


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

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**To cite:** Solvay M, Holfelder P, Klaessens S, *et al.* Tryptophan depletion sensitizes the AHR pathway by increasing AHR expression and GCN2/LAT1-mediated kynurenine uptake, and potentiates induction of regulatory T lymphocytes. *Journal for ImmunoTherapy of Cancer* 2023;**11**:e006728. doi:10.1136/jitc-2023-006728

► Additional supplemental material is published online only. To view, please visit the journal online (<http://dx.doi.org/10.1136/jitc-2023-006728>).

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Accepted 12 May 2023



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## 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 vitro differentiation of naïve CD4<sup>+</sup> 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 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

## 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 on tryptophan depletion.
- ⇒ These mechanisms explain IDO/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<sup>+</sup> 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.<sup>1</sup> 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.<sup>2,3</sup> 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.<sup>4</sup> 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.<sup>5</sup> 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.<sup>6</sup> However, subsequent work showed that GCN2 was not involved in the IDO1-mediated tumoral immune resistance.<sup>7</sup> Another model proposed that IDO1-mediated suppression relied on deactivation of the mTOR pathway due to tryptophan depletion.<sup>8</sup> 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.<sup>9</sup> Moreover, kynurenine itself was then identified as an endogenous ligand for the aryl hydrocarbon receptor (AHR),<sup>10–12</sup> leading to the suggestion that the IDO1/Kyn/AHR axis could play a key role in modulating inflammatory and immune responses.<sup>12,13</sup> 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.<sup>14</sup> AHR activity promotes the induction of peripheral regulatory T lymphocytes (Tregs),<sup>10,15</sup> the upregulation of PD-1 on CD8<sup>+</sup> T cells<sup>16</sup> and the recruitment of immunosuppressive tumor-associated macrophages.<sup>17</sup> 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.*<sup>18</sup> 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.<sup>18</sup> 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.<sup>18</sup> 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.<sup>19</sup>

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 ex 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 Nucleospin Tissue extraction kit (740952.50, Macherey-Nagel). Genotyping was performed by PCR using the Gotaq kit (#M7845, Promega). Primers were purchased from Eurogentec. Gene: *Ahr* forward GGCTAGCGTGCGGGTTTCTC, reverse CTCCGTGTCCCCTAAAGCTTCA, Neomycin resistance-gene forward CGGGAGCGGCCA TACCGTAAAGC, reverse GAAGCGGGAAGGGACT

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.<sup>2,3</sup> TDO-expressing LB159-CRCA cells were derived from a colorectal carcinoma (CRC) patient sample from the Cliniques universitaires Saint-Luc in 1991, Brussels.<sup>2</sup> 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) complemented with 10% FBS (#7524, 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), Kynurenic acid (KA, #K3375), Xanthurenic acid (XA, #D120804), Anthranilic acid (AA, #A89855), 3-hydroxyanthranilic acid (HAA, #148776), 3-Hydroxykynurenine (HK, #H1771), Nicotinamide (NIC, #N9976), 2,3-pyridinedicarboxylic 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.*<sup>20</sup> 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 Tocris 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 (#A4262 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 (#UF-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 (9500g 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 Nu-PAGE 4%–12% Bis-Tris gels (#NP0321BOX and #WG1402Box, 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 ( $\beta$ -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) mouse 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  $\mu$ L of PBS, BCH, or tryptophan were added to appropriate samples before adding 100  $\mu$ L kynurenine 160  $\mu$ M (final concentration: 80  $\mu$ M). The kynurenine uptake was stopped after 4 min by adding 125  $\mu$ L 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<sup>+</sup> T cells and regulatory T-cell differentiation

Naïve CD4<sup>+</sup> T cells were isolated from mice spleen using the CD4<sup>+</sup> CD62L<sup>+</sup> 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  $\mu$ M), penicillin, streptomycin and treated with 5 ng/mL rhTGF- $\beta$  (#240-B, Bio-technique), 100 U/mL IL-2 (#2012-12, Peprotech) and 80  $\mu$ M 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 Verse (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.<sup>21</sup> 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,<sup>22</sup> of which the QUANTISEQ immune cell type deconvolutions were used in this study.

