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STATE-OF-THE-ART REVIEW

Indoleamine 2,3-dioxygenase 1 (IDO1): an up-to-date overview of an eclectic immunoregulatory enzyme

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Indoleamine 2,3-dioxygenase 1 (IDO1) catalyzes the initial rate-limiting step in the degradation of the essential amino acid tryptophan along the kynurenine pathway. When discovered more than 50 years ago, IDO1 was thought to be an effector molecule capable of mediating a survival strategy based on the deprivation of bacteria and tumor cells of the essential amino acid tryptophan. Since 1998, when tryptophan catabolism was discovered to be crucially involved in the maintenance of maternal T-cell tolerance, IDO1 has become the focus of several laboratories around the world. Indeed, IDO1 is now considered as an authentic immune regulator not only in pregnancy, but also in autoimmune diseases, chronic inflammation, and tumor immunity. However, in the last years, a bulk of new information—including structural, biological, and functional evidence—on IDO1 has come to light. For instance, we now know that IDO1 has a peculiar conformational plasticity and, in addition to a complex and highly regulated catalytic activity, is capable of performing a nonenzymic function that reprograms the expression profile of immune cells toward a highly immunoregulatory phenotype. With this state-of-the-art review, we aimed at gathering the most recent information obtained for this eclectic protein as well as at highlighting the major unresolved questions.

Introduction

Indoleamine 2,3-dioxygenase 1 (IDO1) is a cytosolic, monomeric, heme-containing enzyme that catalyzes the initial rate-limiting step in the degradation of the essential amino acid L-tryptophan (L-Trp) along a pathway known as the kynurenine pathway (KP) (Fig. 1). This pathway is a cascade of enzymatic steps that produces several biologically active molecules, such as L-kynurenine (L-Kyn), and can lead to the endogenous production of nicotinamide adenine dinucleotide (NAD^+). IDO1 is normally an inducible enzyme and its most important inducer is the cytokine interferon-γ (IFN-γ).

IDO1 was discovered in the 60' by a Japanese group led by Osamu Hayaishi, along a series of studies on oxygenases in rabbit intestine [1]. However, the year that changed the perception of IDO1 by the scientific community is 1998, when David Munn and Andrew Mellor performed a pioneering experiment demonstrating that IDO1 is crucially involved in the maintenance of maternal T-cell tolerance [2]. These data raised the

Abbreviations
IDO1, indoleamine 2,3-dioxygenase 1; IDO2, indoleamine 2,3-dioxygenase 2; KP, kynurenine pathway; L-Kyn, L-kynurenine; L-Trp, L-tryptophan; PI3K, phosphoinositide 3-kinase; TDO, tryptophan dioxygenase.

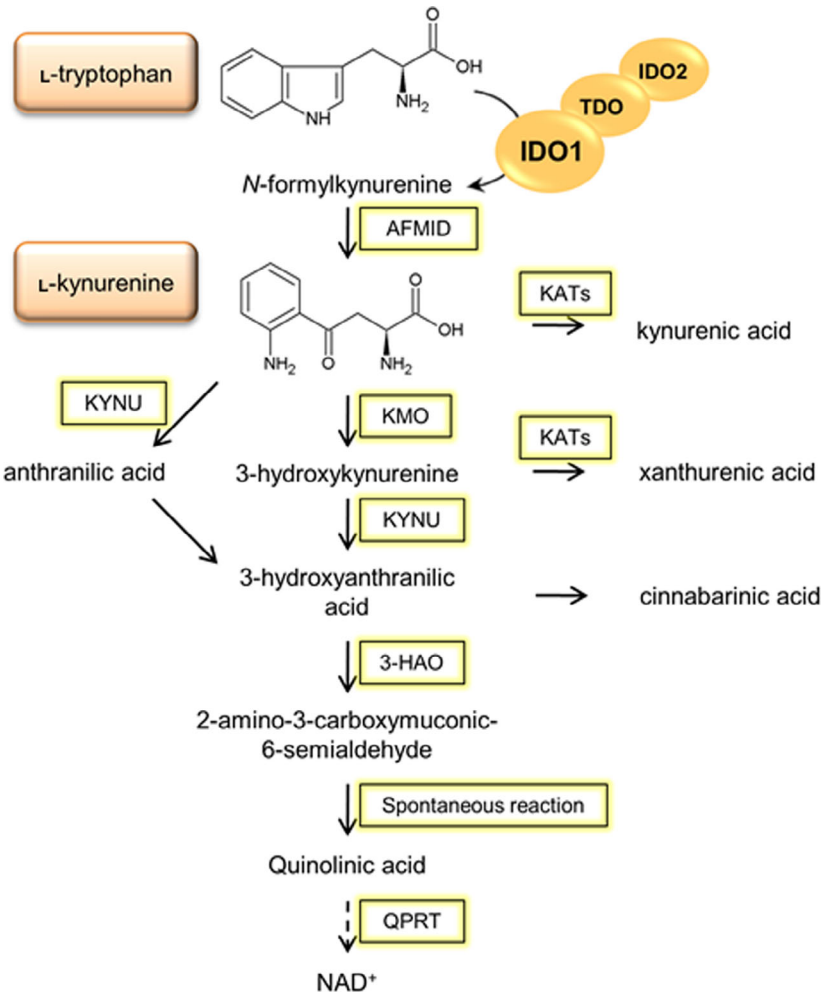

Fig. 1. The KP in mammals. The first and rate-limiting step in the KP is catalyzed by IDO1, IDO2, and TDO. N-formylkynurenine is rapidly metabolized by AFMID into L-kynurenine, which can be transformed by KATs, KYNU, and KMO into kynurenic acid, anthranilic acid, and 3-hydroxykynurenine, respectively. 3-hydroxykynurenine can be then transformed by KATs into xanthurenic acid or KYNU into 3-hydroxyanthranilic acid, which can be converted by 3-HAO into 2-amino-3-carboxymuconic-6-semialdehyde that spontaneously transforms into quinolinic acid. NAD^+, the final product of the pathway, is then obtained by several enzymatic reactions. IDO1, indoleamine 2,3-dioxygenase 1; TDO, tryptophan dioxygenase; IDO2, indoleamine 2,3-dioxygenase 2; AFMID, kynurenine formamidase; KATs, kynurenine aminotransferases; KYNU, kynureninase; KMO, kynurenine monooxygenase; 3-HAO, 3-hydroxyamino oxidase; NAD+, nicotinamide adenine dinucleotide; QPRT, quinolinate phosphoribosyltransferase.

interest on this enzyme to such an extent that the number of total publications on IDO1 has increased enormously since the early '90s.

Recent evidence indicates that the IDO1 biology is more complex than initially assumed. For instance, in human monocyte-derived macrophages and tumor cells either as such or stimulated with IFN-γ, the majority of IDO1 protein molecules do not bind heme, and therefore, a high percentage of IDO1 is in the apo form, whose biology and function are currently unknown [3–6]. Moreover, IDO1 does not only reside in cytosol but can have a distinct intra- and extracellular topology, depending on the cell microenvironment [7]. Perhaps particularly intriguing is the fact that IDO1 is not just an enzyme and, in fact, after phosphorylation of critical tyrosine residues, can act as a mediator of a signaling pathway that profoundly changes the functional phenotype of specific immune cells [8]. Structural studies suggest that the distinct functions of IDO1, that is, catalytic versus signaling, may be associated with distinct protein conformations [9,10].

