Tryptophan depletion sensitizes the AHR pathway by increasing AHR expression and GCN2/LAT1-mediated kynurenine uptake, and potentiates induction of regulatory T lymphocytes

Marie Solvay,1,2 Pauline Holfelder,3,4 Simon Klaessens,1,2 Luc Pilotte,1,2 Vincent Stroobant,1,2 Juliette Lamy,1,2 Stefan Naulaerts,1,2 Quentin Spillier,5 Raphaël Frédéric1,5 Etienne De Plaen,1,2 Christine Sers3,4,7 Christiane A Opitz,4,6 Benoit J Van den Eynde,2,7 Jingjing Zhu1,2

ABSTRACT
Background Indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan-dioxygenase (TDO) are enzymes catabolizing the essential amino acid tryptophan into kynurenine. Expression of these enzymes is frequently observed in advanced-stage cancers and is associated with poor disease prognosis and immune suppression. Mechanistically, the respective roles of tryptophan shortage and kynurenine production in suppressing immunity remain unclear. Kynurenine was proposed as an endogenous ligand for the aryl hydrocarbon receptor (AHR), which can regulate inflammation and immunity. However, controversy remains regarding the role of AHR in IDO1/TDO-mediated immune suppression, as well as the involvement of kynurenine. In this study, we aimed to clarify the link between IDO1/TDO expression, AHR pathway activation and immune suppression.

Methods AHR expression and activation was analyzed by RT-qPCR and western blot analysis in cells engineered to express IDO1/TDO, or cultured in medium mimicking tryptophan catabolism by IDO1/TDO. In vivo differentiation of naïve CD4 T cells into regulatory T cells (Tregs) was compared in T cells isolated from mice bearing different Ahr alleles or a knockout of Ahr, and cultured in medium with or without tryptophan and kynurenine.

Results We confirmed that IDO1/TDO expression activated AHR in HEK-293-E cells, as measured by the induction of AHR target genes. Unexpectedly, AHR was also overexpressed on IDO1/TDO expression. AHR overexpression did not depend on kynurenine but was triggered by tryptophan deprivation. Multiple human tumor cell lines overexpressed AHR on tryptophan deprivation. AHR overexpression was not dependent on general control non-derepressible 2 (GCN2), and strongly sensitized the AHR pathway. As a result, kynurenine and other tryptophan catabolites, which are weak AHR agonists in normal conditions, strongly induced AHR target genes in tryptophan-depleted conditions. Tryptophan depletion also increased kynurenine uptake by increasing SLC7A5 (LAT1) expression in a GCN2-dependent manner. Tryptophan deprivation potentiated Treg differentiation from naïve lymphocytes.

WHAT IS ALREADY KNOWN ON THIS TOPIC
⇒ In preclinical models, indoleamine 2,3-dioxygenase 1 (IDO1) or tryptophan-dioxygenase (TDO)-induced tryptophan degradation was shown to restrict inflammation and promote T-cell tolerance to immunogenic tumor antigens, thereby inducing tumorous resistance to immune rejection.
⇒ Kynurenine was proposed to act as an endogenous ligand for the aryl hydrocarbon receptor (AHR) and modulate inflammatory and immune responses.
⇒ Recent studies challenge the notion that kynurenine is a potent AHR agonistic ligand, and the relative role of tryptophan depletion versus kynurenine production in immune suppression remains unknown.

WHAT THIS STUDY ADDS
⇒ Tryptophan depletion strongly increases AHR expression, potentiating its activation by weak agonists such as kynurenine and derivatives.
⇒ Tryptophan depletion also increased cellular kynurenine uptake by increasing SLC7A5 (LAT1) expression in a general control non-derepressible 2-dependent manner, thereby also contributing to potentiating the capacity of kynurenine to activate the AHR upon tryptophan depletion.
⇒ These mechanisms explain IDO1/TDO-mediated AHR activation and immune suppression, resulting in better induction of regulatory T cells.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY
⇒ The study highlights the importance of considering the relative role of tryptophan depletion versus kynurenine production in immune suppression.
⇒ This study emphasizes the importance of considering the complex interplay between tryptophan depletion, kynurenine production, and AHR activation in immune regulation, which may inform the design of future cancer immunotherapies.
CD4+ T cells isolated from mice bearing an AHR allele of weak affinity similar to the human AHR.

Conclusions Tryptophan deprivation sensitizes the AHR pathway by inducing AHR overexpression and increasing cellular kynurenine uptake. As a result, tryptophan catabolites such as kynurenine more potently activate AHR, and Treg differentiation is promoted. Our results propose a molecular explanation for the combined roles of tryptophan deprivation and kynurenine production in mediating IDO1/TDO-induced immune suppression.

INTRODUCTION

Indoleamine 2,3-dioxygenase 1 (IDO1) and tryptophan-2,3-dioxygenase (TDO) are two enzymes responsible for catabolizing the essential amino acid tryptophan into kynurenine. Expression of these enzymes is frequently observed in advance-stage cancers and is associated with poor disease prognosis. In preclinical in vivo models IDO1 and TDO were found to induce tumoral resistance to immune rejection, by restricting inflammation and promoting T-cell tolerance to immunogenic tumor antigens. Despite their ability to promote immune rejection of tumors in preclinical models, IDO1 inhibitors so far have failed to improve the outcome of immunotherapy in human melanomas. This calls for a better understanding of IDO1-mediated immune suppression in both mouse and human systems. Indeed, the mechanism that translates tryptophan catabolism into T-lymphocyte malfunction within the tumor microenvironment (TME) remains uncertain. Several hypotheses have been put forward to explain IDO1-mediated immune suppression, but today it remains unclear whether the major driver for suppression is the local depletion of tryptophan or the production of kynurenine and derivatives. One of the first models proposed was based on the observed proliferation arrest of T lymphocytes exposed to low (<1 μM) tryptophan concentrations. This arrest was found to depend on an integrated stress response triggered by general control non-derepressible 2 (GCN2), a kinase that is sensitive to transfer RNAs that are not bound to their amino-acid cargo, whose levels increase on amino-acid depletion. However, subsequent work showed that GCN2 was not involved in the IDO1-mediated tumoral immune resistance. Another model proposed that IDO1-mediated suppression relied on deactivation of the mTOR pathway due to tryptophan depletion. The alternative view that the production of kynurenine and derivatives is the major driver of immunosuppression was supported by initial observations that kynurenine and its catabolites could trigger apoptosis of T lymphocytes. Moreover, kynurenine itself was then identified as an endogenous ligand for the aryl hydrocarbon receptor (AHR), leading to the suggestion that the IDO1/Kyn/AHR axis could play a key role in modulating inflammatory and immune responses. Present in cells of various organs, AHR acts as an intracellular receptor sensitive to a variety of aromatic molecules potentially toxic to cells. Once activated, AHR is translocated into the nucleus and induces transcription of detoxifying enzymes, such as cytochrome P450 encoded by CYP1A1, involved in the catabolism of aromatic compounds. AHR activity promotes the induction of peripheral regulatory T lymphocytes (Tregs), the upregulation of PD-1 on CD8+ T cells and the recruitment of immunosuppressive tumor-associated macrophages. However, despite considerable advances in the understanding of immunosuppressive mechanisms mediated by AHR, a recent study challenged the notion that kynurenine is a genuine AHR agonistic ligand. Seok et al. observed that kynurenine dissolved in dimethyl sulfoxide (DMSO) spontaneously undergoes chemical conversion into an oxidized dimeric derivative that is an exceptionally strong AHR agonist and whose trace presence in kynurenine preparations may account for the reported weak agonistic activity of kynurenine, which was usually only observed at high concentrations well above the physiological concentration. In addition, the chemical structure of kynurenine does not make it an optimal ligand, as it is highly polar and also too small for good binding in the AHR ligand binding site, which rather accommodates ligands with more than one aromatic ring. Kynurenine metabolites such as anthranilic acid (AA), kynurenic acid (KA) and 3-hydroxykynurenine (HK), which have a structure more apt to bind AHR, have also been proposed as AHR ligands, but their concentration in human serum is 10–50 times lower than kynurenine.

In this work, we further explored and clarified the association between IDO1/TDO activity and AHR activation. Unexpectedly, we observed that tryptophan depletion strongly increased AHR expression, thereby potentiating its activation by weak agonists and the induction of regulatory T cells. These results provide a mechanistic rationale supporting a combined effect of both tryptophan depletion and production of tryptophan catabolites in the local immunosuppression triggered by IDO1/TDO activity.