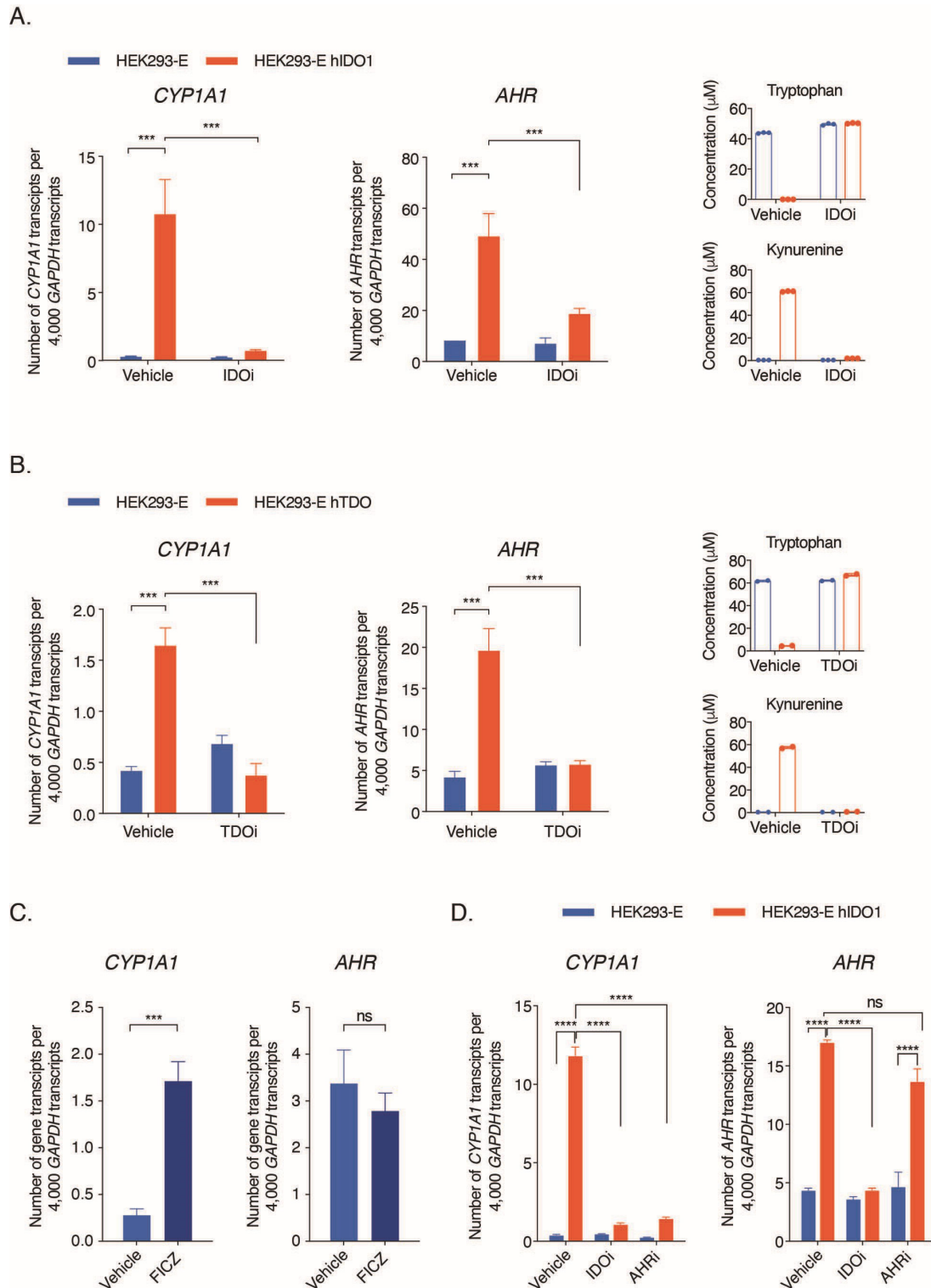
### Statistical method

Statistical analysis was performed with Graphpad prism (V.9.4.0) software. To compare the mRNA transcript, protein quantification or percentage of CD4<sup>+</sup>/FoxP3<sup>+</sup> 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*,<sup>23</sup> *TIPARP*, *EREG* and *IL4I1*<sup>24</sup> (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  $\mu$ M) 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  $\mu$ M). 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  $\mu$ M) for 120 hours. (D) RT-qPCR analysis of *CYP1A1* and *AHR* in HEK293-E or HEK293-E-IDO1 cells treated or not with IDO1 inhibitor (Epacadostat, 2.5  $\mu$ M) or AHR inhibitor (CH-223191, 10  $\mu$ M) for 120 hours. The mRNA levels of different genes were measured by quantitative RT-qPCR and normalized to *GAPDH*. Mean  $\pm$  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,<sup>25</sup> 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 FICZ<sup>26</sup> (figure 1C). Moreover, the increased *AHR* expression observed in IDO1-expressing cells was not abolished on treatment with *AHR* inhibitor CH223191<sup>27</sup> (figure 1D). Although IDO1 was previously shown to be induced by *AHR* activation in dendritic cells,<sup>28</sup> 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

