# Indoleamine 2,3-dioxygenase suppresses humoral alloimmunity via pathways that different to those associated with its effects on T cells

MARIA SOUNIDAKI*, GEORGIOS PISSAS*, THEODOROS ELEFTHERIADIS, GEORGIA ANTONIADI, SPYRIDON GOLFINOPOULOS, VASSILIOS LIAKOPOULOS and IOANNIS STEFANIDIS

Department of Nephrology, Faculty of Medicine, University of Thessaly, Larissa 41110, Greece

Received July 10, 2018; Accepted January 18, 2019

DOI: 10.3892/br.2019.1212

**Abstract.** Chronic antibody-mediated rejection remains a major cause of late graft loss. Regarding cellular alloimmunity, the immunosuppressive properties of indoleamine 2,3-dioxygenase (IDO) have been well investigated; however, little is known of its effects on humoral alloimmunity. Therefore, the present study aimed to evaluate the effects of IDO on humoral alloimmunity. We developed a method for the induction of humoral alloimmunity in a one-way mixed lymphocyte reaction (MLR), which was measured with an antibody-mediated complement-dependent cytotoxicity assay using resting cells, which are similar to the stimulator cells of the aforementioned MLR. In parallel, cellular alloimmunity was assessed in two-way MLRs. The IDO inhibitor 1-methyl-DL-tryptophan was used for evaluating the role of IDO. In order to investigate whether the pathways known to serve a role in the effects of IDO on T cells are applied in humoral alloimmunity, the general control nonderepressible-2 (GCN-2) kinase activator tryptophanol and the aryl hydrocarbon receptor (AhR) inhibitor CH223191 were employed. The IDO inhibitor was revealed to increased cellular autoimmunity, but was decreased by the GCN-2 kinase activator. Unexpectedly, the AhR inhibitor decreased cellular alloimmunity. In addition, the IDO inhibitor was observed to suppress humoral alloimmunity, which may occur in manners independent of GCN-2 kinase AhR. The present study proposed that IDO may decrease humoral alloimmunity in primary human peripheral blood mononuclear cells via pathways that differ to those associated with its effect on T cells.

*Correspondence to:* Dr Maria Sounidaki, Department of Nephrology, Faculty of Medicine, University of Thessaly, Neo Ktirio, Mezourlo Hill, Biopolis, Larissa 41110, Greece
E-mail: maria_sounidaki@hotmail.com

*Contributed equally

*Key words:* indoleamine 2,3-dioxygenase, humoral alloimmunity, control nonderepressible-2 kinase, aryl hydrocarbon receptor, transplantation

## Introduction

Kidney transplantation is the most effective treatment for end-stage renal disease. The widespread use of crossmatch assays and improved immunosuppressive regiments to prevent acute antibody-mediated rejection and acute cellular rejection respectively, have increased early graft survival significantly; however, late graft outcome remains fairly poor in part due to chronic antibody-mediated rejection, which is the result of *de novo* production of antibodies against the graft (1,2). Thus, further investigation into humoral alloimmunity is imperative in order to reduce late graft loss.

Indoleamine 2,3-dioxygenase (IDO) is an immunomodulatory enzyme. Its immunosuppressive properties were originally identified by the detection of its role in preventing semi-allogenic fetal rejection (3). Subsequently, the immunosuppressive effects of IDO have been extensively studied and confirmed in various models of transplantation; autoimmunity and tumor escape from immunosurveillance have also been reported (4,5). Upon inflammation, IDO expression is upregulated in antigen presenting cells resulting in L-tryptophan degradation via the kynurenine pathway (4,5). L-tryptophan depletion suppresses T cells by activating general control nonderepressible-2 (GCN2) kinase (6); products of the kynurenine pathway favor naïve $CD4^+$ T cell differentiation towards a regulatory state instead of an effector phenotype by activating the aryl-hydrocarbon receptor (AhR) (7). The effects of IDO on T cells are mediated by alterations in the expression level of numerous transcription factors and are partial associated with alterations in cell metabolism (8-12).

The role of IDO in preventing acute cellular rejection of allografts has been reported in various models of solid organ transplantation (13-22); however, the effect of this enzyme on humoral alloimmunity requires further investigation. In the present study, we developed a method for assessing *de novo* antibody production during an *in vitro* alloimmune response. In order to evaluate the role of IDO in humoral alloimmunity, the specific inhibitor 1-methyl-DL-tryptophan (1-MT) was employed. 1-MT is a competitive, non-toxic IDO

inhibitor (23), which has been successfully used for suppressing immune tolerance in models of semi-allogenic pregnancy (3), transplantation (24), autoimmunity (25) and cancer (26). To further understand the molecular mechanisms by which IDO may affect humoral alloimmunity, the GCN2 kinase activator tryptophanol (TRP) was used. TRP is a competitive inhibitor of tryptophanyl-tRNA synthetase and by raising the pool of uncharged tRNA, it acts as a pharmacological activator of GCN-2 kinase (6,27). Thus, the AhR inhibitor CH223191 was used. CH223191 does not have detectable AhR agonist-like activity and protects mice from dioxin toxicity (28).

## Materials and methods

*Subjects*. Blood was collected from 4 unrelated healthy subjects (aged 32.5±7.05-year-old) from a blood vessel in the arm, inside of the elbow or wrist, at the laboratory of the Nephrology Department, University of Thessaly. In order to exclude any pre-sensitization event, all subjects were males without a history of blood transfusion. Written informed consent was obtained from each individual enrolled; the present study was approved by the Ethics Committee of the Faculty of Medicine, University of Thessaly (Larissa, Greece) (approval no. 558/10-2-2017).

*Peripheral blood mononuclear cell (PBMC) isolation and culture*. PBMCs were isolated from whole blood samples by Ficoll-Hypaque density gradient centrifugation at 600 x g for 25 min at 18-20˚C using Histopaque-1077 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The interface was collected and washed with RPMI-1640 medium (Sigma Aldrich; Merck KGaA). To count the isolated PBMCs, a Neubauer chamber (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) and an optical microscope at x40 (x4 objective) were used. Cell viability was assessed using the trypan blue exclusion assay (Sigma-Aldrich; Merck KGaA) and for each PBMC sample cells were counted in the four fields of the Neubauer chamber. All cell cultures were performed using RPMI-1640 medium, supplemented with 2 mM L-glutamine, 10 mM HEPES, 10% fetal bovine serum (Sigma-Aldrich; Merck KGaA) and 1% antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B (Sigma-Aldrich; Merck KGaA). Cultures were incubated at 37˚C in an atmosphere of 95% relative humidity and 5% $CO_2$.