MATERIALS AND METHODS

Mice

C57BL/6 mice were bred in the animal facility of the Ludwig Institute for Cancer Research, Brussels, Belgium. C57BL/6 Ahr-KO (Ahr knock-out) mice were obtained from D. Togbe (CNRS UMR7355, Orléans, France). DBA/2 Ahr-KO mice were generated from backcrossing C57BL/6 Ahr-KO mice with DBA/2 mice bred in the animal facility of de Duve Institute (Brussels, Belgium). Ido1-KO mice were purchased from Jackson Laboratory. Mice were used for in vivo experiments at 8–12 weeks of age. Mice were bred and maintained under specific pathogen-free conditions. Genotyping was performed by PCR. DNA was extracted from mice ear tissue using the Gotaq kit (Promega). Primmers were purchased from Eurogentec. Gene: Ahr forward GGCTAGGCTGGGTTTCTC, reverse CTCGGTGTCCTTAAAGCTTCA, Neomycin resistance-gene forward CGGGAGCCGGCGA TACCGTAAAGC, reverse GAAAGGAAAGAGGACT
GGCTGCTA. PCR products were separated on a 1 % agarose gel.

**Cell lines**

HEK293-EBNA (HEK293-E) cell line (Invitrogen) was stably transfected with PEF6/V5-His expression vector encoding human IDO1 and TDO cDNA, as described. TDO-expressing LB159-CRCA cells were derived from a colorectal carcinoma (CRC) patient sample from the Cliniques universitaires Saint-Luc in 1991. HepG2 cells were purchased from Cell Line Service.

**Generation of GCN2-KO and ATF4-KO HEK293-E cells**

GCN2-KO and ATF4-KO HEK293-E cells were generated by transfecting HEK293-E cells with two CRISPR/Cas9 Double Nickase Plasmids targeting each gene: GCN2 (#sc-402313-NIC and #sc-402313-NIC-2, Santa Cruz Biotechnology), activating transcription factor 4 (ATF4) (#sc-400155-NIC and #sc-400155-NIC-2, Santa Cruz Biotechnology). Control scramble (#sc-108062, Santa Cruz Biotechnology) was used as a negative control. Plasmids were diluted in fetal bovine serum (FBS)-free DMEM with Turbofect reagent (Thermo Fisher, #R0531) and incubated at room temperature for 15–20 min. The plasmid/Turbofect mix was then added to the cells in a dropwise fashion, and the plate was gently rocked to achieve even distribution of the plasmids.

Cells were cultured for 72 hours prior to selection with puromycin (10 µg/mL). The knockout of GCN2 and ATF4 was validated by Western blot analysis.

**Culture medium**

Cells were cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) (#21980-032 from Life Technologies) supplemented with 10% FBS (#11180), Sigma, non-essential amino acids (0.55 mM L-arginine, 0.24 mM L-asparagine, 1.5 mM glutamine) and antibiotics (100 µg/mL streptomycin, 100 IU/mL penicillin) unless specified otherwise. In experiments performed using tryptophan-free conditions, we used IMDM medium without L-tryptophan (#ME130013L1, Life Technologies) complemented with non-essential amino acids (0.55 mM L-arginine, 0.24 mM L-asparagine, 1.5 mM glutamine).

**Compounds**

L-tryptophan (Trp, #T0254), L-kynurenine (Kyn, #K8625), Kynurenine acid (KA, #K3757), Xanthurenic acid (XA, #D10804), Anthranilic acid (AA, #A89855), 3-hydroxyanthranilic acid (HAA, #148776), 3-Hydroxykynurenine (HK, #H1771), Nicotinamide (NIC, #N9976), 2,3-pyridinediacarboxylic acid (QA, #P63204), Quinaldic acid (QAA, #160660), and Tetrachlorodibenzo-p-dioxin (TCDD, #48599) were purchased from Sigma-Aldrich. 6-Formylindolo[3,2-b] carbazole (FICZ, #BML-DR206-0100) was purchased from Enzo Life Sciences. The IDO1 inhibitor Epacadostat was obtained from Synnovator, Inc. TDO inhibitor (PF06845102/EOS200809) is described by Schramme et al. and in the published patent application US 20150225367. The AHR antagonist CH-223191 (#182705) was purchased from Merck. LAT1 inhibitor BCH (#5027) was purchased from Torcis and Selleckchem, respectively. All metabolites were solubilized in HBSS medium except for HAA and KA, which required to be dissolved in DMSO. Each compound was aliquoted immediately after reconstitution and was stored at −80°C. Each metabolite was controlled by high performance liquid chromatography (HPLC) after dilution for degraded by-products. Salubrinal (#SML0951) and Isrib (#SML0843) were purchased from Sigma Aldrich. To inhibit the transcription, 5 µg/mL Actinomycin D (#A14262 from Sigma) prediluted in DMSO were added to the medium.

**RT-qPCR**

Total RNA was extracted from cells with the NucleoSpin RNA kit from Macherey Nagel (#740955) and was retro-transcribed to complementary DNA (cDNA) by using the RevertAid kit from ThermoFisher (#K1691). RT-qPCR experiments were performed with the Takyon Rox probe core kit dTTP from Eurogentec (#U-RPCT-C0201) in a StepOnePlus thermal cycler (Applied Biosystems). Primers and probes were purchased from Eurogentec. Primer and probe sequences used are listed in online supplemental file 1.

**Western blot**

Cells were lysed in a complete lysis buffer (0.1% SDS, 1% sodium deoxycholate, 0.5% NP40 supplemented with complete Protease Inhibitor Cocktail (#11697498001, Roche) and if appropriate, 1 tablet of phosphatase inhibitor for 10 mL of lysis buffer (# 04906845001, Roche). Total cell lysates were homogenized by sonication or by passing solutions through a 30G syringe. Lysates were then clarified by centrifugation (9500 g for 10 min). Protein concentrations were evaluated by the bicinchoninic acid assay (#23225, Pierce). Proteins of 20 µg were heated at 70°C for 10 min with loading solutions: NuPAGE Sample Reducing Agent (#NP0009, ThermoFisher) and NuPAGE LDS (lithium dodecyl sulfate) Sample Buffer (#NP0007, ThermoFisher). Samples were loaded on denaturing NuPAGE 4%–12% Bis-Tris gels (#NP0321BOX and #WG1420Box, ThermoFisher) in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (#NP0001-02, ThermoFisher) supplemented with NuPage antioxidant at 1/1000 (#NP0005, ThermoFisher). Proteins were transferred on nitrocellulose membranes (#IB23001, ThermoFisher) by using the iBlot2 Dry Blotting System (ThermoFisher). Nitrocellulose membranes were blocked in phosphate-buffered saline (PBS) or Tris-buffered saline with 5% milk and 0.1% Tween and then incubated with the primary antibodies in PBS/TBS with 5% BSA and 0.1% Tween at 4°C overnight (except for anti-β-actin: 1 hour at room temperature). After three washes, membranes were probed with secondary antibodies conjugated with horseradish peroxidase (HRP) in the same buffer at room temperature for 1 hour. Proteins were detected with the chemiluminescent SuperSignal.
West Pico substrate (#34578, Pierce) or with the Femto substrate (#34096, Pierce). Pictures were captured with the Fusion Fx camera (Vilbert Lourmat). Staining with different antibodies was performed on the same membrane, which was stripped with Restore Western Blot Stripping Buffer (#21063, ThermoFisher) for 10–15 min. The western blot quantification was achieved with Bio1D software from Vilber Lourmat. The protein signal intensity was measured with the optical density grid method. The background was subtracted by using the rolling ball mode. First, the value of each lane was normalized by the mean of all lanes on the same blot to minimize the effects of protein content between experiments and exposure time. Second, AHR signal intensity was normalized by the signal intensity of the housekeeping protein (β-actin or vinculin).

**Antibodies for Western blot**
AHR (clone D5S6H) rabbit monoclonal antibody, ATF4 (clone D4B8) rabbit monoclonal antibody, GCN2 (#3302S) rabbit polyclonal antibody, LAT1 (#5347S) rabbit polyclonal antibody was purchased from Cell Signaling and diluted at 1/1000. Actin mouse monoclonal antibody (#A5441 from Sigma) was diluted at 1/10,000. Vinculin (#V9131) monoclonal antibody was purchased from Sigma-Aldrich and diluted at 1/10,000. Anti-rabbit HRP-linked IgG secondary antibody (#7074 from Cell Signaling) and anti-mouse HRP-linked IgG secondary antibody (#HAF007 from R&D Systems) were used at 1/5000 (except for actin: 1/15,000).

**Kynurenine uptake assay**
Kynurenine, tryptophan and 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) were prewarmed to 37°C. Cells were resuspended in warmed PBS and kept at 37°C. HEK-293-E cells were seeded in a 96U shape plate (100,000 cells per well), 100 µL of PBS, BCH, or tryptophan were added to appropriate samples before adding 100 µL kynurenine 160 µM (final concentration: 80 µM). The kynurenine uptake was stopped after 4 min by adding 125 µL 4% paraformaldehyde for 30 min at room temperature in the dark. After fixation, cells were washed twice in PBS containing 0.5% BSA and kynurenine uptake was measured by flow cytometry analysis using Fortessa (BD Bioscience) and analyzed by FlowJo software (V.10.8.2). The 405 nm laser and 450/50 BP filter or 525/50 BP (Bv510) are used for kynurenine fluorescence detection.

