cells (MDSC). The structurally distinct enzyme tryptophan dioxygenase (TDO) also has been implicated recently in immune escape and metastatic progression. Lastly, emerging evidence suggests that the IDO1-related enzyme IDO2 may support IDO1-mediated iTreg and contribute to B-cell inflammed states in certain cancers. IDO1 and TDO are upregulated widely in neoplastic cells but also variably in stromal, endothelial, and innate immune cells of the tumor microenviroment and in tumor-draining lymph nodes. Pharmacological and genetic proofs in preclinical models of cancer have validated IDO1 as a cancer therapeutic target. IDO1 inhibitors have limited activity on their own but greatly enhance **"**immunogenic**"** chemotherapy or immune checkpoint drugs. IDO/TDO function is rooted in inflammatory programming, thereby influencing tumor neovascularization, MDSC generation, and metastasis beyond effects on adaptive immune tolerance. Discovery and development of two small molecule enzyme inhibitors of IDO1 have advanced furthest to date in Phase II/III human trials (epacadostat and navoximod, respectively). Indoximod, a tryptophan mimetic compound with a different mechanism of action in the IDO pathway has also advanced in multiple Phase II trials. Second generation combined IDO/TDO inhibitors may broaden impact in cancer treatment, for example, in addressing IDO1 bypass (inherent resistance) or acquired resistance to IDO1 inhibitors. This review surveys knowledge about IDO1 function and how IDO1 inhibitors reprogram inflammation to heighten therapeutic responses in cancer.

1. INTRODUCTION

One widespread feature of advanced cancers is elevated tryptophan catabolism, a phenomenon that tracks with tumor burden noticed initially at least several decades ago (Boyland and Williams, 1956). Tryptophan is the rarest amino acid and it is essential in the diet. Thus, its levels are tightly controlled, in part by catabolism along the serotonin and kynurenine pathways which handle this role in the body. The serotonin pathway is better understood and medicinally important in controlling affect (mood) and gut peristalsis. However, only 5% of total tryptophan catabolism occurs through this pathway. The kynurenine pathway is relatively less understood despite its dominant role in tryptophan catabolism. This pathway has been studied mainly in biochemistry and neurology, in the latter case as a source of catabolites contributing to psychogenic disease (Schwarcz and Stone, 2017). Biochemically, indoleamine 2,3-dioxygenase (IDO) and tryptophan dioxygenase (TDO) control the rate-limiting first step in tryptophan catabolism leading to generation of the key enzyme cofactor nicotinamine adenine dinucleotide (NAD). However, NAD is scavenged from the diet to satisfy metabolic needs such that the physiological need of the kynurenine pathway seemed incomplete. TDO encoded by the TDO2

gene has long been known as the predominant liver enzyme mediating catabolism of dietary tryptophan. In contrast, IDO is an inducible enzyme that is more widely expressed. The IDO1 gene encoding IDO was identified in the 1960s as the first interferon-activated gene to be described (Yoshida et al., 1981), but despite some study in the context of infectious disease a fuller impact of this association was not appreciated.

A pivotal conceptual breakthrough with regard to the physiological meaning of tryptophan catabolism occurred in 1998 with the seminal work of Munn, Mellor, and their colleagues who implicated IDO in T celldirected immunosuppression during pregnancy (Munn et al., 1998). Briefly, they proposed that tryptophan deprivation would impair antigen-dependent T cell activation in microenvironments where IDO was active. Initial evidence supporting this concept was offered by studies of how immune tolerance to "foreign" paternal antigens in pregnant mice could be reversed by the IDO pathway inhibitor 1-methyl-D,L-tryptophan (1MT), the administration of which elicited MHC-restricted, T cell-mediated rejection of allogeneic conception (Mellor and Munn, 1999; Munn et al., 1998).

Several reports founded the concept of IDO as a mediator of immune tolerance in cancer. First, overexpression of IDO1 occurs commonly in human tumors (Theate et al., 2015; Uyttenhove et al., 2003). Normally, IDO1 is under the control of the tumor suppressor Bin1 (Jia et al., 2015; Muller et al., 2005a), one of the more commonly attenuated genes in human tumors (Ge et al., 1999; Karni et al., 2007; McKenna et al., 2012; Prendergast et al., 2009; Xu and Lee, 2003). Thus, IDO elevation in cancer cells can be ascribed directly to disruption of a tumor suppressor function for which a powerful selection appears to exist during malignant progression (Prendergast et al., 2009). In immunocompetent mouse models of cancer, 1MT doses that elicited conceptus rejection displayed some limited antitumor effect (Friberg et al., 2002; Uyttenhove et al., 2003). However, the same doses dramatically empowered the efficacy of coadministered immunogenic chemotherapy through a mechanism relying upon CD4+/ CD8+ T cells (Hou et al., 2007; Muller et al., 2005a), offering a more promising perspective on therapeutic utility. As discussed later, the D and L racemers of 1MT act by complex mechanisms of action in vivo that mainly distinct from systemic IDO1 enzyme inhibition (especially in the case of D-1MT, as advanced to clinical trials with the nomenclature indoximod). Thus, the discovery of a true bioactive IDO1 enzyme inhibitor by Muller, Prendergast, and colleagues that could empower chemotherapy offered the first therapeutic proof of concept (Malachowski et al., 2005; Muller and Prendergast, 2005; Muller et al., 2005a,b). Preclinical pharmacological validation was achieved through the study of other structurally distinct bioactive IDO1 enzyme inhibitors (Banerjee et al., 2008; Kumar et al., 2008a,b), including most notably the phenylimidazole and hydroxyamidine chemotypes from which the clinical leads navoximod/NLG919 and epacadostat/INCB024360 were developed, respectively (Kumar et al., 2008a; Yue et al., 2009). Genetic studies in mice deficient in IDO1 strengthened its preclinical validation as a cancer therapeutic target (Muller et al., 2008; Smith et al., 2012). Overall, these efforts helped establish IDO1 as a pivotal mediator of immune escape that is a critical trait of cancer (Prendergast, 2008). At the present time, clinical lead agents indoximod/D-1MT, epacadostat/INCB024360, and navoximod/NLG919 have advanced furthest in human trials. In this chapter, we summarize evidence supporting the concept of IDO/TDO enzymes as inflammatory modifiers involved not only in adaptive tolerance but also in tumor neovascularization and metastasis; the discovery and development of indoximod, epacadostat, and navoximod as lead clinical compounds in the field; and the rationale for and ongoing exploration of TDOinhibitors andmixed IDO/TDOinhibitors based on a broader rationale

involving TDOand IDO2 in driving cancer progression, and their potential roles in IDO1 bypass (inherent resistance) and acquired resistance to IDO1 selective inhibitors currently at the vanguard of clinical development as a unique class of immunometabolic modifiers of cancer-associated inflammation and adaptive immunity. Fig. 1 provides a current perspective on the sites of expression and functional reach of IDO/TDO enzymes in cancer as presented later.

