ORIGINAL ARTICLE
Iran J Allergy Asthma Immunol
February 2018; 17(1):56-67.

Indoleamine 2, 3-dioxygenase Up-regulates Hypoxia-inducible Factor-1α Expression by Degrading L-tryptophan but Not Its Activity in Human Alloreactive T-cells

Theodoros Eleftheriadis, Georgios Pissas, Vassilios Liakopoulos, and Ioannis Stefanidis

Department of Nephrology, Medical School, University of Thessaly, Larissa, Greece

Received: 6 May 2017; Received in revised form: 4 July 2017; Accepted: 24 July 2017

ABSTRACT

Indoleamine 2, 3-dioxygenase (IDO) suppresses T-cell function at least in part by altering cell metabolism. Hypoxia-inducible factor-1 (HIF-1) increases upon T-cell activation and alters cell metabolism favoring their differentiation to effector cells. The effect of IDO on HIF-1α expression and activity was evaluated.

For this purpose, mixed lymphocyte reaction (MLR) was performed using the IDO inhibitor 1-DL-methyl-tryptophan and the p53 inhibitor pifithrin-α. L-tryptophan degradation and cell proliferation were assessed by enzyme-linked immunosorbent assay, whereas the expression of proteins of interest by western blotting.

IDO inhibited cell proliferation, and in MLR-derived T-cells increased HIF-1α and p53, whereas it decreased c-Myc. Inhibition of p53 abrogated IDO-induced HIF-1α upregulation. IDO increased the p53 transcriptional targets p21 and TP53-induced glycolysis and apoptosis regulator. The transcriptional targets of both HIF-1α and c-Myc, hexokinase II and lactate dehydrogenase-A were decreased by IDO. Phosphorylated pyruvate dehydrogenase remained unaffected indicating that pyruvate dehydrogenase kinase, a transcriptional target of HIF-1α, is not affected by IDO.

In human alloreactive T-cells, IDO up-regulates HIF-1α, by inducing p53 overexpression. However, HIF-1α remains transcriptionally inactive.

Keywords: c-Myc; Hypoxia-inducible factor-1 (HIF-1) α; Indoleamine 2, 3-dioxygenase; Metabolism; p53; T-cell

INTRODUCTION

Indoleamine 2, 3-dioxyganase (IDO) is a key immunomodulatory enzyme expressed in antigen presenting cells (APCs),especially under inflammatory conditions. IDO degrades L-tryptophan through the kynurenine pathway.

Corresponding Author: Theodoros Eleftheriadis, MD, PhD; Department of Nephrology, Medical School, University of Thessaly, Neo Ktirio, Mezourlo Hill, 41110 Larissa, Greece. Larissa, Greece, Tel: (+30 24) 1350 1665, Fax: (+30 24) 1350 1667, E-mail: teleftheriadis@yahoo.com

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

In the inflammatory microenvironment, L-tryptophan depletion and/or kynurenine pathway products have been incriminated for the immunosuppressive effects of IDO on T-cells.[1,2] Experiments in models of autoimmunity and transplantation confirmed the immunosuppressive properties of IDO.[1,2] In hemodialysis patients increased IDO levels have been associated with reduced immune response to vaccination against hepatitis B virus,[3] as well as with decreased T-cell count.[4] Apart from APCs, IDO is also expressed in certain types of cancer and in trophoblast, where it plays a role in the escaping of cancer from immunosurveillance and in the success of semi-allogenic pregnancy, respectively.[5,6] Thus, investigation of the mechanisms involved in the immunosuppressive effects of IDO could be proved pharmaceutically interesting.

Recent studies revealed that IDO interferes with T-cell metabolism. IDO-induced L-tryptophan depletion activates the general control nonderepressible-2 (GCN2) kinase and decreases glucose influx, decelerates glycolysis and inhibits glutaminolysis in T-cells.[7] In addition, IDO decreases fatty acid synthesis in human alloreactive CD4+ T-cells.[8] Since, upon T-cell activation aerobic glycolysis and glutaminolysis, as well as fatty acid synthesis, are required for cell proliferation and differentiation to effector cell types,[9,10] it is likely that at least in part the immunosuppressive effects of IDO are mediated by the above alterations in T-cell metabolism. Interestingly, IDO also induces fatty acid oxidation,[11] which favors differentiation of naïve CD4+ T-cells to regulatory T-cells (Treg).[12]Many of the IDO-induced effects on the expression of key transporters and enzymes involved in glucose influx, glycolysis and glutaminolysis have been attributed to the upregulation of the transcription factor p53,[13] as well as to the downregulation of the transcription factor c-Myc.[14] These transcriptions factors play a significant role in T-cell proliferation and apoptosis and concurrently control the expression of many enzymes involved in the above metabolic processes.[15,16]

Another transcription factor that controls cell metabolism is the hypoxia-inducible factor-1 (HIF-1). Under normoxia, the HIF-1β component of this factor is constitutively expressed. However, its HIF-1α counterpart is hydroxylated at its O_2-dependent degradation domain by prolyl hydroxylases domain proteins (PHDs) in a reaction that requires oxygen and α-ketoglutarate. Hydroxylation of HIF-1α facilitates its binding to vonHippel-Lindau protein (pVHL) promotingHIF-1α ubiquitination and degradation by the proteasome. Thus, HIF-1α is expressed under hypoxic conditions.[17]

However, upon T-cell activation and despite the normoxic conditions, HIF-1α increases and plays role in cell proliferation and differentiation to effector Th17 cells instead of Treg.[18] HIF-1 enhances Th17 development through transcriptional upregulation of RAR-related orphan receptor-γt (RORγt). Then a complex of HIF-1, RORγt, and p300 is recruited to the IL-17 promoter increasing the expression of Th17 signature genes. Concurrently, HIF-1 binds to forkhead box P3 (FoxP3) and targets it for degradation by the proteasome. Thereby, HIF-1 inhibits the development of Treg. This regulation occurs under both normoxic and hypoxic conditions.[19] Also the HIF-1α-mediated increased glycolysis favors T-cell differentiation to Th17 instead of Treg.[20] However, others showed that hypoxia through the accumulation of HIF-1α promotes FoxP3 expression and differentiation of T-cells to Treg.[21] This discrepancy between various experimental models indicates that the effect of HIF-1 on T-cells is subjected to different regulatory mechanisms.