**Measurement of tryptophan and kynurenine concentrations by HPLC**
Tryptophan and kynurenine were quantified in the cell culture supernatants by HPLC based on the retention time and the UV absorption (280 nm for tryptophan, 360 nm for kynurenine).

**Isolation of naïve CD4⁺ T cells and regulatory T-cell differentiation**
Naïve CD4⁺ T cells were isolated from mice spleen using the CD4⁺ CD62L⁺ Isolation kit (Miltenyl Biotech #130-093-227). Naïve T cells were cultured in tryptophan-free IMDM medium supplemented with 10% FBS, glutamax, 2-ME (50 µM), penicillin, streptomycin and treated with 5 ng/mL rhTGF-β (#240-B, Bio-technne), 100 U/mL IL-2 (#2012-12, Peprotech) and 80 µg tryptophan. Cells were stimulated using Dynabeads Mouse T-Activator CD3/CD28 (#11456D, ThermoFisher). After 4 days, the cultured cells were harvested for flow cytometric analysis or RT-qPCR analysis. For flow cytometry analysis, cells were first surface stained for viability using fixable viability dye eflour780 (1/2000) (#65-0865-14, ThermoFisher) and anti CD4 antibody-FITC (clone GK1.5, #100406, Biolegend) (1/200) and then fixed and permeabilized with the intracellular fixation/permeabilisation buffer set (#88-882100, ThermoFisher). Following this, cells were intracellularly stained with anti-Foxp3 antibody (clone FJK-16s, #17-5773-82, Invitrogen) (1/100) prior to flow cytometry analysis performed using Verso (BD Bioscience) and analyzed by FlowJo software (V.10.8.2).

**Spearman correlation analysis**
Gene expression data for The Cancer Genome Atlas (TCGA) cancer types was downloaded from Xena. Spearman correlations were calculated with SciPy V.1.6.2 and visualized with matplotlib V.3.5.2 and seaborn V.0.11.1. Precalculated immune deconvolutions for TCGA were obtained from TIMER V.2.0, of which the QUANTISEQ immune cell type deconvolutions were used in this study.

**Statistical method**
Statistical analysis was performed with Graphpad prism (V.9.4.0) software. To compare the mRNA transcript, protein quantification or percentage of CD4⁺/FoxP3⁺ cell population, we applied an unpaired two-tailed Student’s t-test. The p values<0.05 were considered statistically significant. For all figures, the p values are annotated as follows: NS, p>0.05; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

**RESULTS**
**IDO1 and TDO induce AHR activation and overexpression**
We first aimed to confirm the correlation between the enzymatic activity of IDO1/TDO and the activation of AHR in human cells. As shown in figure 1A,B, human embryonic kidney cells (HEK293-E) overexpressing IDO1 or TDO efficiently converted tryptophan into kynurenine (figure 1A,B, right panels), and triggered AHR activity, as indicated by the upregulated expression of AHR-target genes CYP1A1 (figure 1A,B, left panels), CYP1B1, STC2, TIPARP, EREG and IL4I1 (online supplemental figure 1A). This AHR activation was dependent on IDO1/TDO enzymatic activity, as it was abolished in the presence of IDO1 or TDO inhibitors. Unexpectedly, in addition to AHR activation, we observed a strong increase in the expression of the AHR transcript itself, which was also
Figure 1  IDO1 and TDO activity induces AHR activation and upregulation (A) RT-qPCR analysis of CYP1A1 (left panel) and AHR (middle panel) in HEK293-E or HEK293-E hIDO1 cells treated or not with IDO1 inhibitor (Epacadostat, 2.5 µM) for 120 hours. (B) RT-qPCR analysis of CYP1A1 (left panel) and AHR (middle panel) in HEK293-E or HEK293-E hTDO cells treated or not with TDO inhibitor (EOS200809, 5 µM). Kynurenine or tryptophan concentrations were measured by HPLC in cell supernatant (A, B, right panels). (C) RT-qPCR analysis of CYP1A1 and AHR in HEK293-E cells treated with the AHR agonist FICZ (1 µM) for 120 hours. (D) RT-qPCR analysis of CYP1A1 and AHR in HEK293-E or HEK293-E hIDO1 cells treated or not with IDO1 inhibitor (Epacadostat, 2.5 µM) or AHR inhibitor (CH-223191, 10 µM) for 120 hours. The mRNA levels of different genes were measured by quantitative RT-qPCR and normalized to GAPDH. Mean±SD of technical triplicates from one representative experiment out of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.000. AHR, aryl hydrocarbon receptor; FICZ, formylindolo[3,2-b]carbazole; HPLC, high-performance liquid chromatography; IDO1, indoleamine 2,3-dioxygenase 1; ns, not significant; TDO, tryptophan-dioxygenase.
dependent on the enzymatic activity of IDO1 or TDO (figure 1A,B, middle panels).

Because a previous study suggested a possible ‘autoregulation’ of AHR expression by AHR agonists in a positive feedback manner,25 we evaluated whether the increased AHR expression in IDO1/TDO expressing cells was dependent on AHR activity. However, we observed no increased AHR expression in cells treated with the potent AHR agonist FICZ26 (figure 1C). Moreover, the increased AHR expression observed in IDO1-expressing cells was not abolished on treatment with AHR inhibitor CH2339127 (figure 1D). Although IDO1 was previously shown to be induced by AHR activation in dendritic cells,28 no increase of IDO1 expression was found in HEK293E cells cultured under tryptophan-depleted conditions. (online supplemental figure 1B).

Altogether, our results point to a direct regulation of AHR expression by the enzymatic activity of IDO1 and TDO. We next deciphered how the enzymatic activity of IDO1 and TDO regulates AHR expression.

**IDO1/TDO-induced AHR expression is triggered by tryptophan deprivation**

We first wanted to determine whether the increased AHR expression in IDO1-expressing cells was driven by kynurenine production and/or by tryptophan deprivation. When we treated wild-type HEK293-E cells for 72 hours with 80µM fresh kynurenine, we observed no increase in AHR expression (figure 2A). We then repeated this experiment adding kynurenine to the cells, but this time grown in tryptophan-free medium, to mimic the metabolic consequences of tryptophan catabolism mediated by IDO1 and TDO. We found no impact of kynurenine on AHR expression. Surprisingly, however, we found an increased AHR expression in cells cultured in the tryptophan-free medium (figure 2A).

This result suggested that AHR expression was modulated by the tryptophan concentration. Increased AHR expression required very low tryptophan levels and was observed after 72 hours but not after 24 hours of incubation in tryptophan-free medium (figure 2A, B). This likely resulted from the time needed for the cells to completely consume the tryptophan coming with the FBS supplement. Indeed after 24 hours, there was about 2µM residual tryptophan in the medium under these conditions (online supplemental figure 1C). Accordingly, when we cultured the cells in tryptophan-free medium without FBS, we observed an increased AHR expression and activation already at 24 hours (figure 2C, online supplemental figure 1D). To avoid the delayed effect in cells grown with FBS, we then used IDO1-overexpressing HEK293-E cells, which more actively degrade FBS-derived tryptophan. We incubated these cells for 24 hours with different tryptophan concentrations, ranging from 0 to 200µM, and evaluated AHR mRNA (figure 2D) and proteins levels (figure 2E). Tryptophan concentration in the supernatant was measured at the end of the incubation (final Trp) (online supplemental figure 1E) to know the tryptophan concentration at the time when cells were collected for AHR expression analysis. We observed an increase of AHR transcript and protein at low tryptophan levels. This effect was not restricted to HEK293-E cells, as an increase of AHR expression was also observed in CRC (LB159-CRC) and hepatocellular carcinoma (HepG2) cultured in tryptophan-depleted medium (online supplemental figure 2). Analysis of mRNA stability after transcriptional inhibition with Actinomycin D revealed that the increased AHR mRNA level in cells cultured under tryptophan deprivation was not due to increased mRNA stability but rather to increased AHR transcription (figure 2F).