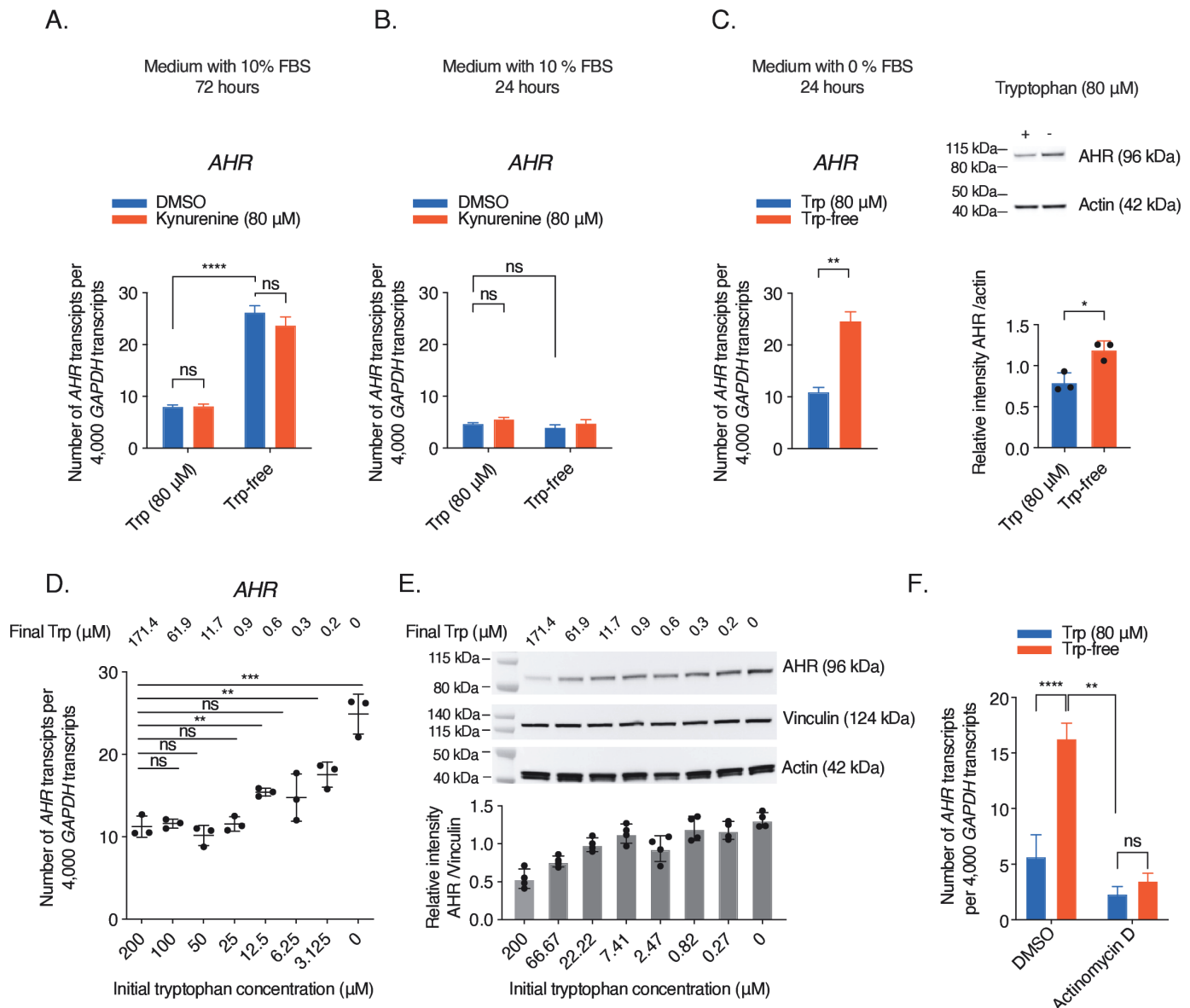
We first wanted to determine whether the increased *AHR* expression in IDO1-expressing cells was driven by kynurenine production and/or by tryptophan deprivation. When we treated wild-type HEK293-E cells for 72 hours with 80  $\mu$ M fresh kynurenine, we observed no increase in *AHR* expression (figure 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 2  $\mu$ M 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 200  $\mu$ M, 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 2 $\alpha$  (eIF2 $\alpha$ ) to shut down cap-dependent translation,<sup>29</sup> while favoring translation of activating transcription factor 4 (ATF4). The latter triggers transcription of genes involved in amino-acid metabolism<sup>30</sup> and transportation,<sup>31</sup> nutrient sensing and protein turnover,<sup>32,33</sup> in order to restore amino-acid homeostasis.<sup>30,34</sup> We confirmed that tryptophan depletion activates the GCN2/ATF4 pathway, as illustrated by increased phosphorylation of eIF2 $\alpha$  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 Salubrinal,<sup>35</sup> an inhibitor of phosphatase ‘growth arrest and DNA damage-inducible protein 34’ (GADD34), which dephosphorylates eIF2 $\alpha$ , thereby mimicking the activation of GCN2. Treating cells with Salubrinal 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,<sup>36</sup> a compound that blocks the GCN2/eIF2 $\alpha$ /ATF4 pathway downstream of eIF2 $\alpha$  (online supplemental figure 3A). As expected, the addition of Isrib to cells cultured under tryptophan deprivation reduced ATF4 expression, indicating a blockage of GCN2-ATF4 signaling. However, Isrib did not prevent induction of *AHR* expression on tryptophan depletion (figure 3B). Finally, to confirm that the GCN2/eIF2 $\alpha$ /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/eIF2 $\alpha$ /ATF4 pathway in the upregulation of *AHR* expression in response to tryptophan depletion.

