THE COEVOLUTION OF IDO1 AND AHR IN THE EMERGENCE OF REGULATORY T CELLS IN MAMMALS

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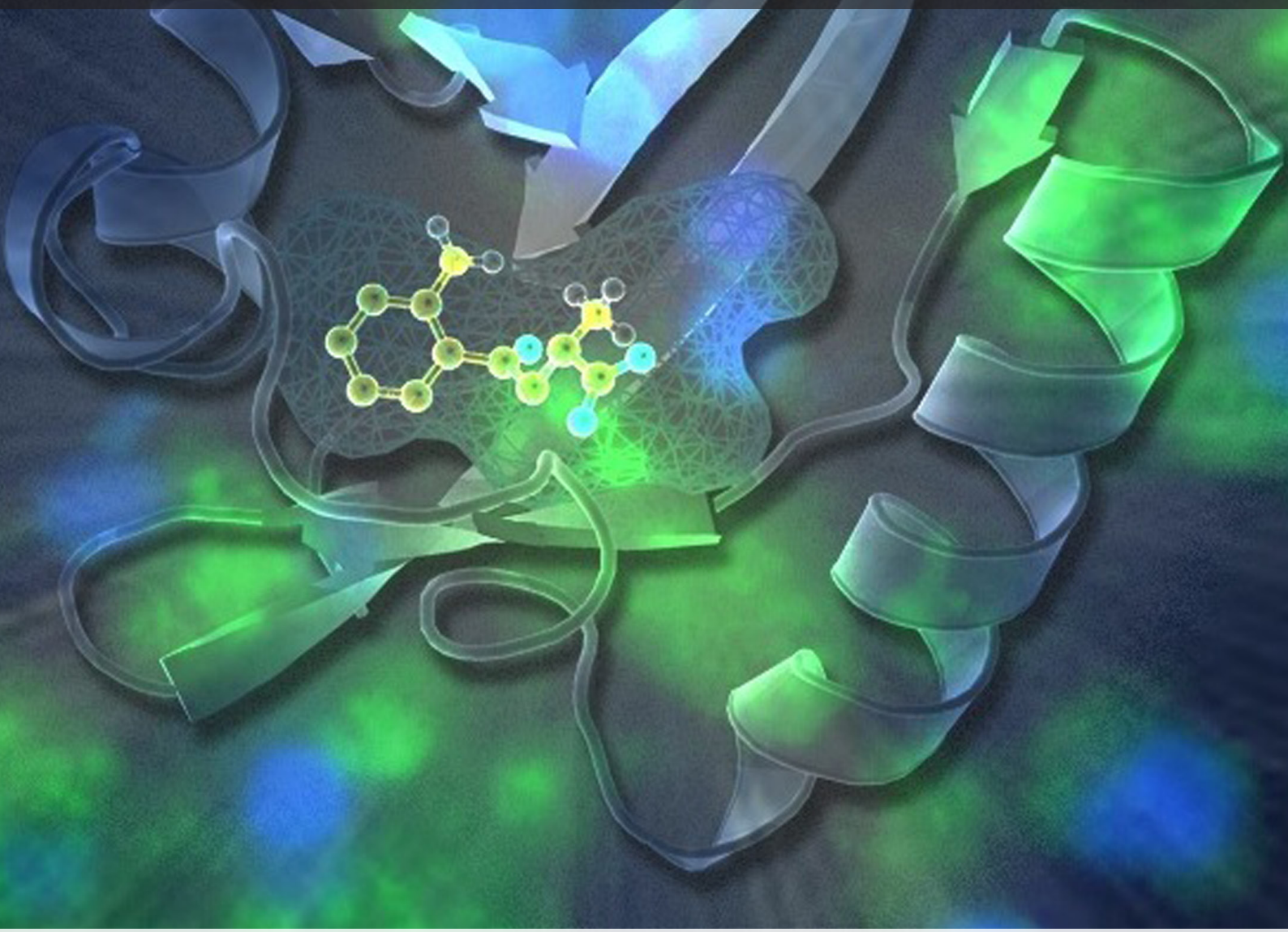

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THE COEVOLUTION OF IDO1 AND AHR IN THE EMERGENCE OF REGULATORY T CELLS IN MAMMALS

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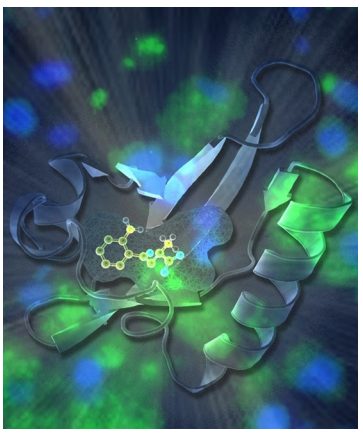

The ligand binding site of AhR. Image by Gianluca Andrielli, University of Perugia

Indoleamine 2,3-dioxygenase (IDO1) is an ancestral enzyme that, initially confined to the regulation of tryptophan availability in local tissue microenvironments, is now considered to play a wider role that extends to homeostasis and plasticity of the immune system. Thus IDO1 biology has implications for many aspects of immunopathology, including viral infections, neoplasia, autoimmunity, and chronic inflammation. Its immunoregulatory effects are mainly mediated by dendritic cells (DCs) and involve not only tryptophan deprivation but also production of kynurenines that act on IDO1$^-$ DCs, thus rendering an otherwise stimulatory DC capable of regulatory effects, as well as on T cells.

The aryl hydrocarbon receptor (AhR) is a ligand-operated transcription factor originally recognized as the effector mediating the pathologic effects of dioxins and other pollutants. However, it is now well established that AhR activation by endogenous ligands can produce immunoregulatory effects.

The IDO1 mechanism appears to have been selected through phylogenesis primarily to prevent overreacting responses to TLR-recognized pathogen-associated molecular patterns, and only later did it become involved in the response to T cell receptor-recognized antigens. As a result, in mammals, IDO1 has become pivotal in fetomaternal tolerance, at a time when regulatory T cells emerged to meet the same need, namely protecting the fetus. IDO1 and regulatory T (Treg) cells may have then coevolved to broaden their function well beyond their initial task of protecting the fetus, such that, in acquired immunity, IDO1 (with its dual enzymic and signaling

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function) has turned into an important component of the peripheral generation and effector function of regulatory T cells. AhR, in turn, which has a role in regulatory T-cell generation, is presumed to have evolved from invertebrates, where it served a ligand-independent role in normal development processes. Evolution of the receptor in vertebrates resulted in the ability to bind structurally different ligands, including xenobiotics and microbiota-derived catabolites. Considering the inability of invertebrate AhR homologs to bind dioxins, the adaptive role of the AhR to act as a regulator of xenobiotic-metabolizing enzymes may have been a vertebrate innovation, to later acquire an additional immune regulatory role by coevolutive pressure in mammals by IDO1 and regulatory T cells.

Thus an entirely new paradigm in immunology, and more specifically in immune tolerance, is the coevolution of three systems, namely, the IDO1 mechanism, AhR-driven gene transcription, and T-cell regulatory activity, that originating from the initial need of protecting the fetus in mammals, have later turned into a pivotal mechanism of peripheral tolerance in autoimmunity, transplantation, and neoplasia.

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The coevolution of IDO1 and AhR in the emergence of regulatory T-cells in mammals

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Keywords: aryl hydrocarbon receptor, tryptophan metabolism, indoleamine 2,3-dioxygenase 1 and 2, tryptophan dioxygenase, immune regulation

The aryl hydrocarbon receptor (AhR) is a ligand-operated transcription factor originally recognized as the mediator of the toxicity of dioxins. AhR is presumed to have evolved from invertebrates, where it served a ligand-independent role in normal development processes. Evolution of the receptor in vertebrates resulted in the ability to bind structurally different ligands, including xenobiotics, such as dioxin, and catabolites derived from the host's own metabolism, or from the microbiota. It is now clear that AhR contributes to immune homeostasis by promoting immunomodulatory and host-protective effects (1, 2). It is likewise clear that the nature of the ligand as well as the tissue specificity [e.g., gut (3), skin (4), and lymphoid tissue] in which AhR engagement occurs largely dictate the outcome of AhR activation.

Indoleamine 2,3-dioxygenase 1 (IDO1) catalyzes the initial rate-limiting step in degrading tryptophan along the kynurenine pathway (5–7). Initially confined to regulation of tryptophan availability in local tissue microenvironments, IDO1 is now considered to play a wider role that extends to homeostasis and plasticity of the immune system. Its effects involve not only tryptophan deprivation but also the production of immunoactive kynurenines, which may act as AhR ligands (8–12). Although two additional enzymes, i.e., IDO2 and tryptophan 2,3-dioxigenase (TDO2), catalyze the same reaction along the kynurenine pathway (13), IDO1 is apparently unique in promoting immunoregulatory effects over the long term, owing to its ability to function as a signaling molecule (14–16). IDO1 first appeared in placental animals by duplication of the *IDO2* gene (17), suggesting that the coexistence of two allogeneic individuals (i.e., mother and fetus) in the same organism would require advanced strategies of immune regulation capable of maintaining T-cell tolerance for prolonged periods of time.

The appearance of higher vertebrates, and specifically mammals, was, in fact, marked by the emergence of regulatory T (Treg) cells (18, 19). Thus, an entirely new paradigm in immunology, and more specifically in immune tolerance, may be the coevolution of three systems, namely, the IDO1 mechanism, kynurenine-driven gene transcription, and T-cell regulatory activity, which, originating from the initial need of protecting the fetus in mammals, have later turned into a pivotal mechanism of peripheral tolerance in autoimmunity, transplantation, and neoplasia.

The present Research Topic brings together 11 articles covering evolutionary aspects of tryptophan catabolic enzymes and AhR, their role in physiology and pathogenesis. In their Review Article, Ball et al. pointed out two interesting features emerging from studies of the dynamic evolution of TDO2, IDO1, and IDO2 (20). The three enzymes, which belong in two distinct superfamilies (TDO and IDO), have *converged* into the same catalytic activity, thus underlining the critical importance of tryptophan metabolism in all organisms. Yet, the IDO superfamily underwent *divergent* evolution, which occurred by gene duplication and led to the expression of an eclectic protein, IDO1, in placental animals. Because *Ido1*$^{-/-}$ mice are mosaic deficient for the IDO2 function possibly owing to an altered mRNA splicing, distinct IDO genes may also influence the expression of each other by a still unclarified mechanism, as suggested by Prendergast et al. (21).

Zelante et al. dealt with the adaptive properties of IDO1 and AhR from a different perspective, i.e., taking into consideration the possibility that tryptophan metabolism by human microbiome has been playing a major role in shaping the coevolution of the AhR/IDO1 axis in immune regulation (22). Interestingly, they recently discovered a tryptophan catabolite selectively produced by certain *Lactobacilli* of the human microbiome (i.e., indole-3-aldehyde) capable of activating AhR and thus inducing the expression of IDO1 and anti-inflammatory responses (3). Tryptophan catabolic enzymes may, however, represent a double-edged sword in the interaction between mammals and pathogenic microbes, as outlined by Schulze et al. (23), because tryptophan depletion exerts bactericidal activity in tryptophan auxotrophs. Microorganisms such as *E. coli* and HIV are known to highjack the immunosuppressive effects of IDO1, though. Intriguingly, Kishimoto et al. discussed the possibility that microRNAs (miRNAs) may regulate the transcriptional expression of IDO-encoding genes, mainly in autoimmunity (24). Because miRNAs have been suggested to be instrumental in the evolution of organismal complexity (25) and AhR has been shown to induce the expression of several miRNAs (26), these observations further underline the critical interdependence of AhR and IDO enzymes in coping with mammalian challenges.

In their Perspective, Orabona et al. proposed an additional level of cross-regulation between the two systems (27), which may occur

via AhR non-genomic effects that imply recruitment of a ubiquitin ligase complex and consequent proteasomal degradation of target proteins, a mechanism also considered by Quintana et al. (28). Because IDO1 is known to be subjected to regulatory proteolysis, AhR may not only induce but also switch off the IDO1 mechanism. Thus, depending on the specific pathologic conditions and timing of events, AhR may represent a friend or foe, and pollutants may play a major role in this regard, as suggested by the Perspective Article by Mezrich et al. (29).

Neoplasia represents a condition where drug targeting of the AhR/tryptophan metabolism axis has made the greatest progress. Already considered as a mechanism of immune escape in tumor progression, the data by Hanks et al. indicate that IDO1, modulated by several factors, is also involved in creating the permissive conditions for early carcinogenetic events (30). Most of these AhR/IDO1 modulating factors were examined by Platten et al. as potentially indirect, yet innovative, drug targets (31). Van den Eynde et al., thanks to the use of a highly specific anti-human IDO1 antibody, elegantly revisited the expression of this immunoregulatory enzyme in almost one thousand tumor specimens, finding that IDO1 is not upregulated in tumor-draining lymph nodes as previously reported, but it is restricted to tumor cells, stroma, and endothelium (32).

The reviews presented in this e-book of Frontiers are meant to provide readers with an overview of the intricacies of AhR functioning in both physiology and pathology, and of the combined effects of AhR ligand – intrinsic and – extrinsic factors, including the local tissue, which may provide a specific set of coactivators and functions bridging the basic transcriptional machinery to the target genes.

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Tryptophan-catabolizing enzymes – party of three

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Indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO) are tryptophan-degrading enzymes that have independently evolved to catalyze the first step in tryptophan catabolism via the kynurenine pathway (KP). The depletion of tryptophan and formation of KP metabolites modulates the activity of the mammalian immune, reproductive, and central nervous systems. IDO and TDO enzymes can have overlapping or distinct functions depending on their expression patterns. The expression of TDO and IDO enzymes in mammals differs not only by tissue/cellular localization but also by their induction by distinct stimuli. To add to the complexity, these genes also have undergone duplications in some organisms leading to multiple isoforms of IDO or TDO. For example, many vertebrates, including all mammals, have acquired two IDO genes via gene duplication, although the IDO1-like gene has been lost in some lower vertebrate lineages. Gene duplications can allow the homologs to diverge and acquire different properties to the original gene. There is evidence for IDO enzymes having differing enzymatic characteristics, signaling properties, and biological functions. This review analyzes the evolutionary convergence of IDO and TDO enzymes as tryptophan-catabolizing enzymes and the divergent evolution of IDO homologs to generate an enzyme family with diverse characteristics not possessed by TDO enzymes, with an emphasis on the immune system.

Keywords: convergent evolution, divergent evolution, indoleamine 2,3-dioxygenase, tryptophan 2,3-dioxygenase, gene duplication, immunoregulation

EVOLUTION OF TRYPTOPHAN-CATABOLIZING ENZYMES

The concepts of convergent and divergent evolution can apply to multi-cellular organisms or at the level of gene families, such as those that encode a particular enzymatic activity [reviewed in Ref. (1)]. In the first case, distinct enzyme superfamilies can evolve to catalyze the same reaction, i.e., functional convergence. Second, sequence divergence within families of enzymes can lead to functional divergence of enzyme homologs. In this review, we bring together and analyze evidence, mostly gathered in the last decade, which shows that the enzymes catalyzing the first step in the kynurenine pathway (KP) are a fascinating example of both these processes (**Figure 1**). The two enzymes, tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygnease (IDO), are structurally distinct proteins that nonetheless have evolved to catalyze the same reaction, the conversion of tryptophan (Trp) to *N*-formylkynurenine. The result of TDO and IDO activity is the depletion of Trp and production of metabolites of the KP. For mammals, Trp is an essential amino acid with most dietary Trp being metabolized through the KP (2). Some Trp is also required for synthesis of protein and the neurochemical serotonin. Activity of the KP can affect the levels of tryptophan, thereby modulating serotonin synthesis or causing suppression of cell proliferation. In addition, the production of metabolites can provide a source of nicotinamide dinucleotide (NAD^+) and have other biological effects, particularly in the immune, reproductive, and central nervous systems. Depending on their expression pattern, TDO and IDO enzymes may have similar or distinct biological activities. Furthermore, in some organisms, gene duplication has resulted in homologs of either TDO or IDO. In the case of IDO homologs, there is evidence of functional divergence that has evolved in the case of one of the duplicated genes.

EVOLUTION OF TDO

Tryptophan 2,3-dioxygenase is widely distributed across species, from metazoans to bacteria, but has not been found in fungi. Its enzymatic activity has been conserved consistently throughout metazoan evolution (Yuasa and Ball, manuscript submitted). A phylogenetic tree showing the distribution of TDO enzymes in metazoan species is presented in **Figure 2**. Gene duplications have resulted in some organisms possessing two TDO genes, e.g., *Danio rerio* (zebrafish) and *Stongylocentrotus purpuratus* (sea urchin). TDO is found in several hundred species/strains of bacteria. They do not form a monophyletic group, and some bacterial TDOs show sequence homology with eukaryotic TDOs. A few bacteria have two TDO genes; however, they are located distant from each other in the phylogenetic tree (**Figure 2**). This suggests that multiple horizontal gene transfer events have occurred in bacterial TDO evolution.

EVOLUTION OF IDO

Indoleamine 2,3-dioxygenase is also widely distributed from bacteria to metazoans and, unlike TDO, can be found in fungi. Gene duplication has occurred in a number of lineages to generate IDO homologs. For example, some Ascomycota fungi possess *IDO*α, *IDO*β, and *IDO*γ, while some Basidiomycota fungi have *IDOa*, *IDOb*, and *IDOc* (5–7). A phylogenetic tree showing the

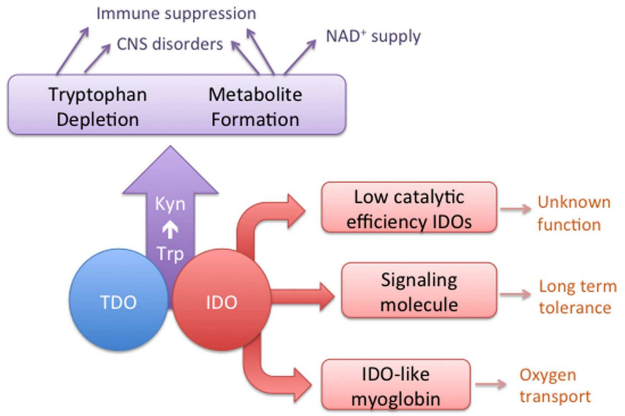

FIGURE 1 | A conceptual diagram depicting the overlapping activities of IDO and TDO enzymes and the distinct characteristics of some IDO homologs.

distribution of IDO enzymes is presented in **Figure 3**. Mammals also possess more than one IDO enzyme, namely, IDO1 and the more recently discovered IDO2 (8–10). Examining the sequence divergence of the IDO1 and IDO2 proteins would suggest a gene duplication event before the origin of the tetrapods (8). However, the presence of two IDO homologs in mammals, but only one IDO2-like enzyme in lower vertebrates, suggests that the ancestral *IDO2* gene was more recently duplicated, before the rise of the mammals (10). Recently, IDO homologs, one with higher homology to mammalian IDO1 compared to IDO2, have been detected in a species of fish and turtle (Yuasa et al., manuscript in preparation). We speculate that this indicates that the duplication event was more ancient and that the *IDO1* gene has been lost in a number of lower vertebrate lineages.

FUNCTIONAL CONVERGENCE OF IDO AND TDO

The genomic structures and sequences of the *IDO* and *TDO* genes do not suggest a common ancestor, rather that the two genes have

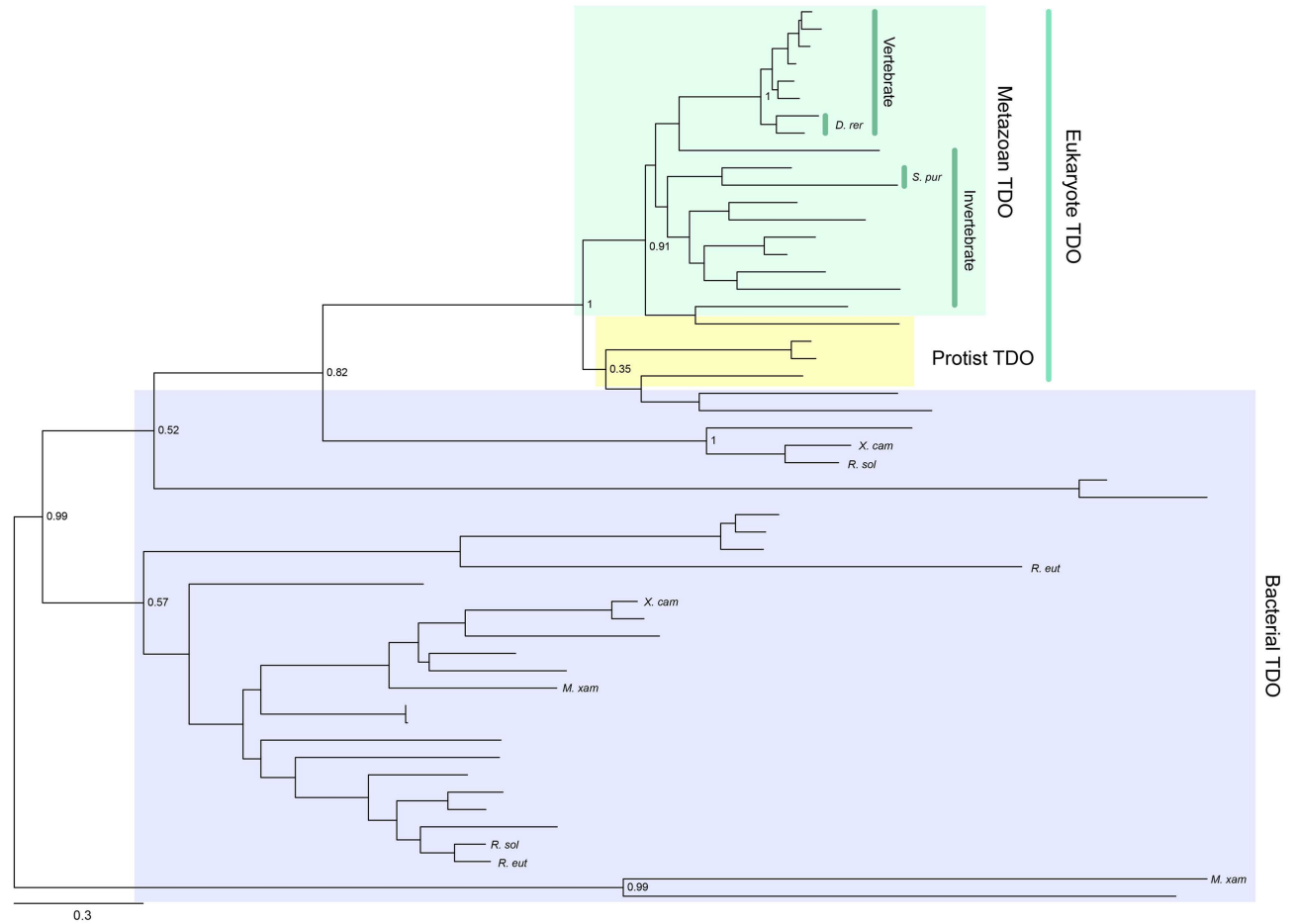

FIGURE 2 | Phylogenetic relationships of known TDOs constructed with the maximum-likelihood method (unrooted tree). Multiple sequence alignment at the amino acid level was generated using the MUSCLE program (3) and the ML tree was constructed using MEGA 6 (4). The internal branch labels are bootstrap values with 100 replications. *D. rerio* (*D. rer*) and *S. purpuratus* (*S. pur*) have two TDOs. A few bacteria, *Myxococcus xanthus* (*M. xan*), *Ralstonia solanacearum* GMI1000 (*R. sal*), *R. eutropha* JMP134 (*R. eut*), and *Xanthomonas campestris* 33913 (*X. cam*), also have two TDOs. A complete tree, with names of all species, is shown in Figure S1 in Supplementary Material.

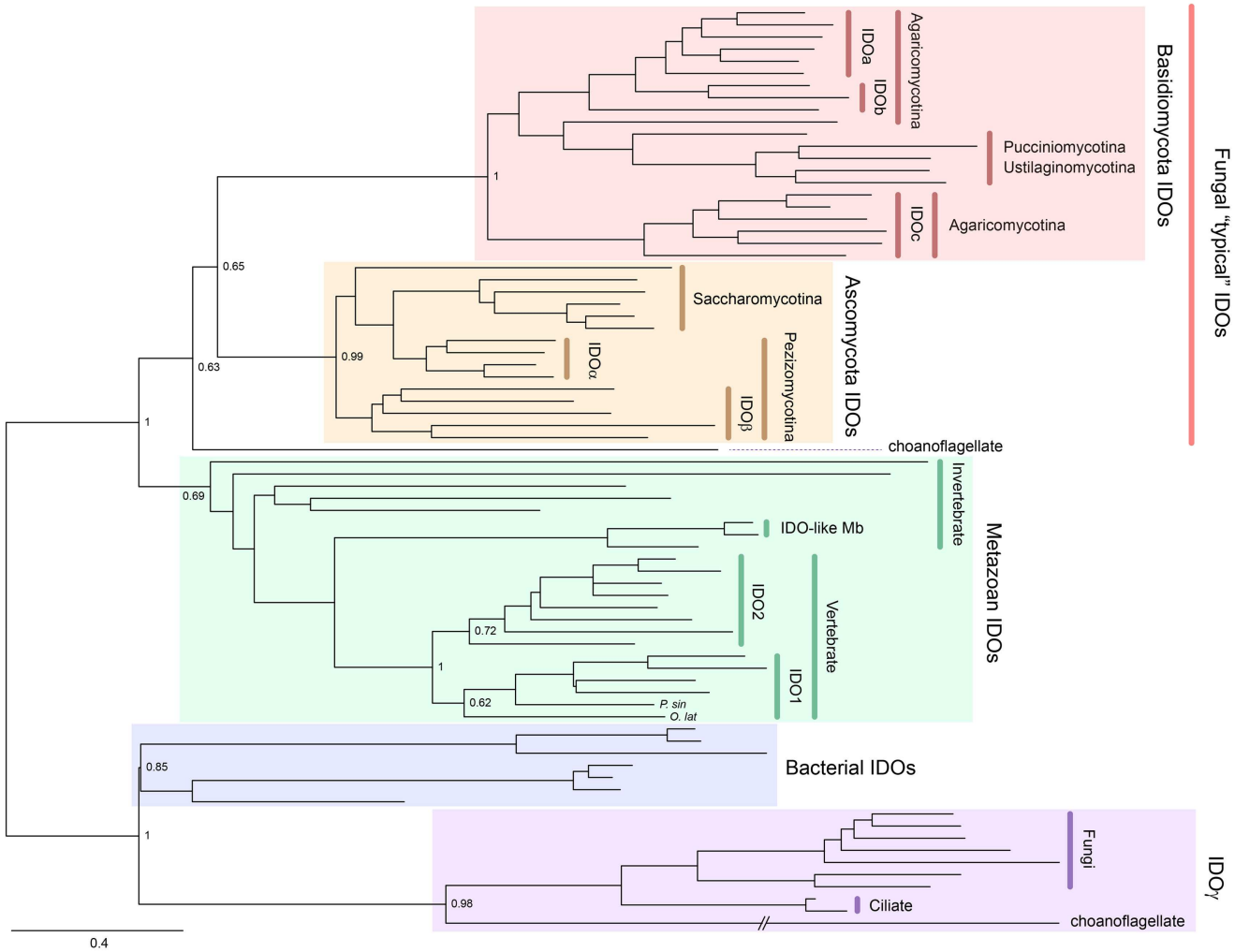

FIGURE 3 | Phylogenetic relationships of known IDOs and IDO-related proteins constructed with the maximum-likelihood method (unrooted tree). Multiple sequence alignment at the amino acid level was generated using the MUSCLE program (3) and the ML tree was constructed using MEGA 6 (4). The internal branch labels are bootstrap values with 100 replications. The Medaka-fish, *Oryzias latipes* (*O. lat*) and a soft-shelled turtle, *Pelodiscus sinensis* (*P. sin*) have a putative IDO1. A complete tree, with names of all species, is shown in Figure S2 in Supplementary Material.

evolved separately to catalyze the same reaction. **Figure 4** depicts amino acid homologies and genomic structures for some selected TDO and IDO proteins and their homologs. Both enzymes are heme-containing proteins and the crystal structures of human IDO1 and bacterial TDOs have been obtained (11–13). IDO1 is a monomeric enzyme while TDO is tetrameric with one heme per monomer. Although there is low sequence identity between the enzymes, the crystal structures reveal similarities in the heme-binding environment and substrate-binding site (13). The step-by-step process by which Trp is converted to *N*-formylkynurenine by heme dioxygenases has been investigated over many years, and the reaction mechanism is thought to be broadly similar between the two enzymes (14).

Structurally-unrelated enzymes that catalyze the same reactions have been termed analogous (rather than homologous) enzymes. Examples of analogous enzymes are found in many classes of enzymes, but are particularly clustered in those involved in synthesis and hydrolysis of polysaccharides, effects of oxygen on cell components, and synthesis/turnover of cell walls (15). A suggested mechanism for the evolution of analogous enzymes is that a changed substrate specificity or reaction mechanism results in the recruitment of an enzyme to perform a new role (15). One difference between mammalian TDO and IDO enzymes is the substrate selectivity; TDO is selective for the L-Trp enantiomer, whereas IDO has a broader substrate range of indole-containing compounds. IDO1 was originally isolated from rabbit intestine as a D-Trp-catabolizing enzyme (16) and its substrates include D- and L-Trp, tryptamine, 5-hydroxytryptophan, and 5-hydroxytryptamine (17). The biological significance of the oxidation of other substrates by IDO1 is not clear and the substrate range of ancestral IDO enzymes is also unknown. We hypothesize that ancestral IDO enzymes were broadly indole-oxidizing

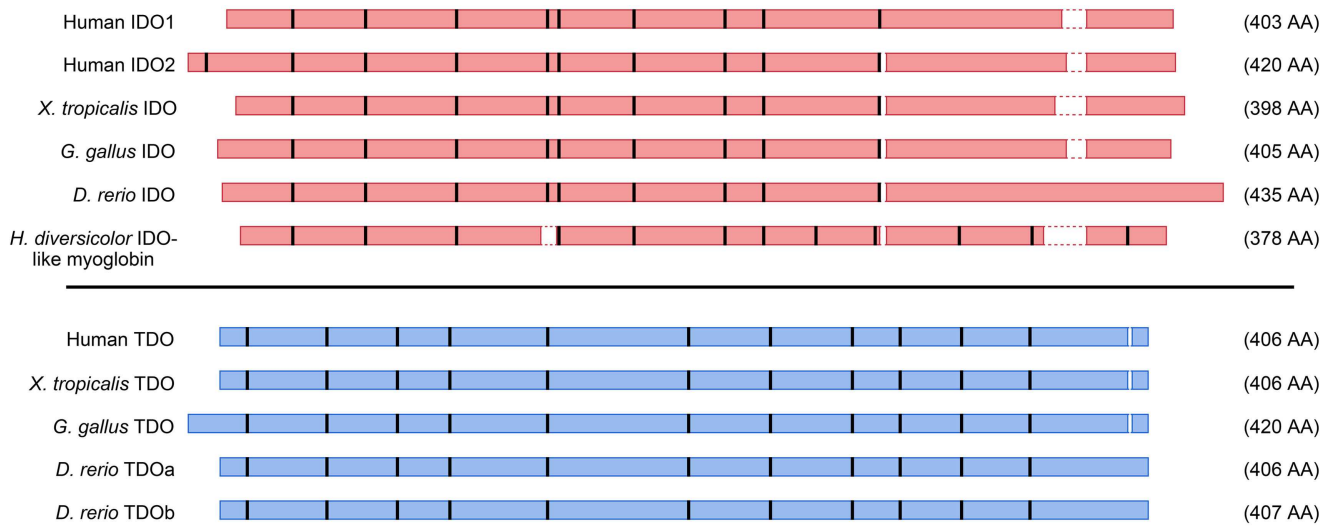

FIGURE 4 | Schematic representation of amino acid alignments for selected IDO and TDO proteins. Amino acid contigs are shown as red (IDO) or blue (TDO) boxes, and positions of introns indicated by thick vertical bars. Gaps less than two amino acids in the alignments were omitted. Selected sequences are from human beings, frog (*Xenopus tropicalis*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*), and abalone (*Haliotis diversicolor*).

enzymes and mammalian IDO1 has evolved into an enzyme with high affinity and efficiency for L-Trp as a substrate, with this activity forming the basis of its biological actions.

It has been observed that analogous enzymes are more likely to have a skewed phylogenetic distribution; for example, one isoform evolves in archaebacteria while the other is present in both metazoan and bacteria (1). The absence of TDO enzymes in fungi suggests that IDO enzymes are responsible for Trp metabolism in this kingdom. In bacteria, some species possess TDO or IDO enzymes and they occur exclusively to each other, suggesting that only one is required for Trp metabolism (18). However, many organisms, including all mammals, possess both TDO and IDO and there is the potential for the enzymes to have overlapping or distinct actions, depending upon their expression patterns. For example, mammalian TDO is expressed in the liver and its activity is upregulated by glucocorticoids and L-Trp (19, 20). IDO1 has constitutive expression in some tissues, such as the lung, but its expression can be widely induced by certain stimuli that are significant in immunity and inflammation, including interferon-γ (IFNγ), lipopolysaccharide (LPS), and tumor necrosis factor (TNF) (21–23). IDO2 has constitutive expression in the liver (8) and can be induced by cytokines in certain cell types (9, 24, 25).

The following sections discuss some examples of IDO and TDO having either overlapping or distinct biological effects based on their common enzymatic activity and distinct expression patterns, with a general focus on pathways and networks that are significant in immunity and/or inflammation. The interactions of KP metabolites with the aryl hydrocarbon receptor (AhR) are described in more detail in other articles in this issue. The roles of Trp-catabolizing enzymes in the placenta and central nervous system also have been reviewed recently (26, 27).

IMMUNE EVASION BY TUMORS

Expression of Trp-catabolizing enzymes, whether in cancer cells or dendritic cells in tumor-draining lymph nodes, has been associated with suppression of the effector T-cell responses toward tumors and, hence, with a poorer prognosis. A number of pathways are implicated [reviewed in Ref. (28)], including suppression of T-cell proliferation through sensing of Trp depletion via the mTOR and/or GCN2 kinase pathways; generation of Treg cells via either the GCN2 kinase pathway and/or kynurenine (Kyn) activating the AhR; and the pro-apoptotic properties of some KP metabolites on T-cells. IDO1 expression, associated with these effects, has been found in plasmacytoid dendritic cells (29) and human tumors (30). Human IDO2 mRNA expression has been detected in gastric, colon, and renal tumors (24) and mRNA/protein in myeloid and plasmacytoid dendritic cells (31). TDO activity in gliomas has been linked to activation of the AhR and reduced antitumor immune responses (32). TDO expression also has been found in a significant proportion of human tumors, and a TDO inhibitor restored the ability of mice to reject TDO-expressing tumors (33). While the relative importance of tumor versus dendritic cell expression is debatable, it is clear that there is the potential for both IDO and TDO enzymes to be involved in the suppression of immune responses toward tumors. Selective inhibitors can distinguish the role of TDO and IDO enzymes, but it is more difficult to define the role of IDO1 versus IDO2 using pharmacological approaches despite some recent progress in that direction (34). There is some confusion regarding the selectivity and mode of action of the enantiomers of the most commonly used IDO inhibitor, 1-methyl-tryptophan [reviewed in Ref. (35)], so genetic approaches are also helpful for defining the function of these enzymes; for example, an IDO inhibitor was ineffective at preventing tolerance of transplanted melanoma in *Ido1*$^{-/-}$ mice, suggesting that the inhibitor acted via IDO1 (36). These *Ido1*$^{-/-}$

mice recently have been found to express an enzymatically inactive form of IDO2 in specific immune cell types (37), which should be considered when drawing conclusions. However, silencing the *Ido1* gene by siRNA resulted in reduced tumor growth in B16F10 tumor-bearing mice (38). In addition, *Ido2*$^{-/-}$ mice did not reproduce the reduced susceptibility to inflammatory skin cancer seen in the *Ido1*$^{-/-}$ mice (37). IDO2 activity may have a role in promoting Treg generation (discussed in Treg Cells and Tolerance); however, it appears that IDO1 is the homolog predominantly associated with immune evasion by tumors. Selective inhibitors of TDO or IDO enzymes have been proposed as adjunctive chemotherapies. The IDO inhibitor 1-methyl-tryptophan can also act as Trp mimetic, signaling amino acid sufficiency in the mTOR pathway (39). This action has the potential to relieve some of the immune suppression caused by all three enzymes, thus modulation of downstream pathways may provide broader efficacy as an adjunctive therapy than would selectively targeting Trp-catabolizing enzymes.

Thus, the evidence for IDO1 modulating host–tumor cell interactions is strong and diverse, but involvement of TDO and IDO2 is much less studied and requires further corroboration.

TREG CELLS AND TOLERANCE

The importance of the activation of the AhR by kynurenines in the development of tolerance is reviewed in Ref. (40) and other articles in this issue. Critical to the development of tolerance is the generation and maintenance of Treg cells, with the kynurenine-mediated activation of the AhR playing an important role (41). As previously mentioned, TDO-mediated activation of the AhR has been linked to reduced immune responses toward gliomas (32). In addition, *Tdo*$^{-/-}$ mice had greater inflammatory responses and mortality after LPS administration, similar to *AhR*$^{-/-}$ mice (42). This suggests that the AhR is activated by kynurenines formed by TDO in certain situations. The longer term development of immune tolerance may depend on sustained kynurenine production in particular microenvironments, such as dendritic cells. Mice develop a tolerance to LPS, as they show reduced inflammatory responses and mortality on re-exposure. This tolerance was observed to be dependent on the combined presence of IDO1 and the AhR, suggesting that IDO1 is important for the longer term modulation of the immune response to LPS (42). This study demonstrated a positive feedback loop where signaling through the AhR induced IDO1 expression, which in turn produced sustained activation of the AhR. This is similar to the transforming growth factor β (TGF β)/IDO1 axis, in which a positive feedback loop results in sustained expression of IDO1, TGFβ, type I interferons, and the generation of Treg cells [reviewed in Ref. (40)]. Interestingly, the establishment of this axis is associated with a non-enzymatic function of IDO1 (see Signaling Properties of IDO1) (43). Kynurenine administration failed to restore tolerance to LPS administration in *Ido1*$^{-/-}$ mice, suggesting that non-enzymatic capabilities of IDO1 may also play a role in establishing tolerance to endotoxins (42).

Many studies have identified IDO1 as the Trp-catabolizing enzyme predominantly involved in the generation of Treg cells, although we emphasize that the pharmacological approaches employed in those studies would not distinguish between IDO1 and IDO2. For example, IDO inhibition with 1-methyl tryptophan was shown to reduce Treg formation by CpG oligonucleotide-stimulated human dendritic cells and in the lungs of *Aspergillus fumigatus*-infected mice (44, 45). Other studies utilize the *Ido1*$^{-/-}$ mouse; however, use of this strain is complicated by cell-specific alternative-splicing leading to some loss of IDO2 activity (37). Two studies employing both *Ido1*$^{-/-}$ and *Ido2*$^{-/-}$ mouse strains showed that the formation of Treg cells either may be modulated by IDO2 (37) or is specific to IDO1 activity (42), depending on the model. In addition, siRNA knockdown of either IDO1 or IDO2 in human dendritic cells reduced the formation of kynurenine and Treg cells (31). The tolerance to LPS administration was specific to IDO1 expression, as IDO2 deletion had no effects on outcomes in either initial or subsequent administrations of LPS (42). The generation of Treg cells in mice treated with CpG oligonucleotide has been defined as IDO1 mediated (46), but the generation of Treg cells was also reduced in *Ido2*$^{-/-}$ mice in this model (37).