Considering the immunosuppressive effect of IDO, partially due to IDO-induced alterations in T-cell metabolism, on one hand, and the role that HIF-1 plays in the regulation of T-cell metabolism and differentiation towards an effective or regulatory lineage on the other hand, the effect of IDO onHIF-1α expression and activity in human T-cells was evaluated. For this purpose, the two-way mixed lymphocyte reaction (MLR), a model T-cell alloreactivity was used.

MATERIALS AND METHODS

Subjects

Blood samples were collected from 5healthy volunteers (3 men and 2 women, 38±6 years old). From the blood samples of these 5 different individuals, up to 10 different couples for MLRs can be created. A written informed consent was obtained from each individual enrolled in the study and the Ethics Committee of the Faculty of Medicine, University of Thessaly (Larissa, Greece) approved the study protocol (No. 558/10-2-2017).

Peripheral Blood Mononuclear Cell Isolation and Culture

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation (Histopaque 1077, Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) and counted by optical microscopy on a Neubauer plaque. Cell viability was assessed by trypan blue assay (Sigma-Aldrich; Merck Millipore, Germany).

PBMCs were resuspended in RPMI 1640 medium with L-glutamine, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and supplemented with 10% fetal bovine serum (Sigma-Aldrich; Merck Millipore) and antibiotic-antimycotic solution (Sigma-Aldrich; Merck Millipore, Germany). All cultures were performed at 37^{o}C in a humidified atmosphere containing 5% CO_2.

Assessment of L-Tryptophan Consumption in MLRs

In MLRs, PBMCs were cultured in 12-well plates with or without the IDO inhibitor 1-DL-methyl-tryptophan (1-MT) (Sigma-Aldrich; Merck Millipore, Germany) at a concentration of 100 μM. This concentration was selected according to previous experiments, which showed efficacy without toxicity.[22,23]

The quantity of PBMCs, from each of the two individuals that contribute to the formation of an MLR couple, was $5x10^5$ cells summing up to $1x10^6$ cells per well. After 7 days, supernatants from each MLR were collected. L-tryptophan consumption was assessed in the supernatants by means of enzyme-linked immunosorbent assay (ELISA) (BlueGene Biotech, Shanghai, China). The sensitivity of the above ELISA kit is 1ng/mL. For assessing L-tryptophan consumption 10 MLRs were performed once.

Assessment of Cell Proliferation in MLRs

Cell proliferation was assessed in PBMCs cultures in 96-well plates and in the presence or not of 100μM 1-MT (Sigma-Aldrich; Merck Millipore). The quantity of PBMCs, from each of the two individuals that contribute to the formation of an MLR couple, was $5x10^4$ cells summing up to$1x10^5$ cells per well. Resting PMBC cultures of $1x10^5$ cells per well from each member of the MLR couple were used as controls. At the end of a 7-day period, cell proliferation was assessed via Cell Proliferation ELISA (Roche Diagnostics, Indianapolis, IN, USA) using bromodeoxyuridine (BrdU) labeling and immunoenzymatic detection according to the manufacturer's protocol. Proliferation index was calculated as the ratio of the optical density (OD) derived from each MLR to the mean of the ODs derived from the control resting PBMC cultures of the two subjects that constituted the specific MLR. For cell proliferation experiments, 10 MLRs were performed in triplicates and the results refer to the mean of the three measurements.

Isolation of T-Cells from the MLRs

In MLRs, PBMCs were cultured in 12well plates for a 7-day period. The quantity of PBMCs, from each of the two individuals that contribute to the formation of an MLR couple, was as previously described. The IDO inhibitor 1-MT at a concentration of 100 μM was used. In MLRs performed with the aim to assess HIF-1α expression in T-cells, also the p53 inhibitor pifithrin-α (PFT) at a concentration of 30μM was used (Santa Cruz Biotechnology, Dallas, TX, USA). This concentration was selected according to previous experiments that showed efficacy without toxicity.[13, 24] Pifithrin-α was re-added to the cell cultures at day 4.

At the end of the 7-day period, non T-cells were indirectly magnetically labeled with a cocktail of biotin-conjugated monoclonal antibodies and were depleted using the Pan T-cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

Assessment of HIF-1*α*, p53 and c-Myc Expression and Certain of Their Transcriptional Targets in MLR-Derived T-Cells

The isolated from the MLRs T-cells were counted via optical microscopy on a Neubauer plaque and cell viability was determined by trypan blue assay (Sigma-Aldrich; Merck Millipore, Germany). Equal numbers of T-cells from each MLR were lysed using the T-PER tissue protein extraction reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich; Merck Millipore, Germany and Roche Diagnostics, USA). Protein was quantified via Bradford assay (Sigma-Aldrich; Merck Millipore, Germany).Equal quantities of protein extracts (10 μg) from each sample were loaded for electrophoresis in precast 4-12% gradient bis-tris polyacrylamide gels (Thermo Fisher Scientific, Inc., USA). Subsequently,

the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Thermo Fisher Scientific, Inc., USA). Blots were blocked in 5% w/v non-fat dry milk (Regilait, Saint Martin Belle Roche, France) diluted in 1X Tris-buffered saline (Thermo Fisher Scientific, Inc., USA) supplemented with 0.1% Tween-20 (Sigma-Aldrich; Merck Millipore, Germany).PVDF blots were incubated at 4^0C with the primary antibody for 16 hours, followed by the secondary antibody incubation (Anti-rabbit IgG, HRP-linked Antibody, dilution 1:1000, Cell Signaling Technology, Danvers, MA, USA) for 30 min at room temperature. In the case of reprobing PVDF blots, the previous primary and secondary antibody were removed by the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc., USA) according to the manufacturer's protocol. The PVDF blot was then reused and western blotting resumed as previously described, using a different primary antibody. Analysis of the westerns blots was performed using the Image J software (National Institute of Health, Bethesda, MD). Expression of the proteins of interest was normalized to the expression of the housekeeping protein β-actin.