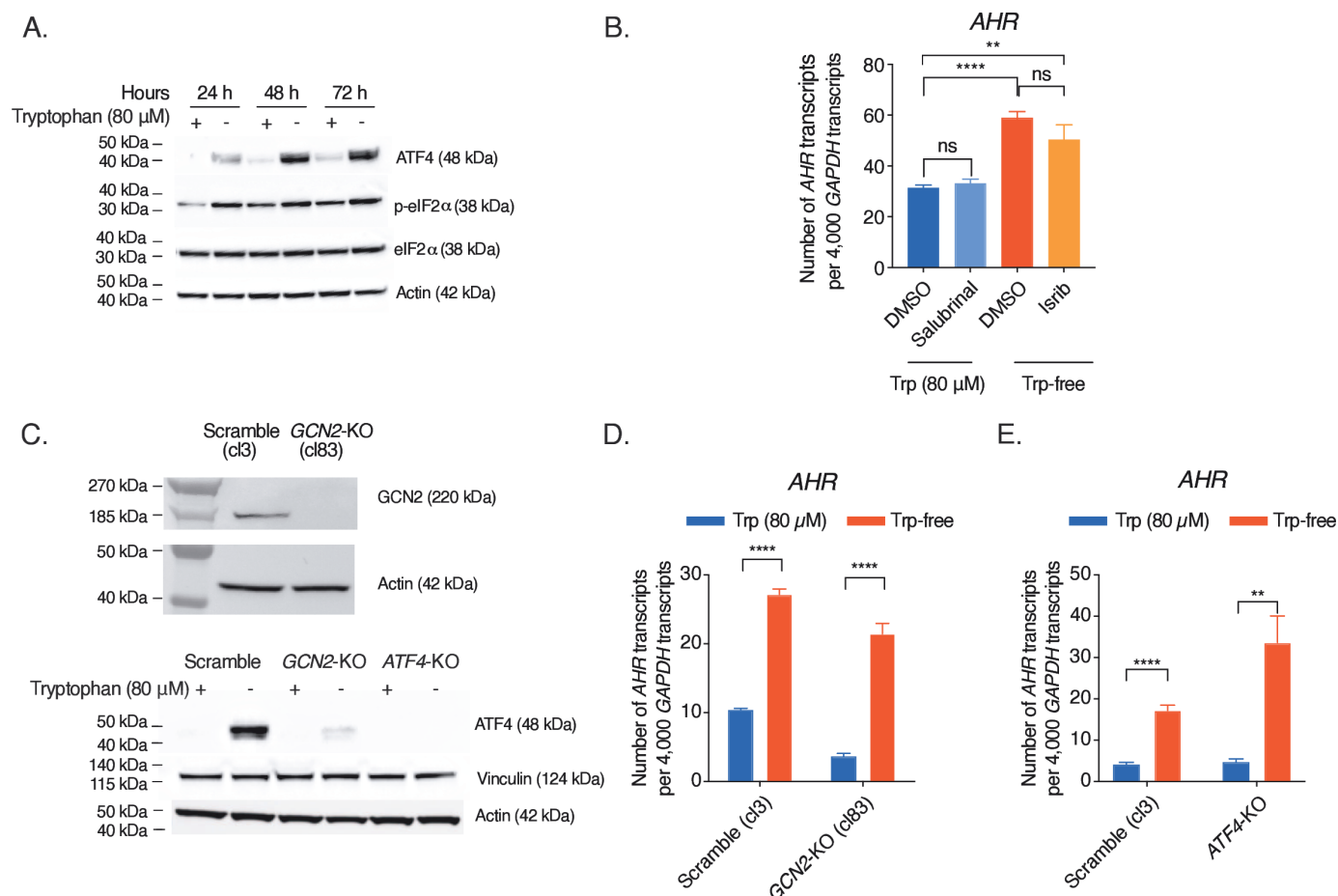


**Figure 2** IDO1/TDO-induced AHR upregulation is induced by tryptophan deprivation. (A, B) HEK293-E cells were treated with kynurenine (80  $\mu$ M) in tryptophan-free medium supplemented or not with 80  $\mu$ M tryptophan in the presence of 10% FBS for 72 hours (A) or 24 hours (B). *AHR* expression was measured by RT-qPCR analysis. Mean  $\pm$  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  $\mu$ M 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  $\pm$  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  $\pm$  SD of technical triplicates from one representative