In summary, all three Trp-catabolizing enzymes have the ability to suppress immune responses. However, the longer term maintenance of the Treg balance may require sustained expression in particular microenvironments. This appears to occur in some TDO- or IDO-expressing tumors and IDO-expressing dendritic cells. It is noteworthy that IDO2 expression was found to be constitutive in human myeloid and plasmacytoid dendritic cells, while IDO1 showed a more restricted expression pattern, dependent on prostaglandin E_2 (31). Overall, we conclude that the different expression patterns of the IDO proteins may determine whether each is tolerogenic in response to specific stimuli.

NAD^+ SUPPLY

NAD^+ is an essential co-factor required by many biochemical processes. It is formed either by a *de novo* synthesis pathway, from metabolites of the KP, or via a salvage pathway using nicotinic acid (NA) and nicotinamide (vitamin B3). Thus, a yeast strain with its only Trp-catabolizing gene (*BNA2*, an IDO homolog) deleted becomes a NA auxotroph. Expressing IDO/TDO enzymes in this mutant strain will rescue the yeast if the enzyme has sufficient Trp-catabolizing activity to provide a source of NAD^+. Many IDO enzymes do rescue the mutant strain, including mouse and human IDO1 enzymes, IDOα/β enzymes from Pezizomycotina, and IDOa/b enzymes from Agaricomycotina (7). As fungi do not possess TDO, it is likely that IDO activity has a role in supplying NAD^+ in these microorganisms. NAD^+ synthesis is not only determined by the activity of the Trp-catabolizing enzymes but also the presence of all the downstream enzymes. One of these enzymes, quinolinic acid phosphoribosyl-transferase, has been found to be active only in the liver and kidney of rodents (47, 48). It has been shown that the KP is the major route of NAD^+ supply in rat hepatocytes (49). Two Trp-catabolizing enzymes are constitutively expressed in mouse liver – TDO and IDO2 (8, 19). TDO is most likely to be involved in NAD^+ supply since TDO enzymes, but not IDO2 enzymes, were able to rescue the NA-auxotrophic yeast strain [Yuasa and Ball, manuscript submitted (7)]. The lack of significant activity of critical downstream enzymes for *de novo* synthesis would suggest that most extrahepatic tissues rely on the salvage pathway for NAD^+ supply. Nevertheless, *in vitro* studies on human brain cells have suggested that the KP pathway can contribute to maintaining NAD^+ and perhaps the distribution and

activity of the enzymes in the pathway has not been fully elucidated (50). In addition, while *Tdo*$^{-/-}$ mice showed significantly reduced levels of circulating NAD^+ when placed on a NA-deficient diet, compared to a complete diet, they still maintained optimal rates of growth (48). The amount of NAD^+ in the liver was consistent in wildtype and *Tdo*$^{-/-}$ mice fed either the complete or NA-deficient diets. Measurements of metabolites in the urine showed that the conversion rate of L-Trp to Kyn was increased in the *Tdo*$^{-/-}$ mice on the NA-deficient diets. This suggests that extrahepatic IDO1 enzyme activity was increasing the circulating pool of Kyn, which could then be further metabolized in the liver to synthesize NAD^+ for recirculation to extrahepatic tissues. We may speculate that IDO2 activity in the liver also contributes to maintaining the levels of NAD^+ in that tissue, in the absence of both NA in the diet and TDO activity.

NAD^+ supply also has modulatory effects in cancer and inflammation. Inhibition of NAD^+ formation has been proposed as a chemotherapeutic target due to high rates of NAD^+ consumption by tumor cells. This is suggested to be predominantly due to increased ADP-ribosylation from polyADP-ribose polymerase activity (51). Although an inhibitor (FK866) of an enzyme in the salvage pathway is efficacious as a chemotherapeutic agent (52), little is known of the contribution of the *de novo* synthesis pathway to tumor cell metabolism. NAD^+ is also critical for mediating the effects of sirtuins, some of which modulate inflammatory pathways. Again, the relative contributions of the *de novo* synthesis pathway and the salvage pathways in sirtuin activation are unclear, although an IDO inhibitor was shown to reduce both NAD^+ levels and sirtuin 1 activity in human primary astrocytes (53). It should be noted that sirtuin 1 activity has been associated with reduced Foxp3 stability and suppression of Treg cell formation (54), in contrast to the Treg-promoting effects of the KP. In summary, while NAD^+ supply regulates tumor cell metabolism and inflammatory responses, the roles of the two supply pathways, as well as each of the enzymes within them, still require elucidation. We suggest that a focus on the KP–NAD^+ axis in tumor and immune cells would provide valuable information both to clarify certain anomalies in the field and to determine whether it is possible to interfere with this pathway in tumor cells without compromising immune cell functions.

DIVERGENT EVOLUTION OF L-Trp-CATABOLIZING ENZYMES

TDO and IDO have evolved to have similar enzymatic functions, and these enzymes coexist in most animals. In addition, gene duplications have resulted in some species having more than one homolog of each enzyme. For example, zebrafish have one IDO enzyme and two TDO enzymes. The TDO enzymes are highly conserved throughout vertebrate evolution (**Table 1**) and zebrafish TDO paralogs have similar enzymatic activity and expression patterns (Jusof et al., manuscript in preparation). *IDO* genes have been duplicated independently in a number of lineages, for example, *IDO1* and *IDO2* in mammals and *IDOa*, *IDOb*, and *IDOc* in Agaricomycotina fungi. Compared with TDO enzymes, there is lower sequence homology between both IDO paralogs and orthologs (**Table 1**). The different biochemical and functional properties observed among IDO enzymes is likely a result of the greater sequence divergence. The following sections describe three features found in certain IDO enzymes that are not shared among all IDO enzymes.

LOW CATALYTIC-EFFICIENCY IDO ENZYMES

IDO enzymatic activity requires the reduction of the heme iron from its ferric form to its ferrous form. Characterization of IDO activity often begins by assessing the activity of the protein in a reaction containing methylene blue (MB) as an electron source, among other constituents including the substrate (55). IDO1

Table 1 | Amino acid identity over the aligned segments of IDO and TDO enzymes using *blastp* (http://blast.ncbi.nlm.nih.gov).

	Human IDO1 (%)	Human IDO2 (%)	*X. tropicalis* IDO (%)	*G. gallus* IDO (%)	*D. rerio* IDO (%)	*H. diversicolor* IDO-like Mb (%)
Human IDO1	100	44	43	44	45	34
Human IDO2	44	100	53	60	50	37
X. tropicalis IDO	43	53	100	55	50	36
G. gallus IDO	44	60	55	100	55	38
D. rerio IDO	45	50	50	55	100	36
H. diversicolor IDO-like Mb	34	37	36	38	36	100

	Human TDO (%)	*X. tropicalis* TDO (%)	*G. gallus* TDO (%)	*D. rerio* TDOa (%)	*D. rerio* TDOb (%)
Human TDO	100	82	84	75	75
X. tropicalis TDO	82	100	86	77	78
G. gallus TDO	84	86	100	77	76
D. rerio TDOa	75	77	77	100	76
D. rerio TDOb	75	78	76	76	100

No significant similarity (E value < 1) was observed when aligning the TDO proteins with IDO proteins. Selected sequences are from human beings, frogs (Xenopus tropicalis), chickens (Gallus gallus), zebrafish (Danio rerio), and abalone (Haliotis diversicolor).

enzymes are highly efficient at metabolizing Trp in the MB assay. In contrast, IDO2 enzymes have a much higher K_m and lower V_{max} for Trp in this reaction (10, 56–58). The physiological reductant of IDO enzymes originally was proposed to be superoxide anion (59) and more recently suggested to be cytochrome b_5 (60, 61). Human IDO1 showed reduced activity in a reaction containing cytochrome b_5 compared with the MB assay (61). In contrast, the Trp-catalyzing efficiency of mouse IDO2 was greatly improved in the cytochrome b_5 reaction, although it was still significantly less efficient than mouse IDO1 (56). The dependence of enzymatic activity on co-factors underscores the difficulty in assessing the biochemical characteristics of IDO homologs. It is certainly possible that the optimal reaction system to observe IDO2 activity has not been developed. Despite this limitation, several other lines of evidence suggest that IDO2 enzymes have lower Trp-metabolizing activity than the IDO1 counterparts. First, the amount of Kyn formed in mammalian cells transfected with mouse IDO2 is lower than in cells transfected with mouse IDO1 (8, 9). In addition, human IDO2 expression produced significantly less Kyn than mouse IDO2 (9, 62). Second, expression of IDO1, but not mouse or human IDO2, rescued a NA-auxotrophic yeast strain (7). This suggests that IDO2 enzymes do not possess sufficient activity to supply the metabolites required for NAD^+ supply in yeast. On the other hand, knockdown of human IDO2 expression in dendritic cells by siRNA significantly suppressed Kyn formation and this was associated with decreased generation of Treg cells (31). Thus, IDO2 may have sufficient enzymatic activity to generate a biological effect in certain systems. The constitutive expression of IDO2 in subsets of dendritic cells, compared with the regulated expression of IDO1, may be an example where the weak enzymatic activity of IDO2 is still able to produce a distinct biological effect, compared with IDO1, by virtue of its unique expression pattern (31).

Ido2$^{-/-}$ mice, while having unaltered plasma Kyn levels, show reduced skin contact hypersensitivity responses and attenuated Treg cell generation (37). In addition, *Ido2*$^{-/-}$ mice have selectively less autoantibody production, compared with overall antibody production, leading to reduced joint inflammation in a spontaneous model of arthritis (63). Interestingly, the effect was unrelated to serum Kyn levels, because these were unchanged in *Ido2* $^{-/-}$ mice while *Ido1* $^{-/-}$ mice had reduced circulating Kyn but no attenuation of joint inflammation. Furthermore, while 1-methyl-tryptophan could inhibit Kyn formation in IDO2-expressing HEK293T cells, it did not affect IDO2-mediated suppression of T-cell proliferation in co-culture experiments (62). Taken together, these findings may point to some effects of IDO2 being mediated via a mechanism unrelated to Trp metabolism/Kyn formation, although, in the mouse model, it is possible that localized enzymatic activity is sufficient to produce the effect.

Interestingly, other lineages outside the metazoan kingdom have independently generated IDO homologs through gene duplication events. Some of these IDO homologs have very low catalytic activities, similar to IDO2 enzymes, as tested in the MB assay (5, 6, 18). The low enzymatic activity of these homologs was confirmed by the lack of rescue of the NA-auxotrophic yeast strain (7). These low catalytic-efficiency enzymes include IDOγ in Perzizomycotina and IDOc in Basidiomycota. The role of the low catalytic-efficiency IDOs in Trp metabolism, or another metabolic process, is not well understood. L-Trp is metabolized by both IDO1 and IDO2 enzymes; however, assessing tryptophan derivatives has revealed substrates, such as 5-methoxytryptophan, which are metabolized by human IDO2, but not human IDO1 (58). Human IDO2 has a higher affinity for some of these substrates than for L-Trp. Additionally, we found that Trp metabolism by mouse IDO2 could be inhibited by a much wider range of compounds compared with mouse IDO1 (34). We conclude that IDO2 enzymes interact with a different, but overlapping, range of substrates/inhibitors compared to IDO1 enzymes.

Recent studies show that IDO1 enzymes can possess heme peroxidase and indole peroxygenase activity in the presence of hydrogen peroxide and relevant substrates (64, 65). These activities have yet to be investigated in other IDO enzymes' paralogs/orthologs. The biological significance of these alternative reactions is unknown. However, as IDO enzymes with relatively low efficiency at converting Trp to Kyn have been maintained in several lineages during evolution, we believe that it would be of interest to investigate whether these enzymes catalyze these alternative reactions more efficiently than the conversion of Trp to *N*-formylkynurenine.

Lymphocytes appeared early in vertebrate evolution, with the emergence of jawed fish, and the adaptive immune system has become increasingly complex as vertebrates have evolved through to mammals. Another feature of mammalian evolution is the development of the chorion into the larger, more complex placenta. We speculate that the development of these two systems has favored acquisition of high Trp-catabolizing efficiency in IDO1 enzymes in order to fulfill particular biological roles. Fungi do not have TDO and it may be that high Trp-catabolizing efficiency IDO enzymes, IDOα/β and IDOa/b, have evolved in fungi to provide a source of NAD^+. The occurrence and conservation of IDO enzymes with lower catalytic activity for Trp metabolism in different kingdoms suggests that these enzymes are still fulfilling a role, albeit one that is less well understood. We conjecture that potential roles may include Trp metabolism in particular microenvironments, or metabolism of other substrates including through an alternative oxidation reaction.

SIGNALING PROPERTIES OF IDO1

It was shown that IDO1 expression, but not IDO1 catalytic activity, was necessary for the immunoregulatory effects leading to longer term self-tolerance of plasmacytoid dendritic cells treated with TGF-β (43). Immunoreceptor tyrosine-based inhibitory motifs (ITIMs) were phosphorylated in mouse IDO1 in response to TGF-β. This was followed by upregulation and recruitment of tyrosine phosphatases, SHP-1 and SHP-2, and the initiation of a cascade of downstream events favoring activation of the non-canonical NF-κB pathway, leading to the sustained expression of IDO1, TGF-β, and interferon-α. Two ITIMs (ITIM1 and ITIM2) were observed to be present in an alignment of human being, rat, dog, and mouse IDO1 proteins. In contrast, a tyrosine to phenylalanine substitution in IDO2 proteins meant that only ITIM2 was present. The presence of only one ITIM was shown to prevent the recruitment of the phosphatase, so that mouse IDO2 does not possess the same signaling capability as mouse IDO1. In addition, the ITIM motifs were demonstrated to regulate the SOCS3-dependent degradation

of the IDO1 protein in response to other cytokines, thus possessing both these motifs also provides an additional mechanism for regulating IDO1 protein turnover (66). The expression of human IDO2 in dendritic cells was found to be SOCS3-independent, correlating with its lack of two ITIM motifs (31). Thus, sequence differences in the IDO1 and IDO2 proteins have resulted in an additional signaling function and mechanism of regulation for one of the homologs.

There is some evidence that IDO2 acts through effector pathways independent of enzymatic activity. As discussed earlier, the attenuation in joint inflammation in *Ido2*$^{-/-}$ mice was unrelated to circulating Kyn levels and an inhibitor of IDO2-catalyzed Kyn formation did not relieve IDO2-mediated suppression of T-cell proliferation (62, 63). Trp depletion induces translation of the liver inhibitory protein via the GCN2 kinase pathway, and this could be reversed by Trp supplementation in IDO1-expressing cells but not in IDO2-expressing cells (9). We suggest that these findings imply that potential alternative signaling properties of IDO2 should be investigated.

IDO-LIKE MYGLOBIN

Hemoglobins and myoglobins from bacteria, plants, and animals are thought to have evolved from a common ancestral gene encoding a 14–16 kDa protein [reviewed in Ref. (67)]. A myoglobin isolated from the abalone *H. diversicolor*, however, was a 39 kDa protein quite different to previous myoglobin proteins and with significant homology to the IDO family of enzymes (68). The IDO reaction mechanism involves the formation of an oxygenated intermediate with an absorption spectrum similar to that of oxymyoglobin (69). IDO enzymes do not act as oxygen carriers as this intermediate is unstable. The IDO-like myoglobin possibly may have evolved to reversibly bind oxygen through amino acid changes in the heme-binding cavity (67). IDO-like myoglobins are found in several families in the Vetigastropoda and, interestingly, the conventional myoglobin is not present in these families (67).

Thus, in an example of functional convergence with the myoglobin family, the IDO-like myoglobins have evolved to fulfill the function of an oxygen carrier where conventional myoglobins have been lost. However, as an example of functional divergence, IDO-like myoglobins have lost the ability to metabolize tryptophan and to function as an IDO enzyme (Yuasa and Ball, manuscript in preparation).

SUMMARY

Convergent evolution usually occurs by two different proteins evolving to fill the same biological role in different organisms. For example, TDO is likely to be the enzyme mostly responsible for Trp metabolism leading to NAD^+ supply in mammals; however, its absence in fungi suggests that IDO enzymes perform this function in fungi. However, both TDO and IDO enzymes, with Trp-catabolizing activity, are present in vertebrates. The functional evolution of IDO proteins is more complex than TDO evolution as efficient Trp-catabolizing activity is not uniformly conserved. Mammalian IDO1s have evolved to be highly efficient at catabolizing Trp. It is possible that the increasing complexity of the vertebrate immune and reproductive systems might have led to selective pressures favoring the acquisition of Trp-catabolizing activity, in particular tissues or situations. Although Trp catabolism, by either IDO or TDO, may have similar effects in some cell types, e.g., tumors, Trp catabolism in dendritic cells that drives tolerance may be an IDO-mediated effect.

Gene duplication is a driving force in evolution as it allows one gene to perform its original function while another, under less selective pressure, may diverge and develop new characteristics and/or lose its original ones. Both *IDO* and *TDO* genes have undergone duplications in some lineages, although IDO proteins show much greater sequence divergence than TDO proteins. This has led to greater diversity within the IDO family, with differing enzymatic activities and signaling capabilities. For example, IDO1 enzymes, but not IDO2 enzymes, possess two motifs that confer a signaling role on the proteins, regulating both expression and stability. Thus, the sequence diversity among IDO proteins leads to a distinct mechanism for sustaining IDO1 expression in dendritic cells and the development of tolerance, in response to specific stimuli. The diverse characteristics and different expression patterns of the Trp-catabolizing enzymes equate to distinct biological roles for the enzymes.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fimmu.2014.00485/abstract

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IDO2 in immunomodulation and autoimmune disease

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IDO2 is a relative of IDO1 implicated in tryptophan catabolism and immune modulation but its specific contributions to normal physiology and pathophysiology are not known. Evolutionary genetic studies suggest that IDO2 has a unique function ancestral to IDO1. In mice, IDO2 gene deletion does not appreciably affect embryonic development or hematopoiesis, but it leads to defects in allergic or autoimmune responses and in the ability of IDO1 to influence the generation of T regulatory cells. Gene expression studies indicate that IDO2 is a basally and more narrowly expressed gene than IDO1 and that IDO2 is uniquely regulated by AhR, which serves as a physiological receptor for the tryptophan catabolite kynurenine. In the established KRN transgenic mouse model of rheumatoid arthritis, where IDO1 gene deletion has no effect, IDO2 deletion selectively blunts responses to autoantigen but has no effect on responses to neoantigen challenge. In human populations, natural variations in IDO2 gene sequence that attenuate enzymatic activity have been reported to influence brain cancer control and adaptive immune responses to the IDO2 protein itself, consistent with the concept that IDO2 is involved in shaping immune tolerance in human beings. Biochemical and pharmacological studies provide further evidence of differences in IDO2 enzymology and function relative to IDO1. We suggest that IDO2 may act in a distinct manner from IDO1 as a set-point for tolerance to "altered-self" antigens along the self-non-self continuum where immune challenges from cancer and autoimmunity may arise.

Keywords: indoleamine dioxygenase, tolerance, kynurenine pathway, aryl hydrocarbon receptor, autoimmunity, rheumatoid arthritis

INTRODUCTION

Of the four tryptophan catabolic enzymes in mammals, IDO2 is the most recently discovered and like the others (IDO1, TDO2, and TPH1), it is implicated in immune control. While all the three dioxygenases in this group (IDO1, IDO2, and TDO2) generate kynurenine as a product, TDO2 represents a structurally distinct multimeric enzyme of divergent origin compared to the monomeric IDO1 and IDO2 enzymes, which are related. The human *IDO2* gene is located downstream of *IDO1* on chromosome 8p21 and these two genes bear close structural and evolutionary relationships. Compared to the other enzymes, IDO2 expression is confined mainly to antigen-presenting immune cells, liver, kidney, brain, and placenta, displaying a unique and relatively more restricted pattern that is consistent with a non-redundant function(s). Early studies of the physiological function of IDO1 by Munn, Mellor, and colleagues pioneered the concept that tryptophan catabolism modulates immunity, based on the discovery that a simple tryptophan mimetic, the IDO inhibitor D,L-1-methyl-tryptophan (1MT), could trigger rejection of allogeneic murine concept (1, 2). Subsequent to this discovery, 1MT has been used in thousands of studies to study IDO function in diverse settings of immune control. However, interpreting these studies may be impacted by the discovery of IDO2, which under various conditions has been found to be inhibited by 1MT like IDO1 (3–7). Thus, while 1MT has been used widely to implicate tryptophan catabolism in numerous chronic inflammatory pathologies, such as cancer, chronic infection, allergy, neurological disorders, and autoimmunity (8, 9), the possible contributions of IDO2 in interpreting the effects of 1MT may be impactful. Another fine review on IDO2 has appeared recently (10). This review summarizes existing knowledge about IDO2 and its functions in immune control and disease.

IDO2 DISCOVERY

IDO2 was discovered independently by groups working in the areas of infectious disease, cancer research, and genomics (3, 11, 12). Ball et al. cloned IDO2 by searching cDNA libraries used in high-throughput sequencing for IDO1-like sequences, identifying in this manner a novel gene they termed INDOL1 (11). Recombinant enzyme was shown to catabolize tryptophan to kynurenine like IDO1 but with a reduced relative activity. Comparative genomics provided evidence that IDO2 arose by gene duplication before the origin of the tetrapods. Expression was documented in kidney, liver, and epididymis, localizing the endogenous IDO2 enzyme to kidney tubular cells and spermatozoa. Distinct functions were suggested by differences in the catalytic expression patterns noted within tissues and during malaria infection.

Metz et al. cloned IDO2 on the basis of partial IDO1 structural homologies that were found downstream of the human IDO1 gene in a region of chromosome 8p12 that was misannotated in early genome compilations (3). This work documented the tryptophan catalytic activity of mouse and human cDNAs, with the mouse isoform exhibiting higher catabolic activity but both isoforms showing less activity compared to IDO1 under similar conditions. Two single nucleotide polymorphisms (SNP) were described in the IDO2 coding region that were widely distributed in human populations, R248W and Y359X, each of which attenuated catalytic activity. A narrow range of IDO2 expression was documented by mouse tissue analysis with highest expression in liver, kidney, and placenta. Complex RNA splicing patterns were revealed in placenta and brain. In human, 293 cells engineered to overexpress IDO1 and IDO2, there were differences in how tryptophan depletion mediated by each enzyme affected translation by regulating eIF2α, with IDO2-expressing cells unresponsive to subsequent tryptophan restoration suggestive of a pseudo-differentiation effect. Moreover, IDO2-expressing cells exhibited a unique susceptibility to catalytic inhibition by the D isoform of 1MT, which selectively impeded the activity of full-length IDO2 but not IDO1 in this setting (3).

Yuasa et al. described a novel mouse IDO2 cDNA identified as an IDO1 paralog in a set of several evolutionary studies of IDO genes that supported the concept of IDO2 functional differences (12). Characterizing the activity of the recombinant mouse enzyme, they noted the relatively lower tryptophan catabolic activity of IDO2 compared to IDO1-like Ball et al. and Metz et al. Based on a phylogenetic analysis, they argued that IDO2 and other low-activity IDO paralogs from non-mammalian organisms were proto-IDO enzymes (12). Interestingly, while IDO-like genes were observed in several lower vertebrates, the genomes from chicken and zebrafish exhibited only one IDO-related gene most similar to IDO2. Accordingly, they argued that IDO1 may have arisen by gene duplication of a more ancient proto-IDO gene before the divergence of marsupial and eutherian (placental) mammals. Given the relatively weaker catalytic activity of IDO2 enzymes, this group suggested that L-Trp may not be a true *in vivo* physiological substrate, although methylene blue rather than physiological reductants (used by all groups in the oxygenase reactions studied) might not provide reliable insights into function, as noted in Ref. (6). This intriguing suggestion is consistent with the finding of Metz et al., who found that IDO2-overexpressing human 293 cells were unresponsive to tryptophan restoration after tryptophan had been depleted in cell culture by IDO2 activity, in stark contrast to IDO1-overexpressing cells, which responded fully.

A subsequent study of fungal IDO homologs by this group further corroborated the hypothesis that IDO2 functions in some unique manner (13). Specifically, this work revealed that the tryptophan catabolic activity of some fungal IDO enzymes was sufficient to supply nicotinamide adenine dinucleotide (NAD), the downstream end product of the kynurenine pathway, whereas other fungal IDO enzymes lacked sufficient tryptophan catabolic activity to supply NAD. Thus, it seems clear that low catalytic efficiency IDO enzymes are only conserved in evolution, but that they also diverged from active IDO enzymes at early times. Overall, initial characterization of IDO2 suggested features arguing for a unique functional role(s) relative to IDO1.

IDO2 EXPRESSION PATTERNS

Several studies have described expression patterns of IDO2 message and protein that suggest unique regulation but also some functional redundancy with IDO1. Whereas IDO1 predominates in colon and epididymis, IDO2 mRNA predominates in cerebral cortex, liver, and kidney. Evidence of redundancy is suggested by the finding that *Ido1* genetic deficiency in mice leads to compensatory upregulation of IDO2 in the epididymis, where IDO1 is relatively more highly expressed normally (14). IDO2 is also expressed like IDO1 in antigen-presenting cells but under somewhat different control. The IDO2 promoter includes a prominent binding site for the transcription factor IRF-7, a master regulator of dendritic cell maturation, suggesting a central role in these professional antigen-presenting cells (15). In this setting, IDO2 appears to be a mainly basally expressed gene, the levels of which vary little by comparison to IDO1 levels that are more robustly regulated. Current information suggests that at the RNA level IDO2 expression is regulated by various pro-inflammatory stimuli, but less robustly than IDO1, including in dendritic cells by interferon-γ (IFN-γ), IL-10, lipopolysaccharide, and prostaglandin E2 (3, 15–18). Interestingly, activation of the aryl hydrocarbon receptor (AhR), a transcription factor, which can serve as a physiological ligand for kynurenine (19), has been reported to upregulate IDO2 in dendritic cells (20, 21). Since activated IDO1 generates kynurenine, this observation presents the intriguing possibility of a downstream mechanism to elevate IDO2 levels in dendritic cells where IDO1 becomes upregulated, a prospect discussed further below. IDO2 has been reported to be overexpressed along with IDO1 in pancreatic cancer (22, 23), and in basal cell skin carcinomas, where its expression appears to be driven by the T-cell-attracting chemokine CXCL11 (16), but neither the extent of IDO2 expression nor knowledge of its regulatory mechanisms in cancer settings are as widely described as IDO1 as yet.

MOUSE GENETIC STUDIES: IDO2 IS CRITICAL FOR IDO1-DEPENDENT Treg GENERATION

Our group constructed and characterized mice that are genetically deficient in *Ido2* to investigate its functions in development, normal physiology, and pathophysiology (24). These mice retain the normal structure and expression of the nearby upstream *Ido1* gene. Interest in generating this strain was reinforced by our discovery that IDO2 RNA levels were attenuated in myeloid cells from $Ido1^{-/-}$ mice due to an altered RNA splicing event that abolishes catalytic function (24). This was a tissue-specific effect insofar as IDO2 RNA splicing was unaffected in livers from $Ido1^{-/-}$ mice. How IDO1 may affect IDO2 RNA processing was unclear but likely to be indirect. Nevertheless, it appeared that in addition to their deficiency in IDO1 function $Ido1^{-/-}$ mice were also mosaic deficient for IDO2 function. This revelation was important since it influences the interpretation of phenotypic results involving myeloid cells from $Ido1^{-/-}$ mice, widely studied in the field, which might conceivably be explained by loss of function in IDO2 rather than IDO1. Indeed, the possibility of an IDO1 → IDO2 genetic pathway in myeloid cells was consistent with expression data from

Bankati et al., who had found that activation of the kynurenine-stimulated transcription factor AhR was sufficient to stimulate IDO2 transcription (21). Overall, we reasoned that mice deficient in IDO2 might not only help define its functions but also help re-interpret of functions previously ascribed to IDO1 (made on the basis of findings from *Ido1*$^{-/-}$ mice).

IDO1 acts to control the activation and differentiation of T regulatory cells (Treg) in a variety of settings, including cancer (25–27). Given evidence of genetic epistasis between IDO1 and IDO2, we asked whether IDO2 loss could affect IDO1-mediated Treg generation in settings where an essential function for IDO1 has been established. In WT or *Ido2*$^{-/-}$ mice treated with CpG oligonucleotides, a critical role for IDO2 in Treg generation was documented in an established T-cell suppression assay (26). Suppression relieved by *Ido2* loss in this assay was reversed by a cocktail of PD1 and PD-L1/PD-L2 antibodies that block PD-1 interaction with PD-L1/PD-L2, a hallmark of IDO1-activated Tregs (26). Strikingly, the effect observed phenocopied the effects of *Ido1* loss in Treg cells generated under the same conditions, directly supporting a functional requirement for IDO2 in Treg generation and offering further evidence of its genetic epistatic interaction with IDO1.

Comparing the response of *Ido2*-deficient mice in a classical assay for contact hypersensitivity led to further support for a role of IDO2 in T-cell-dependent immune responses (24). While *Ido1*$^{-/-}$ and *Ido2*$^{-/-}$ mice both displayed a reduction in contact hypersensitivity, relative to WT control animals, loss of *Ido2* but not *Ido1* was associated with a reduction in systemic levels of cytokines implicated causally in this classical immune response (GM-CSF, G-CSF, IFN-γ, TNF-α, IL-6, and CCL2). Reductions in GM-CSF might be relevant in skin, given its critical role in stimulating AhR-dependent maturation of Langerhans cells (LC) (28), which are thought to be involved in skin tolerance. Since IDO2 is itself an AhR target gene (20, 21), one plausible model is that IDO2 acts downstream of AhR to support local expression of GM-CSF, thereby promoting LC maturation and LC-mediated tolerance through an autocrine loop. While focused mechanistic investigations are needed such models may offer a logical starting point to interpret how IDO2 may act in antigen-presenting cells to influence T-cell function.

Distinct contributions to pathogenic inflammatory processes were likewise indicated in skin carcinogenesis assays, where tumors are induced by a single topical administration of the Ras mutagen DMBA followed by chronic weekly exposure to the pro-inflammatory phorbol ester TPA. Here, while *Ido1* loss was sufficient to blunt tumor formation, as observed previously (29), *Ido2* loss had no effect on the susceptibility to either formation or progression of tumors (24). Taken together, these results offered the first direct physiological evidence that IDO2 helps regulate adaptive immunity, perhaps through contributions to inflammatory control that are at least partly non-redundant with IDO1.

MOUSE GENETIC STUDIES: IDO2 IS CRITICAL FOR AUTOANTIBODY PRODUCTION AND AUTOIMMUNITY

Rheumatoid arthritis (RA) is an autoimmune disorder that has been associated with aberrant IDO activity and defective T-cell function (30–32). In IDO studies conducted in preclinical mouse models of RA, there is complexity in interpreting the contributions of IDO to the disease state, with opposing effects depending on the model used. Nevertheless, as an initial assessment of the possible connections between IDO2 and T-cell function in a genetically defined model, we compared the effects of genetic deletion of IDO1 or IDO2 in the KRN transgenic mouse model of spontaneous RA. The specific pathophysiological relevancy of the KRN model to human disease is justified in part by its mimicry of the elevated tryptophan degradation in RA patients, which has been appreciated clinically for many years [as summarized in Ref. (33)].

In the KRN model of spontaneous RA, we found that IDO2 was crucial for the development of arthritis but that IDO1 was completely dispensable. This finding was provocative in light of earlier observations that D-1MT treatment could attenuate RA in this model (34) and that D-1MT was capable of inhibiting the tryptophan catabolic activity of IDO2 but not IDO1 in human cells (3). Interestingly, while *Ido2* deficiency phenocopied D-1MT treatment, *Ido1* deficiency abolished responses to D-1MT even though *Ido1* was dispensable for RA pathogenicity, providing further support for IDO1-IDO2 genetic interaction in immune control.

Investigations of cellular mechanisms revealed that the decreased joint inflammation displayed by *Ido2*$^{-/-}$ mice relative to control animals was due to a reduction in pathogenic autoantibodies and antibody-secreting B cells. Strikingly, reduced inflammation in *Ido2*$^{-/-}$ mice was associated with a defect in the initiation of autoreactive B cell responses, but not with any overall defect in normal B cell responses: total serum immunoglobulin levels were unaffected in *Ido2*$^{-/-}$ mice, and those mice were fully competent to mount productive antibody responses to model antigens *in vitro* and *in vivo*. *Ido2* deficiency also reduced CD4^{+} helper T-cell responses; however, in this case reciprocal adoptive cell transfer studies showed that this defect was extrinsic to T cells. While interpretation of these results must be tempered in light of distinct effects of IDO signaling in collagen-induced models of arthritis (35), a different preclinical model used in the field, our genetic studies of IDO2 in the mouse nevertheless offer the first direct evidence that it makes unique contributions to the control of adaptive immunity and inflammatory disease.

IDO2 IN HUMAN STUDIES

In human immune physiology, the implication of a genetic linkage between IDO1 and IDO2 is intriguing in light of the broad distribution of two functionally attenuating SNP in the coding region of the IDO2 gene in human populations (3). These SNP variations dramatically reduce or abolish tryptophan catabolic activity, therefore varying the level of this IDO2 function in different individuals, perhaps affecting T-cell-dependent immune control as a result. Since antigen-presenting cells are a primary site of IDO2 expression, further investigation is needed to understand how IDO2 may act to initiate, maintain, fix, or reverse antigen tolerance. Along these lines, a recent study in human DC suggests that IDO2 may help fix basal levels of tolerance, acting differently than IDO1, which unlike IDO2 is induced strongly by prostaglandin E2 (PGE2) and other pro-inflammatory signals in these cells (15). This study compared the patterns of expression and regulation of IDO1 and IDO2 in human circulating DC. At the protein level, IDO1 was expressed only in circulating myeloid DC and was

modulated by PGE2, whereas IDO2 was expressed in both mDC and plasmacytoid DC and was not modulated by PGE2. In circulating DC from healthy subjects, IDO1 expression relied on PGE2 whereas IDO2 expression was constitutive. However, in DC from arthritis patients, circulating DC expressed both IDO1 and IDO2. Notably, mDC and plasmacytoid DC both generated T regulatory cells through a mechanism that relied upon both IDO1 and IDO2 expression, based on the interpretation of RNAi-mediated gene silencing experiments (15). These observations further supported a model for IDO1-IDO2 genetic interaction in antigen-presenting cells. Further, they suggested that IDO2 may act as a downstream basal function in determining "set points" for tolerance determined by IDO1 acting as an upstream inducible function. In any case, this work established that IDO2 is expressed stably in DC under steady-state conditions and that it may contribute to the homeostatic tolerogenic capacity of DC.

The relatively small number of studies of IDO2 in human systems has focused on cancer and where roles in immunosuppression have been hypothesized. In evaluating the use of IDO2 SNP as biomarkers for therapeutic response, Eldredge et al. stratified the response of brain metastasis patients to whole brain radiotherapy (WBRT) when they were orally administered low-dose chloroquine concomitant with therapy (36). This experiment was based on a multipronged rationale. First, patients with brain malignancy who received WBRT were in some cases found to benefit significantly from low-dose chloroquine when administered concomitant with therapy (37). Second, low-dose chloroquine was serendipitously discovered to indirectly inhibit the tryptophan catabolic activity of IDO2 but not IDO1 in cells, apparently by selective interference with the physiologic reductant used by each enzyme in the oxygenase reaction (R. Metz, unpublished observations). Third, the possibility that IDO2 might contribute to cancer immunosuppression in some settings where it is expressed, including brain, similar to the manner in which IDO1 has been implicated widely (9). Fourth, that IDO2 inhibition might relieve immunosuppression in such settings, but only in patients with a functionally active SNP configuration in their IDO2 genes. In a prospective, single-cohort study, WBRT (37.5 Gy in 2.5 Gy daily fractions) administered with concurrent CQ (p.o. 250 mg daily) was safely tolerated in patients with newly diagnosed brain metastases from biopsy-proven, primary lung, breast, or ovarian tumors ($n = 20$). The main finding of this study was a trend toward increased overall survival in patients with wild-type *IDO2* compared to patients with heterozygous or homozygous SNP configurations that ablate *IDO2* enzyme activity (10.4 vs. 4.1 months; $p = 0.07$). In light of evidence that tryptophan catabolism in the brain may influence affective disorders (i.e., mood), it is interesting that a recent study also showed a trend in association of IDO2 region SNP in predicting outcomes to treatment with the anti-depressant drug citalopram (Celexa®) (38).

Additional studies encourage the notion that IDO2 may offer some pathogenic support to advanced cancer in certain settings, albeit less widely than IDO1. In cancer, there are reports of IDO2 overexpression in certain gastrointestinal tumors (39), including frequent overexpression in pancreatic cancer (23). Although there is little exploration of this direction as yet, one study reported that skin administration of IDO2 siRNA was as efficient as IDO1 siRNA in promoting the efficacy of a HER2-based DNA vaccine, in a mouse model of breast cancer (40). In a different and more provocative direction, Sorensen et al. have described naturally occurring anti-IDO2 immune responses in the peripheral blood of both cancer patients and healthy donors, specifically, in the presence of a spontaneous cytotoxic T-cell reactivity directed against the IDO2 protein that can recognize and destroy human tumor cells (39). This work extends an earlier description from the same group of a similar parallel response directed against the IDO1 protein (41). More recent work on the IDO2 response stratified the number of responses based on the IDO2 coding region SNP, highlighting stronger responses to homozygous Y359 alleles that do not truncate the IDO2 protein, and more numerous responses to homozygous 248W alleles that reduce tryptophan catabolic activity relative to the wild-type 248R configuration (42). Thus, IDO2 SNP allelic status, which affects tryptophan catalytic function, appears to influence a self-reactive cytotoxic T-cell-dependent response that is directed against IDO2 protein. The role of IDO1 and IDO2 in cytotoxic T-cell responses directed against self has been reviewed recently with a perspective on regulating this response in the setting of cancer therapy (43).

IDO2 BIOCHEMISTRY AND PHARMACOLOGY

Much of the existing literature on IDO2 relates to questions about its biochemical and signaling properties and a budding interest in its pharmacologic inhibition for therapeutic purpose. As alluded to above, there is a general consensus that the tryptophan catabolic activity of IDO2 and IDO2-like genes in non-mammalian organisms is much weaker than IDO1. Studies of the mouse IDO2 enzyme show it to be more catalytically active than the human enzyme, which is quite weak indeed, yet even in mice genetic knockout does not affect systemic kynurenine levels (i.e., as measured in blood serum) (24). Moreover, in cells where IDO2 or IDO1 are overexpressed to levels that deplete tryptophan, inducing autophagy as a result (44), restoring tryptophan is insufficient to relieve protein translation blockades as monitored by a reversal in the expression of the translation stress-induced transcription factor LIP (3, 44), a pathway with pathophysiologic relevance to IDO-driven cancer (45). All in all, the work to date has fed skepticism that tryptophan is an important physiological substrate for IDO2, as Yuasa et al. originally speculated (6). Supporting this idea, three enzymologic studies comparing tryptophan-like compounds as substrates and inhibitors have presented results arguing that human IDO2 may be somewhat more promiscuous than IDO1 (46–48). A screen of a library of FDA-approved drugs for inhibitory activity against recombinant IDO2 identified the proton pump drug tenatoprazole as a low-micromolar inhibitor ($IC50 = 1.8\ \mu M$), with no IDO1 or TDO2 inhibition up to 50-fold higher drug levels (47). A comparison of recombinant proteins extending the enzymological analysis of human IDO2 reinforced its distinct nature from IDO1, in terms of substrate specificity and affinity, and also based on the identification of tryptophan derivatives that are mutually exclusive as substrates (48). Two groups conducting modeling and experimental testing of novel IDO1 inhibitors demonstrated selectivity against murine IDO2, adding to the evidence of enzymologic differences between IDO1 and IDO2 (49, 50). Going forward, pressing questions to resolve are

whether and where tryptophan may be physiologically relevant as an IDO2 substrate, if at all, and whether there are non-tryptophan substrates that are physiologically or pathophysiologically relevant to IDO2 function, as a growing number of investigators seem to currently suspect.

