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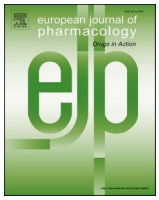

A peripheral blood mononuclear cell-based *in vitro* model: A tool to explore indoleamine 2, 3-dioxygenase-1 (IDO1)

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ABSTRACT

Background: Proinflammatory cytokines powerfully induce the rate-limiting enzyme indoleamine 2, 3-dioxygenase-1 (IDO-1) in dendritic cells (DCs) and monocytes, it converts tryptophan (Trp) into L-kynurenine (KYN), along the kynurenine pathway (KP). This mechanism represents a crucial innate immunity regulator that can modulate T cells. This work explores the role of IDO1 in lymphocyte proliferation within a specific pro-inflammatory milieu.

Methods: Peripheral blood mononuclera cells (PBMCs) were isolated from buffy coats taken from healthy blood donors and exposed to a pro-inflammatory milieu triggered by a double-hit stimulus: lipopolysaccharide (LPS) plus anti-CD3/CD28. The IDO1 mRNA levels in the PBMCs were measured by RT-PCR; the IDO1 activity was analyzed using the KYN/Trp ratio, measured by HPLC-EC; and lymphocyte proliferation was measured by flow cytometry. Trp and epacadostat (EP) were used as an IDO1 substrate and inhibitor, respectively. KYN, which is known to modulate Teffs, was tested as a positive control in lymphocyte proliferation.

Results: IDO1 expression and activity in PBMCs increased in an *in vitro* pro-inflammatory milieu. The lymphoid stimulus increased IDO1 expression and activity, which supports the interaction between the activated lymphocytes and the circulating myeloid IDO1-expressing cells. The addition of Trp decreased lymphocyte proliferation but EP, which abrogated the IDO1 function, had no impact on proliferation. Additionally, incubation with KYN seemed to decrease the lymphocyte proliferation.

Conclusion: IDO1 inhibition did not change T lymphocyte proliferation. We present herein an *in vitro* experimental model suitable to measure IDO1 expression and activity in circulating myeloid cells.

1. Introduction

The Kynurenine pathway (KP) is the main route (about 90–95%) of degradation of the essential amino acid L-Tryptophan (Trp) (van der Goot and Nollen, 2013). Other Trp routes include: the serotonin pathway, protein synthesis, decarboxylation (to tryptamine), and transamination (to indol-3-yl pyruvic acid) (Badawy, 2017b). The degradation of Trp by the KP occurs over several steps and generates several metabolites with biological functions including immunomodulatory activity (Platten et al., 2019; Sadok et al., 2017). KP dysregulations have been linked to several inflammation-driven pathologies including: neurodegenerative (eg. Parkinson's Disease), oncological, and cardiovascular diseases (Braidy and Grant, 2017; Shen et al., 2023). Importantly, the rate-limiting enzyme indoleamine 2,3-dioxygenase-1

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(IDO-1), which converts Trp into L-kynurenine (KYN), is powerfully induced by proinflammatory cytokines, such as interferon (IFN)-γ, tumor necrosis factor (TNF)-α, interleukin (IL)-6 and LPS(Vandenbon et al., 2014; Wang et al., 2015). KYN feeds the two main branches of KP leading to the formation of two relevant metabolites: quinolinic acid (QUIN) and kynurenic acid (KYNA) with neuroactive and immunomodulatory properties (Maddison and Giorgini, 2015). A brain imbalance between the neuroprotective KYNA and the neurotoxic QUIN is well established in neurodegenerative diseases; however, the role of periphery KP metabolites in these diseases has been almost completely ignored (Gao et al., 2023; Lim et al., 2017; Marszalek-Grabska et al., 2021). IDO1, which can be upregulated in dendritic cells (DCs) and monocytes (0.1–1% and 1–6% of leukocytes in the human peripheral blood) (Nares and Wahl, 2005; Orsini et al., 2012), is a crucial innate immunity regulator that seems to play a role in suppressing effector T-cell (Teffs) cells and activating regulatory immune responses in inflammatory microenvironments (Liu et al., 2018; Muller et al., 2023; Orsini et al., 2012). IDO1 presents immunomodulatory functions related to Trp starvation and increased KYN levels. For example, the starvation of Trp leads to cell cycle arrest and T cell apoptosis whereas KYN activates the transcription factor aryl hydrocarbon receptor (AhR) which induces the differentiation of $CD4^+$ T cells into Tregs (Le Naour et al., 2020; Mellor and Munn, 2004; Salminen, 2023). IDO1 modulation has been indicated as an object of study in autoimmune and neuroinflammatory diseases because of its anti-inflammatory and immunoregulatory effects, both at the periphery (peripheral blood mononuclear cells - PBMCs) and the central nervous system (CNS) (Mondanelli et al., 2020; Moroni et al., 2012). Therefore, targeting IDO1 might be an important strategy to control the Teff-mediated pro-inflammatory process in the neuroimmune interface and to manage neurodegenerative diseases (Appel et al., 2010; Stone and Williams, 2023). We explored, therefore, the impact of IDO1 on lymphocyte proliferation within a specific pro-inflammatory milieu. To reach this goal, PBMCs were subjected sequentially to lipopolysaccharide (LPS, myeloid stimulus), which can induce IDO1, and to anti-CD3/CD28 (lymphoid stimuli), which increase T cell proliferation[4-8].

2. Material and methods

2.1. Subjects

PBMCs were isolated from the buffy coats (BC) of healthy donors and collected in the Immunohematology and Transfusion Medicine service of *Ospedale di Circolo e Fondazione Macchi di Varese*, Italy. There is no information concerning the gender or age of the individuals. Fig. 1 illustrates the work timeline.

2.2. Reagents

Bovine serum albumin (BSA) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were purchased from Sigma, Italy. RPMI 1640, heat-inactivated fetal bovine serum (FBS), glutamine, and penicillin/streptomycin were obtained from Euroclone, Italy. Ficoll-Paque Plus was obtained from Pharmacia Biotech (Uppsala, Sweden, GEH1714403). The phosphate buffer saline (PBS (g/L) NaCl 8.0, KCl 1.5, Na_2HPO_4 1.44, KH_2PO_4 0.24, pH 7.4) used for the isolation and cell culture of PBMCs, as well as the lysis buffer ((g/L) NH_4Cl 8.248, $KHCO_3$ 1.0, EDTA 0.0368), also used for the isolation of PBMCs, were prepared with reagents from Sigma-Aldrich (Saint Louis, MO, USA). Trypan Blue solution 0.5% (ECM0990D-100 mL) was purchased from Euroclone, Italy. Purified mouse ab anti-human CD3 (555330, clone UCHT1, Mouse IgG1, κ) and purified mouse ab anti-human CD28 (clone CD28.2, Mouse C3H x BALB/c, IgG1, κ) were obtained from Becton Dickinson, Italy. The cell Proliferation Dye, eFluor 670 (CPD) was obtained from eBioscience-Prodotti Gianni, Italy (65–0840). Lipopolysaccharide (LPS, Standard lipopolysaccharide from *E. coli* 0111:B4 strain; TLR4 ligand, tlrl-eblps) was obtained from Invivogen). Tryptophan (Trp, 73-22-3) and kynurenine (KYN, 2922-83-0) were from Sigma-Aldrich (Saint Louis, MO, USA). Epacadostat (EP, 1204669-58-8) was obtained from Cayman Chemical Company. BD Cytofix/Cytoperm (554714, Becton Dickinson, San Diego, CA, USA).