*Assessment of 1-MT or TRP effect on lactate dehydrogenase (LDH) release following treatment with CH223191*. The concentrations of 1-MT (100 $\mu$M), TRP (0.25 mM) and CH223191 (3 $\mu$M) were selected according to previous studies (8,29,30). In particular, the concentration of 100 $\mu$M for 1-MT was selected according to the results that were repeatedly obtained from several of our previous studies, which revealed efficacy in inhibiting IDO and reduced L-tryptophan consumption in mixed lymphocyte reactions (MLRs) without toxicity (9-12,29). In addition, an LDH release assay for the selected concentrations of the aforementioned substances was performed in resting PBMCs seeded in 96-well plates ($1x10^5$ cells/well) and cultured at 37˚C for a 7-day period. The LDH release assay was performed using the Cytotox Non-Radioactive Cytotoxic Assay kit (cat no. G1780; Promega Corporation, Madison, WI, USA) according to the manufacturer's protocols. LDH release was calculated by the following equation: LDH release (%) = (LDH in the supernatant / total LDH) x 100. All experiments were performed in triplicate and the results were presented as the mean of the three measurements.

Of note, our previous study originally planned to apply the sodium 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)-carbonyl]-2H-tetrazolium (XTT) assay to analyze cell proliferation. This assay comprises a colorimetric technique that assesses actual cytosolic NADH content, a key compound in the mitochondria, in which the tricarboxylic acid cycle takes place (31). The results revealed that IDO inhibition by 1-MT significantly decreased mitochondrial function in PBMCs stimulated with lymphocyte-specific stimulus tetanus toxoid (TT), which was unexpected. In addition, compared with untreated cells, TT stimulation notably increased the optical density (OD) value from the XTT assay, indicating enhanced mitochondrial function. On the contrary, in cells treated with TT and 1-MT, the XTT assay presented a significantly lower OD value compared with TT-treated cells. This decrease suggested that IDO enhances mitochondrial function in stimulated PBMCs, and its inhibition by 1-MT may markedly abrogate this effect. Additionally, as 1-MT did not alter the proliferation of TT-stimulated lymphocytes, the use of tetrazolium dyes for assessing cell proliferation may not be reliable (32).

*Assessment of cellular alloimmunity in two-way MLRs*. MLRs are *ex vivo* cellular immunoassays that occur between two genetically distinct allogeneic lymphocyte populations of the same species. In a one-way MLR, only one lymphocyte population can respond or proliferate. In a two-way MLR, the two populations can proliferate. MLRs are performed to assess the response of T cells to external stimuli. The assay comprises the purification of responder lymphocytes from peripheral blood followed by co-culturing with stimulator cells. Stimulator cell populations that also contain T cells (two-way MLR) can replicate in the presence of the responder cells. Therefore, for a one-way MLR, stimulator cells are prevented from replicating by mitomycin C, a DNA crosslinker to restricT cell replication. The maximal measurable cellular proliferation occurs ~5-7 days of the MLR (33).

In the present study, two-way MLRs were performed in 96-well plates for 7 days in the presence or absence of 100 $\mu$M 1-MT, 0.25 mM TRP or 3 $\mu$M CH223191. A total of 6 different pairs of PBMC samples of the 4 different healthy aforementioned individuals. For each pair, lymphocytes from a subject were mixed with lymphocytes from a different subject. In particular, PBMCs from subject #1 were co-cultured with PBMCs from subject #2, 3 or 4; thus three different MLRs could be obtained. PBMCs from subject #2 were cultured with PBMCs from subject #3 or 4, which provided two more MLRs. Additionally, PBMCs from subject #3 were cultured with PBMCs from subject #4 yielding only one MLR that differed from the others. The number of PBMCs from each member of the MLR pairs was $5x10^4$, which comprised $1x10^5$ PBMCs in each well. Cultures of $1x10^5$ resting PMBCs per well were used as controls. At the end of the 7-day period, cell proliferation was assessed by chemiluminescence with

Cell Proliferation ELISA (Roche Diagnostics, Indianapolis, IN, USA) using bromodeoxyuridine (BrdU) labeling overnight at 37˚C and immunoenzymatic detection according to the manufacturer's protocols. For determining the results of the BrdU Cell Proliferation Assay, the fluorescence intensity was measured and analyzed using an EnSpire® Multimode Plate Reader (PerkinElmer, Inc., Waltham, MA, USA). The proliferation index was calculated by the following equation: Proliferation index (%) = (OD derived from each MLR / the mean OD derived from the control resting PBMCs cultures of the two subjects that constituted the specific MLR) x 100. All aforementioned MLRs were performed in triplicate and the results were representative of the mean of three measurements.

*Assessment of humoral alloimmunity*. In order to evaluate humoral alloimmunity, the following method was developed. One-way MLRs were performed in 24-well plates. Mitomycin-C-treated PBMCs ($0.5x10^6$ cells) from one subject were used as stimulator cells. For the mitomycin-C treatment, PBMCs were incubated for 30 min at 37˚C with 50 $\mu$g/ml mitomycin C (Sigma Aldrich; Merck Merck KGaA) and then washed three times with complete RPMI-1640 medium supplemented with 2 mM L-glutamine and 10% FBS. As responder cells, $0.5x10^6$ PBMCs from another individual were used. A total of 12 different pairs of PBMC samples of the 4 healthy aforementioned subjects using one-way MLR cultures. For each pair, lymphocytes from a responder subject were mixed with inactivated lymphocytes from a stimulator subject and cultured as follows: Pair 1, responder subject #1 + stimulator subject #2; pair 2, stimulator subject #1 + responder subject #2; pair 3, responder subject #1 + stimulator subject #3; pair 4, stimulator subject #1 + responder subject #3; pair 5, responder subject #1 + stimulator subject #4; pair 6, stimulator subject #1 + responder subject #4; pair 7, responder subject #2 + stimulator subject #3, pair 8, stimulator subject #2 + responder subject #3; pair 9, responder subject #2 + stimulator subject #4; pair 10, stimulator subject #2 + responder subject #4; pair 11, responder subject #3 + stimulator subject #4 and pair 12, stimulator subject #3 + responder subject #4. MLRs lasted for 7 days in culture at 37˚C in the presence or absence of 100 $\mu$M 1-MT, 0.25 mM TRP or 3 $\mu$M CH223191. Of note, a 7-day period was reported as adequate for the production of IgM and IgG alloantibodies in human mixed lymphocyte cultures (34). Following this period, the supernatants from each one-way MLR were harvested and expected to contain antibodies produced against the stimulator PBMCs.