Several studies have addressed the inhibitory properties of 1MT as an IDO2 inhibitor (5–7, 46, 48, 51), particularly with regard to the racemic selectivity of D-1MT in whole cells as originally reported by our group (3). This area of investigation continues to be fraught not only with concerns about suitable physiological substrates, as noted above, but also about suitable physiological reductants involved in the oxygenase reaction(s) that can be mediated by IDO2. We have discussed this issue recently at some length elsewhere (9). Briefly, we have argued that use of methylene blue as a non-physiological reductant obscures the core challenge of how to interpret the ability of 1MT racemers to inhibit IDO2 activity in cells where it may make relevant contributions to normal physiology or pathophysiology, for example, in cancer or autoimmunity. In exploring other reductants, Austin et al. employed cytochrome *b* in oxygenase reactions of recombinant IDO2 but still found it less active than IDO1 and poorly inhibited by either 1MT racemer (46). In examining IDO2 contributions in a human T-cell system, Qian et al. reported that IDO2 could suppress cell growth but that neither 1MT racemer exhibited potency in inhibiting this effect (4). In *Ido2*-deficient mice, we found that genetic ablation of IDO2 but not IDO1 could phenocopy the effect of D-1MT in the context of the KRN model of RA autoimmunity (33, 34). This system offers a murine setting where biochemical studies can be connected to a genetic and pathobiological context, perhaps encouraging investigations in a human setting that can rule in or rule out the relevancy of 1MT as an IDO2 inhibitor. In closing, we propose that the use of IDO2-deficient mice will be useful to advance studies of how immunometabolism mediates tolerance in normal physiology and disease; to gain mechanistic insights into how IDO pathways direct pathogenic inflammation in diverse settings; and to help inform clinical development of IDO and TDO inhibitors being developed to treat cancer and other inflammatory disorders, where early clinical trials have suggested therapeutic promise.

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Tryptophan feeding of the IDO1-AhR axis in host–microbial symbiosis

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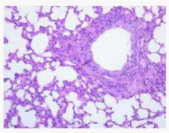
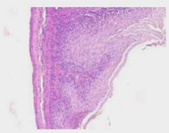
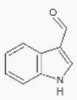
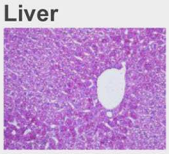
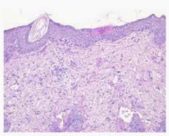
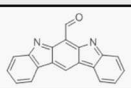
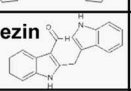
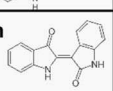
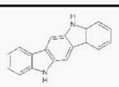
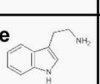
The large variety of microbial species in the human microbiome plays an important role in human health by affecting tissue differentiation, modulation of the immune system, as well as the general response against infectious pathogens. The aryl hydrocarbon receptor (AhR) contributes to immune homeostasis as having an antimicrobial role on the one hand – owing to AhR-dependent IL-22 transcription – and, on the other, an anti-inflammatory role in that it mediates the differentiation of regulatory T cells (Tregs). Here, we have examined the multifaceted physiological role of AhR as resulting from the vast array of recently described AhR ligands and of the multiplicity of AhR-expressing cells in host-microbial symbiosis in mammals.

THE PROMISCUOUS NATURE OF AhR AGONISTS

Aryl hydrocarbon receptor is a ligand-dependent transcription factor activated by a variety of synthetic and natural molecules. In particular, ligands of AhR include hydrocarbons, heterocyclic amines, and indole-derived compounds. Dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD) represents the prototypical environmental and most potent AhR ligand known (1). In addition, a variety of herbal extracts – such as ginseng, licorice, and gingko biloba – stimulate AhR DNA binding and the downstream transcription of numerous genes, thus indicating that AhR might have evolved to respond to mainly dietary products to which animals and humans are chronically exposed (2). Interestingly, ginseng, saponins (gingenosides) have been defined as potent AhR agonists or antagonists (3, 4). Despite the ability of environmental chemicals or other products in diet to bind and subsequently activate AhR, former studies have also shown that natural endogenous ligands may bind and mediate AhR-dependent downstream effects as well (5). Thus the evolution of the AhR – some 450 million years ago – underlies the concept that the original AhR ligands emerged prior to the anthropogenic introduction of polyaromatic hydrocarbons (2). In addition, AhR has an exceptionally promiscuous ligand-binding pocket, which explains the extreme variety of molecules binding AhR with agonist or antagonistic activity (6). Indeed, it has been shown that both bilirubin and biliverdin represent good examples of endogenous ligands in liver with agonist activity for AhR (7, 8). The induction of AhR in the liver by those ligands induces upregulation of *Ugt1a1* to prevent jaundice in neonates and to regulate antioxidant AhR effects in the adult liver (9). The intricacies of AhR activation also relate to the mode of application of a ligand and not only to its nature. Thus systemic administration of 6-formylindolo [3,2-*b*]carbazole (FICZ) reduced clinical signs in a murine model of encephalomyelitis (EAE), while local injection of FICZ, incorporated into the antigen emulsion for induction of EAE, seemed to more directly target and promote Th17 cells, thereby exacerbating pathology in EAE (10).

Recently, many studies have shown that the microbiome represents a consistent source of AhR endogenous ligands with disparate effects on immune homeostasis (**Figure 1**). Thus, moving to the context of the microbiota, the nature of both ligands and target cells vary consistently according to the microbe niche, providing a more complex scenario of AhR's impact on immune homeostasis. In the human skin, commensals such as *Malassezia* yeasts secrete AhR agonists, such as indirubin, FICZ, indolo[3,2-*b*]carbazole (ICZ), malassezin, pityriacitrin, and tryptamine, which are all potent AhR ligands (11). When skin extracts are isolated from patients with ongoing skin infection, AhR is potently activated, and an increased concentration of AhR ligands in the skin has been linked to the development of *Malassezia*-associated skin diseases. Interestingly, some of the isolated molecules are able to convert to other AhR ligands such as ICZ, which is released under conversion of malassezin (12). Therefore, *Malassezia*-derived AhR ligands may have a significant impact on skin homeostatic immune mechanisms and disease development. Indeed, indirubin and ICZ significantly augmented AhR-mediated *Cyp1a1* and *Cyp1b1* gene expression in dendritic cells, while reducing Toll-like receptor (TLR)-induced dendritic cell maturation and T-lymphocyte proliferation (13). In line with this finding, FICZ has been used to dampen the inflammatory response in both mouse and human skin (14). Through the activation of AhR in non-hematopoietic skin cells, administration of FICZ ameliorated the inflammatory profile of psoriasiform human and murine skin specimens. Of interest, a key aspect of tryptophan-derived metabolites is related to their molecular dynamics of interconversion. For example, tryptamine serves as a proligand for AhR, and its activation depends mainly on monoamine oxidases (15), which eventually convert tryptamine

Tissue	AhR Ligand	Source	Function	Pathway	Ref
Perypheral Lymph nodes	L-Kynurenine 	Human Myeloid cells	Antimicrobial effects	?	20
		Murine Myeloid cells	Immunoregulation in fungal infections	Treg/Th17 homeostasis	22, 23, 24
Lung 	Phthiocol 	*Mycobacterium tubercolosis*	Increased pathogen clearance	CYP1A1, CYP1B1, AHRR	17
	Phenazine 	*Pseudomonas aeruginosa*	Increased pathogen clearance	CYP1A1, CYP1B1, AHRR	17
	L-Kynurenine 	Murine Myeloid cells	Immunoregulation in CGD	Treg/Th17 homeostasis	22, 23, 24
Stomach 	3-IAld 	*Lactobacillus reuteri*	Colonization resistance against *C.albicans*	IL-22	19
Vagina 	3-IAld 	*Lactobacillus acidophilus*	Colonization resistance against *C.albicans*	IL-22	19
Liver 	L-Kynurenine 	?	Prevention of infection-associated immunopathology	CYP1A1 TGFβ IDO1 TDO2	6
Skin 	FICZ 	*Malassezia furfur* Tryptophan UV exposure	Protection against psoriatic inflammation	CYP1A1 CYP1B1	11, 12, 13
	Malassezin 	*Malassezia furfur*	?	CYP1A1	11, 12, 13
	Indirubin 	*Malassezia furfur*	Dampening DC maturation	CYP1A1 CYP1B1	11, 12, 13
	ICZ 	*Malassezia furfur*	Dampening DC maturation	CYP1A1 CYP1B1	11, 12, 13
	Tryptamine 	*Malassezia furfur*	?	CYP1A1	11, 12, 13
	3-IAld 	*Malassezia furfur*	?	?	11, 12, 13

3-IAld, Indole-3-aldehyde
FICZ, 6-formylindolo(3,2-b)carbazole
ICZ, Indolo[3,2-b]carbazole

AHRR, Aryl-hydrocarbon Receptor Repressor
CGD, Chronic Granulomatous Disease

FIGURE 1 | Tryptophan-derived AhR activating molecules with antimicrobial activity.

to other AhR ligands, such as the indole-3-aldehyde (3-IAld) and eventually by spontaneous dimerization to FICZ (15). Importantly, intestinal microbiota will also convert tryptophan to tryptamine by decarboxylation. In doing so, and by modulating the colonic ion secretion, tryptamine affects the transit of food particles and bacterial cells through the gut lumen (16). More recently, *Pseudomonas aeruginosa* as well as *Mycobacterium tuberculosis* showed an ability to activate AhR in the lung through the release of pigmented virulence factors, such as phenazines and phthiocol, respectively, pointing to AhR as a sensor of a new class of pathogen-associated molecular patterns. Upon AhR binding, an AhR-controlled metabolic circuit was activated and the virulence factors degraded with consequent pathogen clearance (17).

We found that highly adaptive lactobacilli in the gut, in particular *Lactobacillus reuteri*, by switching from sugar to tryptophan as an energy source, were expanded and produced an AhR ligand, 3-IAld, active in innate lymphoid cells (ILCs) where it would contribute to mucosal resistance against the opportunistic pathogen *Candida albicans*. IL-22 is the main downstream product of AhR activation upon 3-IAld stimulation in ILCs, regulating the release of antimicrobial peptides in the gut epithelia. Of notice, IL-22^{+} ILCs are also able to limit segmented filamentous bacteria colonization in the gut (18). A similar effect was found in the murine vaginal tissue, where *Lactobacillus acidophilus* will degrade tryptophan to 3-IAld and protect mice from *C. albicans* vaginitis (19). Pivotally, these antimicrobial effects were more evident under conditions of higher tryptophan availability in mucosal tissues, as it occurs in mice fed with a tryptophan-enriched diet or in mice bearing deficiency of a tryptophan catabolic enzyme.

In addition to microbial derived ligands, mammalian cells in the liver, as well as in peripheral lymph nodes, activate enzymes such as tryptophan-2,3-dioxygenase (TDO2) and indoleamine-2,3-dioxygenase 1 (IDO1), able to generate tryptophan derivatives such as kynurenines, which also notably act as ligands for AhR (6). Kynurenines have long been known for their ability to exert specific antimicrobial activities (20). Thus, the recent findings provide mechanistic insight into the interplay between IDO1-dependent metabolism and AhR activation in colonization resistance and tolerance induction at the host/microbe interface (6) (**Figure 1**).

THE IDO1-AhR-TREG AXIS IN MAMMALS: THE CO-EVOLUTION OF A TOLEROGENIC DEFENSE STRATEGY

Humans have evolved with microbes, and crucial factors for survival include prompt recognition of invading pathogens, acquisition of controlled immune response, fine-tuned pathogen eradication and return to homeostasis. Co-evolution with hosts had a particularly strong impact on the immune system, which needed to develop an ability to discriminate between resident microbes – maintaining a homeostatic balance – and invasive pathogens, which it must respond to. This complexity could be achieved by integrating two major immune defense mechanisms: infection resistance and disease tolerance (21). Induction of immune tolerance and the maintenance of homeostatic balance provide a series of benefits, including avoidance of tissue injury and para-inflammatory side effects, such as chronic infection and inflammation, which are major epigenetic and environmental factors that contribute to metabolic diseases and autoimmunity, and, in specific settings, to cancer. Conversely, the induction of immune resistance reflects opposite intents, such as the avoidance of infection and control of microbial burden (22, 23).

This paradigm has been epitomized in fungal commensalisms where immune protection must oppose fungal infectivity and ensure survival, while limiting collateral damage and restoring a homeostatic environment (also referred to as "protective tolerance") (22, 24). Primordial resistance against fungi is mainly mediated by naturally occurring IL-22/IL-17A-producing cells, highly prevalent at mucosal sites, and activated by AhR (19, 25). The tryptophan metabolic pathway appeared to play a key and decisive role in fostering tolerance by means of IDO1 activation, tryptophan starvation, the production of immunomodulatory kynurenines, and the activation of Tregs that are strictly required for the generation of protective tolerance to fungi (26, 27). As AhR activation leads to the activation of IDO1 (25), the regulatory loop involving AhR and IDO1 may have driven the co-evolution of commensal fungi with the mammalian immune system and the microbiota, to the benefit of host survival and fungal commensalism.

More recently, the cross-regulatory circuit between IDO1 and AhR has been elegantly shown to mediate disease tolerance (6). Among IDO1 secondary metabolites, L-kynurenine has been identified as an AhR ligand (6). In turn, the AhR-associated Src activity was responsible for IDO1 phosphorylation, TGF-β production, and Treg cell expansion, thus allowing for endotoxin tolerance to occur. Importantly, the activation of the IDO-AhR-Treg axis prevented *Salmonella typhimurium* infection and significantly reduced clinical signs of *Streptococcus* arthritis (6).

More interestingly, the interaction between IDO1 and AhR may have important roles in the context of autoimmune diseases. Autoimmune diseases are indeed multifactorial, depending on intrinsic or environmental components, including diet, infections, and microbial exposure. The role of AhR in autoimmunity is even more interesting because of evidence on protection to EAE by TCDD, which exerts anti-inflammatory effects through induction of Tregs (1). In this context, it is interesting to note that FICZ also protects against EAE when systemically administered (10). Therefore, future studies are needed to elucidate the possible role of AhR ligands of microbial origin in protecting from autoimmune diseases.

In conclusion, the development of a highly specialized symbiosis requires iterative sets of mutual adaptation between and among symbionts and their hosts. This demands moving beyond surveys of microbial diversity to identify host/microbial metabolites that directly target the IDO1-AhR axis for the promotion of infection control and immune homeostasis.

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New insights into IDO biology in bacterial and viral infections

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Initially, indoleamine-2,3-dioxygenase (IDO) has been introduced as a bactericidal effector mechanism and has been linked to T-cell immunosuppression and tolerance. In recent years, evidence has been accumulated that IDO also plays an important role during viral infections including HIV, influenza, and hepatitis B and C. Moreover, novel aspects about the role of IDO in bacterial infections and sepsis have been revealed. Here, we review these recent findings highlighting the central role of IDO and tryptophan metabolism in many major human infections. Moreover, we also shed light on issues concerning human-specific and mouse-specific host–pathogen interactions that need to be considered when studying the biology of IDO in the context of infections.

Keywords: IDO, viral infection, bacterial infection, depressive disorders, kyn metabolites

INTRODUCTION

Indoleamine-2,3-dioxygenase (IDO) is an intracellular, non-secreted enzyme, which catabolizes the production of kynurenine (Kyn) derivates from tryptophan (Trp). Anti-proliferative features of IDO on bacteria, protozoa, and tumor cells have been first described in 1984 by Pfefferkorn (1) as well as Taylor and Feng (2). Induction of IDO in cells of the immune system by IFNγ was introduced for the first time in the late 1980s (3, 4). Today, IDO is thought to be part of a fast local immune regulatory mechanism called "metabolic immune regulation" to protect the host from over-reactive immune reactions via induction of systemic immune tolerance [reviewed elsewhere (5)]. It participates in a broad spectrum of immune responses during chronic infections, immune-escape of cancer cells, tissue inflammation, transplantation, and maternal tolerance toward the fetus and autoimmunity (6). Interestingly, accumulating evidence also connects enhanced Trp metabolism to mental disorders based on serotonin starvation.

Exogenous inflammatory stimuli induce the expression of IDO in antigen-presenting cells (APCs), such as dendritic cells (DC) (7), macrophages (4), and B-cells. Gene expression of IDO was found to be regulated by interferon-α (IFNα) and interferon γ (IFNγ) and also TNFα and prostaglandins (8, 9). As mode of action for IDO, $O_2^{\cdot}$-radical scavenging (2, 10) and later suppression of T-cell responses (11, 12) were discussed. Since many microbial organisms rely on the essential amino acid Trp, its degradation by IDO-expressing cells of the innate immune system was favored as the major IDO-mediated mechanism against infections (13). In infectious disease states, IDO has been shown to exert pleiotropic effects, even with opposing outcomes. On the one hand, IDO directly suppresses the replication of certain parasites and bacteria (1, 14–16), or at least prevents viral spread (17–20), on the other hand, it also acts on host cells to suppress immune reactions thereby promoting infectious diseases (21, 22).

Besides Trp depletion, production of Trp metabolites with bactericidal activity, like Kyn, were identified in human macrophages upon infection with diverse bacteria species as another defense mechanism mediated by IDO (23). IFNγ induced Trp degradation leading to anti-toxoplasmosis activity in infected human fibroblasts was first described by Pfefferkorn in 1984 (1). Only a few years later, several studies linked this effect to enhanced IDO activity against pathogens like *Toxoplasma gondii*, certain *Chlamydia psittaci* strains and *Leishmania donovani* (14–16). In these initial studies, IDO-expressing immune cells were described as macrophages. A contribution of IDO in containment of viral infections was suggested by *in vitro* experiments demonstrating that the inhibition of human cytomegalovirus (CMV) replication was induced by IFNγ and IFNβ (18). This virostatic effect could be reverted by addition of exogenous Trp indicating an involvement of IDO (17, 19, 20). Interestingly, the activity of inducible nitric oxide synthetase (iNOS) was suggested to be able to substitute for the IDO-mediated anti-viral mechanism (18, 24). Since then, it was demonstrated that other viruses, such as herpes simplex virus type 2 (HSV-2) (17), measles virus (19), and vaccinia virus (20), are sensible to IDO-induced Trp depletion.

Apparently, pathogens are able to highjack the immunosuppressive effects of IDO and make use of them to facilitate their own life cycle. For instance, uropathogenic *Escherichia coli* (UPEC) induce IDO in epithelial cells of the urinary tract and in polymorphonuclear leukocytes (21). The dampened immune response upon IDO induction enables a successful colonization of urinary epithelium by UPEC. In addition, viruses like human immunodeficiency virus 1 (HIV) use the immunosuppressive activity of IDO to drive HIV infection into the chronic phase (25). In the following chapters, we will focus on new insights into the role of IDO and Kyn derivates in major viral and bacterial infections in mice and men.

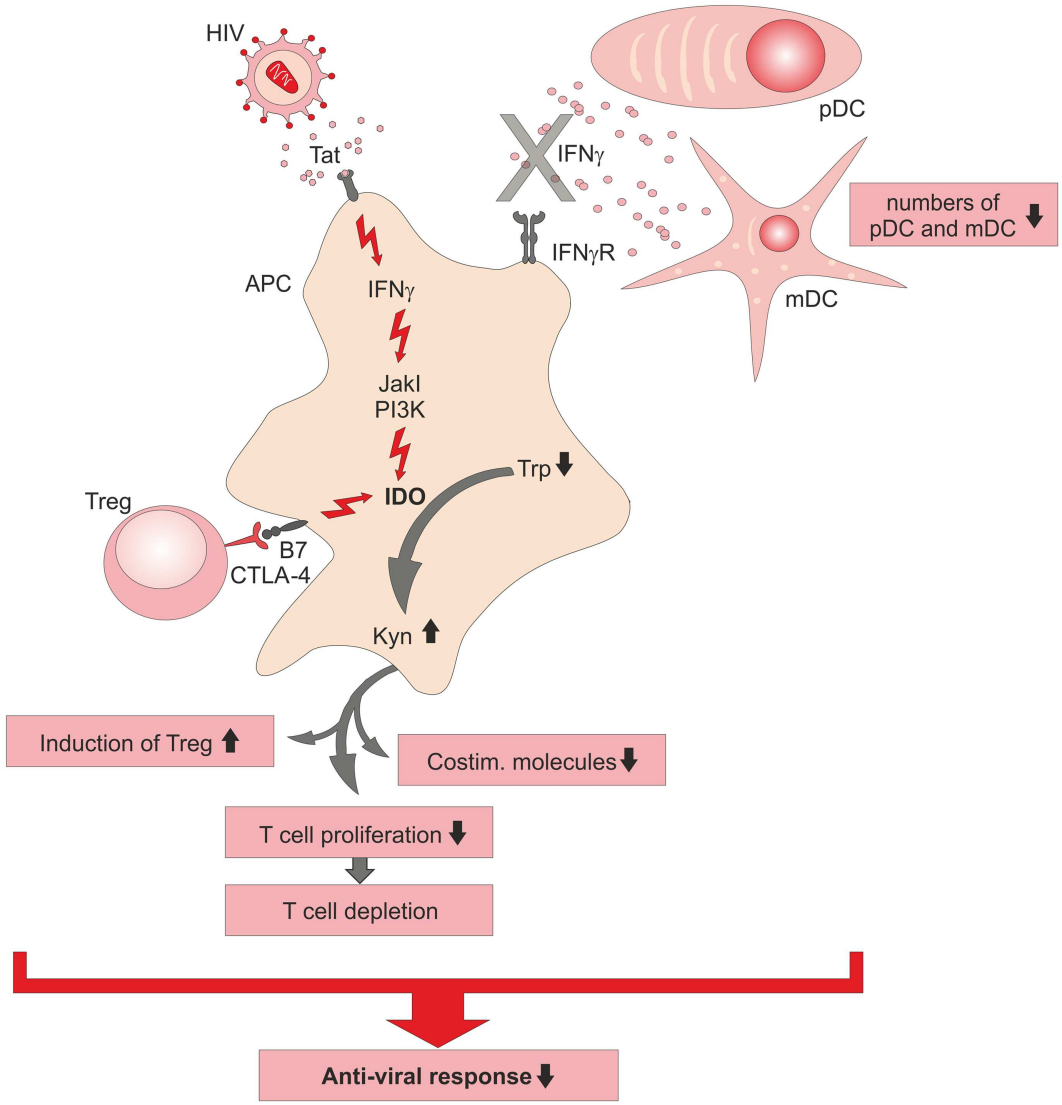

FIGURE 1 | Schematic summary of immunosuppressive functions of indoleamine-2,3-dioxygenase (IDO) during HIV infection. Direct induction of IDO in antigen-presenting cells (APC) by viral Tat protein is established via an intracellular signaling cascade including kinases (JakI, PI3K) or CTLA-4-B7 interaction on regulatory T (Treg)-cells with B7 co-receptor on the APC, which leads in consequence to a breakdown of tryptophan (Trp) into kynurenine (Kyn). Diminished anti-viral immune responses during chronic HIV infection is caused by an impaired T-cell response, the lack of potent IFNγ secreting DC, and the induction of immunosuppressive IDO$^+$ APC. pDC, plasmacytoid DC; mDC, myeloid DC.

ROLE OF IDO IN VIRAL INFECTIONS

ROLE OF IDO IN HIV INFECTION

Infection with HIV causes a severe impairment of T-cell responses by loss of proliferative capacity of T-cells accompanied by a depletion of functionally competent CD4$^+$ T helper cells and by induction of regulatory T-cells (Treg) during the chronic phase of HIV infection (**Figure 1**). The exact T-cell impairing mechanism is still not completely understood, but inhibitory molecules on T-cell function have been investigated intensely [reviewed elsewhere (26)]. Elevated serum levels of IFNγ (27, 28) and Kyn (29) in HIV patients pointed toward a participation of IDO in suppression of T-cell function, yet molecular mechanisms were unknown. Further support came from increased IDO mRNA levels measured in peripheral blood mononuclear cells (PBMCs) of HIV-infected patients (30). *In vitro* infection of PBMC led to the secretion of IFNα and IFNβ by plasmacytoid dendritic cells (pDC) (31). While both CD4$^+$ and CD8$^+$ T-cells expressed the activation markers CD69 and CD38, they failed to proliferate and were insensitive to T-cell receptor stimulation, a status described as division arrest anergy (32). While CD4$^+$ T-cells were arrested in G1/S phase, CD8$^+$ T-cells downregulated the costimulatory receptor CD28. When the enzymatic activity of IDO was inhibited by 1-methyl tryptophan (1-MT), CD4$^+$ and CD8$^+$ T-cells regained their ability to proliferate (30, 31). In monocyte-derived DC (moDC), the N-terminal domain of HIV-1 transactivator regulatory protein (Tat) induced IFNγ and IDO expression and therefore further led to a

suppression of T-cell proliferation. Here, 1-MT was also able to reconstitute T-cell proliferation (33). IDO expression was initially induced by Tat and followed by the induction of IFNγ leading to a feed forward loop. Interestingly, IFNγ signaling pathways leading to IDO expression could be blocked by JAKs and PI3K inhibitors but Tat-induced IDO expression could not be inhibited, suggesting a novel so far not characterized mechanism of IDO induction by Tat proteins in HIV infection (33).

In simian immunodeficiency virus (SIV)-infected macaques, treatment with a combination of antiretroviral therapy (ART) and 1-MT successfully diminished viral loads in plasma and lymph nodes and restored Trp levels but did not reduce Kyn (34). It is worth to mention that 1-MT alone was not able to restrain viremia in this animal model. Probably, IDO was only partially inhibited since reconstituted Trp levels were accompanied with elevated Kyn levels in sera of treated animals. Further, a compensatory counterregulatory mechanism for 1-MT was suggested due to increased IDO and TGFβ production in lymph nodes of treated animals. Decreased numbers of $CD4^+$ T-cells during the course of HIV infection are accompanied by a loss of type I IFN producing cells, like pDC (35, 36). One reason for the low pDC numbers might be their redistribution to peripheral lymph nodes, as observed in the acute phase of SIV-infected macaques (36). Also, numbers of myeloid DC (mDC) are diminished in blood of HIV patients during primary infection (37). Yet, little is known about IDO expression in HIV-infected mDC. To investigate, if HIV alters the function of infected mDC, PBMC-derived DC were transfected with a HIV containing vector construct as a model system (38). This resulted in the induction of IDO in immature and matured DC, accompanied by increased levels of Kyn. In addition, elevated levels of TNFα and IFNγ were secreted by these DC with mature DC secreting the highest amounts. Further, HIV-transduced mature DC induced only modest T-cell proliferation in mixed-lymphocyte reactions, which might be due to IDO activity depleting Trp, a necessary molecule for T-cell function. The addition of 1-MT restored the immunostimulatory capacity of these DC, suggesting a central role of IDO in the suppression induced by HIV-infected DC.

Another mechanism of IDO expression in APCs is mediated by regulatory T-cells (Treg) (39, 40). In HIV patients, an elevated enzymatic activity of IDO in APCs was associated with a reduced anti-viral T-cell response [reviewed elsewhere (41)], while depletion of Treg cells reconstituted anti-HIV immune responses (42). Similarly, in SIV-infected macaques, the expression of the Treg markers CTLA-4 and FoxP3 was increased in T-cells of mesenteric lymph nodes, spleen, and colon, organs with high viral load (43). Simultaneously, IDO expression in spleen and gut-associated lymphoid tissues was suggested to support immunological suppression in favor of viral replication. Therefore, therapeutic targeting of Treg in HIV patients thereby reducing IDO expression in APCs and subsequently immunosuppression seemed promising. However, when blocking CTLA-4 signaling in SIV-infected macaques, an unexpected increase in IDO expression and Kyn levels was observed (44). Moreover, even under conditions of increased T-cell activation due to loss of the regulatory CTLA-4 signaling, viral replication was still promoted. Increased IDO levels were suggested to be a consequence of viral replication. In another attempt to refine ART by combining CTLA-4-blockade and 1-MT treatment, severe side effects causing acute pancreatitis with massive lymphocyte infiltration into the pancreas and loss of Langerhans islets were induced (45). Moreover, all tested animals developed diabetes and hyperglycemic coma while SIV-specific responses were not observed. These results clearly illustrate that the reversal of immunosuppression by targeting CTLA-4 in chronic viral infection is not a promising approach.

The influence of IDO expression on viral loads in mice infected with the retroviral leukemia virus LP-BM5 is controversially discussed. In an earlier study, higher numbers of pDC in IDO KO mice correlated with increased levels of type I IFN and reduced viral load (46). However, in a more recent study using the same model, IDO had no impact on disease progression (47). Viral loads of IDO KO mice were comparable to those of wild-type B6 mice and both IDO KO and WT mice showed decreased responsiveness to B-cell and T-cell mitogens. One might postulate that an important denominator of efficacy targeting IDO might simply be the time when IDO is induced during the course of the infection. Clearly, more work is necessary to determine the role of IDO in murine retroviral infections.

An interesting link between chronic inflammatory diseases and neurological disorders has been recently made with IDO being involved. Patients suffering from chronic inflammatory diseases show often signs of depressive mood behavior (**Figure 2**). In patients with chronic HIV infection, the elevated Trp catabolism maintained by IDO expression is associated with reduced levels of free serum Trp (48, 49). This correlates with a reduction of serotonin (5-HT) and serotonin transporter (5-HTT) expression, as well as an accumulation of neurotoxic Trp metabolites (50). Especially, Kyn and quinolinic acid (QA) can be detected in cerebrospinal fluids of HIV patients and are linked to the development of neuropsychiatric disorders, as part of the Neuro-AIDS complex of symptoms (50). HIV-1 associated dementia (HAD) is correlated to IDO and Kyn induced by Tat of HIV-1 clade B in human primary astrocytes (51) while Tat of HIV-1 clade C does not induce IDO activity in human primary astrocytes and is not associated with HAD. Further evidence for the role of Tat for IDO expression in the brain came from experiments injecting Tat protein intracerebroventricularly into different mice strains. Further, when injecting Tat, induction of IDO and several other pro-inflammatory cytokines in the brain was associated with reduced mobility and depressive-like behavior (52), demonstrating the important role of IDO in the pathophysiology of HIV infection. Blockade of IDO or upstream events of IDO induction in chronic infection might be a novel approach to treat chronic HIV infections.

ROLE OF IDO IN INFLUENZA INFECTION

So far, research of IDO function has focused mainly on murine influenza infection models. Infection with murine influenza virus PR8 has been shown to induce IDO expression in mouse lung tissue (53, 54) and lung-associated lymph nodes (54). IDO activity increased during influenza infection and peak expression correlated with increased lymphocyte numbers in the respiratory tract, albeit the study did not discriminate between T-cell and B-cell subpopulations (53). In another study, inhibition of IDO

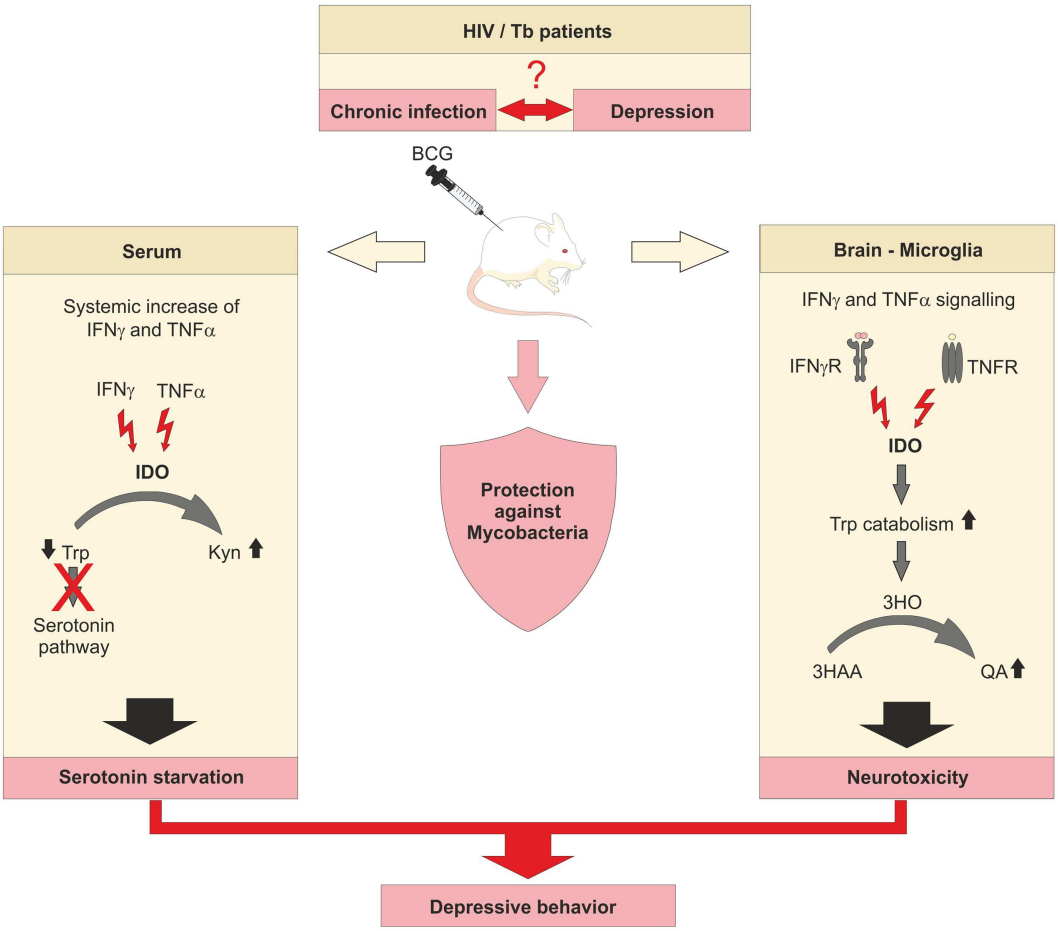

FIGURE 2 | IDO links chronic viral and bacterial infections to cases of depression. Underlying mechanisms of the connection between chronic infection with viruses or bacteria and the onset of mental disorders in men are unknown. Insufficient levels of serotonin in chronically infected and depressive patients are thought to be the consequence of constitutively elevated levels of IDO. Murine models of chronic infections are established to decipher the involvement of IDO in the development of depressive-like behavior.

by 1-MT treatment in influenza-infected mice led to increased numbers of virus-specific memory $CD8^+$ T-cells and functionally activated effector $CD4^+$ T-cells (55). In a follow-up study, 1-MT-treatment improved memory T-cell responses correlated with increased secretion of IFNγ by $CD4^+$ and $CD8^+$ T-cells, accelerated Th1 responses, and a broader virus- and epitope-specific repertoire of $CD8^+$ T-cells (56). Besides, 1-MT treatment led to improved repair of lung tissue. These results indicate that inhibition of IDO might improve flu vaccine activity, might aid in heterosubtypic immunity and support a faster recovery. This view is also supported by data reporting reduced morbidity rates in IDO KO mice when challenged with RP8 or X31 influenza viruses (54). Especially, IDO deficiency led to enhanced development of memory T-cells, which protected mice from lethal virus infection. Further, it was demonstrated that non-hematopoietic cells from lung-draining lymph nodes were major IDO producing cells in response to X31-induced IFNγ secretion. As a feed forward loop, IFN type I and II induced subsequently IDO activity in hematopoietic cells. As a next step, it will be important to translate these findings to human influenza infection and design clinical studies that would allow testing IDO blockade in context of infection and/or vaccination.

ROLE OF IDO IN HBV AND HCV CHRONIC INFECTION

Indoleamine-2,3-dioxygenase has also been linked to chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) (57). Despite an appropriate T-cell response during the acute phase of HCV infection and subsequent viral clearance, T-cell responses in the chronic phase are weak [summarized by Hiroishi et al. (58)]. A massive amount of HCV-specific CD8 T-cells are recruited to the liver; however, recognition of viral epitopes is barely present. Similarly, in chronic HBV infection, cytotoxic T lymphocytes (CTLs) show only a weak response against the HBV surface antigen (HBsAg). While the molecular mechanisms of the tolerogenic state in chronic HBV and HCV infection are not completely understood, an increased IDO expression in the liver of patients with chronic HBV or HCV infection has been observed (57). Moreover, high systemic Kyn/Trp ratios in chronically infected patients

indicate increased IDO activation. In a recent study including 176 patients suffering from chronic HCV infection and 37 healthy controls, it could be shown that Kyn levels correlated with advanced liver inflammation and fibrosis (59). Furthermore, monocytes isolated from PBMCs of HCV patients differentiated into IDO$^+$ DC were more potent in inducing Treg cells when activated with LPS or IFNγ than those of the control group. Along these lines, *in vitro* stimulation of the HCV-infected hepatocellular carcinoma cell line Huh7 with IFNγ led to an induction of IDO, yet HCV replication was not altered by IDO activity (57).

Yet another mechanism for induction of IDO expression was demonstrated in a murine hepatitis model. Treatment with α-galactosylceramide (α-GalCer), a specific agonist for natural killer (NK) cells was able to induce IDO (60). It was speculated that IDO suppresses an overactive immune response triggered by TNFα-producing NK cells and macrophages infiltrating the liver. Following the idea of re-establishing immunocompetent CTLs, wild-type and IDO KO mice were immunized with a combination of α-GalCer and HBsAg (61). Upon immunization, expression of the cytokines IL-2 and IL-12b were only increased in IDO KO mice leading to the induction of HBsAg-specific CTLs. Major IDO-expressing cells were CD11b$^+$ Ly6G$^+$ myeloid-derived suppressor cells (MDSCs) from spleen, which increased in numbers after immunization. They directly inhibited the proliferation of HBsAg-specific CTLs. To assess the role of genes induced by IFNα and/or IFNγ, Mao et al. co-transfected HepG2 cells with the HBV core promoter and 37 different expression plasmids for IFNα- and/or IFNγ-induced genes (62). Only IDO, APOBEC3G, PKR, and ISG20 reduced HBV DNA levels of more than 60%. IDO was considered as the major mediator of the IFNγ-induced anti-viral response, since it mediated Trp depletion followed by suppression of HBV replication.

ROLE OF IDO IN OTHER CHRONIC VIRAL INFECTIONS

Indoleamine-2,3-dioxygenase might also play a role in several other viral infections. While infection with Epstein–Barr virus (EBV) normally causes a self-limited polyclonal lymphoproliferation, it was shown recently that EBV-transformed B-cells express elevated levels of IDO causing Trp degradation to Kyn, which – in turn – suppressed the expression of the activating receptor NK group 2, member D (NKG2D) receptor on the surface of NK cells (63). This might be important since NK cells have been suggested to control the proliferation of EBV-infected B-cells in the acute phase. In the same report, it was shown that IL18 suppressed the effect of Kyn on NKG2D expression. It might be speculated that the suppression of NK cell activation by IDO-expressing EBV-infected B-cells serves as an escape strategy of the virus. Recently, it was shown that *in vitro* generated macrophages expressed IDO after infection with EBV and displayed T-cell suppressive activities (64). IDO expression induced by TNFα and IL6 signaling was mediated by NFκB and the MAP kinase signaling cascade. Both factors were able to further increase IDO expression, thereby suppressing the proliferative capacity of CD4$^+$ and CD8$^+$ T-cells as well as dampening their cytolytic activity.