Therefore, IDO1 should be considered a moonlighting protein [11,12], that is, capable of mediating distinct functions in response to distinct cellular needs, and as such should be taken into account as drug target for a more effective immunotherapy.

Insights into IDO1 key structural elements

Several crystallographic structures of human IDO1 are available in different ligand-bound and unbound forms [13]. Overall, they show a conserved fold of the enzymic primary sequence that is composed of a large domain containing the catalytic cleft and a small domain featuring two functional immunoreceptor tyrosine-based inhibitory motifs (ITIMs; Y111, Y249) that are associated with the noncatalytic signaling function of the enzyme (Fig. 2) [8,10,14]. A further

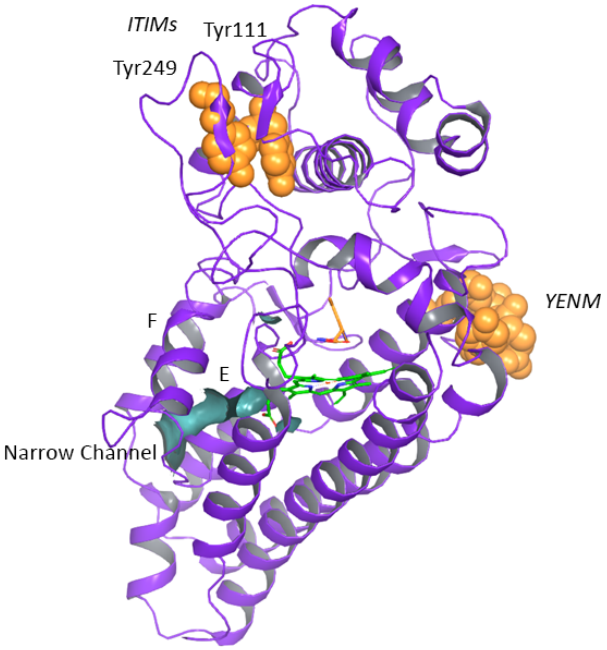

Fig. 2. Crystal structure of substrate-bound IDO1 (pdb code: 5WMU). Key structural elements are highlighted and labeled: ITIMs (Tyr111, Tyr249, orange CPK atoms); YENM motif (orange CPK atom sticks); narrow channel (cyan surface). Heme cofactor (green carbon atoms) and L-Trp (orange carbon atom sticks).

conserved post-transcriptional regulatory site is located at a YENM motif (residues 145–148), which extends from the small domain toward the large domain. Upon phosphorylation, it triggers the interaction with class IA phosphoinositide 3-kinases (PI3Ks) that, once activated, promote a localization shift of IDO1 from the cytosol to early endosomes (see below) [7].

Large and small domains are connected by a loop (DE-loop; residues 260–268) above the sixth coordination site of the heme cofactor. A large flexible loop (JK-loop; residues 360–382) controls the access to the catalytic site, adopting distinct closed, intermediate, and open conformations that are involved in mediating substrate recognition [9,15–18]. Another structural element of IDO1 is a narrow channel formed by α-helices E and F that extends from the solvent-exposed surface of the protein to the heme group [3]. This channel is relevant for the catalytic activity as it is thought to mediate the shuttling of the co-substrate oxygen to the catalytic cleft for promoting the oxidative cleavage of the indole ring of L-Trp [3]. At odds with its functionally related enzyme tryptophan dioxygenase (TDO), IDO1 shows a wide recognition of substrates beyond L-Trp, including many indole-bearing compounds such as serotonin, melatonin, and tryptamine [19], suggesting that IDO1 activity may translate into additional functions as compared to the L-Trp-specific enzyme TDO.

Recent crystallographic studies of human IDO1 in complex with the substrate cast a light on the binding modes of L-Trp into the catalytic cleft and an accessory site, with the latter being associated with the inhibition by substrate phenomenon [3,20]. Upon oxygen binding to the ferrous active form of IDO1 [21–24], L-Trp binds to the catalytic pocket on the sixth coordination site of the heme cofactor, yielding the catalytically active ternary complex (Fig. 3). According to this binding mode, the indole ring engages S167 with a water-mediated hydrogen bond and establishes face-to-edge π-stacking and hydrophobic interactions with F163 and Y126. The ammonium group of L-Trp is hydrogen-bonded to T379 and the 7-propionate group of the heme, while the carboxylic moiety makes three hydrogen bonds with R231 and T379. This kind of interactions is frequently observed for primary amine and carboxylic moieties in the crystal structures of ligand-bound protein complexes [25,26].

High concentration of L-Trp inhibits the catalytic activity of IDO1, causing the inhibition by substrate phenomenon [19,27]. This is ascribed to the interaction of L-Trp to an accessory site that is located below the fifth coordination site of the heme cofactor as observed in the crystal structure of the F270G variant of IDO1 (Fig. 4) [19]. According to this binding mode, L-Trp engages the side chains of V170, V269, L339, and L342 with extensive hydrophobic contacts, yielding a poor dissociation constant, that is, in the millimolar range of potency (K_d = 26 mM) [21]. Noteworthy, positive allosteric modulators (PAMs) of IDO1 such as indoleamine-ethanol (IDE) and N-acetylserotonin (NAS), as well as some uncompetitive inhibitors such as mitomycin C, have been proposed to bind into this accessory site of the enzyme [3,28,29].

Beside the canonical catalytic cleft and the substrate accessory site, other cryptic binding pockets of L-Trp have been conjectured in the structure of IDO1. However, their functional relevance is still elusive. In this regard, computational and biophysical studies have suggested the presence of a metastable binding pocket that is located on K238 and promotes a first interaction of L-Trp to the enzyme along the substrate approaching pathway from the solvent to the catalytic cleft [9]. In the same study, another cryptic site was suggested using microscale thermophoresis to investigate binding interactions between L-Trp and IDO1. Specifically, multiple binding events of the substrate to the enzyme were observed in both the heme-bound and apo states of IDO1, yielding high and low

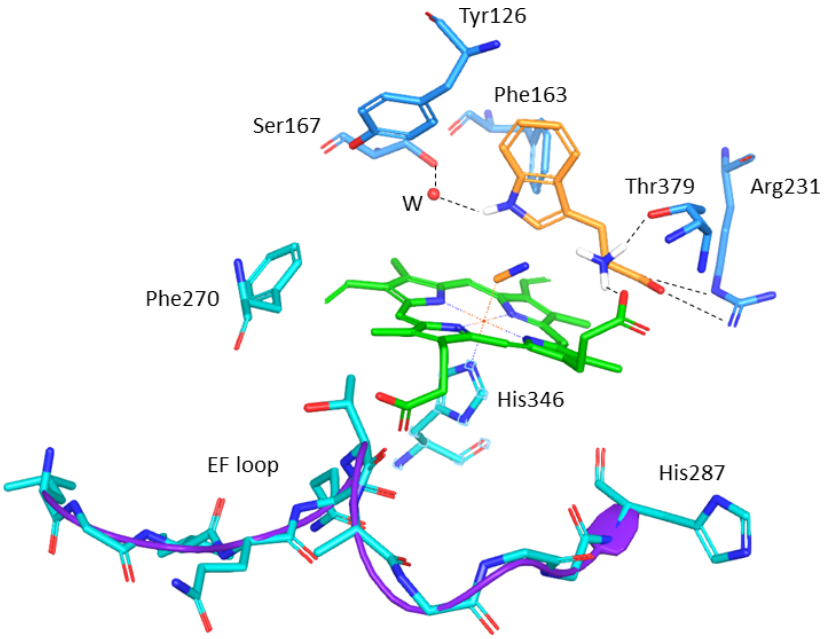

Fig. 3. Binding mode of L-Trp (orange carbon atom sticks) into the catalytic site of IDO1 (pdb code: 5WMU). Hydrogen bond interactions between L-Trp and binding site residues are shown in black dashed lines. The EF loop is highlighted with a purple cartoon.