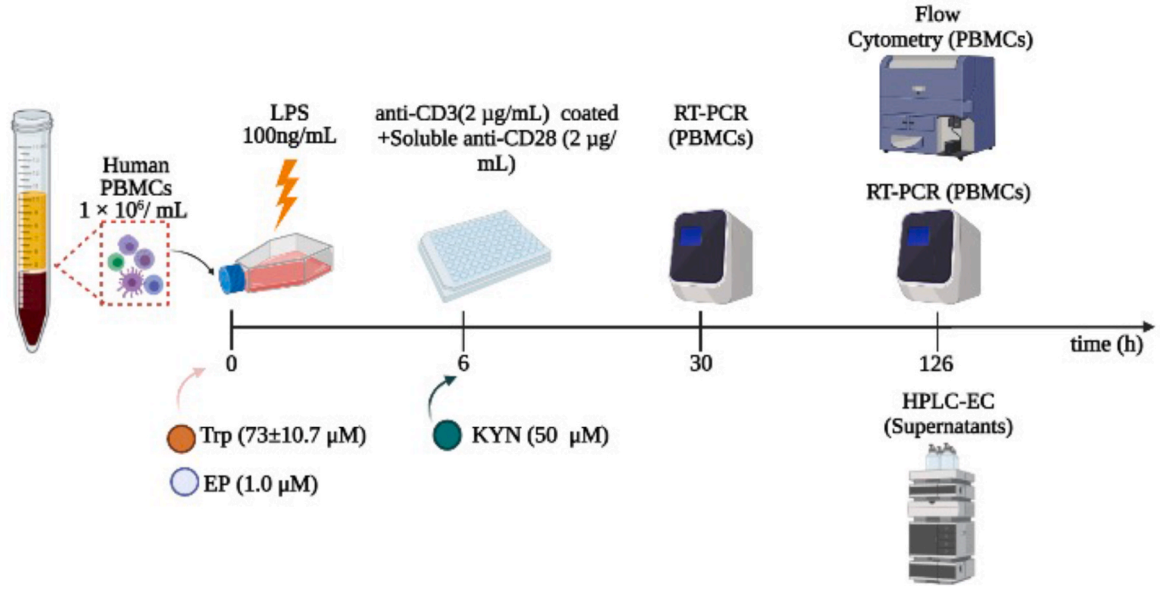

Fig. 1. Schematic representation of the work timeline created in BioRender.com. Isolated peripheral blood mononuclear cells (PBMCs) were incubated with lipopolysaccharide (LPS) during 6h at 37 °C, 5%CO_2 in the presence or absence of tryptophan (Trp) and/or Epacadostat (EP). Control cells were unstimulated. The unstimulated PBMCs and the ones pre-incubated with LPS were then transferred to a 96 well plate coated with anti-CD3 (2 µg/mL) whereas soluble anti-CD28 (2 µg/mL) was added simultaneously in the presence or absence of Kynurenine (KYN). Real-time PCR (RT-PCR) was used to analyse IDO1 mRNA levels of PBMCs 24 or 120h after incubation with anti-CD32/CD28 at 37 °C, 5%CO_2. After 120h of incubation at 37 °C, 5%CO_2, cells were collected to analyse lymphocytes proliferation by flow cytometry and supernatants were collected to measure Trp and KYN metabolites in PBMCs supernatants (HPLC-EC).

2.3. Isolation of PBMCs

BC were diluted (1:1) in PBS ((g/L) NaCl 8.0, KCl 1.5, Na_2HPO_4 1.44, KH_2PO_4 0.24, pH 7.4) and then the PBMCs were isolated by Ficoll-Paque Plus density gradient centrifugation. The cells were resuspended and any residual contaminating erythrocytes were lysed by the addition of 5 mL of the lysis buffer ((g/L) NH_4Cl 8.248, $KHCO_3$ 1.0, EDTA 0.0368), followed by immediate centrifugation at 100*g* for 10 min at room temperature (RT). The cells were washed in PBS/FBS 2% and centrifuged at 300 g for 10 min at RT and resuspended at the final concentration of 10 $\times$ 10^6 cells in 10 ml of RPMI for subsequent culture. Cell viability was assessed by trypan blue and purity was assessed by turk solution [(v/v): gentian violet 0.02%+acetic acid 3% prepared in water]. The viability of the cultured cells was $\geq$95%. The PBMC suspension obtained by this method contained at least 90% of lymphocytes and a few contaminant polymorphonuclear (PMN) cells ($\leq$5%), as confirmed by flow cytometry.

2.4. Cell culture

2.4.1. LPS stimulus

The PBMCs were resuspended at the concentration of 1 $\times$ 10^6/mL in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin and activated with LPS (100 ng/mL) in a cell culture flask, for 6h at 37 °C in a moist atmosphere of 5% CO2. These cells were then transferred to the 96-well plates coated with anti-CD3. This time-point was chosen based on a time-course evaluating IDO1 induction in monocytes and DCs across 24h-stimulation of total blood (Fig. 1aS).

2.4.2. Anti-CD3/CD28 stimuli

First, the 96-well flat bottom plate was coated with 100 μL of anti-CD3 antibody (2 μg/mL in sterile PBS 1X). The lid-covered plate was incubated for 2h at 37 °C with 5% of CO_2. Then the unbound antibody was washed twice with 200 μL of sterile 1X PBS. 250 μL of resuspended PBMCs (1 $\times$ 10^6/mL in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin) pre-stimulated or not with LPS (100 ng/mL for 6h) were added to each well and stimulated with soluble anti-CD28 (2 μg/mL) for 24 or 120h at 37 °C in a moist atmosphere of 5% CO_2.