**AHR upregulation due to tryptophan depletion does not result from GCN2 signaling**

To further elucidate how tryptophan deprivation induces AHR expression, we assessed the involvement of GCN2, which senses unbound transfer RNA, indicating amino-acid shortage, and phosphorylates eukaryotic translation initiation factor 2α (eIF2α) to shut down cap-dependent translation,29 while favoring translation of activating transcription factor 4 (ATF4). The latter triggers transcription of genes involved in amino-acid metabolism30 and transportation,31 nutrient sensing and protein turnover,32 33 in order to restore amino-acid homeostasis.34 35 We confirmed that tryptophan deprivation activates the GCN2/ATF4 pathway, as illustrated by increased phosphorylation of eIF2α and expression of ATF4 (figure 3A). To test the involvement of the GCN2-ATF4 pathway in the regulation of tryptophan-deprivation-induced AHR expression, we first used Salubrilin,35 an inhibitor of phosphatase ‘growth arrest and DNA damage-inducible protein 34’ (GADD34), which dephosphorylates eIF2α, thereby mimicking the activation of GCN2. Treating cells with Salubrilin increased ATF4 expression (Suppl figure 3A) without tryptophan deprivation, indicating an activation of the GCN2-ATF4 pathway. However, it did not increase AHR expression (figure 3B). We also used Isrib,36 a compound that blocks the GCN2/eIF2α/ATF4 pathway downstream of eIF2α (online supplemental figure 3A). As expected, the addition of Isrib to cells cultured under tryptophan deprivation reduced ATF4 expression, indicating a blockade of GCN2-ATF4 signaling. However, Isrib did not prevent induction of AHR expression on tryptophan deprivation, indicating an activation of the GCN2-ATF4 pathway. However, it did not increase AHR expression (figure 3B).

Finally, to confirm that the GCN2/eIF2α/ATF4 pathway was not involved in the regulation of AHR expression, we generated GCN2-knockout and ATF4-knockout HEK293-E cells (figure 3C) and assessed AHR expression in these cells under tryptophan-depleted conditions. As shown in figure 3D, E, AHR expression was still increased on tryptophan depletion, despite the absence of GCN2 and/or ATF4. Altogether, our results excluded the involvement of the GCN2/eIF2α/ATF4 pathway in the upregulation of AHR expression in response to tryptophan depletion.
AHR activation by kynurenine and derivatives is enhanced in the absence of tryptophan

Kynurenine has been widely described as a weak AHR agonist, although controversy remains due to its atypical, highly polar, and non-ligand-like structure. Given that tryptophan shortage increases AHR expression, we

Figure 2  IDO1/TDO-induced AHR upregulation is induced by tryptophan deprivation. (A, B) HEK293-E cells were treated with kynurenine (80 μM) in tryptophan-free medium supplemented or not with 80 μM tryptophan in the presence of 10% FBS for 72 hours (A) or 24 hours (B). AHR expression was measured by RT-qPCR analysis. Mean±SD of technical triplicates from one representative experiment out of three independent experiments. (C) HEK293-E cells were cultured for 24 hours in tryptophan-free medium supplemented or not with 80 μM tryptophan in the absence of FBS. AHR expression was measured by RT-qPCR analysis (left panel) and western blot analysis (right panels). The quantification of AHR protein is shown in the lower right panel, each point representing a technical replicate. Mean±SD of technical triplicates from one representative experiment out of three independent experiments (left panel). (D, E) HEK293-E IDO1 cells were cultured in 2% FBS with decreasing concentrations of tryptophan for 24 hours. AHR expression was measured by RT-qPCR analysis (D) and western blot analysis (E). The concentration of tryptophan at the end of the 24 hours incubation was measured by HPLC (final Trp). Mean±SD of technical triplicates from one representative experiment out of three independent experiments for panel D and mean±SD of technical duplicates from one representative experiment out of two independent experiments for panel E. In figure 2E, the quantification of AHR is shown in the lower panel. Each point represents a technical replicate. (F) HEK293-E cells treated with DMSO or Actinomycin D (5 μg/mL) were cultured for 24 hours with or without tryptophan in medium without FBS. AHR expression was measured by RT-qPCR analysis. The mRNA levels of different genes were measured by quantitative RT-qPCR and normalized to GAPDH. Mean±SD of technical triplicates from one representative experiment out of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. AHR, aryl hydrocarbon receptor; FBS, fetal bovine serum; IDO1, indoleamine 2,3-dioxygenase 1; ns, not significant; TDO, tryptophan-dioxygenase.
assessed whether it would potentiate AHR activation by kynurenine in HEK293-E cells, by analyzing the expression of the AHR target gene CYP1A1. We observed that, under normal tryptophan conditions, kynurenine did not activate AHR in these cells (figure 4A, online supplemental figure 3B). However, kynurenine clearly activated AHR in tryptophan-free conditions (figure 4A, online supplemental figure 3B), even at low concentrations (figure 4B). Also in HepG2 cells did tryptophan depletion reveal the AHR agonistic activity of kynurenine (online supplemental figure 3C). The effect was AHR-dependent since adding an AHR inhibitor abolished the induction of AHR target gene expression by kynurenine under tryptophan deprivation (figure 4A).

Although in contrast with several reports in the literature,10–12 our observation that kynurenine does not activate AHR in normal conditions is in line with the report of Seok et al indicating that kynurenine requires subsequent modifications to act as a potent AHR agonist.18 The authors showed that kynurenine stored in DMSO transforms into an oxidized dimeric derivative with a very potent AHR agonistic activity. We also observed a strong increase in the agonistic activity of our kynurenine solutions prepared and stored in DMSO (online supplemental figure 4). We identified the same derivative in our old solutions, and verified that it was much more potent in activating AHR as compared with kynurenine (online supplemental figure 4). These results suggest on one hand

---

**Figure 3** Tryptophan-depletion induced AHR overexpression is independent of GCN2. (A) Western blot analysis of HEK293-E cells cultured with or without tryptophan for 24, 48 and 72 hours for the expression of phospho-eIF2α, eIF2α, and ATF4. One representative experiment out of two independent experiments. (B) RT-qPCR analysis of AHR expression in HEK293-E cells treated for 24 hours with Salubrinal (20 µM), Isrib (5 µM) or vehicle (DMSO) with or without tryptophan in medium without FBS. Mean±SD of technical triplicates from one representative experiment out of two independent experiments. (C) Upper panel: GCN2-KO efficiency. Lower panel: ATRF expression in GCN2-KO, ATRF-KO or wild-type cells cultured for 24 hours without FBS in medium with or without tryptophan. One representative experiment out of two independent experiments. (D) RT-qPCR analysis of AHR expression in scramble or GCN2-KO HEK293-E cells cultured for 24 hours without FBS in medium with or without tryptophan. Mean±SD of technical triplicates from one representative experiment out of three independent experiments. (E) RT-qPCR analysis of AHR expression in wildtype or ATRF-KO HEK293-E cells cultured for 24 hours without FBS in medium with or without tryptophan. Mean±SD of technical triplicates from one representative experiment out of two independent experiments. *p<0.05, **p<0.01, ****p<0.0001. AHR, aryl hydrocarbon receptor; FBS, fetal bovine serum; ns, not significant.
Figure 4  AHR activation by kynurenine and its derivatives is enhanced in the absence of tryptophan. (A) HEK293-E cells were treated for 72 hours with kynurenine (80 µM), AHR inhibitor (CH223191, 10 µM) or both in tryptophan-free medium supplemented or not with 80 µM tryptophan in the presence of 10% FBS. CYP1A1 expression was measured by RT-qPCR analysis. (B) HEK293-E cells were treated for 24 hours with different concentrations of kynurenine in tryptophan-free medium supplemented or not with 80 µM tryptophan in medium without FBS. CYP1A1 expression was measured by RT-qPCR analysis. (C) Flow cytometric evaluation of kynurenine uptake in HEK293-E cells. Kynurenine fluorescence in HEK293-E cells treated with 80 µM kynurenine, in the presence or absence of the System L inhibitor, BCH (10 mM) in PBS at 37°C or 4°C. (D) Kynurenine fluorescence in HEK293-E cells treated with 80 µM kynurenine and different concentrations of tryptophan in PBS for 4 min. (E, F) RT-qPCR analysis (E) and western blot analysis (F) of SLC7A5 expression in wildtype or GCN2-KO HEK293-E cells cultured for 24 hours in tryptophan medium or tryptophan-free medium. The quantification of LAT1 protein expression is shown in the lower panel, each point representing a technical replicate. (G) Scramble or GCN2-KO HEK293-E cells were cultured overnight in tryptophan-free medium supplemented or not with 80 µM tryptophan without FBS. Cells were then collected and incubated with or without 80 µM kynurenine and treated for 4 min in PBS without tryptophan. Kynurenine uptake was measured by flow cytometry analysis of kynurenine fluorescence. (H) RT-qPCR analysis of CYP1A1 expression in wild-type (upper panel) or GCN2-KO (lower panel) HEK293-E cells treated for 24 hours with kynurenine (80 µM) or FICZ (1 µM) in tryptophan-free medium supplemented or not with 80 µM tryptophan in the absence of FBS. (I) HEK293-E cells were treated for 72 hours with 80 µM kynurenine metabolites in tryptophan-free medium supplemented or not with 80 µM tryptophan in the presence of 10% FBS. CYP1A1 expression was measured by RT-qPCR analysis. (J) HEK293-E cells were treated for 24 hours with different concentrations of kynurenic acid in medium with or without tryptophan and without FBS. CYP1A1 expression was measured by RT-qPCR analysis. (K) RT-qPCR analysis of AADAT expression in wildtype or ATF4-KO HEK293-E cells cultured for 72 hours in tryptophan-free medium supplemented or not with 80 µM tryptophan in the presence of 10% FBS. The mRNA levels of different genes were measured by quantitative RT-qPCR and normalized to GAPDH. Means±SD of technical triplicates from one representative experiment out of three independent experiments, except for panel F and K, which are Means±SD of technical replicates from one representative experiment out of two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. AHR, aryl hydrocarbon receptor; FBS, fetal bovine serum; ns, not significant; PBS, phosphate-buffered saline.
that some reports about AHR activation by kynurenine may have been affected by the presence of this strong artificial agonist. On the other hand, although there is no evidence that kynurenine can produce the same derivative ‘in cellulo’, it is possible that kynurenine acts as a ‘pro-ligand’ requiring further metabolization to become a good AHR agonist. In the experiments reported here, we used kynurenine freshly dissolved in medium, and we observed no AHR activation in normal conditions.