Additional evidence for an important role for IDO in viral infection was provided in studies of human papilloma viruses (HPV). Mucosa-tropic HPV are tumorigenic viruses causing genital cancer, e.g., cervical cancer by inducing epithelial hyperplasia (65). Immunotherapy against the tumor is often inefficient because of the existence of a local immunosuppressive tumor milieu with impaired tumor cell antigen presentation and resistance of tumor cells against effector mechanisms of T-cells. The immunosuppressive milieu might be created during the stage of cervical intraepithelial neoplasia (CIN), since numbers of IDO, IFNγ, IL10, and FoxP3 expressing cells are elevated in CIN compared to normal cervical tissue (66). When HPV16 E7, an envelope- and oncoprotein of HPV was expressed under the keratin-14 promoter (K14E7) in a skin grafting model, graft rejection was prevented suggesting that HPV16E7 induced an immunotolerogenic environment (67). As previously shown, tolerance against E7-expressing skin grafts is based on the induction of IFNγ producing natural killer T (NKT)-cells, which not only reduce the capacity of CD11c$^+$ DC to cross-present antigens to CD8 T-cells (68) but also seem to induce IDO expression. In fact, IDO seems to have a pivotal role in K14E7 graft tolerance, since IDO inhibition by 1-MT leads to rejection of K14E7 skin grafts (67). Furthermore, it was observed that skin grafts recruit higher numbers of DC, which expressed elevated levels of IFNγ receptor (IFNγR). Especially, dermal Langerin$^+$ (CD207$^+$) DC expressed IDO and aided in the recruitment of further DC to the side of transplantation. Clearly, this murine model strongly suggests an important participation of IDO in HPV evasion from host immunity.

Overall, IDO seems to play an important role in chronic viral infections mainly by contributing to the establishment of an immunotolerogenic microenvironment. New strategies to target IDO itself or upstream mechanisms inducing IDO might help in the development of therapeutic drugs for patients with chronic viral infections.

NOVEL ASPECTS OF IDO IN MAJOR BACTERIAL INFECTIONS

ROLE OF IDO IN MYCOBACTERIA INFECTIONS

Tuberculosis (Tb), caused by *mycobacteria*, is one of the major human infectious diseases. We and others have linked IDO expression to this disease (69–72). In Tb patients elevated levels of anti-inflammatory molecules in the sputum, amongst them IDO have been detected (70). These immune suppressive mediators, also including IL10, TGFßRII, and IL1 receptor antagonist (IL1Rn), have been suggested as biomarkers for Tb. It was assumed that all these inhibitory molecules dampen Th1 responses in the lung thereby contributing to immune-escape of the bacteria. While the direct molecular mechanism of IDO induction in Tb patients is unresolved, it is clear that IFNγ and TNFα play pivotal roles in the containment of *Mycobacterium tuberculosis* (*M. tuberculosis*) in humans and in mice (**Figure 3**). Deficiency of IFNγ or TNFα expression, or the lack of the respective receptors cause severe courses of Tb in mice (73–77). From *in vitro* studies, there is evidence that TNFα might play a role during the acute and chronic phase of Tb (9, 76). Moreover, treatment of patients with anti-TNFα antibodies can lead to exacerbation of Tb and the induction of Tb sepsis (78). *In situ* studies clearly demonstrated that granuloma formation after *M. tuberculosis* infection in humans is associated with high expression of IDO in cells of the center and the ring wall structure surrounding the center of the granuloma

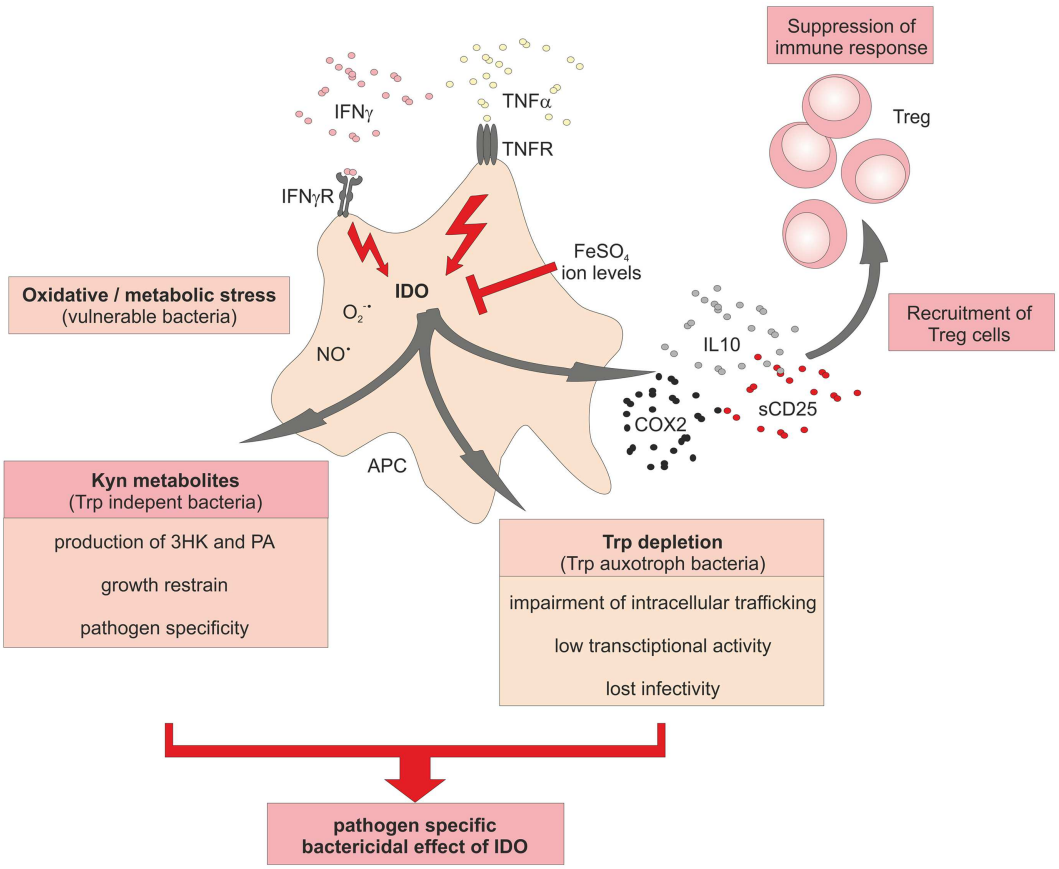

FIGURE 3 | Schematic overview of the central role of IDO in immune responses to bacterial infections. Activation of IDO activity in bacteria-infected cells induces a potent bactericidal growth restrain to fight against spreading of the infection. Besides the induction of reactive oxygen species (ROS) and nitric oxide radicals, activity of IDO aids in Trp degradation to starve Trp auxotroph bacteria. A further species-specific bactericidal effect of increased IDO activation is the production of toxic Kyn metabolites. Recruitment of Treg cells and increasing levels of free ions might help as negative feedback loop to terminate inflammatory responses.

(9, 71, 79). Predominant IDO-expressing cells were identified as $CD68^+$ macrophages and in fewer numbers as $CD11c^+$ $S100^+$ DC. Both IDO-expressing myeloid cell populations were surrounded by $CD3^+$ T-cells. The induction of a tolerogenic milieu including the recruitment of $FoxP3^+$ regulatory T-cells by IDO-expressing cells together with depletion of Trp most likely aids in the restriction of bacterial spread. IDO seemed to be absent in biopsies of *Mycobacterium leprae* (*M. leprae*) infected skin, suggesting that IDO is not necessarily involved in all granulomatous diseases. However, there is still controversy about this, since a recent report demonstrated expression of IDO in macrophages of leprotic skin lesions (80).

Further controversy about the role of IDO in Tb comes from a recent report demonstrating no essential role for IDO in a murine model of Tb (81). Wild-type as well as IDO KO mice showed comparable bacterial burden, T-cell responses, and survival rates, leading to the conclusion that IDO activity is not required for the control of *M. tuberculosis*. The authors present no evidence IDO is indeed expressed in the myeloid cell compartment, like in human disease, indicating that this mouse model might not reflect the human situation. In contrast, another report demonstrates a direct connection between IFNγ and IDO expression in non-hematopoietic cells, during the chronic phase of murine *M. tuberculosis* infection (82). In this model, IDO was expressed in endothelial and epithelial cells, and IFN receptor deficiency reduced the levels of expression of IDO in these cells thereby leading to an impaired long-term control of *M. tuberculosis* (82). However, it was also not shown to which extent IDO activity directly controls *M. tuberculosis*.

There have been numerous studies during the last 20 years demonstrating a correlation between Tb and depressive disorders in patients (**Figure 2**) (83–86), but many of the underlying molecular mechanisms remain unclear. To shed some light on the mechanism, O'Connor et al. utilized a BCG vaccination model (87). Mice that showed a depressive-like behavior also represented increased expression of IFNγ, TNFα, and IDO. When studying IFNγ-R deficient mice, many inflammatory mediators were still elevated while the expression of TNFα was attenuated and IFNγ and IDO were absent in lung or brain tissue. Furthermore, the lack of IDO activity in IFNγ-R deficient mice resulted in diminished

plasma ratios of Kyn/Trp. The authors assumed a synergistic effect of IDO and TNFα on microglia of BCG-treated mice since pretreatment of mice with a TNFα antagonist was able to attenuate TNFα expression and to abrogate depressive-like behavior. Further studies need to clarify whether TNFα is actually upstream of IDO and blockade of TNFα can reduce IDO expression and function. Downstream of IDO, BCG also increased the expression of 3-hydroxyanthranilic acid oxygenase (3-HAO), which participates in the generation of the neurotoxic Kyn metabolite QA (88). Application of 1-MT also prevented the development of depressive-like behavior after BCG inoculation in mice, suggesting that elevated IDO, like TNFα, contributes to the onset of depressive disorders during chronic inflammation caused by *mycobacteria*.

ROLE OF IDO IN *CHLAMYDIA* INFECTIONS

Indoleamine-2,3-dioxygenase was also shown to be important in the defense against the ubiquitous intracellular bacterium *Chlamydia pneumoniae* (*C. pneumoniae*), which causes respiratory tract infections and is associated with chronic diseases like asthma. In general, the life cycle of *Chlamydia* species can be divided into two phases: (1) a stage of infectious but metabolically inactive form directly after uptake of the bacteria into the host cell, and (2) a stage of differentiation and multiplication enabled by an active metabolism. It can be assumed that immune responses of host cells might differ according to the life cycle of *Chlamydia*. Njau et al. recently demonstrated that *C. pneumoniae* infection of human moDC-induced IDO expression in a TNFα-dependent manner and IDO was sufficient to restrain bacterial growth (89). TNFα-dependent bactericidal effects on *C. pneumoniae* were abrogated after supplementation of Trp. Since *C. pneumoniae* is Trp auxotroph, the authors concluded from these experiments that Trp depletion is detrimental for *C. pneumonia* growth. In *C. pneumoniae*-infected THP-1 cells, an induction of IDO, TNFα, and neopterin could be demonstrated (90). However, IDO activation in combination with increased Trp degradation as well as IFNγ treatment had no effect on numbers or growth of *C. pneumoniae* in THP-1 cells. This observation was explained by the ability of *C. pneumoniae* to survive even under conditions of low Trp concentrations, going into a latent state without proliferation and differentiation. An interesting aspect of this study was the comparison of chlamydia infection in THP-1 cells and human endothelial cells. While IDO was already induced in monocytic cells upon infection and was further elevated by IFNγ, infected and non-infected endothelial cells required IFNγ stimulation to induce IDO expression. Only after IFNγ-treatment, suppression of proliferation of *C. pneumoniae* in endothelial cells was observed. The authors also argued that the differences between the two cell types might also reflect different stages of the *C. pneumoniae* infectious cycle. The bacteria might use immune competent monocytes as transportation vessels for systemic dissemination, while endothelial cells might serve as habitats for differentiation and proliferation, especially under iron rich conditions.

An interesting link between IDO and iron metabolism has been suggested by Krausse-Opatz and colleagues (91). In a hepatic cell line infected with *Chlamydia trachomatis* (*C. trachomatis*), they could demonstrate that increased intracellular levels of ferrous iron $FeSO_4$ attenuated IFNγ-induced IDO expression leading to increased infectious yields. Similarly, the human monocytic cell line THP-1 has been shown to be sensitive to low iron concentrations, which showed an inhibitory effect on IFNγ signaling resulting in decreased Trp metabolism (92). This observation led to the hypothesis that immune cells retain iron during inflammatory diseases to enable efficient IFNγ driven immune responses. On the other hand, reduction of iron levels by deferoxamine did not reconstitute IDO activity, but still suppressed bacterial growth (90, 91). Leonhardt et al. addressed whether IDO-mediated Trp depletion is responsible for suppression of bacterial growth of *C. trachomatis* (93). They used HeLa cells, an immortalized cervical carcinoma cell line, as the model for infection. Interestingly, the lack of available Trp in IDO competent HeLa cells led to an impairment of intracellular bacteria trafficking toward the perinuclear microtubule-organizing center after entrance into the host cell. Bacteria displayed low transcriptional activity, lost their infectivity, and remained scattered in the periphery of the HeLa cells. Since most of the recent work concerning the influence of iron ions has been performed in cell line models, it remains an unanswered question, whether these observations will hold true, once primary human cells are used for analysis.

ROLE OF IDO IN COMMUNITY-ACQUIRED PNEUMONIA AND SEPSIS

Another area of great interest during the last years has been the function of IDO during community-acquired pneumonia (CAP) and sepsis. Already in 2005, increased Kyn levels in trauma patients with bacteremic sepsis, respiratory distress syndrome, or multi organ dysfunction/failure pointed toward a role for IDO in sepsis (94). In a larger cohort-study, Suzuki and colleagues investigated 129 patients and 64 healthy controls and revealed increased Kyn/Trp ratios as prognostic marker for severity and morbidity of CAP caused by multiple pathogens (95). Along these lines, several independent studies reported increased IDO activity as predictor of severity and mortality of sepsis (96–98). Elevated Kyn/Trp ratios as indicator of IDO activity in plasma of sepsis patients also correlated with elevated levels of IFNγ and IL10, which were also shown to further trigger IDO activity (96). Furthermore, these patients show reduced $CD4^+$ and $CD8^+$ T-cell counts similarly pointing to an overall impairment of immune functions during sepsis. There was also an inverse correlation between elevated Kyn/Trp ratios and NO-dependent microvascular reactivity as a surrogate marker for endothelial cell function, as mean arterial or diastolic blood pressure were reduced. Direct measurement of IDO activity in patients with sepsis or septic shock showed a gradual increase of IDO activity with severity of sepsis, which was directly associated with mortality (97). The major sources of IDO were circulating $CD14^+$ monocytes, which were increased both absolutely and relatively compared to other white blood cells during sepsis. Interestingly, *ex vivo* stimulation of monocytes from septic patients with IFNγ led to the induction of functional IDO and was found to be independent of NFκB signaling while other TLR agonists like LPS-induced IDO expression in an NFκB dependent fashion (97).

Along these lines, hints for a novel IDO-inducing mechanism were found in a murine mouse model in which the induction of IDO in context of sepsis was investigated. Mice which are deficient in serine/threonine kinase, general control non-derepressible 2 (GCN2) gene were protected from endotoxic shock (99). This

was associated with a rapid induction of IDO in spleen and an increased Kyn/Trp ratio in serum suggesting that elevated IDO levels in sepsis might actually be beneficial. However, this does not seem to be necessarily the case since IDO deficient mice also are resistant against LPS-induced septic shock (100). Similarly, inhibition of IDO activity by 1-MT during endotoxin shock *in vivo* was associated with increased survival (100). Whether these results can be translated to humans has to be proven. In this context, it is interesting to mention that application of GMCSF in sepsis patients was associated with suppression of IDO activity and reduction of free Kyn metabolites (101). Experimental proof for concomitant improvement of antibacterial defense has to be further investigated.

Overall, IDO also seems to play a role during clinically relevant infections such as CAP and sepsis. Whether IDO might become a therapeutic target in these patients is still to be investigated.

ROLE OF IDO IN *LISTERIA* INFECTIONS

During the last years, we have defined the role of IDO in human listeriosis (8, 9, 23, 69, 79, 93, 102). Listeriosis is a foodborne disease caused by oral infection with *Listeria monocytogenes* (*L. monocytogenes*). Newborn infants are prone to infections due to incompletely developed cell-mediated immunity. Early on, we could demonstrate that induction of IDO in myeloid cells after *L. monocytogenes* infection is TNFα dependent (9). Chronic listeriosis is characterized by the development of granulomatous structures encapsulating bacteria-infected cells to restrain bacterial spreading. IDO-expressing $CD68^+$ macrophages and $S100^+CD11c^+$ DC, but not T- or B-cells, are part of the ring wall structure in these granuloma. When analyzing the effect of *L. monocytogenes* infection on DC function, we observed an inhibitory effect of these DC on T-cell proliferation and cytokine expression. Furthermore, we demonstrated that the inhibitory effect is mediated by IDO and the TNFα-dependent production of suppressive molecules like IL10, COX2, and soluble CD25 (69). Initial experiments addressing the IDO-mediated inhibitory effects on T-cells suggested that IDO-mediated Trp depletion and also the production of toxic metabolites were responsible for the observed loss of T-cell proliferation and cytokine production (69, 102). More recently, we were interested to understand whether bactericidal effects exerted by IDO-expressing human myeloid cells is mainly due to Trp depletion or toxic metabolites (23). Earlier work in mice and in human cells, as well as in other bacterial infections showed conflicting results concerning the role of these two bactericidal mechanisms. Using primary human macrophages and DCs, we could show unequivocally that it is not Trp depletion but rather Kyn metabolites that exert the bactericidal effect against *L. monocytogenes*. Especially, 3-hydroxy-kynurenine (3HK) was the most potent Kyn metabolite. Moreover, this bactericidal effect was also seen for other Listeria strains including *Listeria innocua*. It is important to note that these findings only reflect IDO biology in humans, since we could also clearly show that IDO is not induced in murine DC and macrophages after infection with *L. monocytogenes*. In contrast, murine *L. monocytogenes* infected myeloid cells produced iNOS instead of IDO. One explanation of the observed species-specific differences might be a differential expression of the iNOS cofactor tetrahydrobiopterin, which is expressed by murine primary macrophages as well as murine macrophage cell lines after activation with IFNγ and LPS (103, 104). In fact, murine IFNγ stimulated macrophages showed a strong correlation between tetrahydrobiopterin and NO levels (104). In contrast, tetrahydrobiopterin was shown to be expressed only at very low levels in human monocytes and macrophages (104, 105). A further explanation for this divergent usage of IDO and NO between mice and men came from a study, which revealed an inhibitory effect of NO on IDO in primary IFNγ-activated human peripheral mononuclear cells and macrophages (106). An interaction of NO with the heme iron at the active site of IDO preventing the conversion of ferric iron, necessary for IDO activity, has been proposed to be responsible for the inhibitory effect of NO on IDO (107). IDO inhibition might also rely on rapid removal of oxygen radicals by NO, which are required for IDO activity (108, 109). Such a clear-cut species-specific difference in usage of effector molecules as well as diverging susceptibilities against pathogens between mice and men are not too surprising since both species have evolved in different habitats, being exposed to completely different sets of pathogens. These observations further support the notion that one needs to be very careful when studying murine IDO in infection models and translating gained knowledge back to human diseases such as listeriosis or Tb.

SUMMARY/CONCLUDING REMARKS

Recent findings have clearly revealed that elevated IDO expression is a hallmark of major human viral infections including HIV, HBV, HCV, or influenza, and also major bacterial infections such as Tb, CAP, listeriosis, or sepsis. Besides Trp depletion, production of ROS or the modulation of Trp metabolism by iron ions via IFNγ have to be considered as part of a complex network participating in the fight against pathogens. An elevated Kyn/Trp ratio as a surrogate for IDO activity seems to be a hallmark for these infectious diseases and as demonstrated for sepsis patients seem to correlate with the severity of disease. So far, the major role of elevated IDO activity has not been definitely established. Both anti-pathogen and immunosuppressive mechanisms have been suggested. In this scenario, IDO activity might be a dual sword, fighting the pathogen directly by a metabolic mechanism, while keeping an overwhelming immune response in check. Whether this delicate balance between pathogen defense and host protection from lateral damage induced by IDO could be utilized therapeutically to further optimize the host response during infection remains to be seen. So far, it is not clear whether a further increase of IDO activity would indeed be beneficial. It might enhance the anti-pathogenic effect, but further elevating immunosuppression might actually be detrimental to the host during infection.

In fact, data from septic patients as well as studies from murine sepsis models utilizing LPS to induce septic shock would actually speak against enhancing IDO activity. On the contrary, in these models, depletion of IDO function is actually beneficial to the host. As exemplified above, interpreting the role of IDO for human Tb might be very misleading when based on murine models that do not reflect human disease properly. Moreover, we have clearly established that IDO is differentially regulated in murine and human myeloid cells in response to infection

with *L. monocytogenes* and probably also other viral and bacterial infections. Particularly when studying the myeloid compartment, we strongly recommend to study regulation and function of IDO first in humans. If IDO is involved, the findings should be translated into the murine model. However, if there is no evidence for IDO regulation in the same cellular compartment in the murine infection model, then it will be necessary to either use other more informative animal models or to apply other approaches in the human setting. For future research, it will be extremely important to develop structured datasets that can be quickly interrogated to understand whether gene function observed in human disease is also apparent in major animal models, particularly the murine system. So far, such data are missing.

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Aryl hydrocarbon receptor and kynurenine: recent advances in autoimmune disease research

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Aryl hydrocarbon receptor (AHR) is thought to be a crucial factor in the regulation of immune responses. Many AHR-mediated immunoregulatory mechanisms have been discovered, and this knowledge may enhance our understanding of the molecular pathogenesis of autoimmune inflammatory syndromes such as collagen-induced arthritis, experimental autoimmune encephalomyelitis, and experimental colitis. Recent findings have elucidated the critical link between AHR and indoleamine 2,3-dioxygenase (IDO) in the development of regulatory T cells and Th17 cells, which are key factors in a variety of human autoimmune diseases. Induction of IDO and IDO-mediated tryptophan catabolism, together with its downstream products such as kynurenine, is an important immunoregulatory mechanism underlying immunosuppression, tolerance, and immunity. Recent studies revealed that induction of IDO depends on AHR expression. This review summarizes the most current findings regarding the functions of AHR and IDO in immune cells as they relate to the pathogenesis of autoimmune diseases in response to various stimuli. We also discuss the potential link between AHR and IDO/tryptophan metabolites, and the involvement of several novel related factors (such as microRNA) in the development of autoimmune diseases. These novel factors represent potential therapeutic targets for the treatment of autoimmune disorders.

Keywords: dioxin receptor, indoleamine 2,3-dioxygenase, transcription factor, autoimmunity, immune regulation

ROLES OF AHR IN THE IMMUNE SYSTEM

Aryl hydrocarbon receptor (AHR) is a ligand-activated member of the Per–Arnt–Sim (PAS) family of basic helix–loop–helix (HLH) transcription factors. AHR mediates cellular responses to toxins or its ligands, including TCDD, 6-formylindolo[3,2-b]carbazole (FICZ), kynurenine, and 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) (1–4). AHR forms an active complex in the cytoplasm with chaperone proteins such as heat shock protein 90 (HSP90), AHR-interacting protein (AIP), and p23 (5–7). Once bound to its ligands, the AHR complex translocates to the nucleus and binds AHR nuclear translocator (Arnt). The resultant AHR–Arnt heterodimers bind specific motifs, called dioxin-responsive elements (DREs), in the promoter region of target genes. These targets, the so-called AHR battery genes, include *CYP1A1*, *CYP1A2*, *CYP1B1*, and other members of cytochrome P450 family (8–11). Several pathways are involved in the regulation of AHR, including proteasomal degradation of AHR, ligand metabolism by CYP1A1, and formation of the AHR–Arnt complex (12, 13). One of these pathways involves an inhibitory peptide. Mimura et al. isolated a cDNA clone that encode a polypeptide with high similarity to the sequence of the bHLH/PAS of AHR (14). This polypeptide can repress the transcriptional activity of AHR by competing with AHR for binding to Arnt and by binding to the enhancer sequence XRE, upstream of the AHRR gene; therefore, this peptide is designated AHR repressor or AHRR. Expression of AHRR is induced by the AHR/Arnt heterodimer through binding to XRE, resulting in feedback inhibition of AHR. In addition, several transcription factors can interact and regulate AHR signaling; these include STAT-1, STAT3, STAT5, Pai-2, Sp1, c-maf, and Bach2 in certain cell types (15–24). AHR is activated in many immune cell types, including T cells, B cells, NK cells, macrophages, and dendritic cells (DCs), as well as in epithelial cells, Langerhans cells, innate lymphoid cells, intraepithelial lymphocytes, and microglia (15, 16, 20, 21, 25–40). Depending on the presence of specific ligands, AHR activation may suppress or exacerbate experimental autoimmune diseases. For examples, TCDD and ITE can suppress experimental autoimmune encephalomyelitis (EAE), a model of multiple sclerosis (MS), whereas FICZ exacerbates disease development (17, 41–43). Differences in the stability and structure of these ligands, as well as their affinity for AHR, should be taken into account when considering their mode of action in the activation of AHR. In addition, AHR seems to be regulated by unraveled factors such as transcription factors, tryptophan metabolites, feedback regulation of the cytokine network, and microRNA (miR). Below, we will discuss in detail the factors that may interact with AHR to modulate immune responses.

IDO AND TRYPTOPHAN METABOLITES

Indoleamine 2,3-dioxygenase (IDO) is the rate-limiting enzyme in extrahepatic catabolism of the essential amino acid tryptophan

via the kynurenine pathway (44). IDO is constitutively expressed in placenta, but is also highly expressed in epididymis, gut, lymph nodes, spleen, thymus, and lung (45). IDO converts tryptophan into kynurenine and other downstream metabolites, some with neuroactive properties, such as kynurenic acid, 3-hydroxykynurenine, quinolinic acid (QUIN), and serotonin. Deficiency or overexpression of IDO can result in changes in the levels of these neuroactive IDO-mediated metabolites, ultimately leading to neuronal disorders. For instance, elevated levels of QUIN, a potently neurotoxic *N*-methyl-*D*-aspartate (NMDA) receptor agonist, can cause not only neurodegenerative conditions such as Huntington's disease (46) and Parkinson's disease (47), but also infections of the central nervous system and psychiatric diseases such as depression (48). On the other hand, in pregnant mice, IDO may play a role in preventing the rejection of allogeneic fetuses (49). In addition, blocking IDO activity with the competitive inhibitor 1-methyl tryptophan (1-MT) selectively disrupts the maintenance of pregnancy in mice (49, 50). Consistent with the observations that IDO induces tolerance in allogeneic fetuses, IDO expression, especially in DCs, suppresses the T cell response (49, 51, 52). Various mediators can induce IDO expression and activity, including IFN-γ, TNF-α, IL-1β, and IL-6 (53–58). Recent work showed that the induction of IDO depends on AHR (31, 59, 60). Furthermore, kynurenine has been identified as a potent AHR agonist (2, 61, 62).

Immunoregulatory IDO activity and tryptophan metabolites participate in the regulation of many cell types, including T cells, DCs, monocytes, macrophages, and microglia, that play specific roles in immune responses and regulate the development of immune-mediated inflammatory diseases (62–66). Alberati-Giani et al. showed that IFN-γ induced the expression of IDO activity in immortalized murine macrophages (MT2) and microglial (N11) cells. IFN-γ-treated MT2 cells, but not N11 cells, were able to produce detectable amounts of radiolabeled 3-hydroxyanthranilic and QUIN from L-[5-^{3}H] tryptophan. In addition, Heyes et al. demonstrated that increased activities of IDO, kynurenine-3-hydroxylase, and kynureninase in infiltrating primary macrophages may accelerate the synthesis of QUIN, L-kynurenine, and kynurenic acid in conditions of brain inflammation (48). Taken together, these findings suggested that infiltrating macrophages may contribute high amount of cerebral QUIN in brain inflammation (48, 67). Besides, IDO has been demonstrated to be constitutively expressed in DCs (68). Particularly, CD8α^{+}DC exhibited high functional activity of IDO while CD8^{-} fraction of DC did not exhibit significant enzyme activity (68). Moreover, it has been shown that the expression of IDO makes CD8^{+}DC treated with IFN-γ capable of affecting apoptosis of T helper type 1 (Th1) cells (68, 69). Fallarino et al. also found that tryptophan metabolites such as 3-hydroxyanthranilic and QUIN induce the selective apoptosis *in vitro* of murine Th1 cells at relatively low concentrations of kynurenines (70). Together, IDO and kynurenines metabolites are able to be induced in various cell types under different stimuli regulating many immune responses. Modulation of IDO activity and/or kynurenine pathway may develop therapeutics for inflammatory diseases. In addition to IDO, tryptophan 2,3-dioxygenase (TDO) originated from liver and neuron, is also an important rate-limiting enzyme in the tryptophan metabolism. TDO plays a pivotal role in tumor development and various diseases in the brain (61, 71–73).

The transcriptional regulation of IDO is mediated by two main pathways, IFN-γ-dependent and -independent, which involve transcription factors such as NF-κB, STAT-1, and IRF-1 (74). We will discuss the translational regulation of IDO by AHR in the following section.

TRANSLATIONAL REGULATION OF IDO

Although the regulatory roles of IDO in tryptophan metabolism in immune regulation have been extensively studied, the mechanisms by which IDO is controlled at the pre-, co-, and post-translational levels are poorly understood. Fujigaki et al. demonstrated that IDO activity is regulated by post-translational modification: specifically, IDO activity is inhibited by peroxynitrite due to the nitration of tyrosine resides (Tyr15, Tyr345, and Tyr353) (75). The same group also demonstrated that an N-terminal alanine of IDO is acetylated in IFN-γ-stimulated THP-1 cells, but the biological significance of this modification has not been fully investigated (76, 77). In addition, another group found that Tyr115 and Tyr253 in mouse IDO can be phosphorylated, and these phosphorylations are required for formation of the IDO/SOCS3 complex (78). This complex is ubiquitinated and subsequent proteasomally degraded in DCs. These studies indicate that post-translational modifications such as nitration and phosphorylation of tyrosine in IDO may affect IDO/kynurenine-mediated immune regulation. Recently, we showed that the miR-132/212 cluster participates in AHR-dependent generation of Th17 cells (79). miRs, 20–22-nucleotide non-coding RNAs, are a new class of regulators of gene expression at the posttranscriptional level. miRs binding to the 3′UTR of target mRNAs, leading to translational inhibition and/or degradation of the targets (80). Although numerous immunoregulatory genes are controlled by miRs, to date no miRs targeting IDO have been identified. According to a widely used computational miR target prediction tool, microRNA.org, several miRs potentially regulate IDO mRNA (http://www.microrna.org/microrna/home.do). For example, miR-203 has a putative binding site in the 3′UTR of IDO in mouse. Interestingly, this miR is induced by AHR ligands such as TCDD and BaP, and it negatively affects AHR expression in human cancer cell lines (81). In addition, it has been reported that miR-203 in macrophage RAW264.7 cells negatively regulates LPS-induced IL-6 and TNF-α by targeting MyD88 (82). Considering that AHR inhibits pro-inflammatory cytokines production such as IL-6 and TNF-α in LPS-stimulated macrophages as similarly to miR-203, miR-203 may also be involved in AHR-mediated regulation of inflammatory responses in macrophages. Although experimental verification is necessary in order to confirm this possible relationship between miR and IDO mRNA, identification of miR-mediated regulation of the IDO pathway may shed light on novel mechanisms of immune regulation.

AHR/IDO AXIS IN PATHOGENESIS IN AUTOIMMUNE DISEASE

For several decades, AHR has been studied as an important transcription factor involved in regulation of a large superfamily of genes encoding cytochrome p450 proteins, which are xenobiotic metabolizing enzymes. Two independent groups demonstrated that AHR controls the generation of regulatory T (Treg) cells

and/or IL-17-producing T helper (Th17) cells in EAE, a mouse model of MS (17, 41). Because the balance between Treg and Th17 cells is now considered to be more important than the Th1/Th2 balance in regard to the onset of autoimmunity, AHR has attracted increased attention in the context of immunology. To investigate the pathophysiological roles of AHR in autoimmunity, several groups have studied mouse models of autoimmune disease, such as colitis and rheumatoid arthritis, using AHR-KO mice and/or AHR ligands (83–85). Meanwhile, the roles of AHR in various immune cells such as DCs and macrophages have also been investigated (16, 25, 60). In DCs, AHR positively regulates IDO expression and subsequent kynurenine production (60). In addition, Kimura et al. observed reduced phosphorylation of STAT-1 in AHR-deficient macrophages; by contrast, AHR acts as a negative regulator of STAT-1 activation in T cells under Th17-polarizing conditions (15, 16). Thus, the effect of AHR on STAT-1 status is complex, and may be cell type- or stimulus-dependent. Given that expression of IDO is predominantly controlled by the IFN-γ/STAT-1 axis, AHR may positively control STAT-1 activation and subsequent IDO expression in DCs. More importantly, kynurenine is an agonist of AHR, and may participate in a positive-feedback loop in AHR signaling. In both plasma and CSF from MS patients, tryptophan levels were significantly reduced (86). In addition, IFN-β treatment, a first-line immunomodulatory treatment for MS, causes elevation of IDO mRNA and plasma or serum kynurenine (87, 88). As well as the observation in MS patients, EAE induction leads to alteration of the ratio of kynurenine and tryptophan or IDO expression in brain and spinal cord (89, 90). On the other hand, in spite of evidence that IDO is induced by IFN-γ, IDO expression is negatively correlated with IFN-γ mRNA levels in brain and spinal cord. This observation suggests that IDO activity inhibits the generation of IFN-γ-producing Th1 cells, a primary subset of pathogenic T cells (89). Consistent with these findings, deletion of IDO in mice and treatment with the IDO inhibitor 1-MT result in exacerbated disease symptoms in association with elevated levels of Th1 cells (89–91). We previously showed that LPS- or CpG-stimulated AHR-deficient BMDCs co-cultured with naïve T cells contain a smaller proportion of Treg cells and elevated levels of Th1 and Th17 (60). Furthermore, the aberrant generation of each Th-cell subset can be rescued by kynurenine. These observations indicate that kynurenine generates a tolerogenic condition by controlling not only Th1, but also Th17 and Treg cells. In addition, it has been reported that AHR participates in type 1 regulatory T (Tr1) cell generation *in vitro* and *in vivo* (18, 92, 93). Thus, we cannot exclude the possibility that not only TCDD and FICZ, but also kynurenine regulates Tr1 cell induction. Taken together, further investigation of the roles of kynurenine in T cell differentiation may reveal potential therapeutic strategies for MS. Thus, further investigation of the roles of kynurenine in T cell differentiation may reveal potential therapeutic strategies for MS. Immune regulation through the AHR/IDO axis is summarized in **Figure 1**.

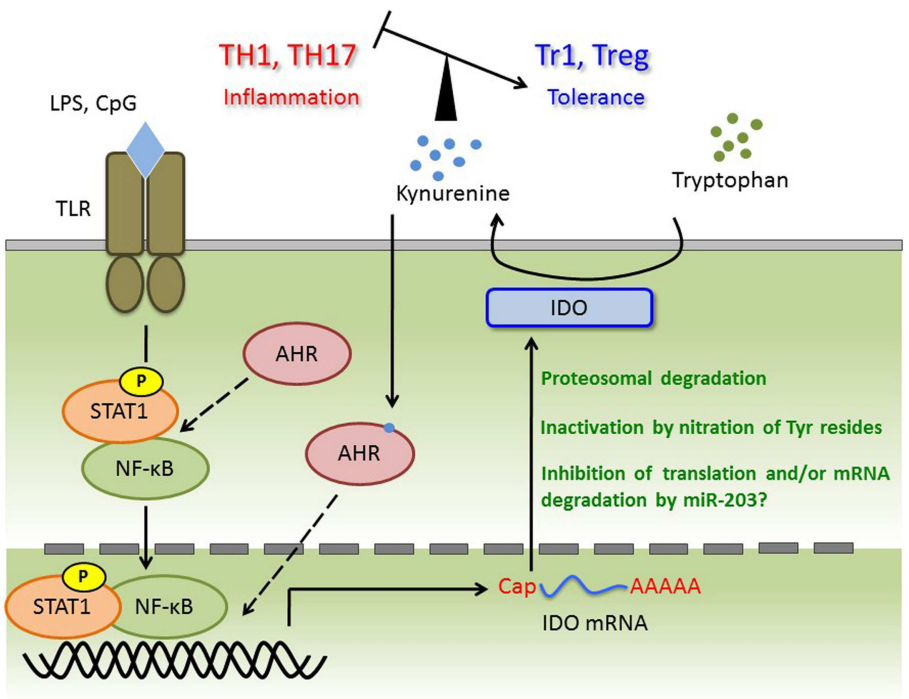

FIGURE 1 | TLR ligands trigger transcriptional activation of STAT-1 and NF-κB, and then induce IDO mRNA. Although AHR forms complex with STAT-1 and NF-κB in macrophages under pro-inflammatory cytokines production, whether this complex is appeared in DC or required for IDO expression is not known. Induced IDO mRNA may be controlled by miR-203 (not investigated), and the activity or amount of IDO protein is regulated at post-translational modification such as nitration of Tyr and ubiquitin ligation. Kynurenine catalyzed by IDO induces tolerance via regulating the balance of TH1, TH17, Tr1, and Treg. Kynurenine may activate the AHR for IDO induction with autocrine manner, and form AHR/Kynurenine positive-feedback loop.

PERSPECTIVES OF AHR/IDO AXIS: FROM BENCH TO BEDSIDE

Aryl hydrocarbon receptor and tryptophan metabolites participate in experimental models of autoimmune diseases (4, 17, 26, 41, 42, 61, 85, 94–96). However, the possible role of AHR in dioxin-exposed people is still unknown, particularly in autoimmune disorders. Therefore, it will be interesting to examine the expression of AHR and AHR-related genes in dioxin-affected people with the aim of identifying the potential link between AHR induction and dioxin-related autoimmune diseases. Future investigations should focus on determining whether activation of AHR leads to stimulation of IDO expression, and consequently promotes production of tryptophan metabolites such as kynurenine, which potentially mediate the neurological disorders and/or autoimmune diseases in dioxin-exposed people. Promising therapeutics based on intervention in the AHR/IDO axis may help to improve the health outcomes of dioxin exposure.

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AhR-mediated, non-genomic modulation of IDO1 function

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The evolutionary process has conferred a dual – enzymatic and signaling – function on the ancestral metabolic enzyme indoleamine 2,3-dioxygenase 1 (IDO1), which has long been known for converting the essential amino acid tryptophan (TRP) into neuroactive and immunoactive catabolites (kynurenines). In addition to TRP catabolic activity, phosphorylated immunoreceptor tyrosine-based inhibitory motifs, present in the IDO1 protein, act as docking sites for different molecular partners, which activate positive (transcriptional) or negative (post-translational) modulation of IDO1 protein. The ligand-operated transcription factor aryl hydrocarbon receptor (AhR) contributes to *Ido1* transcription, and it can be operated by both exogenous and endogenous ligands, including L-kynurenine itself, the first byproduct of TRP catabolism. Ligand-bound AhR is also a component of a ubiquitin ligase complex responsible for regulatory proteolysis of different target proteins. Because IDO1 half-life is controlled by the ubiquitin–proteasome system, we here discuss the possibility that AhR, in addition to enhancing *Ido1* transcription, contributes to IDO1 regulation by a non-genomic mechanism affecting the protein's half-life.

Keywords: IDO1, AhR, ITIM, ubiquitin ligase complex, tryptophan metabolism, L-kynurenine, immunoregulation, dendritic cells

INTRODUCTION

Fine tuning of immune reactivity is guaranteed by the recruitment of enzymes with disparate and pleiotropic functions. Indoleamine 2,3-dioxygenase 1 (IDO1) was first recognized as an immune regulator in pregnancy (1), and subsequently, in numerous experimental and clinical settings, including autoimmune diseases, chronic inflammation, transplantation, and neoplasia (2). IDO1 catalyzes degradation of the essential amino acid tryptophan (TRP) along a pathway that causes TRP starvation and yields several biologically active catabolites, collectively known as kynurenines. Similar to other metabolic enzymes, IDO1 is endowed with a second ("moonlighting") function, which makes it a signal-transducing molecule owing to the presence of two immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (3, 4). When phosphorylated, those motifs act as docking sites for distinct molecular partners, which can either prolong IDO1's half-life – thus sustaining immunoregulatory effects – or shorten its half-life, so favoring inflammatory responses (5).

The aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that mediates dioxin toxicity (6), is of vital importance in the regulation of immune responses. Unbound AhR is sequestered in the cytosol by the Hsp90/XAP2 chaperon complex (7–10). Ligand binding to AhR induces conformational changes that promote nuclear translocation of the receptor. In association with the AhR nuclear translocator (Arnt), AhR modulates the transcription of target genes through AhR-responsive elements (AHREs) (7, 8, 10). A wide array of distinct exogenous and endogenous ligands bind AhR, including indole-containing molecules and TRP metabolites such as L-kynurenine (L-kyn), the upstream metabolite generated via TRP degradation (11, 12). The immunoregulatory effects mediated by AhR have been known to mostly involve its genomic activity, contributing to *Ido1* transcription (13). However, AhR is also involved in a non-genomic pathway, being a component of an atypical ubiquitin ligase complex, which regulates the proteasomal degradation of target proteins (14–16).

REGULATORY PROTEOLYSIS OF IDO1 ENZYME

Intracellular proteins are continually "turning over" as they become hydrolyzed to their constituent amino acids and replaced by new synthesis, which absolves to several important homeostatic functions. Cells contain multiple proteolytic systems to carry out the degradation process and complex regulatory mechanisms, to ensure that the continual proteolytic processes are highly selective. In all tissues, the ubiquitin–proteasome system presides over the degradation of the majority of intracellular proteins (17). In dendritic cells (DCs), under inflammatory conditions, IDO1 itself undergoes proteasomal degradation by associating with suppressor of cytokine signaling 3 (SOCS3) through tyrosine phosphorylated ITIMs present in an IDO1 domain distinct from that mediating its enzymatic function (3).

The crystal structure of the enzyme has indeed been solved in its catalytically inactive conformation (18), unveiling the presence of two folding domains, namely, a large domain and a small domain. The former contains the heme-binding site, forming the catalytic cleft of the enzyme, while the latter contains two highly conserved ITIMs, which, once tyrosine phosphorylated, can act as docking sites for the association with different molecule partners (5). Remarkably, the inspection of the crystal structure of the enzyme (pdb code: 2D0T, 2D0U) shows that the phosphorylable ITIM tyrosines are unexposed to the solvent, and thus, poorly compatible with the experimentally observed interaction with SOCS3.

Multiple conformational states of enzymes have been reported to play a role in molecular recognition, catalysis, and allosteric modulation (19–21). Compliant to this paradigm, conformational fluctuations of the large and small domains of IDO1 may exist and be triggered by ligand binding and/or by post-translational modifications. Hence, the aforementioned tyrosine phosphorylation of IDO1's ITIMs may promote a specific conformational state of IDO1, amenable the interaction with SOCS3.

Suppressor of cytokine signaling 3 represents the first IDO1 partner identified so far, capable of binding the ITIM docking sites in the enzyme. As a member of the SOCS protein family, it acts as a feedback inhibitor, blocking JAK/STAT signaling in response to pro-inflammatory cytokines, such as IL-6. SOCS3 possesses a Src homology 2 (SH2) domain, binding phosphotyrosine-containing peptides, and a SOCS box, which recruits an E3 ubiquitin ligase complex and targets several signaling proteins, disparate in nature, for ubiquitination and proteasomal degradation (22, 23). We have previously provided evidence that the SH2 domain in SOCS3, by anchoring phosphorylated IDO1's ITIMs, brings the enzyme close to the E3 ubiquitin complex that ubiquitinates and targets IDO1 for proteasomal degradation. This mechanism provides an explanation for the observed, inverse relationship between SOCS3 and IDO1 expression in DCs. In fact, in DCs lacking SOCS3, the immunoadjuvant effect of the immunogenic fusion protein CD28-Ig is lost, and the latter behaves much like cytotoxic T lymphocyte-associated antigen 4 (CTLA4)-Ig, which is immune suppressive in nature (24). This has been traced to CD28-Ig's unique ability to trigger IL-6 and SOCS3 activities, a property unshared by CTLA4-Ig. IL-6-induced SOCS3 is indeed responsible for degrading the protein product of *Ido1*, whose transcriptional activation is mediated by IFN-γ (25). Mutations of the phosphorylable tyrosines in IDO1's ITIM domains completely abolish the ability of the enzyme to bind SOCS3, thus preventing its targeting for proteasomal degradation (3, 26).

Therefore, the IDO1/SOCS3 association in DCs represents a molecular mechanism whereby IDO1-positive ($IDO1^+$) DCs, expressing a tolerogenic phenotype, can turn into immunostimulatory antigen presenting cells (APCs), according to environmental needs (3). According to the variety of pathophysiologic contexts that DCs must face, proteasomal degradation of IDO1 could represent a non-genomic mechanism of modulation of the enzyme in order to promptly turn IDO^+ DCs into immunogenic IDO-negative DCs under conditions in the local microenvironment that require activation of the immune response. Regulatory proteolysis of IDO1 by the ubiquitin–proteasome system may be a more common event than previously appreciated, and it may involve other pathway besides that of IL-6-induced SOCS3 activation.

LIGAND-DEPENDENT E3 UBIQUITIN LIGASE ACTIVITY OF AhR

Although AhR has traditionally been defined as a transcription factor involved in adaptive xenobiotic and in environmental pollutant responses – including polycyclic and halogenated aromatic hydrocarbons, such as 2,3,7,8-tetrachorodibenzo-*p*-dioxin (TCDD, "dioxin") – the direct transcriptional activity of AhR alone does not fully explain its toxicological and physiological effects. Accumulating evidence suggests that AhR exhibits its regulatory functions by "cross-talking" with a variety of signaling pathways, including estrogen (ER) and androgen (AR) receptors (27, 28). In addition to its genomic activity, AhR is also capable of mediating non-genomic effects, by assembling an atypical E3 ubiquitin ligase complex, $CUL4B^{AhR}$ that includes cullin 4B (CUL4B) (14, 28). E3 ubiquitin ligases act in the last step of a sequential reaction, also involving E1 and E2 ligases, and culminating in the ubiquitination of protein substrates.

Similar to the aforementioned ubiquitin ligase activity, mediated by SOCS3 with IDO1 being the target protein, the ubiquitin ligase activity of $CUL4B^{AhR}$ complex has been reported to target several types of protein for proteolysis. Besides the transcriptional regulation of ERs and ARs, ligand-operating AhR has recently been shown to promote proteasomal degradation of the very same receptors, by assembling the $CUL4B^{AhR}$ E3 ubiquitin ligase complex (15). Similarly, ligand-based assembly of the $CUL4B^{AhR}$ E3 ligase complex has been found to promote ubiquitination of β-catenin (16), a transcription factor downstream from the Wnt signaling pathway, leading to proteasomal degradation of β-catenin in colon tumor cell lines. Interestingly, AhR-deficient mice frequently develop colon tumor with abnormal accumulation of β-catenin protein. Inversely, administration of AhR ligands efficiently suppressed colon cancer in an established mouse model of familial adenomatous polyposis. These findings suggest that AhR ligands can be used to successfully prevent intestinal tumors, where increased stabilization and accumulation of β-catenin may be responsible for the initiation of intestinal carcinogenesis. Notably, the substrates of $CUL4B^{AhR}$ ubiquitin ligase complex – ER, AR, and β-catenin – all promote cellular proliferation in their target tissues, suggesting that one putative biological role of the ubiquitin ligase function of AhR could be the antiproliferative activity through degradation of those transcription factors, promoting cell proliferation. This raises the possibility of developing selective AhR ligands in cancer therapy, by promoting ubiquitin ligase function.

Besides a direct role of AhR in assembling an ubiquitin ligase complex, there is evidence that AhR may indirectly promote ubiquitination of target proteins. One such recent mechanism has been described for SOCS2-induced proteasomal degradation of tumor necrosis factor receptor-associated factor (TRAF)6 in a model of *Toxoplasma gondii* infection. In this study, both L-kyn and the lipoxin LXA_4 were found to induce SOCS2-dependent ubiquitination and proteasomal degradation of TRAF6, hindering pro-inflammatory cytokine expression by DCs. In this case, the mechanism was mediated by the transcriptional activity of AhR, leading to SOCS2 expression that, in turn, promoted TRAF6 polyubiquitination and proteasomal degradation of the adapter proteins (29).

Taken together, the results provide compelling evidence of a prototypic indirect mechanism by which AhR, through AHRE promoter-carrying genes (which include *Socs2* and, interestingly, *Socs3*) can mediate the proteasomal degradation of target proteins. Notably, L-kyn is a TRP metabolite generated by IDO1 in tolerogenic DCs (30), and it acts as endogenous ligand of AhR in promoting IDO1 phosphorylation, leading to TGF-β production (13). Moreover, the interaction of L-kyn with AhR can generate regulatory T cells (12). Overall, these observations shed light on the crucial relationship between TRP metabolism and both the genomic and non-genomic activities of AhR in modulating immune responses.

PERSPECTIVE ON AhR-MEDIATED, NON-GENOMIC MODULATION OF IDO1

Several experimental models dissected the tight relationship between TRP metabolism and the activity of AhR in modulating the immune response. It is well established that L-kyn, the upstream metabolite generated by TRP-degrading enzymes, acts as an endogenous AhR ligand, leading to generation of regulatory T cells, and participating in immune homeostasis (12, 31). The IDO1–AhR axis has been described in several settings of immune tolerance, including maternal–fetal tolerance (32), immune suppression induced by several human cancers (33), and endotoxin tolerance (13). Therefore, the molecular dissection of the mechanisms that sustain the immunoregulatory IDO1–AhR axis has become a compelling need.

As a transcription factor, AhR promotes IDO1 expression in response to structurally disparate ligands such as L-kyn – in conventional DCs in a model of endotoxin tolerance (13) – and TCDD, during maturation of bone marrow-derived DCs (34), making *Ido1* as an AhR-responsive gene. Moreover, an autocrine signaling loop involving IL-6, STAT3, and AhR was found to sustain the constitutive expression of IDO1 in human cancer cells (33). The genomic modulation of IDO1 by ligand-operating AhR also involves non-canonical mechanisms mediated by kinase activity. TCDD-activated AhR was independently reported to initiate a rapid non-genomic signaling cascade, culminating in the activation of Src and Erk kinases (35–37). Recently, L-kyn–bound AhR was found to promote IDO1 phosphorylation, through Src kinase-mediated activity, which activates the signaling function of IDO1, leading to the *de novo* synthesis of the enzyme via TGF-β production (4, 13). Interestingly, in a model of murine vulvovaginal candidiasis an increased expression of AhR was observed in the vagina of both naïve and infected IDO1-deficient mice, suggesting a further mechanism of mutual transcriptional regulation between IDO1, the source of L-kyn, and its sensor AhR (38).

Besides its transcriptional activity, the non-genomic modality of action of AhR could represent a further mechanism whereby TRP metabolism and AhR cross their pathways. Analogous to sex hormone receptors, regulated by AhR in both transcriptionally and non-genomically fashions, IDO1 could represent another substrate for the ubiquitin ligase activity of $CUL4B^{AhR}$.

Proteasome-mediated degradation of IDO1 has been described in DCs under IL-6-driven pro-inflammatory condition (3). The ubiquitination of the enzyme is mediated by SOCS3 protein that signals the enzyme to the proteasome. The mechanism is particularly active in inflammatory DCs, where SOCS3 is highly expressed and the cells are not required to manifest an immunoregulatory phenotype. Regulatory proteolysis of IDO1 via the ubiquitin–proteasome system may represent a non-genomic means of switching off the enzyme. Similar to SOCS3, the $CUL4B^{AhR}$ complex could promote the ubiquitination of IDO1, targeting it for proteasomal degradation. Physiologically, the non-genomic modulation of IDO1 by ligand-bound AhR could be taken as a typical negative feedback loop of enzyme regulation, where the same trigger (liganded AhR) of its transcriptional expression can also act as a quencher of the protein function, by promoting proteasomal degradation. Such a mechanism would contribute to a fine modulation of IDO1-based immunoregulatory response.

The hypothetical view of AhR-driven proteasomal degradation of IDO1 is based on several observations that include the finding that the enzyme is a proteasome substrate (3). Interestingly, both IL-6 and SOCS3 (the trigger of the ubiquitination of IDO1) were independently reported to be induced by activated AhR (33, 39). In addition, SOCS2 – a member of the SOCS family in which SOCS3 likewise belongs – is induced by L-kyn–activated AhR, and it promotes the ubiquitination of the adapter protein TRAF6 (29). Overall, these findings suggest that activated AhR, through its transcriptional activity, is capable of inducing all of the components (i.e., IL-6, SOCS3, and IDO1) of a putative feedback loop, promoting intracellular conditions ensuring IDO1 ubiquitination and proteasomal degradation. In addition, post-translational modifications of substrates, such as phosphorylation, typically serve for recruiting E3 ubiquitin ligases. In fact, tyrosine phosphorylation of the two ITIM motifs in the small domain of IDO1 is needed for anchoring SOCS3 protein that bridges E3 ubiquitin ligase (3). Interestingly, IDO1 phosphorylation may be promoted by AhR activity in conventional DCs in a model of endotoxin tolerance (13).

In this hypothetical scenario, AhR would play the canonical role of transcription factor, capable of inducing the stimuli (IL-6 and SOCS3) responsible for ubiquitination of IDO1 enzyme, and would promote – through a non-canonical pathway – the phosphorylation of IDO1 required for anchoring SOCS3. It is also likely that the ubiquitin ligase activity ascribed to AhR might directly act on IDO1 as a substrate, bypassing the "bridging" function mediated by SOCS3 (**Figure 1**). If so, the direct molecular association of $CUL4B^{AhR}$ with IDO1 enzyme should be characterized. All of the previously described complexes of $CUL4B^{AhR}$ with both ER/AR and β-catenin are localized into the nucleus, as involving the association of ligand-bound AhR with the specific nuclear translocator Arnt. A major question relates to the subcellular location where $CUL4B^{AhR}$ would bind the substrate IDO1. There is no evidence of a nuclear localization of IDO1 enzyme or of a cytosolic localization of $CUL4B^{AhR}$. Thus, one should postulate that AhR assembles an ubiquitin ligase complex that involves different molecular partners, not necessarily requiring the nuclear translocation. In this regard, the C-terminus of Hsp70-interacting protein (CHIP), a quality-controlling ubiquitin ligase, reportedly promotes degradation of AhR. Since both CHIP and unliganded AhR are mainly located in the cytosol, the degradation of AhR through CHIP is likely to occur in the cytosol (40).

A second question relates to the nature of the AhR ligand that could target IDO1 as a substrate for ubiquitination. In view of a fine cross-talk between the TRP metabolism and AhR activity, L-kyn and the downstream TRP metabolites could be good candidates for playing this role in a negative feedback loop, aimed at controlling IDO1 enzymatic activity. As it holds true of ER/AR signaling, in which AhR appears to modulate the receptors both positively and negatively, although L-kyn has been shown to transcriptionally induce IDO1 expression via AhR, later in an inflammatory context, the same molecules generated by IDO1 enzymatic activity could also promote IDO1 ubiquitination and proteasomal degradation.

Thus, L-kyn and its derivatives along the kynurenine pathway, exploiting the genomic and non-genomic modality of action of

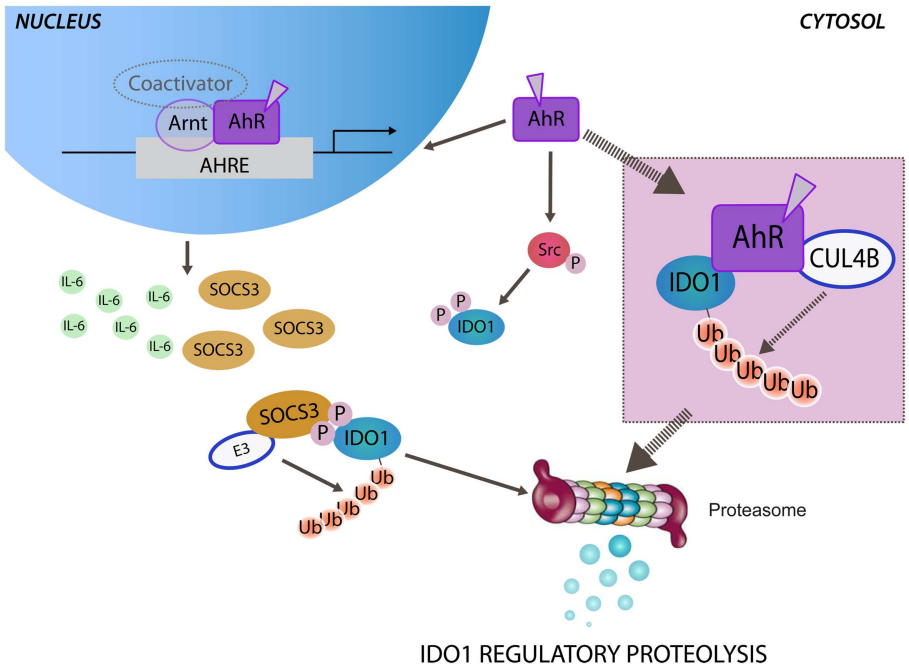

FIGURE 1 | Aryl hydrocarbon receptor-mediated regulatory proteolysis of IDO1. Ligand-operating aryl hydrocarbon receptor (AhR) promotes both genomic and non-genomic activity. After nuclear translocation, ligand-bound AhR dimerizes with the AhR nuclear translocator (Arnt) and activates the transcription of target genes through AhR-responsive elements (AHREs). Both IL-6 and SOCS3 are AhR-responsive genes and may be independently induced by the genomic activity of AhR. In the cytosol SOCS3, by anchoring tyrosine phosphorylated IDO1, brings the enzyme close to the E3 ubiquitin complex (E3) that promotes the polyubiquitination and the proteasomal degradation of IDO1. The non-genomic activity of ligand-bound AhR promotes Src kinase-mediated phosphorylation of IDO1, required for anchoring SOCS3. Ligand-bound AhR can assemble an atypical E3 ubiquitin ligase complex, involving cullin 4B (CUL4B), namely, $CUL4B^{AhR}$. A direct association of $CUL4B^{AhR}$ with IDO1 protein has been prospected (inset) in determining the polyubiquitination and the proteasomal degradation of IDO1.

the receptor AhR, could tightly control IDO1 activity in a sort of negative feedback loop. By construing the ubiquitin ligase activity of AhR as a sensor of environmental stress, as suggested in sex hormone signaling (41), several inflammatory adverse effects of dioxin-type ligands of AhR could be, at least in part, attributed to the accelerated degradation of IDO1 that physiologically prevents overreacting responses. In this regard, the appreciation of exogenous/endogenous ligands that selectively activate the non-genomic pathway of AhR might shed light on the biological role of AhR-based modulation of IDO1.

The involvement of the atypical ubiquitin ligase activity of AhR in the quenching of IDO1 activity may represent an attractive therapeutic perspective. Translated into the clinic, the non-genomic control of IDO1 by activated AhR becomes of great interest in neoplasia. The main strategy currently envisioned to tackle IDO1 clinically is by inhibiting its enzymatic activity. The post-translational modification of the enzyme, promoting its ubiquitination and proteasomal degradation, could represent a valid alternative or a complementary approach to the enzymatic inhibition. In this regard, the purine analog, fludarabine, currently used as a chemotherapeutic agent, has recently been proposed to act as a promoter of proteasome-mediated degradation of IDO1 in tumors (42). Noteworthy, the ubiquitin ligase function of AhR has also been contextualized to the antiproliferative activity resulting from proteasomal degradation of transcription factors (ER/AR, β-catenin) that promote cell proliferation in target tissues. Although IDO1 does not possess a transcriptional activity of its own like other substrates of $CUL4B^{AhR}$, it is noteworthy that, in cancer tissues, IDO1 plays a proliferative action (43–45), and therefore, the putative $CUL4B^{AhR}$-mediated degradation of IDO1 may result in antiproliferative activity.

CONCLUSION

Dissecting the molecular mechanism of ubiquitin ligase activity of AhR might lead to a better understanding of the diverse biological effects induced by exogenous/endogenous AhR ligands. Specifically, clarifying this mechanism in relationship to the AhR–IDO1 axis might be of great interest in providing innovative IDO1-based therapeutic targets. AhR-mediated non-genomic modulation of IDO1 might provide druggable targets in cancer therapy, in alternative to or in combination with the already available enzyme inhibitors. Thus, the identification of selective "non-toxic" AhR ligands, activating the non-canonical pathway of the receptor, represents an emerging area of research.

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Immunological relevance of the coevolution of IDO1 and AHR

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The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor initially identified because of its role in controlling the cellular response to environmental molecules. More recently, AHR has been shown to play a crucial role in controlling innate and adaptive immune responses through several mechanisms, one of which is the regulation of tryptophan metabolism. Indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) are considered rate-limiting enzymes in the tryptophan catabolism and play important roles in the regulation of the immunity. Moreover, AHR and IDO/TDO are closely interconnected: AHR regulates IDO and TDO expression, and kynurenine produced by IDO/TDO is an AHR agonist. In this review, we propose to examine the relationship between AHR and IDO/TDO and its relevance for the regulation of the immune response in health and disease.

Keywords: aryl hydrocarbon receptor, 2,3-dioxygenase, tryptophan-2,3-dioxygenase

AHR SIGNALING PATHWAYS

Aryl hydrocarbon receptor belongs to the family of basic-helix–loop–helix/Per–Arnt–Sim transcription factors. It is abundantly expressed in numerous tissues, such as liver, lung, and placenta (1, 2). Interestingly, AHR is highly conserved through evolution (3), highlighting its importance across the animal kingdom. Originally, AHR was studied in the context of the biological response to environmental toxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). However, it was later found that AHR has an important role in the regulation of immune responses by small molecules provided by the diet, the commensal flora, and metabolism. In its inactive state, AHR resides in the cytosol as part of a complex that includes other proteins such as the 90 kDa heat shock protein (HSP90), the AHR-interacting protein, p23, and the c-SRC protein kinase (4–7). It is thought that HSP90 and p23 protect the receptor from proteolysis and maintain a conformation suitable for ligand binding (8).

Aryl hydrocarbon receptor is activated by ligands binding the PAS-B domain (9), triggering a conformational change that results in the dissociation of AHR from the chaperone proteins and the exposure of its nuclear localization sequence (10). Ligand activation of AHR elicits genomic and non-genomic AHR-dependent signaling pathways. Genomic AHR signaling involves the interaction of AHR with other transcription factors and co-activators to directly regulate the transcription of target genes (7). After ligand activation, AHR translocates to the nucleus where it dimerizes with the AHR nuclear translocator (ARNT) (11) to form an active DNA-binding complex and control the expression of target genes containing xenobiotic response elements (XREs) in their regulatory regions (9). The AHR–ARNT complex can promote or inhibit the expression of its target genes. Moreover, ChIP-seq and microarray studies with different cell types and ligands (12–14) suggest that the AHR target genes in a specific cell are determined by the ligands, and also the identity and developmental stage of the target cells (15).

Non-genomic AHR signaling is more diverse and encompasses, for example, the release of c-SRC from its complex with AHR, resulting in the phosphorylation of c-SRC cellular targets (7). In addition, AHR can promote the degradation of specific target proteins such as estrogen and androgen receptors by the proteasome. This ability to trigger the degradation of specific proteins results from its E3 ligase activity, by which AHR selects proteins for ubiquitination by E2 ubiquitin-conjugating enzymes. The resulting ubiquitinated proteins are then recognized by the 26S proteasome and degraded (16–18). Indeed, following activation AHR itself is eventually exported out from the nucleus and degraded by the 26S proteasome pathway (19–21).

Structure–activity relationship studies showed that AHR's ligand binding pocket is promiscuous and able to accommodate numerous hydrophobic planar compounds (22). From an historic point of view, AHR can be seen as an endocrine-disrupting chemicals (EDCs) receptor, as it is known that EDCs affect the endocrine system either directly by AHR-dependent changes in gene expression or indirectly via AHR cross-talk with endocrine signaling pathways (23). However, both endogenous and exogenous AHR ligands have been identified. Classical AHR ligands include synthetic aromatic and polycyclic aromatic hydrocarbons (HAHs and PAHs) as well as natural ligands tetrapyrroles, flavonoids, tryptophan derivatives, and dietary carotinoids (24). Interestingly, some of the natural AHR ligands, such as resveratrol (25) and 7-ketocholestrol (26) can act as antagonists rather than agonists. Within the endogenous AHR ligands, tryptophan-derived metabolites have become one of the most interesting and utmost studied group (7). It should be noted that AHR activation

in the absence of ligand binding has also been described. Although the relevance of this observation for vertebrates is not completely understood, the ligand-independent activation of AHR might play a physiological role in invertebrates (see below).

AHR EVOLUTION

Aryl hydrocarbon receptor homologs have been identified in most major groups of animals, including the two main clades of protostome invertebrates as well as deuterostomes (27, 28) highlighting the biological importance of AHR throughout the animal kingdom. AHR homologs identified in invertebrates share similarities with their vertebrate counterparts, such as the interaction with ARNT to recognize XRE (29–31). However, invertebrate AHR homologs do not bind known AHR ligands like TCDD or β-naphthoflavone (29, 31). Indeed, it was recently reported that the metabolic response to xenobiotics in *Caenorhabditis elegans* is not controlled by AHR (32).

In *C. elegans*, the orthologs of AHR and ARNT are encoded by the AHR-related (*ahr-1*) and *ahr-1* associated (*aha-1*) genes, respectively. AHR-1 and AHA have HSP90 binding properties comparable to those of their mammalian counterparts (31). AHR-1 shares 38% amino acid identity with the human AHR over the first 395 amino acids. Furthermore, AHR-1 contains a PAS domain with both PAS-A and PAS-B repeats as well as a bHLH domain where specific residues mediating the recognition of mammalian XREs are conserved (31). However, AHR-1 does not have a glutamine-rich transcriptional activation domain similar to the one present in mammals.

Notably, mutations in AHR-1 affect several aspects of neuronal development determining, for example, the fate of GABAergic neurons in the L1 larval stage, regulating both cell and axon migrations as well as specifying the fate of AVM light touch sensory neuron (33–35). In addition, AHR-1 is involved in social feeding (36), in which nematodes form groups on the border of the bacterial lawn (37).

Recent studies have also demonstrated a role for AHR-1 in regulating the synthesis of long-chain unsaturated fatty acids that eventually produce lipid signaling molecules (38). This finding is consistent with findings in mouse models, where ligand activation of AHR has been linked to alterations in gene expression of fatty acid metabolism (39, 40).

The homologs of mammalian AHR and ARNT are encoded by *spineless* and *tango* in *Drosophila melanogaster* (41, 42). In agreement with observations made in *C. elegans*, *spineless* does not bind TCDD or β-naphthoflavone (29). In addition, sequence alignments suggest that key residues required for the interaction of mammalian AHR with TCDD are not conserved in *spineless* (29, 41). Thus, although it is still possible that the localization and/or the activity of *spineless* are modified by unknown endogenous ligands, it appears that this protein does not bind classical AHR ligands functional in mammalian systems. Moreover, in certain cells spineless appears to be constitutively active (43). *Spineless* plays a role in several aspects of antenna and leg development (41, 44), photoreceptor cell differentiation (45), and in controlling the morphology of sensory neurons (46).

Not surprisingly, most of our knowledge on mammalian AHR comes from studies on human beings and mice. Key features characterizing mammalian AHR are (1) in contrast to other vertebrates (47) all studied mammalians have a single AHR gene and (2) AHR in mammals is not only involved in the toxic effects of environmental pollutants (48, 49), it also has important roles in development (50–53) and immune responses [reviewed in Ref. (7)]. Indeed, it has been hypothesized that the original function of the AHR might have been developmental regulation and that AHR's ability to bind HAHs, PAHs, and mediate adaptive responses involving induction of xenobiotic-metabolizing enzymes is a vertebrate innovation (3, 47).

KYNURENINE PATHWAYS TDO/IDO AND IMMUNE REGULATION

Tryptophan metabolites have become one of the most interesting groups of endogenous AHR ligands. Especially kynurenine, an immediate tryptophan metabolite, has been extensively studied in recent years. The metabolic fate of tryptophan is conversion into a range of neuroactive substances, such as serotonin and melatonin. In addition, tryptophan can be catabolized into kynurenine metabolites. Indoleamine-2,3-dioxygenase (IDO1), tryptophan-2,3-dioxygenase (TDO), and recently discovered IDO-related enzyme IDO2 (54) are the first and rate-limiting enzymes converting tryptophan to *N*-formylkynurenine (55, 56) which is then metabolized to L-kynurenine. Both TDO and IDO1 are thought to be intracellular enzymes (57, 58). Therefore, ATP-binding cassette (ABC) transporter (59), enzyme facilitating cellular entry of tryptophan, is considered to be another rate-limiting factor in tryptophan catabolism (60). L-Kynurenine can be catabolized by three different ways: (1) kynurenine monooxygenase, kynureninase, and 3-hydroxyanthranilic acid oxidase catalyze the synthesis of anthranilic acid, 3-hydroxyanthranilic acid, quinolinic acid, and 3-hydroxykynurenine. (2) Kynurenine aminotransfereases catalyze the synthesis of kynurenic acid. (3) Kynurenine monooxygenase and kynurenine aminotransfereases catalyze the synthesis of xanthurenic acid (**Figure 1**) [reviewed in Ref. (61)].

In human beings, IDO1 is expressed in various tissues and cell subsets following cytokine stimulation during infection, transplantation, pregnancy, autoimmunity, and neoplasia (62–64). IDO1 is constitutively expressed in many human tumors, creating an immunosuppressive microenvironment as a result of tryptophan depletion and the synthesis of immunosuppressive metabolites such as kynurenine (65, 66). Surprisingly, the expression of IDO1 is controlled by AHR (67) via an autocrine AHR-IL6-STAT3 signaling loop (68). In addition, tryptophan starvation caused by IDO1 activity, together with IDO1-dependent tryptophan catabolism, inhibits the proliferation and activation of antigen-specific T lymphocytes and induces immune tolerance (69–72). In addition, strong evidence suggests that tryptophan catabolism can inhibit T-cell based adaptive immunity by inducing the differentiation of regulatory T cells (Treg) in tumors (62, 73–75). Interestingly, kynurenine is also indicated to promote the differentiation of Tregs (76) while suppressing antigen-specific T-cell responses (77).

In mammals, TDO2 is expressed primarily in the liver (78–80) but can also be detected in other tissues such as the brain (79, 81–83). TDO2 is constitutively expressed and activated in gliomas (84). Recently, lipopolysaccharide was demonstrated to induce TDO2 expression and via consequent production of kynurenine

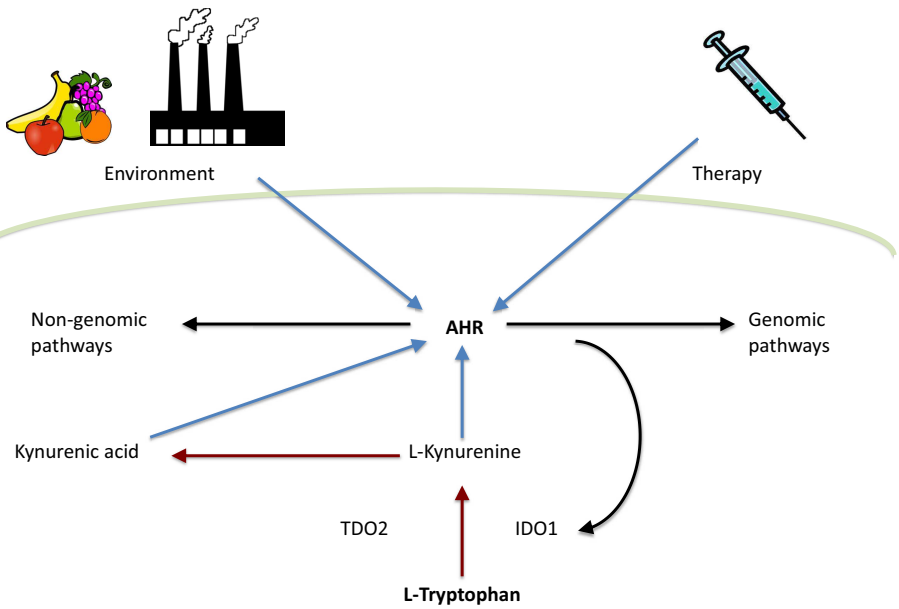

FIGURE 1 | Interplay between tryptophan metabolism and AHR. Tryptophan is metabolized by TDO2 and IDO1 to L-kynurenine, which is further converted to kynurenic acid. Both L-kynurenine and kynurenic acid can activate AHR. In addition, AHR activity is influenced by environment and therapy. Finally, AHR can activate either genomic or non-genomic AHR-dependent signaling pathways. Red arrows indicate tryptophan catabolism pathways, blue arrows indicate AHR activation, and black arrows indicate pathways activated by AHR.

activate AHR-dependent pathways leading to protection against endotoxin challenge (85). In addition, this study also reported that endotoxin tolerance is also mediated by AHR as it was demonstrated that AHR activation by kynurenine elicits the c-SRC dependent phosphorylation of IDO1, which further regulates TGFβ1 production by dendritic cells as well as limits immunopathology triggered by both *Salmonella typhimurium* and group B *Streptococcus* (85). Furthermore, TDO2 derived kynurenine has been demonstrated to suppress antitumor immune responses as well as promote survival and motility of tumor cells via AHR in an autocrine manner (84). Note that kynurenic acid can also activate AHR signaling (86).

IDO/TDO EVOLUTION

Unfortunately, not much is know about the kynurenine pathway in nematodes. However, the study of intestinal autofluorescence in relation to tryptophan catabolism revealed that nematodes having a mutated *flu-1* gene show altered gut granule autofluoresence as well as decreased kynurenine hydroxylase activity (87). Whereas, *flu-2* mutants have reduced kynureninase and gut granule autofluorescence (87). In support of these observations, the *C. elegans* genome has homologs of kynurenine hydroxylase and kynureninase in the vicinity of *flu-1* and *flu-2* loci (88).

Additional putative kynurenine pathway related genes have been identified in the *C. elegans* genome (89) (**Figure 2**). The knock down of *tdo-2*, for example, abrogated the gut granule fluorescence (90, 91). Involvement of the *C. elegans* kynurenine pathway has been demonstrated in neurodegeneration and aging: in a *C. elegans* model of Parkinson's disease; RNAi knock down of *tdo-2* reduced α-synuclein aggregation-induced toxicity and increased life span (92). However, these effects were proven to be a result of increased tryptophan rather than changed levels of kynurenines (92).

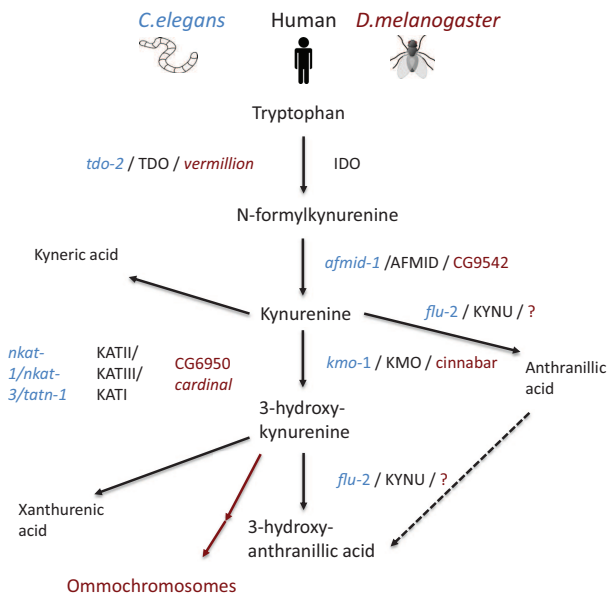

FIGURE 2 | Schematic representation of the kynurenine pathway in human beings (mammal), *C. elegans*, and *D. melanogaster*. Pathway tree demonstrates differences between mammalian and invertebrate tryptophan catabolism. Mammalian enzymes are depicted in black, *C. elegans* enzymes in blue, and *D. melanogaster* in red. Modified from Ref. (89)

In *D. melanogaster,* tryptophan catabolism takes place in pigmented eyes (93–95). Remarkably, the role of kynurenine pathway in eye function is conserved from flies to mammals, as it plays an essential role in protecting the lens from ultraviolet irradiation (96). *D. melanogaster* TDO2 is encoded by *vermillion.* Flies having the *vermillion* mutation lack brown pigment in their eyes and have been thought to be deficient for TDO2 activity (93, 94, 97, 98). This was verified when kynurenine pathway and related genes were described in full in 2003 (99). In the same way, as in *C. elegans*, loss of *vermillion* function has been demonstrated to be neuroprotective in *D. melanogaster* model of Huntington's disease (100). In addition, loss of *vermillion* function extend the life span of *D. melanogaster* (101, 102) while resulting gradual memory decline (103). Furthermore, white eye mutants having impaired ABC transport show extended life spans (102). In addition, other *D. melanogaster* mutants, cardinal and cinnabar, resulting in excess of 3-hydroxykynurenine and neuroprotective kynurenic acid, have been demonstrated to modify the brain plasticity (104).

CONCLUSION

Aryl hydrocarbon receptor, a member of the dHLH-PAS superfamily, has been identified both in invertebrates and vertebrates, suggesting that the ancestral AHR gene arose over 500 million years ago (3). In vertebrates, especially in mammals, the activity of AHR is mostly regulated by its interactions with ligands. However, in invertebrates (e.g. *C. elegans*) AHR does not seem to interact with TCDD or any other known ligand (105), and it is constitutively localized in the nuclei of certain cells suggesting ligand-independent activation (34). Similar observations have been made for *D. melanogaster´s spineless* (29). Although one cannot rule out the possibility that invertebrates require a different kind of AHR ligands than vertebrates, it has been speculated that in early metazoans AHR might have had a ligand-independent roles in development. Thus, the ability of AHR to interact with ligands, bind HAHs and PAHs, and regulate xenobiotic-metabolizing enzymes has been postulated to be a vertebrate novelty (3, 47).

Aryl hydrocarbon receptor signaling modulates development and immune function in mammals (7). Fairly recently, the involvement of tryptophan metabolism has been implicated in regulating both innate and adaptive immune responses. Most importantly, kynurenine produced by TDO or IDO1 during tryptophan catabolism has been identified as an AHR ligand, linking IDO/TDO to AHR. Considering the evolutionary conservation of the kynurenine pathway, it is tempting to speculate that the cross-talk between AHR and IDO/TDO immunoregulatory pathways is a recent evolutionary innovation aimed at providing a mechanism to fine tune the immune response in response to environmental cues provided by the tissue microenvironment. This interpretation suggests that approaches targeting both AHR and IDO/TDO are likely to provide efficient new avenues for the therapeutic manipulation of the immune response.

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The aryl hydrocarbon receptor meets immunology: friend or foe? A little of both

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The aryl hydrocarbon receptor (AHR) has long been studied by toxicologists as a ligand-activated transcription factor that is activated by dioxin and other environmental pollutants such as polycyclic aromatic hydrocarbons (PAHs). The hallmark of AHR activation is the upregulation of the cytochrome P450 enzymes that metabolize many of these toxic compounds. However, recent findings demonstrate that both exogenous and endogenous AHR ligands can alter innate and adaptive immune responses including effects on T-cell differentiation. Kynurenine, a tryptophan breakdown product, is one such endogenous ligand of the AHR. Expression of indoleamine 2,3-dioxygenase by dendritic cells causes accumulation of kynurenine and results in subsequent tolerogenic effects including increased regulatory T-cell activity. At the same time, PAHs found in pollution enhance Th17 differentiation in the lungs of exposed mice via the AHR. In this perspective, we will discuss the importance of the AHR in the immune system and the role this might play in normal physiology and response to disease.