2.4.3. IDO1 activation by Trp supplementation

We adjusted the Trp concentration present in RPMI (25 μM) to human physiologic values according to Geisler et al. (2015) (67.4 $\pm$ 10.2 μM) (Geisler et al., 2015) for the assays with Trp supplementation. Trp was added conjointly with LPS. The final concentration of Trp (73.00 $\pm$ 10.70 μM) in the culture supernatants was determined after 126h of cell culture at 37 °C with 5% of CO_2 by HPLC-EC.

2.4.4. IDO1 inhibition by epacadostat

Epacadostat (EP) is an IDO1 inhibitor that acts as a Trp-competitive inhibitor of the catabolic activity of human IDO1 in cell-based assays (IC_{50} = 12 nmol/L) with >100-fold selectivity exhibited against IDO2 and TDO2 (Prendergast et al., 2017). The EP was dissolved in DMSO and used at a concentration of 1.0 μM, which mimics the serum concentration observed in patients receiving 300 mg BID, where there was >90% inhibition of IDO1 (Jochems et al., 2016). The EP was added conjointly with LPS and Trp.

2.4.5. KYN incubation

KYN was directly added to the cell culture after 6h of incubation with LPS (100 ng/mL) and immediately before the anti-CD3/CD28 stimuli at a concentration of 50 μM(Greene et al., 2019). The final concentration of KYN (47.69 $\pm$ 4.32 μM) in the culture supernatants was determined after 120h of cell culture at 37 °C with 5% of CO_2 by HPLC-EC and may reflect the exogenous addition plus the KYN released by the cells into the medium in the pro-inflammatory environment.

2.5. IDO1 expression by real Time-PCR (RT-PCR)

Cells were collected after 24 or 120h of cell culture (counting from the last stimuli – anti-CD3/CD28) at 37 °C with 5% CO_2 for IDO1 expression analysis. RT-PCR of IDO1 was performed according to a previously reported method but with modifications (Kustrimovic et al., 2016). Briefly, *circa* 50,000 PBMCs were resuspended in PerfectPure RNA lysis buffer (5 Prime Gmbh, Hamburg, Germany) to isolate the RNA. Total RNA was extracted by PerfectPure RNA Cell KitTM (5 Prime Gmbh), and the amount of RNA extracted was estimated by spectrophotometry at λ = 260 nm. The total mRNA obtained from the PBMCs was reverse-transcribed using a random primer, high-capacity cDNA RT kit (Applied Biosystems). The cDNA was amplified with the SsoAdvanced™ Universal SYBR® Green Supermix (BIORAD) for analysis and then assayed on the StepOne® System (Applied Biosystems). The linearity of the real-time PCR assays was tested by constructing standard curves using serial 10-fold dilutions of a standard calibrator cDNA for the gene, and the regression coefficients (r2) were always >0.999; a melting curve was also performed to check for the specificity of IDO1. The gene expression level in any given sample was represented as $2-\Delta Ct$ where Δ Ct = [Ct (sample) – Ct (housekeeping gene)]. The relative expression was determined by normalization to 18 S cDNA. Analysis of the data was performed by StepOne software™ 2.2.2- Applied Biosystems.

2.6. Proliferation assay

T lymphocyte proliferation was measured after 120h of cell culture after the last stimuli at 37 °C with 5% CO_2, using standard staining with the proliferation dye CPD and flow cytometric analysis [BD FACSCelesta flow cytometer, Becton Dickinson, Milan, Italy with BD FACSDiva software (version 8.0.1.1)]. Resting and activated lymphocytes were identified according to their typical morphological parameters in the Forward and Side Scatter dot-plot (FSC vs. SSC plot) and a sample of a minimum of 20,000 cells was acquired at the gate (Table S1 and Fig. 3S, supplementary files). CPD is a red fluorescent dye that binds to any cellular protein containing primary amines and as cells divide the dye is distributed equally between daughter cells resulting in a successive halving of the fluorescence intensity during cell division. The lymphocyte proliferation can be therefore calculated as a percentage (%) of the CPD^{low} cells collected at the gate. The necessary amount of PBMCs, including the cells to be stained with CPD (CPD^+) and the cells that were not stained with CPD (CPD^-), were counted by using a cellometer (cellometer Auto T4, Nexcelom Bioscience, Euroclone). The cells were then washed with PBS/FBS 0.1% to a volume of 5 mL and centrifuged at 1200 g for 5 min at RT. The supernatants were carefully removed and CPD^- cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin at a concentration of 1x10^6 cells/mL. These cells were incubated at 37 °C with 5%CO_2 until CPD staining of the remaining cells was completed. CPD (2.5 μM) was added to the remaining cells that were resuspended in 1 mL of PBS/FBS 0.1% (for up to 10x10^6 cells), the suspension was immediately mixed using vortex and then incubated at RT for 8 min, protected from light. The reaction was stopped by the addition of an equal volume of pre-warmed (37 °C) FBS (1 mL) and incubated at 37 °C for 10 min. Next, PBS/FBS 10% was added to a volume of 10 mL. The suspension was centrifuged at 1200 g, for 5 min at RT and washed twice with 10 mL PBS/FBS 10% at 1200 g for 5 min. The cells were resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 U/mL penicillin/streptomycin at a concentration of 1x10^6 cells/mL before being cultured.

2.7. High-performance liquid chromatography -electrochemical (HPLC-EC) measurements of Trp and KYN

Supernatants were collected after 120h of cell culture with the last stimuli at 37 °C with 5% CO_2 and diluted (1:1) with perchloric acid ($HClO_4$) 0.2 N. Then, the solution was centrifuged at 10,000g and 4 °C. The supernatants were recovered and stored at −80 °C until quantification. The supernatants were then thawed and centrifuged at 10,000g at 4 °C immediately before the analysis. These KP metabolites (Table S3, supplementary files) were assayed by HPLC with electrochemical detection, using a previously described method, with modifications (Cosentino et al., 2000). Briefly, the HPLC system consisted of a pump (model LC10ADVp, Shimadzu, Kyoto, Japan), a Waters X-Bridge Shield C18 RP (150*4,6 mm, 3,5 µm, 80A) column, an autosampler (model SIL9A, Shimadzu), and an electrochemical detector (ESA Couloarray 5800A, ESA, Bedford, MA, USA) with an analytical coulometric cell with 4 electrodes (model 6210, ESA). The chromatograms were collected, stored, and processed with a computerized integrator (ESA Coularray version 3.1, ESA, Bedford, MA, USA). The optimal composition of the mobile phase was ultrapure water/acetonitrile (90.7:9.3 v/v), 0.6264 mM octansulfonic acid, 0.349 mM Na_2EDTA and 0.049 mM potassium phosphate monobasic with an adjusted (using H_3PO_4) pH of 3.2. The freshly prepared mobile phase was filtered (GSWP 04700, 0.45 mm, Millipore, Bedford, MA) and degassed in a vacuum for 10 min. The flow rate was 0.9 ml/min. The volume of the sample injected was 30 µl. The system was running continuously with the mobile phase being recycled back into the reservoir. Trp was detected in channel 1 (600 mV) and KYN was detected in channel 2 (500 mV) simultaneously. Linearity and sensitivity of the method (detection limit: 0.1 µM, for both metabolites) were tested by constructing standard curves using serial dilutions of the standards (Trp and KYN) and the regression coefficients (r^2) were >0.98 (Table S2, supplementary files). Trp and KYN were reconstituted according to the manufacturer's specifications.

