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Amino-acid sensing and degrading pathways in immune regulation

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ABSTRACT

Indoleamine 2,3-dioxygenases (IDOs) – belonging in the heme dioxygenase family and degrading tryptophan – are responsible for the *de novo* synthesis of nicotinamide adenine dinucleotide (NAD^+). As such, they are expressed by a variety of invertebrate and vertebrate species. In mammals, IDO1 has remarkably evolved to expand its functions, so to become a prominent homeostatic regulator, capable of modulating infection and immunity in multiple ways, including local tryptophan deprivation, production of biologically active tryptophan catabolites, and non-enzymatic cell-signaling activity. Much like IDO1, arginase 1 (Arg1) is an immunoregulatory enzyme that catalyzes the degradation of arginine. Here, we discuss the possible role of amino-acid degradation as related to the evolution of the immune systems and how the functions of those enzymes are linked by an entwined pathway selected by phylogenesis to meet the newly arising needs imposed by an evolving environment.

1. Introduction

The bidirectional interaction between the immune system and whole-body metabolism has been well recognized for many years. *Via* effects on a multiplicity of cells, immune cells can modulate whole-body metabolism and, reciprocally, host nutrition and commensal-microbiota–derived metabolites modulate immunological homeostasis. A major focus is thus being placed on 'immunometabolism' [1], which focuses on how the cell-intrinsic metabolic properties of accessory cells – in particular, dendritic cells (DCs) – of the immune system 'sense' the environment, and affect whole-body metabolism while shaping the most appropriate immune response. We particularly focus on pathways of amino-acid sensing and degradation *via* specific enzymes in accessory cells that shape immune responses so to best accommodate the needs of a changing environment.

2. Sensing amino acids for proteogenesis and proteostasis

Like acrobats balancing on the wire, eukaryotic cells control their protein components through a tight regulation of concentration, conformation, binding interactions and localization of individual proteins. The consequence of these interacting activities is a delicate state of dynamic equilibrium, known as proteome homeostasis, or proteostasis. Perturbations in the mechanisms modulating protein structure and function can lead to protein dysfunction, as well as deleterious cell processes and disease onset.

Proteostasis is influenced by a complex network of biological pathways, involved in protein synthesis, folding, localization and degradation. Cells modulate protein folding and degradation through extensive signaling networks to avoid the accumulation of misfolded species. Several checkpoints interconnect pathways responsible for the maintenance of the correct protein structure, starting from synthesis, where regulators of the ribosomal activity and controllers of the translation supervise the first steps of a protein's lifetime, proceeding along with chaperones and enzymes assisting protein folding and trafficking, up to the biological degradation, controlled by the ubiquitin-proteasome system and by autophagy and apoptosis mechanisms [2]. A partial decline in proteostatic control occurs during aging, partially explaining why many proteome-related diseases are of late-age onset [3].

A crucial step in the maintenance of proteostasis is the synthesis of newborn proteins, necessary for cellular growth and proliferation. Many factors regulate the complex phenomenon of protein synthesis; nutrients and growth factors availability are integrated with anabolic responses to trigger the synthesis of essential cellular building blocks, such as proteins. A key signaling hub in this process is represented by the mechanistic target of rapamycin complex 1 (mTORC1) [4], which activates the metabolic pathways that ultimately drive cell growth; more in detail,

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mTORC1 controls both protein translation and autophagy [5]. Another important mechanism activated by eukaryotic cells to sense nutrient, and more specifically amino-acid availability, is represented by the kinase GCN2, which binds to uncharged tRNA and regulates adaptive changes to perceived amino-acid deficiency. The amino acid-sensing pathways, like all the nutrient-sensing systems – composed of sensors, transporters, and signaling proteins – are utilized by cells to monitor and respond to fluctuations in environmental nutrient levels; the influx of amino acids is especially critical to meet the increased demands for protein synthesis during cellular lifetime. Furthermore, amino acids can serve as sources for metabolites that enter into metabolic processes, such as the tricarboxylic-acid cycle [6].

Amino acids are considered to be the basic chemical building blocks during the whole process of the origin of life, or biogenesis; thus, "proteogenesis" (the origin of proteins) is a key part of biogenesis, principally because proteins are uniquely capable of performing the main processes necessary to maintain living systems. An accepted theory proposes that a limited set of α-amino acids was initially present on the pre-biotic earth, produced or delivered by abiotic chemical and physical processes [7]. Such prebiotic amino acids provided the raw material for the very first proteogenesis prior to the emergence of any biosynthetic pathway. According to this theory, some minimum alphabet of 10 amino acids was required for the synthesis of properly folded essential proteins; thus, the redundant set of 20 common amino acids was not fully required for the first proteogenesis processes. The analyses of potential sources of abiotic organics identify a consensus set of 10 racemic α-amino acids, including alanine (Ala), aspartic acid (Asp), glutamic acid (Glu), glycine (Gly), isoleucine (Ile), leucin (Leu), proline (Pro), serine (Ser), threonine (Thr), and valine (Val) [8]. There is a general consensus that aromatic amino acids were essentially absent when life first emerged [9]. The aromatic amino acids are the largest and most complex of the common α-amino acids and prebiotic aromatic amino-acid synthesis appears highly inefficient. Attempts at reconstructing the order of amino-acid incorporation into the genetic code are in agreement: coevolution theory identifies the aromatics as being part of a later phase, with the three aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp) as being the last amino acids to be incorporated into the genetic code, along with aliphatic methionine (Met). Aromatic amino-acid biosynthesis thus appears as a key adaptation acquired sometime after the emergence of life, separate from the initial proteogenesis event. During the evolution, most mammals abandoned producing 9 of the 20 amino acids needed for the protein biosynthesis—the so-called essential amino acids (i.e., Phe, Val, Thr, Trp, Met, Leu, Ile, Lys, and histidine or His). Currently, in mammals, cells of the immune system are auxothrophs for most amino acids, including non-essential ones. Amino-acid auxotrophy, namely the need for external supply of amino acids, became an immunoregulatory control point not only to orchestrate the essential mechanism of protein synthesis and the delicate proteostasis events, but also to reduce the microbial burden, thus controlling the damage caused by the growth of pathogenic microorganisms, and, more importantly, to shape the immune response. In fact, amino-acid sensing influences immunologic responses to inflammatory and antigenic cues by generating new compounds, amino-acid catabolites, with immune modulatory properties. Then, it appears very clear that the environmental availability of certain amino acids (i.e., Trp, Arg, and Gly) became during the evolution a crucial requirement not only for the synthesis of the major components of living cells, but also for the maintenance of the proteome, and, more in general, for cellular homeostasis, in order to avoid disease onset and to maintain the balance between host and the microbial environment unchanged.