In parallel to the aforementioned one-way MLRs, resting untreated PBMCs were cultured in 6-well plates. At the end of the 7-day one-way MLRs, resting PBMCs similar to those used as stimulator cells in MLRs but untreated, were counted using a Neubauer chamber (Paul Marienfeld GmbH & Co. KG) and an optical microscope at x40 (x4 objective). Cell viability was assessed using the trypan blue exclusion assay and for each PBMC sample, cells were counted in the four different fields of the Neubauer chamber. Then, cells were placed in 96-well plates at a number of $0.5x10^5$ in a volume of 50 $\mu$l of RPMI-1640 medium, supplemented with L-glutamine, 10 mM HEPES, 10% fetal bovine serum and 1% antibiotic-antimycotic solution containing penicillin, streptomycin and amphotericin B. Cultures were incubated at 37˚C in an atmosphere of 95% relative humidity and 5% $CO_2$. For assessing antibody-mediated complement-dependent cytotoxicity (CDC) a modified protocol, which was initially developed for the assessment of antigen specific antibodies in serum samples was conducted (35). In brief, 50 $\mu$l of each supernatant collected from each one-way MLR, undiluted or diluted 1:2 with complete RPMI-1640, was added into 96-well plates that were pre-seeded with the resting target PBMCs. The plates were incubated on ice for 30 min. Subsequently, 11 $\mu$l of rabbit complement (Low-Tox-H rabbit complement, Cedarlane Corporation, Burlington, Canada) was added to each well at a final concentration of 10%. The 96-well plates were incubated for another 2 h at 37˚C. As a control, 50 $\mu$l of complete RMPI-1640 was added instead of the one-way MLR supernatant, along with 11 $\mu$l of rabbit complement.

As cell-mediated cytotoxicity occurs in MLRs (36) and the used compounds (1-MT, TRP and CH223191) affected the intensity of MLRs leading to the release of various quantities of LDH in the supernatants, antibody-mediated CDC induced by these supernatants in cells was not investigated directly. Instead, the present study analyzed cell survival. As the exact time-points of cell death for each of the differenT cell types involved in MLRs have not been clearly defined yet, Annexin V staining for the detection of apoptosis was not selected in the present study; however, it is considered to be the gold standard for the analysis of cell survival/death. Therefore, cell survival was assessed colorimetrically by measuring the reduction of XTT, a yellow tetrazolium salt, to orange formazan via the metabolic targeting of cells. Target cells were incubated with XTT reagent for 1 h at 37˚C. For this purpose, the TACS XTT assay kit (Trevigen, Inc., Gaithersburg, MD, USA) was used according to manufacturer's protocols. Cell survival was calculated by the following equation: Cell survival (%) = (XTT assay OD of the control / XTT assay OD of the evaluated condition) x 100. A total of twelve experiments were performed, each in triplicates and the results were representative of the mean of the three measurements.

*Statistical analysis*. Statistical analyses were performed using SPSS Statistics, Version 20 (IBM Corp., Armonk, NY, USA). The normality of the evaluated variables was assessed and confirmed by the one-sample Kolmogorov-Smirnov test. For the comparison of means, a paired t-test or one-way repeated measures analysis of variance followed by Bonferroni's correction test were used. The results were expressed as the mean ± standard deviation. $P<0.05$ was considered to indicate a statistically significant difference.

In some cases, to avoid the violation of the prerequisite for normal distribution of the compared variables when applying parametric statistical tests, we compared the OD values as derived from the aforementioned assays prior to normalization to the control; however, the results were expressed and depicted following the normalization of values to the control group.

## Results

*1-MT, TRP and CH223191 does not affect LDH release in resting PBMCs*. At the concentrations of 100 $\mu$M for 1-MT,

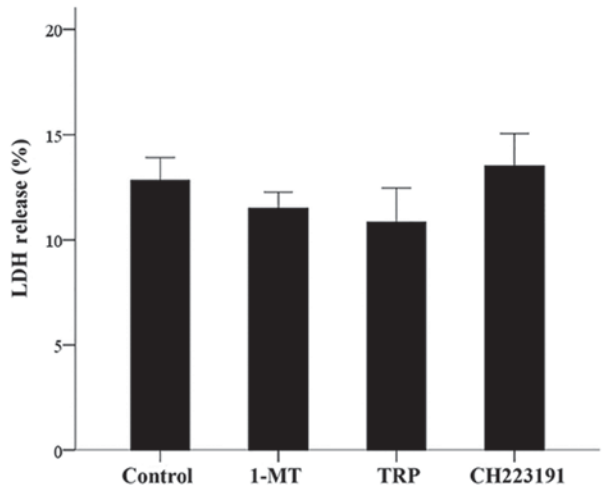


Figure 1. 1-MT, TRP and CH223191 does not affect LDH release in resting PBMCs. At 7 days of resting PBMC culture, an LDH release assay revealed that 100 $\mu$M 1-MT, 0.25 mM TRP or 3 $\mu$M CH223191 did not induce a statistically significant change in LDH release. In particular LDH release was 12.83±1.01% for control, 11.50±0.75% for 1-MT, 10.83±1.64 for TRP and 13.50±1.56% for CH223191. Error bars represent standard deviation. 1-MT, 1-methyl-DL-tryptophan; LDH, lactate dehydrogenase; TRP, tryptophanol.