2.8. Statistical analysis

Data are reported as means ± SD of the indicated number of experiments. Data came from independent experiments. Statistical analysis and graphic illustrations were performed using GraphPad Prism 8.0.1 software (San Diego, CA). Paired two-tailed Student's t-tests, ANOVA with posthoc analysis: Tukey test and Friedman test with posthoc analysis and the Dunn test were used to calculate p values. A p-value of less than 0.05 was considered significant.

3. Results

The pro-inflammatory milieu increased IDO1 expression and activity. Different stimuli were applied to the isolated PBMCs from healthy blood donors to study the impact of a pro-inflammatory environment on the IDO1 expression and activity *in vitro*: they were LPS

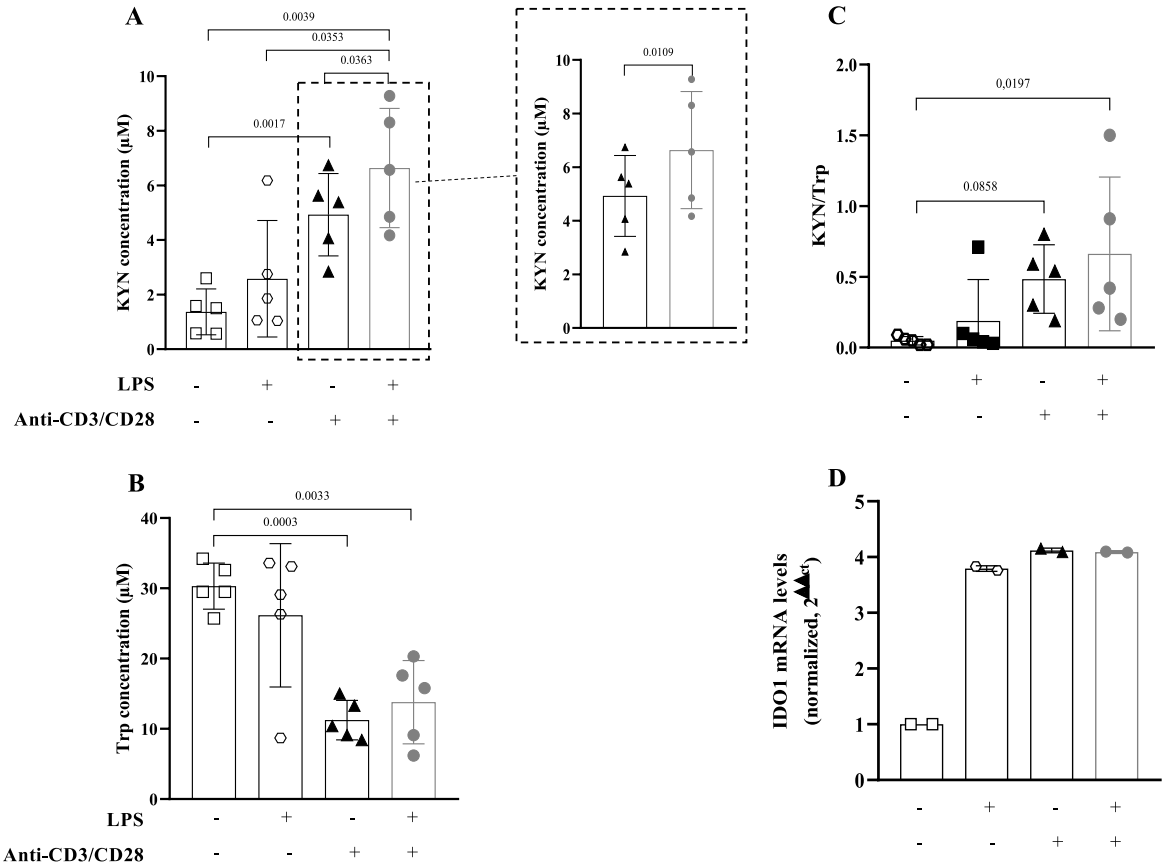

Fig. 2. A pro-inflammatory milieu increased IDO1 expression and activity. Different pro-inflammatory stimuli changed IDO1 mRNA levels and activity. LPS (100 ng/mL) was applied 6h before the anti-CD3/CD28 (2 µg/mL) stimuli. (A) *In vitro* kynurenine (KYN) and (B) tryptophan (Trp) concentration (µM) measured in peripheral blood mononuclear cells (PBMCs) culture supernatants (HPLC-EC). Statistics: one-way ANOVA, post-hoc analysis: Tukey test, n = 5. Inset – the effect of LPS in KYN levels achieved by the anti-CD3/CD28 stimuli. Statistical analysis: t-student, n = 5. (C) IDO1 activity given by kynurenine (KYN)/Tryptophan (Trp) ratio measured in supernatants of PBMCs by HPLC-EC. Statistics: Friedman test, post-hoc analysis - Dunn test, n = 5. (D) normalized IDO1 mRNA levels were measured in PBMCs lysates by RT-PCR. No statistical analysis, n = 2. All measurements were performed after 120h incubation of PBMCs in the presence or absence of anti-CD3/CD28 following a pre-exposure with LPS (6h) at 37 °C, 5%CO_2. IDO1 mRNA absolute values ($2^{\Delta ct}$) resting condition were $3.69x10^{-6} \pm 1.63x10^{-7}$, represented as mean ± SD.

