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

THEODOROS ELEFTHERIADIS\(^1\), GEORGIOS PISSAS\(^1\), MARIA SOUNIDAKI\(^1\), KONSTANTINA TSOGKA\(^1\), NIKOLAOS ANTONIADIS\(^2\), GEORGIA ANTONIADI\(^1\), VASSILIOS LIAKOPoulos\(^1\) and IOANNIS STEFANIDIS\(^1\)

\(^1\)Department of Nephrology, Medical School, University of Thessaly, Larissa; 
\(^2\)Organ Transplant Unit, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece

Received May 14, 2016; Accepted September 9, 2016

DOI: 10.3892/ijmm.2016.2750

Abstract. Indoleamine 2,3-dioxygenase (IDO) is expressed in antigen-presenting cells and by degrading L-tryptophan along the kynurenine pathway suppresses CD4\(^+\) T-cell proliferation, induces apoptosis and promotes differentiation towards a regulatory as opposed to an effector phenotype. Recent findings revealed that the above effects may be mediated through alterations in T-cell metabolism. In this study, the effect of IDO on fatty acid \(\beta\)-oxidation in CD4\(^+\) T-cells was evaluated in human mixed lymphocyte reactions (MLRs) using the IDO inhibitor, 1-DL-methyl-tryptophan. Protein analysis of CD4\(^+\) T-cells isolated from the MLR showed that L-tryptophan degradation acts by activating the general control non-derepressible 2 kinase and aryl-hydrocarbon receptor in T-cells. In the absence of IDO inhibition, fatty acid oxidation increased along with increased activity of carnitine palmitoyltransferase I (CPT1), the latter due to the increased expression of CPT1 isoenzymes and alterations in acetyl-CoA carboxylase 2, the enzyme that controls CPT1 activity. Increased fatty acid oxidation due to the action of IDO was accompanied by an increased expression of forkhead box P3 (FoxP3) and a decreased expression of the kynurenine pathway suppresses CD4\(^+\) T-cells by affecting the expression and differentiation of CD4\(^+\) T-cells. (20,21). While many intermediate events remain unexplained, the presence of IDO in APCs leads to decreased proliferation and increased apoptosis, and promotes the differentiation of CD4\(^+\) T-cells towards a regulatory instead of effector (Teff) phenotype (20,21). While many intermediate events remain to be elucidated, recent research indicates that IDO may exert these effects by affecting the metabolism of CD4\(^+\) T-cells. Indeed, IDO suppresses aerobic glycolysis and glutaminolysis in human alloreactive CD4\(^+\) T-cells by affecting the expression of glucose transporter 1 and various glycolytic and glutamino-lytic enzymes (14-16). It also downregulates key enzymes involved in fatty acid synthesis (17). The above-mentioned
metabolic pathways are prerequisites for rapid T-cell proliferation following T-cell receptor stimulation, as well as for differentiation towards effector cell lineages instead of Tregs. Following T-cell activation, T-cells reprogram their metabolic pathways from pyruvate via the Krebs cycle to the glycolytic and glutaminolytic pathways in order to fulfill the bioenergetic and biosynthetic demands of proliferation (22-24). In parallel, fatty acid synthesis is upregulated during the activation of CD4+ T-cells enhancing their proliferation and promoting their differentiation into T helper 17 cells (Th17) instead of Tregs (25).

Another metabolic pathway that plays a significant role in the CD4+ T-cell response and differentiation is fatty acid β-oxidation. More precisely, Tregs are dependent on fatty acid oxidation for its differentiation, whereas, Teff populations on aerobic glycolysis (26). The effect of IDO on fatty acid oxidation in CD4+ T-cells remains to be investigated, and constitutes the aim of the present study.

For the purposes of the present study, two-way mixed lymphocyte reaction (MLR) was used as a model of allogreactivity (27), along with the specific IDO inhibitor, 1-DL-methyl-tryptophan (1MT) (4,7). In order to evaluate fatty acid oxidation, cells were cultured in a medium containing oleate. The effect of IDO on carnitine palmitoyltransferase I (CPT1), the tightly regulated enzyme that controls the entry of fatty acids into the mitochondria for oxidation (28,29), was assessed as well. Treatment of CD4+ T-cells with the CPT1 inhibitor, etomoxir, has been shown to abrogate differentiation into Tregs (26). The effects of IDO on the end-points of CD4+ T-cell function, proliferation, apoptosis and differentiation were also evaluated.