Here, as an example, we would like to make the case that the occurrence of a "tryptophan-to-kynurenine-nicotinamide 'immune tolerance' pathway" may not only represent an important phylogenetic biochemical and immunological switch in terms of human evolution – as previously suggested [10] – but it may also represent a means of adaptation to environmental changes exploited by living organisms that are auxotroph for Trp, including mammals. Vitamin B3 (nicotinamide) is a redox cofactor used by all living organisms and cells. Nicotinamide, indeed – as an essential component of the NAD/NADH redox pair – drives the electron transport chain, converting the free energy of the electromotive force into a proton gradient across the mitochondrial inner membrane, driving ATP production and controlling pH and other voltage-coupled processes. Likewise, many NAD-coupled redox reactions are known to be important for cell development, repair, and ageing: NAD is a master controller of the amount of metabolism necessary either for a living organism to cope with environmental needs [10] or for a cell to cope with its basal needs and functions. Under conditions of nutrient abundance, functioning of the NAD/NADH redox pair is dependent on nicotinamide intake from the environment. Under conditions in which the availability of external nicotinamide is deficient, the organism resorts to the *de novo* synthesis of NAD, which requires that Trp — an essential amino acid and the rarest one – be degraded along the kynurenine (Kyn) pathway. It is interesting to note that bacteria that eat worms use NAD as a 'food signal' to open their mouths but, if NAD is unavailable, they stop reproducing and enter a developmental and reproductive arrest phase, mediated by serotonin, to survive [11]. Much like those bacteria, sensing a nutrient-deficient environment by an organism might inevitably imply an impaired proteogenesis, and that would be accomplished *via* the subtraction of the rarest amino acid, Trp. The same metabolic pathway, namely, Trp degradation would then accomplish the double objective of supplying the necessary and sufficient amount of NAD^+ and force the cell not to engage in NAD-consuming anabolic processes, such as proteogenesis. In general terms, the process of catabolic consumption of essential amino acids may meet the needs of nutrient and energy constraints to the benefit of maintaining living systems.

When contextualized to the functioning of the mammalian immune system, this might imply that sensing of nicotinamide deficiency by antigen-presenting cells (APCs) of the immune system will activate L-Trp conversion to L-Kyn, turning those cells from immunogenic to tolerogenic, as will be discussed in more detail later on. This would, indeed, be followed by the Kyn-dependent activation of the transcription factor Aryl hydrocarbon Receptor (AhR), necessary for the generation of regulatory T (Treg) cells, thus increasing the overall 'tolerance' mechanisms of the host to a variety of potentially immunogenic antigens of both environmental (*e.g.*, symbionts and microbes) and endogenous origin [12].

3. Evolution of amino-acid regulatory systems: to each its own

Both in microbes and higher organisms the availability of amino acids – endogenously synthesized or acquired from the environment – is fundamental to cell survival and proliferation. Most microorganisms can produce the amino acids necessary for their growth *ex novo*, while some others, such as *Chlamydia*, *Mycobacterium*, and fungi, have lost this biosynthetic ability and thus become auxotrophic for several such nutrients. Because humans also need to acquire essential amino acids from the diet and/or microbiota, the competition for those protein-building blocks has become a strategy exploited by both microorganisms and vertebrates. In particular, mammals have learnt to control pathogen infection by increasing amino acid catabolism, thus restricting local – mostly intracellular – nutrient availability to invading pathogens.

This apparently paradoxical evolutionary countermeasure has further expanded during phylogenesis, so to not only result in pathogen starvation and control of infection, but also become a means of regulating the host's own inflammatory reactivity in response to infection [13–15]. Thus, in mammals, the crosstalk between catabolic mechanisms of essential amino acids and a well-structured immune system represents much more than a survival strategy, ultimately proving to be an important mechanism of fine-tuning immune reactivity – and thus avoiding hyperinflammation – in innate, adaptive, and regulatory responses to infections, a mechanism crucial for the host to avoid exaggerated inflammation and infection-driven immunopathology. Of all amino acids, Trp and arginine (Arg) represent perhaps the best-known examples of catabolic pathways aimed at modulating immune reactivity. Phe, glutamine (Gln) and cysteine (Cys) are also being considered as amino acids involved in the balance of immune reactivity [16]. In general, successful host responses to pathogens in mammals involve a combination of 'infection resistance' (aimed at eradicating infection *via* inflammatory responses) and 'disease tolerance' (aimed at preserving host's fitness in the face of potentially harmful hyperinflammatory responses, without necessarily eradicating the pathogen) [12]. Convergent evolution of microorganisms and their hosts has thus opted for a compromise that ultimately benefits both components of the 'symbiotic' pair.