The primary antibodies used in western blotting were specific for HIF-1α(Cell Signaling Technology; cat. no. 3716; dilution 1:500), p53 (Cell Signaling Technology; cat. no. 9282; dilution 1:500), c-Myc (Cell Signaling Technology; cat. no. 5605; dilution 1:500), p21 (Cell Signaling Technology; cat. no. 2947; dilution 1:500), activated cleaved caspase-3 (Cell Signaling Technology; cat. no. 9664; dilution 1:500), TP53-induced glycolysis and apoptosis regulator (TIGAR) (Santa Cruz Biotechnology; cat. no. sc-67273; dilution 1:500), pyruvate dehydrogenase (PDH) (Cell Signaling Technology; cat. no. 2784; dilution 1:1000;), PDH phosphorylated at serine 393 (p-PDH) (Biorbyt, San Francisco, CA, USA; cat. no. orb6670; dilution 1:100), hexokinase II (HKII) (Cell Signaling Technology; cat. no. 2867; dilution 1:1000), lactate dehydrogenase-A (LDH-A)(Cell Signaling Technology; cat. no. 2012; dilution 1:1000) and β-actin (Cell Signaling Technology; cat. no. 4967; dilution, 1:5000). For assessing protein expression in MLR-derived T-cells 10 MLRs were performed once.

Isolation of T-cells from PBMCs; Their Culture and Stimulation

T-cells were isolated from the PBMCs of 5 healthy volunteers using the Pan T-cell Isolation Kit (Miltenyi Biotec GmbH, Germany). Isolated T-cells were counted by optical microscopy on a Neubauer plaque. Cell viability was assessed by trypan blue assay (Sigma-Aldrich; Merck Millipore, Germany). T-cells were cultured for 72h without stimuli or stimulated with anti-CD2, anti-CD3 and anti-CD28 conjugated beads using the T-Cell activation/expansion kit (Miltenyi Biotec GmbH, Germany) in a bead to cell ratio of 1:2. Stimulated T-cells were also cultured in the presence or not of the GCN2 kinase activator tryptophanol (TRP) at a concentration of 0.25mM. This TRP concentration was selected according to previous experiments that showed efficacy without toxicity.[13, 22]

Assessment of the GCN2 Kinase Activator Tryptophanol Effect on Proliferation of Stimulated Isolated T-Cells

In T-cells cultured in 96-well plates ($1x10^5$/well), cell proliferation was assessed by Cell Proliferation ELISA (Roche Diagnostics, USA).Proliferation index was calculated as the ratio of the ODs derived from stimulated T-cells to the ODs derived from unstimulated T-cells. For these cell proliferation experiments, 5 separate experiments were performed in triplicates and the results refer to the mean of the three measurements.

Assessment of the GCN2 Kinase Activator Tryp tophanol Effect on HIF-1αLevel in Stimulated Isolated T-Cells

Protein was extracted from T-cells cultured in 12 well plates ($1x10^6$ cells/well) and Western blotting was performed as described above in order to assess HIF-1α level using a specific primary antibody (Cell Signaling Technology). For assessing HIF-1αexpression in T-cells, 5 separate experiments were performed once.

Statistical Analysis

The SPSS software (version 13; SPSS Inc, Chicago, IL, USA) was used for statistical analysis. The normality of the evaluated variables was assessed and confirmed by the one-sample Kolmogorov-Smirnov test. For comparison of means paired-sample t-test or one-way repeated-measures ANOVA were used, and the results were expressed as mean±SD. A $p<0.05$ was considered statistically significant.

For the western blotting, results were expressed as optical densities (OD), so *p* values were calculated by comparing the means of OD. Statistical analysis

relative to the control OD values was avoided to preventing violation of the prerequisite for normal distribution of the compared variables when applying parametric statistical tests. Results were depicted according to the ODs. However, for the reader's convenience, in the text, the results were expressed after normalization of means for the control group.

RESULTS

IDO increases L-tryptophan Degradation in MLRs

In MLRs, the IDO inhibitor 1-MT decreased significantly the degradation of L-tryptophan. Specifically, in untreated MLRs L-tryptophan concentration was 2.62±0.53 μg/mL, whereas in the presence of 1-MT was 6.24±0.73 μg/mL (p<0.001). L-tryptophan concentration in the culture medium with 10% FBS was measured to be 7.20 μg/mL. L-tryptophan consumption was calculating by subtracting its concentrations in the supernatants from the experiments from the concentration of 7.20 μg/mL (Figure 1).This effect of the IDO inhibitor indicates that IDO increases L-tryptophan degradation in MLRs.

IDO Suppresses Cell Proliferation in MLRs

Treatment of MLRs with the IDO inhibitor 1-MT increased proliferation index from 2.06±0.16 to 3.33±0.53 (p<0.001) (Figure 2A). This effect of the IDO inhibitor indicates that IDO suppresses alloreactive T-cell proliferation.

The Effect of IDO on the Level of the Transcription Factors HIF-1α, p53 and c-Myc in MLR-derived T-Cells

Treatment of MLRs with the IDO inhibitor 1-MT decreased HIF-1α level to 0.35±0.11 of its level in untreated MLRs (p<0.001) (Figures 2B & C). Thus, IDO increases HIF-1α expression in alloreactive T-cells. Treatment of MLRs with the p53 inhibitor PFT decreased HIF-1α expression to 0.26±0.24 of its level in untreated MLRs (p=0.001) (Figures 2B & C).

Consequently, p53 increases HIF-1α expression in alloreactive T-cells.

Treatment of MLRs with the IDO inhibitor 1-MT decreased p53 level to 0.67±0.05of its level in untreated MLRs (p<0.001) (Figures2B & D). Thus, IDO increases p53 expression in alloreactive T-cells.

Treatment of MLRs with the IDO inhibitor 1-MT increased c-Myc level by a factor of 1.54±0.39 (p<0.001) (Figures2B & E). Hence, IDO decreases c-Myc expression in alloreactive T-cells.

The Effect of IDO on the Levels of Certain Transcriptional Target Proteins of HIF-1α, p53 and c-Myc in MLR-Derived T-cells

Treatment of MLRs with 1-MT decreased p21 expression in MLR-derived T-cells to 0.53±0.11 of its level in untreated MLRs (p<0.001) (Figures 3A & B). Also, 1-MT decreased the expression of activated cleaved caspase-3 to 0.62±0.14 of its level in untreated MLRs (p<0.001) (Figures 3A & C). In addition, 1-MT down-regulated the expression of TIGAR to 0.48±0.10 of its level in untreated MLRs (p<0.001) (Figures 3A & D). Consequently, IDO up-regulates p21 and TIGAR, which are transcriptional targets of p53. Also, it activates caspase-3, the final caspase of the apoptotic pathways.