alone, anti-CD3/CD28, and LPS combined with anti-CD3/CD28 (LPS+anti-CD3/CD28). IDO1 expression was increased at 126h in the LPS condition (2.8-fold vs. unstimulated; Fig. 2D). However, the KYN and Trp levels (Fig. 2 A and B) and KYN/Trp (Fig. 2C) were not altered, at the same timepoint in the culture medium in the LPS condition, when compared to the unstimulated condition. On the other hand, the anti-CD3/CD28 stimuli increased the KYN levels (1.37 ± 0.84 μM vs. 4.93 ± 1.51 μM, p = 0.0017; Fig. 2A), decreased the Trp levels (29.5 ± 3.28 μM vs. 10.40 ± 2.82 μM, p = 0.0003; Fig. 2B) and increased the IDO1 activity (0.05 ± 0.03 vs. 0.48 ± 0.24, p = 0.0858; Fig. 2C) and IDO1 expression (3.1-fold; Fig. 2D), when compared to the unstimulated condition. The combination of both stimuli (LPS and anti-CD3/CD28) also increased KYN levels (1.37 ± 0.84 μM vs. 6.64 ± 2.18 μM, p = 0.0039; Fig. 2A), decreased Trp levels (29.50 ± 3.28 μM vs. 15.80 ± 5.93 μM, p = 0.0033; Fig. 2B) and increased IDO1 activity (0.05 ± 0.03 vs. 0.66 ± 0.54, p = 0.0197; Fig. 2C) and IDO1 expression (3.1-fold, Fig. 2D) when compared to the unstimulated condition. Moreover, KYN levels in the LPS+anti-CD3/CD28 condition were significantly higher when compared to anti-CD3/CD28 (6.64 ± 2.18 μM vs. 4.93 ± 1.51 μM, p = 0.0109, t-student; Fig. 2A, inset).

LPS attenuated anti-CD3/CD28-induced lymphocyte proliferation. Anti-CD3/CD28, but not LPS, increased lymphocyte proliferation (51.96 ± 10.05% vs. 1.30 ± 0.84%, p = 0.0011; Fig. 3). Interestingly, pre-incubation with LPS attenuated anti-CD3/CD28-induced lymphocyte proliferation (37.46 ± 11.20 vs. 51.96 ± 10.05%, p = 0.0327, t-student; Fig. 3, inset).

IDO1 inhibition did not change lymphocyte proliferation. We supplemented the culture medium with Trp (IDO1 substrate) reaching physiological values of 73.00 ± 10.70 μM to increase IDO1 activity, thus feeding KP. Trp addition further attenuated lymphocyte proliferation (37.46 ± 11.20 vs. 16.76 ± 7.33%, p = 0.0630; Fig. 4D) without reaching a statistical significance. However, Trp showed a tendency to decrease Kyn levels (Fig. 4C) without IDO1 mRNA alterations (Fig. 4A). Further, we used EP, which is an IDO1 catalytic inhibitor, to restore lymphocyte proliferation. In fact, EP reduced IDO1 mRNA levels (3.3-fold; Fig. 4B) and KYN levels (3.80 ± 2.49 vs. 0.68 ± 0.11 μM, p = 0.0498; Fig. 4C). However, the IDO1 inhibitor did not restore lymphocyte proliferation (19.94 ± 18.56 vs. 21.08 ± 15.42%, p = 0.5942; Fig. 4D).

The addition of a higher concentration of KYN decreased lymphocyte proliferation without reaching significance. As a positive control, a higher concentration of KYN (48.95 ± 2.51 μM) was added to the cell culture in our specific pro-inflammatory milieu LPS+anti-CD3/CD28. KYN was able to decrease the proliferation of lymphocytes further, reaching levels that were less than half of the values obtained with LPS+anti-CD3/CD28. However, statistical significance was not reached (37.46 ± 11.20 vs. 15.21 ± 17.31%, p = 0.1250; Fig. 5).

4. Discussion

First we need to emphasize that the aim of the *in vitro* model was to reproduce the pro-inflammatory milieu seen in pathological contexts and which enabled us to simultaneously determine IDO1 expression (mRNA levels by RT-PCR) and activity (KYN/Trp ratio by HPLC-EC), as well as lymphocyte proliferation by flow cytometry (Badawy and Guillemin, 2019).

IDO1 modulation by different pro-inflammatory stimuli. The KP imbalance that occurs in several pathologies has been associated with inflammation (Joisten et al., 2021). PBMCs, namely circulating myeloid cells, can use KP to orchestrate peripherally and central (CNS) immune responses (Mondanelli et al., 2020). Thus, KP has inflammatory modulation properties (Savitz, 2020). It has been suggested that tryptophan-derived catabolites, including KYN, are responsible for the inhibition of T-cell proliferation induced by IDO1[50]. Therefore, we used a pro-inflammatory milieu to induce IDO1 in PBMCs and to study the impact of IDO1 manipulation on lymphocyte proliferation. Importantly, we show herein that this model is suitable to measure IDO1 expression and activity. We used double-hit stimuli that combined two pro-inflammatory stimuli: LPS (DCs and monocytes) and anti-CD3/CD28 (T cells). Innate cells, including monocytes and DCs, can be activated through pattern recognition receptors (PRRs) with pathogen-associated molecular patterns (PAMPs) such as LPS(Fallarino et al., 2015; Murakami et al., 2013). Furthermore, LPS is known to induce IDO1 in DCs and monocytes (Murakami et al., 2013; Salazar et al., 2016). This is consistent with our flow cytometry data showing that LPS (100 ng/ml) induced IDO1 in DCs and monocytes in a time-dependent fashion, in total blood samples (supplementary files Fig. S1b). We specifically showed that 34% of DCs and 43% of monocytes were IDO1^{+} at 6h. Moreover, DCs and monocytes showed their maximum IDO1 induction at 12h (55.4 and 95%, respectively). This level of induction lasted remained up to 24h (Fig. 1BS). Accordingly, our gene expression analysis showed that LPS increased IDO1 in PBMCs lysates. However, LPS failed to significantly increase KYN levels and KYN/Trp (this ratio has been validated as an *in vitro* measure of IDO1 activity for several studies) (Badawy and Guillemin, 2019). On the other