experiment out of three independent experiments for panel D and mean  $\pm$  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  $\mu$ g/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  $\pm$  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.

### AHR activation by kynurenine and derivatives is enhanced in the absence of tryptophan

Kynurenine has been widely described as a weak AHR

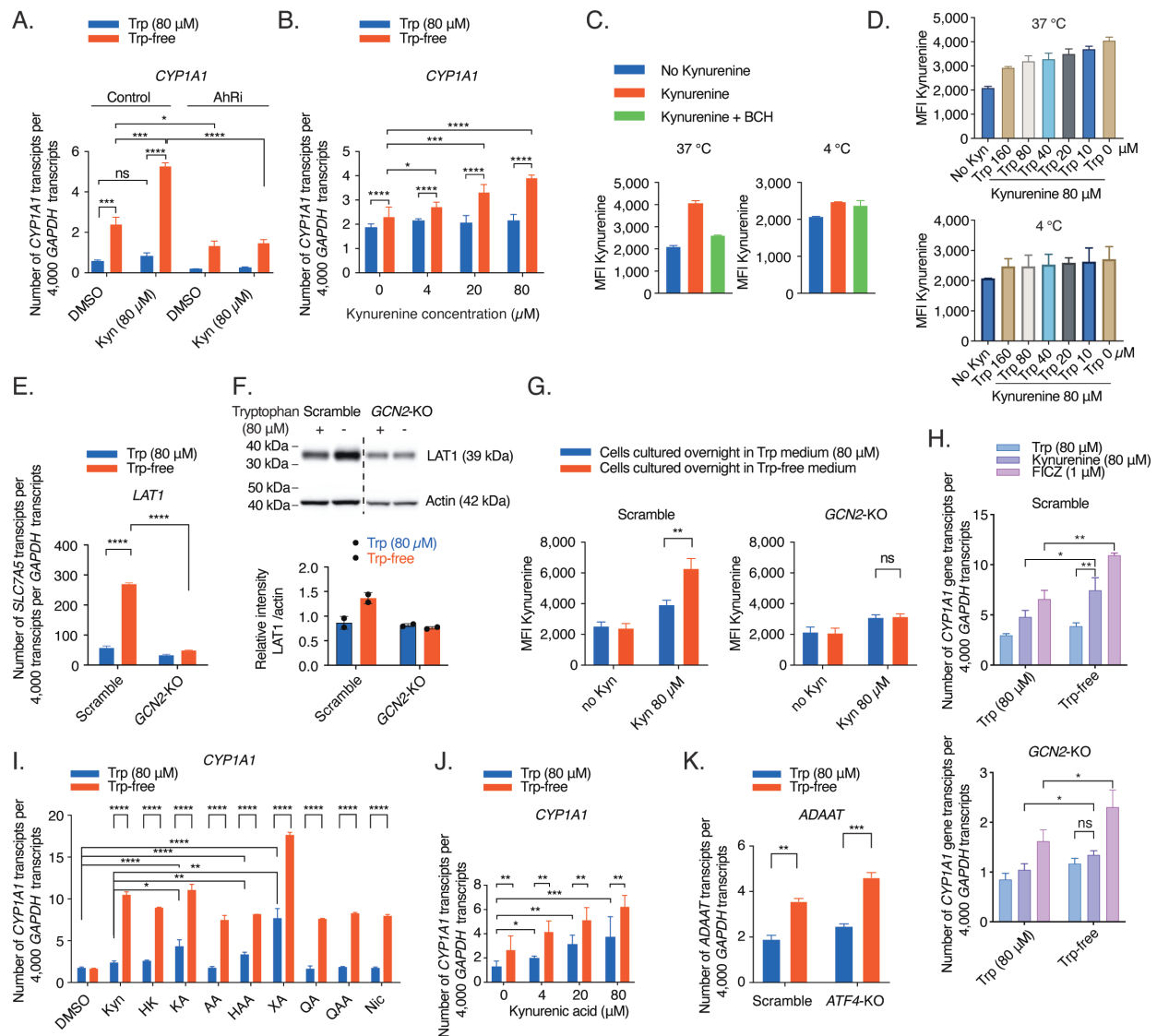
agonist, although controversy remains due to its atypical, highly polar, and non-ligand-like structure.<sup>18</sup> Given that tryptophan shortage increases *AHR* expression, we