Materials and methods

Subjects. Blood samples were collected from 5 healthy volunteers (3 males and 2 females, 37±7 years of age). Informed consent was obtained from each individual enrolled in the study and the Ethics Committee of the University Hospital of Larissa (Larissa, Greece) approved the study protocol.

Cell culture conditions. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by the Ficoll-Hypaque density gradient centrifugation (Histopaque 1077; Sigma-Aldrich, St. Louis, MO, USA) and counted using an optical microscope (Axiovert 40 C; Carl Zeiss AG, Oberkochen, Germany) on a Neubauer plaque. Cell viability was assessed by trypan blue assay (Sigma-Aldrich). The above concentration was chosen according to the manufacturer's protocol. The PVDF blot was performed using the ImageJ software (National Institute of Health, Bethesda, MD, USA).

Cell proliferation in two-way mixed lymphocyte reactions. Cell proliferation enzyme-linked immunosorbent assay (ELISA) (Roche Diagnostics, Indianapolis, IN, USA), based on bromodeoxyuridine (Brdu) labeling and immunoenzymatic detection, was used to examine cell proliferation. The 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. These experiments were performed in triplicate and the results refer to the mean of the three measurements.

L-tryptophan and oleate consumption in MLRs. L-tryptophan consumption was assessed by measuring its concentration in the supernatants of MLRs by means of ELISA (BlueGene Biotech, Shanghai, China). The sensitivity of the above ELISA kit is 1 ng/ml.

Similarly, oleate consumption was assessed by measuring its concentration in the supernatants of MLRs colorimetrically using the Free Fatty Acid Quantification kit (Abcam, Cambridge, UK). The detection limit of the above kit was 2 µM.

Expression of certain proteins in CD4+ T-cells isolated from the MLRs. The expression of certain proteins in CD4+ T-cells was assessed by western blot analysis. Isolated CD4+ T-cells were counted via optical microscopy on a Neubauer plaque and cell viability was determined by trypan blue assay (Sigma-Aldrich). Equal numbers of T-cells from each MLR were lysed using the T-PER tissue protein extraction reagent (Thermo Fisher Scientific Inc., Rockford, IL, USA) supplemented with protease and phosphatase inhibitors (Sigma-Aldrich and Roche Diagnostics). Protein was quantified using the Bradford assay (Sigma-Aldrich) and 10 µg from each sample were used for western blot analysis. The blots were incubated with the primary antibodies for 16 h, followed by the secondary antibody (anti-rabbit IgG, HRP-linked antibody; Cell Signaling Technology, Danvers, MA, USA) incubation for 30 min. In case of reprobing PVDF blots, the previous primary and secondary antibodies were removed using the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The PVDF blot was then reused and western blot analysis resumed as previously described, using a different primary antibody. Analysis of the western blots was performed using the ImageJ software (National Institute of Health, Bethesda, MD, USA).

The primary antibodies used in western blot analysis were specific for eIF2α phosphorylated at serine 51 (p-eIF2α;
IDO increases fatty acid oxidation in MLRs and CPTI enzymatic activity in MLR-derived CD4+ T-cells. In MLRs, IDO increased fatty acid oxidation since its inhibitor 1MT increased the oleate concentration in the supernatants from 0.51±0.05 mM to 0.82±0.03 mM (p<0.001, paired t-test) (Fig. 2A).

In CD4+ T-cells derived from 1MT-treated MLRs CPT1 activity was at the 56.61±7.32% of the activity found in cells derived from the control MLRs (p<0.001, paired t-test), indicating that IDO enhanced CPT1 enzymatic activity in CD4+ T-cells (Fig. 2B).

IDO increases CPT1A, CPT1B and CPT1C expression in MLR-derived CD4+ T-cells. Unblocked IDO activity in MLRs increased CPT1A expression in MLR-derived CD4+ T-cells since the treatment of MLRs with the IDO inhibitor, 1MT, altered the CPT1A level by a factor of 0.74±0.05 (p<0.001, paired t-test) (Fig. 3A and B). This was even more profound in the case of CPT1B, which was altered due to 1MT by a factor of 0.57±0.13 (p<0.001, paired t-test) (Fig. 3A and C), and of CPT1C, which was altered by a factor of 0.44±0.13 (p<0.001, paired t-test) (Fig. 3A and D).