The essential amino acid Trp is an important precursor for the synthesis of proteins and several molecules involved in fundamental and diverse biological processes, i.e., Kyn, NAD, and the neurotransmitter serotonin [17]. The majority of Trp is metabolized by the Kyn pathway (Fig. 1), while a less relevant amount

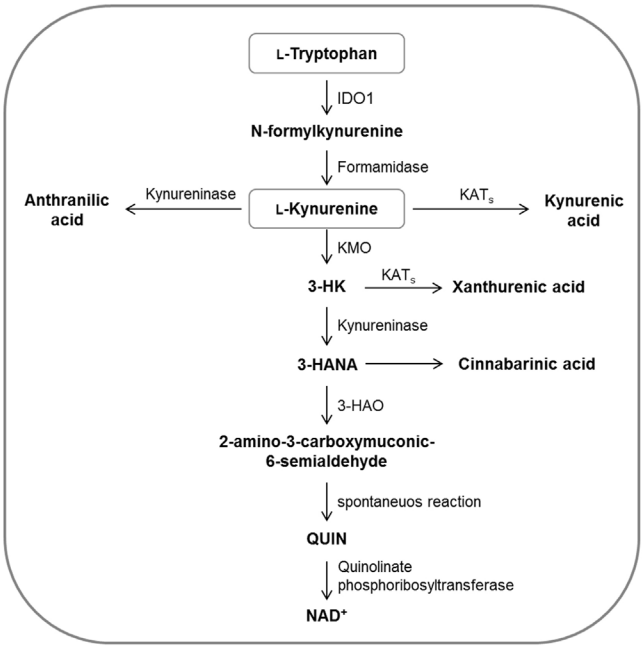

Fig. 1. The kynurenine (Kyn) pathway in mammalian cells. The Kyn pathway is initiated by the transformation of Trp into N-formylkynurenine by IDO1. N-formylkynurenine is rapidly degraded by formamidase to yield the L-Kyn metabolite. L-Kyn is then converted into kynurenic acid, 3-hydroxykynurenine (3-HK) or anthranilic acid *via* kynurenine aminotransferase (KATs), kynurenine 3-monooxygenase (KMO), and kynureninase, respectively. Additional lateral branches of the Kyn pathway lead to the formation of other terminal products, such as xanthurenic acid. Mammalian kynureninase preferentially recognizes 3-HK over L-Kyn, catalysing the formation of 3-hydroxyanthranilic acid (3-HANA) that is converted by 3-hydroxyamino oxidase (3-HAO) to 2-amino-3-carboxymuconate-6-semialdehyde. Under physiological conditions, 2-amino-3-carboxymuconic-6-semialdehyde spontaneously rearranges to form quinolinic acid (QUIN), making this enzyme one of the gatekeepers for the synthesis of NAD^+.

enters the methoxyindole pathway. Along the Kyn pathway, Trp degradation occurs by two different enzymes, indoleamine-2,3-dioxygenase 1 (IDO1) and tryptophan-2,3-dioxygenase (TDO), catalyzing the conversion of Trp into Kyn, an amino acid itself. Alternatively, acting as a substrate for tryptophan hydroxylase-1, Trp can be transformed into 5-hydroxytryptophan, a precursor for serotonin, which is a precursor in melatonin biosynthesis [14,18]. The Kyn pathway provides several important catabolites – collectively called kynurenines –endowed with different biological activities [19]. Some of Kyn, such as quinolinic acid (QUIN) and 3-hydroxyanthranilic acid (3-HANA) can act as immunosuppressive factors inhibiting T-cell proliferation [20,21]. L-Kyn (an amino acid itself) has an important role in immune regulation, because, acting as an endogenous ligand for AhR, affects the biology of immune cells as well as of cancer cells [22]. The enzymatic cascade downstream of L-Kyn generates several neuroactive intermediates, including the neurotoxic and free-radical generators 3-hydroxykynurenine (3-HK) and 3-HANA, the excitotoxin QUIN and the neuroprotectant picolinic acid (PIC) [23]. The NAD cofactor is the final product of the pathway, being produced from QUIN after several metabolic steps, and has fundamental roles in redox reactions essential for mitochondrial function [19].

Arg, though being a dispensable (nonessential) amino acid for healthy humans, has notable nutritional and physiological significance. It is a precursor for the synthesis of proteins, is catabolized to urea and creatine, and serves for the synthesis of signaling molecules such as Glu, nitric oxide, and agmatine [24]. Four distinct enzymes operate Arg catabolism in mammals, namely, nitric oxide synthase (NOS), arginase 1 and 2 (Arg1 and (Arg2), arginine decarboxylase (ADC), and arginine glycine amidinotransferase (AGAT) [25]. NOS transforms Arg to nitric oxide (NO) and citrulline. NO is an important cardiovascular, immunological and neurological signaling molecule mediating powerful vascular dilatation and antimicrobial cytotoxic effect, and also playing a neurotransmitter role. Arg1 and Arg2, which hydrolyse Arg into urea and ornithine (Orn), contribute to ammonia detoxification through the urea cycle in ureotelic organisms and provide a precursor, Orn, for Glu, proline (Pro) and polyamine synthesis, necessary for energy metabolism, cell proliferation and repair, and modulation of inflammation [14]. The ADC pathway produces agmantine, which, already long known to be a source of energy, has recently been found to act a as neuromodulator for several types of neurotransmitter receptor, where it exerts cytoprotective effects, not only in neurons, but also in kidney, heart, and stomach [26]. AGAT catalyzes the first reaction for the conversion of Arg and glycine to creatine, with Orn as a byproduct. Creatine and its phosphorylated form phosphocreatine contribute to the transient intracellular storage of metabolic energy and the recycle of ADP to ATP, particularly in tissues with high energetic needs, such as muscle and brain [27].