**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: ATF4 expression in GCN2-KO, ATF4-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 ATF4-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. 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 two independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.0001$ . AHR, aryl hydrocarbon receptor; FBS, fetal bovine serum; ns, not significant.

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,<sup>10–12</sup> 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.<sup>18</sup> 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 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  $\mu$ M), AHR inhibitor (CH223191, 10  $\mu$ M) or both in tryptophan-free medium supplemented or not with 80  $\mu$ M 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  $\mu$ M 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  $\mu$ M 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  $\mu$ M 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  $\mu$ M tryptophan without FBS. Cells were then collected and incubated with or without 80  $\mu$ M kynurenine during 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  $\mu$ M) or FICZ (1  $\mu$ M) in tryptophan-free medium supplemented or not with 80  $\mu$ M tryptophan in the absence of FBS. (I) HEK293-E cells were treated for 72 hours with 80  $\mu$ M kynurenine metabolites in tryptophan-free medium supplemented or not with 80  $\mu$ M 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  $\mu$ M tryptophan in the presence of 10% FBS. The mRNA levels of different genes were measured by quantitative RT-qPCR and normalized to *GAPDH*. Mean  $\pm$  SD of technical triplicates from one representative experiment out of three independent experiments, except for panel F and K, which are Mean  $\pm$  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 metabolism 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.<sup>37,38</sup> 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 hepatocellular 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).<sup>39,40</sup> 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.*<sup>40</sup> 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, online supplemental figure 6B). The increase of LAT1 expression was regulated via the GCN2 pathway, as it was not observed in GCN2-KO cells (figure 4E,F, online supplemental figure 6B). 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).

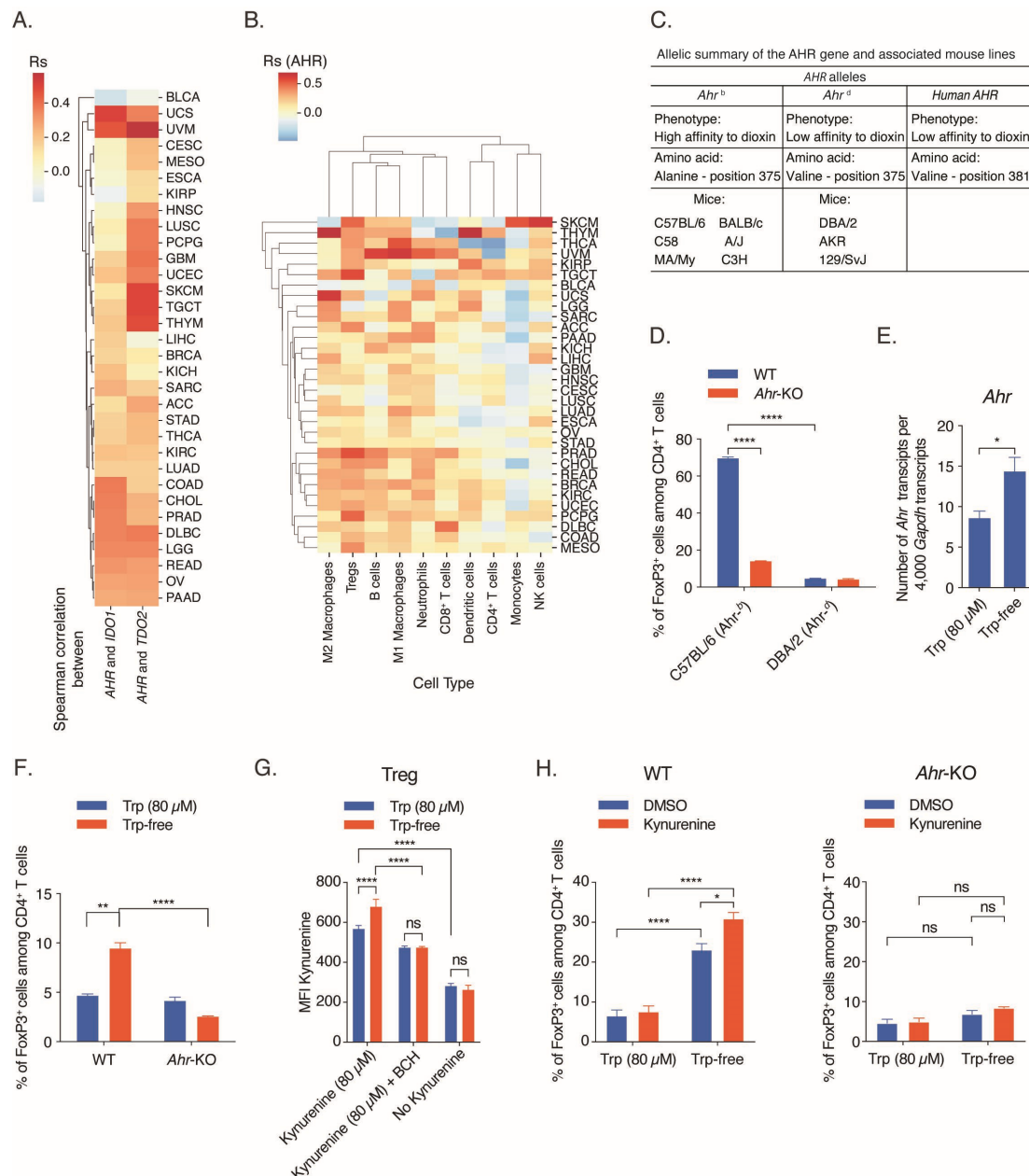
Tryptophan deprivation potentiates AHR activation by kynurenine in wild-type HEK293-E cells, as measured by *CYP1A1* expression. This increase of AHR activation by kynurenine is partly due to the increased kynurenine