Note that there is a basal level of AHR activation in HEK293-E cells in the standard medium (figure 4A), which might be due to the presence of aromatic AHR ligand-like compounds in the culture medium. In line with this hypothesis, tryptophan depletion further increased this basal level of AHR activity, possibly due to increased AHR expression (figure 4A). Similar observations were made with CRC cells LB159-CRCA and hepato-cellular carcinoma cells HepG2 (online supplemental figure 5).

Our results thus show that, under tryptophan deprivation, kynurenine is more potent to activate AHR, possibly due to increased AHR expression. Another factor that might potentiate the agonistic activity of kynurenine is its intracellular concentration. Cells have been reported to uptake both tryptophan and kynurenine in a competitive manner through the same System L transporter, SLC7A5 (LAT1). A decreased tryptophan concentration might thereby potentiate kynurenine uptake into the cells. To test this hypothesis, we adopted the flow cytometry-based assay developed previously by Sinclair et al, which can directly monitor the intracellular level of kynurenine based on its fluorescence spectral properties. We further optimized the method and found a better signal detection when using a band pass filter 525/50 BP (Bv510) (online supplemental figure 6A, figure 4C). Indeed, an increased fluorescence signal was detected when cells were incubated with kynurenine at 37°C but not at 4°C, indicating an active entry of kynurenine into the cell. In addition, the increase of kynurenine fluorescence signal was attenuated when we treated the cells with BCH, a LAT1 inhibitor, confirming LAT1 as the transporter for kynurenine entry into the cells (figure 4C). As shown in figure 4D, decreasing tryptophan concentration indeed potentiated kynurenine entry into the cells in a dose-dependent manner. In addition, we observed an increased expression of transporter SLC7A5 (LAT1) under tryptophan deprivation (figure 4E,F). The increase of LAT1 expression was regulated via the GCN2 pathway, as it was not observed in GCN2-KO cells (figure 4E,F). These GCN2-KO cells also failed to increase kynurenine uptake in a tryptophan-free medium, indicating that this increase largely depended on LAT1 overexpression (figure 4G, online supplemental figure 6B).

Tryptophan deprivation potentiates AHR activation by kynurenine in wild-type HEK293-E cells, as measured by CYP1A1 expression. This increase of AHR activation by kynurenine is partly due to the increased kynurenine entry mediated by the increased LAT1 expression on tryptophan-deprivation, as a milder increase of CYP1A1 is observed in GCN2-KO HEK293-E cells (figure 4H). For other agonists that are not dependent on LAT1 transportation such as FICZ, tryptophan-deprivation induced AHR overexpression appears to be a major contributor to enhance their AHR activation ability (figure 4H). Altogether, our results indicate a multifaceted regulation of AHR activation by kynurenine under tryptophan deprivation, involving both GCN2-dependent and GCN2-independent pathways.

We also tested other kynurenine pathway metabolites and observed that tryptophan deprivation revealed a potent AHR agonistic activity for all of them (figure 4I). Among them, KA and XA showed already a more potent AHR agonistic activity in standard medium as compared with kynurenine. They became very potent in tryptophan-free medium (figure 4J). To determine which kynurenine metabolite was produced in HEK293-E cells, we evaluated expression of the different enzymes of the pathway in these cells (online supplemental figure 6C). We found that kynurenine formamidase (AFMID) and kynurenine aminotransferase (AADA1) were expressed, but kynurenine 3-monoxygenase (KMO) and kynureninase (KNU) were not. Therefore, the increased AHR activity in IDO1-expressing or TDO-expressing HEK293-E cells or in kynurenine-treated HEK293-E cells cultured in tryptophan-free medium was possibly mediated by kynurenine and/or KA, but not by XA. Interestingly, we also observed a GCN2-ATF4 independent increased expression of kynurenine aminotransferase under tryptophan deprivation (figure 4K). Altogether, the increased AHR activation by kynurenine under tryptophan deprivation might result from the combined effect of increased AHR expression, increased kynurenine import, and increased production of its metabolites.

AHR upregulation favors in vitro differentiation of CD4+ FoxP3+ Treg cells

Analyzing The Cancer Genome Atlas (TCGA) patient tumor transcriptome data (https://www.cancer.gov/tcga) revealed a correlation between the expression of AHR and that of IDO1/TDO2 in multiple tumor types, confirming the physiological and translational relevance of our findings (figure 5A, online supplemental figure 7A). Among different immune cells in the TME, a strong correlation between AHR expression and the presence of Tregs was observed in various patient tumors (figure 5B). It was previously observed that IDO1-expressing tumors had significantly more Tregs and that the presence of AHR was required to optimally generate Treg cells in vitro. We, therefore, evaluated the role of tryptophan-deprivation induced AHR overexpression in Treg differentiation using murine wild-type and Ahr−/− mice. Two alleles encoding different Ahr variants exist among laboratory mouse strains (figure 5C). Compared with human AHR (hAHR), the mouse Ahrb (mAhrb) variant has a ~10fold higher affinity for 2,3,7,8...
**Figure 5** AHR upregulation favors in vitro differentiation of CD4+ FoxP3+ regulatory T lymphocytes. (A) Spearman correlation in bulk TCGA RNAseq data between expression of AHR and IDO1 (left column) and TDO2 (right column) per TCGA cancer type. (B) Spearman correlation between expression of the AHR gene and various QUANTISEQ immune-deconvoluted populations per cancer type in the TCGA patient cohort. (C) Allelic summary of the AHR gene in human and mouse lines. (D) Naïve CD4+ CD62L+ T cells were isolated from WT (wild-type) and Ahr-KO mice from C57BL/6 or DBA/2 background, and were cultured for 5 days with anti-CD3 anti-CD28 beads, TGF-β (5 ng/mL) and IL-2 (100 U/mL). The percentage of Treg differentiation was determined by flow cytometry analysis of cell surface CD4 and intracellular Foxp3 expression. (E) RT-qPCR analysis of Ahr expression in Tregs differentiated from CD4+ T cells isolated from wild-type DBA/2 mice in medium containing or not 80 µM tryptophan. The mRNA levels of different genes were measured by quantitative RT-qPCR and normalized to Gapdh. Mean±SD of triplicates from one out of three experiments. (F) Treg differentiation (same as D) of naïve CD4+ CD62L+ T cells isolated from WT or Ahr-KO DBA/2 mice in medium containing or not 80 µM tryptophan. Mean±SD of technical triplicates from one representative experiment out of three independent experiments. (G) Kynurenine fluorescence in differentiated murine Treg cells treated with 80 µM kynurenine in PBS supplemented or not with tryptophan for 5 min. To demonstrate the LAT1-dependent kynurenine cell entry, we pretreated the cells with BCH (10 mM) in some conditions, as indicated. Cells treated without kynurenine were used as a negative control. Mean±SD of technical triplicates from one representative experiment out of two independent experiments. (H) Treg differentiation (same as D) of naïve CD4+ CD62L+ T cells isolated from WT or Ahr-KO DBA/2 mice in medium containing or not 80 µM tryptophan in the presence or absence of 80 µM kynurenine. Mean±SD of technical triplicates from one representative experiment out of three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. AHR, aryl hydrocarbon receptor; IDO1, indoleamine 2,3-dioxygenase 1; ns, not significant; TCGA, The Cancer Genome Atlas; PBS, Phosphate-buffered saline.
-tetrachlorobenzo-p-dioxin (TCDD). Most in vivo or ex vivo murine data published regarding the role of IDO1/TDO and AHR in immunosuppression result from experiments performed in C57BL/6 mice, which have the mAhrβ variant. Using naïve CD4+ T cells derived from C57BL/6 WT or Ahr-KO mice, we confirmed the critical role of AHR for Treg differentiation induced by TGF-β in vitro. We observed that significantly less Tregs were generated by Ahr-KO as compared with WT CD4+ T cells (figure 5D).