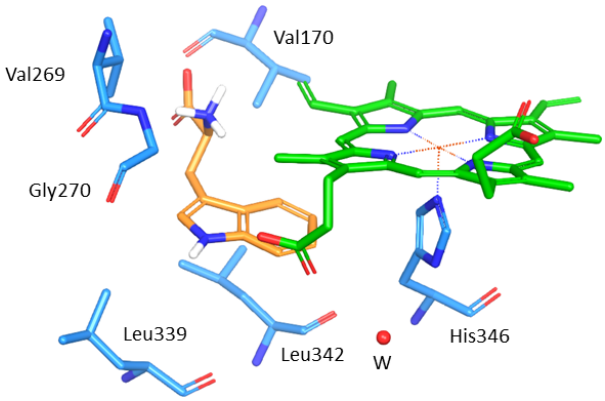

Fig. 4. Binding mode of L-Trp (orange carbon atom sticks) into the accessory site of the Phe270Gly variant of IDO1 (pdb code: 5WMW). L-Trp engages a cluster of nonpolar residues (Val170, Val269, Leu342) in hydrophobic contacts, whereas no polar interactions are observed.

dissociation constants. Although the high dissociation constants were in agreement with previous studies on the binding interaction of L-Trp to the catalytic active site [13,21,29,30], the low dissociation constants were clue for the existence of an additional site of L-Trp in the structure of IDO1, which has still to be characterized.

Overall, these findings suggest a high conformational plasticity of IDO1, with at least two distinct conformations that may be stabilized by the interaction of the substrate and/or ligands to distinct pockets of the enzyme, including the catalytic site. The stabilization of each of these conformations may affect the ability of IDO1 to recruit protein partners, thus driving enzymic or nonenzymic functions.

Cellular mechanisms of enzymic and nonenzymic functions of IDO1

Over the years, the central role of IDO1 in orchestrating immune responses has acquired interesting aspects. L-Trp starvation and generation of immunoactive kynurenines by $IDO1^{+}$ dendritic cells (DCs) contribute to create an immunosuppressive microenvironment, characterized by impaired T effector cell functions and enhanced activity of regulatory T (Treg) cells [31,32]. In particular, the degradation and thus starvation in microenvironments of L-Trp (i.e., an essential amino acid) activates general control nonderepressible 2 (GCN2) [31], a kinase that shuts down the expression of several genes, including the pro-inflammatory cytokine interleukin-6 (IL-6), via the inactivation of the eukaryotic translation initiation factor 2A (eIF2A). This effect leads to anergy in effector T cells and also blocks the conversion of Treg cells into pro-inflammatory T helper type 17 (Th17) cells [33,34]. Moreover, L-Kyn, the main product of IDO1 catalytic activity, is an endogenous agonist of the aryl hydrocarbon receptor (AhR; Fig. 5) [35], a ligand-activated transcription factor that, besides induction of xenobiotic metabolism genes in the liver, can promote the differentiation of effector T cells into Treg cells [36]

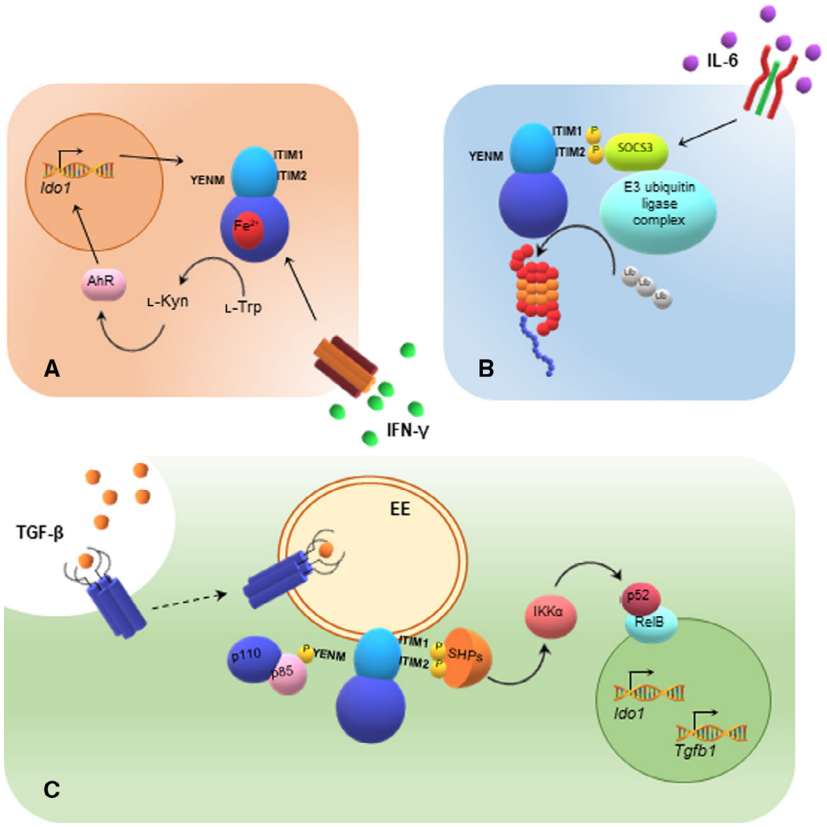

Fig. 5. Intracellular dynamics of IDO1 in DCs. Depending on the microenvironment, IDO1 shapes its conformation and acquires functions suitable for cellular needs. (A) In acute inflammation, the pro-inflammatory cytokine IFN-γ induces IDO1 enzymic activity, promoting transformation of L-Trp into L-Kyn, an agonist of AhR, a ligand-activated transcription factor that moves from cytosol to the nucleus and, via a positive feedback loop, upregulates *Ido1* gene expression. (B) Pro-inflammatory contexts driven by IL-6 are characterized by the upregulation of SOCS3, which associates with phosphorylated ITIM2 via its SH2 domain, recruits the E3 ubiquitin ligase complex, and directs cytosolic IDO1 to proteasomal degradation. (C) In a microenvironment dominated by immunoregulatory TGF-β, IDO1 is phosphorylated in ITIM (ITIM1 and ITIM2) and YENM domains, which bind and activate SHPs and PI3Ks, respectively. PI3K activation implies IDO1 direct binding to the p85 regulatory subunit that in turn promotes activation of p110, the PI3K catalytic subunit. PI3K binding favors IDO1 anchoring to EE and signaling function. SHP binding promotes IKKα-dependent activation of the noncanonical pathway of NF-κB that, via the p52/RelB heterodimer translocating to the nucleus, upregulates the expression of *Ido1* and *Tgfb1* genes. This mechanism establishes a positive immunoregulatory circuitry that ensures IDO1 long-term expression in dendritic cells. IDO1, indoleamine 2,3-dioxygenase 1; IFN-γ, interferon γ; L-Trp, L-tryptophan; L-Kyn, L-kynurenine; AhR, aryl hydrocarbon receptor; IL-6, interleukin-6; SOCS3, suppressor of cytokine signaling 3; ITIM, immune tyrosine-based inhibitory motif; TGF-β, transforming growth factor β; PI3Ks, phosphoinositide 3-kinases; EE, early endosome; SHPs, Src homology 2 domain phosphatases; IKKα, inhibitory-κB kinase α.

and also upregulation of IDO1 in DCs [37], rendering these potent antigen-presenting cells immunosuppressive. Very recently, blockade of the AhR was demonstrated to restrict an immunosuppressive axis mediated by Treg cells and tumor-associated macrophages (TAM) [38].