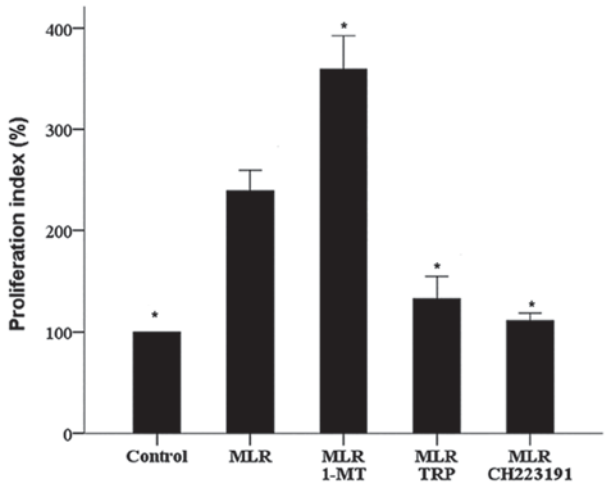


Figure 2. 1-MT increases cell proliferation, but is decreased by TRP and CH223191. In two-way MLRs, the indoleamine 2,3-dioxygenase inhibitor 1-MT increased cellular alloimmunity as assessed by cell proliferation. On the contrary, the general control nonderepressible-2 kinase activator TRP and the aryl hydrocarbon receptor inhibitor CH223191 decreased cell proliferation. *P<0.05 vs. MLR. Error bars represent standard deviation. 1-MT, 1-methyl-DL-tryptophan; TRP, tryptophanol.

0.25 mM for TRP or 3 $\mu$M for CH223191, none of the evaluated compounds induced a statistically significant change in the LDH release of resting PBMCs. The LDH release assay revealed a value of 12.83±1.01% for the control, 11.50±0.75% for 1-MT (P>0.05), 10.83±1.64% for TRP (P>0.05) and 13.50±1.56% for CH223191 (P>0.05; Fig. 1).

*1-MT increases cellular alloimmunity, but is decreased by TRP and CH223191*. The IDO inhibitor 1-MT increased cellular alloimmunity, as assessed by cell proliferation in two-way MLRs. The proliferation index in 1-MT-treated MLRs was significantly higher than that of untreated MLRs

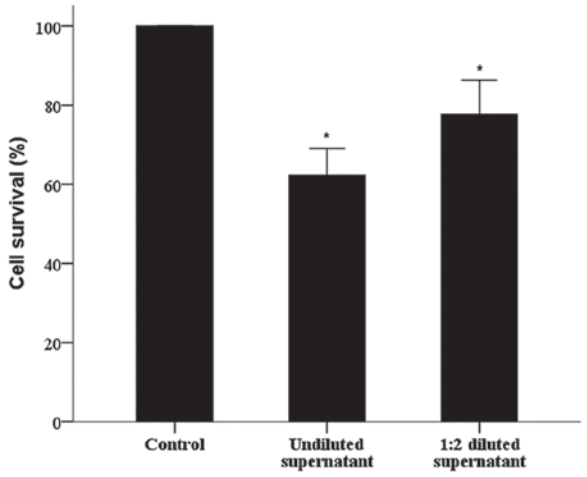


Figure 3. In one-way MLR, antibodies against stimulator PBMCs are produced and secreted in the supernatant. One-way MLRs were performed, and then the supernatants were collected and used in an antibody-mediated CDC assay against resting target PBMCs similar to those employed as stimulator cells in the respective MLRs. The supernatants induced antibody-mediated CDC. Compared with the control, the survival of target cells of the antibody-mediated CDC assay was significantly decreased when undiluted supernatants from the MLRs were used. When 1:2 diluted supernatants were used cell survival was also significantly decreased compared with the control, but to a lesser extent than in the case of the undiluted supernatant. *P<0.05 vs. control. Error bars represent standard deviation. CDC, complement-dependent cytotoxicity; MLR, mixed lymphocyte reaction; PBMC, peripheral blood mononuclear cell; TRP, tryptophanol.

(359.37±32.93 vs. 238.99±20.55% respectively, P<0.001; Fig. 2).

On the contrary, compared with MLRs of untreated cells, treatment with the GCN2 kinase activator TRP significantly decreased the proliferation index to 132.89±21.65% (P<0.001; Fig. 2). Similarly, the AhR inhibitor CH223191 significantly reduced the proliferation index to 111.07±7.36% (P<0.001; Fig. 2).

*Supernatants from one-way MLRs contain specific antibodies against the stimulator PBMCs*. The antibody-mediated CDC assay revealed that one-way MLR-antibodies specific for the stimulator PBMCs were produced by the responder PBMCs. The cell survival of untreated PBMCs, similar to stimulator cells, decreased to 62.25±6.76% compared with the control when supernatants from the respective MLRs were undiluted (P<0.001), and to 77.71±8.62% when diluted 1:2 (P<0.001). Interestingly, the diluted supernatants exhibited less antibody-mediated CDC against the untreated PBMCs, similar to stimulator cells, target PBMCs (P<0.001; Fig. 3).

*1-MT increases humoral alloimmunity, but is not affected by TRP and CH22319*. The antibody-mediated CDC assay revealed that the treatment of one-way MLRs with 1-MT increased the production of specific for the stimulator PBMCs antibodies. When undiluted supernatants from untreated MLRs were used, the survival of target PBMCs was 62.25±6.76%, which was significantly decreased to 35.39±7.75% following treatment with 1-MT (P<0.001; Fig. 4A). Similar results were obtained from the 1:2 diluted supernatants compared with the control; however, decreased antibody-mediated CDC was