Treatment of MLRs with the IDO inhibitor 1-MT increased HKII level in MLR-derived T-cells by a factor of 1.89±0.23 (p<0.001) (Figures 3A & E). Also, 1-MT increased the expression of LDH-A by a factor of 1.69±0.38 (p<0.001) (Figures 3A & F). Thus, IDO down-regulates HKII and LDH-A levels in alloreactive T-cells, which are transcriptional targets of both c-Myc and HIF-1α. 1-MT did not affect the levels of PDH and

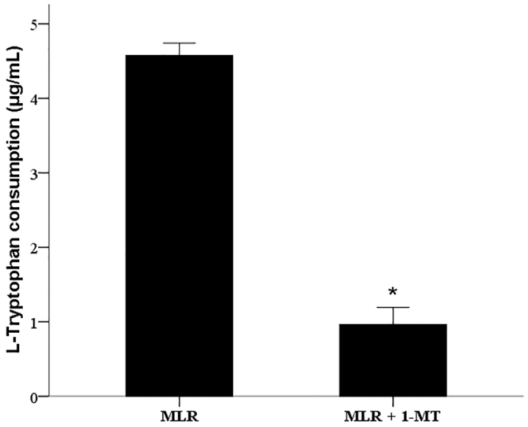

Figure 1. The effect of indoleamine 2, 3-dioxygenase on L-tryptophan degradation in mixed lymphocyte reactions
Mixed lymphocyte reactions (MLRs) were performed in the presence or lack of the indoleamine 2,3-dioxygenase (IDO) inhibitor1-DL-methyl-tryptophan (1-MT). L-tryptophan consumption decreased in the presence of the inhibitor indicating that IDO increases L-tryptophan degradation. Ten separate MLRs were performed once. Asterisk corresponds to p<0.001 compared to the MLR control group. Error bars correspond to 2SD.

p-PDH since their expression altered only by a factor 1.07±0.08 (p=0.06) and 1.07±0.07 (p=0.063) respectively (Figures 3A & G and 3A & H, respectively). Phosphorylation of PDH is catalyzed by PDK, which is a HIF-1α transcriptional target.

Cell Proliferation and HIF-1α Level in Isolated from PBMCs T-Cells and the Effect of TRP

Activation of T-cells with anti-CD2, anti-CD3 and anti-CD28 coated beads resulted in a proliferation index of 5.41±0.42. Concurrent treatment with the GCN2 kinase activator TRP decreased the proliferation index to 2.23±0.44 (p<0.01) (Figure 4A).

Compared to resting T-cells, the level of HIF-1α increased significantly in activated T-cells by a factor of 1.71±0.36 (p<0.01) (Figures 4B & C). Concurrent treatment of activated T-cells with TRP increased HIF-1α expression further. Compared to resting T-cells, the level of HIF-1α in TRP-treated activated T-cells increased by a factor of 3.97±3.63 (p<0.01) (Figures 4B & C).

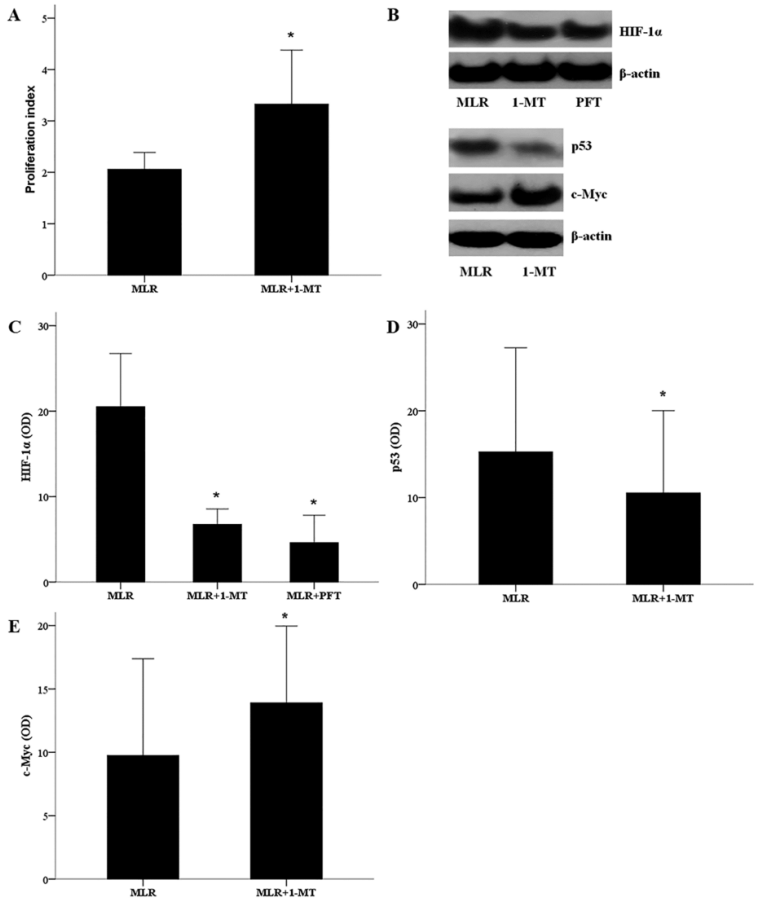

Figure 2. The effect of indoleamine 2,3-dioxygenaseon cell proliferation and the levels of the transcription factors hypoxia-inducible factor-1α, p53, and c-Myc in alloreactive T-cells

Mixed lymphocyte reactions (MLRs) were performed in the presence or lack of the indoleamine 2,3-dioxygenase(IDO) inhibitor1-DL-methyl-tryptophan (1-MT). In MLRs, the IDO inhibitor 1-MT increased cell proliferation (A). Ten separate cell proliferation experiments were performed in triplicates. MLRs were performed in the presence or not of the IDO inhibitor 1-MT and in some cases of the p53 inhibitor pifithrin-α (PFT). Then T-cells were isolated in order to evaluate the levels of hypoxia-inducible factor-1α (HIF-1α), p53 and c-Myc. A representative experiment is depicted in panel B. In T-cells derived from the MLRs treated with 1-MT, HIF-1α (C) and p53 (D) levels were reduced, while c-Myc level was increased (E). The p53 inhibitor PFT also decreased HIF-1α level (C). For assessing the expression of these transcription factors 10 separate MLRs were performed once. Asterisk corresponds to $p \leq 0.001$ compared to the MLR control group. Error bars correspond to 2SD.

