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ORIGINAL PAPER

Indoleamine 2, 3-dioxygenase Regulates the Differentiation of T Lymphocytes to Promote the Growth of Gastric Cancer Cells through the PI3K/Akt/mTOR Pathway

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Abstract

To investigate the regulatory mechanism of indoleamine 2, 3-dioxygenase (IDO) in T lymphocyte differentiation and its role in promoting the growth of gastric cancer (GC) cells through the PI3K/Akt/mTOR pathway. GC cell lines (MFC and NCI-N87) and PBMC cells were co-cultured and IDO inhibitor 1-methyl-tryptophan (1-MT) was added. The proliferation was detected by CCK-8, the apoptosis was detected by flow cytometry, and the contents of TNF-α, IL-1β, IL-6, IL-8, and INF-γ were detected by ELISA. The expression levels of PI3K, p-PI3K, Akt, p-Akt, mTOR, and p-mTOR were tested using Western blot, and the proportion of $CD4^+/CD8^+$, $CD4^+CD25^+Foxp3^+$Treg cells was detected by flow cytometry. C57BL/6 mice were used to establish the MFC GC mouse model and treated with 1-MT. The changes in body weight and tumor diameter were measured. Ki-67, $CD4^+$, $CD8^+$, and $CD25^+$ expressions were detected by immunohistochemistry. IDO promoted the proliferation of MFC and NCI-N87 cells, inhibited apoptosis, and decreased the levels of TNF-α, IL-1β, IL-6, IL-8, and INF-γ in the supernatant after co-culture with BPMC. The expressions of p-AKT, p-mTOR, and p-PI3K increased after 1-MT treatment. The proportion of $CD4^+/CD8^+$ cells was increased and the proportion of Treg cells was decreased in PBMC cells after the addition of 1-MT. Overexpression of IDO suppressed T cells differentiation by inhibiting the PI3K/Akt/mTOR pathway. In vivo, 1-MT treatment reduced the tumor size and weight, increased $CD4^+$ and $CD8^+$ positive area proportion, and decreased Ki-67 and $CD25^+$ positive area proportion. Co-culture of GC cells and immune cells promotes the proliferation of GC cells and inhibits apoptosis, which can be reversed by 1-MT. IDO may suppress the proliferation of T lymphocyte through inhibiting the PI3K/Akt/mTOR signaling pathway. This provides new evidence for the potential of exploiting IDO inhibitors for GC treatment.

Keywords Gastric cancer · PI3K/Akt/mTOR · Indoleamine 2, 3-dioxygenase · T lymphocytes · Immunotherapy · Tumor microenvironment

Introduction

Gastric cancer (GC) refers to the malignant tumor that occurs in the stomach, which is one of the most common malignant tumors [1]. The biological properties of gastric tumor cells are quite complex, and they can evade the surveillance of the immune system in all aspects [2]. Studies have shown that there is an increased proportion of $CD4^+CD25^+$Treg cells in peripheral blood, lymph nodes, and tumor-infiltrating area tissues of patients with GC, and the increased level is significantly correlated with the progression of disease and clinicopathology, which may play an important role in tumor escape and promote tumor growth [3]. Indoleamine 2, 3-dioxygenase (IDO) can regulate the proliferation and activation of $CD4^+$, $CD8^+$, $CD25^+$, and other T cells, thus playing a role in the proliferation, metastasis, and immune escape of tumor cells [4, 5]. Previous studies have shown that IDO induces immune tolerance of T cells to tumor antigen stimulation, and then indirectly inhibits the anti-tumor immune effect mediated by T cells, thus forming immune escape of GC cells [6]. In 1991, Candy's group reported the first

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competitive inhibitor of IDO, 1-methyl-tryptophan (1-MT) [7]. In 2003, Uyttenhove et al. [8] reported that a large number of tumor cell lines and primary tumors expressed IDO. Study also found that tumor cells transfected with IDO could prevent the rejection reaction of inoculated mice. Along with the lack of tumor site-specific T lymphocytes, 1-MT could partially inhibit these effects. It has confirmed that IDO expressed by tumor cells inhibited the proliferation of antigen-specific T lymphocytes to escape immune attack. IDO inhibitor is an immune checkpoint inhibitor of research value, which can enhance the function of tumor immunotherapy.