2. IDO1 IN IMMUNE ESCAPE FROM T CELL IMMUNITY

The prevailing view among cancer biologists of the determinative importance of intrinsic tumor cell characteristics was encapsulated in a highly influential categorization of the hallmarks of cancer (Hanahan and Weinberg, 2000). In this broad conceptualization, even metastasis and angiogenesis, the two recognized hallmarks with clear host dependence, were considered from a tumor-centric perspective, and no consideration was given to possibility that interactions with host immunity might also play an instrumental role in cancer outcomes. The case for including immune escape within the pantheon of critical hallmarks was first promulgated in a 2008 review on IDO1 (Prendergast, 2008), and eventually gained general acceptance as the hallmark designations were reassessed in light of recognition of the importance of host environmental factors such as immunity and inflammation (Hanahan and Weinberg, 2011; Luo et al., 2009).

IDO1 induction in DC and macrophages promotes immune tolerance by suppressing effector T cells, converting naı¨ve T cells to FoxP3+ Tregs, and elevating the suppressive activity of “natural” Tregs, a topic that has been reviewed in detail elsewhere (Munn and Mellor, 2013). Extratumoral induction of IDO1 was reported initially in a subset of cancer patients and preclinical tumor graft models (Friberg et al., 2002; Munn et al., 2004). In the mouse B16 melanoma model, IDO1 was not detectable directly in the tumors that formed, but rather was elevated in tumor-draining lymph nodes (TDLN) where it was localized to a specific subset of DC characterized for T cell suppressive activity (Munn et al., 2004). Several different IDO1 inhibitory compounds have since been identified that can produce highly significant B16 tumor growth suppression that relies both on intact T cell immunity and host IDO1 function (Banerjee et al., 2008; Kumar et al., 2008b; Muller et al., 2010a), providing pharmacological support for an extratumoral role of IDO1 in limiting antitumor immunity. In like manner, the first genetic validation of IDO1’s involvement in driving autochthonous tumor development came from studies in classical two-stage models of skin carcinogenesis, where there was no evidence of IDO1 expression in the developing lesions: similar to the B16 model, IDO1 expression and activity were highly elevated in DC within the TDLN (Muller et al., 2008). In this context, where tumor initiation and promotion are distinctly separable, IDO1 was found to be elevated in the tumor-promoting inflammatory environment, even in the absence of tumor initiation, clearly indicating that extratumoral IDO1 elevation is an early event that occurs before initiation in programming a protumorigenic inflammatory microenvironment (Muller et al., 2010b).

3. IDO1 IN INFLAMMATORY PROGRAMMING: MDSC DEVELOPMENT AND METASTASIS

Myeloid-derived suppressor cells (MDSC) found to rely upon IDO1 support are another key player in the establishment of an immunosuppressive tumor microenvironment. MDSC are an immature population of bone marrow-derived hematopoietic cells functionally defined by their ability to suppress T cell activity (Munn and Bronte, 2016). In response to inflammatory signals, MDSCs migrate to the lymph node, spleen and tumor tissue to create local immune suppression. Among the mechanisms utilized by MDSC to exert their T cell suppressive effects

(Hanson et al., 2009; Nagaraj et al., 2007; Rodriguez et al., 2004; Serafini et al., 2008; Sinha et al., 2007; Srivastava et al., 2010; Yu et al., 2013), there is evidence that IDO1 activity is a critical factor. This connection was first revealed by genetic studies in Ido1 _/_ mouse models of de novo lung carcinoma and metastases (Smith et al., 2012). Ido1 _/_ mice resisted the outgrowth of lung tumors and MDSC obtained from tumor-bearing animals were impaired for suppression of CD8+ and CD4+ T cells. Moreover, IDO1 loss caused an attenuation of IL-6, a major driver of MDSC, and ectopic expression of IL-6 was sufficient to rescue impairment of the T cell suppressive activity of MDSC as well as the resistance to pulmonary metastasis in Ido1 _/_ mice (Smith et al., 2012). Thus, IDO1 exerted regulatory control over MDSC suppressive function by its ability to influence the inflammatory milieu. Other studies show that IDO1 is needed for MDSC recruitment to tumors (Holmgaard et al., 2015, 2016). In light of the pivotal role of IDO1 in supporting MDSC function, it is notable that no compelling evidence exists in mouse models that IDO1 is expressed directly in MDSC. In contrast, human studies have identified populations of IDO1-expressing MDSC and associated the IDO1 expression in those cells with immunosuppressive function (Mougiakakos et al., 2013; Yu et al., 2013). Overall, in addition to regulating MDSC function and recruitment, IDO1 may act through additional mechanisms to support MDSC activity.

4. IDO1 IN INFLAMMATORY PROGRAMMING: PATHOGENIC NEOVASCULARIZATION AND METASTASIS

The critical importance of neoangiogenesis for supporting tumor outgrowth is well established (Hanahan and Folkman, 1996). Although angiogenesis is sometimes used to refer broadly to all blood vessel development, its specific meaning is the formation of new vessels from the preexisting vascular network in contrast to vasculogenesis which refers to vessel formation through recruitment of new cells such as bone marrow-derived endothelial precursor cells. While vasculogenesis has been predominantly associated with embryogenesis and angiogenesis with adult vessel formation, the picture is likely to be more complex and the distinction between the two processes may not be absolute. A combination of vasculogenesis and angiogenesis has been implicated in the vascularization of organs of both mesodermal and endodermal origin such as lung, heart, pancreas, and liver, while for organs of ectodermal origin, such as brain, kidney, thymus, and limb bud, angiogenesis appears to be predominant (Ribatti et al., 2001). Importantly, these observations suggest that the operative processes for forming new blood vessels may not be the same between different tissue environments, which may be a factor influencing the outgrowth of tumors and metastases at different sites in the body.

Neovascularization refers to the excessive and disorganized growth of blood vessels induced by ischemia in tissues such as the retina and lungs. Neovascularization is also a distinguishing characteristic of growing tumors. In experimental models of ischemia, immune cells have been reported to be important for pruning the excess vasculature and limiting neovascularization (Ishida et al., 2003), suggesting that immunity might play an important antineovascular role in tumors as well. In particular, the inflammatory cytokine IFNγ has been shown to trigger antineovascular activity that results in tumor cell killing. In a series of studies, IFNγ-mediated elimination of vessels was implicated as the primary mechanism for both CD4 and CD8 T celldependent tumor rejection (Qin and Blankenstein, 2000; Qin et al., 2003). However, inflammation is a complex process that can also promote neovascularizarion. In particular, the inflammatory cytokine IL6 has been shown to be important for ischemia-induced neovascularization (McClintock and Wagner, 2005) and has been demonstrated to promote

aberrant angiogenesis through a signaling process that does not require VEGF (Gopinathan et al., 2015). IL6 is also generally regarded as protumorigenic as opposed to IFNγ which is regarded as antitumorigenic. This raises the possibility that the cytokine balance in an inflammatory environment may influence tumor outgrowth by how it impacts neovascularization.