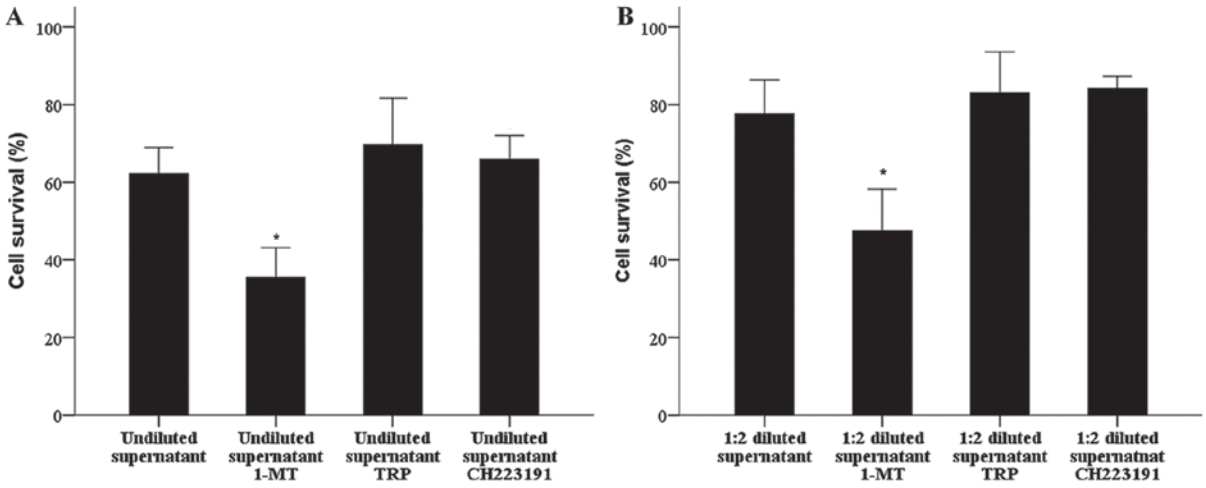


Figure 4. 1-MT increases humoral alloimmunity, but is notably affected by TRP and CH223191. One-way MLRs were performed in the presence or absence of indoleamine 2,3-dioxygenase inhibitor 1-MT, the GCN-2 kinase activator TRP or the aryl hydrocarbon receptor inhibitor CH223191. (A) Then the supernatants were collected and used in an antibody-mediated CDC assay against resting target PBMCs similar to those used as stimulator cells in the respective MLRs; (B) supernatants were diluted 1:2. When undiluted supernatants were employed, those derived from 1-MT-treated MLRs exhibited greater antibody-mediated CDC than those derived from untreated MLRs. On the contrary, supernatants from TRP- or CH223191-treated MLRs did not exhibit notably different antibody-mediated CDC, than those from untreated MLRs. *P<0.05 vs. untreated supernatant. Error bars represent standard deviation. 1-MT, 1-methyl-DL-tryptophan; CDC, complement-dependent cytotoxicity; MLR, mixed lymphocyte reaction; TRP, tryptophanol.

observed, (cell survival of diluted supernatant, 77.71±8.62% versus diluted supernatant with 1-MT, 47.48±10.72%, P<0.001; Fig. 4B).

In one-way MLRs, the GCN-2 kinase activator TRP did not markedly affect the production of specific antibodies against the stimulator PBMCs. The antibody-mediated CDC assay revealed that when undiluted supernatants from untreated MLRs were used, the survival of untreated, target PMBCs, which are similar to stimulatory cells, was 62.25±6.76%, while in the case of undiluted supernatants from TRP-treated MLRs cell survival was 69.63±12.01% (P>0.05; Fig. 4A). In the case of 1:2 diluted supernatants, cell survival was 77.71±8.62 and 83.04±10.58% in the untreated as TRP-treated groups, respectively (P>0.05; Fig. 4B).

Similarly, in one-way MLRs, the AhR inhibitor CH223191 did not notably alter the production of specific antibodies against the stimulatory PBMCs. The antibody-mediated CDC assay demonstrated that the cell survival of untreated target PMBCs, which are similar to stimulatory cells, was 62.25±6.76%, while that of undiluted supernatants from CH223191-treated MLRs, cell survival was 65.87±6.17% (P>0.05; Fig. 4A). In the case of 1:2 diluted supernatants, cell survival was 77.71±8.62 and 84.13±5.21%, in the untreated and CH223191-treated groups, respectively (P>0.05; Fig. 4B).

## Discussion

Developments in immunological assays and immunosuppressive medications have reduced the incidence of acute antibody-mediated rejection and acute cellular rejection; however, little is known of the pathophysiology and treatment of chronic antibody-mediated rejection, which notably contributes to late graft loss (1,2). The aim of the present study was to evaluate the effects of IDO, an immunomodulatory enzyme (4,5), on humoral alloimmunity. In addition, cellular alloimmunity was also assessed.

Cellular alloimmunity was investigated by measuring cell proliferation in two-way MLRs. As expected, inhibition of IDO by 1-MT at a non-toxic concentration, increased cell proliferation. The immunosuppressive effects of IDO on cellular adaptive immunity has been extensively studied (4,5), and numerous experimental models of transplantation have revealed the protective role of IDO in acute cellular rejection (13-22). IDO may affect T cell function via L-tryptophan depletion and GCN-2 kinase activation (6,29). In accordance with this, MLR treatment with non-toxic concentrations of the GCN-2 kinase activator TRP inhibited cell proliferation in the present study.

In addition, IDO affects T cell function and favors naïve $CD4^+$ T cell differentiation toward a regulatory phenotype via the activation of AhR by L-tryptophan degradation products (7). On the contrary, the AhR inhibitor CH223191 decreased cell proliferation. Generally, there are contradicting results regarding the role of AhR in cellular alloimmunity. For instance, a previous study revealed that the prototype AhR activator 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) decreased cell proliferation in MLRs (37), while the same activator enhanced T cell proliferation by increasing the expression of MHCII and cluster of differentiation 86 in allogenic bone marrow-derived dendritic cells (38). It has been also been proposed, that under different conditions, the activation of AhR may affect the differentiation of naïve $CD4^+$ T cells into the Th17 or Treg cell lineages (39). The effects of AhR activation on cellular alloimmunity requires further investigation, taking into consideration that AhR regulation differs between human and murine cells (40). Thus, primary human PBMCs were employed for analysis in the present study.

To assess humoral alloimmunity, we developed a simple experimental model using one-way MLRs and an antibody-mediated CDC assay. The results confirmed that in the 7-day period of MLR culture, specific antibodies against the stimulator cells were produced and secreted into the

supernatant by the responder cells. These antibodies were identified and quantified via an antibody-mediated CDC assay of resting PBMCs derived from an individual conferring stimulator PBMCs for the one-way MLR.