Published by Tehran University of Medical Sciences (http://ijaai.tums.ac.ir)

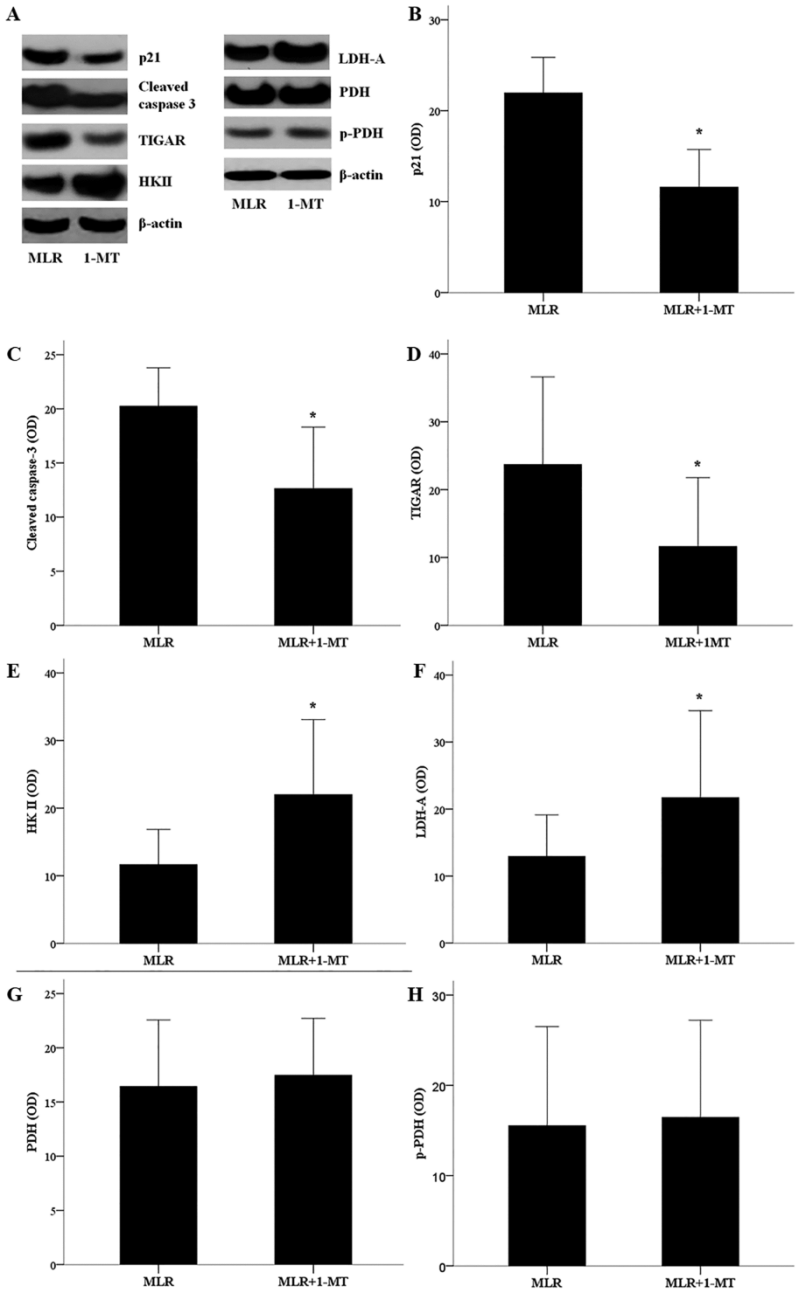

Figure 3. The effect of indoleamine 2, 3-dioxygenaseon the levels of certain targets of the evaluated transcription factors in alloreactive T-cells

Mixed lymphocyte reactions (MLRs) were performed in the presence or lack of the indoleamine 2, 3-dioxygenase (IDO) inhibitor1-DL-methyl-tryptophan (1-MT). Then T-cells were isolated in order to evaluate the levels of certain proteins that are transcriptional targets of hypoxia-inducible factor-1α (HIF-1α), p53 and/or c-Myc. A representative experiment is depicted in panel A. In T-cells derived from the MLRs treated with 1-MT, the levels of p21 (B), activated cleaved caspase-3 (C) and TP53-induced glycolysis and apoptosis regulator (TIGAR) (D) were decreased. Treatment of the MLRs with 1-MT increased the levels of hexokinase II (HKII) (E) and lactate dehydrogenase-A (LDH-A) (F) in alloreactive T-cells. 1-MT did not affect the levels of pyruvate dehydrogenase (PDH) (G) and PDH phosphorylated at serine 393 (p-PDH) (H) in alloreactive T-cells. For assessing the expression of these proteins 10 separate MLRs were performed once. Asterisk corresponds to $p<0.001$ compared to the MLR control group. Error bars correspond to 2SD.