DBA/2 mice carry the Ahrδ variant, which is structurally closer to hAHR, as it contains the same key valine residue in the active site. The mAhrβ variant also has a lower affinity than mAhrβ for TCDD, similar to that of hAHR. We observed that CD4+ T cells from DBA/2 mice produced very few Tregs after in vitro induction (figure 5D). This might result from the low affinity of AHRδ for its ligands. When we repeated the experiment with DBA/2 CD4+ T cells in tryptophan-free medium, we observed an increased AHR expression (figure 5E) and an enhanced Treg differentiation, which was abolished when we used CD4+ T cells from Ahr-KO/DBA/2 mice (figure 5F). In addition, like in HEK293-E cells, we observed an increased LAT1-dependent kynurenine entry into the differentiated Treg cells when there was no tryptophan (figure 5G). Both Ahr activation (online supplemental figure 7B) and Treg differentiation (figure 5H) were further enhanced on addition of kynurenine, but only in tryptophan-free conditions, in line with our previous observation that tryptophan deprivation potentiated AHR activation by kynurenine. Like in HEK293-E cells, no increase of Ido1 expression was found in Treg cells cultured under tryptophan deprivation (online supplemental figure 7D,E). Neither blocking Ido1 activity nor using naïve CD4 T cells from Ido1-KO mice showed any impact on Treg differentiation.

Altogether, our data confirm that AHR is required for Treg induction and indicate that tryptophan deprivation improves Treg induction by upregulating AHR expression. This new role of tryptophan deprivation appears to be key in CD4+ T cells that carry a low-affinity AHR variant, as is the case in DBA/2 mice, but also in humans.

DISCUSSION
Our results show the impact of tryptophan deprivation on AHR regulation, demonstrating an increased expression of AHR at the transcript level, resulting in higher protein levels and a strong sensitization of the AHR pathway. A direct consequence is that weak AHR agonists, including kynurenine and its derivatives, become stronger agonists under tryptophan deprivation. Interestingly, in one of the first reports identifying kynurenine as an endogenous AHR agonist, kynurenine dissolved in aqueous solution was added to TDO-expressing cells cultured in tryptophan-free medium to study the effects of defined kynurenine concentrations on AHR activity by excluding endogenous production of kynurenine. In this study, it was already observed that the induction of the AHR target gene CYP1A1 by kynurenine was stronger in cells cultured in tryptophan-free than in tryptophan-containing medium, but the underlying mechanisms remained elusive. The expression of AHR is tightly regulated by a variety of mechanisms, including transcriptional regulation by specific transcription factors. Sp1, a transcription factor that binds to the AHR promoter, is required for basal AHR expression and can enhance its expression in response to certain stimuli. Inflammation-induced nuclear factor-kB (NF-kB) can induce AHR expression by binding to its promoter. The AHR repressor, a transcription factor that binds to the AHR promoter, can negatively regulate AHR expression. Nuclear factor erythroid 2-related factor 2 is a transcription factor that plays a critical role in cellular responses to oxidative stress. In addition to its well-established antioxidant functions, Nrf2 has been shown to influence the expression and activity of AHR. Earlier studies revealed that ligand-activated AHR could modulate the expression of Nrf2 and vice versa. Nrf2 was reported to directly affect AHR gene transcription by binding to one antioxidant response element found in the −230 bp region of the promoter of AHR. Although involved in different signaling pathways, all these potential regulators of AHR play important roles in cellular adaptation to stresses, and have been shown to cross-regulate each other. Understanding how they regulate AHR transcription under tryptophan deprivation requires further investigation.

Although its chemical structure does not make it an optimal AHR ligand, kynurenine has been proposed as an endogenous AHR agonist. It was also proposed that kynurenine could act as a prolongand of AHR, requiring further metabolism to produce more potent agonist(s). Seok et al. reported that kynurenine dissolved in DMSO spontaneously converts into aromatic condensation products that better fit the AHR ligand binding site and act as highly potent AHR agonists. We also observed that aged kynurenine solutions activated AHR much more strongly than fresh ones, and we identified the very same condensation derivative as a very potent AHR agonist in old kynurenine solutions. It is not clear at the moment whether this metabolite can be produced in vivo inside the cell. However, since kynurenine is often dissolved in DMSO, it is possible that the agonistic activity of kynurenine reported in the literature is partly due to this artificial contaminant.

When using fresh kynurenine solutions, we observed no AHR activation in HEK293-E cells. However, under tryptophan depletion, fresh kynurenine displayed AHR agonistic properties. This can result from several factors. Probably a dominant factor is the overexpression of AHR that we report here, which sensitizes the AHR pathway to kynurenine and its metabolites. A second factor could be the overexpression of LAT1, the transporter used by both tryptophan and kynurenine to enter cells. We show that contrary to AHR overexpression, LAT1 overexpression is dependent on the GCN2/ATF4 pathway. LAT1...
overexpression results in increased import of kynurenine into the cell, potentiating its effect.

Besides LAT1, other transporters of kynurenine have been described, although their impact on kynurenine entry in our experimental setting seems minor, as the LAT1 inhibitor BChl almost completely blocked kynurenine entry. It will, nevertheless, be interesting to evaluate the impact of tryptophan deprivation on other kynurenine transporters, such as SLC36A4, SLC7A8, and SLC7A11, using relevant cell lines. Lastly, we observed overexpression of kynurenine aminotransferase II under tryptophan deprivation in HEK293-E cells. This enzyme may increase production of kynurenine metabolites such as KA, which may be more potent agonists than kynurenine itself.

Our results also point to the modulation of both amino-acid and kynurenine uptake by cells in the TME, through changes in both amino acid concentration and transporter expression. The latter is increased on amino acid shortage in a GCN2-dependent manner (our results and Timosenko et al.41). Furthermore, since kynurenine and hydrophobic amino acids all compete for LAT1 as an uptake transporter, kynurenine uptake is favored by the lack of tryptophan, at least in PBS that lack other amino acids. It remains to be determined whether the same is true in the TME, which contains other hydrophobic amino acids. Of note, local deprivation of other amino acids could also increase cellular uptake of kynurenine and tryptophan. Likewise, IDO1-expressing tumors produce high amounts of kynurenine, which could, in turn, affect the bioavailability of other amino acids transported by LAT1, and thereby regulate the function of immune cells in the TME. These interesting questions are worth further exploration.

The induction of AHR expression on tryptophan deprivation has important consequences on immunoregulation. Indeed, we found that Treg differentiation induced by TGF-β was inefficient in DBA/2 mice in normal conditions but was strongly favored in tryptophan-depleted conditions. This was paralleled by an increased expression of Ahr. In Ahr-KO DBA/2 mice, differentiation of Tregs did not occur, even under tryptophan depletion. Altogether, this indicates that Treg differentiation requires a high expression of AHR to sensitize the pathway to weak agonists, and this can be obtained by tryptophan deprivation. In this protocol of in vitro Treg differentiation, there is no addition of exogenous kynurenine and no involvement of IDO1 (online supplemental figure 7), so that the exact nature of the AHR ligand is unclear. It could be weak agonists present in the culture medium, as we observed (figure 4A) and as reported in.37 38 Addition of exogenous kynurenine further increased Treg differentiation in tryptophan-depleted conditions. Fallarino et al reported already in 2006 that the induction of Treg in vivo by IDO1-expressing dendritic cells required both tryptophan depletion and kynurenine production.92 Yet no molecular explanation has been proposed so far for this observation. Opitz et al have shown that tumors expressing TDO can produce kynurenine which activates the AHR pathway and thereby induces immunosuppression.12 Mezrich et al demonstrated that kynurenine could induce Treg differentiation via AHR activation.10 Our study has demonstrated for the first time that in addition to kynurenine production, tryptophan deprivation increases AHR expression, further potentiating AHR activation by kynurenine, thereby inducing Treg differentiation.

Our results provide a mechanistic explanation for this dual requirement for Treg induction: tryptophan depletion is needed to increase AHR and LAT1 expression, and thereby potentiating the weak AHR agonistic activity of kynurenine and derivatives. In the in vivo model of Fallarino et al, Treg induction was found to be dependent on GCN2, which suggests that the induction of LAT1 played a dominant role over the induction of AHR in these conditions.

Finally, we observed that the expression of AHR was a key limiting factor for Treg differentiation of CD4+ T cells from DBA/2 mice, which express the Ahrβ allele that has a low affinity for classical AHR ligands. CD4+ T cells from C57BL/6 mice, which express the high affinity allele Ahrβ, do not need tryptophan depletion to differentiate into Tregs. The functional consequences of this allelic difference have been overlooked so far, as most studies analyzing the role of AHR in immunoregulation made use of C57BL/6 mice. Importantly, the human AHR has the same valine residue as the murine AHRβ in the ligand binding site, and both have a low affinity for TCDD.44 Therefore, the AHR results obtained in C57BL/6 mice should be extrapolated with caution to humans and might benefit from further validation in mice with an Ahrβ allele. Because our observation that Treg differentiation requires tryptophan depletion was made in DBA/2 mice, it is likely relevant to humans.