In this context, the finding that loss of IDO1 resulted in diminished pulmonary vascularization (Smith et al., 2012) suggested the hypothesis that the induction of IDO1 by IFNγ might be working in a negative feedback capacity to limit the antiangiogenic impact of IFNγ and that this might be an important factor accounting for the ability of IDO1 to counteract immune-based restriction of tumor outgrowth. IDO1 loss was also associated with attenuated induction of the inflammatory cytokine IL6, and it was demonstrated in a pulmonary metastasis model that ectopic expression of IL6 could overcome the resistance metastatic tumor outgrowth exhibited by Ido1 _/_ mice. These findings led to the hypothesis that IDO1 acts downstream of IFNγ and upstream of IL6 from the very onset of tumor initiation to shift the inflammatory environment toward angiogenesis and tumor promotion.

As predicted by this model, pulmonary metastases that developed in Ido1 _/_ mice exhibited significantly reduced neovascularization relative to their WT counterparts (Mondal et al., 2016). However, since overall metastatic tumor outgrowth in Ido1 _/_ mice was also significantly reduced, it was not clear if the reduction blood vessel formation was a direct effect of IDO1 loss. To test the idea that IDO1 is important for supporting neovascularization outside other possible confounding effects within the tumor microenvironment, studies were conducted in a mouse OIR (oxygen-induced retinopathy) model, a well established, reproducibly quantifiable surrogate system for studying neovascularization (Palmer et al., 2012; Stahl et al., 2012). As predicted, Ido1 _/_ mice exhibited a significant reduction in OIR-induced retinal neovascularization relative to their WT counterparts (Mondal et al., 2016). Loss of the related IDO2 isoform had no demonstrable effect on OIR-induced retinal neovascularization, indicating that the effect is specific to IDO1 (Mondal et al., 2016). No difference in the normal retinal vascularization that develops under normoxic conditions was observed between Ido1 _/_ and WT groups and reduction of the avascular region (Mondal et al., 2016), indicative of normal revascularization, was actually higher in the Ido1 _/_ animals indicative of an improvement in normal vascular regrowth occurring inmice lacking IDO1. The reduction in OIR-induced retinal neovascularization observed in mice lacking Ido1 genetically was recapitulated by siRNA-mediated knockdown of Ido1 expression in the retina (Mondal et al., 2016), demonstrating that the effect of IDO1 loss on neovascularization could be elicited both locally and acutely. Likewise, pharmacologic inhibition of IDO1 with the clinical agent epacadostat reducedOIR-induced retinal neovascularization when delivered systemically to neonates (Mondal et al., 2016). In parallel studies, epacadostat administration in the pulmonary metastasis model resulted in rapid elimination of the existing neovasculature (Mondal et al., 2016), validating the potential therapeutic relevance of these findings in the cancer setting.

Having established the importance of IDO1's role in supporting neovascularization, studies were carried out to test the hypothesis that IDO1 produces this effect through its integration at the regulatory interface between the inflammatory cytokines IFNγ and IL6. Consistent with the hypothesis that IDO1 supports neovascularization primarily by counteracting the antiangiogenic activity of IFNγ, the concurrent elimination of IFNγ in double knockout Ifng _/_ Ido1 _/_ mice reverted the level of neovascularization in both the OIR and pulmonary metastasis models backto wild type levels (Mondal et al., 2016). Conversely, Il6 _/_ mice, as predicted, exhibited a reduction in neovascularization in both the OIR and metastasis models

similar to that observed in Ido1 _/_ mice (Mondal et al., 2016). The effect of IL6 loss on neovascularization was likewise reversed by the concomitant elimination of IFNγ in double knockout Ifng _/_ Il6 _/_ mice (Mondal et al., 2016), consistent with the hypothesis that the upstream potentiation of the proangiogenic activity of IL6 may be an important contributing factor in IDO1's ability to support neovascularization. In all cases, neovascularization tracked closely with overall survival in the pulmonary metastasis model (Mondal et al., 2016), indicating that the impact on tumor neovascularization may be a meaningful consequence of treatment IDO1 inhibitors that should be taken into consideration as part of the ongoing clinical development of these agents.

5. IDO2 IN B-CELL INFLAMED STATES AND CERTAIN IDO1 FUNCTIONS: CONNECTIONS AND QUESTIONS

Although relatively little studied as yet, IDO2 is a structural relative of IDO1 also implicated in modulating immunity through tryptophan catabolism, particularly autoimmunity (Prendergast et al., 2014a). The IDO2 gene is located immediately downstream of IDO1 in the mouse and human genomes, and structural studies suggest a more ancestral function for IDO2 (Yuasa et al., 2007). Deletion of the Ido2 gene in the mouse does not appreciably affect embryonic development, hematopoiesis, or immune character, nor does it affect tryptophan or kynurenine levels in blood (Metz et al., 2014). IDO2 enzyme activity clearly relies upon conditions that differ from IDO1, for example, in differing requirements for a physiological coreductant system (Eldredge et al., 2013). Indeed, earlier characterizations of human IDO2 as inactive simply reflect nonoptimal biochemical conditions which when corrected confer demonstrable activity (Li et al., 2016; S.-R. Yeh, personal communication; L. Laury-Kleintop, J. DuHadaway, and G.C.P., unpublished observations). Thus, the lack of significant effects of Ido2 deletion on systemic blood levels in the mouse may reflect the far narrower normal range of IDO2 expression relative to IDO1 and TDO, which are relatively more broadly and strongly expressed.

Mouse genetic experiments establish a function for IDO2 in immunomodulation (Prendergast et al., 2014a). One notable feature of Ido2-deficient mice is a deficiency in their ability to support IDO1-induced T regulatory cells (Metz et al., 2014). Parallel evidence of a similar tolerizing function for IDO2 in human dendritic cells has been reported (Trabanelli et al., 2014). Ido1-deficient mice have also been found to be mosaicdeficient for Ido2 function, strengthening clues of IDO1–IDO2 interaction in immune control (Metz et al., 2014). Fig. 2 summarizes this feature of IDO2 and a model which captures its potential implications in cancer. Interestingly, in a mouse model of autoimmune arthritis, indoximod (D-1MT) administration phenocopied the reduced disease severity associated with Ido2 deletion and this therapeutic effect was abolished by Ido1 deletion (Merlo et al., 2014), aligning with earlier evidence that indoximod can selectively disrupt IDO2 enzyme activity (Metz et al., 2007). However, these connections may be contextual having yet to be extended in other systems (van Baren and Van den Eynde, 2015a), including humans where common genetic variations in IDO2 that reduce tryptophan catabolic activity may be relevant (Metz et al., 2007).