Phe catabolism occurs through an oxidative deamination catalyzed by the interleukin 4-inducible 1 (IL4I1) enzyme, possibly secreted or localized in lysosomes and producing equimolar amounts of an α-ketoacid (phenylpyruvate), H_2O_2, and NH_3 [28]. H_2O_2 antibacterial toxic effects, potentiated by NH_3-dependent basification of the medium, are accompanied by Phe depletion, a condition resulting in growth control of bacterial strains auxotrophic for this amino acid, together with an immunosuppressive activity directed towards T lymphocytes, as observed in other essential amino-acid degrading systems [29].

Although Gln is considered non-essential in healthy individuals, it is regarded as essential for the proliferating cells, including lymphocytes, thymocytes, and colonocytes, where it is highly consumed for a plethora of metabolic processes. Gln is degraded by glutaminase to Glu, further transformed to such molecules as γ-amino butyrate, reduced glutathione, and folic acid. Gln is

engaged for the synthesis of peptides, nucleotides and amino sugars, acts as a nitrogen donor, and fuels energy needs of the cell by providing intermediates for the tricarboxylic-acid cycle, thus participating in processes critically impacting the efficiency of immune cell responses [30,31].

Cys is another essential amino acid strictly required for T-cell proliferation. Because T cells lack the enzyme converting methionine to cysteine, these cells have to import cysteine *via* their neutral amino-acid transporter system. Cys availability is therefore critical for T-cell functions, with cells either providing (as is the case for APCs) or sequestering (in particular, myeloid-derived suppressor cells or MDSCs) this amino acid, so to result in stimulatory or suppressive effects, respectively. Cys dioxygenase (CDO) addresses Cys catabolism to cysteine sulfinic acid, further catabolized to hypotaurine and then to taurine, or to pyruvate and sulfite. Thus CDO not only removes Cys cytotoxic excess, but is also necessary for the production of hypotaurine/taurine and sulfite/sulfate from cysteine [32].

4. The Arg breakdown system

In healthy humans, the semi-essential amino acid Arg is endogenously synthesized to meet the basal metabolic demand, but in some pathological states (including sepsis, trauma and cancer), the need exceeds the physiological production, making it necessary to rely on external supply [33]. Arg biosynthesis is achieved by two major pathways. The first one, known as 'intestinal-renal axis', begins in epithelial cells of the small intestine and uses dietary Pro, Glu, and Gln for synthesizing Orn and citrulline. Intestinal citrulline, once released into the circulation, is taken up by the proximal tubules of the kidney and converted into Arg. The second pathway, referred to as the 'citrulline-NO cycle', provides immune cells with a strategy to ensure a constant provision of Arg to be used for the synthesis of NO, a key player in host immune responses [34].

Because of its cationic feature, Arg enters the cell *via* specialized transporters belonging in the solute carrier family 7 (SLC7), i.e., CAT-1 (cationic amino acid transporter), CAT-2, and CAT-3. As opposed to CAT-1, which is constitutively expressed by most tissues, CAT-2 is induced in murine macrophages under conditions of both T-helper 1 (Th1) and Th2 cell stimulation, [35], and in murine DCs as well by retinoic acid [36]. In those cells, enhanced Arg import *via* CAT-2 is coupled to the induction of Arg catabolizing enzymes, indicating that the amino acid transporter is exploited not only for nutrient supply, but it is also involved in more complex metabolic networks [36].

In immune cells, Arg breakdown can be performed by the inducible form of NOS and by Arg1. Through the differential modulation of those enzymes, myeloid cells regulate the immune responses against viruses, bacteria and tumor cells. Three NOS isozymes have been identified, namely, NOS1 (also known as neuronal; nNOS), NOS2 (referred as inducible NOS; iNOS) and NOS3 (known as the endothelial form; eNOS) [37]. Although these enzymes catalyze the same reaction, they differ in distribution and regulation. While NOS1 and NOS3 are constitutive enzymes, iNOS is induced by several pro-inflammatory cytokines (including IFN-γ and IL-1β) and microbial products. The released NO acts as a stimulator of guanylate cyclase, so to generate cyclic GMP within the target cell. NO also promotes the nitrosylation of proteins thus altering their functions, and reacts with a variety of molecules to generate other reactive nitrogen species. Thanks to the cytotoxic and cytostatic activity of NO, iNOS-expressing macrophages are crucial for the initial phase of immune responses against foreign materials.

The story of arginase started more than 130 years ago, when the enzyme was isolated and identified as a component of animal proteins [38]. Later on, Krebs and Henseleit described arginase as a key enzyme of the hepatic urea cycle [38]. Only 50 years later, it was shown that arginase not only has a metabolic role in ammonia detoxification, but it is also expressed by immune cells and participates in the regulation of several pathophysiologic conditions [39,40]. Arginase exists in two isoforms, named Arg1 and Arg2, which catalyze the same reaction, yet have different intracellular localization and tissue distribution. Arg1 is a cytosolic enzyme whereas Arg2 is expressed in mitochondria of cells from kidney, small intestine, and brain [39]. Because Arg1 is restricted to a more specialized species, it has been proposed that mitochondrial Arg2 is a surviving form of the ancestral enzyme, indicating that the primitive function of that protein was the regulation of cellular Arg, rather than ammonia detoxification [41].