**Author affiliations**
de Duve Institute, UCLouvain, Brussels, Belgium
2Ludwig Institute for Cancer Research, de Duve Institute, Brussels, Belgium
3Faculty of Bioscience, Heidelberg University, Heidelberg, Germany
4DKTK Division of Metabolic Crosstalk in Cancer, German Cancer Research Center, DKFZ, INF 280, 69120 Heidelberg, Germany
5Louvain Drug Research Institute, UCLouvain, Brussels, Belgium
6Partner Site Berlin, German Cancer Consortium, Heidelberg, Germany
7German Cancer Consortium Partner Site Berlin, German Cancer Research Center, Heidelberg, Germany
8Neurology Clinic and National Center for Tumor Diseases, Heidelberg, Germany

**Contributors** MS, PP, SK and LP performed experiments, analysed data, and wrote materials and methods and figure captions. VS designed and performed HPLC analysis. VS, JL, QS and RF designed and performed chemistry analysis. VS designed and performed bioinformatics analyses. CS contributed to data interpretation and scientific discussions. JZ and BVdE supervised the study, designed and analysed experiments, and wrote the manuscript. All the authors read, revised, and approved the manuscript. Guarantors: JZ and BVdE.

**Funding** This work was supported by Ludwig Cancer Research, de Duve Institute (Belgium), and Université catholique de Louvain (Belgium). This work was also supported by grants from: Le Fonds de la Recherche Fondamentale Stratégique—WELBIO (Walloon Excellence in Life Sciences and Biotechnology), Belgium (grant...
REFERENCES


13. Gutiérrez-Vázquez C, Quintana FJ. Regulation of the immune response by the aryl hydrocarbon receptor. Immunity 2018;48:51074–7613(17)30570-8:19-33:...


33. Adam I, Dewi DL, Mocowier J, et al. Upregulation of tryptophanyl-tRNA synthetase adapts human cancer cells to nutritional...


45 Fitzgerald CT, Nebert DW, Puga A. Regulation of mouse Ah receptor (AHR) gene basal expression by members of the sp family of transcription factors. *DNA Cell Biol* 1998;17:811–22.


Supplementary Figure 1

A. 

CYP1B1  

<table>
<thead>
<tr>
<th></th>
<th>HEK293</th>
<th>HEK293-hIDO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td>IDOi</td>
<td>0.8</td>
<td>0.6</td>
</tr>
</tbody>
</table>

STC2  

<table>
<thead>
<tr>
<th></th>
<th>HEK293</th>
<th>HEK293-hIDO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>IDOi</td>
<td>1.2</td>
<td>1.0</td>
</tr>
</tbody>
</table>

B. 

TIPARP  

<table>
<thead>
<tr>
<th></th>
<th>HEK293</th>
<th>HEK293-E hIDO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>150</td>
<td>120</td>
</tr>
<tr>
<td>IDOi</td>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

EREQ  

<table>
<thead>
<tr>
<th></th>
<th>HEK293</th>
<th>HEK293-E hIDO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>IDOi</td>
<td>1.5</td>
<td>1.3</td>
</tr>
</tbody>
</table>

IL411  

<table>
<thead>
<tr>
<th></th>
<th>HEK293</th>
<th>HEK293-E hIDO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.5</td>
<td>2.2</td>
</tr>
<tr>
<td>IDOi</td>
<td>2.0</td>
<td>1.8</td>
</tr>
</tbody>
</table>

C. 

iDO1  

<table>
<thead>
<tr>
<th></th>
<th>HEK293</th>
<th>HEK293-E hIDO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trp (80 μM)</td>
<td>0.1</td>
<td>0.02</td>
</tr>
<tr>
<td>Trp-free</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

D. 

CYP1A1  

<table>
<thead>
<tr>
<th></th>
<th>HEK293</th>
<th>HEK293-E hIDO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMISO</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>PFOZ</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

E. 

<table>
<thead>
<tr>
<th>Initial Trp Conc (μM)</th>
<th>Final Trp Conc (μM)</th>
<th>Final Kyn Conc (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>17.1</td>
<td>31</td>
</tr>
<tr>
<td>1.0</td>
<td>61.9</td>
<td>31.1</td>
</tr>
<tr>
<td>1.0</td>
<td>11.7</td>
<td>31.4</td>
</tr>
<tr>
<td>1.0</td>
<td>0.9</td>
<td>16.4</td>
</tr>
<tr>
<td>1.0</td>
<td>0.6</td>
<td>8.4</td>
</tr>
<tr>
<td>1.0</td>
<td>0.3</td>
<td>5.5</td>
</tr>
<tr>
<td>1.0</td>
<td>0.2</td>
<td>2.9</td>
</tr>
<tr>
<td>1.0</td>
<td>0.0</td>
<td>1.3</td>
</tr>
</tbody>
</table>
Supplementary Figure 1: IDO1 and TDO activity induces AHR activation

A. qRT-PCR analysis of \textit{CYP1B1}, \textit{TIPARP}, \textit{STC2}, \textit{EREG} and \textit{IL4I1} expression in HEK293-E or HEK293-E hIDO1 cells treated or not with IDO1 inhibitor (Epacadostat, 2.5 µM) for 120 h. The mRNA levels of different genes was measured by quantitative qRT-PCR and normalized to \textit{GAPDH}. Mean ± SD of technical triplicates from one representative experiment out of three independent experiments. **** significant with p<0.0001, *** significant with p<0.001, ** p<0.01, * p<0.05 and NS= not significant.  

B. qRT-PCR analysis of \textit{IDO1} in HEK293-E cells cultured in medium with or without 80µM tryptophan for 24 h. HEK293-E hIDO1 cell culture in completed medium was used as a positive control for \textit{IDO1} expression. Mean ± SD of technical triplicates from one representative experiment out of three independent experiments. 

C. Tryptophan concentrations measured by HPLC in cell supernatant from Fig 2A and 2B. 

D. qRT-PCR analysis of \textit{CYP1A1} expression in HEK293-E cells cultured for 24 h without FBS in medium with or without tryptophan and treated with or without FICZ. Mean ± SD of technical triplicates from one representative experiment out of three independent experiments. 

E. HPLC quantification of kynurenine or tryptophan concentrations in the supernatant of HEK293-E IDO1 cells from the experiment shown in Fig 2D and E.

Supplemental material placed on this supplemental material which has been supplied by the author(s)

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance on the material contained in this supplement and excludes all warranties with regard to fit for purpose.

BMJ Publishing Group Limited (BMJ) disclaims all liability and responsibility arising from any reliance on the material contained in this supplement and excludes all warranties with regard to fit for purpose.
Supplementary Figure 2: Tryptophan deprivation induces AHR expression in human tumor cells of different histological types
Colorectal carcinoma (LB159-CRC) and hepatocellular carcinoma (HepG2) cells were cultured for 72 h in tryptophan-free medium supplemented or not with 80 μM tryptophan, in the presence of 10 % FBS. AHR expression was measured by quantitative qRT-PCR analysis and normalized to GAPDH. Mean ± SD of technical triplicates from one representative experiment out of three independent experiments. **** significant with p<0.0001, *** significant with p<0.001, ** p<0.01, * p<0.05 and NS= not significant.
Supplementary Figure 3: Western blot analysis of the integrated stress response (ISR) in cells treated with Salubrinal or Isrub

A. Western blot analysis of the expression of ATF4 in HEK293-E cells treated for 24 h with Salubrinal (20 μM), Isrub (5 μM) or vehicle (DMSO) in tryptophan-free medium supplemented or not with 80 μM tryptophan in the absence of FBS. One representative out of three independent experiments. B. HEK293-E cells were treated for 72 h with kynurenine (80 μM), AHR inhibitor (CH233191, 10 μM) or both in tryptophan-free medium supplemented or not with 80 μM tryptophan in the presence of 10 % FBS. AHR expression was measured by qRT-PCR analysis. Mean ± SD of technical triplicates from one representative experiment out of three independent experiments. C. HepG2 cells were treated for 24 h with or without kynurenine (80 μM) or FICZ in tryptophan-free medium supplemented or not with 80 μM tryptophan. CYP1A1 and AHR expression was measured by qRT-PCR analysis. Mean ± SD of technical triplicates from one representative experiment out of two independent experiments.
Supplementary Figure 4

A. Kynurenine «Old»
- Kynurenine «New»
- PICZ
- 2,3,7,8 TCDD
- DMSO

CYP1A1
Luciferase Assay

% results to 30 μM TCDD
Log (Concentration M)

Fold Change Ratio To NT

B. HPLC - Kynurenine «New»
DMSO
Kynurenine

Retention time (min)

A
210nm (AU)

C. Luciferase Assay

Fold Change Ratio To NT

Fraction

Kynurenine «new»
Kynurenine «old»

D. 9H-benzo[h]quinolino[4,3,2-de][1,6]naphthyridine

E. UV spectrum
- K270 and QS006 -

Absorbance (AU)

Wavelength (nm)

F. Luciferase Assay

Fold Change Ratio To NT

Concentration (nM) (pM for TCDD)

G. CYP1A1

Number of CYP1A1 transcripts per 4,000 GAPDH transcripts

Number of CYP1A1 transcripts per 4,000 GAPDH transcripts

Number of CYP1A1 transcripts per 4,000 GAPDH transcripts

H. CYP1A1

** Fold Change Ratio To NT

* Fold Change Ratio To NT

Supplementary Figure 4: A kynurenine derivative spontaneously produced in DMSO potently activates AHR

Kynurenine is commonly dissolved in DMSO. However, in DMSO kynurenine is transformed into an oxidized dimeric derivative that potently activates AHR. Hereunder we refer to the transformed kynurenine as «Old» kynurenine (stored at RT for at least 5 days) and freshly dissolved kynurenine as «New».