Recent studies of the reduced susceptibility of Ido2 _/_ mice to autoimmune arthritis have revealed that IDO2 functions in B cells where it acts to support B-cell inflammed states (Merlo et al., 2014, 2016, 2017). These findings are interesting in light of evidence that certain cancers rely upon B-cell inflamed states for their development (Affara et al., 2014; Schioppa et al., 2011). While IDO2-deficient mice are unchanged with regard to their susceptibility to

inflammatory skin carcinogenesis (Metz et al., 2014), they resist the development of K-Ras-induced pancreatic cancers (G.C.P. and A.J.M., unpublished data). IDO2 enzymology differs from IDO1 in requiring different reductant systems, especially for the human enzymes, but recent elucidation of these differences confirms that IDO2 has demonstrable tryptophan catabolic activity (Li et al., 2016). While small molecule inhibitors of mouse or human IDO2 have been reported (Austin et al., 2010; Bakmiwewa et al., 2012; Li et al., 2016; Pantouris et al., 2014; R€ohrig et al., 2016), they are not bioactive or for other reasons have not been studied in vivo as yet. Interestingly, a B cell-penetrating bioactive antibody against IDO2 has been reported recently that phenocopies the antiarthritic effects of Ido2 genetic deficiency in the mouse (Merlo et al., 2017).

In normal tissues IDO2 expression is more narrow than IDO1 or TDO, being confined mainly to liver, kidney, brain, placenta, and antigenpresenting cells (APCs) including B cells. Cancers do not tend to overexpress IDO2 although it has been reported in melanoma and gastric, brain and pancreatic tumors, in the latter case rather widely (Witkiewicz et al., 2009). The IDO2 gene is regulated by the aryl hydrocarbon receptor (AhR) (Bankoti et al., 2010; Simones and Shepherd, 2011), which binds kynurenine as a endogenous ligand produced by the more active IDO1 enzyme (Opitz et al., 2011). Thus, given clues of IDO1–IDO2 genetic interaction (Metz et al., 2014), it is conceivable that locoregional IDO1 activity may increase levels of IDO2 in roving APCs in the tumor microenvironment, perhaps contributing to a tolerized state that contributes to Treg formation in TDLN. Fig. 2 presents a model in which IDO2 functions on the Kyn effector pathway downstream of IDO1/TDO to positively modify decisions made in the TDLN to set tolerance to “altered-self ” antigens, along the self-nonself continuum where immune challenges from autoimmunity and cancer arise (Prendergast, 2015).

6. TDO IN INFLAMMATORY PROGRAMMING: IMMUNE ESCAPE, ANOIKIS RESISTANCE, AND METASTASIS

TDO expression in liver is responsible for homeostasis of tryptophan levels in the blood. Similar to IDO1, some tumors overexpress TDO as a means of immune escape (Platten et al., 2012, 2014; van Baren and Van den Eynde, 2015b). Thus, there has been growing interest in small molecule inhibitors of TDO as a parallel immunomodulatory strategy to attack tumors (Abdel-Magid, 2017; Dolusic et al., 2011; Pantouris and Mowat, 2014; Pilotte et al., 2012; Salter et al., 1995; Wu et al., 2015), the rationale for which has been reviewed in detail recently by pioneers in this area (Platten et al., 2014; van Baren and Van den Eynde, 2015b). The initial bioactive lead structure developed in the 1990s termed 68OC91 (Salter et al., 1995) has been used for mouse studies, but compounds optimized for potency and more favorable pharmacological profiles have been reported (Dolusic et al., 2011; Pantouris and Mowat, 2014; Pilotte et al., 2012; Wu et al., 2015). Deletion of the TDO-encoding gene Tdo2 in the mouse causes higher concentrations of L-tryptophan to accumulate in blood, with some neurologic alternations perhaps attributable to a coordinate elevation in blood/brain levels of serotonin in these mice (Kanai et al., 2009). Interestingly, mice treated with the Tdo2 inhibitor 680C91 will phenocopy Tdo2 _/_ mice in showing an increased sensitivity to endotoxin-induced shock, implicating TDO in inflammatory programming (Bessede et al., 2014). However, despite this parallel with IDO1, as in the case with IDO2 there are differences in the inflammatory characteristics that appeared to be conferred by TDO, despite the common role of these enzymes in tryptophan catabolism (Larkin et al., 2016). While enzymological differences may help explain these different roles, it would also seem likely they reflect differences in locoregional

control in the production of kynurenine and its metabolites, or in the relative availability or efficiency of kynurenine effector mechanisms (AhR, kynurenine pathway catabolic enzymes, etc.). With regard to TDO, while there is evidence of its contribution to tumoral immune escape established preclinically with selective bioactive inhibitors (Opitz et al., 2011; Pilotte et al., 2012), neither a genetic proof in mice nor an understanding of the nature or extent of its expression in tumor cells or the tumor microenvironment has been established as yet. Moreover, TDO inhibitors pose different safety concerns from IDO1 inhibitors, including in the liver and central nervous system, as carefully discussed recently elsewhere (Platten et al., 2014). That said, the rationale for developing TDO inhibitors as well as IDO/TDO combined inhibitors as nextgeneration modalities in the field continues to strengthen.

Recent emerging evidence suggests that TDO contributes to cancer associated inflammatory programming like IDO1. Specifically, upregulation of TDO in cancer cells has been found to contribute to tumor cell survival and metastatic prowess beyond its role in immune escape (D'Amato et al., 2015). Resistance to anoikis—a type of apoptosis triggered by cell adhesion deprival—is a key step in metastatic progression (Hanahan and Weinberg, 2011). In a seminal study of aggressive "triple-negative" breast cancer (TNBC), D'Amato and colleagues showed how TDO upregulation in forced suspension culture was essential for anoikis resistance and metastatic capacity of TNBC cells (D'Amato et al., 2015). Similar to its role in immune escape (Opitz et al., 2011), kynurenine induction resulting from TDO pregulation was sufficient to activate the AhR signaling pathway, and pharmacological inhibition or genetic attenuation of TDOor AhR was each sufficient to restore anoikis sensitivity and reduce the invasive character of TNBC cells. Supporting these observations, tumor-bearing mice treated with the TDO inhibitor 680C91 exhibited reduced pulmonary metastasis. Lastly, elevated expression of TDO in clinical TNBC specimens was associated with increased disease grade, estrogen receptor-negative status, and shorter overall survival (D'Amato et al., 2015). These findings extend the concept that TDO acts like IDO1 to drive a pathogenic inflammatory program(s) in cancer that extends beyond their contributions to enabling adaptive immune tolerance. Fig. 3 summarizes ways in which IDO/ TDO inhibitors may be used to leverage immune checkpoint therapy and chemotherapy through their effects on inflammatory programming and adaptive antitumor immune responses.