In our model, the IDO inhibitor 1-MT increased humoral alloimmunity, which suggests that IDO inhibits humoral alloimmunity. Providing this may be simply attributed to 1-MT-induced increased cellular autoimmunity, it would be expected that humoral alloimmunity would be reduced in the presence of TRP or CH223191, as these substances decreased cellular immunity. Additionally, neither the GCN-2 kinase activator TRP, nor the AhR inhibitor CH223191 exerted any significant effects on humoral alloimmunity. These pathways have been evaluated mainly in T cells and in models where IDO1 is upregulated in antigen presenting cells due to inflammation (4-7). The effects of IDO and the involved molecular pathways on humoral immunity require further investigation. For instance, IDO is generally considered to possess immunosuppressive properties; however, IDO1 expression in B cells increased antibody production against T cell-independent (TI) antigens (41). In addition, *in vitro* activation with Toll-like receptor ligands or B cell receptor crosslinking rapidly induced IDO1 expression and activity in purified B cells, whereas IDO1$^{-/-}$ B cells exhibited enhanced proliferation and survival, associated with increased immunoglobulin and cytokine production compared with wild type B cells. Murine B cells purified from the spleen of knockout or wild type mice activated with TI type I antigens LPS and CpG, or anti-IgM B cell receptor exhibited crosslinking to mimic TI-2 antigen responses (41). On the contrary, to the best of our knowledge, we are the first to examine the role of the IDO pathway in B cell responses in a more physiologically relevant setting of MLRs using human cells and 1-MT as a treatment in order to inhibit IDO activity. Furthermore, IDO inhibition with 1-MT has been reported to exacerbate autoimmune diseases (25,42), whereas other studies revealed opposing findings (43,44). This could be due to the inhibitory effects of 1-MT on IDO1 and IDO2. IDO2 is a low-efficiency L-tryptophan catabolizing enzyme, which was associated with immunomodulation; its molecular mode of action remains unknown (45). A recent study demonstrated that IDO2 expression in B cells enhances humoral autoimmunity by supporting cross talk between autoreactive T and B cells; however, humoral immune responses to T cell-dependent antigens were unaffected (46). Our study revealed that IDO inhibition via 1-MT increased humoral alloimmunity; this may provide insight into the prevention of chronic antibody-mediated rejection. The lack of effects from TRP and CH223191 may indicate that other molecular pathways are responsible for the inhibitory effects of IDO in humoral alloimmunity, which may differ to the mechanisms reported in T cells. Thus, IDO2 may serve as a promising candidate for further research.

Another possible explanation for the lack of effects of the AhR inhibitor CH223191 on humoral alloimmunity in the present study may be due to the activation of AhR that directly affects B cells. An endogenous tryptophan catabolism-derived AhR agonist has been shown to suppress the differentiation of B cells into immunoglobulin-secreting plasma cells (47). A recent study revealed that the prototypical AhR activator TCDD decreases antibody production by promoting an interaction between AhR and an essential transcriptional element in the gene of the immunoglobulin heavy chain (48). Thus, inhibition of AhR may be proposed to increase humoral alloimmunity. This may explain our finding of steady humoral alloimmunity in CH223191-treated one-way MLRs despite the decreased T cell response detected in two-way MLRs treated with the same inhibitor. In MLRs with human cells, 1-MT-induced decreases in L-tryptophan degradation reduced AhR activation (12), supporting the finding of the present study in which that 1-MT increased humoral alloimmunity.

Of note, inter-species variation should be taken into consideration when interpreting the results of the aforementioned studies. Additionally, AhR regulation differs between human and murine cells (40). For instance, in humans, the antibody response to a vaccine against the surface antigen of hepatitis B is decreased in association with increased IDO levels (49), whereas administration of the same antigen in mice along with IDO inhibitor 1-MT resulted in a suppressed humoral immune response (50). Nevertheless, the use of primary human PBMCs in the present study due to certain inter-species differences in AhR regulation yields several limitations. In particular, primary human PBMCs have a short lifespan in culture (51); thus, experiments with overexpression of IDO, which may provide further insight into underlying mechanisms, would be rather ineffective in this particular study.

In conclusion, IDO decreased humoral alloimmunity in a GCN-2 kinase-dependent manner, which may occur independently of AhR in humans. As humoral alloimmunity may induce chronic antibody-mediated rejection, understanding the major cause of late kidney allograft dysfunction and the underlying mechanisms may contribute to developments in prolonging kidney allograft survival.

**Acknowledgements**

The present study was based on a previously published abstract by Eleftheriadis *et al* in The Journal of Immunology, volume 146, pages 292-300 (2015) entitled as: 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.

**Funding**

No funding was received.

**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

All of the authors contributed to the preparation of the manuscript and have read and agree to the manuscript as written. MS and GP performed the experiments; TE made substantial contributions to the conception of the present study; MS, GP, TE, GA, SG, and VL analyzed the data; MS, GP, and TE wrote the manuscript; IS supported all stages.

## Ethics approval and consent to participate

Written informed consent for the use of blood samples was obtained from all participants. The present study was approved by the Ethics Committee of the Faculty of Medicine, University of Thessaly (Larissa, Greece; approval no. 558/10-2-2017).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interest.