Keywords: indoleamine 2,3-dioxygenase, Th17 Cells, Treg cells, immunomodulation, kynurenine, aryl hydrocarbon receptor

INTRODUCTION

Our laboratory has been actively investigating the role of the aryl hydrocarbon receptor (AHR) in the immune system, and the variable effects seen after binding endogenous and exogenous ligands. Study of this receptor traditionally was in the domain of toxicologists, as it was originally defined as a receptor to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (1). It was further identified that additional toxins, including polycyclic aromatic hydrocarbons (PAHs), also bind to the AHR (2–4). As the mechanism of AHR activation and function was characterized, the importance of this receptor as a transcription factor was realized. The AHR is a cytosolic receptor that after binding migrates to the nucleus where it becomes a transcription factor for cytochrome P450 genes that encode enzymes that metabolize toxins, including those same toxins that bind to the receptor (5). A connection to the immune system had been previously recognized, primarily based on the knowledge that exposure to TCDD led to rapid thymic involution, and animals and human beings exposed to TCDD were known to be immunosuppressed. Effects of TCDD on dendritic cells (DCs) and T-cells have been recognized for years (6), and generation of regulatory T-cells by an AHR-dependent mechanism was identified in 2005 (7). But in general, immunologists and those studying autoimmunity did not become interested in the AHR until 2008, when two high impact papers identified the role of the AHR in T-cell differentiation, with certain ligands enhancing Treg generation, and others enhancing Th17 differentiation, both *in vitro* and *in vivo* (8, 9). Since that time, there has been a flood of reports on the role of the AHR in the adaptive immune system and animal models of disease, particularly autoimmune disease (10–12).

The role of the AHR in responding to toxins is thought to have evolved, as invertebrate forms of the AHR do not bind TCDD (13), and the toxins associated with the AHR are man made and were generated long after the origins of the receptor. It has been postulated that the initial importance of the AHR was in embryologic development, and in addition there was (and remains) a requirement for binding of this receptor to endogenous ligands early in development. This is based on the abnormal phenotype of AHR null mice that display a patent ductus venosus (14), as well as the necessity for the hypomorphs to bind AHR ligand early in development to prevent the patent ductus venosus in these transgenic mice (15). In addition to binding to pollution and endogenous ligands, the AHR binds to numerous ligands present in the diet including flavonoids, which are ubiquitous in fruits and vegetables. There has been an ongoing search for relevant endogenous ligands in animals and human beings.

Around the time that the AHR was first recognized to play some role in the acquired immune system by toxicologists, immunologists were investigating indoleamine 2,3-dioxygenase (IDO_1), initially recognized as the rate-limiting enzyme in tryptophan metabolism. In 1998, it was reported that tryptophan catabolism by IDO_1 and other enzymes was responsible for prevention of allogeneic fetal rejection in mice, and it was further revealed that this enzyme was generated by DCs, and in some way increased the differentiation of Tregs (16). The mechanism was unclear, but the two leading theories were that either tryptophan deprivation from its metabolism decreased the generation and division of effector cells (17), or certain breakdown products of tryptophan, termed kynurenines, were acting through some target on T-cells or other cells to favor Treg differentiation (18, 19). In our own lab, we considered the hypothesis that kynurenine or one of its breakdown products was working through the AHR in T-cells to enhance Treg generation. This was based on the knowledge

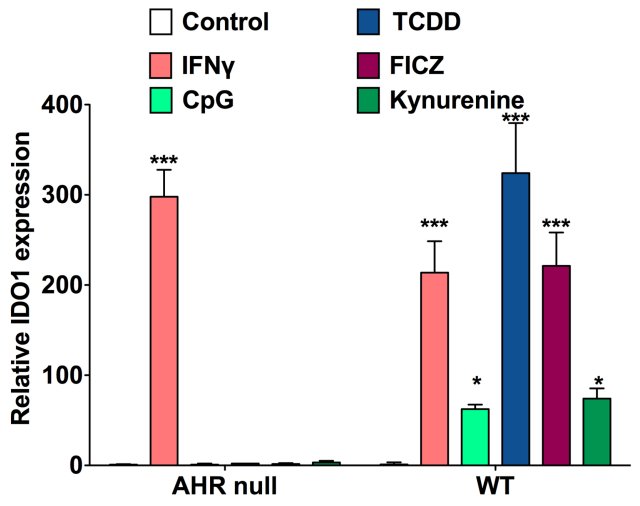

FIGURE 1 | AHR ligands increase IDO$_1$ expression in BMDCs. qRT-PCR analysis of IDO$_1$ mRNA expression in bone marrow-derived DCs treated for 48 h with IFN-γ (10 μg/ml), CpG (2.5 μM), TCDD (10 nM), FICZ (200 nM), or kynurenine (50 μM). Data are from three independent experiments.

that indoles and other tryptophan derivatives are often ligands of this receptor, making kynurenine a good candidate as an endogenous ligand. We identified that not only does kynurenine bind to the AHR in the cytosol of T-cells and enhance Treg generation *in vitro* but additionally kynurenine and other AHR ligands act on BMDCs to upregulate IDO expression in an AHR-dependent manner (20) (**Figure 1**). The effects of AHR ligands on differentiation and function of natural versus induced Tregs has not yet been delineated.

One of the controversial and yet unanswered questions about the role of the AHR in the immune system is whether it is probable that different ligands bind to the AHR and lead to different, almost opposite outcomes, perhaps based on some conformational change or transcription of different genes, a finding that would almost be unprecedented in physiology. We theorize that some combination of strength of binding, duration of binding, route of exposure, and surrounding milieu determines whether Treg or Th17 differentiation is enhanced. Although early experience seemed to suggest that ligands were either "regulatory" (meaning they favored differentiation of Tregs) or "effector" (meaning they favored differentiation of effector Th17 cells) (8), more recent studies and our own experience has suggested that this is not likely the case. For instance, the ligand 6-formylindolo[3,2-b]carbazole (FICZ) was first described as enhancing Th17 differentiation *in vitro* and *in vivo*, and when administered subcutaneously worsened autoimmunity in an experimental autoimmune encephalomyelitis (EAE) model in mice. However, it has recently been shown that when this same ligand is administered intraperitoneally, Treg differentiation is favored and there is a reduction in EAE disease severity (21, 22). Our own experience has also shown that depending on the culture conditions or inflammatory status of the animals, similar ligands can enhance either Th17 or Treg generation. We have also found that every ligand we have tested stimulates T-cells to generate IL-22, and it has been demonstrated that the AHR is required for IL-22 generation in the majority of scenarios (23–25). IL-22 is an interesting cytokine that is generated by various types of T-cells, but its receptor is found on epithelial cells. In general, IL-22 is thought to be protective against immune damage and helpful in epithelial cell repair (26–28). We have recently shown that particulate matter (PM) in the form of urban dust particles (UDP) from the National Institute of Standards and Technology (NIST; SRM1649b), containing PAHs, increases the Th17 response both in the lung *in vivo* and in T-cells *in vitro* (29). The *in vitro* response was shown to be AHR dependent. Therefore, a connection between the AHR and the immune system seems real, but the effects are complicated and dependent on the model or conditions being examined. This relationship of the AHR with the immune system, where binding of this receptor with different ligands in differing milieus can lead to seemingly opposing responses in T-cell differentiation has allowed us to come up with the following hypothesis:

THE AHR AS A SENSOR

The AHR serves as a sensor that responds to signals, both from the outside environment or internal milieu, to modulate an immune response. In the setting of an endogenous immune response, the AHR helps contain the inflammation and decreases collateral damage, so the response does not cascade in strength and lead to autoimmune destruction. In responding to exogenous ligands, the AHR functions as a sensor to "danger" signals, such as dioxins, PAHs, and other components of pollution. This is of evolutionary benefit for individuals exposed to man-made pollution or other toxins, as illustrated by the following scenario. When someone is exposed to inhaled atmospheric pollution, damage to bacterial clearance mechanisms and the epithelial lining of the airway puts the individual at risk for endogenous bacterial invasion and activation of a reactive airway response. The AHR present in immune cells residing in the lung binds to fractions of PM (PAHs and others), leading to an enhancement of Th17 differentiation that can serve as an immune barrier to the endogenous bacteria found in the lining of the lung. Simultaneously, AHR activation leads to generation of IL-22 that helps with epithelial cell repair and maintenance of tight junctions. Finally, activation of the AHR causes an upregulation of cytochrome P450 enzymes that metabolize the harmful toxicants. Taken together, the AHR aids in tissue protection, repair, and toxin clearance (**Figure 2**, left).

ENDOGENOUS LIGANDS

We hypothesize that as the adaptive immune system developed, it became crucial that there be a way for the body to dampen overly robust responses, thereby preventing reactivity to self-proteins and ensuing autoimmunity. Kynurenine, the first breakdown product of tryptophan in the IDO pathway, became the transmitter that interacts with the AHR in T-cells and enhances the differentiation of Tregs that reduce an immune response. At the same time, kynurenine also interacts with the AHR in DCs to enhance the generation of IDO, leading to further tryptophan break down. This response is self-limited, as once the tryptophan in the inflammatory milieu is dissipated, kynurenine is no longer generated and Treg generation is decreased. This push toward a regulatory

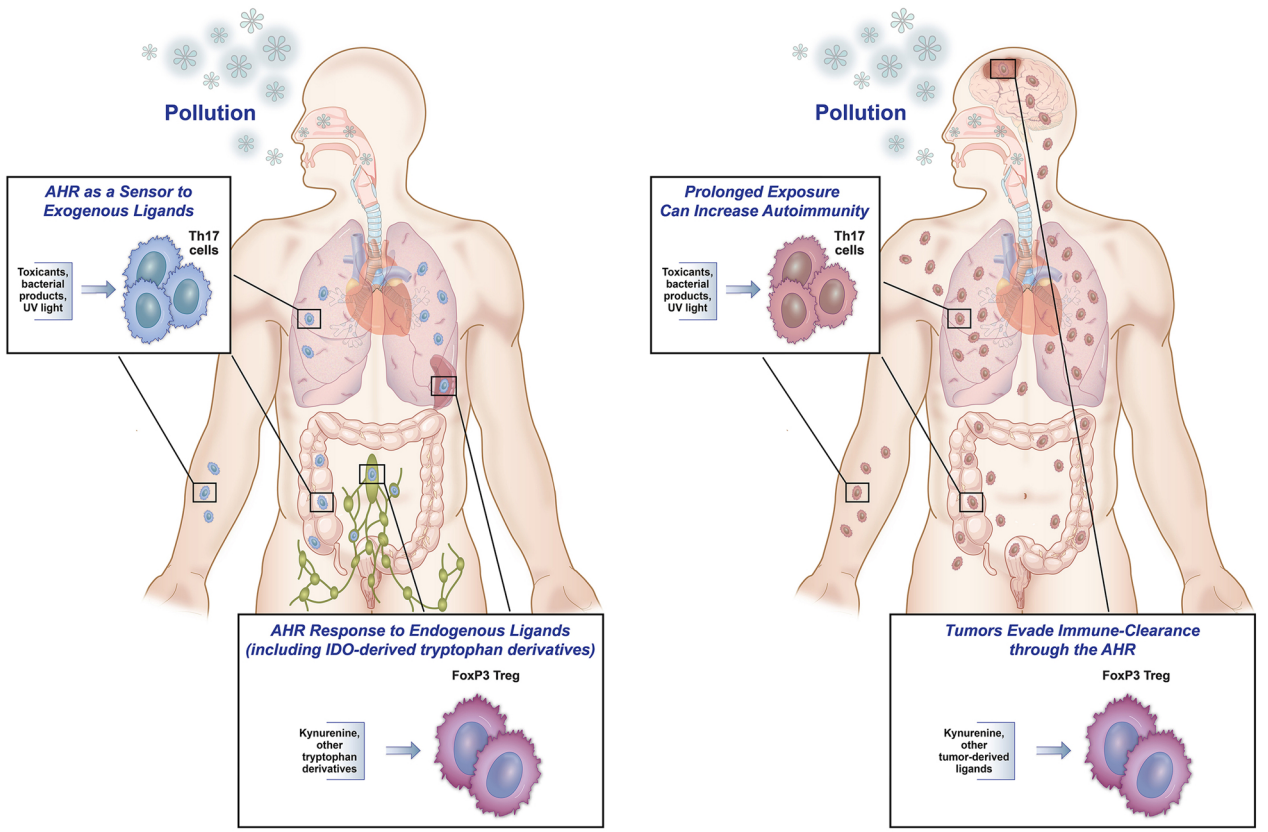

FIGURE 2 | The AHR as friend and foe. This figure represents the hypothesis that the AHR can be both protective and pathologic in responding to endogenous and exogenous ligands. **On the left**, exposure to inhaled pollution (as well as ingested or topical) can bind to the AHR in immune cells, and in an inflammatory setting, lead to increased Th17 differentiation, IL-22 generation, and cytochrome P450 enzyme production. All of these responses can help protect the host from pathologic inflammation and invasion by endogenous bacteria. Similarly, in response to endogenous ligands during inflammation (in the spleen, lymph node, or throughout the body), AHR ligands can lead to increased IDO production and differentiation of Tregs by binding of kynurenine to the AHR in T-cells, hence controlling an immune response. **On the right**, examples of binding of the AHR leading to increased pathology are shown. Tumors (in the brain or elsewhere) have been shown to generate TDO, leading to kynurenine production that binds to the AHR in tumor cells, causing increased regulation and decreased tumor destruction by the native immune system. In addition, prolonged exposure to external pollutants can lead to over-differentiation of Th17 cells and aggravation of autoimmunity in the lungs, gut, skin, and elsewhere in susceptible people.

response from endogenous ligands makes evolutionary sense, as internal inflammatory responses would need to be controlled to prevent damage to surrounding epithelial and parenchymal cells at the sight of an immune response. Additionally, binding of the AHR to ligand leads to the generation of IL-22 and engenders epithelial cell homeostasis, proliferation, and anti-microbial peptide production (30), further protecting the bystander tissue.

EXOGENOUS LIGANDS

In contrast to the role that the AHR plays in regulating autoimmunity when presented with endogenous ligands, at interface organs such as the lung, gut, and skin, AHR activation leads to an effector response. At these locations, toxin binding to the AHR in immune cells leads to Th17 differentiation and a heightened inflammatory response. We view this interaction as a "danger" signal that allows activation of the immune system at the same time that P450 enzymes are upregulated to metabolize toxins. When a host is presented with a potentially harmful exposure, early signaling and activation of the immune system occurs concurrently with ligand clearance. We believe that this is the role of exogenous activation of the AHR in immune cells.

It is important to again point out that we do not believe that endogenous ligands such as kynurenine always favor Treg generation, and exogenous ligands including fractions of pollution always favor Th17 differentiation. However, we do believe that in the *in vivo* inflammatory milieu where exogenous ligands are exposed to interface organs during a toxic environmental exposure, Th17 differentiation is favored, and similarly in the endogenous *in vivo* milieu of an immune response in the presence of IDO and tryptophan, Treg generation is favored. The mechanistic details of these

effects on T-cell differentiation require further delineation and are an exciting area of research.

PATHOLOGIC RESPONSE

While we hypothesize that the AHR and its role in the immune system evolved to be protective, there are examples where this interaction actually increases pathology. In the setting of an overwhelming exposure to exogenous ligands, generation of ligands by tumors, or changes in the microbiome altering physiologic interactions between products of bacteria and receptors in the gut, the AHR can worsen pathology (**Figure 2**, right).

CANCER

Certain cancers have developed the ability to evade their own clearance through use of the AHR. In gliomas, constitutive activation of tryptophan 2,3-dioxygenase (TDO-2) leads to high levels of kynurenine production. The TDO-2-derived kynurenine then acts through the AHR in tumor cells in an autocrine manner and promotes tumor survival as well as altering the regional immune response by favoring Treg development. These Tregs hamper the ability of the immune system to clear the tumor. This is particularly effective as the interaction between kynurenine and T-cells in the vicinity of the tumor leads to local regulation in the very location and milieu that the tumor resides in Ref. (31). In addition, there are some examples in human cancer where the tumors show increased copies of the AHR (32), which could increase the autocrine effect of preventing tumor clearance.

AUTOIMMUNITY

The immune system is in a constant balance of regulation and inflammation, and loss of this equilibrium can lead to pathology. We hypothesize that exogenous ligands of the AHR found in pollution can aggravate autoimmune conditions in an AHR-dependent mechanism. For many years, there has been speculation that environmental exposures can aggravate or even cause autoimmune disease, and a recent analysis of epidemiologic data has supported this hypothesis. The specific exposures that lead to disease and the mechanisms through which they impart pathology remain unclear. Recent data suggest that modulation of the Treg/Th17 balance may be at the center of environmentally induced autoimmunity. An NIEHS workshop concluded that "dysregulated Th17 activity can lead to pathology" in various autoimmune diseases, and that both smoking and "AHR binding by aromatic hydrocarbons favors differentiation of Th17 cells and can exacerbate autoimmunity" (33). We recently identified that inhaled PM leads to an increase in Th17-driven inflammation (29). In our resultant model, when an individual is exposed to inhaled PM for a prolonged period of time, there is a shift in the Treg/Th17 balance toward Th17 cells, aggravating or initiating autoimmunity in susceptible people. This ubiquitous mechanism will be applicable to multiple environmentally induced autoimmune diseases.

TRANSPLANT REJECTION

One of the novel theories we have been studying in our lab is the possibility that exposure to pollution is altering the Treg/Th17 balance and leading to chronic rejection after solid organ transplant. This may be most relevant to lung transplantation, where the transplanted organ is at the interface with the outside environment (34). There is growing evidence that inhaled exposure to elevated levels of atmospheric pollution, with increased PM, increases chronic rejection in lung transplants in human beings (35, 36). Bronchiolitic obliterans syndrome (BOS) is the major form of chronic rejection in lung transplantation, and it has been well documented that Th17 cells and the cytokine IL-17 are required for pathology (37–39). Given the link between IL-17 and BOS, the fact that pollution accelerates BOS, and our own work demonstrating that pollutants contain AHR ligands, which favor a Th17 response, it is possible that the AHR is a key factor in pollutant accelerated rejection in lung transplantation.

GUT IMMUNITY

Some of the highest impact papers regarding the AHR in the last couple of years have described the relationship between gut immunity and the AHR. The presence of this receptor is necessary for the establishment of certain populations of immune cells in the gut. Furthermore, both dietary ligands of the AHR as well as ligands produced by gut bacteria interact with and alter the gastrointestinal immune system (40, 41). The healthy microbiome generates ligands that interact with the AHR to maintain gut immune structure and stability. Loss of some of these ligand-producing bacteria may increase the risk of autoimmune colitis (42).

SKIN

The AHR is highly expressed in multiple cell types found in skin. As skin is one of the organs at the interface with the outside environment, it is likely that inflammatory disorders are altered by interaction with toxins and other environmental factors. A recent publication did document the protective role of various AHR ligands in reducing inflammation in a model for psoriasis (22). At the same time, exposure to AHR antagonists increased inflammation. This is clinically relevant, as it is known that certain toxicants contain fractions that may inhibit the AHR or cytochrome P450 enzymes, and certain weak AHR agonists actually inhibit activation by other agonists that may be found in different fractions of a given exposure (43). This implies that in clinically relevant mixtures of chemicals, the overall response could be one of antagonism of the AHR and worsening of skin inflammation.

AHR MEETS IMMUNOLOGY: FRIEND OR FOE?

So does the relationship between the AHR and the immune system make us healthier and more able to withstand interactions with the environment, or leave us at risk for pathologic manipulation of the immune system in response to external and internal signals? We assert that the AHR does make us healthier, and allows us to survive in the environment that currently exists. The relationship of the endogenous ligand kynurenine and the AHR allows a feedback loop that dampens an immune response to prevent too much inflammation and autoimmunity, allowing a response to be self-limited. Exogenous ligands serve as "danger" signals that alert the immune system to toxic insults from the environment, allowing immune cells to play an early role in containing damage done by these exposures, helping repair local barriers, and containing local endogenous infectious exposures. At the same time, there are examples where disease processes have taken advantage of this

connection to increase pathology. This scenario, where normal physiology or "protective" responses become overwhelmed and lead to increased severity of illness, is well documented in immune responses to pathology, including systemic inflammatory response syndrome in response to infection, multiple forms of autoimmunity, and resistance of cancer to its own destruction. As we better understand the mechanisms and signals behind the role of the AHR in the immune system, we will be able to manipulate this receptor to treat or prevent diseases as diverse as cancer, autoimmunity, and transplant rejection.

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Early carcinogenesis involves the establishment of immune privilege via intrinsic and extrinsic regulation of indoleamine 2,3-dioxygenase-1: translational implications in cancer immunotherapy

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Although prolonged genetic pressure has been conjectured to be necessary for the eventual development of tumor immune evasion mechanisms, recent work is demonstrating that early genetic mutations are capable of moonlighting as both intrinsic and extrinsic modulators of the tumor immune microenvironment. The indoleamine 2,3-dioxygenase-1 (IDO) immunoregulatory enzyme is emerging as a key player in tumor-mediated immune tolerance. While loss of the tumor suppressor, BIN-1, and the over-expression of cyclooxygenase-2 have been implicated in intrinsic regulation of IDO, recent findings have demonstrated the loss of TβRIII and the upregulation of Wnt5a by developing cancers to play a role in the extrinsic control of IDO activity by local dendritic cell populations residing within tumor and tumor-draining lymph node tissues. Together, these genetic changes are capable of modulating paracrine signaling pathways in the early stages of carcinogenesis to establish a site of immune privilege by promoting the differentiation and activation of local regulatory T cells. Additional investigation of these immune evasion pathways promises to provide opportunities for the development of novel strategies to synergistically enhance the efficacy of the evolving class of T cell-targeted "checkpoint" inhibitors.

Keywords: indoleamine 2,3-dioxygenase, dendritic cells, tumor immune evasion, β-catenin, tumor immunotherapy, Wnt5a, type III TGF-β receptor, COX-2

INTRODUCTION

Indoleamine 2,3-dioxygenase-1 (IDO) is a heme-containing enzyme known to catalyze the rate limiting step in the degradation of the essential amino acid tryptophan to its metabolic byproducts known collectively as the kynurenines (1). Although initially felt to play primarily an anti-microbial role, pioneering work eventually showed this biochemical pathway to impact the immune system by inhibiting T cell proliferation and driving the differentiation and activation of regulatory T cells (Tregs) (2–6). While IDO has been broadly implicated in the progression of several cancers by aiding tumors to evade the host immune system, the mechanisms utilized by cancers to regulate IDO activity have remained poorly characterized (7, 8). Recent work in preclinical models has revealed novel mechanisms utilized by cancers to manipulate IDO activity within the tumor microenvironment. Interestingly, these mechanisms have been found to be regulated by previously defined tumor suppressors and oncogenes, which undergo genetic alteration relatively early during tumorigenesis. In contrast to the cancer immunoediting hypothesis, which proposes that immune-mediated selective pressure by the adaptive immune system is necessary before active immune tolerization mechanisms develop (9), this observation suggests that subversion of the immune system is necessary at relatively early stages of tumor development and, in fact, occur concurrently with the process of malignant transformation (**Figure 1**). This review discusses our understanding of IDO regulation, highlights mechanisms utilized by cancers to control IDO activity in the tumor immune microenvironment, and outlines pharmacological strategies for reversing these processes to ultimately augment our immunotherapeutic strategies for managing cancer patients.

REGULATION OF IDO EXPRESSION AND ENZYMATIC ACTIVITY

Several factors have been shown to regulate the expression of IDO in a cell type-specific manner in monocytes, macrophages, endothelial cells, fibroblasts, some tumor cells, and various populations of dendritic cells (DCs). Initial studies focused on the process of inflammation, showing interferon-γ (IFN-γ) to be a potent inducer of IDO expression in many cell types and demonstrating this pathway of IDO induction to provide protection from intracellular pathogens by depleting local tryptophan levels (10, 11). Other inflammatory factors that have been shown to regulate IDO expression include IL-1, tumor necrosis factor-α (TNF-α), and lipopolysaccharide (LPS) (12, 13). Interestingly, prostaglandin E2 has been observed to induce the transcription of IDO in human monocyte-derived DCs; however, additional activation with TNF-α or LPS was noted to be necessary to achieve full enzymatic activity (14). This is consistent with observations by other investigators who have found that two-signals are often necessary to induce maximal IDO expression by specific DC subsets

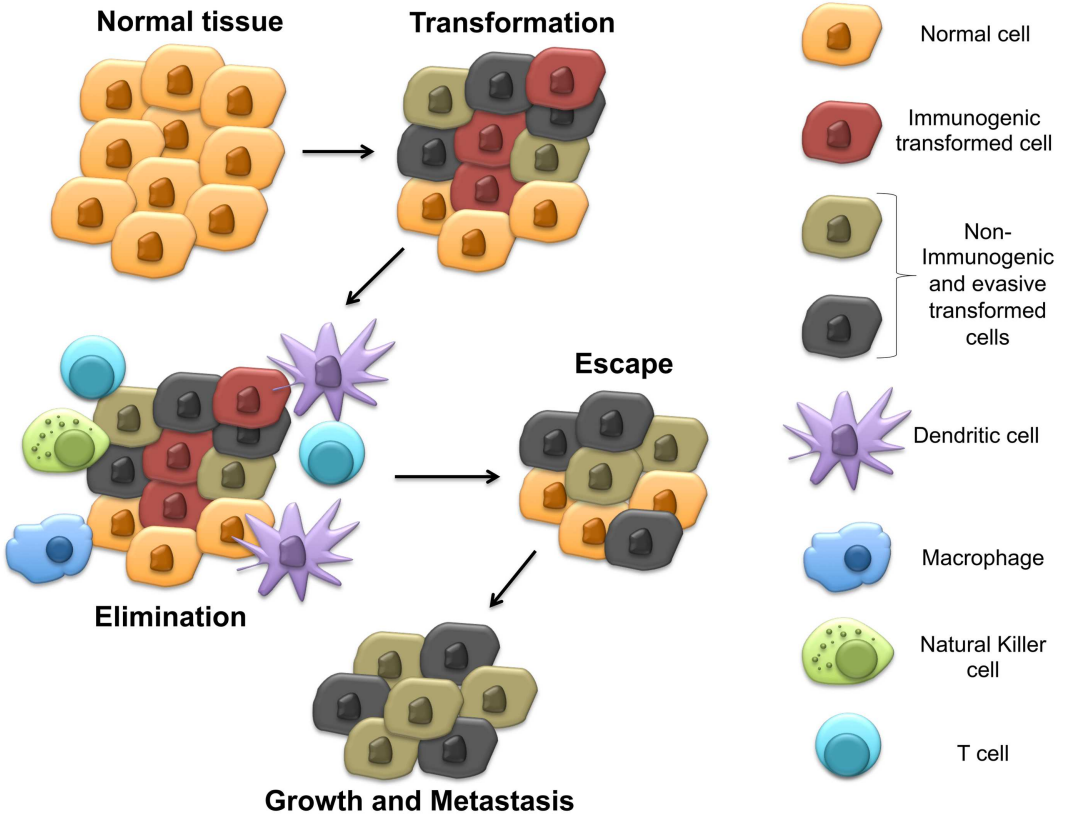

FIGURE 1 | Early steps in carcinogenesis include immune evasion by upregulating IDO expression in the tumor microenvironment. This model proposes the development of immune evasion mechanisms early during transformation, which stimulate local IDO activity prior to the development of adaptive anti-tumor immunity and generation of the selective pressure responsible for cancer immunoediting.

(5). Additional T cell-derived signals have also been demonstrated to play a role in regulating IDO expression including reverse signaling via B7 co-stimulatory molecule (CD80/CD86) cross-linking on the surface of antigen-presenting cells (APCs) (15–18). Using this mechanism, Tregs constitutively expressing CTLA-4 condition DCs by stimulating IDO expression and, in turn, suppressing local T cell proliferation, thereby establishing a state of immune tolerance. Later work revealed this B7 reverse signaling mechanism to be dependent upon the activation of the non-canonical NFκB signaling pathway, a mechanism also responsible for the induction of IDO following stimulation by other cell surface receptors including CD40 and the glucocorticoid-induced TNF receptor (GITR) (19).

It has been generally proposed that IDO activation signals have evolved to provide a negative feedback mechanism to dampen local inflammatory processes and prevent immune-mediated pathology. However, more recent studies have shown the immunosuppressive cytokine, TGF-β, to induce novel IDO functionality by specific subtypes of DCs including the murine $CD8^+$ splenic DC subset as well as the plasmacytoid DC (pDC) population (20, 21). These studies revealed TGF-β to stimulate the expression and Fyn-dependent phosphorylation of IDO, enabling this protein to also serve as a scaffolding molecule for downstream signaling ultimately leading to the expression of both TGF-β and IDO itself. As opposed to the rapid and short-lived induction of IDO expression by IFN-γ, the stimulation of IDO expression by TGF-β is thought to be durable and to serve as a mechanism for generating long-term immune tolerance. This post-translational modification of IDO by TGF-β also has additional implications in terms of its regulation. In inflammatory conditions, exposure to IL-6 promotes the degradation of IDO by upregulating suppressor of cytokine signaling 3 (SOCS3), which binds to a phosphorylated immunoreceptor tyrosine-based inhibitory motif (ITIM) in IDO and promotes its proteosomal degradation (22).

Additional post-translational regulatory mechanisms have been elucidated, which are also capable of contributing to the regulation of IDO activity. This includes the nitration of various IDO tyrosine residues by peroxynitrite, a byproduct of nitric oxide (NO) and superoxide, which has also been shown to dampen IDO enzyme activity in macrophages (23). This is consistent with other findings showing NO to directly inhibit IDO activity by binding to its active site heme moiety (24). Indeed, previous work indicates that the reduction–oxidation status of the cell is capable of modulating activity of the IDO holoenzyme by interfering with the heme biosynthetic pathway (25).

It is clear that there are multiple mechanisms, which may regulate IDO on both the transcriptional and post-translational levels. However, the biological contexts in which these regulatory

mechanisms affect IDO activity remain unclear. This is particularly true for the process of carcinogenesis, which occurs in a biochemically altered environment. Several studies have supported an important role for IDO in the generation of an immunotolerant tumor microenvironment that facilitates tumor progression (26–28). These findings indicate that the mechanisms utilized by cancers to modulate IDO expression and activity may be central to understanding the highly complex process of carcinogenesis. Here, we discuss recent studies investigating the mechanisms that cancers utilize to manipulate local IDO activity within the immune microenvironment in order to facilitate their metastatic progression.

TUMOR-MEDIATED REGULATION OF INTRINSIC IDO1 EXPRESSION

The expression of IDO by many cancer types has been correlated with inferior progression-free and overall survival (29). However, the regulation of IDO expression by malignant tissues has been poorly understood. In 2005, the first mechanism by which several solid tumors can regulate the intrinsic expression of IDO was described. This work focused on the BAR adapter-encoding gene BIN-1, a tumor suppressor previously found to be downregulated in several transformed cell lines and demonstrated to suppress the transformational activity of MYC by interacting with its N-terminus (30). Subsequent studies revealed BIN-1 to interfere with malignant transformation utilizing several mechanisms beyond the suppression of MYC activity and additional work showed BIN-1 to play the role of a tumor suppressor in several cancer types including melanoma, breast cancer, colon cancer, and prostate cancer (31–33). After noting that BIN-1 seemed to suppress the development of a transformed epithelial tumor model via an immune-dependent mechanism, Muller and colleagues noted that the deletion of *Bin-1* significantly enhanced the IFN-γ-mediated upregulation of IDO expression by tumor cells (34). Indeed, the observed enhancement in tumor growth following *Bin-1* deletion was reversed in the presence of the 1-methyltryptophan (1-MT) IDO inhibitor only in the setting of an intact immune system. These authors concluded that BIN-1 was capable of modulating IDO expression by regulating the STAT1 and NFκB signaling pathways that have been previously implicated in promoting the transcription of *Indo*, the IDO encoding gene (**Figure 2A**). This represented the initial study linking IDO regulation to an intrinsic tumor suppressor pathway by showing that the loss of *Bin1* tumor expression contributes to tumorigenesis by driving cellular proliferation while simultaneously concealing itself from detection and destruction by the host immune system. This work prompted us to conjecture that early phases of tumor initiation and growth will often require the evolution of multifunctional genes, which regulate both cell division and/or survival, as well as elements of the local immune microenvironment.

Cyclooxygenase-2 (COX2), another modulator of IDO expression and activity, has also been implicated in the process of tumorigenesis and may be consistent with this hypothesis (35). COX2 represents the inducible isoform of the cyclooxygenases and plays a critical role in eicosanoid biosynthesis, including the prostaglandins and leukotrienes. The initial data linking COX2 biology with carcinogenesis was provided by genetic studies showing that when $APC^{\Delta 716}$ mice, which model the colon cancer syndrome, familial adenomatous polyposis, are crossed with mice carrying an inactivating mutation in the *Pgst2* gene encoding COX2, offspring develop exhibiting a diminished number of intestinal polyps (36). Since this study, several pre-malignant and malignant tissues have been shown to express COX2 at relatively early time points of tumorigenesis and several pro-tumorigenic functions have been ascribed to COX2 including the promotion of tumor-mediated angiogenesis, anti-apoptosis, and the generation

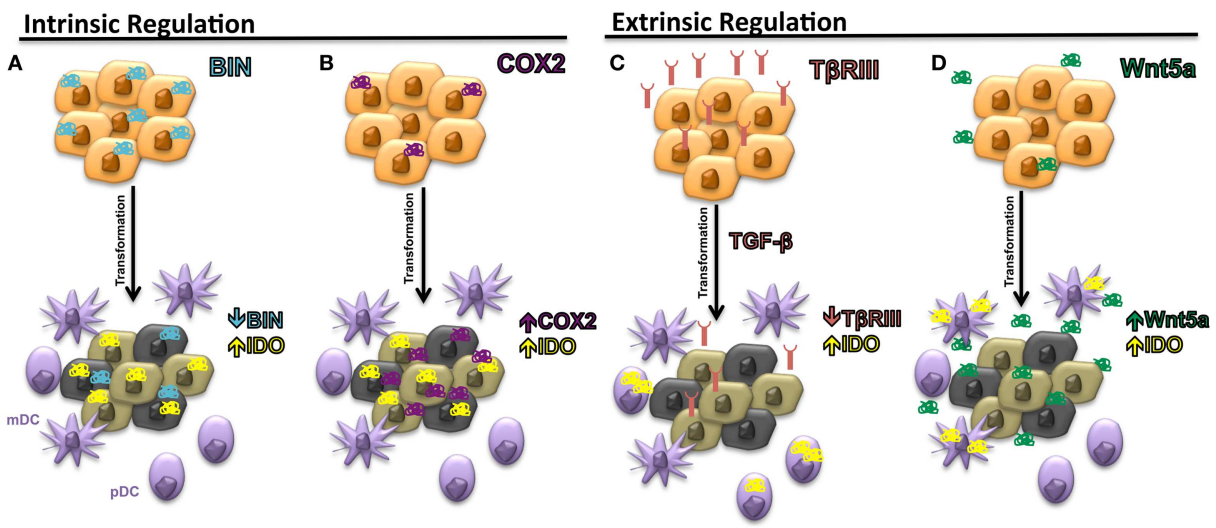

FIGURE 2 | Intrinsic and extrinsic mechanisms of IDO regulation in the tumor microenvironment. **(A)** Downregulation of *Bin1* expression leads to enhanced expression of IDO by tumor cells. **(B)** Upregulation of cyclooxygenase-2 (COX-2) expression by tumor cells stimulates intrinsic tumor expression of IDO. **(C)** Loss of *TβRIII*, the gene encoding the type IIII TGF-β receptor (TβRIII), allows for increased TGF-β paracrine signaling in the tumor microenvironment and the upregulation of IDO by local plasmacytoid DCs (pDCs). **(D)** Increased soluble Wnt5a secretion upregulates IDO expression by local myeloid DCs (mDCs).

of the epidermal growth factor receptor ligand, amphiregulin (37). One of the downstream products of COX2 activity, prostaglandin E2 (PGE2), has been previously demonstrated to interfere with T cell and DC function (38). Additional studies have shown COX2 and IDO expression to correlate in both human breast cancer cell lines and human breast cancer primary tissues while other investigators have found PGE2 to directly stimulate IDO expression (14, 39). Interestingly, COX2 inhibitors enhance the anti-tumor effects of DC-based vaccines and promote tumor-specific T cell responses in the MMTV-*neu* autochthonous murine mammary carcinoma model further suggesting an immunologic role for COX2 in cancer (35). Further studies have also shown COX2 inhibitors to augment a MUC1-based vaccine in a transgenic pancreatic cancer model in a manner that depended on suppressed IDO activity within tumor tissues (40). Similar roles for COX2 in promoting Tregs in non-small cell lung cancer and in elevating IDO expression in acute myeloid leukemia have also been described (41, 42). Together, these studies suggest that COX2 represents an important regulator of IDO function within malignant tissues (**Figure 2B**). While these studies focused on investigating the relationship between COX2 expression and the intrinsic regulation of IDO expression by tumor cells, a more recent study has shown a COX2-expressing MCF-7 breast cancer cell line to induce IDO expression by co-cultured fibroblasts, suggesting that paracrine IDO regulatory networks may also be relevant during the process of carcinogenesis (43).

CHARACTERIZING TUMOR-MEDIATED REGULATION OF EXTRINSIC IDO1 EXPRESSION

Although it is unclear if the cell type expressing the IDO enzyme may affect its ultimate immunologic impact in the setting of cancer, previous investigators have shown a relationship between local DC expression of IDO and poor clinical prognosis in patients with melanoma (28, 44). In light of these data, we reasoned that tumor-derived soluble factors may have evolved to manipulate local DC expression of this critical immune regulatory mechanism. Further, in light of the dual role of the BIN-1 tumor suppressor described above, we searched for soluble factors already described to have a pro-tumorigenic role in the literature. These criteria led us to the type III TGF-β receptor (TβRIII) that functions as a co-receptor for the TGF-β signaling pathway by binding and presenting all three TGF-β isoforms to the type I and II TGF-β receptor signaling complex (45). TβRIII has been independently implicated in suppressing cellular migration in several experimental systems through a β-arrestin2-mediated mechanism (46, 47). Consistent with the criteria discussed above, earlier studies also revealed TβRIII to be shed at the cell surface and for its soluble form, sTβRIII, to bind and suppress downstream TGF-β-mediated signaling, effectively functioning as a molecular sink for the TGF-β cytokine (48, 49). Additional work has demonstrated human breast cancers to downregulate TβRIII expression by loss of heterozygosity and for TβRIII to impede metastatic progression of the 4T1 murine breast cancer model (50). This loss of TβRIII expression has since been shown to occur during the progression of several additional cancers including pancreatic, lung, and prostate cancers (51–53). Notably, the downregulation of TβRIII has also been demonstrated to occur at a relatively early stage of tumorigenesis, exemplified by the loss of TβRIII in tissue specimens histologically characterized as ductal carcinoma *in situ*, an early precursor to invasive ductal carcinoma of the breast. Additional studies showing the loss of TβRIII to promote the epithelial-to-mesenchymal transition (EMT) in pancreatic cancer further suggests that this genetic alteration occurs at a relatively early time point during carcinogenesis (53).