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,<sup>40</sup> 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 4I,J). 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 (*AADAT*) were expressed, but kynurenine 3-monooxygenase (*KMO*) and kynureninase (*KYNU*) 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<sup>+</sup> FoxP3<sup>+</sup> 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<sup>41</sup> and that the presence of AHR was required to optimally generate Treg cells in vitro.<sup>10</sup> We, therefore, evaluated the role of tryptophan-deprivation induced AHR overexpression in Treg differentiation using murine wild-type and *Ahr*-KO mice. Two alleles encoding different *Ahr* variants exist among laboratory mouse strains<sup>42</sup> (figure 5C). Compared with human AHR (hAHR), the mouse *Ahr*<sup>b</sup> (*mAhr*<sup>b</sup>) variant has a ~10fold higher affinity for 2,3,7,8



**Figure 5** AHR upregulation favors in vitro differentiation of CD4<sup>+</sup> FoxP3<sup>+</sup> 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<sup>+</sup> CD62L<sup>+</sup> 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- $\beta$  (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<sup>+</sup> T cells isolated from wild-type DBA/2 mice in medium containing or not 80  $\mu$ M tryptophan. The mRNA levels of different genes were measured by quantitative RT-qPCR and normalized to *Gapdh*. Mean $\pm$ SD of triplicates from one out of three experiments. (F) Treg differentiation (same as D) of naïve CD4<sup>+</sup> CD62L<sup>+</sup> T cells isolated from WT or *Ahr*-KO DBA/2 mice in medium containing or not 80  $\mu$ M tryptophan. Mean $\pm$ SD of technical triplicates from one representative experiment out of three independent experiments. (G) Kynurenine fluorescence in differentiated murine Treg cells treated with 80  $\mu$ M 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 $\pm$ SD of technical triplicates from one representative experiment out of two independent experiments. (H) Treg differentiation (same as D) of naïve CD4<sup>+</sup> CD62L<sup>+</sup> T cells isolated from WT or *Ahr*-KO DBA/2 mice in medium containing or not 80  $\mu$ M tryptophan in the presence or absence of 80  $\mu$ M kynurenine. Mean $\pm$ SD of technical triplicates from one representative experiment out of three independent experiments. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\*\* $p$ <0.0001. AHR, aryl hydrocarbon receptor; IDO1, indoleamine 2,3-dioxygenase 1; ns, not significant; TCGA, The Cancer Genome Atlas; PBS, Phosphate-buffered saline.

-tetrachlorodibenzo-p-dioxin (TCDD).<sup>43</sup> 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 *mAhr<sup>b</sup>* variant. Using naïve CD4<sup>+</sup> T cells derived from C57BL/6 WT or *Ahr*-KO mice, we confirmed the critical role of AHR for Treg differentiation induced by TGF- $\beta$  in vitro. We observed that significantly less Tregs were generated by *Ahr*-KO as compared with WT CD4<sup>+</sup> T cells (figure 5D).