7. LEAD CLINICAL AGENTS: INDOXIMOD, EPACADOSTAT, AND NAVOXIMOD

7.1 Indoximod (D-1MT/NLG-8189)

A detailed discussion of the preclinical studies and rationale to embark upon clinical evaluation of this simple 1-methyl derivative of D-tryptophan has been published elsewhere (Prendergast et al., 2014b,c). By far, the most commonly employed molecular probe to study IDO in the preclinical literature has been the D,L racemic mixture of 1-methyl-tryptophan (1MT). L-1MT is a weak substrate of IDO rather than a true inhibitor (Prendergast et al., 2014b,c); D-1MT is neither substrate nor inhibitor of IDO, though in multiple model systems it exhibits relatively greater antitumor properties associated with inhibition of IDO-mediated tryptophan catabolismin human dendritic cells (Hou et al., 2007). Thus, neither are selective probes. As the first compound to enter Phase I trials, indoximod was found to be well tolerated as a single agent or in combination with chemotherapy in studies which defined a dose of 1200mg/ day for ongoing evaluation in multiple Phase II trials (Soliman et al., 2014, 2016). Among this work, three notable trials focus on breast cancer patients in combination with taxotere (chemotherapy combination); prostate cancer patients in combination with the dendritic

cell vaccine sipuleucel-T (vaccine combination); and melanoma patients in combination with anti-PD1 (immune checkpoint combination) (http://clinicaltrials.gov/ct2/results?term**.**IDO&Search**.**Search; Vahanian et al., AACR 2017 late-breaking abstract). While the precise mechanism of action of indoximod has not yet been established definitively, striking cell-based experiments reveal that the mTORC1 pathway interprets indoximod at clinically relevant nanomolar concentrations as a mimetic of L-tryptophan (Prendergast and Metz, 2012). Thus, indoximod may act in part by relieving the inhibitory effects of IDO/TDO-mediated tryptophan deprivation on mTOR signals needed in T cells for antitumor activity. As further work reveals the precise mechanism of action, the low toxicity of indoximod as a simple D-tryptophan derivative remains an appealing feature of its clinical development, alongwith the opportunity it affords to leverage IDO1 and IDO/TDO enzymatic inhibitors.

7.2 Epacadostat (INCB024360)

Epacadostat, developed under the code name INCB024360, is the lead clinical agent from a hydroxylamidine series of IDO1 selective inhibitors pioneered by Incyte Corporation which is furthest in clinical development. Details regarding the chemistry effort that led to the development of epacadostat are covered in the recent publication of its identification and structure by the team at Incyte that spearheaded the project (Yue et al., 2017). In preclinical studies, epacadostat selectively inhibited the tryptophan catabolic activity of human IDO1 in cell-based assays (IC50 **.**10nM) with little activity against IDO2 and TDO2. In cocultures of human allogeneic lymphocytes with DC or tumor cells, epacadostat promoted the growth of effector T cells andNKcells, reduced conversion of naı¨ve T cells to Tregs, and increased the number of CD86high DC (Liu et al., 2010). Consistent with these effects, administration of epacadostat to tumor-bearing syngeneic mice inhibited kynurenine levels _90% in both plasma and tumor and reduced tumor growth in immunocompetent but not immunocompromised mice, confirming that drug efficacy relies upon functional immunity. Further, in the B16 melanoma model, epacadostat was found to enhance the antitumor effects of anti-CTLA4 or anti-PDL1 antibodies, where increased IL-2 production and CD8+ T cell proliferation was suggestive of greater pronounced T cell activity than either agent alone (Spranger et al., 2014).

Clinical evaluation of epacadostat opened with a first-in-human Phase I study to investigate safety and maximum-tolerated dose, pharmacokinetics, pharmacodynamics, and antitumor activity (Beatty et al., 2017). In this study, epacadostat was generally well tolerated, effectively normalized plasma kynurenine levels and was maximally inhibitory to IDO1 activity at doses of >100mg BID. While no objective responses were detected, stable disease lasting _16 weeks was observed in 7/52 patients (Beatty et al., 2017). A study coadministering epacadostat in combination with ipilimumab was conducted in patients with advanced melanoma (Gibney et al., 2015). Doses of epacadostat at 25mg BID and 50mg BID were generally well tolerated. Of note was a 31%ORR by immune response Recist Criteria (irRC) including 3/32 patients with complete responses. While uncontrolled, the median PFS by irRC was 8.2 months in patients who had not received prior immune therapy. The efficacy endpoints compared favorably with historical controls reported prevously for ipilimumab, which demonstrated 11% ORR with a median PFS of 2.86 months (Hodi et al., 2010).

Epacadostat is currently being studied in a total of 14 tumor types as coadministered with anti-PD-1 antibodies (nivolumab or pembrolizumab) or anti-PD-L1 antibodies (atezolizumab and duvalumab). Early pembrolizumab combination data indicated that the combination was well

tolerated with promising clinical activity (Gangadhar et al., 2016). Among 19 treatment-naıve advanced melanoma patients, 4 CR, 7 PR, and 3 SD were reported resulting in 58% ORR and 74% DCR, with responses in all epacadostat dose cohorts _50mg BID and at all target lesion sites including in liver, lung, and lymph nodes. All responses reported at presentation were confirmed and ongoing and median PFS had not been reached (Gangadhar et al., 2016). These results compare favorably with pembrolizumab monotherapy or nivolumab–ipilimumab combination therapy in melanoma patients (Postow et al., 2015; Robert et al., 2015). In the epacadostat–pembrolizumab combination study, responses were observed in patients previously treated for advanced melanoma (**n.**3; 1 CR, 1 SD) and in patients with NSCLC(**n.**12;5PRs, 2 SDs),RCC(**n.**11; 3PRs, 5 SDs), endometrial adenocarcinoma (**n.**7; 1 CR, 1 PR), TCC (**n.**5; 3 PRs), TNBC (**n.**3; 2 SDs), SCCHN(**n.**2; 1 PR, 1 SD). Based on these results, a Phase III randomized double-blind, placebo-controlled study investigating pembrolizumab in combination with epacadostat or placebo for first-line treatment of patients with advanced or metastatic melanoma was initiated in June 2016 (ECHO-301 [NCT02752074]). Additional studies in lung, renal, head and neck, and bladder cancers are expected to open in 2017.