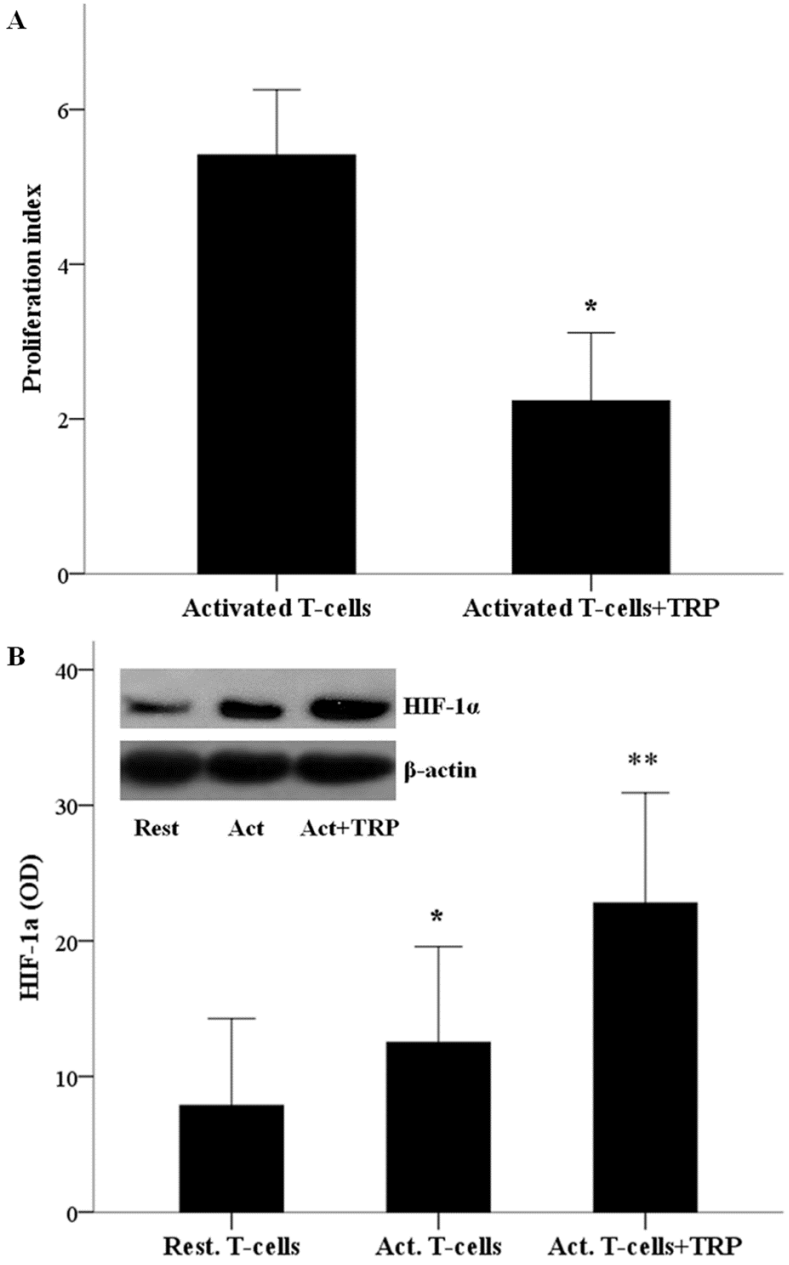

Figure 4. The effect of general control nonderepressible-2kinase activation on T-cell proliferation and on the level of hypoxia-inducible factor-1α

T-cells were isolated from peripheral blood mononuclear cells of 5 individuals and activated with anti-CD2, anti-CD3 and anti-CD28 coated beads in the presence or not of the general control nonderepressible-2 (GCN2) kinase activator tryptophanol (TRP). Stimulation of T-cells increased cell proliferation, whereas in the presence of TRP cell proliferation was significantly attenuated (A). Five separate cell proliferation experiments were performed in triplicates. Asterisk corresponds to $p \leq 0.001$ compared to the activated T-cells group. Error bars correspond to 2SD. Expression of hypoxia-inducible factor-1α (HIF-1α) was evaluated by western blotting as it is depicted in the lane which corresponds to one representative experiment (B). HIF-1α increased in activated T-cells and increased further in case of concurrent treatment with TRP (B). For assessing HIF-1α expression in T-cells 5 separate experiments were performed once. Asterisk corresponds to $p<0.01$ compared to the resting T-cells group and double asterisks to $p<0.01$ compared to activated T-cells group. Error bars correspond to 2SD.

DISCUSSION

Indoleamine 2, 3-dioxygenase is a key immunomodulatory enzyme, which through L-tryptophan depletion and/or kynurenine pathway products suppresses T-cell proliferation and differentiation to effector cells.[1,2]. It exerts its effect at least in part by affecting T-cell metabolism through an increase in p53 or a decrease c-Myc level.[7,13,14] Another transcription factor that is up-regulated upon T-cell activation and is known to affect cell metabolism is HIF-1. Upon T-cell activation, the HIF-1αcomponent of HIF-1 is up-regulated even under normoxia.[18-20] In this study, the effect of IDO on HIF-1α level and function in T-cells was evaluated.

The MLR, a model T-cell alloreactivity in which APCs are present, was used. For evaluating the effect of IDO, its inhibitor 1-MT was used. Indeed, 1-MT decreased L-tryptophan consumption confirming its activity as an IDO inhibitor in our experimental model. As expected, 1-MT increased cell proliferation in MLRs. Like in previous studies,[13,14] in T-cells isolated from the MLRs, 1-MT decreased p53 and increased c-Myc. Surprisingly, 1-MT decreased HIF-1α level, which means that despite the known HIF-1α upregulation upon T-cell activation,[18-20] IDO, an immunosuppressive enzyme,[1,2] up-regulates HIF-1α.

Because IDO up-regulates p53, through GCN2 kinase activation,[13] and the interactions between this transcription factor and HIF-1α are well documented,[25] MLRs were performed in the presence or not of the p53 inhibitor PFT. In T-cells isolated from the MLRs, inhibition of p53 decreased HIF-1αlevel. This indicates that IDO-induced HIF-1α upregulation may be the result of the IDO-mediated increase of p53. p53 can affect HIF-1α level in many ways. For instance, p53 can up-regulate HIF-1αthrough downregulation of pVHL resulting in decreased HIF-1αubiquitination and proteasomal degradation,[26] or through upregulation of murine double minute 2 (MDM2),[15] which by a direct interaction increases HIF-1α.[27] Another possible explanation for the increase of HIF-1αby IDO may reside in the metabolic effects of this enzyme. IDO-induced p53 upregulation results to decrease glucose influx into the T-cells, as well as to deceleration of glycolysis leading to less pyruvate production for entry into the Krebs's cycle.[13] In parallel IDO reduces glutaminolysis, through downregulation of c-Myc, preventing this anaplerotic for the Krebs's cycle pathway.[14] Hence, by reducing the Krebs's cycle supply with new substrates, IDO may reduce the level of the intermediate α-ketoglutarate, which is required for the hydroxylation of HIF-1α and its proteasomal degradation,[17] resulting in HIF-1α accumulation.