Approximately 10 years ago, a novel function of IDO1 was discovered [8]. The nonenzymic function of IDO1 depends on the presence of two ITIMs, namely ITIM1 and ITIM2, located in the small, noncatalytic domain of the enzyme. The ITIM sequence, I/V/L/SxYxxL/V/F (where x indicates any amino acids), when tyrosine is phosphorylated, acts as a docking site for diverse molecular partners containing Src homology 2 (SH2) domains [39,40], thus fulfilling diverse immunological needs. In fact, when DCs face the immunosuppressive cytokine transforming growth factor β (TGF-β), Src homology 2 domain phosphatase-1 (SHP1) and SHP2 are upregulated and preferentially associate with ITIM1 rather than ITIM2 (Fig. 5) [8,14]. This event translates into SHPs' interaction with the interleukin-1 receptor-associated kinase (IRAK),

thus favoring the activation of noncanonical, anti-inflammatory NF-κB rather than canonical, pro-inflammatory NF-κB [8]. In turn, noncanonical NF-κB, driven by activated inhibitory-κB kinase α (IKKα) and in the form of p52-RelB heterodimer, translocates to the nucleus and induces the expression of the *Ido1* gene itself [8,41–43]. Because the IDO1 induction is accompanied by upregulation of *Tgfb1* [7,8,44,45], this mechanism will ultimately establish a positive feedback loop that confers a long-term immunoregulatory phenotype on both conventional and plasmacytoid mouse DCs (cDCs and pDCs, respectively) [8,46].

Suppressor of cytokine signaling (SOCS) proteins are critical modulators of immune responses (11) that possess an SH2 domain, binding phosphotyrosine-containing peptides, and a SOCS box. The latter domain participates in the formation of an E3 ubiquitin ligase complex that targets several signaling proteins for proteasomal degradation [47,48]. In the presence of a microenvironment dominated by pro-inflammatory interleukin-6 (IL-6), upregulated SOCS3 preferentially associates with phosphorylated IDO1 ITIM2, recruits the E3 ubiquitin ligase complex, and targets IDO1 to proteasomal degradation [14,49]. Therefore, SOCS3 interaction with phosphorylated ITIM2 will reduce IDO1 half-life, interrupt tolerogenic mechanisms, and favor a pro-inflammatory phenotype in the DCs (Fig. 5).

A crucial point in these processes is the tyrosine kinase responsible for the phosphorylation of IDO1 ITIMs. A meta-analysis of pDC gene expression public data sets indicated that the most widely represented is Fyn, a kinase belonging to the Src family, and that Fyn inhibition decreases IDO1 ITIM phosphorylation by TGF-β [8]. In cDCs, the most important kinase involved in this mechanism is instead Src [41,46,50]. However, the possible existence of a preferential tyrosine phosphorylation of IDO1 ITIM1 versus ITIM2 by these kinases in distinct conditions has not been clarified yet.

Therefore, on the basis of these observations, IDO1 may be considered a moonlighting protein [12], that is, endowed with an additional function besides catalytic activity. Moonlighting proteins are very peculiar in that they will switch between functions by changing their conformational state, as may occur in response to altered environmental conditions [11]. Those include the redox state of the cell, temperature, but also post-translational modifications (such as phosphorylation), changes in cellular localization, and interactions with other polypeptides. The additional function of IDO1 is compatible with that of a signal transducer leading to genomic effects and reprogramming DC functions. The interesting thing is that phosphorylable ITIMs can either prolong or reduce IDO1 expression, depending on specific cellular microenvironments and distinct partnerships [51], thus underlining the plasticity of IDO1 biology.

Modulation of IDO1 expression

In humans, IDO1 is constitutively expressed in a restricted set of cells, including placental and pulmonary endothelial cells, epithelial cells inside the female genital tract, and antigen-presenting cells, such as mature DCs in secondary lymphoid organs [52,53]. IDO1 is also highly expressed in β-cells of pancreatic islet of healthy individuals but is absent in the residual β-cells of patients with autoimmune diabetes [54]. Several human tumors constitutively express IDO1. In this regard, the IDO1 protein can be found in neoplastic cells themselves but also in fibroblasts and myeloid and endothelial cells present in the tumor bed [55,56]. In some tumor cells, prostaglandin E2 (PGE2) and interleukin-1β (IL-1β) contribute to the maintenance of IDO1 basal expression [57]. Additionally, constitutive IDO1 expression in human cancer may be sustained by an autocrine signaling loop involving IL-6 and AhR [58], suggesting that IL-6 in tumors may behave differently from normal DCs in which the cytokine promotes IDO1 proteasomal degradation [49].

One of the major features of IDO1 is its inducibility [59]. IFN-γ is considered the main inducer of IDO1 in several types of cells, including but not limited to immunocytes and fibroblasts. Indeed, the promoter region of the *IDO1* gene (present in human chromosome 8p22) contains several IFN-stimulated elements (ISREs) and gamma activation sequences (GAS) [60] that respond to transcription factors such as signal transducer and activator of transcription 1 (STAT1) and interferon-regulatory factors (IRFs). IDO1 induction by IFN-γ may represent a counteracting response in inflammatory conditions [61–63], a mechanism also exploited by tumors to favor their immune escape [63]. Indeed, some neoplastic cells downregulate the expression of *Bin1* (encoding a Myc-interacting protein with features of immunosuppressor), with consequent elevation of STAT1-dependent expression of IDO1 [64]. Type I IFNs, that is, IFN-α and IFN-β, also represent IDO1 inducers, although, at variance with IFN-γ (type II IFN), activate a pathway that leads to transcription factors that bind ISREs but not GAS and are thus less effective than IFN-γ [65]. Involvement of type I IFNs is mainly observed in pDCs, which are the cells that produce the highest levels of these cytokines.

Over the years, several other signals have been identified as IDO1 inducers. These include the cytokine TGF-β that, however, favors the signaling rather than catalytic function of IDO1 [8,51]. In cDCs, activation of the Src kinase necessary for IDO1 ITIM phosphorylation can be directly promoted by spermidine, a polyamine produced downstream arginase 1 (ARG1; induced by TGF-β earlier than IDO1) [46], a finding that linked for the first time two potent immunosuppressors such as IDO1 and ARG1 [66,67] as well as polyamines and kynurenines [68]. The signaling function of IDO1 and consequent immunosuppressive effects can also be triggered by incubation of cDCs with ADX188, a positive allosteric modulator (PAM) of metabotropic glutamate receptor 4 (mGluR4), via potentiation of protein G-independent mechanisms [41]. Other cytokines, that is, tumor necrosis factor α (TNF-α), IL-6, and IL-10, can synergize with each other to increase IDO1 expression [69].