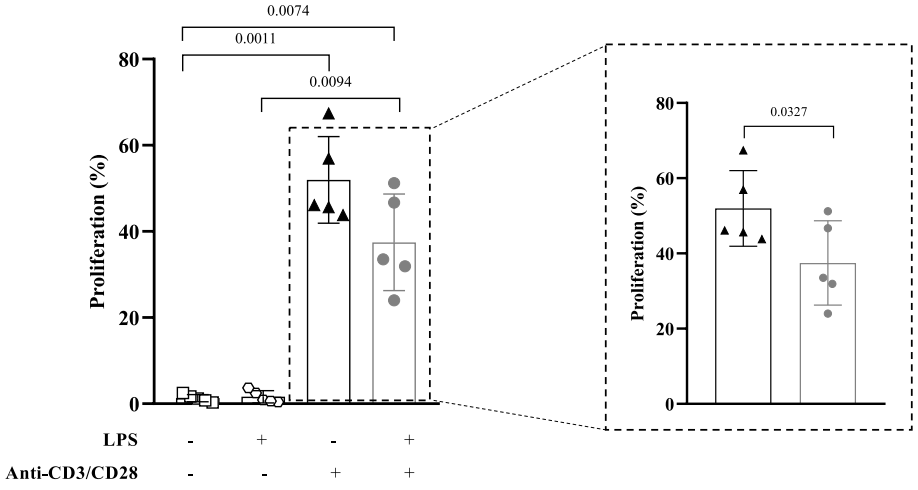

Fig. 3. LPS attenuated anti-CD3/CD28-induced lymphocyte proliferation. Lymphocytes proliferation was measured by Flow cytometry after 120h incubation of PBMCs with anti-CD3/CD28 (2 μg/mL) following a pre-exposure to LPS (100 ng/mL, 6h) at 37 °C, 5%CO_2. Statistical analysis: one-way ANOVA, post-hoc analysis - Tukey test; n = 5. Inset – the effect of LPS in lymphocyte proliferation triggered by the anti-CD3/CD28 stimuli. Statistical analysis: t-student, n = 5.

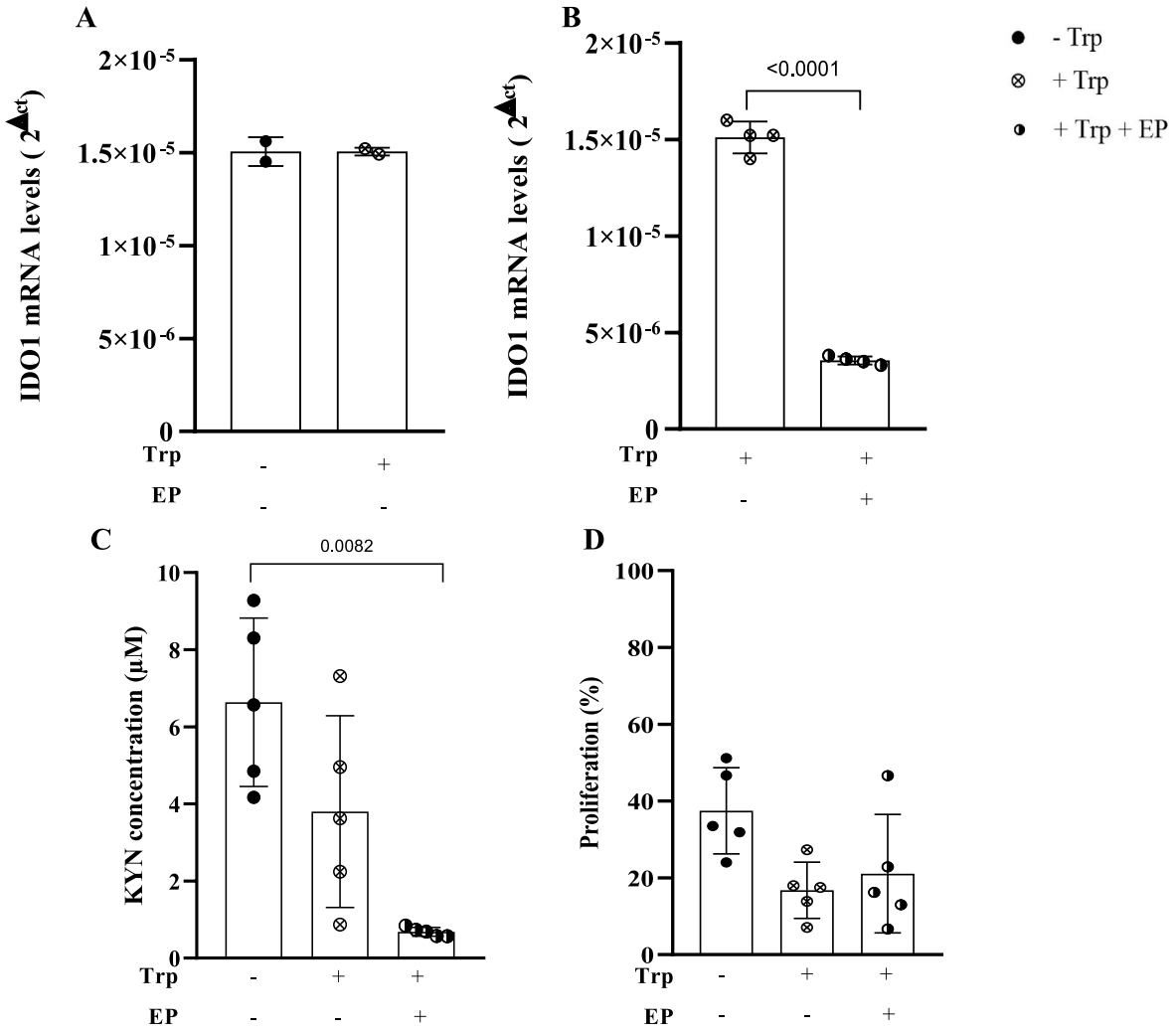

Fig. 4. IDO1 inhibition did not change T lymphocyte proliferation. The pro-inflammatory milieu used was the following: pre-incubation with LPS (100 ng/mL, 6h) in combination with anti-CD3/CD28 (2 µg/mL, during 120h) 37 °C, 5%CO_2. The impacts of tryptophan (Trp; 73.00 ± 10.70 µM) and epacadostat (EP, 1 µM; IDO1 inhibitor) addition are shown. (A) IDO1 mRNA levels were measured in lysates from PBMCs cultured in media Trp supplementation for 126h (RT-PCR). Data are expressed as 2Δct. No statistical analysis, n = 2. (B) IDO1 mRNA levels were measured in lysates from PBMCs cultured in medium supplemented with Trp in the presence or absence of EP for 30h (RT-PCR). Data are expressed as 2Δct. Statistical analysis: t-student, n = 4. (C) *In vitro* kynurenine (KYN) levels (concentration, µM) were measured in PBMCs supernatants (HPLC-EC). Statistical analysis: one-way ANOVA, n = 5. (D) Lymphocytes proliferation was measured by Flow Cytometry. Statistical analysis: one-way ANOVA, n = 5.