A. HepG2 cells were treated with 4 different concentrations (100 μM, 25 μM, 5 μM, 1 μM) of kynurenine «Old» (●) and kynurenine «New» (■). Known AHR ligands 2,3,7,8 TCCD ( ●) and FICZ (■) were used as controls at 30 nM and at 1 μM, 250 nM, 50 nM and 10 nM respectively. CYP1A1 expression was measured by qRT-qPCR after 24 h (left). Data presented are the means ± SD and are representative of 3 independent experiments. AHR activation was measured in a luciferase assay after 16 h (right). A construct containing 4 repeats of the target sequence of AHR followed by the sequence of the reporter gene firefly luciferase was stably transfected in HepG2 cells (HepG2 PGudLuc6.1). Data presented are the means ± SD and are representative of 3 independent experiments. *** significant with p<0.001, ** p<0.01, * p<0.05 and NS= not significant.

B. The two kynurenine solutions were analysed by HPLC on a C18 column. New peaks were detected by UV in the «Old» solution in addition to the two major peaks corresponding to DMSO and kynurenine, while the kynurenine peak was reduced by a factor 1.7 in the «Old» solution. This indicated the presence of other molecules than kynurenine in the «Old» solution.

C. To identify the molecule responsible for activating AHR in the «Old» solution, HPLC was used to isolate forty fractions from the «Old» (red curve, white bars) and the «New» (blue curve, black bars) solutions. These fractions were tested in an AHR luciferase assay as described in A. Control (CTRL) corresponds to the unfractionated kynurenine solution. These data are representative of 3 independent experiments. The intact kynurenine present in fraction 3-4 had no activity. Fraction 28 was the most active in 3 independent experiments. The blue and red curves represent the UV spectrum of «New» and «Old» kynurenine, respectively.

D. Putative structure of the compound (K270) that activates AHR in fraction 28 based on kynurenine structure and chemical analysis.

E. Electrospray ionization (ESI-MS) analysis of «Old» kynurenine fraction 28 provided accurate mass measurement of the ion [M+H]^+ at m/z 270.1027, and the molecule was therefore named K270. Based on the structure prediction, the compound was synthesised following a four-steps procedure involving the initial formation of the ethyl 4-methyl-2-oxo-1,2-dihydroquinoline-3-carboxylate by condensation and cyclisation of the commercially available 2-aminoacetophenone with the ethyl 3-chloro-3-oxopropanoate. The cyclized compound was next chlorinated with oxalyl chloride to afford the ethyl 2-chloro-4-methylquinoline-3-carboxylate derivative. In the next step, the tetracyclic 12-methylidibenzof6,g][1,8]naphthyridin-11(6H)-one was obtained by reaction of the latter with aniline and polyphosphoric acid (PPA). The final 9H-benzo[h]quinolino[4,3,2-de][1,6]naphthyridine compound was obtained by reaction of this tetracyclic precursor with Bredereck’s reagent. It should be noted that the final compound (QS006) was only obtained in poor yield because of the difficulty of the purification step that could only isolate the pure product after low-efficiency of HPLC separations. Figure shown is the spectroscopy analysis of K270 and QS006 (synthesized), with perfectly superimposed results confirming that QS006 (blue line) is identical or highly similar to K270 (red line).
F. The two molecules K270 and QS006 were tested for their ability to activate AHR under normal tryptophan (80 µM) conditions in a luciferase reporter assay described in A. TCDD was used as a control for AHR activation. NT: not treated.

G. HepG2 cells were incubated with K270 or QS006 at two concentrations (78 nM and 39 nM) for 24 h and the induction of *CYP1A1* was measured by qRT-PCR. TCDD was used as a control for AHR activation.

Data presented are the mean ± SD of technical triplicates from one representative experiment out of three independent experiments. *** significant with p<0.001, ** p<0.01, * p<0.05 and NS= not significant.

H. HepG2 cells were treated for 24 h with kynurenine (80 µM) or K720 (200 nM), in tryptophan-free medium supplemented or not with 80 µM tryptophan. *CYP1A1* expression was measured by qRT-PCR analysis. Mean ± SD of technical triplicates from one representative experiment out of two independent experiments.
Supplementary Figure 5

A. LB159-CRC

B. HepG2

Supplementary Figure 5: Tryptophan deprivation induces AHR activation in human tumor cells of different types

Colorectal carcinoma LB159-CRC cells or hepatocarcinoma HepG2 cells were cultured for 72 h in tryptophan-free medium supplemented or not with 80 µM tryptophan in the presence of 10 % FBS. CYP1A1 and TIPARP expression were measured by qRT-PCR analysis. The mRNA levels of different genes were measured by quantitative qRT-PCR and normalized to GAPDH. Mean ± SD of technical triplicates from one representative experiment out of three independent experiments. **** significant with p<0.0001, *** significant with p<0.001, ** p<0.01, * p<0.05 and NS= not significant.
Supplementary Figure 6: Tryptophan deprivation induces LAT1 expression via the GCN2 pathway

A. Flow cytometric evaluation of kynurenine uptake in HEK293-E cells. HEK293-E cells were treated with different concentrations of kynurenine in PBS for 4 minutes at 37°C. The reaction was stopped after 4 minutes by adding 125 μl 4% PFA for 30 minutes at room temperature in the dark. Data was acquired using 405 nm excitation (violet laser) and bandpass filter 525/50 BP (Bv510) on a BD LSRII (Fortessa).

B. Western blot analysis of LAT1 expression for Fig 4G.

C. qRT-PCR analysis of the expression of enzymes AFMID (kynurenic formamidase), ADAAT (kynurenic aminotransferase), KMO (kynurenine 3-monooxygenase), and KYNU (kynureninase), which are involved in kynurenine metabolism, in HEK293-E cells. Human liver samples were used as a positive control. Mean ± SD of technical triplicates from one representative experiment out of three independent experiments. **** significant with p<0.0001, *** significant with p<0.001, ** p<0.01, * p<0.05 and NS= not significant.
Supplementary Figure 7: Impact of IDO1 on CD4+ FoxP3+ Treg differentiation

A. Spearman correlations between expression (counts) of indicated genes in 9195 TCGA primary tumour samples. Spearman correlation values are indicated between brackets. The following cancer types were present: ACC: 77, BLCA: 407, BRCA: 1091, CESC: 303, CHOL: 36, COAD: 285, DLBC: 47, ESCA: 181, GBM: 152, HNSC: 518, KICH: 66, KIRC: 530, KIRP: 288, LGG: 508, LIHC: 369, LUAD: 513, LUSC: 498, MESO: 87, OV: 420, PAAD: 178, PCPG: 176, PRAD: 494, READ: 91, SARC: 258, SKCM: 102, STAD: 413, TGCT: 132, THCA: 504, THYM: 119, UCEC: 180, UCS: 57, UVM: 79. B. Differentiated murine Treg cells (from naïve CD4+ T cells of DBA/2 mice) from were treated for 24 h with or without kynurenine (80 µM) in tryptophan-free medium supplemented or not with 80 µM tryptophan. CYP1A1 expression was measured by qRT-PCR analysis. Mean ± SD of technical triplicates from two independent experiments. C. *Ido1* expression measured by qRT-PCR in differentiated murine Treg cells. EG7-OVA tumor cells were used as a positive control. Mean ± SD of technical triplicates from one representative experiment out of two independent experiments. D, E. Treg differentiation (same as Fig 5D) of naïve CD4+ CD62L+ T cells isolated from WT C57BL/6 mice treated or not with IDO1 inhibitor (Epaeadostat, 2.5µM) during Treg differentiation. E. Treg differentiation (same as Fig 5D) of naïve CD4+ CD62L+ T cells isolated from WT or *Ido1-KO* C57BL/6 mice. Mean ± SD of technical triplicates from one representative experiment out of three independent experiments. **** significant with p<0.0001, *** significant with p<0.001, ** p<0.01, * p<0.05 and NS= not significant.