7.3 Navoximod (NLG919)

Prior to 2005, there was little motivation to develop inhibitors of IDO1, an unremarkable tryptophan-catabolizing enzyme. This situation changed with the first preclinical evidence of a role for IDO1 in cancer and of IDO1 inhibitor efficacy when combined with chemotherapy (Malachowski et al., 2005; Muller and Prendergast, 2005; Muller et al., 2005a,b). In 2005 the only bioactive IDO inhibitor was 1MT with a reported Ki of 34M (Cady and Sono, 1991; Peterson et al., 1994). One of the few other reported IDO inhibitors at the time was 4-phenyl-imidazole (4-PI) identified in 1989 as a weak noncompetitive inhibitor of IDO1 by Sono and Cady (1989). Interestingly, although 4-PI showed noncompetitive inhibition kinetics through impressive spectroscopic studies Sono and Cady showed that 4-PI was actually binding to the heme iron at the active site. Subsequently, the first crystal structure of IDO1 to be reported (Sugimoto et al., 2006) confirmed this finding by showing 4-PI bound to the heme iron (Fig. 4A). This confirmation along with the rich crystal structure information facilitated the first structure-based drug design activities of Malachowski and colleagues, seeding work in the phenylimidazole series from which the clinical lead navoximod (NLG919) was later derived (Kumar et al., 2008a).

In early foundational work (Kumar et al., 2008a), Malachowski and colleagues explored 4-PI analogs to probe the active site of IDO1 with structural modifications that were focused on exploiting three binding interactions within the IDO active site: (1) the active site entrance region decorated with the heme 7-propionic acid; (2) the interior of the active site, in particular interactions with C129 and S167; and (3) the heme iron-binding group. The enhancement of IDOinhibition of 4-PI structures through interactions at the active site entrance focused on the N-1, C-2, and N-3 positions of the imidazole ring (Fig. 4B). All three positions were substituted with the goal of appending groups that would occupy the active site entrance. In the crystal structure of 4-PI with IDO, this region contains an N-cyclohexyl-2- aminoethanesulfonic acid (CHES) buffer molecule whose alkyl portion forms hydrophobic interactions with F163 and F226. In addition, the amino group of the CHES molecule forms an ion pair with the heme 7-propionic acid.

N-1 substituted 4-PI derivatives were completely devoid of inhibitory activity, which, not surprisingly, confirmed the binding of the N-1 nitrogen to the heme iron and, more importantly,

demonstrated that the N-3 nitrogen of the imidazole cannot substitute to bind at the heme iron. However, N-3 benzyl-substituted derivatives (Fig. 4C, **1**) were unexpectedly found to be roughly equipotent to 4-PI, thereby demonstrating that imidazole ring substitution was tolerable. The N-3 benzyl-substituted compound identified the correct imidazole ring location and spatial tolerance, likely occupying the active site entrance where the CHES buffer molecule sits in the IDO-4-PI crystal structure (Sugimoto et al., 2006). This discovery was consistent with the pharmacophore developed in studies of IDO1 inhibition by brassinin derivatives, i.e., a heme iron-binding group flanked by two large aromatic or hydrocarbon structures (Gaspari et al., 2006). This insight proved to be important to subsequent development regarding the backbone structure for the clinical candidate navoximod/NLG919 (Fig. 4C). The backbone as shown extended from the same N-3 position to situate a similar hydrocarbon moiety in the active site entrance of IDO1.

Analysis of the crystal structure of 4-PI bound to IDO1 (Sugimoto et al., 2006) indicated that S167 and C129 were in close proximity to the phenyl ring of 4-PI in the interior of the active site. Systematic evaluation of ortho, meta, and para substitutions of the phenyl ring with oxygen, sulfur, and fluorine were undertaken to ascertain if specific protein–ligand interactions could be exploited. The 2‘-hydroxy (ortho-substituted) modification afforded the most success generating a 10-fold increase in potency relative to 4-PI (Fig. 4D). Two possibilities existed for this increased activity: intermolecular H-bonding with S167 or intramolecular H-bonding with N-3 to lock the phenyl and imidazole rings. The 2‘,6‘-dihydroxy-phenyl derivative, which presents a hydroxy group to S167 or N-3 imidazole in either rotamer was also synthesized and it was roughly equipotent to the 2‘-hydroxy derivative, thereb demonstrating that there was no additional benefit from both events. NewLink Genetics introduced a hydrocarbon bridge that replicates the H-bond to the N-3 imidazole; i.e., locking the conformation of the benzene and imidazole ring into one plane.

Modification of the critical heme iron-binding imidazole ring and its effect on IDO1 inhibition was also explored by Malachowski and colleagues. To probe the effect of heterocycle binding to the heme iron, alternative aromatic rings were subsituted for the imidazole of 4-PI. These changes almost universally led to less potent compounds relative to 4-PI. For instance, pyridine, thiazole, pyrazole, and furan all failed to demonstrate any inhibition. Presumably the thiazole, pyrazole, and furan fail to bind to the heme iron with the same affinity as the imidazole, a well-known iron ligand in nature, e.g., histidine. The replacement of the phenyl group of 4-PI with thiophene was permitted, although there was approximately a fivefold loss in activity. Only when the hydroxy groups of the phenyl ring were returned was activity restored or modestly improved over 4-PI. This, again, was consistent with the hydroxyl group forming an intramolecular H-bond with the pyrazole nitrogen and locking the two rings in the same plane, as previously noted. Although these studies demonstrated that the imidazole group was optimal in terms of both iron binding strength and shape complimentarity, subsequent work illustrated that triazoles have related activity (Rohrig et al., 2012). Overall, early studies yielded three critical discoveries about the phenylimidazole series leading to development: (1) N-3 substitution was permitted and a rather large space existed in the active site to accommodate hydrocarbon moieties in this position; (2) incorporation of an orthohydroxyl group was beneficial; (3) the imidazole ring was optimal for binding to the heme iron.

Preclinical studies of NLG919 illustrate its potency as an IDO1 inhibitor with EC50 **.**75nM in cell-based assays and a 10- to 20-fold selectivity against TDO (Mautino et al., 2013). NLG919 is orally bioavailable and has a favorable pharmacokinetic and toxicity profile. Oral administration was shown to reduce plasma kynurenine levels by approximately 50% in mice. In

human IDO1+ DCs in an allogeneic mixed lymphocyte reaction, NLG919 blocked IDO1-induced T cell suppression and restored T cell responses in vitro. In the B16 melanoma mouse model, coadministration of with pmel-1 T cells and gp100 peptide vaccination reduced relative tumor size _95% within 4 days of vaccination. Additionally, in the EMT6 syngeneic model, combining NLG919 with an anti-PD-L1 antibody improved relative antitumor efficacy (Spahn et al., 2015). The combination led to an increased CD8+ T/Treg ratio and higher plasma levels of interferon-γ. Treatment also resulted in the activation of intratumoral macrophages and DC in the model.

In the clinical setting, NLG919 has been studied as monotherapy so far in patients with recurrent/advanced solid tumors and the safety, pharmacokinetic, and pharmacodynamic results from the Phase 1a study were reported (Nayak et al., 2015). Overall, NLG919 was well tolerated up to 800mg BID on a 21/28 day cycle. The best response observed was SD in 7/17 patients. Plasma exposures ofNLG919 increased from50 to 800mg in a doseproportional manmer and plasma kynurenine levels were _30% decreased transiently 4h after dosing in a manner consistent with the predicted drug half-life. It was reported that safety, pharmacokinetics, and pharmacodynamics of NLG919 are being evaluated on a continuous dosing schedule (BID, 28/28 days). NLG919 is also being studied in combination with the anti-PD-L1 antibody atezolizumab (NCT0271846).