As already noted, IDO may exert its immunosuppressive effect through L-tryptophan depletion and GCN2 kinase activation or through kynurenine pathway products. GCN2 kinase activation alone has been shown to be adequate for the IDO-induced upregulation of p53.[13] In order to elucidate whether GCN2 activation alone is capable of increasing HIF-1α level, isolated T-cells were stimulated in the presence or not of the GCN2 kinase activator TRP. This system lacks APCs and consequently large amounts of IDO and can be considered as kynurenine free. As expected, T-cell activation induced T-cell proliferation, which was accompanied by increased HIF-1α level. Concurrent treatment of activated T-cells with TRP abrogated cell proliferation but increased HIF-1α expression further. Thus, GCN2 kinase activation by IDO-induced L-tryptophan depletion seems to be an adequate insult for increasing HIF-1α level.

Certainly, the reason for the IDO-induced upregulation of HIF-1αis interesting and beyond GCN2 kinase activation and p53 upregulation, the detailed mechanisms remain to be elucidated. However, the fact that an immunosuppressive enzyme increases a transcription factor that is known to be up-regulated upon T-cell activation and to drive T-cell differentiation toward the effector Th17 instead of the Treg lineage is intriguing.[1,2,18-20] Also it is known that HIF-1α induces glycolysis,[20] whereas IDO favors the opposite.[7,13,14] Since experiments with proteasome inhibitors have shown that accumulated HIF-1α is not always active,[28] we evaluated whether the increased due to IDO HIF-1α is functional. For this purpose, the expression of certain targets of the transcription factors p53, c-Myc and HIF-1α, which are affected by IDO, was evaluated in MLR-derived T-cells.

The p53 target p21 is up-regulated by IDO and may contribute to decreased T-cell proliferation by inducing G1 phase cell cycle arrest.[15] Also activated cleaved caspase-3, in which the apoptotic pathways converge, increased by IDO. The apoptotic effect of IDO maybe mediated by p53, which is a known pro-apoptotic factor.[15] Also, IDO increased TIGAR, which decelerates glycolysis and is a p53 target.[29] Thus, the

upregulated by IDO p53 is transcriptionally active.

HKII and LDH-A are the first and the last enzymes of glycolysis respectively. Both were decreased by IDO and are transcriptional targets of c-Myc and HIF-1α.[9,14,30,31]As IDO down-regulates c-Myc, but up-regulates HIF-1α, it is likely that the IDO-induced decrease in HKII and LDH-A levels found in this study are mediated by the decreased c-Myc, and that HIF-1α does not exert its transcriptional activity. Another HIF-1α target is the PDK.[32] PDK phosphorylates and inactivates the PDH, which enters pyruvate into the Krebs's cycle by converting it to acetyl-CoA.[32] The fact that IDO did not affect PDH and p-PDH expression, while it increased HIF-1α, also supports that HIF-1α is transcriptionally inactive. A possible mechanism may reside in the p53/HIF-1α interaction. Experiments in other cell types have shown that HIF-1α transcriptional activity is attenuated when p53 is accumulated due to a competition between p53 and HIF-1α for limited amounts of the transcriptional co-activator p300.[33,34] Production of kynurenine due to IDO-induced L-tryptophan degradation may also be responsible. Kynurenine binds and activates the cytosolic transcription factor aryl hydrocarbon receptor (AhR) leading to its translocation into the nucleus and dimerization with the HIF-1βcomponent of HIF-1. Such a competition between AhR and HIF-1α for HIF-1β has been confirmed in various cell lines using AhR ligands and hypoxia or hypoxia mimetics.[35, 36]This antagonism between AhR and HIF-1α for HIF-1β may reduce HIF-1 activity and it would be interesting to be studied in the context of the known kynurenine-mediated immunosuppression.[37]

Certainly, further studies are required in order to reveal in more detail the molecular mechanisms that govern the effect of IDO on HIF-1α expression and function. Also, the study of this phenomenon on the different T-cell subsets and its consequences would add to our knowledge about the immunomodulatory properties of IDO.

In conclusion, the results of our study support that in human alloreactive T-cells, IDO up-regulates HIF-1α, by inducing p53 overexpression. However, HIF-1α remains transcriptionally inactive.

REFERENCES

1. King NJ, Thomas SR. Molecules in focus: Indoleamine 2, 3-dioxygenase. Int J Biochem Cell Biol 2007;39(12):2167-72.
2. Curti A, Trabanelli S, Salvestrini V, Baccarani M, Lemoli RM. The role of indoleamine 2,3-dioxygenase in the induction of immune tolerance: Focus on hematology. Blood 2009;113(11):2394-401.
3. Eleftheriadis T, Liakopoulos V, Antoniadi G, Stefanidis I, Galaktidou G. Indoleamine 2,3-dioxygenase is increased in hemodialysis patients and affects immune response to hepatitis b vaccination. Vaccine 2011;29(12):2242-7.
4. Eleftheriadis T, Yiannaki E, Antoniadi G, Liakopoulos V, Pissas G, Galaktidou G, et al. Plasma indoleamine 2,3-dioxygenase and arginase type i may contribute to decreased blood t-cell count in hemodialysis patients. Ren Fail 2012;34(9):1118-22
5. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, et al. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science. 1998;281:1191-3.
6. Munn DH, Mellor AL. Indoleamine 2,3-dioxygenase and tumor-induced tolerance. J Clin Invest 2007;117(5):1147-54.
7. Eleftheriadis T, Pissas G, Yiannaki E, Markala D, Arampatzis S, Antoniadi G, et al. Inhibition of indoleamine 2,3-dioxygenase in mixed lymphocyte reaction affects glucose influx and enzymes involved in aerobic glycolysis and glutaminolysis in alloreactive t-cells. Hum Immunol 2013;74(12):1501-9.
8. Eleftheriadis T, Pissas G, Antoniadi G, Liakopoulos V, Stefanidis I. Indoleamine 2,3-dioxygenase depletes tryptophan, activates general control nonderepressible 2 kinase and downregulates key enzymes involved in fatty acid synthesis in primary human cd4+ t-cells. Immunology 2015;146:292-300.
9. Wang R, Dillon CP, Shi LZ, Milasta S, Carter R, Finkelstein D, et al. The transcription factor myc controls metabolic reprogramming upon t lymphocyte activation. Immunity 2011;35(6):871-82.
10. Berod L, Friedrich C, Nandan A, Freitag J, Hagemann S, Harmrolfs K, et al. De novo fatty acid synthesis controls the fate between regulatory t and t helper 17 cells. Nat Med 2014;20(11):1327-33.
11. Eleftheriadis T, Pissas G, Sounidaki M, Tsogka K, et al. Indoleamine 2,3-dioxygenase, by degrading l-tryptophan, enhances carnitine palmitoyltransferase i activity and fatty acid oxidation, and exerts fatty acid-dependent effects in human alloreactive cd4+ t-cells. Int J Mol Med2016;38(5):1605-13.
12. Michalek RD, Gerriets VA, Jacobs SR, Macintyre AN,