Arg1 can be found in mouse macrophages, MDSCs, DCs and innate lymphoid group 2 cells in response to Th2-type cytokines, such as IL-4 and IL-13 [38,42]. The Th2–mediated induction of Arg1 is regulated by the signal transducer and activator of transcription 6 (STAT6) that, in conjunction with CCAAT/enhancer binding protein β (C/EBPβ), binds to an enhancer located 3 kb upstream of the basal promoter [43]. Multiple other factors modulate the expression of Arg1, including macrophage and granulocyte/macrophage colony-stimulating factors (M-CSF and GM-CSF, respectively), cyclic AMP, toll-like receptor (TLR) agonists and several tumor-derived soluble factors, including interleukin 6 (IL-6) and tumor-growth factor (TGF)-β [38,44]. Human Arg1 has been detected in peripheral blood mononuclear cells (PBMCs) from subjects after injury and in activated monocytes of patients with autoimmune disease [45]. In healthy humans, Arg1 is constitutively present in the granular compartment of granulocytes, where it is inactive and becomes activated upon extracellular release [46,47].

Arg hydrolysis by Arg1 yields the non-essential amino acid Orn, which is further metabolized to citrulline by the activity of Orn transcarbamylase (OTC), and to polyamines (putrescine, spermidine, and spermine) and Pro by Orn decarboxylase (ODC) and Orn amino transferase (OAT), respectively (Fig. 2). Citrulline is recycled back to Arg, while Pro is used for synthesis of collagen. The fine balance between consumption of Arg by iNOS and that by Arg1 determines the course of an immune response. If, on the one hand, NO-producing macrophages dominate the initial phase of the immune reaction to eradicate pathogens, on the other hand, Arg1 meets the requirement of ensuring enough Pro and polyamines for the subsequent tissue events leading to resolution and repair. The small polycationic molecules, i.e., polyamines, are important for neural growth, development, and regeneration [48]; they are involved in the pathogenesis of cancer and in regulating the translation *via* hypusination of the putative translation factor eIF52A [49]. Of note, polyamines can also act at the crossroad between Arg and Trp metabolism in DCs as detailed below. In particular, spermidine triggers a series of molecular events related to the activation of IDO1 signaling and confers on DCs a long-lasting tolerogenic phenotype [50].

5. The Trp catabolic system: from Trp depletion to signaling function in IDO1 and the generation of intracellular messengers and ligands

IDO1 represents one of the most interesting molecule that links an ancient metabolic pathway with immune regulation. IDO1 is a monomeric, heme-containing enzyme that catalyzes the initial, rate-limiting step in the degradation of the essential amino acid L-Trp into L-Kyn, which represents the upstream metabolite along the so-called Kyn pathway [51–53]. The immunoregulatory effect of IDO1, mainly expressed by DCs, is linked to its catalytic activity that causes Trp deprivation and the generation of immunoactive

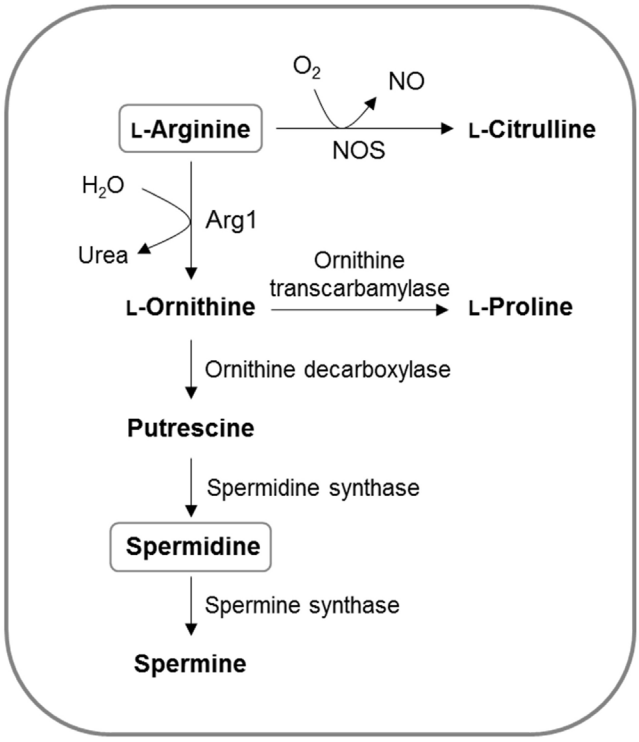

Fig. 2. The Arg metabolic pathway. Arg breakdown can be performed by two enzymes, namely iNOS and Arg1. Arg1 catalyzes the conversion of Arg in ornithine and urea, while iNOS into NO and citrulline. Orn acts as a substrate of ornithine decarboxylase and ornithine amino transferase to yield putrescine and proline, respectively. Putrescine is sequentially converted into spermidine and spermine, through the action of spermidine synthase and spermine synthase.

Kyn. Both mechanisms, related to a change of Trp and Kyn levels in the extracellular milieu, have a short-term effect suitable for controlling acute inflammation. As a result, IDO1-competent DCs mediate multiple effects on T lymphocytes, including inhibition of proliferation, apoptosis, and differentiation towards a regulatory phenotype [54,55]. These effects are induced by the cytokine IFN-γ, which activates intense but short-term IDO1-mediated immunosuppressive activity.

In 2011, we demonstrated that IDO1 also acts as a signal-transducing molecule, an effect that relies on the presence of two immunoreceptor tyrosine-based inhibitory motifs (namely, ITIM1 and ITIM2) in the noncatalytic, small domain of IDO1. Tyrosine-phosphorylated ITIM1 and ITIM2 in IDO1 act as relevant docking sites, allowing the association of the enzyme to different types of molecular partners (i.e., to SHP phosphatases or, in alternative, to the suppressor of cytokine signaling-3 (SOCS3) molecule), which determine the half-life of the IDO1 protein and thus the duration of its immunoregulatory activity [56]. Specifically, in a TGF-β–-dominated environment, phosphorylated IDO1 interacts with the tyrosine phosphatases SHP-1 and SHP-2 and this leads to the activation of the non-canonical NF-κB pathway [57–59]. Those molecular events activate, in turn, the transcription of both *Ido1* and *Tgfb1* genes in a feedforward loop, which sustains a long-term immunoregulatory program in plasmacytoid dendritic cells and thus provides a suitable means of controlling autoimmune or chronic inflammatory conditions [59,60]. Maneuvers aimed at modulating IDO1 induction and/or catalytic activity (either in a negative or positive fashion) may therefore represent an important therapeutic option under conditions such as neoplasia and viral infections–where IDO1 is one of the main causes of immune unresponsiveness [61–64]–as well as in autoimmune [65,66] and chronic inflammatory diseases [67], characterized by a defective activity of IDO1.