IDO decreases ACC2 expression, whereas it increases the level of phosphorylated ACC2 in MLR-derived CD4+ T-cells. IDO activity in the MLRs decreased the total ACC2 expression in MLR-derived CD4+ T-cells since the treatment of MLRs with the IDO inhibitor, 1MT, led to alterations in the levels of ACC2 by a factor of 1.24±0.18 (p=0.001, paired t-test) (Fig. 4A and B). The effect of IDO on the level of p-ACC2 was more profound since in the CD4+ T-cells derived from the 1MT-treated MLRs, the level of p-ACC2 was altered by a factor of 0.37±0.18 (p<0.001, paired t-test) (Fig. 4A and C). Thus, IDO, by degrading L-tryptophan in the MLRs, increased the content of the inactivated phosphorylated form of ACC2 in CD4+ T-cells.

IDO does not affect cell proliferation in MLRs nor activated caspase-3 in MLR-derived CD4+ T-cells. Using culture media containing oleate, IDO did not affect cell proliferation in MLRs, since the addition of 1MT did not affect the proliferation index significantly. More precisely, the proliferation index was 4.87±0.27 in the untreated MLRs and 4.75±0.34 in the 1MT-treated MLRs (p=0.313, paired t-test) (Fig. 5A).

Similarly, in the presence of oleate, IDO did not affect the content of activated caspase-3 in CD4+ T-cells, which is the terminal caspase of the apoptotic pathways. Compared to the activated caspase-3 level in CD4+ T-cells derived from the control MLRs, its level did show a negligible variation only by a factor of 0.98±0.11 in CD4+ T-cells derived from 1MT-treated MLRs (p=0.523, paired t-test) (Fig. 5B and C).
**IDO, particularly in the presence of oleate, induces FoxP3 expression, but suppresses RORγt expression in MLR-derived CD4+ T-cells.** Unblocked IDO activity in MLRs increased FoxP3 expression in MLR-derived CD4+ T-cells as the treatment of MLRs with the IDO inhibitor, 1MT, altered the FoxP3 level by a factor of 0.33±0.21 (p<0.001, paired t-test) (Fig. 6A and B).

The opposite was observed with the expression of RORγt. Treatment of the MLRs with the IDO inhibitor, 1MT, induced a significant increase in RORγt levels by a factor of 2.24±0.41 (p<0.001, paired t-test) (Fig. 6A and C). Thus, by degrading L-tryptophan, IDO decreased RORγt expression in CD4+ T-cells.

In the absence of oleate, treatment of the MLRs with 1MT also resulted in a decrease in FoxP3 expression in CD4+ T-cells.
by a factor of 0.59±0.11 (p<0.001) (Fig. 6D and E). However, this decrease was significantly less than that observed in MLRs in the presence of oleate (p=0.003, unpaired t-test) suggesting that oleate is beneficial for FoxP3 expression.
In the absence of oleate from the MLRs, 1MT treatment also resulted in an increase in RORγt expression in CD4+ T-cells by a factor of 2.11±1.28 (p<0.001) (Fig. 6D and F). This increase did not differ from the increase observed in MLRs performed in the presence of oleate (p=0.772, unpaired t-test).

Discussion

Indoleamine 2,3-dioxygenase is expressed in APCs and by degrading L-tryptophan in the microenvironment where the immune response occurs, it suppresses CD4+ T-cell function by inhibiting cell proliferation, inducing apoptosis and promoting differentiation into Tregs (20,21).

In order to define which of the described pathways are involved in the effect of IDO on CD4+ T cells (12-14,17-19), a model of alloreactivity, the MLR, was used. In this model, IDO induced L-tryptophan degradation. Decreased L-tryptophan activated the GCN2K pathway since the phosphorylation of its substrate eIF2α was increased when IDO activity was not blocked by 1MT. This observation is in accordance with previous studies (12-14). Additionally, the present study recapitulates the results of other studies that failed to detect an effect on the other amino-acid sensing system, the mTORC1, since the level of phosphorylation of its substrate, p70S6K, remained unaffected by 1MT (12-14). This is in accordance with findings showing that mTORC1...
is sensitive to the depletion of certain amino acids; and more precisely of leucine, isoleucine, valine and possibly arginine, but not of tryptophan (31). Furthermore, L-tryptophan depletion and its degradation by IDO results in the production of kynurenine, which may affect CD4\(^+\) T-cell function (18,19).

In the MLR-derived CD4\(^+\) T cells, the expression of CYP1A1, a transcriptional target of AhR, was increased in the absence of 1MT, indicating that the IDO kynurenine AhR pathway is associated with our experimental model.