Pro-inflammatory signals capable of upregulating IDO1 include pathogen-associated molecular patterns (PAMPs) [70]. PAMPs are present in diverse organisms but absent in the host and provide exogenous signals that alert the immune system to the presence of pathogens, thereby promoting immunity. They are recognized by and activate Toll-like receptors (TLRs), normally contributing to inflammatory processes but also immune tolerance [71]. Lipopolysaccharide (LPS) and oligonucleotides containing unmethylated CpG motifs (CpG-DNA) are PAMPs that can modulate IDO1 activity via activation of TLR4 and TLR9, respectively. TLR4 is an intriguing receptor that, by virtue of its surface membrane or early endosome (EE) localization, can recruit either MyD88 or TRIF signaling adaptors, mediators of inflammatory or tolerogenic effects, respectively [72]. After repeated stimulation, LPS can in fact generate a state of immune suppression, known as endotoxin tolerance. Bessede *et al.* showed that IDO1 is highly expressed in cDCs repeatedly stimulated with low doses of LPS *in vitro* and that administration of LPS-treated DCs or L-Kyn alone can confer protection in recipient mice from endotoxic shock via activation of AhR and IDO1 upregulation in cDCs [50]. Human DCs conditioned with LPS exhibit upregulation of anti-inflammatory IL-10 that in turn activates the noncanonical NF-κB pathway, with overexpression of RelB in an IDO1-dependent manner [73]. Activation of TLR9 by CpG-oligodeoxynucleotides induces the expression of IDO1 in splenic $CD19^+$ DC, which acquire suppressive functions [74]. Moreover, in pDCs, the tolerogenic signaling via TLR9 that leads to IDO1 expression is dependent on the dosage of CpG-ODN administration (i.e., low dose being immunostimulatory and high dose immunoregulatory) and requires the involvement of the TRIF adaptor [75].

An intriguing issue is the capacity of membrane-anchored molecules to induce IDO1 in DCs via 'reverse signaling'. This phenomenon occurs when a molecule normally operating as a receptor and expressed on the surface membrane of another cell activates a signaling pathway via engagement of a membrane molecular counterpart normally acting as a ligand [42]. By binding to CD80 and CD86 on DCs, cytotoxic T lymphocyte antigen 4 (CTLA-4) engenders an IFN-γ-dependent induction of IDO1 [76]. Because Treg cells constitutively express CTLA-4, this mechanism represents an important functional bridge between Treg cells and regulatory DCs in immune tolerance [77]. In addition to CTLA-4, Treg cells possess the glucocorticoid-inducible TNF receptor (GITR) [78]. Engagement of its ligand, GITRL, by a soluble form of GITR activates a reverse signaling in pDCs resulting in the activation of the noncanonical pathway of NF-κB, increased IDO1 expression, and IDO1-dependent immunosuppressive effects *in vivo* [79]. Because GITR and GITRL are under the control of glucocorticoids, these data unveiled an important mechanism of action of such potent compounds.

Therefore, IDO1 expression and functions appear to be under the control of a great variety of stimuli that can act either directly or indirectly and can sometimes behave very differently, depending on the circumstances.

Intra- and extracellular localization of IDO1 and role of PI3Ks

The bulk of literature has repeatedly indicated a cytosolic localization for the IDO1 enzyme in several types of cells [80,81]. This localization may favor IDO1 intense and transient catalytic activity, possibly because of the easier access to substrate, that is, L-Trp. However, until recently, no information was available regarding the intracellular topology of IDO1 as a signaling molecule. A critical discovery was that, in addition to ITIMs, IDO1 contains a consensus binding site for the p85 regulatory subunit of PI3K [7]. This motif, namely YENM, is characterized by a YxxM sequence (where 'x' indicates any amino acid). Alignment of the amino acid sequences reveals that the YENM sequence of IDO1 in mammals is completely conserved [7] but is not present in mammalian indoleamine 2,3-dioxygenase 2 (IDO2), the IDO1 paralogue whose gene is considered an ancient version of *IDO1* itself (see next paragraph) [82].

When the tyrosine residue within YENM becomes phosphorylated, IDO1 associates with p85 that in turn interacts with the p110 catalytic subunits of PI3Ks [7]. PI3Ks are a family of enzymes subdivided into three classes. Among them, class IA PI3Ks are heterodimeric proteins consisting of a regulatory subunit (p85α, p85β, or p55γ) and a catalytic subunit (p110α, p110β, or p110δ) [83]. Although p110α and p110β subunits are ubiquitously expressed, p110δ expression is generally restricted to cells of the immune system [83] and promotes anti-inflammatory effects in DCs and macrophages [72]. As a consequence of IDO1 binding to p85, all class IA p110 catalytic subunits can be recruited, although the greater extent is observed for p110δ in cell transfectants expressing mouse IDO1 [7]. Class I PI3Ks possess important roles in several physiologic conditions as well as in disease [84–86]. By activating multiple signaling pathways and involving a plethora of downstream effectors, they can orchestrate both pro-inflammatory and anti-inflammatory mechanisms to maintain effective immunity while protecting host tissues. Interestingly, among their multiple functions, members of the PI3K family can also affect intracellular trafficking of vesicles and proteins [87].

Several activated receptors and signaling proteins accumulate in EE, which therefore can be defined as a platform hosting intracellular signaling [88]. Signals deriving from receptors and other proteins located in EE have different roles, such as control of growth, differentiation, survival, inflammation, and immunity. Efficient TGF-β signaling—also responsible for IDO1 signaling activation—requires internalization of the TGF-β receptor in EE [89,90]. In a very recent study, treatment of pDCs with IFN-γ or TGF-β determined a dominant localization of the IDO1 protein in cytosol or EE, respectively [7]. Moreover, IDO1 anchoring to EE was mediated by binding of class I PI3Ks [7], an effect favoring IDO1 signaling rather catalytic activity in pDCs. These observations indicated, for the first time, an IDO1 localization distinct from cytosol and how the intracellular switch can occur. It is interesting to note that, in the absence of any stimulus, in both primary fresh cultures of pDCs and tumor transfectants highly expressing IDO1, IDO1 is mainly present in the cytosol but discrete quantity of the protein can be detected in EE as well, suggesting the *a priori* existence in $IDO1^+$ cells of a pool of IDO1 proteins with distinct topology and possibly ready for distinct functions. Furthermore, although not demonstrated yet, IDO1 in EE may not contain heme. In fact, in unstimulated human ovarian cancer cells, IDO1 is dynamically bound to its heme cofactor and, perhaps most importantly, at least 85% of IDO1 exists in the apo form, that is, catalytically inactive, that can be nevertheless activated by exogenous heme added in the form of hemin [6]. Likewise, hemin increases IDO1 activity 2-fold in IFN-γ-activated human monocyte-derived macrophages [4], suggesting that cellular heme is limiting for IDO1 activity and that a significant portion of IDO1 in these cells is present as apoprotein. Along this line of evidence, transfected mouse IDO1 with tyrosine in ITIM1 substituted with glutamate (i.e., mimicking phosphorylated tyrosine) does not show any catalytic activity [14].