DBA/2 mice carry the *Ahr<sup>d</sup>* variant, which is structurally closer to hAHR, as it contains the same key valine residue in the active site.<sup>44</sup> The *mAhr<sup>d</sup>* variant also has a lower affinity than *mAhr<sup>b</sup>* for TCDD, similar to that of hAHR.<sup>44</sup> We observed that CD4<sup>+</sup> T cells from DBA/2 mice produced very few Tregs after in vitro induction (figure 5D). This might result from the low affinity of AHR<sup>d</sup> for its ligands. When we repeated the experiment with DBA/2 CD4<sup>+</sup> 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<sup>+</sup> 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<sup>+</sup> 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.<sup>12</sup> 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,<sup>12</sup> 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.<sup>45</sup> Inflammation-induced nuclear factor- $\kappa$ B (NF- $\kappa$ B) can induce AHR expression by binding to its promoter.<sup>46 47</sup> The AHR repressor, a transcription factor that binds to the AHR promoter, can negatively regulate AHR expression.<sup>48</sup> Nrf2 (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.<sup>49 50</sup> 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.<sup>49</sup> 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.<sup>12</sup> It was also proposed that kynurenine could act as a proligand of AHR, requiring further metabolism to produce more potent agonist(s).<sup>18</sup> 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.<sup>18</sup> 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 BCH 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,<sup>16</sup> and SLC7A11,<sup>51</sup> 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.*<sup>31</sup>). 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- $\beta$  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.<sup>37,38</sup> 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.<sup>52</sup> 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.<sup>12</sup> Mezrich *et al.* demonstrated that kynurenine could induce Treg differentiation via AHR activation.<sup>10</sup> 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 potentiate 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<sup>+</sup> T cells from DBA/2 mice, which express the *Ahr*<sup>d</sup> allele that has a low affinity for classical AHR ligands. CD4<sup>+</sup> T cells from C57BL/6 mice, which express the high affinity allele *Ahr*<sup>b</sup>, 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 AHR<sup>d</sup> in the ligand binding site, and both have a low affinity for TCDD.<sup>44</sup> 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 *Ahr*<sup>d</sup> allele. Because our observation that Treg differentiation requires tryptophan depletion was made in DBA/2 mice, it is likely relevant to humans.

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**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 of kynurenine derivative spontaneously produced in DMSO. SN designed and performed bioinformatics analyses. CS contributed to data interpretation and scientific discussions. ED supervised the initial part of the study. CAO analysed data and contributed to scientific discussions. JZ and BJVD supervised the study, designed and analysed experiments, and wrote the manuscript. All the authors read, revised, and approved the manuscript. Guarantors: JZ and BJVD.

**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 (Wallon Excellence in Life Sciences and Biotechnology), Belgium (grant

number: WELBIO-CR-2019C-05); Fonds pour la Recherche Scientifique—FNRS, Belgium (grant number: EOS 0000518F and PDR T.0091.18); Fondation contre le Cancer, Belgium (grant number: 2018-090); and the European Union's Horizon 2020 Research and Innovation Programme (Grant Agreement No. 754688, MESI-STRAT (Systems Medicine of Metabolic-Signaling Networks) to CS, CAO and BJvDE. Fonds pour la Recherche Scientifique (FNRS), Belgium (Grant 23638468), Fédération Wallonie-Bruxelles (ARC 14/19-058). Q.S. was supported by a fellowship from FNRS-Télévie (grant 22846630). CAO acknowledges support from the BMBF e:Med initiative Gliopath (01ZX1402), the German Research Foundation (SFB1389 UNITE-Glioblastoma; project No. 404521405) and the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement number 101045257). PP was supported by the German Research Foundation (SFB1389 UNITE-Glioblastoma; project No. 404521405). JZ was supported by Fondation Contre le Cancer (Grant number 2019-094).

**Competing interests** CAO is founder and managing director of cAHRmeleon Bioscience. CAO has patents on AHR inhibitors in cancer (WO2013034685); a method to multiplex tryptophan and its metabolites (WO2017072368); a transcriptional signature to determine AHR activity (WO2020201825); Interleukin-4-induced gene 1 (IL4I1) as a biomarker (WO2020208190) Interleukin-4-induced gene 1 (il4i1) and its metabolites as biomarkers for cancer (WO2021116357). BJvDE is cofounder of iTeos Therapeutics.

**Patient consent for publication** Not applicable.

**Ethics approval** Housing and experimental procedures were approved by the Animal Ethical Committee of UCLouvain (2019/UCL/MD/019).

**Provenance and peer review** Not commissioned; externally peer reviewed.

**Data availability statement** All data relevant to the study are included in the article or uploaded as online supplemental information.

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