## References


1. Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, Birk PE, Campbell PM, Cascalho M, Collins AB, Demetris AJ, *et al*: Banff '05 Meeting Report: Differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy ('CAN'). Am J Transplant 7: 518-526, 2007.
2. Solez K, Colvin RB, Racusen LC, Haas M, Sis B, Mengel M, Halloran PF, Baldwin W, Banfi G, Collins AB, *et al*: Banff 07 classification of renal allograft pathology: Updates and future directions. Am J Transplant 8: 753-760, 2008.
3. Munn DH, Zhou M, Attwood JT, Bondarev I, Conway SJ, Marshall B, Brown C and Mellor AL: Prevention of allogeneic fetal rejection by tryptophan catabolism. Science 281: 1191-1193, 1998.
4. King NJC and Thomas SR: Molecules in focus: Indoleamine 2,3-dioxygenase. Int J Biochem Cell Biol 39: 2167-2172, 2007.
5. Curti A, Trabanelli S, Salvestrini V, Baccarani M and Lemoli RM: The role of indoleamine 2,3-dioxygenase in the induction of immune tolerance: Focus on hematology. Blood 113: 2394-2401, 2009.
6. Munn DH, Sharma MD, Baban B, Harding HP, Zhang Y, Ron D and Mellor AL: GCN2 kinase in T cells mediates proliferative arrest and anergy induction in response to indoleamine 2,3-dioxygenase. Immunity 22: 633-642, 2005.
7. Mezrich JD, Fechner JH, Zhang X, Johnson BP, Burlingham WJ and Bradfield CA: An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. J Immunol 185: 3190-3198, 2010.
8. Eleftheriadis T, Pissas G, Yiannaki E, Markala D, Arampatzis S, Antoniadi G, Liakopoulos V and Stefanidis I: 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 74: 1501-1509, 2013.
9. Eleftheriadis T, Pissas G, Antoniadi G, Spanoulis A, Liakopoulos V and Stefanidis I: 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 26: 673-684, 2014.
10. Eleftheriadis T, Pissas G, Antoniadi G, Liakopoulos V and 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 146: 292-300, 2015.
11. Eleftheriadis T, Pissas G, Antoniadi G, Tsogka K, Sounidaki M, Liakopoulos V and Stefanidis I: Indoleamine 2,3 dioxygenase downregulates T cell receptor complex ζ chain and c Myc, and reduces proliferation, lactate dehydrogenase levels and mitochondrial glutaminase in human T cells. Mol Med Rep 13: 925-932, 2016.
12. Eleftheriadis T, Pissas G, Sounidaki M, Tsogka K, Antoniadis N, Antoniadi G, Liakopoulos V and Stefanidis I: 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 Med 38: 1605-1613, 2016.
13. Vavrincova-Yaghi D, Deelman LE, Goor H, Seelen M, Kema IP, Smit-van Oosten A, Zeeuw D, Henning RH and Sandovici M: Gene therapy with adenovirus-delivered indoleamine 2,3-dioxygenase improves renal function and morphology following allogeneic kidney transplantation in rat. J Gene Med 13: 373-381, 2011.
14. Sun X, Gong ZJ, Wang ZW, Li T, Zhang JY, Sun HC, Liu S, Huang L, Huang C and Peng ZH: IDO-competent-DCs induced by IFN-γ attenuate acute rejection in rat liver transplantation. J Clin Immunol 32: 837-847, 2012.
15. Iken K, Liu K, Liu H, Bizargity P, Wang L, Hancock WW and Visner GA: Indoleamine 2,3-dioxygenase and metabolites protect murine lung allografts and impair the calcium mobilization of T cells. Am J Respir Cell Mol Biol 47: 405-416, 2012.
16. Hosseini-Tabatabaei A, Jalili RB, Khosravi-Maharlooei M, Hartwell R, Kilani RT, Zhang Y and Ghahary A: Immunoprotection and Functional Improvement of Allogeneic Islets in Diabetic Mice, Using a Stable Indoleamine 2,3-Dioxygenase Producing Scaffold. Transplantation 99: 1341-1348, 2015.
17. Xie FT, Cao JS, Zhao J, Yu Y, Qi F and Dai XC: IDO expressing dendritic cells suppress allograft rejection of small bowel transplantation in mice by expansion of Foxp3+ regulatory T cells. Transpl Immunol 33: 69-77, 2015.
18. He Y, Zhou S, Liu H, Shen B, Zhao H, Peng K and Wu X: Indoleamine 2,3-Dioxgenase Transfected Mesenchymal Stem Cells Induce Kidney Allograft Tolerance by Increasing the Production and Function of Regulatory T cells. Transplantation 99: 1829-1838, 2015.
19. Ebrahimi A, Kardar GA, Teimoori-Toolabi L, Ghanbari H, Sadroddiny E and Sadroddiny E: Inducible expression of indoleamine 2,3-dioxygenase attenuates acute rejection of tissue-engineered lung allografts in rats. Gene 576: 412-420, 2016.
20. Li C, Liu T, Zhao N, Zhu L, Wang P and Dai X: Dendritic cells transfected with indoleamine 2,3-dioxygenase gene suppressed acute rejection of cardiac allograft. Int Immunopharmacol 36: 31-38, 2016.
21. Na N, Luo Y, Zhao D, Yang S, Hong L, Li H, Miao B and Qiu J: Prolongation of kidney allograft survival regulated by indoleamine 2, 3-dioxygenase in immature dendritic cells generated from recipient type bone marrow progenitors. Mol Immunol 79: 22-31, 2016.
22. Khosravi-Maharlooei M, Pakyari M, Jalili RB, Kilani RT and Ghahary A: Intraperitoneal injection of IDO-expressing dermal fibroblasts improves the allograft survival. Clin Immunol 174: 1-9, 2017.
23. Jia L, Schweikart K, Tomaszewski J, Page JG, Noker PE, Buhrow SA, Reid JM, Ames MM and Munn DH: Toxicology and pharmacokinetics of 1-methyl-d-tryptophan: Absence of toxicity due to saturating absorption. Food Chem Toxicol 46: 203-211, 2008.
24. Alexander AM, Crawford M, Bertera S, Rudert WA, Takikawa O, Robbins PD and Trucco M: Indoleamine 2,3-dioxygenase expression in transplanted NOD Islets prolongs graft survival after adoptive transfer of diabetogenic splenocytes. Diabetes 51: 356-365, 2002.
25. Sakurai K, Zou J-P, Tschetter JR, Ward JM and Shearer GM: Effect of indoleamine 2,3-dioxygenase on induction of experimental autoimmune encephalomyelitis. J Neuroimmunol 129: 186-196, 2002.
26. Uyttenhove C, Pilotte L, Théate I, Stroobant V, Colau D, Parmentier N, Boon T and Van den Eynde BJ: Evidence for a tumoral immune resistance mechanism based on tryptophan degradation by indoleamine 2,3-dioxygenase. Nat Med 9: 1269-1274, 2003.
27. Lowe G and Tansley G: An investigation of the mechanism of activation of tryptophan by tryptophanyl-tRNA synthetase from beef pancreas. Eur J Biochem 138: 597-602, 1984.
28. Kim SH, Henry EC, Kim DK, Kim YH, Shin KJ, Han MS, Lee TG, Kang JK, Gasiewicz TA, Ryu SH, *et al*: Novel compound 2-methyl-2H-pyrazole-3-carboxylic acid (2-methyl-4-o-tolylazo-phenyl)-amide (CH-223191) prevents 2,3,7,8-TCDD-induced toxicity by antagonizing the aryl hydrocarbon receptor. Mol Pharmacol 69: 1871-1878, 2006.
29. Eleftheriadis T, Pissas G, Antoniadi G, Liakopoulos V, Tsogka K, Sounidaki M and Stefanidis I: Differential effects of the two amino acid sensing systems, the GCN2 kinase and the mTOR complex 1, on primary human alloreactive $CD4^+$ T-cells. Int J Mol Med 37: 1412-1420, 2016.