IDO1 is not confined into the cells but can be secreted in extracellular vesicles (EVs), including exosomes. In fact, stem cells from amniotic fluid treated with the main IDO1 inducer IFN-γ produce immunoregulatory EVs containing IDO1 [91]. EVs from human semen express high levels of IDO1 transcripts, suggesting a mechanism whereby these vesicles induce tolerance [92]. Also, malignant glioblastoma cells generate immunosuppressive EVs containing IDO1, suggesting that tumors can exploit both intracellular and extracellular IDO1 as immune escape mechanism [93]. Exosomes derived from IDO1-overexpressing rat bone marrow mesenchymal stem cells promote immunotolerance of cardiac allografts [94]. Although we do not know how and when IDO1 is transferred outside the cells via vesicles yet, these observations further confirm the complexity and dynamics of IDO1 in physiologic and pathologic conditions.

As a whole, IDO1 expression in cytosol, EE, and EVs—and, perhaps most importantly, the distinct IDO1 functions in cytosol versus EE—further confirm the hypothesis of IDO1 being a moonlighting protein [95]. In fact, the localization of a protein in a new cellular microenvironment could promote unpredicted molecular interactions by many potential new binding partners and contribute to generate new functions, as occurring for IDO1 [7]. Distinct activities mediated by STAT3 and Tgase2 are associated with different localization, including exosomes [96,97].

In conclusion, the IDO1 catalytic and signaling functions are spatially segregated. Further studies are necessary to define the role of the IDO1 signaling function and its subcellular localization in pathological conditions, such as cancer, in order to optimize the development of new and more effective therapies [98].

IDO1, IDO2, and TDO: similar or distinct enzymes?

In addition to IDO1, there are two other mammalian enzymes capable to convert L-Trp to L-Kyn, that is,

IDO2 and TDO. Since its discovery in 2007 [82], IDO2 represents the 'enigmatic version' of IDO1. In fact, the two IDOs are encoded by genes located in tandem on chromosome 8, in both humans and mice [99], and share a high level of sequence similarity at the amino acid level. Nevertheless, IDO2 catalytic activity is negligible *in vitro* [82]. Moreover, IDO2 knockout does not contribute to the reduction in L-Kyn sera levels *in vivo* as instead shown by IDO1 depletion [100] and the pattern of the expression of the two molecules is consistently different, being IDO2 mainly expressed in the liver under physiologic conditions [101].

Interestingly, lower vertebrates generally possess only IDO2, and many fish species have both IDO1 and IDO2; as a possible explanation, molecular phylogenetic analyses showed that the gene duplication occurred before the divergence of vertebrates, with IDO1 having been lost in a number of lower vertebrate lineages [102]. Sequence analysis of the human *IDO2* showed a peculiar and wide distribution of two single nucleotide polymorphisms (SNPs), which are rs10109853, leading to a > 90% reduction in IDO2 catalytic activity (R248W), and rs4503083, generating a premature stop codon (Y359X) [103]. However, the exact functional significance of the two common IDO2 variants is still far from being deciphered, as is the understanding of IDO2 physiological role. In fact, the putative structural analogies between IDO1 and IDO2, suggested by their high amino acid sequence homology, seem to not correspond to similar functions.

In vivo studies demonstrated a contrasting role for IDO2, with experiments in preclinical models of autoimmune arthritis suggesting a pro-inflammatory role driving the disease [104]. In neoplastic diseases, IDO2 is much less expressed in tumor cells than IDO1 and its function is still matter of debate. In humans, pancreatic ductal adenocarcinoma (PDAC) [105] and non-small-cell lung cancer (NSCLC) [106] show particularly high levels of IDO2 expression. Nevertheless, the presence of a genotype compatible with an inactive form of IDO2 significantly associates with an improved disease-free survival in patients with PDAC [107] but increases the risk of developing NSCLC [108].

Many studies have demonstrated that the knockout of the *Ido2* gene can inhibit tumor growth in multiple animal models [109]. However, the use of IDO2 specific inhibitors could be a failure strategy, as it was in case of IDO1 [98]. In fact, the inhibition of IDO1 enzyme activity only may be not sufficient to exploit an effective therapeutic function, considering the complex functional dynamics of IDO1 and its additional signaling function. Regarding IDO2, we might hypothesize that it could possess a completely different function other than the poor enzymatic one, presumably relying on some still unidentified partnership with other proteins and consequent signaling activity. The presence of one ITIM motif (i.e., ITIM2) in both human and mouse IDO2 amino acid sequences pleads for a potential role of this molecule as a signaling molecule, although its function is still unknown.

Unlike IDO2, the physiologic role of TDO is better defined. TDO is mainly located in the liver and uses L-Trp as substrate, thus showing a higher substrate specificity compared to IDO1 [110]. In the liver, TDO degrades excess dietary L-Trp in order to maintain systemic stable levels of the amino acid [111]. Interestingly, seric L-Kyn, the main product of both TDO and IDO1, is increased in TDO knockout mice but is totally absent in mice lacking IDO1, indicating that circulating L-Kyn is mainly produced by IDO1. This is likely because the L-Kyn produced by TDO is further degraded in the liver, which expresses all the enzymes of the L-Kyn pathway, whereas inflammatory and immune cells that express IDO1 in the periphery may not express these enzymes [112].

TDO expression is also detected in the decidualized endometrium, where its function is unknown [113], and in the brain, contributing to the synthesis of neuroactive compounds [114]. TDO is thought to participate in the pathogenesis of several neurodegenerative diseases, such as Alzheimer, Parkinson, and Huntington disease (AD, PD, and HD, respectively), via regulation of proteotoxic events [115]. Significantly high TDO immunoreactivity has been observed in the AD patients' hippocampus [116]. Moreover, in fly models of AD and PD, TDO inhibition improves motor performance and helps survival, and increased kynurenic acid relative to 3-hydroxykynurenine alleviates HD, indicating that shifting the KP to kynurenic acid rather than 3-hydroxykynurenine synthesis can reduce neurodegeneration [117].

TDO2 mRNA can be also found in a number of human tumor samples, including hepatocarcinomas, glioblastomas, melanomas, and bladder carcinomas [118] and in many human tumor cell lines, including glioblastoma, colorectal, head and neck, lung, and gall bladder carcinoma cell lines, where its protein expression and activity have been confirmed [119]. Like IDO1, TDO plays an immunoregulatory role in tumors via L-Kyn activation of AhR [35,120]. TDO-transfected mouse mastocytoma P815 cells resist immune rejection by mice immunized against P1A, the dominant antigen expressed in these tumors, and tumor rejection is restored upon treatment of mice

with a TDO inhibitor [119], thus pinpointing TDO as a potential target for the development of selective antitumoral drugs.

Physiologic versus pathologic effects of IDO1

Physiologic roles of IDO1

The IDO1 protein is a physiologic checkpoint that guarantees immune homeostasis in our organism by finely modulating a balanced immune response orchestrated by antigen-presenting cells, such as DCs and macrophages. It plays a key role in supporting immune privilege [121], including the feto-maternal interface in allogeneic pregnancy to protect the fetus from the maternal immune system [2] and further immune niches as cornea [122] and brain [123]. L-Trp metabolites generated by IDO1 along the KP serve as signaling molecules for fine-tuning the host immune response, mainly via AhR engagement of AhR [124–126]. The large evidence of a nonenzymic function of IDO1 that co-operates with its catalytic activity renders more intricate the mechanisms of IDO1-based immune tolerance [8,51] that, nevertheless, opens the generation of innovative strategies targeting IDO1.