The identification of the ITIM-related functions of IDO1 has been paving the way to new strategies aimed at modulating IDO1 expression and activity at different levels. Particularly, the activation of the TGF-β/IDO1/SHPs axis could be one of the mechanisms used by tumors for evading immune surveillance ('tumor escape'). In fact, all of the molecules involved in IDO1 signaling activity have an established role in the immunobiology of cancer. On the one hand, the tumor-promoter or –suppressor role of the TGF-β pathway in cancer is still a matter of debate, owing to its differential effects at the early and late stages of carcinogenesis. On the other, at the microenvironmental level, the TGF-β pathway does contribute to the generation of a favorable milieau for tumor growth and metastasis throughout all steps of carcinogenesis [68]. Moreover, both the kinases responsible of IDO1-ITIM phosphorylation (Fyn and Src) [12,69] and the tyrosine phosphatases SHP-1 and SHP-2 are strongly upregulated as to expression and function in several cancer types, such that their inhibition is a new strategy in cancer therapy [70–73]. It follows that drug targeting of Fyn/Src, SHP-1 or SHP-2 may be a proper means of interfering with IDO1 signaling. Along this direction, the discovery of molecules capable of modifying IDO1 binding to its molecular partners may, likewise, pave the way to the identification of a new class of anti-cancer drugs targeting tumor escape mechanisms.

The phosphorylated docking sites of IDO1 can –in a different local tissue microenvironment– anchor the SOCS3 partner, leading to an opposite outcome. In an IL-6–rich inflammatory milieu, IL-6–induced SOCS3 is capable of associating phosphorylated IDO1 through its Src homology 2 (SH2) domain and thus promoting ubiquitination and proteasomal degradation of the enzyme [74]. This mechanism is largely sustained by the inflammatory context, often characterized by high-level production of IL-6, which operates through SOCS3, a negative regulator of both IDO1's enzymatic and signaling functions. One major effect of reduced Trp catabolic function of IDO1 could be down-regulation of the short-term regulatory effects of IDO1 (i.e., those associated with Trp starvation and Kyn generation). Later in time, one major effect of a defective IDO1 catalytic activity could be the poor generation of Kyn-type ligands of the AhR, which is involved in the generation of Treg cells [12,55]. In addition, any accelerated proteolysis of IDO1 will interrupt the positive circuit sustaining the long-term immunoregulatory effect of the enzyme associated with its signaling function. Overall, the SOCS3-mediated proteolysis of IDO1 could be considered a post-translational mechanism of negative regulation of IDO1, which occurs in an early inflammatory context at a time when inflammation is still a necessity to cope with an environmental challenge.

Recently, Albini et al. demonstrated a distinct role of the two phosphorylated ITIM1 (pITIM1) and pITIM2 of IDO1, by using stably recombinant phospho-(Tyr)-mimetic IDO1 mutants [75]. Specifically, the pITIM1-mimic mutant was found to bind SHP phosphatases and confer immunosuppressive effects on DCs, while the pITIM2-mimic mutant would preferentially bind SOCS3 and shorten IDO1's half-life. Although such non-overlapping, if not divergent, functions of the pITIMs of IDO1 have been observed in an artificial system with stable IDO1 mutants, these data would establish non-redundant biological roles for the two ITIMs in IDO1, thus providing the enzyme with different ITIM-conditioned fates and functions so to meet the needs of the local microenvironment. It is thus IDO1 plasticity that confers opposite functional phenotypes on DCs, either immunoadjuvant or tolerogenic in nature.

Of note in this regard, the available crystal structures of human IDO1 show a poor solvent exposure of the two IDO1 ITIMs, suggesting that ITIM phosphorylation requires a major

conformational change in IDO1 that could heavily affect the catalytic cleft of the enzyme. In fact, both the single and double phospho-mimetic IDO1 mutants completely lose the catalytic activity, but not their ability to associate with IDO1's molecular partners [75]. It is tempting to speculate that there might occur a dynamic balance between different conformational states of IDO1, making the protein best suited to either catalytic or signaling function. Overall, the identification of mechanisms that diametrically control the functions and fate of IDO1 opens up new perspectives for the pharmacologic control of IDO1 functioning.

6. The convergent evolution of different amino acid breakdown systems: a case for Arg1 and IDO1, and its functional implications

Although as far as 50 years ago physicians and scientists started to be aware of the tight association between metabolic disorders and systemic inflammation, only the last decade has witnessed a sharpened focus on the convergence of metabolism and immune functions, i.e., a field now known as 'immunometabolism' [76]. Many layers of evolutionarily conserved interactions have been identified between immune responses and metabolism, particularly in the promotion and maintenance of chronic metabolic inflammation, i.e., 'metaflammation', occurring in several metabolic diseases such as type 2 diabetes and obesity [77]. These intersections may thus be the result of a convergent evolution of the two systems.