MacIver NJ, Mason EF, et al. Cutting edge: Distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory cd4+ t cell subsets. J Immunol 2011;186(6):3299-303.
13. Eleftheriadis T, Pissas G, Antoniadi G, Spanoulis A, et al. Indoleamine 2,3-dioxygenase increases p53 levels in alloreactive human t cells, and both indoleamine 2,3-dioxygenase and p53 suppress glucose uptake, glycolysis and proliferation. Int Immunol 2014;26(12):673-84.
14. Eleftheriadis T, Pissas G, Antoniadi G, Tsogka K, Sounidaki M, Liakopoulos V, et al. Indoleamine 2,3- dioxygenase downregulates t cell receptor complex chain and cmyc, and reduces proliferation, lactate dehydrogenase levels and mitochondrial glutaminase in human t cells. Mol Med Rep2016;13(1):925-32.
15. Brady CA, Attardi LD. P53 at a glance. J Cell Sci. 2010;123:2527-32.
16. Miller DM, Thomas SD, Islam A, Muench D, Sedoris K. C-myc and cancer metabolism. Clin Cancer Res 2012;18:5546-53.
17. Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, et al. Targeting of hif-alpha to the von hippel-lindau ubiquitylation complex by o2-regulated prolyl hydroxylation. Science 2001;292(5516):468-72.
18. Pan F, Barbi J, Pardoll DM. Hypoxia-inducible factor 1: A link between metabolism and t cell differentiation and a potential therapeutic target. Oncoimmunology 2012;1(4):510-5.
19. Dang EV, Barbi J, Yang HY, Jinasena D, Yu H, Zheng Y, et al. Control of t(h)17/t(reg) balance by hypoxia-inducible factor 1. Cell 2011;146(5):772-84.
20. Shi LZ, Wang R, Huang G, Vogel P, Neale G, Green DR, et al. Hif1alpha-dependent glycolytic pathway orchestrates a metabolic checkpoint for the differentiation of th17 and treg cells. J Exp Med 2011;208(7):1367-76.
21. Clambey ET, McNamee EN, Westrich JA, Glover LE, Campbell EL, Jedlicka P, et al. Hypoxia-inducible factor-1 alpha-dependent induction of foxp3 drives regulatory t-cell abundance and function during inflammatory hypoxia of the mucosa. Proc Natl Acad Sci U S A 2012;109(41):E2784-93.
22. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D, et al. Gcn2 kinase in t cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity 2005;22(5):633-42.
23. Eleftheriadis T, Pissas G, Antoniadi G, Liakopoulos V, Stefanidis I. Indoleamine 2,3-dioxygenase depletes tryptophan, activates general control non-derepressible 2 kinase and down-regulates key enzymes involved in fatty acid synthesis in primary human cd4+ t cells. Immunology 2015;146(2):292-300.
24. Komarov PG, Komarova EA, Kondratov RV, Christov-Tselkov K, Coon JS, Chernov MV, et al. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. Science 1999; 285(5434):1733-7.
25. Sermeus A, Michiels C. Reciprocal influence of the p53 and the hypoxic pathways. Cell Death Dis 2011;2:e164.
26. Sutton TA, Wilkinson J, Mang HE, Knipe NL, Plotkin Z, Hosein M, et al. P53 regulates renal expression of hif-1{alpha} and pvhl under physiological conditions and after ischemia-reperfusion injury. Am J Physiol Renal Physiol 2008;295(6):F1666-77.
27. Nieminen AL, Qanungo S, Schneider EA, Jiang BH, Agani FH. Mdm2 and hif-1alpha interaction in tumor cells during hypoxia. J Cell Physiol 2005;204(2):364-9.
28. Kaluz S, Kaluzova M, Stanbridge EJ. Does inhibition of degradation of hypoxia-inducible factor (hif) alpha always lead to activation of hif? Lessons learnt from the effect of proteasomal inhibition on hif activity. J Cell Biochem 2008;104(2):536-544.
29. Bensaad K, Tsuruta A, Selak MA, Vidal MN, Nakano K, Bartrons R, et al. Tigar, a p53-inducible regulator of glycolysis and apoptosis. Cell 2006;126(1):107-20.
30. Iyer NV, Kotch LE, Agani F, Leung SW, Laughner E, Wenger RH, et al. Cellular and developmental control of o2 homeostasis by hypoxia-inducible factor 1 alpha. Genes Dev 1998;12(2):149-62.
31. Valera A, Pujol A, Gregori X, Riu E, Visa J, Bosch F. Evidence from transgenic mice that myc regulates hepatic glycolysis. Faseb J 1995;9(11):1067-78.
32. Kim JW, Tchernyshyov I, Semenza GL, Dang CV. Hif-1-mediated expression of pyruvate dehydrogenase kinase: A metabolic switch required for cellular adaptation to hypoxia. Cell Metab 2006;3(3):177-85.
33. Schmid T, Zhou J, Kohl R, Brune B. P300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (hif-1). Biochem J 2004;380(pt 1):289-95.
34. Datta K, Li J, Bhattacharya R, Gasparian L, Wang E, Mukhopadhyay D. Protein kinase c zeta transactivates hypoxia-inducible factor alpha by promoting its association with p300 in renal cancer. Cancer Res 2004;64(2):456-62.
35. Vorrink SU, Domann FE. Regulatory crosstalk and interference between the xenobiotic and hypoxia sensing pathways at the ahr-arnt-hif1alpha signaling node. Chem Biol Interact 2014;218:82-8.
36. Eleftheriadis T, Pissas G, Antoniadi G, Liakopoulos V, Stefanidis I. Kynurenine, by activating aryl hydrocarbon

receptor, decreases erythropoietin and increases hepcidin production in hepg2 cells: A new mechanism for anemia of inflammation. Exp Hematol 2016;44(1):60-67 e61.

37. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ, Bradfield CA. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory t cells. J Immunol 2010;185(6):3190-8.