IDO1 catalytic activity also controls the *de novo* synthesis of NAD^+, in addition to the salvage pathway that supplies the cell with the highest levels of NAD^+ cofactor. The metabolic relevance of the kynurenine in NAD^+ supplementation in both health and disease is not well defined, although the contribution of L-Trp metabolism to NAD^+ formation in the central nervous system (CNS) and in the tumor microenvironment (TME) is more evident than in other tissues [127,128].

A growing body of evidence has demonstrated that IDO1 becomes a pathogenic factor overarching a wide range of human pathologies, including infections, neoplasia, autoimmunity, and neurodegeneration [55,129–131], as outlined below.

IDO1 in infectious diseases

L-Trp metabolism is an important regulator of immune host-pathogen interactions. Several PAMPs can induce IDO1 expression by acting as TLR ligands [75,132].

In infectious diseases, L-Trp depletion caused by IDO1 activity has long been known to induce antimicrobial actions for auxotroph pathogens [133–135]. Several *in vitro* studies reported that measles, influenza, cytomegalovirus, and herpes simplex viral infections are susceptible to L-Trp levels [132,135]. However, L-Trp starvation can reprogram several auxotroph invaders to re-acquire the capacity to synthesize this essential amino acid, as in the case of *Mycobacterium tuberculosis* [136]. At the same time, generation of downstream kynurenines by IDO1 acts as a double-edge sword in infectious diseases, creating a state of immunosuppression that may impair clearance of the microorganism.

Combined antiviral therapy with IDO1 blockade significantly reduces the simian immunodeficiency virus (SIV) load in plasma and lymph nodes of treated rhesus macaques [137], indicating that, in this case, immunoregulatory IDO1 is at work in the disease and its inhibition favors recovery of infected animals. Recently, Gautam *et al.* [138] demonstrated in macaques affected by active tuberculosis that inhibition of IDO1 activity leads to a reduced *M. tuberculosis* burden, pathology, and improved animal survival. Currently, it is unclear yet whether elevated IDO1 is causative of the progression to active infection or a compensatory response to the microbe. However, these observations suggest that a potential for using IDO1 inhibitors as an effective anti-microbial therapy may exist.

In addition to the control of pathogen load, IDO1 activation can restrain pathogenic immune activation that would ultimately worsen the infection. Indeed, in experimental fungal infections, IDO1 blockade greatly exacerbates the disease and the associated inflammatory pathology, as a result of dysregulated innate and adaptive immune responses to the fungi [139]. Interestingly, fungi express IDO1 and produce kynurenines that are involved in fungal but also host fitness [140].

IDO1 in cancer

In tumors, activation of L-Trp catabolism by any of the L-Trp-degrading enzymes leads to a local generation of immunosuppressive kynurenines and L-Trp depletion that determines a poor immune response. Based on this postulate, a fervid drug discovery activity in the development of anticancer small molecules targeting both IDO1 and TDO enzymes has characterized the last two decades [141–143]. A variety of catalytic inhibitors of IDO1 have been developed with favorable properties for a drug candidate (i.e., higher selectivity, potency, oral bioavailability, and safe profile) and with different mechanism of inhibition (i.e., competitive, noncompetitive, and heme-displacing molecules) [69,144]. An enormous attention has developed around targeting IDO1 in cancer immunotherapy, encouraged by promising results coming from preclinical studies that demonstrated, in a number of animal models, the antitumor effect of IDO1 blockade

in monotherapy or in combination with other immune checkpoint inhibitors [145,146].

Based on the correlation of IDO1 expression with worse prognosis in a wide range of human tumors [147,148], the most promising compound, that is, epacadostat, reached clinical trial phase 3. Disappointingly, the largest phase 3 clinical trial (ECHO-301) in advanced melanoma with epacadostat and pembrolizumab (an anti-PD1 antibody) failed. The trial was suspended because the therapeutic strategy did not show any increased benefit compared with placebo plus pembrolizumab in melanoma patients [149]. To date, none of the IDO1 inhibitors has been approved for anticancer therapy yet.

Despite the failed experience with epacadostat, a strong translational rationale still exists for targeting IDO1 and, more widely, L-Trp metabolism in cancer immunotherapy. Since it became evident that standalone IDO1 blockade is not effective in tumor disease progression, numerous novel molecules with dual inhibitory activity have been developed. Dual TDO/IDO1 inhibitors have been designed for tumors where IDO1 inhibition can play the by-pass to L-Kyn generation by TDO. TDO might provide compensatory mechanisms to L-Trp depletion and production of L-Kyn metabolites. Therefore, dual or even pan-inhibition of L-Trp-degrading enzymes (i.e., also targeting IDO2) might be beneficial and complementary for efficacy improvement in immunotherapy [119].

Novel L-Trp–L-Kyn–AhR axis inhibitors, as L-Kyn-degrading enzymes, direct AhR antagonists, and L-Trp mimetics are also advancing in preclinical development [150]. Although multitargeting of L-Trp metabolism in cancer appears a rational strategy, potential side effects should of course be considered. Lastly, the nonenzymatic function of IDO1 that promotes in DCs a long-term immunosuppressive phenotype [8,46] could contribute to restrain the antitumor immune response in TME. Conformations that mediate both enzymatic and nonenzymatic (i.e., signaling) activities of IDO1 are in a dynamic balance in the cell [14]. Computational studies performed by Mammoli *et al.* on the crystal structures of IDO1, with or without bound inhibitors, suggest that diverse inhibitors can induce different conformational changes in the small domain of IDO1 and thus affect not only its catalytic but also the signaling function [10]. In patients with noncurable glioblastoma, the advanced age was associated with increased IDO1 expression, decreased immunotherapeutic efficacy, and was not reversed by IDO1 enzyme inhibition [146], proposing that targeting the signaling activity of IDO1 in combination with its catalytic function may improve IDO1-targeting immunotherapy. Indeed, the nonenzymatic function could provide either an interpretation of the IDO1 inhibitors' failure in the trials or suggest new strategies for targeting IDO1 more effectively.

IDO1 in autoimmunity

The pleiotropic mechanisms of IDO1 are deeply involved in the pathogenesis of autoimmune conditions featured by an aberrant immune response against self-antigens. Contrary to what happens in cancer, a poor activity of IDO1 facilitates the over-activation of immune effectors in autoimmunity [151]. A critical role is played by $IDO1^+$ DCs that, in physiological conditions, may control immune responses by inhibiting effector T cells and promoting Treg lymphocytes in an antigen-specific manner [42,61,62].

In type 1 diabetes (T1D), a defective IDO1 activity has been observed in DCs from nonobese diabetic (NOD) mice [44,152,153], in a majority of peripheral blood mononuclear cells (PBMCs) isolated from pediatric diabetic patients [154], and in β-cells of human pancreas from adult diabetic donors [54]. In both DCs from NOD mice and PBMCs from diabetic children, poor IDO1 activity is caused by a rapid turnover of the protein triggered by an IL-6-driven pro-inflammatory milieu. Indeed, the IDO1 defect can be corrected by blocking the IL-6 receptor (IL-6R) by means of tocilizumab (TCZ). The drug inhibits IDO1 degradation and restores normoglycemia in diabetic NOD mice via an IDO1-dependent mechanism [155]. Similarly, TCZ can restore L-Trp metabolism in PBMCs from a subgroup of T1D children characterized by a deregulated IL-6R-SOCS3 axis and a distinct frequency of a single nucleotide polymorphism (SNP), that is, *IDO1* rs7820268. These observations are consistent with the data reported by Antequil *et al.* that describe a gradual decay of IDO1 in pancreatic β-cells during T1D pathogenesis, ranging from autoantibody-positive donors to recent-onset T1D patients, and a complete absence of IDO1 in insulin-deficient islets [54].