30. Eleftheriadis T, Pissas G, Antoniadi G, Liakopoulos V and 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 44: 60-7.e1, 2016.
31. Berridge MV, Herst PM and Tan AS: Tetrazolium dyes as tools in cell biology: New insights into their cellular reduction. Biotechnol Annu Rev 11: 127-152, 2005.
32. Eleftheriadis T, Pissas G, Karioti A, Antoniadi G, Liakopoulos V, Dafopoulou K, Pournaras S, Koukoulis G and Stefanidis I: The indoleamine 2,3-dioxygenase inhibitor 1-methyl-tryptophan suppresses mitochondrial function, induces aerobic glycolysis and decreases interleukin-10 production in human lymphocytes. Immunol Invest 41: 507-520, 2012.
33. Meo T: The MLR test in the mouse. In: Immunological methods. Lefkovits I and Pernis B (eds). Academic Press, New York, NY, pp227-239, 1979.
34. Rümke HC, Terpstra FG, Huis B, Out TA and Zeijlemaker WP: Immunoglobulin production in human mixed lymphocyte cultures: Implications for co-cultures of cells from patients and healthy donors. J Immunol 128: 696-701, 1982.
35. Konishi E, Kitai Y and Kondo T: Utilization of complement-dependent cytotoxicity to measure low levels of antibodies: Application to nonstructural protein 1 in a model of Japanese encephalitis virus. Clin Vaccine Immunol 15: 88-94, 2008.
36. Sato T, Deiwick A, Raddatz G, Koyama K and Schlitt HJ: Interactions of allogeneic human mononuclear cells in the two-way mixed leucocyte culture (MLC): Influence of cell numbers, subpopulations and cyclosporin. Clin Exp Immunol 115: 301-308, 1999.
37. Cai LJ, Yu DW, Gao Y, Yang C, Zhou HM and Chen ZK: Activation of aryl hydrocarbon receptor prolongs survival of fully mismatched cardiac allografts. J Huazhong Univ Sci Technolog Med Sci 33: 199-204, 2013.
38. Lee JA, Hwang JA, Sung HN, Jeon CH, Gill BC, Youn HJ and Park JH: 2,3,7,8-Tetrachlorodibenzo-p-dioxin modulates functional differentiation of mouse bone marrow-derived dendritic cells Downregulation of RelB by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Lett 173: 31-40, 2007.
39. Julliard W, Fechner JH and Mezrich JD: The aryl hydrocarbon receptor meets immunology: Friend or foe? A little of both. Front Immunol 5: 458, 2014.
40. Panchanathan R, Liu H and Choubey D: Activation of p53 in Human and Murine Cells by DNA-Damaging Agents Differentially Regulates Aryl Hydrocarbon Receptor Levels. Int J Toxicol 34: 242-249, 2015.
41. Shinde R, Shimoda M, Chaudhary K, Liu H, Mohamed E, Bradley J, Kandala S, Li X, Liu K and McGaha TL: B cell-Intrinsic IDO1 Regulates Humoral Immunity to T cell-Independent Antigens. J Immunol 195: 2374-2382, 2015.
42. Criado G, Simelyte E, Inglis JJ, Essex D and Williams RO: Indoleamine 2,3 dioxygenase-mediated tryptophan catabolism regulates accumulation of Th1/Th17 cells in the joint in collagen-induced arthritis. Arthritis Rheum 60: 1342-1351, 2009.
43. Scott GN, DuHadaway J, Pigott E, Ridge N, Prendergast GC, Muller AJ and Mandik-Nayak L: The immunoregulatory enzyme IDO paradoxically drives B cell-mediated autoimmunity. J Immunol 182: 7509-7517, 2009.
44. Xu H, Oriss TB, Fei M, Henry AC, Melgert BN, Chen L, Mellor AL, Munn DH, Irvin CG, Ray P, *et al*: Indoleamine 2,3-dioxygenase in lung dendritic cells promotes Th2 responses and allergic inflammation. Proc Natl Acad Sci USA 105: 6690-6695, 2008.
45. Merlo LMF, Pigott E, DuHadaway JB, Grabler S, Metz R, Prendergast GC and Mandik-Nayak L: IDO2 is a critical mediator of autoantibody production and inflammatory pathogenesis in a mouse model of autoimmune arthritis. J Immunol 192: 2082-2090, 2014.
46. Merlo LMF, DuHadaway JB, Grabler S, Prendergast GC, Muller AJ and Mandik-Nayak L: IDO2 Modulates T cell-Dependent Autoimmune Responses through a B Cell-Intrinsic Mechanism. J Immunol 196: 4487-4497, 2016.
47. Yoshida T, Katsuya K, Oka T, Koizumi S, Wakita D, Kitamura H and Nishimura T: Effects of AhR ligands on the production of immunoglobulins in purified mouse B cells. Biomed Res 33: 67-74, 2012.
48. Wourms MJ and Sulentic CEW: The aryl hydrocarbon receptor regulates an essential transcriptional element in the immunoglobulin heavy chain gene. Cell Immunol 295: 60-66, 2015.
49. Eleftheriadis T, Liakopoulos V, Antoniadi G, Stefanidis I and Galaktidou G: Indoleamine 2,3-dioxygenase is increased in hemodialysis patients and affects immune response to hepatitis B vaccination. Vaccine 29: 2242-2247, 2011.
50. Eleftheriadis T, Sparopoulou T, Antoniadi G, Liakopoulos V, Stefanidis I and Galaktidou G: Suppression of humoral immune response to hepatitis B surface antigen vaccine in BALB/c mice by 1-methyl-tryptophan co-administration. Daru 19: 236-239, 2011.
51. Patel AA, Zhang Y, Fullerton JN, Boelen L, Rongvaux A, Maini AA, Bigley V, Flavell RA, Gilroy DW, Asquith B, *et al*: The fate and lifespan of human monocyte subsets in steady state and systemic inflammation. J Exp Med 214: 1913-1923, 2017.