Recent studies have confirmed that IDO may exert its effect on CD4\(^+\) T cells by affecting their metabolism (13-16). Specifically, L-tryptophan degradation by IDO has been shown to decrease aerobic glycolysis, glutaminolysis and fatty acid synthesis (13-16), all required for rapid CD4\(^+\) T-cell proliferation and differentiation towards Teff lineages (22-25). The results of the present study confirmed that L-tryptophan degradation by IDO increased fatty acid consumption in MLRs. In parallel, the activity of CPT1 in MLR-derived CD4\(^+\) T cells increased. Fatty acid β-oxidation occurs in the mitochondrial matrix. However, acyl-CoAs cannot pass the inner mitochondrial membrane, unless they are converted to acylcarnitine in the cytoplasmic surface of the inner mitochondrial membrane. This reaction is catalyzed by CPT1, which, by controlling the entry of fatty acid into the mitochondrial matrix regulates the rate of fatty acid oxidation (28,29).

The reason for the increased CPT1 activity in CD4\(^+\) T cells derived from MLR without the IDO inhibitor, 1MT, may be due to the increased levels of the three CPT1 isoenzymes iden-
tified in the current study. A possible explanation may depend on the confirmed effect of IDO-induced L-tryptophan degradation in transcription factors such as p53 and cMyc that control cell metabolism in CD4+ T cells (14,16). However, the exact mechanism for this IDO-related increase in CPT1A, CPT1B and CPTIC expression remains to be elucidated.

In addition to CPT1 expression, the activity of this enzyme is tightly regulated and more precisely, is allosterically inhibited by malonyl-CoA. Malonyl-CoA is produced by ACC2, an enzyme associated with the outer mitochondrial membrane (28,29). When IDO activity was not inhibited in MLRs, ACC2 expression in the MLR-derived CD4+ T cells decreased. In addition, possibly due to AMPK-activated protein kinase (AMPK) (32), the phosphorylated inactivated form of ACC2 increased markedly. This IDO-induced alteration in ACC2 is expected to lead to decreased ACC2 activity, decreased malonyl-CoA production and increased CPT1 activity and fatty acid β-oxidation.

We also evaluated the effect of IDO-induced L-tryptophan degradation on two terminal points of CD4+ T-cell immune response, proliferation and apoptosis. Contrary to what has been shown in a similar experimental model (13-16), IDO did not affect cell proliferation in MLRs, or CD4+ T-cell apoptosis as assessed by activated caspase-3, the terminal caspase at which all the apoptotic pathways converge (33). However, the presence of oleate in the culture medium in the present study yielded different results. Oleate, as a fatty acid, along with the IDO-induced increase in fatty acid oxidation may protect CD4+ T-cells from energy deprivation, since IDO is known to decrease glucose influx in the cell, aerobic glycolysis and glutaminolysis (13-16). The presence of a fatty acid in the culture medium may protect cells from energy deprivation, thus also preventing the inhibition of cell proliferation and the induction of apoptosis. These results also raise the question of whether it is more appropriate to perform immunological experiments using more ‘normal’ culture medium, which contains fatty acids.

The effect of IDO-induced L-tryptophan degradation on the expression of the Treg signature transcription factor FoxP3, and of the Th17 signature transcription factor, RORγt, was evaluated (34). The two CD4+ T-cell lineages are formed reciprocally as regards fatty acid metabolism. Fatty acid synthesis favors differentiation into the Th17 lineage, whereas fatty acid oxidation favors differentiation into Tregs (25,26). According to what is generally considered (35-37), IDO increased FoxP3, but decreased RORγt expression in MLR-derived CD4+ T-cells. In order to define the effect of the presence of fatty acid in the culture medium, we repeated the experiments without oleate. No difference was detected regarding RORγt; however, the presence of oleate IDO induced a greater increase in FoxP3 expression. The reason remains to be defined, since various aspects regarding the mechanisms that connect fatty acid metabolism with CD4+ T-cell function, such as post-translational protein modification by lipids or the availability of acetyl-CoA for epigenetic modifications, are under investigation (38). Thus, this raises the question of whether a culture medium containing fatty acids more closely mimics the in vivo conditions and may thus be more suitable for lymphocyte culture studies.

In conclusion, the present study demonstrated that IDO, by degrading L-tryptophan, enhanced CPT1 activity and fatty acid oxidation, and exerted fatty acid-dependent effects in human alloreative CD4+ T cells.

References