Recently, Mondanelli *et al.* [28] proposed an innovative therapeutic indication for NAS, a L-Trp-derived metabolite produced along the serotonin pathway, in experimental autoimmune encephalomyelitis (EAE; an animal model of human multiple sclerosis, MS). For the first time, NAS has been described as a PAM of the IDO1 enzyme, capable of implementing the production of L-Kyn that in turn activates AhR. As a consequence, NAS reinforces the immunoregulatory IDO1-L-Kyn-AhR axis in DCs and restrains the pathogenic neuroinflammation that characterizes MS. NAS

protects mice from EAE in an IDO1- and AhR-dependent manner. In PBMCs isolated from patients with relapsing-remitting MS [28,156], NAS increases L-Kyn release in IFN-γ-stimulated conditions without modulating IDO1 expression.

The analysis of IDO1 SNPs in both autoimmune diabetes and MS cohorts displayed a different distribution of the rs7820268 SNP among autoimmune patients and healthy subjects. Although several SNPs in *IDO1* have been associated with the occurrence of autoimmune/chronic inflammatory diseases, including T1D, Crohn's disease, systemic sclerosis, and MS [28,151,157,158], the majority of them affects intronic regions or the *IDO1* promoter and lacks a cause–effect relationship. A bottleneck in developing IDO1 as a predictive biomarker and promising therapeutic target in both T1D and MS is in fact the lack of a genotype-to-protein association. To date, a unique rs751360195 single variant in the IDO1 coding sequence has been identified in a 30-year-old woman affected by coeliac disorder, selective IgA deficiency, thyroiditis, and thrombocytopenia [159]. Interestingly, the single nucleotide variant (SNV) rs751360195 encodes a missense mutation (K257E) very closed to the ITIM2 motif that shortens IDO1 half-life in PBMCs. The recent identification of a specific IDO1 genotype encoding for a short-lived protein IDO1 in a patient affected by a broader dysimmune disorder extends the investigative field of defective IDO1 in further autoimmune conditions and reinforce the perspective to develop IDO1 as a predictive biomarker that will contribute to precision medicine in the autoimmunity field.

IDO1 in neurodegeneration

KP metabolites have been closely linked to the pathogenesis of several neurodegenerative diseases, including AD, PD, and HD [131,160]. In AD, the amyloid peptide upregulates IDO1 expression and increases the production of quinolinic acid in human macrophages and microglia [161]. Furthermore, human neurons treated with quinolinic acid upregulate genes involved in tau phosphorylation, the mechanism crucially involved in the formation of pathogenetic neurofibrillary tangles [162]. In HD, high levels of L-Kyn lead to high production of neurotoxic quinolinic acid and very low levels of neuroprotective kynurenic acid [163], thus suggesting the existence of an imbalance between distinct L-Trp metabolites that may play a causative role in HD pathogenesis. Such imbalance has also been observed in PD patients in dopaminergic neurons and surrounding microglia [164].

As compared to other pathological conditions, targeting IDO1 and other L-Trp-metabolizing enzymes in neurodegenerative diseases is still in its infancy. Nevertheless, it appears that efforts in this direction might very likely be worth making.

Concluding remarks

By comparing the effects of carbon monoxide on the conformations of human IDO1 versus human TDO, Syun-Ru Yeh and colleagues very recently found that IDO1 exhibits remarkable conformational plasticity as compared to the more rigid TDO [165]. The authors hypothesized that this structural fingerprint may be relevant for IDO1 being a multitasking protein under a harsh inflammatory environment [165]. Lacking completely in TDO, the small domain and interconnecting loop may thus represent the main structural parts of IDO1 modulating its conformational as well as functional plasticity. As a matter of fact, all identified motifs allowing IDO1 to establish a partnership with other molecules, that is, SHPs, PI3Ks, and SOCS3, have been localized in its small domain. Therefore, a peculiar overall architecture could imprint IDO1 with the flexible capacity to promote Trp metabolism, its expression, or its proteasomal degradation, meeting specific cellular needs in specific microenvironmental conditions. In this regard, it should be mentioned that several multitasking proteins undergo structural transformation when performing distinct functions [166,167]. An additional level of complexity derives from the IDO1 ability to catalyze other catalytic reactions in addition to its dioxygenase activity, including peroxygenase [168], peroxidase [169], and nitrite reductase [170] activities as well as the generation of singlet oxygen [171]. Of note, the production of singlet oxygen by IDO1 leads to the formation of a tricyclic hydroperoxide that decreases blood pressure [171]. Therefore, the versatility of IDO1 likely extends to the catalysis of several biologically relevant enzyme reactions.

Although we do not know where binding of SOCS3 and ubiquitination may occur yet (but we may hypothesized the cytosol, given the cytosolic localization of the proteasome complex), IDO1 enzymic and nonenzymic activities take place in the cytosol and EE, respectively, at least in DCs and IDO1-transfected P815 mouse mastocytoma cells [7]. An important question would be how the apo form of IDO1 influences responsiveness to specific cellular microenvironments (i.e., cytokines, TLR ligands, Trp, and O_2 concentration, pH), and thus, the choice of a specific function or fate. Vice versa, specific microenvironments may

change the balance between holo- and apo-IDO1. Moreover, which is the intra- (and possibly extra-) cellular topology of apo-IDO1? Presumably, because tumor transfectants expressing a mouse IDO1 mutant mimicking tyrosine-phosphorylated ITIM1 perform IDO1 signaling activity but do not bind heme and thus do not produce L-Kyn [14], the apoprotein may be associated with EE. Therefore, a systematic study of the percentages of apo-IDO1, dynamics of cellular localization, and performance of a specific function, either in basal conditions or in response to specific stimuli, should be undertaken in both primary cells (including DCs but also macrophages, fibroblasts, and epithelial cells known to express IDO1) and tumors, of mouse and human origin.

The delineation of the biologic and functional profile of IDO1 in tumor contexts may lead to the generation of novel drugs targeting this important protein in neoplastic patients and, perhaps, most importantly, could be more effective than the currently available catalytic inhibitors. In this regard, it would be very interesting to know whether the catalytic inhibitors targeting apo-IDO1 [6]—and preventing heme binding—do also influence the IDO1 signaling activity, which may be more important in a chronic disease like neoplasia. In chronic inflammation and autoimmunity, drug targeting of IDO1 (and enzymes in general) appears to be a more difficult task. In fact, in these conditions, IDO1 function/s should be potentiated rather than inhibited. However, the very recent discovery of VIS351 [172], an inducer of IDO1 signaling, and NAS [28], an enhancer of IDO1 catalytic activity protecting from neuroinflammation, may bring some fresh air in IDO1-based immunotherapies.

Therefore, the great amount of work that has been performed on IDO1 in the most recent years raises new biologic questions but also opens new exciting perspectives for therapeutic advances in several disease areas.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

All authors contributed to the article and approved the submitted version.

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