hand, the activation of T cells with anti-CD3/CD28, both in the presence or absence of LPS, increased all the IDO1 parameters measured: gene expression, KYN levels, and KYN/Trp. Moreover, all experimental conditions increased IDO1 mRNA levels similarly. Importantly, our flow-cytometry data also showed that LPS, as well as anti-CD3/CD28 and the combination of both stimuli are unable to induce IDO1 in B, T, and NK cells (supplementary files Fig. S2b). This clearly shows that circulating myeloid cells are the main source of IDO1 in PBMCs cultures. Therefore, this suggests that myeloid cells are the chief contributors to IDO1 data in this study. Additionally, our data suggest that T-cell activation is necessary to sustain IDO1 activity in the circulating myeloid cells in our model. This is consistent with what was reported in cell cultures with high myeloid/T cell ratios, that mimic tumor microenvironments: interferon-gamma (IFN-γ) released by activated T cells triggers the monocytes to express IDO1 (Silberman et al., 2012). Indeed, we are working with PBMCs that comprise both myeloid cells and T cells. T cell activation with the eventual production of IFN-γ might interact with IDO1-expressing myeloid cells in our cultures, thus driving IDO1 induction and activity. This cell-cell interaction was also explored by Lawlor et al., 2020, in a holistic study of circulating immune cell responses where the authors observed that anti-CD3/CD28 activated all classes of lymphocytes either directly (T cell subset) or indirectly (B and NK cell subsets (Lawlor et al., 2020)). One should not forget that NK cells also release IFN, thus also contributing to IDO1 modulation (Lin et al., 2022).

LPS attenuated lymphocyte proliferation. The ability of T cells to proliferate in response to antigens has been used as an indicator of the presence of antigen-specific T cells. Thus, measuring T cell functions in terms of lymphocyte proliferation is used as an immunological marker in the present work (Deenadayalan et al., 2013). We noted that LPS attenuated lymphocyte proliferation while there were increased levels of KYN in supernatants of LPS+anti-CD3/CD28 when compared with anti-CD3/CD28 alone. The mechanisms whereby LPS attenuated proliferation herein may rely on monocyte priming prior to the lymphoid stimulation. This may include: a reduction in the production of pro-inflammatory cytokines, an increased expression of anti-inflammatory cytokines, a decrease in the antigen-presenting capacity partly due to a reduced HLA-DR (major histocompatibility complex cell surface receptor) expression and the upregulation of numerous mechanisms that negatively regulate toll-like receptor (TLR)-associated

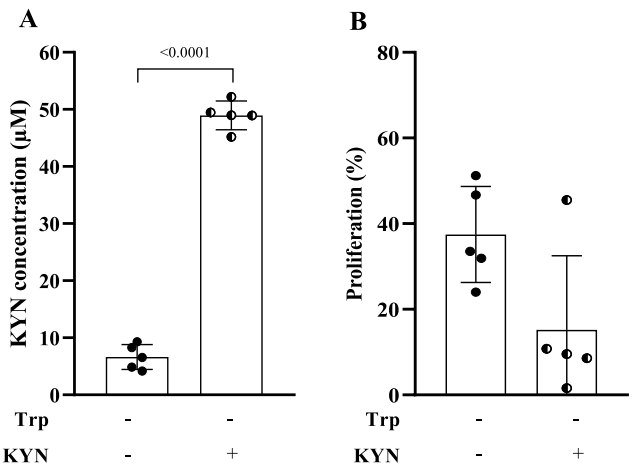

Fig. 5. Addition of exogenous kynurenine (KYN) seemed to decrease lymphocyte proliferation. The pro-inflammatory milieu was set by using a pre-incubation with LPS (100 ng/mL, 6h) in combination with anti-CD3/CD28 (2 μg/mL, during 120h). (A) *In vitro* KYN levels (concentration, μM) were measured in peripheral blood mononuclear cells (PBMCs) culture supernatants, after the addition of 50 μM of KYN to the culture medium (HPLC-EC); t-student, n = 5. (B) the impact of the addition of 50 μM of KYN in PBMCs proliferation was measured by Flow Cytometry, t-student, n = 5. Both measurements were made after 120h incubation of PBMCs with anti-CD3/CD28 following a pre-incubation with LPS (6h) at 37 °C, 5%CO_2.

signaling pathways (Turrel-Davin et al., 2011). Overall, this altered monocyte function can, in part, contribute to a weaker activation of the T cell response with a consequent reduction in lymphocyte proliferation.

IDO1 inhibition does not affect lymphocyte proliferation. Since the double-hit stimuli both increased IDO1 expression and changed the lymphocyte proliferation, we investigated the role of IDO1 in these proliferation changes. We decided first to feed KP by supplementing the culture medium with Trp to reach physiological values (Geisler et al., 2015). Interestingly, Trp supplementation further aggravated the effects of LPS on lymphocyte proliferation without changing IDO1 mRNA. Moreover, TRP also seemed to decrease KYN levels, without a reaching statistical significance. There are important points that need to be considered: 1) mRNA levels do not necessarily reflect IDO1 protein levels because IDO1 is also controlled by post-transcriptional mechanisms (Glubb et al., 2014; Gargaro et al., 2022); 2) KYN/Trp, which is a proxy for IDO1 activity, could not be compared between conditions with and without Trp supplementation, given that the initial amount of Trp is different between conditions; 3) IDO1 may be inhibited by an excess of Trp (Badawy, 2017a). In fact, some authors have stated that Trp concentrations higher than 50 μM might inhibit the KYN/Trp ratio and IDO1 activity (Badawy and Guillemin, 2019). On the one hand, we used a Trp concentration which is *circa* 5x TRP KM (~15 μM) (Lu et al., 2010), on the other hand normal Trp plasma levels are between *circa* 46–83 μM. However, others have shown that supplementing the culture medium with 100 μM of Trp triggered an increase in extracellular levels of KYN in an *in vitro* increment approach (Jürgens et al., 2009; Grohmann et al., 2001), and 3) Trp could be feeding other immune modulator pathways (Badawy and Guillemin, 2019; Geisler et al., 2015; Mondanelli et al., 2020; Wirthgen et al., 2018). For example, Trp could be feeding the serotonin pathway, and this indolamine has immunoregulatory properties by acting on serotonin receptors that are present in peripheral immune cells (Herr et al., 2017). Nonetheless, information about the immunomodulator mechanisms of serotonin in peripheral immune cells is controversial and still scarce (Wu et al., 2019). Finally, there is an alternative KP route that includes the enzyme interleukin 4-induced 1 (IL4I1) that degrades Trp into an indole metabolite (indole-3-pyruvic acid) which is converted into KYNA and other derivatives that activate AhR, thus exerting immune modulatory action and anti-inflammatory activity (Sadik et al., 2020). We next used IDO1 inhibition as a strategy to further understand the impact of IDO1 on lymphocyte proliferation. The EP concentration used herein corresponds to the plasmatic EP concentration seen in melanoma patients treated with EP 300–400 mg BID (metastatic melanoma trial). One should point out that this trial failed since only one third of the patients achieved 90% inhibition of IDO1 at these dose levels. It was argued that this failure was due to low levels of EP (Van Den Eynde et al., 2020). Nonetheless, we showed that EP triggered a robust decrease in IDO1 mRNA (at 24h) and KYN levels (at 120 h). EP is, usually, described as a Trp-competitive inhibitor of the catabolic activity of human IDO1 (Yue et al., 2017; Mckinnirey et al., 2021). However, we show that it also reduced IDO1 gene expression. This may have strongly contributed to the decreased levels of KYN. In addition to catalytic activity, IDO1 is also endowed with nonenzymatic functions that contribute to reprograming the immunoregulatory phenotype of immune cells (Pallotta et al., 2021). We have shown that both catalytic and non-enzymatic activities were nearly abolished by EP. However, this did not have an impact on lymphocyte proliferation. On the contrary, others have suggested that EP, at concentrations lower than the one we used, have actually increased human lymphocyte proliferation[39,40]. The apparent contradictions regarding EP as well as Trp data may stem from cell culture conditions, incubation times, and stimuli which are different across studies (Jiang et al., 2020; Torres Crigna et al., 2020). Overall, our study clearly shows that IDO1 inhibition did not change lymphocyte proliferation in this *in vitro* experimental model.