Pathways exerting negative effects on both metainflammation and adaptive immune responses are generally less clear than those promoting their potentiation. However, an exception may be represented by Arg1 and IDO1, whose biologic knowledge has rapidly advanced in recent years as outlined above [14]. As a whole, the bulk of available data on these two immunometabolic enzymes would suggest that they might operate in quite distinct spatial (i.e., cells) and mechanistic modes, namely *via* either amino acid starvation itself (as is the case for Arg1) or *via* the combined effects of immunoregulatory Kyn and signaling activity (IDO1). Thus Arg1 and IDO1 may have separate functions (to possibly cope with distinct environmental needs) and/or have evolved to complement, rather than integrate, each other. This hypothesis is sustained by the fact that IL-4– or IFN-γ–dominated cellular environments specifically promote Arg1 (mainly in macrophages/MDSCs) [44] or IDO1 (DCs) alone expression [54], respectively (Fig. 3).

TGF-β is a cytokine that appeared quite recently over evolution, i.e., in metazoans, and is often marked by potent immunosuppressive effects, particularly in tumor settings [78]. We recently demonstrated that TGF-β, at variance with IL-4 and IFN-γ, upregulates the expression and activity of both Arg1 and IDO1 in the same cells, i.e., DCs, and that both enzymes are required for the *de novo* immunoregulatory properties acquired by DCs in response to the cytokine [50]. The co-expression of both enzymes could be observed in the presence of TGF-β alone and not in combination with either IL-4 or IFN-γ, conditions that skew towards the upregulation of either Arg1 or IDO1 alone, respectively (Fig. 3) [50]. Perhaps more importantly, we identified a 'relay' pathway in DCs based on the sequential actions of TGF-β, Arg1, and IDO1, whereby the cytokine upregulates first Arg1 activity and resulting Orn is transformed into the polyamine spermidine that in turn promotes IDO1 phosphorylation by the Src kinase, thus triggering the IDO1 signaling pathway and consequent immunosuppressive effects over the long-term (Fig. 4). Spermidine may therefore represent a novel, two-sided node responsible for an important intersection between the immunometabolic pathways of Arg1 and IDO1 that would allow the immune system to translate an initial short-term (Arg1-mediated; typical of early-acting

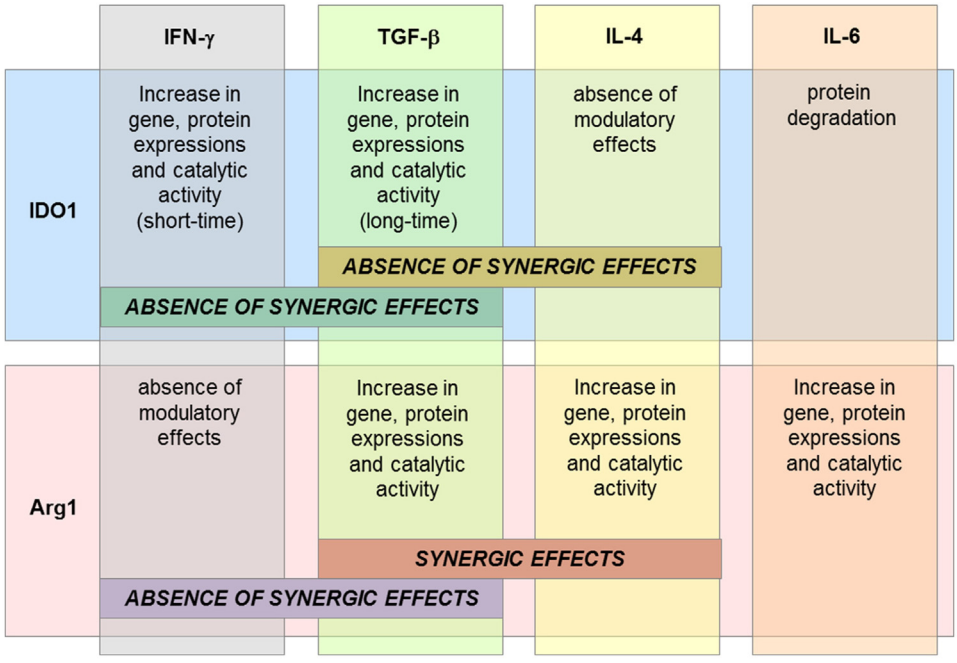

Fig. 3. Effects of IL-4, IFN-γ, and TGF-β on Arg1 and/or IDO1 expression/s in mouse DCs. TGF-β alone, but not its combination with IL-4 or IFN-γ, upregulates both Arg1 and IDO1 in DCs. IFN-γ or IL-4 alone induces the expression and activity of IDO1 or Arg1, respectively. Although IL-4 and TGF-β have synergistic effects in upregulating Arg1, the same combination inhibits the ability of TGF-β to induce IDO1 due to the antagonistic effect of IL-4 on *Ido1* transcripts [83]. On the other hand, the co-presence of TGF-β and IFN-γ dampens the Arg1-inducing potential of TGF-β, leading to a reduced upregulation of IDO1, possibly because IFN-γ does not promote the IDO1 signaling and thus the consequent positive feedback loop self-promoting IDO1 expression. The pro-inflammatory cytokine IL-6 triggers the proteasomal degradation of IDO1 [74], while it induces the expression of Arg1 in bone marrow-derived DCs [84].