8. OTHER IDO/TDO INHIBITOR CLINICAL CANDIDATES

Additional IDO1 inhibitory compounds have been reported to be entering clinical testing. PF-06840003 is a tryptophan noncompetitive, nonheme-binding IDO1 inhibitor licensed by iTeos SA to Pfizer for clinical development (Wythes et al., SITC 2016, poster 253). This compound is predicted to have favorable human PK characteristics, a prolonged human half-life that may allow single dose daily administration, and CNS penetration properties that may enable efficient access to brain metastases. In preclinical study, PF-06840003 enhanced the antitumor efficacy of anti-PD1/PDL1 axis blockade. A first-in-patient study was initiated in 2016 in malignant gliomas (NCT02764151). BMS-986205 is an IDO1 inhibitor licensed by Flexus Inc. to Bristol–Myers Squibb for clinical development. This compound is reported to have improved potency and pharmacokinetics relative to epacadostat. In 2015 it entered a Phase 1 study in solid tumors both as monotherapy and in combination with nivolumab (NCT02658890). Several other IDO1 inhibitors are reported in late preclinical stages of development with little information disclosed to date.

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FIGURES

Fig. 1. Sites of IDO/TDO expression and action in cancer. Expression of IDO1, IDO2, and TDO documented in various cells in the tumor microenvironment (including metastatic sites) and in tumor-draining lymph nodes (TDLN) is indicated, including in tumor, stromal, vascular, and immune cells. Both tryptophan deprivation and kynurenine production mediated by IDO1 and TDO has been implicated in inflammatory processes and immune escape (antigenic tolerance). Effects of IDO/TDO activity on the function of T cells and MDSC are shown. APC, antigen-presenting cell (e.g., dendritic cell); MDSC, myeloid-derived suppressor cell; TAM, tumor-associated macrophage; TAN, tumorassociated neutrophil; Teff, T effector cell; Treg, T regulatory cell.

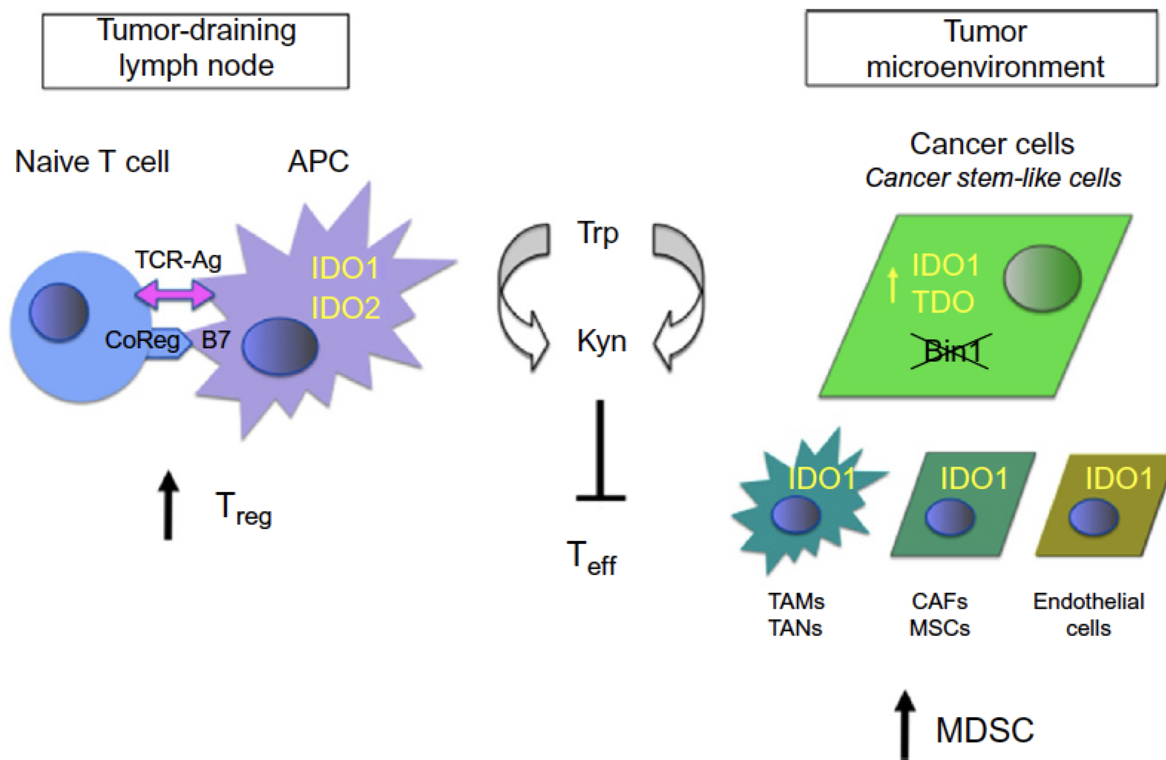

Fig. 2. IDO2 as a contributor to IDO1-mediated immune tolerance. (A) Ido2-deficient mice are defective in a PD1-dependent mechanism of IDO1-mediated Treg induction, in support of other evidence of Ido1–Ido2 genetic interaction in the mouse (Metz et al., 2014). (B) Model. IDO2 expression activated by kynurenine/AhR signaling in APC acts to distally propagate tolerance signals produced locally by IDO1 in tumor and tumor stromal cells (gray or pink cells in blue tumor, respectively). Local IDO1 expression blunts antigen-specific T effector cells mediated by kynurenine production and tryptophan deprivation (Metz et al., 2012). IDO2 expression is upregulated in roving APC through the IDO1-mediated production of kynurenine, which acts through its receptor AhR to drive IDO2 transcription in APC (Vogel et al., 2008). IDO2 activity is licensed by IDO1 through transcriptional and posttranscriptional mechanisms (Prendergast et al., 2014a). APC are tolerized by kynurenine (Nguyen et al., 2010) and IDO2 is evoked as an effector in this model. IDO2 reinforces tolerance in APC by irreversible signals that differ from IDO1 signals which are reversible (Metz et al., 2007). APC programmed by IDO2 rove to tumor-draining lymph nodes (green TDLN) or other metastatic sites where they reinforce IDO1-dependent Treg formation (IDO1 is also expressed in APC but not shown for clarity). This model is compatible with the latest model for IDO1 function in Treg formation (Munn and Mellor, 2013), invoking IDO2 as a required intermediate function based on studies in Ido2-deficient mice (Metz et al., 2014).