TGF-β has been described as a pleiotropic regulator of the immune system capable of modulating both IDO activity and several additional immunosuppressive pathways (54). By inhibiting both T cell and NK cell proliferation and activation, as well as promoting the differentiation of Treg populations, the TGF-β cytokine plays an important role in maintaining peripheral immune tolerance. We, therefore, hypothesized that the loss of TβRIII expression by developing malignancies would allow for enhanced TGF-β-dependent signaling in the tumor microenvironment, thereby inhibiting local immune surveillance mechanisms and ultimately promoting tumorigenesis (55). Using the 4T1 murine breast cancer model, we initially determined that TβRIII expression suppressed metastatic progression only in immunocompetent hosts. Consistent with this observation, further work revealed the loss of TβRIII to be associated with the development of an immunotolerant microenvironment characterized by a decrease in the number of $CD8^+$ T cells and a corresponding increase in the $CD4^+FoxP3^+$ Treg population in both breast cancer and melanoma model systems.

These findings led to the identification of an association between TβRIII expression and suppressed levels of IDO in both the tumor bed and within the tumor-draining lymph nodes (TDLNs). We determined that the loss of TβRIII expression correlated with the upregulation of IDO by pDC populations within TDLN tissues, the same cell population that was previously noted to be important for the expression of IDO within TDLN tissues (**Figure 2C**) (21, 27). These results also corresponded with diminished pDC and whole TDLN tissue IDO enzyme activity when recovered from mice bearing TβRIII-expressing tumors. Since sTβRIII is an effective inhibitor of downstream TGF-β signaling, we hypothesized that TGF-β was a major mediator of pDC IDO activity in the tumor microenvironment and confirmed the findings of Pallotta and colleagues by showing TGF-β treatment of purified pDCs to enhance IDO expression and enzymatic activity (21). We then demonstrated this paracrine signaling mechanism to be functionally relevant by demonstrating purified pDCs derived from mice harboring TβRIII-downregulated tumors, to suppress *in vitro* T cell proliferation in an IDO-dependent manner. Further, using a doxycycline-inducible system, we showed that the earlier the alteration in TβRIII expression by a developing tumor, the more profound the effect on the local tumor microenvironment. These results suggest that modulation of the immune microenvironment at earlier stages of tumorigenesis is associated with a greater impact on tumor progression.

The work described above illustrates the potential for genetic alterations within the tumor to impact local DC function and to subvert immunosurveillance. Given the critical role that these APCs play in orchestrating the anti-tumor immune response, it follows that the evolution of various mechanisms to convert local DCs into a tolerogenic state would be quite advantageous for a developing tumor. The pathways for driving DC tolerogenesis in

the setting of cancer remain poorly characterized; however, the β-catenin signaling pathway has emerged as a potentially important component of this process. This is supported by data showing that activation of the β-catenin pathway in myeloid DCs (mDCs) conditioned these APCs to promote the generation of IL-10-expressing $CD4^+$ T cells capable of suppressing the autoimmune phenotype of a mouse model of multiple sclerosis (56). These findings were further substantiated by *in vivo* experiments using the CD11c-cre × β-$cat^{lox/lox}$ transgenic model demonstrating the DC-specific β-catenin pathway as an important regulator of Treg differentiation in gastrointestinal tissues (57). These studies raised the possibility that tumors may promote DC tolerization in the tumor microenvironment via the expression of soluble Wnt ligands. Interestingly, several members of the Wnt ligand family have been noted to play a role in carcinogenesis. Indeed, mechanistic studies have revealed Wnt5a to promote melanoma cell migration and invasion, ultimately leading to disease metastases (58). In addition, increased Wnt5a expression levels in melanoma tissues as well as diminished levels of the soluble Wnt antagonist, Dkk-1, have been associated with an inferior clinical outcome in patients with advanced melanoma (59–63).

Together, the above reports led us to screen the conditioned media of several human melanoma cell lines for their ability to stimulate downstream β-catenin signaling activity. This work consistently showed that melanoma-derived conditioned media was capable of inducing this signaling pathway in both reporter cell lines and primary DCs *in vitro* (64). Further work using the *Tyr:CreER;*$Braf^{CA}$*;*$Pten^{lox/lox}$ inducible transgenic melanoma model, showed that TDLN DCs and tumor-infiltrating DCs associated with developing melanomas expressed elevated levels of known β-catenin target genes including *Axin2*, *Ccnd1*, *C-myc*, and *Tcf-7* relative to DCs derived from more distant lymph node tissues. This local paracrine β-catenin signaling effect was then confirmed *in situ* within the melanoma stroma and within TDLN tissues by confocal microscopy using the Tg(TCF/Lef1-HIST1H2BB/EGFP)61Hadj/J transgenic reporter strain, which encodes an EGFP reporter downstream of a β-catenin-responsive promoter containing tandem TCF/LEF1 transcription factor binding elements (65).

After verifying that this signaling pathway could be induced within mDCs in an autochthonous model of melanoma, we demonstrated that β-catenin was promoting tolerogenic DC development by regulating the downstream expression of IDO. Although recent studies had shown Wnt-mediated signaling to stimulate expression of the immunosuppressive factor IL-10 by DCs, the effects on IDO expression were unknown (61). Using a variety of methods including pharmacological inhibitors, promoter–reporter systems, immunofluorescence microscopy, and chromatin immunoprecipitation, we were able to demonstrate that Wnt ligands robustly induced the durable upregulation of both IDO expression and enzymatic activity by bone marrow-derived DCs in a β-catenin-dependent manner. By performing blocking experiments, we further noted that the soluble Wnt5a ligand was the dominant factor in melanoma-conditioned media in the induction of IDO by DCs and that Wnt5a was capable of promoting IDO expression in an IFN-γ-independent manner (**Figure 2D**). Importantly, as opposed to several other stimuli including Wnt3a, additional work revealed Wnt5a conditioned DCs to significantly promote the differentiation and expansion of naïve $CD4^+$ T cells into Tregs in an IDO-dependent manner. Indeed, further *in silico* gene expression analysis has revealed a significant association between *Wnt5a* and *Foxp3* gene expression levels in human melanomas. Similar to BIN-1, COX2, and TβRIII above, this work implicates Wnt5a as a factor with dual roles in carcinogenesis including tumor invasion and metastasis, as well as the suppression of local immune surveillance. Similar to TβRIII, Wnt5a has also been implicated in the promotion of EMT in both pancreatic cancer and gastric cancer (66, 67). This finding implies that Wnt5a is upregulated and is capable of modulating the immune microenvironment at an early time point during the process of carcinogenesis suggesting that Wnt5a likely has an impact on the establishment of immune privilege during the earliest stages of transformation.

THERAPEUTIC IMPLICATIONS OF IDO REGULATORY PATHWAYS IN CANCER-MEDIATED IMMUNE EVASION

Although recent advances in immunotherapy have made substantial strides in improving clinical responses in patients with advanced cancers, a significant fraction of these patients continue to fail therapy. In light of the diverse array of immune evasion mechanisms that individual cancers are able to employ to escape detection and destruction by the host immune system, it seems that combinatorial therapies, which target different aspects of immune suppression will be necessary to fully realize the promise of immunotherapy. While the majority of immunotherapy development has targeted T cell-expressed negative regulators such as CTLA-4 and PD-1 (68, 69), few agents are currently under investigation, which are capable of modulating tolerogenic DCs in the tumor microenvironment. An exception to this includes the IDO inhibitor, 1-methyltryptophan, which was shown to enhance chemotherapeutic effects in both the murine orthotopic 4T1 and the autochthonous MMTV-*neu* breast cancer models (70). Given these encouraging findings, high throughput screening has been employed to identify improved compounds for further clinical trial development (71–73). Although single agent efficacy has been modest (74), recent reports are showing encouraging clinical responses when combined with anti-CTLA-4 monoclonal antibody therapy while several other combination studies are ongoing (75–79) (**Table 1**). These results suggest that immunotherapeutic regimens employing a combinatorial approach including T cell-targeted immune checkpoint inhibitors and agents capable of reversing tumor-mediated immune evasion mechanisms have great promise.

In addition to targeting the IDO immunoregulatory enzyme itself, pharmacological strategies designed to interfere with the previously discussed regulatory pathways of IDO have theoretical advantages (**Figure 3**). First, evidence showing that TGF-β-induced IDO in specific DC subsets is capable of maintaining immune tolerance via a signaling mechanism that is independent of its enzymatic activity suggests that inhibitors targeting the active site of IDO may have limited clinical benefit (21). Second, IDO is likely to only be a component of the tolerogenic DC program induced by specific tumor-derived mediators, implying that inhibition of the upstream signals that activate this program

Table 1 | Active clinical trials investigating the activity of IDO inhibitors in advanced cancer.

Agent	Regimen	Disease	Sponsor	ClinicalTrials.gov Identifier	Phase of development	Reference
NLG-919	Monotherapy	Advanced solid tumors	New link genetics	NCT02048709	I	(72)
Indoximod	Temozolomide	Glioblastoma multiforme	New link genetics	NCT02052648	I/II	(75)
Indoximod	Docetaxel	Breast cancer	New link genetics	NCT01792050	II	(76)
Indoximod	Sipuleucel-T	Prostate cancer	New link genetics	NCT01560923	II	(77)
Indoximod	Ipilimumab	Melanoma	New link genetics	NCT02073123	I/II	(78)
INCB024360	Ipilimumab	Melanoma	Incyte Corp.	NCT01604889	I/II	(74)
INCB024360	Pembrolizumab	Lung cancer and other solid tumors	Incyte Corp./Merck & Co.	NCT02178722	I/II	clinicalTrials.gov
INCB024360	Anti-DEC-205/NY-ESO-1 vaccine and poly-ICLC	Ovarian, fallopian, peritoneal cancers	Fred Hutchinson Cancer Center/CITN/ Celldex Therapeutics	NCT02166905	I/II	clinicalTrials.gov
INCB024360	MELITAC multipeptide vaccine	Melanoma	Fred Hutchinson Cancer Research Center/CITN/ Incyte Corp.	NCT01961115	II	clinicalTrials.gov

Ipilimumab, anti-CTLA-4 monoclonal antibody (Bristol-Myers Squibb). Pembrolizumab, anti-PD-1 monoclonal antibody (Merck & Co.). anti-DEC-205-NY-ESO-1, DC-targeted antibody-peptide fusion vaccine (CDX-1401, Celldex Therapeutics). Poly-ICLC, polyinosinic-polycytidylic acid and poly-L-lysine double-stranded RNA TLR3 agonist (Oncovir, Inc.). MELITAC multipeptide vaccine, emulsion of a mixture of 12 class I MHC-restricted melanoma peptides (University of Virginia), CITN, Cancer Immunotherapy Network.

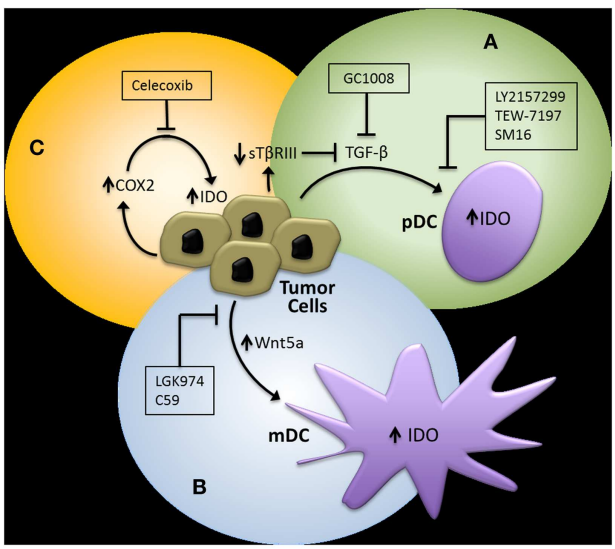

FIGURE 3 | Potential pharmacological strategies for suppressing IDO expression in the tumor microenvironment. **(A)** TGF-β inhibitors. GC1008, pan-TGF-β isoform monoclonal antibody (Genzyme), LY2157299, type I TGF-β receptor serine/threonine kinase small molecule inhibitor (Eli Lilly), TEW-7197, type I TGF-β receptor serine/threonine kinase small molecule inhibitor (MedPacto, Inc.). **(B)** Wnt inhibitors. LGK974, Porcn acyl transferase small molecule inhibitor (Novartis). **(C)** Cyclooxygenase-2 (COX2) Inhibitors. Celecoxib (Pfizer, Inc.). pDC, plasmacytoid DC; mDC, myeloid DC.

are more likely to have greater clinical efficacy. This is exemplified by TGF-β, which is known to induce other immunosuppressive pathways involving a variety of cellular targets (54).

Since our studies have indicated that tumor-derived TGF-β is capable of inducing IDO expression by pDCs and that this effect is enhanced upon loss of TβRIII in a murine breast cancer model, we investigated the ability of an oral type I TGF-β receptor serine/threonine kinase inhibitor (SM16) to augment the immunologic response of a Her2/neu vaccine. This work confirmed that a type I TGF-β receptor serine/threonine kinase inhibitor could synergistically enhance the $CD8^+$ T cell host immune response to Her2/neu and effectively suppress the progression of a Her2/neu-expressing 4T1 breast cancer model (55). Given these findings as well as the supporting data suggesting that the loss of TβRIII in murine melanoma models also promotes the development of an immune suppressive microenvironment, we investigated the combination of another oral type I TGF-β receptor kinase inhibitor currently in clinical trial development, LY2157299 monohydrate (80, 81), with anti-CTLA-4 monoclonal antibody therapy in the *Tyr:CreER;Braf^{CA};Pten$^{lox/lox}$* transgenic melanoma mouse model (82). Consistent with our previous findings, this combinatorial treatment approach also generated a synergistic anti-tumor response, effectively enhancing the tumor-infiltrating $CD8^+$ T cell/Treg ratio and suppressing both primary melanoma development and the establishment of distant metastasis.

As described previously, emerging data suggest that the tumor-mediated expression of Wnt5a contributes to the generation of an immunotolerant microenvironment. We, therefore, hypothesized

that the inhibition of Wnt5a-mediated signaling would also augment immunotherapy efficacy in melanoma. Several strategies to inhibit the Wnt-β-catenin pathway have been investigated; however, one of the more promising approaches is targeting the membrane-bound *O*-acyltransferase, Porcn, which catalyzes the palmitoylation of all vertebrate Wnt ligands, a step necessary for effective secretion of the Wnt soluble protein family (83, 84). This work has led to the introduction of LGK974, a small molecule Porcn acyltransferase inhibitor, into early phase clinical trials (85). To determine if the inhibition of Wnt secretion by targeting Porcn would be an effective approach for reversing melanoma-mediated immune suppression, we genetically silenced Porcn expression by the B16 murine melanoma model and performed several *in vivo* tumor assays. This new B16-PORCN^{KD} cell line was found to exhibit impaired Wnt secretion, suppressed tumor growth *in vivo*, and for this to be associated with both enhanced levels of infiltrating CD8$^+$ T cells and suppressed levels of PD-1-expressing tumor-infiltrating lymphocytes. With this data, we then evaluated the ability of a commercially available pharmacological inhibitor of Porcn, C59, to reverse B16-mediated immune suppression (86). Given as monotherapy to mice bearing B16 melanomas, this agent did not seem to exhibit a significant anti-tumor effect, however, when combined with anti-CTLA-4 monoclonal antibody therapy, a synergistic enhancement in activated 41BB$^+$ TRP2-specific CD8$^+$ tumor-infiltrating lymphocytes were observed along with B16 tumor growth suppression. Together, these data support the use of combinatorial immunotherapy strategies that involve agents capable of interfering with tumor immune evasion pathways including the upregulation of local IDO expression.

CONCLUSION

Studies focused on understanding the interplay between tumor development and the host immune system are now revealing an intimate relationship between the processes of tumor invasion and metastasis and the active induction of immune tolerance. Rather than developing as a response to immune-mediated selective pressure, we hypothesize that some immune evasion mechanisms are capable of developing at a very early stage in carcinogenesis and simultaneously promoting tumor invasion while also interfering with tumor detection by the host immune system. The pathways that we have found to meet these criteria are induced by intrinsic genetic alterations, resulting in the downregulation of both the BIN-1 and TβRIII tumor suppressors and the upregulation of the pro-tumorigenic factors, COX2 and Wnt5a. Interestingly, this body of work also highlights important differences in cell-specific IDO expression kinetics. While IFN-γ is a rapid inducer of IDO expression in many cell types, studies are now demonstrating that the loss of *TβRIII* in a TGF-β^{hi} tumor microenvironment promotes durable IDO expression by pDCs while the upregulation of the Wnt5a oncogene results in durable IDO expression by mDC populations (**Figures 2C,D**). We expect for several other tumor-mediated soluble factors or perhaps exosome-derived factors to also regulate IDO expression via similar paracrine signaling mechanisms. Their identification will be important for therapeutic development as well as for the establishment of predictive biomarkers to determine when these novel therapeutic strategies would be most effectively employed. Importantly, pre-clinical experimental investigation, to date, suggests that the use of a pharmacologic agent to inhibit these tumor-mediated evasion pathways which target IDO activity effectively synergize with immune checkpoint blockade. These data strongly support the physiologic relevance of these novel immune evasion pathways, which target IDO activity within the tumor microenvironment.

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Cancer immunotherapy by targeting IDO1/TDO and their downstream effectors

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The tryptophan (TRP) to kynurenine (KYN) metabolic pathway is now firmly established as a key regulator of innate and adaptive immunity. A plethora of preclinical models suggests that this immune tolerance pathway – driven by the key and rate-limiting enzymes indoleamine-2,3-dioxygenase and TRP-2,3-dioxygenase – is active in cancer immunity, autoimmunity, infection, transplant rejection, and allergy. Drugs targeting this pathway, specifically indoleamine-2,3-dioxygenase, are already in clinical trials with the aim at reverting cancer-induced immunosuppression. In the past years, there has been an increase in our understanding of the regulation and downstream mediators of TRP metabolism, such as the aryl hydrocarbon receptor as a receptor for KYN and kynurenic acid. This more detailed understanding will expand our opportunities to interfere with the pathway therapeutically on multiple levels. Here, we discuss the perspective of targeting TRP metabolism at these different levels based on reviewing recent insight into the regulation of TRP metabolism and its downstream effectors.

Keywords: IDO, TDO, AhR, tumor immunity, tryptophan metabolism

INTRODUCTION

The catabolism of the essential amino acid tryptophan (TRP) is a central pathway maintaining the immunosuppressive microenvironment in many types of cancers. The classic concept proposes that tumor cells or myeloid cells in the tumor microenvironment or draining lymph nodes express high levels of indoleamine-2,3-dioxygenase 1 (IDO1), which is the first and rate-limiting enzyme in the degradation of TRP. This enzymatic activity results in the depletion of TRP in the local microenvironment and subsequent inhibition of T cell responses. T cells sense low TRP levels via uncharged tRNAs and subsequently activating the kinase general control non-derepressible 2 (GCN2) and initiating an amino acid starvation response resulting in cell cycle arrest and cell death. This rather non-specific metabolic pathway exerts immunosuppression in the local microenvironment as T cells are particularly sensitive to low TRP levels. This IDO1-centered concept is supported by numerous preclinical studies in models of tumor immunity, autoimmunity, infection, and allergy. More recent preclinical studies, however, propose an alternative route of TRP degradation in tumors via the enzyme TRP-2,3-dioxygenase 2 (TDO), which was previously believed to be liver- and neuron-specific. Tumor cells and possibly specialized myeloid cells may express and catabolize TRP via TDO instead of or in addition to IDO1. Thus, TDO may represent an additional target for cancer immunotherapy, while both enzymes ought to employ identical downstream effectors, such as GCN2.

The effector function of GCN2 in the context of cancer immunity, however, is less well understood and established. In addition, several studies have proposed that immunosuppression by TRP degradation is not solely a consequence of lowering local TRP levels but also of accumulating high levels of TRP metabolites. This alternative or additional concept is supported by studies demonstrating that T cell responses are inhibited by TRP metabolites, mainly by binding to the aryl hydrocarbon receptor (AHR), a cytoplasmic transcription factor, previously believed to be solely responsible for detoxification of polyaromatic hydrocarbons. The importance of the AHR in regulating autoimmunity and tumor immunity is supported by preclinical studies and analyses of human tumor tissue demonstrating that binding of the TRP metabolite kynurenine (KYN) to the AHR results in reprograming the differentiation of naïve CD4+ T-helper (Th) cells favoring a regulatory T cells phenotype (Treg) while suppressing the differentiation into interleukin-17 (IL-17)-producing Th (Th17) cells. Notably, activation of the AHR also results in promoting a tolerogenic phenotype on dendritic cells (DC). The AHR seems to be required for the induction of IDO in dendritic cells (1) and stimulation with the poisonous AHR-agonist 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) was shown to induce IDO expression in dendritic cells (DC) (2), suggesting a feed-forward loop of immunosuppressive TRP metabolism. The role of the AHR on CD8+ effector T cells is less well understood.

While first clinical trials with IDO1 inhibitors are underway, this review aims at putting the recent advances in understanding the immunobiology of TRP catabolism via IDO1/TDO in therapeutic perspective for cancer immunotherapy.

MULTIPLE THERAPEUTIC TARGETS IN TRYPTOPHAN CATABOLISM

Current preclinical studies suggest that the opportunity to interfere with immunosuppressive TRP catabolism goes well beyond

restoring TRP levels by inhibiting the enzymatic activity of IDO1. First, tumors may catabolize TRP by alternative enzymatic routes such as TDO. A survey of cancer cell lines indicates that 16% of tumor cell lines are IDO1 positive, while 19% are TDO positive and 15% express both TDO and IDO1 (3). These observations suggest that targeting TDO may complement IDO1 inhibition. Remarkably, IDO1 inhibitors available to date do not cross-inhibit TDO and vice-versa, probably due to low sequence homology of these two enzymes despite similar enzymatic properties. Second, several studies have indicated that IDO1 is intricately linked to an oncogenic signaling pathway, opening new therapeutic avenues to inhibit IDO on a more upstream transcriptional or translational level. Promising upstream targets of IDO1 include KIT, signal transducer and activator of transcription 3 (STAT3), and the tumor suppressor Bin1 (4–6). Also, there is new evidence of IDO1 promoting self-tolerance via non-enzymatic signaling pathways involving transforming growth factor-β (TGF-β) (7). This non-enzymatic activity of IDO1 would only be targeted by strategies interfering with upstream pathways regulating IDO1 transcription and/or translation. Third, low TRP levels, which are believed to mediate to a relevant extent the immunosuppressive activity of IDO1, are sensed by the stress kinase GCN2 activated in T cells in low TRP conditions. The specific pathways transducing the immunosuppressive signals by activated GCN2 in T cells are yet to be identified. Alternatively or in addition, low TRP levels may be sensed by the signaling complex mammalian target of rapamycin (mTOR), which may provide a TRP sufficiency signal not only in cancer cells but also in T cells. Fourth, TRP metabolites such as KYN, 3-hydroxy-kynurenine (3-HK), and kynurenic acid (KA), which may accumulate in the local microenvironment due to high activity of IDO1 and/or TDO, actively suppress T cell responses. These activities are – at least in part – mediated by binding to the AHR, which is – among other effects – involved in the control of differentiation and activation of Tregs. The role of the orphan G protein-coupled receptor GPR35 as a receptor for KYN and KA in immunity is currently unclear. Fifth, transcellular transport systems responsible for shuttling TRP and its metabolites in and out of its target cell include several promiscuous but possibly other more specific transporters whose function in regulating immune responses are not well understood, but, which may serve as potential therapeutic targets, for instance, with the aim at maintaining high intracellular TRP levels in immune effector cells despite low extracellular TRP levels.

Conceptually, these five distinct hubs may serve as potential therapeutic targets interfering with immunosuppressive TRP catabolism in the context of cancer and possibly other immune-mediated diseases associated with an activation of TRP metabolism (**Figure 1**). The potential therapeutic opportunities and challenges associated with these hubs are discussed in the following chapters.

TARGETING IDO1: FINDING THE RIGHT COMBINATION PARTNER

IDO1 is now firmly established target of drug discovery in cancer immunotherapy. The first IDO1-inhibitor, 1-methyl-TRP, is a mixture of the two racemic isoforms 1-methyl-L-TRP (1-L-MT) and 1-methyl-D-TRP (1-D-MT). While 1-L-MT is the classic non-competitive inhibitor of IDO1, 1-D-MT has been suggested to be less active in inhibiting IDO1 (8), but showing higher potency in reversing IDO-mediated T cell suppression (9, 10). 1-D-MT is being developed clinically as an IDO-inhibitor (indoximod, NLG8189) for the treatment of several cancers with the aim at reversing cancer-associated immune suppression. Reversal of tumor-associated immune suppression by 1-D-MT appears to be dependent on host IDO1 expression in preclinical models (9). In addition or alternatively to direct IDO inhibition, 1-D-MT may interfere with transcellular TRP transport (11), thereby providing via mTOR a TRP sufficiency signal to the cell (12) and by additional off-target effects (13). As 1-L-MT is principally capable of exerting the same effects, it is not yet entirely clear why 1-D-MT is more effective in restoring T cell activity under physiological conditions (12). 1-D-MT was reported to preferentially target IDO2 (8). IDO2 is an IDO-related enzyme with a different expression pattern. The physiological relevance of IDO2 particularly in humans remains unclear (8, 10, 14); further studies are required to determine whether it may serve as a suitable target for cancer immunotherapy.

In addition to directly inhibiting IDO enzymatic activity, second-generation IDO1 inhibitors such as INCB024360 may have a more favorable pharmacokinetic profile. While phase I clinical trials with these orally available compounds have demonstrated safety (15) and indicated biological efficacy based on serum parameters demonstrating reversal of TRP depletion and KYN accumulation, it is questionable whether these compounds will be effective by themselves. The major challenge for designing future clinical trials will be to find the appropriate combination partner. In this respect, preclinical studies have provided valuable insight into potential strategies to amplify the efficacy of IDO1 inhibitors. Initial experiments have applied 1-MT in combination with chemotherapy (6). Consequently, clinical phase I trials have combined indoximod with chemotherapy (16–18). Based on early observations that IDO1 is induced in DCs following ligation of B7 molecules by cytotoxic T-lymphocyte-associated protein 4 (CTLA4) (19–21), a recent preclinical study suggested that IDO1 is a critical resistance mechanism attenuating the efficacy of anti-CTLA4 antibodies in cancer immunotherapy. Interestingly, in this study this crucial role as a resistance factor is not restricted to anti-CTLA4 antibodies but also antibodies to programed cell death 1 (PD-1) and programed death-ligand 1 (PD-L1) (22). Trials combining indoximod (23) or INCB024360 (24) with the anti-CTLA4 antibody ipilimumab in patients with melanoma are underway. Conceptually and also supported by preclinical studies, IDO1 inhibition may enhance the efficacy of active cancer vaccines as it may break cancer-induced tolerance. Two phase II studies are currently evaluating this combination approach (25, 26).

It has been shown that prostaglandin E2 (PGE2) expression in a cancer setting induces regulatory T cells, promotes T cell anergy through direct effects on T cells and indirect effects via antigen presenting cells (APCs) (27), thus, like IDO, shifting the immune system toward a tolerogenic phenotype and promoting tumor progression. Cyclooxygenase 2 (COX2), key enzyme in the production cascade of PGE2, like IDO, is expressed at low levels by most somatic cells but is upregulated in many types of cancer cells and tumor-infiltrating APC (27). Interestingly, PGE2 may

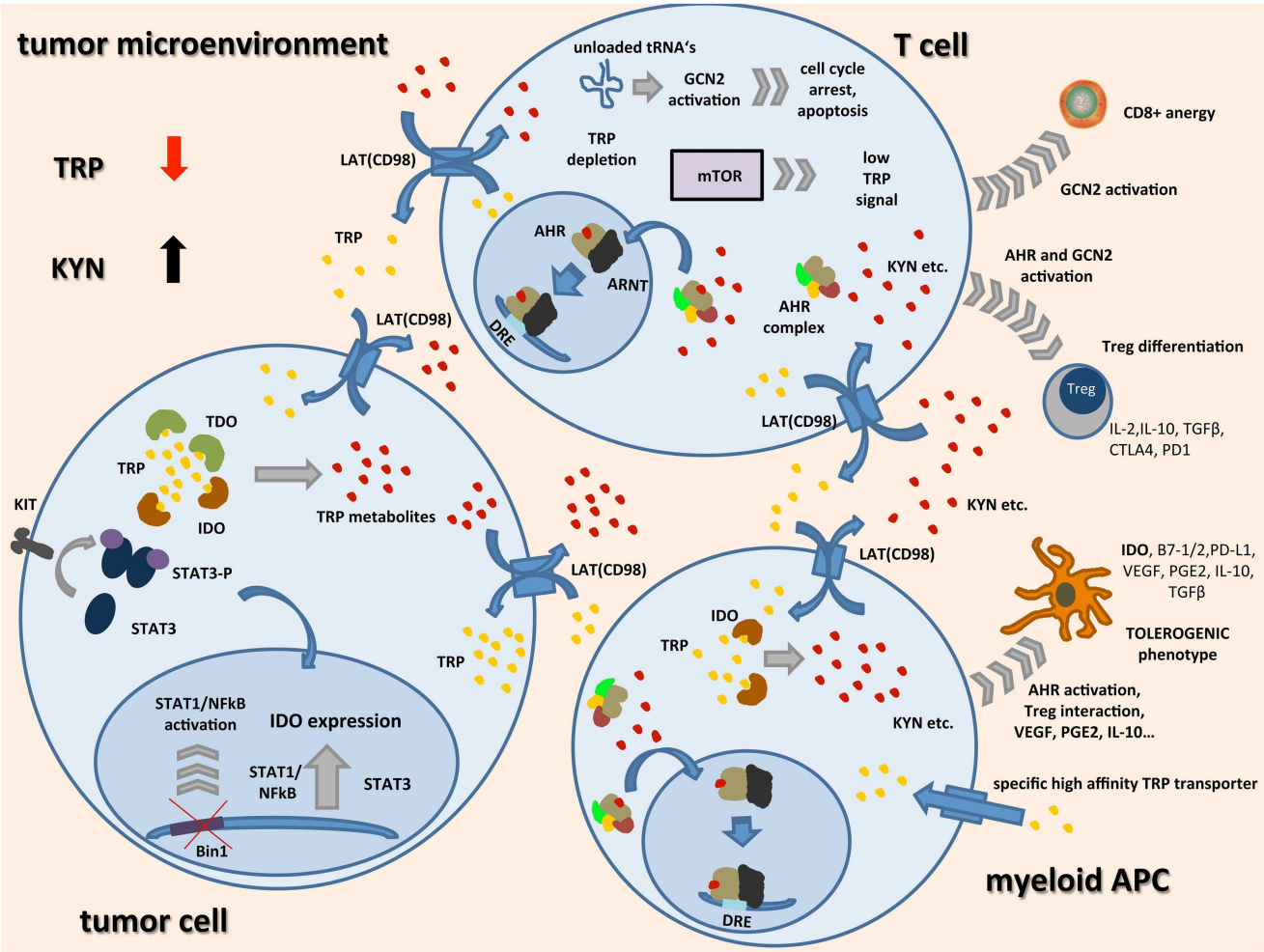

FIGURE 1 | Schematic representation of TRP catabolism in the tumor microenvironment: expression of indoleamine-2,3-dioxygenase (IDO) and tryptophan-2,3-dioxygenase (TDO) by tumor cells and infiltrating immune cells leads to depletion of the essential amino acid tryptophan (TRP) while metabolites such as kynurenine (KYN) accumulate. Upon binding of TRP catabolites, the aryl hydrocarbon receptor (AHR) translocates to the nucleus, dimerizes with AHR nuclear translocator (ARNT), and induces the expression of its target genes by binding to dioxin-responsive elements (DRE). In tumor cells, expression of IDO seems to be linked to oncogenic signaling pathways such as loss of tumor suppressor gene Bin1 with subsequent STAT1 and NFκB activation, and STAT3 activation through mutated receptor tyrosine kinases such as KIT. IDO expression in APCs seems to be associated with a regulatory phenotype and is among others driven by AHR activation, cell–cell contact with tumor cells, and Tregs through PD-1/PD-L1 or B7/CTLA4 interaction (not shown), and soluble factors such as PGE2, IFNγ, VEGF, and IL-10. In most cell types, transcellular TRP transport is handled by light chain glycoprotein-associated amino acid transporters (LAT) functioning as TRP/KYN antiporters. IDO expressing tumor cells and APCs additionally express high-affinity TRP transporters leading to a shift of remaining TRP away from highly TRP-dependent T cells. There, general control non-derepressible 2 (GCN2) activation through accumulation of unloaded tRNAs, mTOR signaling, and AHR activation lead to inhibition of CD8+ effector T cells while differentiation of Tregs is enhanced. These Tregs together with tolerogenic APCs further inhibit an effective anti-tumor immune response by release of immunosuppressive cytokines and inhibitory cell surface proteins.

be part of the immunosuppressive KYN-AHR feed-forward loop by driving IDO and TDO [Ref. (28) and unpublished observations]. It seems reasonable that suppression of anti-tumor immunity via PGE2 and IDO are not separately working mechanisms but rather contribute synergistically to tumor immune evasion. Thus, a combinatorial approach with IDO- and COX2-inhibitors might be an interesting option to break suppression of anti-cancer immunity.

Besides finding the right combination partner, a future challenge will certainly be the identification of cancer types and patients who will benefit from an IDO1 inhibitory approach. Current trials do not select patients based on IDO1 expression in tumor tissue or assessment of systemic IDO1 activity by analysis of TRP and its metabolites in patients' serum. It is conceivable that this approach may be most successful in cancer types, which are immunogenic *per se*, such as malignant melanoma. Another challenge will certainly be the assessment of possible escape strategies that cancers may develop. There is essentially no data on such potential evasion strategies in preclinical studies.

NON-ENZYMATIC TARGETING OF IDO1

In the past years, it has become increasingly clear that IDO1 is part of an oncogenic signature in cancer supporting the concept that cell-autonomous pathways driving cancer cells are intricately linked to an immunosuppressive phenotype (29). For instance, IDO1 is controlled by the tumor suppressor gene *Bin1* encoding the Myc-box-encoding protein 1. Loss of Bin1 in tumors results in transcriptional upregulation of IDO1 via STAT1 and *nuclear factor* (NF)-kB and subsequent escape from T cell-dependent anti-tumor immunity (6). In addition, a recent study suggests that IDO1 is also driven by oncogenic KIT signaling in gastrointestinal stromal tumors (GIST). Treatment of experimental tumors with imatinib resulted in the reversal of IDO1-mediated immunosuppression and thus activation of T effector cells and suppression of Tregs, which was dependent on IDO1, which was suppressed by imatinib (4). These data suggest that this targeted agent may derive its remarkable clinical effects in this tumor entity from its profound immunological effects and advocate for conducting preclinical studies in tumor models involving immunocompetent hosts. Based on the link between CTLA4 and IDO1, a rational therapeutic consequence of this observation is to combine imatinib with an anti-CTLA4 approach in GIST, which is currently tested in a clinical trial (30). IDO1 expression, which is classically induced by proinflammatory cytokines, is tightly controlled by STAT molecules. These pathways are also activated by oncogenic signaling pathways. For instance, STAT3, which is activated by KIT and also growth factors such as epidermal growth factor (EGF) and cytokines such as IL-6 transcriptionally activates IDO1 by binding to its promoter (5). As new compounds targeting, for instance, activated STAT3 are now in clinical trials (31), there is a great opportunity for investigating, whether the beneficial and potentially immunostimulatory effects of these agents are also dependent on IDO1. The same mechanisms that mediate transcriptional or translational activation of IDO1 may affect its stability. For instance, the STAT antagonist suppressor of cytokine signaling (SOCS) 3 promotes the active protein to bind IDO1 and promote its proteasomal degradation (32). This intriguing observation further strengthens the rational to interfere with IDO protein expression in addition or alternatively to IDO1 enzyme inhibition. Along this line are preclinical and clinical studies targeting IDO1 by a peptide vaccine (33). Here, it will be fascinating to see how elimination of IDO-expressing cells will alter the immunosuppressive tumor microenvironment and allow for a more efficient anti-tumor immunity. We are only beginning to understand the complex network interacting with IDO1. This complexity is even increased by recent observations that IDO1 may act as a signaling molecule mediating or sustaining immune tolerance independent of its enzymatic activity (7). This observation further supports the concept that IDO1 in cancer immune therapy ought to be targeted not only at the enzymatic level.

TARGETING TDO: AIMING AT A LIVER ENZYME

With two recent studies demonstrating that TRP metabolism via TDO represents an alternative route to IDO1 activity employed by tumors (3, 34), there is an interest in pharmacological targeting of TDO for cancer immunotherapy. This interest is fueled by the fact that currently available IDO1 inhibitors do not target TDO. Based on the lead structure of 680C91 (35), the indole LM10 has recently been developed with a more favorable pharmacokinetic profile (36). One concern of systemic TDO inhibition is safety. In contrast to IDO1, TDO is strongly expressed constitutively in the liver, where it is believed to be responsible for maintaining systemic TRP levels, and – albeit at lower levels – in neurons. There are two lines of evidence that TDO may be targeted safely with a specific inhibitor: currently, preclinical studies have not documented relevant liver toxicity using LM10 (3) and TDO-deficient mice develop normally and display an unremarkable phenotype except for an increased neurogenesis and a less anxious phenotype (37). The latter may be due to increased levels of 5-hydroxy-TRP (5-HTP) in the hippocampus. While these may be beneficial effects in some diseases, CNS-specific side effects ought to be closely monitored in future preclinical and clinical studies. It also needs to be taken into consideration that systemic TDO inhibition will result in increased levels of TRP metabolites such as KYN due to increased availability of TRP for IDO1 as suggested by the TDO-deficient mice. If TRP metabolites are relevant in mediating the immunosuppressive and tolerogenic effects of TRP catabolism in cancer, a dual approach needs to be considered to combine an anti-TDO strategy with inhibitors of KYN. In addition to identifying novel TDO inhibiting compounds, it is logical to test – based on the experiences with IDO1 – existing anti-cancer compounds for their potential to inhibit TDO (38). Here, the understanding of the signaling pathways driving constitutive TDO expression in tumor cells is key to interfere with this pathway. First studies aiming at deciphering these pathways have just been published and reveal fundamental differences in the regulation of TDO in cancer cells versus untransformed cells (39).

TARGETING AHR: CHALLENGING A PROMISCUOUS RECEPTOR

Several metabolites of TRP including photoproducts such as 6-formylindolo-[3,2-b]carbazole (FICZ) (40), bacterial products such as indole-3-aldehyde, phenazines, and naphthoquinones (41, 42), and plant products such as indoles, flavonoids, and polyphenoles (43) have been shown to be ligands of the AHR. Since the discovery that AHR-deficient mice are prone to autoimmunity (44, 45) and that mice expressing a constitutively active AHR are prone to develop tumors (46), it has been speculated that endogenous TRP metabolites are responsible for inducing AHR-mediated tolerance. Indeed, a recent study has demonstrated that TRP metabolites produced by IDO1 and/or TDO induce tolerance to bacterial products via the AHR (47). This study is important as it supports the concept that endogenous TRP levels produced by IDO1/TDO accumulate at levels sufficient to activate the AHR. Of note, the IC50 of KYN and KA for the AHR is in the low micromolar range (34, 48). As tumors produce high levels of these metabolites in the order of 30–50 μM, it comes as no surprise that the levels are sufficient to activate the AHR (34). As preclinical studies also suggest that the AHR is responsible for mediating – at least in part – the immunosuppressive effects of cancer-derived TRP metabolites (34, 48), the AHR represents a logical pharmaceutical target for cancer immunotherapy. Several challenges have to be met in developing AHR antagonists to cancer immunotherapy: first, the AHR is a promiscuous receptor binding

several structurally diverse molecules with different affinity. Many studies evaluating AHR ligands rely on luciferase assays measuring the transcriptional activity of dioxin responsive elements (DRE) bound by the AHR. Often times, however, compounds, which induce AHR-dependent DRE activity, are not direct AHR ligands but rather facilitate its activity as a transcription factor. Here, the fact that it has not been possible to date to crystallize the AHR for receptor–ligand interaction studies has been an important hurdle in the development of drugs binding to the AHR. Clearly, due to its promiscuity, screens based on cellular luciferase assays have to cope with a high hit rate. On the other hand, there are compounds already available with AHR antagonistic activity. Whether these compounds are capable of blocking all AHR ligands including TRP metabolites is unclear. Second, there are safety concerns with respect to pharmacological AHR inhibition. While AHR knockout mice develop normally and display only mild immunological aberrations including deficiency of specific resident immune cells in the gut and skin, challenge of these mice with drugs, which are metabolized via the AHR, may result in toxicity, which would also be observed in patients treated with AHR antagonists. Third, it is not entirely clear, which cellular and molecular mechanisms are involved in AHR-mediated tolerance to tumors. As AHR activation has been shown to induce a tolerogenic phenotype in DCs and modulate Treg differentiation, it may well be possible that the AHR acts at multiple levels of the immune compartment. Clearly, further studies are warranted to clarify these challenges before moving AHR antagonistic strategies to the clinic.