Inhibition of lymphocyte proliferation is dependent on KYN concentration. Finally, we added KYN (50 μM) directly to the PBMCs culture, as a positive control. KYN reached a final concentration of 47.69 ± 4.32 μM in the culture supernatants. This may reflect the exogenous addition plus the KYN released by the cells to the medium. KYN supplementation reduced lymphocyte proliferation by more than half of the values obtained with LPS+anti-CD3/CD28. However, this reduction did not reach statistical significance (all but one sample showed a robust proliferation reduction). Nonetheless, our data seems to be consistent with others showing: 1) that T cell proliferation is dose-dependently inhibited by KYN and 2) that KYN inhibits the proliferation of PBMCs at lower concentrations in the absence of Trp (Frumento et al., 2002; Greene et al., 2019; Terness et al., 2002). Others showed that KYN used at 50 μM induced primary human CD8 T-cell death[22]. Therefore, one can argue that KYN can reduce T cell proliferation in a concentration-dependent manner and the KYN concentrations triggered by our double-hit protocol were not sufficient to inhibit lymphocyte proliferation.

Study limitations and future directions. It has been suggested that both gender and age (immunoaging) have an impact on the responses of the immune system in acute and chronic inflammatory diseases (Jaillon et al., 2019; Lopez-lee et al., 2023; Taneja, 2018; Tansey and Romero-Ramos, 2019). Furthermore, monocytes, one of the cell types expressing IDO1 in our experimental model, showed a sex-dependent response to LPS(Campesi et al., 2022). Generally, there is a lack of information and consensus about the role of gender in IDO1 expression. A study using *in vitro* PBMCs showed increased levels of basal IDO1 expression in males when compared with females (Da Pozzo et al., 2018). On the other hand, IDO1 expression on colorectal cancer and solid tumors in humans may not be related to gender (Ma et al., 2018; Yu et al., 2018). Interestingly, aging seems to foster the activation of the IDO1-KYN-AhR pathway which might be related to the suppression of effector immune cells and immunosenescence (Salminen, 2022). All together, we can conclude that sex and age might affect both the immune profile of cells and IDO1 expression. Based on this, we consider that the lack of availability of information concerning biological sex and age regarding BC is a strong limitation of this study. We consider that, in the future, this model must also address gender and age differences, including the phase of the menstrual cycle and the use of oral contraceptives. Additionally, we also consider it important to explore this

model in the context of different diseases, such as neurodegenerative diseases to explore the role of IDO1 with regard to these patients' PBMCs. Therefore, we suggest that this model is also suitable to explore other pharmacological agents with an immunomodulatory function.

5. Conclusion

The undoubted importance of KP regarding cancer, associated with the discovery the immune properties of KP metabolites has led to a growing interest in exploring KP in other diseases with inflammatory components. Several disruptions in metabolite balance have been associated with specific diseases, namely neurological ones. However, as referred to by Joisten et al. (2021), it has been hard to understand the physio pathological role of IDO1 immunoregulatory mechanisms: do they drive pathological states or are they compensatory mechanisms? We were able to create a pro-inflammatory environment where IDO1 is increased and could be manipulated. We observed that the changes in the lymphocyte proliferation parameter seem to be IDO1-independent. Moreover, our study also highlights the importance of further investigation of the Trp immunomodulatory IDO1-independent properties. Importantly, the suggestion that KYN impacts on proliferation in a concentration- and environment-dependent manner increases the importance of carefully analyzing KP imbalances for disease staging (diagnostics) and therapeutic purposes in the context of neurodegenerative diseases.

Ethics statement

All blood samples were collected from healthy individuals after they signed an informed consent to participate in the present study approved by the Ethical Committee of the Coimbra Hospital and University Centre (Portugal; CHUC-131-19) (supplementary methods).

Disclosers

No conflicts of interest, financial or otherwise, are declared by the authors.

CRediT authorship contribution statement

Milene Gonçalves: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft. **Alessia Furgiuele:** Methodology. **Emanuela Rasini:** Formal analysis, Methodology. **Massimiliano Legnaro:** Formal analysis, Methodology. **Marco Ferrari:** Formal analysis, Methodology. **Alessandra Luini:** Formal analysis, Methodology. **Paulo Rodrigues-Santos:** Funding acquisition, Methodology, Resources. **Francisco Caramelo:** Formal analysis. **Franca Marino:** Writing – review & editing. **Frederico C. Pereira:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing. **Marco Cosentino:** Conceptualization, Funding acquisition, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Abbreviations

AhR	transcription factor aryl hydrocarbon receptor
BC	buffy coats
CNS	central nervous system
DCs	dendritic cells
EPA	Epacadostat
HPLC-EC	High Performance Liquid Chromatography -Electrochemical
IDO1	Indoleamine 2, 3-dioxygenase 1
IFN	γ interferon gamma
IL4I1	interleukin 4-induced 1
KP	kynurenine pathway
KYN	Kynurenine
KYNA	kynurenic acid
LPS	Lipopolysaccharide
NK	Natural-killer
PAM	positive allosteric modulator
PAMPs	pathogen-associated molecular patterns
PBMCs	peripheral blood monocytes cells
PRRs	pattern recognition receptors
QUIN	quinolinic acid
RF	relative frequency
RT-PCR	Real time polymerase chain reaction
Teffs	effector T
TLR	toll-like receptor
Tregs	T regulatory cells
Trp	Tryptophan

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejphar.2024.176420.

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