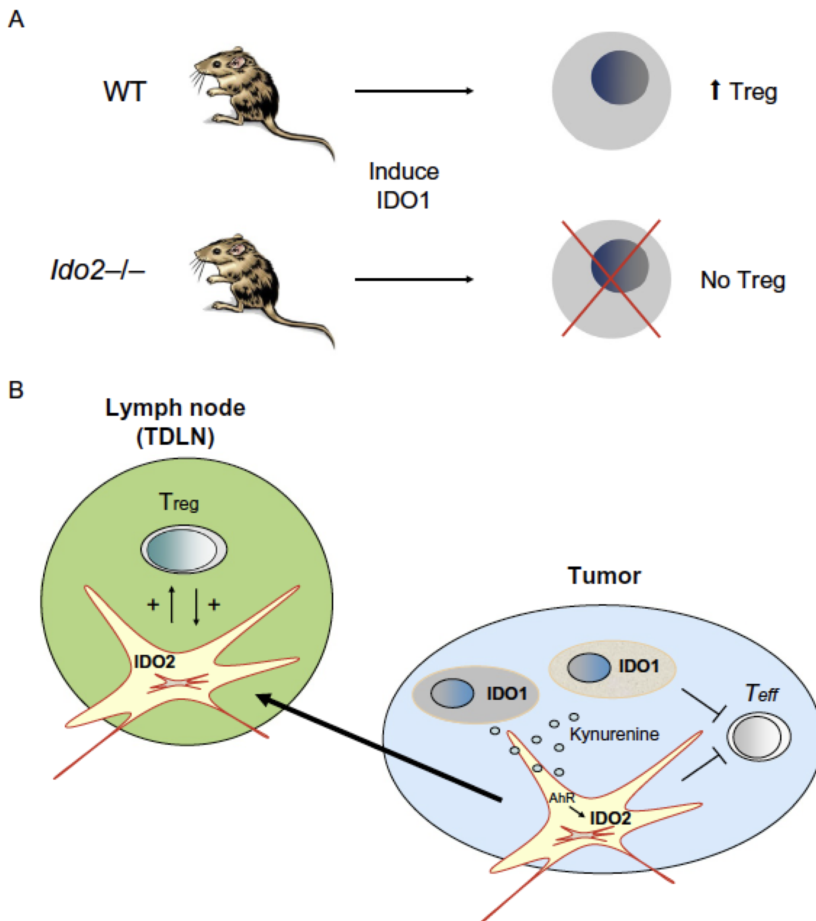

Fig. 3. IDO/TDO inhibitors to leverage immune checkpoint therapy and chemotherapy. IDO/TDO inhibitors are effective only in combination therapeutic regimens, acting as immunomodulators to relieve immune escape and promote adaptive immune escape, but also to ablating or reprogramming inflammatory processes which can leverage the efficacy of chemotherapy as well as immune checkpoint therapy.

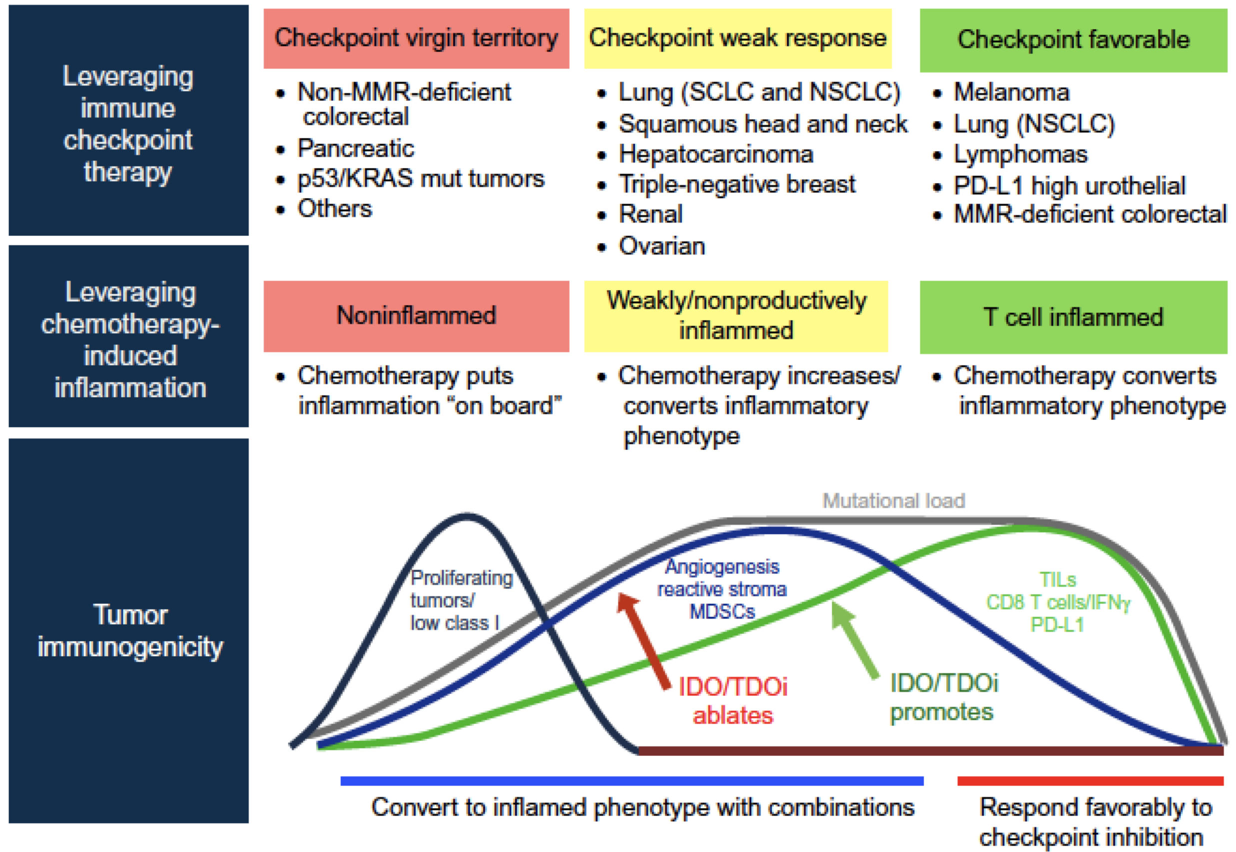

Fig. 4. Phenylimidazoles rooted in navoximod/NLG919 development. (A) 4-PI bound to heme iron of IDO1. C129 is located above the 4-PI phenyl ring, while S167 resides in the back of the binding site. The buffer molecule CHES (yellow) is bound at the entrance of the active site of the IDO crystal structure. Graphics generated with PyMOL 1.0, [http://wwwpymolorg] an open-source molecular graphics system developed, supported, and maintained by DeLano Scientific LLC http://www.delanoscientific.com. (B) Ring numbering of 4-phenylimidazole structures. (C) Structure of N-3 benzyl-substituted 4-PI and root Markush structure of NLG-919/GDC-0919. (D) Two possible benefits of 2**0**-OH substitution of 4-PI core.