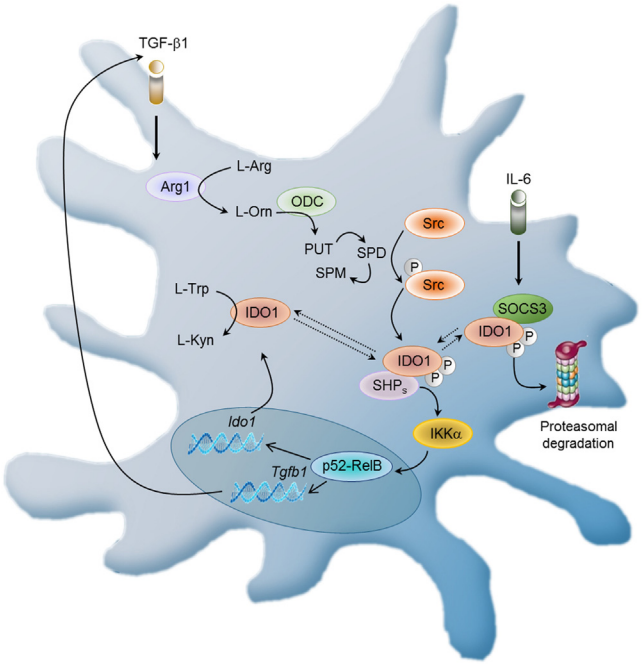

Fig. 4. Scheme of the relay pathway between Arg1 and IDO1 in DCs. Arg1, induced by the cytokine TGF-β, catalyzes the conversion of Arg in urea and ornithine (Orn), which is further metabolized by ornithine decarboxylase (ODC) into polyamines (PUT, putrescine; SPD, spermidine; and SPM, spermine). SPD, through the activation of the Src kinase, promotes the phosphorylation of IDO1 and thus favors the initiation of immunoregulatory signaling events in DCs. Once phosphorylated, IDO1 recruits tyrosine phosphatases (SHPs) and activates the non-canonical NF-κB pathway by inducing phosphorylation of the kinase IKKα and nuclear translocation of p52-RelB complexes. The p52-RelB dimer in turn activates genes encoding for IDO1 and TGF-β, creating a self-sustaining circuitry responsible for the maintenance of immune tolerance. On the contrary, in the presence of IL-6, SOCS3 is induced and it associates with the IDO1 protein, which is then targeted for proteasomal degradation. The reduction of IDO1 lifespan subverts the tolerogenic program of DCs, promoting immune activation.

MDSCs and regulatory macrophages [79] into a sustained regulatory response (*via* IDO1; typical of long-term acting tolerogenic DCs [80].

Therefore, once IDO1 appeared in mammals, the evolutionary pressure may have favored a functional convergence not only between metabolic and immunologic pathways but also between distinct amino acid catabolizing enzymes to cope better with the multiple needs of high vertebrates. A consequence of this could be that tumors, considered to be the result of an evolutionary process [81], have become particularly apt to co-opt metabolic and immunosuppressive networks to propel their generation and progression and therefore the simultaneous inhibition of two immune checkpoints such as Arg1 (https://clinicaltrials.gov/show/NCT02903914) and IDO1 [82] could represent a successful strategy in the immunotherapy of tumors dominated by the expression of TGF-β.

7. Conclusion

Imbedded in immune cell physiology are metabolic pathways and metabolites that not only provide energy and substrates for growth and survival, but also instruct effector functions, differentiation, and gene expression. Focusing on the specific aspect of amino-acid sensing and degradation in immunometabolism, we have made the case for Trp- and Arg-catabolic pathways – and the inter-cross thereof – to exemplify the choices that environments impose on the metabolism and function of immune cells and highlight their consequences during homeostasis and disease.

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Ursula Grohmann, Pharm.D. (Pharmaceutical Chemistry and Technology) and Ph.D. in Experimental Medicine, has been working at the Pharmacology Section of the Department of Experimental Medicine (DEM; University of Perugia, Italy) since 1986, first as post-doc fellow, then as senior researcher and associate professor. Since 2006, she has been appointed as full professor of Pharmacology at DEM, University of Perugia, and, since July 2014, as Visiting Professor at the Albert Einstein College of Medicine, New York, NY (http://www.einstein.yu.edu/faculty/14465/ursula-grohmann/). In 1987-88 and 1990, she accomplished part of her research project at the National Cancer Institute, National Institutes of Health, Bethesda, MD under the supervision of Drs. Michael Mage and Ettore Appella. In 2001, with other members of the DEM Pharmacology group, she discovered a crucial immunosuppressive role in tumor immunity for the enzyme indoleamine 2,3-dioxygenase 1 (IDO1) expressed by dendritic cells. Since then, she continued to study many aspects of IDO1's biology not only in experimental tumor systems, but also in autoimmune and metabolic diseases, allergy, and chronic inflammation. In 2011, she discovered that IDO1 is not just an enzyme catabolizing tryptophan but also a signaling molecule capable of reprogramming dendritic cells towards an immunoregulatory profile. Grohmann's group currently constitutes one of the top world leaders in research related to IDO1 and immune regulation. She is author of more than 120 published articles *in extenso* appearing in the NCBI database and inventor of two patents. The H index of her global career is equal to 51 (Google Scholar), with more than 11,800 citations, and her name is included in the list of Top Italian Scientists in the world. She has been and is the principal investigator of several national and international projects, including the ongoing *Dynamics of IDO*, awarded by the prestigious European Research Council (ERC; years 2014–2019; 2,492,000 Euro). She has been recently awarded as Top Italian Woman Scientist 2016 (http://www.ondaosservatorio.it/cerimonia-top-italian-women-scientists-2016/). She is member of several scientific societies, including the international European Macrophage & Dendritic Cell Society and American Association of Immunologists and is an active reviewer of national and international grants as well as oustanding journals, including Nature Medicine, Immunity, Science Signaling, Journal of Clinical Investigation, EMBO Journal, among many others. She has been recently nominated by the Italian Ministry of Education and University Research as member of the national committee for the 2016 selection of PRIN (research projects of national interest). She is the coordinator of the Doctorate in Systems Biology in Infectious and Immune Diseases (University of Perugia), which also includes Prof. Maikel Peppelenbosch from the Erasmus Medical Center, Rotterdam, The Netherlands, and the Official Research Delegate of DEM.