TARGETING GCN2: INTERFERING WITH MULTIPLE PATHWAYS

The classical effector pathway of immunosuppressive TRP metabolism involves the activation of the stress kinase GCN2. GCN2 is activated by uncharged tryptophanyl tRNAs accumulating in conditions of low TRP levels. Interestingly, although GCN2 has been suggested as a key mediator of the T cell suppressive effects of low TRP conditions (49), remarkably little is known about how GCN2 regulates T cell function. While in low TRP environments, GCN2 in CD8+ effector T cells is important for induction of anergy following TCR stimulation (49), in CD4+ T cells GCN2 appears to be important for the expansion and activation of regulatory T cells (50). Of note, GCN2 is also expressed in DCs where it appears to aid antigen presentation by regulating autophagy. While it is in principle conceivable that GCN2 may be a therapeutic target in the immunosuppressive TRP pathway, there are several challenges ahead: first, GCN2 is ubiquitously expressed and important in regulating response not only to amino acid deprivation but also to other forms of cellular stress including UV irradiation. Again, GCN2-deficient mice are remarkably normal but display abnormalities in regulating body weight owing to a crucial role of neuronal GCN2 in regulating eating behavior in response to nutritional cues. As cellular stress is a crucial hallmark of cancer affecting tumor cells and the tumor stroma, further studies are required to delineate the role of GCN2 in this context. Also remarkably, there is no evidence that host GCN2 is relevant in regulating tumor immunity in transplantable or spontaneous syngeneic tumor models not requiring adoptive transfer of antigen-specific T cells. Clearly, these studies have to be performed, also to enable the identification of key signaling pathways involved in GCN2-mediated alteration of T cell function in response to tumors with active TRP metabolism.

TRP TRANSPORT PATHWAYS – AN UNDERRECOGNIZED VARIABLE

Induction of TRP dioxygenase enzyme activity in general may result in a dramatic drop in extracellular TRP levels. The observation, for instance, that IDO-mediated immune suppression can be reversed in certain paradigms by supplementing TRP has led to the hypothesis that the depletion of extracellular TRP suppresses T cell function (51). This hypothesis, however, is based on the presumption that extra- and intracellular TRP pools are equilibrated. Transmembrane TRP transport is chiefly regulated by two distinct systems: the T-system (T-type amino acid transporter, TAT) and the L-system (light chain glycoprotein-associated amino acid transporter, LAT). Most cell types use the L-system to transport TRP across the cell membrane. The placenta, for instance, which is an organ with high IDO activity, solely relies on the L-system to achieve TRP influx (52). The L-system is a heterodimeric transmembrane receptor consisting of a heavy chain (4F2hc, CD98hc) and a light chain (LAT1 or LAT2), the latter representing the catalytic subunits. Interestingly, the L-system is identical with CD98, a cell surface receptor originally identified as an antigen expressed on the cell surface of tumor cells and activated T cells (53). CD98hc interacts with and modulates the cell adhesion properties of integrins (54). System L is commonly overexpressed in tumor cells and seems to be the main route for transcellular TRP transport in T cells (55). Myeloid APCs have been shown to express an additional high-affinity TRP transport mechanism (55) thus being able to take up TRP efficiently in a low TRP containing microenvironment. We have previously shown that System L functions as a TRP/KYN antiport system using a FRET-based TRP sensor (56). While T cells respond to low extracellular TRP levels with growth arrest and anergy, IDO-expressing tumor cells and myeloid APCs might maintain sufficient intracellular TRP levels through KYN/TRP exchange and high-affinity TRP transport. Hence, under TRP depleting conditions, such as cancer, it seems likely that T cells are more affected by TRP starvation and TRP is efficiently being shifted toward TRP consuming cells. The L-system not only binds TRP but also structurally related molecules. The commonly used IDO-inhibitor 1-methyltrypophan (1-MT), for instance, binds LAT1 in breast cancer cells and suppresses TRP influx and enhances TRP efflux (11).

By employing the same transcellular transport mechanisms, 1-D-MT may act as a TRP mimetic, as outlined above, and provide an intracellular TRP sufficiency signal maintaining mTOR activity also in T cells, thus restoring their activity (12). Whether cancer cells rely on TRP metabolism to maintain their NAD levels or whether this pathway represents a rescue system when *de novo* NAD synthesis is not sufficient to provide energy under certain circumstances has not been analyzed.

Collectively, these observations indicate that transmembrane TRP transport by the L-system may play a fundamental role in regulating T cell responses in low TRP environments.

Transcellular transport systems of TRP and its metabolites may thus offer an additional, yet underrecognized potential to influence

the consequences of immunosuppressive TRP metabolism at the T cell level to revert cancer-associated immune suppression.

SUMMARY

Recent advances in understanding the regulation as well as the cellular and molecular targets of TRP metabolism have expanded the opportunity to interfere with this pathway well beyond inhibiting IDO. TDO is actively pursued as a target and multiple approved drugs have been shown to interfere with IDO expression in cancer. With our increased knowledge, future therapeutic strategies will have to take downstream targets such as the AHR but also TRP transport mechanisms into consideration, particularly as these may be more easily and specifically targeted. At the same time, preclinical studies have made clear that such pathway inhibitors may not be active enough as a stand-alone therapeutic approach. Thus, the rational combination with already available and/or yet to be identified immunomodulatory strategies such as cancer vaccines or checkpoint inhibitors based on thorough basic research is warranted. Continuing basic research on this highly conserved and versatile pathway will expand not only our view on its pathophysiological relevance but will also open novel therapeutic avenues for defining novel therapeutic targets for diseases associated with an aberrant immune tolerance, such as cancer, autoimmunity, allergy, transplantation rejection, and infection.

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Tryptophan-degrading enzymes in tumoral immune resistance

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Tryptophan is required for T lymphocyte effector functions. Its degradation is one of the mechanisms selected by tumors to resist immune destruction. Two enzymes, tryptophan-2,3-dioxygenase and indoleamine 2,3-dioxygenase 1, control tryptophan degradation through the kynurenine pathway. A third protein, indoleamine 2,3-dioxygenase 2, was identified more recently. All three enzymes were reported to be expressed in tumors, and are candidate targets for pharmacological inhibition aimed at restoring effective anti-tumoral immunity. In this review, we compare these three enzymes in terms of structure, activity, regulation, and expression in healthy and cancerous tissues, in order to appreciate their relevance to tumoral immune resistance.

Keywords: indoleamine 2,3-dioxygenase, tryptophan-2,3-dioxygenase, dendritic cells, tumor, immunosuppression, tryptophan, adaptive resistance

INTRODUCTION

In the last decade, tryptophan catabolism has emerged as a powerful mechanism of peripheral immune tolerance, contributing to maintain homeostasis by preventing autoimmunity or immunopathology that would result from uncontrolled and overreacting immune responses. This is achieved through the action of enzymes catalyzing the first and rate-limiting step of tryptophan degradation along the kynurenine pathway, including indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan-2,3-dioxygenase (TDO). As a result, tryptophan is locally depleted while tryptophan catabolites accumulate, including kynurenine and its derivatives, depending on the presence of downstream enzymes in the kynurenine pathway. Although IDO1 and TDO are located in the cytosol, the metabolic modifications they induce extend to the extracellular microenvironment because tryptophan and kynurenine derivatives readily cross the plasma membrane through specific transporters (1–3). These metabolic modifications result in a local microenvironment becoming profoundly immunosuppressive, as a result of various mechanisms whose respective role remains incompletely characterized. A first mechanism is based on tryptophan depletion: T lymphocytes are extremely sensitive to tryptophan shortage, which causes their arrest in the G1 phase of the cell cycle (4). This is due, at least partly, to the induction of an integrated stress response triggered by GCN2, a stress kinase that is activated by elevations in uncharged tRNAs (5). Tryptophan shortage can also stop T-cell proliferation through inactivation of the mTOR pathway (6). A second mechanism depends on the accumulation of tryptophan catabolites: some of them, such as 3-hydroxyanthranilic and quinolinic acids, can induce T-cell apoptosis (7, 8), while other kynurenine derivatives can induce the differentiation of regulatory T-cells (9), possibly through activation of the aryl hydrocarbon receptor (AhR) (10).

One of the key hallmarks of cancer is the ability to evade immune destruction (11). To do so, tumors often hijack one or several of these tryptophan-catabolizing enzymes endowed with immunosuppressive properties. The best studied enzyme in this respect is IDO1. In 2003, our group showed for the first time that IDO1 was expressed in human tumors, and that, in a mouse tumor model, IDO1 protected tumors against immune rejection, an effect that could be reversed by pharmacological IDO1 blockade (12). Numerous subsequent studies have confirmed these findings and demonstrated, in mouse models, the benefit of IDO1 inhibitors, either alone or in combination with chemotherapy (13, 14). Several IDO1 inhibitors currently undergo clinical development. More recent work from our group demonstrated that also TDO was expressed in human tumors and showed a similar tumor-protective effect against immune rejection (15). Tumor rejection was restored by pharmacological TDO inhibition, making TDO a second attractive target for cancer therapy. More recently, indoleamine 2,3-dioxygenase 2 (IDO2), a third enzyme potentially involved in tryptophan catabolism, was identified through its high homology with IDO1 (16, 17). Its precise activity and its involvement in tumoral immune resistance have not been clearly established.

Tryptophan-2,3-dioxygenase, IDO1, and possibly IDO2 catalyze the same reaction of oxidative breakdown of the indole group of tryptophan. However, they differ in a number of important aspects such as structure, activity, regulation, and tissular expression. In this review, we discuss the aspects of these enzymes that are relevant to their potential immunosuppressive role in the context of tumoral immune resistance, in view of recent data

Abbreviations: DC, dendritic cells; IDO1, indoleamine 2,3-dioxygenase 1; IDO2M, indoleamine 2,3-dioxygenase 2; IFNγ, interferon-gamma; MoDC, monocyte-derived DC; TDO, tryptophan-2,3-dioxygenase.

highlighting their expression profile in normal and cancerous human tissues.

TRYPTOPHAN-2,3-DIOXYGENASE

Tryptophan-2,3-dioxygenase is a 167 kDa tetrameric heme-containing enzyme with constitutive and specific L-tryptophan-catabolizing activity. It is selectively expressed in the liver, where it regulates the levels of blood tryptophan. Consistent with this regulatory function, human TDO has a K_m of 190 μM for L-tryptophan, which allows it to efficiently degrade this amino acid at concentrations above its physiological level (around 80 μM) (18). As the main enzyme responsible for the metabolism of dietary tryptophan, TDO is positively regulated by tryptophan, which dramatically increases TDO expression and/or activity when present in the blood at supraphysiological concentrations (19). These features appear optimized to fulfill the main function of TDO, i.e., to maintain tryptophan homeostasis. This principal function of TDO was confirmed by the observation of a 10-fold increased tryptophan level in the blood of TDO-knockout as compared to wild-type mice (20).

As compared to liver, the expression of TDO in other normal tissues is negligible. This is illustrated on **Figure 1A**, where we compiled whole transcriptome data from the Genome Tissue Expression project (GTEx)[1] (21). In contrast, a number of human tumors express significant levels of TDO. **Figure 1A** also shows a compilation of *TDO2* expression data in 9,169 human tumors, retrieved from whole transcriptome data from The Cancer Genome Atlas (TCGA)[2] (22). Importantly, we used the same approach (DESeq) (23) to normalize the RNA-Seq data from normal and tumoral tissues, so as to allow comparison of expression levels, despite the distinct origins of the two data sets.

As expected because of its expression in normal liver, *TDO2* is expressed at high levels in hepatocarcinoma (**Figure 1A**). It is also expressed in many other tumor types, although at weaker levels and only in a fraction of the samples. These data are in line with published RT-qPCR data obtained in a smaller series of human tumor samples (15), and corroborate reported findings of constitutive TDO expression in a number of established human tumor cell lines of various histologies, including glioblastoma, colorectal carcinoma, head-and-neck carcinoma, and gallbladder carcinoma (15, 24). The precise cell type(s) expressing TDO in liver and tumors remain(s) to be identified, awaiting the availability of a reliable validated antibody.

Given the immunosuppressive effect of tryptophan catabolism, this TDO expression in human tumors prompted us to evaluate whether TDO favors tumor growth by promoting resistance to immune rejection. Due to the lack of mouse tumors naturally expressing TDO, we resorted to TDO-transfected tumor lines, and observed that TDO-positive P815 tumors were no longer rejected by mice immunized against P1A, a MAGE-type tumor antigen naturally expressed by this mastocytoma (15). Moreover, based on a previously published scaffold (25), we developed a new TDO inhibitor with a better bioavailability after oral administration (26), and observed that mice treated with this compound recovered their ability to reject TDO-expressing tumors (15). The treatment was not associated with any noticeable toxicity nor elevation of liver enzymes. These results made the proof of concept for the use of TDO inhibitors as immune modulators for cancer therapy. In parallel, another group provided evidence for a tumor cell autonomous effect of TDO expression in glioblastoma, promoting tumor progression through AhR activation by tryptophan catabolites, resulting in increased tumor cell survival and motility, and reduced anti-tumor immune responses (24). The notion that the effects of TDO on tumor growth and anti-tumor immunity would be primarily mediated by tryptophan catabolites – as opposed to tryptophan depletion – fits with the high K_m of TDO for tryptophan, which makes this enzyme more apt at producing significant amounts of tryptophan catabolites than at depleting tryptophan down to the submicromolar levels needed to impair T-cells.

Besides IDO, these results establish TDO as another immunosuppressive enzyme involved in tumor progression, and make it a promising drug discovery target. In addition, the high expression of TDO in the liver raises interesting questions regarding its potential immunosuppressive role in this organ. Liver is known as an immune tolerant organ: as opposed to other transplanted tissues, HLA-mismatched liver allografts are usually well tolerated in humans and require less immunosuppressive therapy (27). It will be interesting to evaluate whether TDO plays a role in this phenomenon and to determine whether TDO inhibition could alter the course of liver infections, primary liver tumors, or liver metastases. In line with the notion of a protective effect of TDO against excessive liver immunity and/or inflammation, TDO was recently found to play a key role in the protection against endotoxic shock: TDO-knockout mice died after injection of a LPS dose that was only sublethal in wild-type animals (28). Interestingly, AhR-knockout mice were equally sensitive to sublethal LPS, in line with the notion that a tryptophan catabolite produced by TDO – possibly kynurenine – was responsible for the protective effect by activating AhR.

INDOLEAMINE 2,3-DIOXYGENASE 1

Indoleamine 2,3-Dioxygenase has been known for more than four decades as an intracellular tryptophan-degrading enzyme whose expression is strongly induced by IFNγ in most cells. Although IDO1 catalyzes the same reaction as TDO, the two enzymes are unrelated in terms of primary structure and also differ in quaternary structure. Both are heme-containing enzymes requiring a reduced iron atom in the catalytic site, but IDO1 functions as a monomer while TDO is a tetramer of four heme-containing subunits (29). The enzymes further differ in terms of expression profile (see below) and substrate specificity, with IDO1 acting on a larger variety of substrates of the indoleamine family. Lastly, IDO1 has a much lower K_m for tryptophan (about 20 μM), making this enzyme able to significantly deplete tryptophan down to the submicromolar range (30). As such IDO1 was long considered as one of the effector molecules of IFNγ, acting to limit the growth of intracellular pathogens by depriving tryptophan. In the late 90s, the immunosuppressive function of IDO1 was discovered in mice by studying the placenta, which constitutively expresses high levels of IDO1: tryptophan catabolism by placental IDO1 was

[1] http://www.gtexportal.org
[2] http://cancergenome.nih.gov

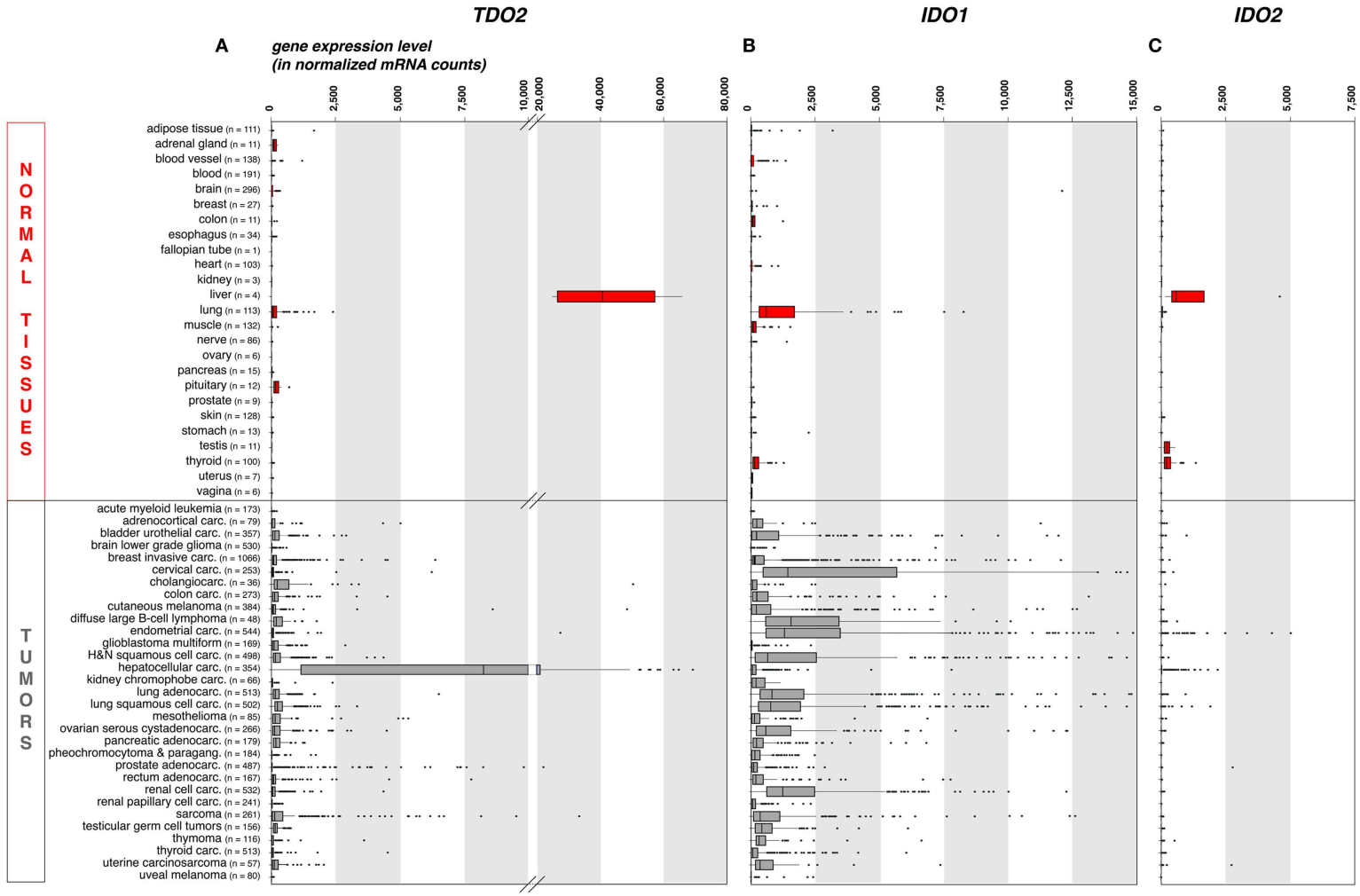

FIGURE 1 | *TDO2, IDO1,* and *IDO2* gene expression in common human normal and tumoral tissues (panels A, B and C, respectively). We used publicly available whole transcriptome data to assess the expression of these three genes in large series of human normal and cancerous tissues. We retrieved raw mRNA counts from the Genome Tissue Expression project (GTEx, see text footnote 1) and The Cancer Genome Atlas (TCGA, see text footnote 2) databases, respectively, normalized the values according to the DESeq approach (23), and represented the results as boxplot graphs for each normal tissue and tumor type, using the R statistical software and the Bioconductor package. The DESeq normalization approach allows to correct the data for the sequencing depth, which affects the number of aligned reads to the gene. It (i) computes the geometric mean for each gene; (ii) divides raw counts for each gene by the corresponding geometric mean; (iii) computes for each sample the median of the obtained ratios; and (iv) divides each gene count by the computed median for the sample. The vertical bars in each box represent, from left to right, the first quartile, median, and third quartile of the indicated sample population. The left and right edge of the horizontal line represents the minimum and maximum values, respectively, after exclusion of the outliers, displayed as individual dots. Several ($n = 37$) IDO1 outlier values >15,000 have been omitted for the clarity and concision of the graphical display. This approach provides a robust mean to assess the expression of specific genes in the context of malignant diseases, because RNA-Seq data are more precise and have less background than microarray data, and because large series of highly controlled data of various sample types are available from public databases. Carc, carcinoma; H&N, head-and-neck; Paragang, paraganglioma.

found instrumental in protecting the fetus from maternal immune rejection (31). Subsequent studies amply confirmed this immunosuppressive role of IDO1, first in dendritic cells (DC), where IDO1 can be induced by a number of stimuli that drive DC toward a tolerogenic program (32, 33), but also in tumors, which often express IDO1 and thereby resist immune rejection (12). These findings shed a new light on the function of IFNγ-induced IDO1: more than an effector mechanism of IFNγ, IDO1 now appears as part of a retrocontrol mechanism responsible for the termination of immune responses and the prevention of immunopathology, which would result from overreacting responses. Numerous preclinical studies have subsequently documented the therapeutic potential of IDO1 inhibitors in cancer therapy, either alone or in combination with chemotherapy or immunotherapy (13, 14, 34). Inhibition of the IDO1 pathway therefore represents a promising therapeutic approach, and clinical trials evaluating the first IDO1 inhibitors have started. IDO1 inhibition does not cause obvious toxicity in mouse models, and IDO1-knockout mice do not display abnormal phenotypic features (35, 36), suggesting that the approach is safe. As expected, IDO1–KO mice displayed increased sensitivity to the induction of inflammatory and autoimmune reactions (on-target effects) (37). They also displayed pericardiac calcifications, but this was observed in only 30% of females of only one strain of mice (37). Because the IDO1 expression profile differs between mouse and man, a better prediction of potential side effects of IDO1 inhibition requires a careful evaluation of IDO1 expression in human tissues. We recently developed and validated a new highly specific monoclonal antibody against IDO1, and used it to perform an extensive profiling of IDO1 expression in normal and tumoral tissues by immunohistochemistry (38). We discuss the main findings of this work in the following paragraphs.

IDO1 EXPRESSION IN HUMAN NON-CANCEROUS TISSUES

In normal human tissues, the IDO1 protein was observed in mature DC located in lymphoid organs, in some epithelial cells of the female genital tract, as well as in endothelial cells of term placenta and, surprisingly, lung parenchyma (38). These data are consistent with the expression profile of the *IDO1* transcript that we obtained from the GTEx database using the same approach as for *TDO2* (**Figure 1B**). The *IDO1* gene is weakly or not expressed in most normal tissues, with the noticeable exception of lung. Note that placenta and secondary lymphoid organs, which also contain IDO1-expressing cells, were not represented in the GTEx data set that we used.

Healthy non-lymphoid tissues

Indoleamine 2,3-Dioxygenase 1 is constitutively expressed in human and mouse placenta. In the latter, its involvement in fetal protection against maternal immune rejection has been demonstrated experimentally (31). This function cannot be readily extrapolated to human pregnancies, because the cell types that express IDO1 differ between the two species. In the mouse, IDO1 expression was observed in trophoblast cells (35, 39) whereas in human placenta it was found in endothelial cells (38, 40). The functional consequences of endothelial IDO1 expression in terms of tryptophan degradation and immune protection are not known. The same applies to lung endothelium. It is hard to consider that tryptophan catabolism by cells exposed to the blood flow can impose an immunosuppressive flavor to the microenvironment. Therefore, a cell-intrinsic function is more likely. In this regard, it is interesting to note that the density of the pulmonary vasculature was reported to be reduced in IDO1-deficient as compared to wild-type mice, suggesting a role for IDO1 in supporting lung vascular development (41). In mice, inflammation-induced IDO1 expression in endothelial cells was also reported to induce vasodilation and contribute to reduced blood pressure during severe inflammation (42). Apart from IDO1 vascular expression, lung and placenta have another exclusive feature in common. Both are respiratory organs, in which the oxygen and carbon dioxide gradients are inverted as compared to peripheral organs. Whether there is a link between these two features, and whether these gradients affect the redox status of IDO1, which controls its enzymatic activity, is not known.

Constitutive expression of the IDO1 protein has also been reported in epithelial cells from the female genital tract (38, 43). It has been hypothesized that this expression helps fight genital infections through local depletion of tryptophan, required for pathogen growth. In the mouse, one of the tissues with the highest expression of IDO1 is the epididymis. This specific tissular expression was not observed in man (38).

Healthy lymphoid tissues

Interstitial cells expressing IDO1 were observed in human lymphoid tissues, including lymph nodes, spleen, tonsils, Peyers's patches, the gut lamina propria, and the thymic medulla (38). These cells were further characterized in lymph nodes and lamina propria, and were identified as mature conventional DC. They all expressed maturation markers DC-LAMP and CD83, and lacked markers of other DC or myeloid subtypes such as CD1a, langerin, CD123, and CD163. In lymph nodes, about 50% of mature conventional DC expressed IDO1, while neither plasmacytoid DC (pDC) nor any other cell type did. This parallels the IDO1 induction observed *in vitro* during maturation of human monocyte-derived DC (MoDC), which happens during the terminal phase of the DC maturation program, and likely represents a negative feedback loop of retrocontrol of the immune response (38). Various maturation stimuli can induce IDO1 in MoDC, including LPS and the cocktail of cytokines [interleukin-(1)-beta, interleukin-6, tumor necrosing factor-alpha, and prostaglandin-E2 (PGE2)] commonly used to produce DC-based vaccines for clinical immunotherapy (44). Among the cytokines present in this maturation cocktail, PGE2 plays the key role for IDO1 induction (45). Interestingly, omitting PGE2 from the cytokine cocktail results in fully mature MoDC lacking IDO1 expression, which should be more efficient as a vaccine platform (38).

Elegant mechanistic studies have characterized the induction and function of IDO1 in murine DC, particularly in pDC (46, 47). These studies showed a key role for IDO1 in controlling tolerogenic properties of pDC, and uncovered a complex regulation integrating transcriptional induction of IDO1, proteasome-mediated IDO1 degradation triggered under inflammatory conditions by IL-6 and SOCS3, and IDO1-mediated signaling leading to long-term tolerance via transforming-growth factor beta production. It

is unclear at the present time whether those findings also apply to human pDC, which do not express IDO1 (38, 48).

IDO1 EXPRESSION IN TUMORS

At the tumor site

Tumors have diverted the immunosuppressive function of IDO1 to their own benefit in their continued efforts to resist immune rejection. A number of human tumor cell lines express IDO1 in a constitutive manner, and most other tumor lines start expressing IDO1 when exposed to IFNγ (12). In human tumor samples, IDO1 expression is commonly observed both at the RNA and protein levels (**Figure 1B**) (38, 49). Murine tumors expressing IDO1 are resistant to immunization-dependent rejection, a phenomenon that can be partially reverted by IDO1 inhibition (12). In human tumors, IDO1 expression usually correlates with a poor prognosis and is linked to a more aggressive tumor phenotype, a reduced tumor infiltrate, and an increased number of regulatory T-cells at the tumor site (49).

Our recent immunohistochemistry study analyzed 866 human tumors of 15 common types: about 56% expressed IDO1 (38). There was a remarkable match between the hierarchy of our IDO1 protein expression profile per tumor type and that of the corresponding mRNA retrieved from the TCGA database (**Figure 1B**). In both datasets, endometrial and cervical carcinomas emerged as the tumors with the highest and most frequent IDO1 expression, followed by kidney and lung carcinomas. The lowest values were in both cases observed in glioblastomas. Three distinct cellular expression patterns emerged, individually or in combination. IDO1 was expressed by tumor cells (20% of the samples), by interstitial cells in lymphocyte-rich areas in the tumor stroma (46% of the samples), or by endothelial cells (14% of the samples). Part of the IDO1 expression by tumor cells might result from an ongoing immune response involving T lymphocytes producing IFNγ. This is exemplified by cervical carcinoma, where IDO1-positive tumor cells were often located at the periphery of tumor nodules, which were surrounded by T lymphocytes. This is reminiscent of the expression profile observed for PD-L1, another protein involved in tumoral immune resistance, which is also induced by IFNγ and often observed in T-cell infiltrated tumors. This PD-L1 expression profile, indicative of an adaptive resistance mechanism, was found to predict clinical responses to PD1/PD-L1 blocking reagents (50–52). In a similar manner, IDO1 expression in inflamed tumors might also indicate an adaptive resistance mechanism. In line with this, IDO1 expression in human melanoma was found to correlate with T-cell infiltration (53). Moreover, and quite paradoxically, IDO1 belongs to the group of genes whose expression in tumors prior to immunotherapy is predictive of a better clinical response, along with T-cell specific genes and other IFNγ-induced genes (54). Here, the key predictive factor is most likely the presence of tumor-infiltrating lymphocytes (TILs) inside the tumor, and IDO1 is secondarily induced in response to IFNγ produced by those TILs. In our study mentioned above (38), the IDO1 expression that was often observed in the tumor stroma also likely resulted from adaptive resistance. Such a pattern was dominant, for example, in colorectal carcinomas.

In contrast, a subset of tumors expressed IDO1 within tumor cells in the absence of any inflammation. This is the case in endometrial carcinomas, which often contain IDO1-expressing tumor cells scattered within tumor nodules in the absence of obvious T-cell infiltration (38). Constitutive IDO1 expression is also observed in a number of human tumor lines (12), and is likely triggered by oncogenic events, whose characterization will be of great interest (55). Tumor-intrinsic constitutive IDO1 expression might contribute to tumoral immune resistance by preventing T-cell infiltration, a mechanism conceptually different from adaptive resistance, where IDO1 expression would represent a negative feedback mechanism induced by the T-cell response. Intriguingly, constitutive IDO1 expression has not been observed in murine tumors so far. Many murine tumors (with the notable exception of B16 melanoma) express IDO1 upon exposure to IFNγ, but none of them expresses IDO1 in the absence of IFNγ. Therefore, preclinical models commonly used to evaluate IDO1 inhibitors investigate IDO1-related adaptive resistance but not intrinsic resistance (13). The latter, which is relevant to the human situation, can only be evaluated using models based on murine tumors stably transfected with IDO1 (12, 56). Recent evidence in mouse models indicates that both tumor cells and host-derived cells contribute to IDO1-mediated immune resistance to anti-CTLA4 therapy (56).

The last pattern of IDO1 expression observed in human tumors, which is particularly striking in kidney cancer, is restricted to endothelial cells (38). As discussed above, the biological function of endothelial IDO1 remains to be defined. Intriguingly, endothelial IDO1 expression in kidney tumors was reported to be associated with a better prognosis, while in most other tumor types, IDO1 expression is associated with a worse clinical outcome (57). Of note, as opposed to many other tumor types, T-cell infiltration of kidney tumors is associated with a bad prognosis (58). Further studies will be required to understand those unexpected features of kidney tumors.

In tumor-draining lymph nodes

Several publications have reported increased proportions of IDO1-expressing DC in tumor-draining lymph nodes (TDLN) as compared to normal lymph nodes, mostly in mouse tumor models but also in human tumors, suggesting an important role of TDLNs in shaping tumoral immune tolerance (59–62). However, the recent study mentioned above, which used a carefully validated monoclonal antibody against human IDO1, did not confirm an increased proportion of IDO1-expressing DC in a series of 30 human TDLNs obtained from human melanomas and breast carcinomas: these TDLNs expressed IDO1 at the same level as normal lymph nodes (38). These results suggest that the IDO1 expression that is relevant to tumor immunosuppression is located at the tumor site rather than in TDLNs. This is in line with recent findings showing that the use of IDO1 inhibitors in a mouse model barely affected the priming of new anti-tumor T-cells, but strongly reactivated effector T-cells *in situ* at the tumor site (34).

INDOLEAMINE 2,3-DIOXYGENASE 2

Indoleamine 2,3-dioxygenase 2 was identified based on its structural homology with IDO1 (16, 17). The corresponding genes are highly homologous and located adjacent to each other on the same chromosome, suggesting that they resulted from gene duplication.

Both proteins share 43% identity at the amino acid level, including conserved residues that are important for IDO1 enzymatic activity. IDO2 is less well characterized than IDO1. Even though IDO2 has been reported to have enzymatic L-tryptophan degradation activity *in vitro* (17, 63), its K_m for this amino acid is much higher than that of IDO1 and TDO, around 6.8 and 12 mM for the human and mouse enzymes, respectively (30, 64). These values are more than a 100-fold higher than the physiological L-tryptophan concentrations, making it unlikely that IDO2 plays a direct role in the degradation of this amino acid. They rather suggest that IDO2 has a different natural substrate (63).

Constitutive expression of Ido2 mRNA and protein has been detected in mouse kidney, liver, epididymis and brain (16, 17, 65). Upregulation by inflammatory stimuli was observed *in vitro* in murine DCs and mesenchymal stem cells treated with IFNγ, and *in vivo* in brain following *Toxoplasma gondii* infection (17, 66–68). *Ido2*-knockout mice have a normal embryologic development and do not show major phenotypic abnormalities, but some aspects of their inflammatory and immune response to antigenic stimulation are reduced, including skin contact hypersensitivity reactions, generation of regulatory T-cells, and production of pro-inflammatory cytokines (69). *Ido2*-/- mice also display decreased joint inflammation in a mouse model of rheumatoid arthritis (70). These experimental observations are difficult to reconcile with the proposed model of IDO2 functioning as an immunosuppressive enzyme, and suggest a more subtle immunoregulatory role for IDO2, also in line with its genetic epistatic interaction with IDO1, suggested by the altered IDO2 splicing observed in IDO1-knockout mice (69).

In contrast with the mouse, little is known about IDO2 expression and function in humans. This assessment is rendered difficult by the lack of a strictly validated antibody and by the complexity of human *IDO2* transcription. The human *IDO2* gene contains two functional polymorphisms in its coding sequence. The R248W polymorphism drastically reduces the measured enzymatic activity of IDO2, and the Y359X polymorphism generates a truncated, enzymatically inactive protein. The high prevalence of these polymorphic alleles results in a non-functional IDO2 enzyme in up to 50% of persons (17). In addition, the *IDO2* gene generates at least five alternative transcripts, with only one encoding the full IDO1-related protein. IDO2 mRNA was detected by RT-PCR in liver, small intestine, spleen, placenta, thymus, lung, brain, kidney, and colon, but the full-length transcript only in placenta and brain (17). Expression of the *IDO2* gene was detected by RT-PCR in circulating myeloid DC and pDC and in *in vitro*-matured MoDC (48, 71). However, it is not known whether the detected IDO2 transcripts encoded a functional IDO2 protein, as the PCR primers were chosen to amplify exons 9 and 10, which are present in all 5 IDO2 transcripts. Using PCR conditions that amplify exons 4–9, which are only present in the full-length IDO2-encoding mRNA, we failed to detect IDO2 expression in *in vitro*-matured MoDC (38).

There are insufficient experimental arguments to support the view that IDO2 is expressed in human tumors. Transcription of *IDO2* was detected by RT-PCR in a high proportion of a small number of gastric, colon, and renal carcinoma samples, as well as in several tumor cell lines treated with IFNγ, but again using primers amplifying exons 9 and 10 (72). The IDO2 protein was detected by Western blot in three IFNγ-incubated tumor cell lines (73). However, despite the fact that one of these lines was derived from a patient homozygous for the Y359X polymorphism, which results in a truncated protein, the size of the corresponding IDO2 band on the immunoblot was identical to the two others, raising doubts about the specificity of the antibody used. In addition, it is unclear whether this antibody was directed against IDO2 or IDO1. In the same study, IDO2 was detected by immunohistochemistry in the tumor cells from 12 out of 12 pancreatic carcinoma samples, but not in normal pancreatic tissue, using an anti-IDO2 polyclonal antibody. Here also, the antibody stained tumor samples from Y359X homozygous patients. Its specificity controls were not provided.

Our whole transcriptome analysis retrieved from public RNA-Seq databases displayed in **Figure 1C** shows that, among healthy human tissues, only liver, testis, and thyroid express the *IDO2* gene, whereas the overwhelming majority of human tumor samples (>99%) are negative. Because these data do not take the complexity of the alternative splicing of IDO2 transcripts into consideration, we verified the absence of IDO2-matched RNA-Seq reads in 20 melanoma metastases sequenced in our laboratory (data not shown). We also confirmed the absence of detectable *IDO2* expression by RT-qPCR in 128 human tumor samples and 25 human tumor cell lines of various histological types using primers amplifying exons 4–9 (data not shown).

Altogether, the frequent occurrence of non-functional *IDO2* alleles in the population, the lack of experimental evidence for a biologically relevant tryptophan catalyzing activity of IDO2 and the absence of detectable IDO2 expression in most tumors make it highly unlikely that IDO2 plays a significant role in tryptophan-related tumoral resistance against immune rejection in humans. This is supported by the observed trend toward increased overall survival in patients with wild-type IDO2 compared to patients with heterozygous or homozygous polymorphisms that ablate IDO2 activity, in a recent study of brain metastatic patients receiving radiotherapy combined with chloroquine (74).

SUMMARY

Three enzymes with different features have been implicated in the first step of tryptophan degradation along the kynurenine pathway. TDO is a tetrameric enzyme expressed at a high level in the liver, and responsible for degrading dietary tryptophan and maintaining constant levels of blood tryptophan. Besides this physiological role, which is supported by a high K_m and a positive regulation of TDO by tryptophan, TDO appears to have an immunoregulatory role, probably mediated mostly by tryptophan catabolites. In contrast, IDO1 is an unrelated monomeric enzyme, whose expression is mostly inducible in most tissues, and which plays a key role in immunoregulation and the retrocontrol of immune responses. Its low K_m allows IDO1 to effectively deplete tryptophan in the local microenvironment and thereby impair T-cell mediated immune responses. Both IDO1 and – albeit less frequently – TDO are expressed in human tumors and appear to play a role in tumoral immune resistance, which warrants ongoing drug discovery efforts aimed at the clinical development of IDO and TDO inhibitors for cancer therapy. Much less is known about

the role of IDO2, the third enzyme of the pathway. IDO2 appears to be expressed at a low level in the liver, testis and thyroid. It is not significantly expressed in human tumors. The tryptophan-degrading activity of IDO2 is particularly low, with a K_m at least 100-fold higher than the physiological concentration of tryptophan. This, together with the frequent occurrence of polymorphisms affecting the assumed catalytic activity of human IDO2, makes it unlikely that IDO2 by itself plays a direct role in tryptophan catabolism, but rather supports another function for this protein.

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