PI3K/Akt/mTOR is an important survival signal transduction pathway in cells, which plays an extremely important biological function in the process of cell growth, survival, proliferation, apoptosis, angiogenesis, etc [9]. The abnormality of this signaling pathway may lead to diseases such as cancer and autoimmune dysfunction. It has been found that MTORC1-dependent cholesterol biosynthesis mainly controls the proliferation of $CD4^+CD25^+$Treg cells by up-regulating the expression of $CD4^+CD25^+$Treg cell inhibitory molecules CTLA4 and ICOS [10]. IDO-mediated tryptophan catabolism inhibits immunomodulatory kinase mTOR, blocks tryptophan-sensitive inflammatory signals, and promotes tumor T cell tolerance [11, 12].

Therefore, this study aims to explore the mechanism of IDO regulating T lymphocyte differentiation and inhibiting the growth of GC cells through the PI3K/Akt/mTOR pathway, which is expected to be a new approach for tumor prevention and treatment.

Materials and Methods

Cell Culture

Mouse forestomach carcinoma (MFC) (Item No. ICell-M035) cells were purchased from iCell Bioscience Inc. (Shanghai, China). Human gastric cancer cell lines (NCI-N87) (Item No. CL-0169) were purchased from Wuhan Pricella Biotechnology Co., Ltd. (Wuhan, China). Cells were cultured in RPMI1640 complete medium containing 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin, and 100 U/mL penicillin.

Isolation of Primary PBMC and T lymphocytes

Primary PBMC and T lymphocytes were isolated by density centrifugation using mouse peripheral blood mononuclear cell isolation solution KIT (No. LDS1090, Tbdscience, China) and mouse peripheral blood lymphocyte separation KIT (No. LTS1092, Tbdscience, China), respectively. In brief, 2 mL of mouse blood samples were slowly added to 2 mL of the solution and centrifuged for 30 min at 450 g. After centrifugation, the centrifuge tube was divided into four layers from top to bottom. The ring-shaped milky mononuclear cell layer, or lymphocyte layer, in the centrifuge tube was carefully aspirated and transferred to a new centrifuge tube, and 10 mL washing solution was added. The cells were centrifuged at 250 g for 10 min, the supernatant was discarded, repeated twice, and the resulting cells were resuspended.

Cell Co-culture and Cell Grouping

The MFC, NCI-N87, PBMC, and T cells at the logarithmic growth stage were inoculated into 96-well plates with a density of 4×10^4/mL (100 µL/well), and cultured in a 5% CO_2 incubator at 37 °C and 95% humidity. A co-culture system between PBMC and GC cells was developed. The ratio of NCI-N87 or MFC to PBMC was 1:1, and the total number of cells per well was 5×10^4, and then co-cultured for 24 h under the above conditions. 1-MT (No. HY-16724, MedChemExpress, USA) and LY294002 (No. HY-10108, MedChemExpress, USA) were purchased from MedChemExpress.

Groups 1: (1) control group (MFC cells or NCI-N87 cells); (2) GC cells (MFC cells or NCI-N87 cells) + IDO inhibitors (adding 1-MT at the same time, 5 mmol/L, incubation for 24 h) group; (3) GC cells (MFC cells or NCI-N87 cells) + PBMC co-culture (MFC or NCI-N87 to PBMC ratio 1:1, incubation for 24 h) group; (4) GC cells (MFC cells or NCI-N87 cells) + PBMC co-culture + IDO inhibitor (1-MT, 5 mmol/L, incubation for 24 h) group, 4 replicates per group, and constant temperature culture at 37 °C and 5% CO_2.

Groups 2: (1) T cell group; (2) T cell + oe-IDO group; and (3) T cell + oe-IDO + LY294002 (PI3K pathway inhibitor) group.

CCK-8 Assay Detection for Cell Proliferation

MFC, NCI-N87, PBMC, and T cells at the logarithmic growth stage were taken and washed with PBS and collected after trypsin digestion. After PBMC suspension, cell suspension was collected and centrifuged at 250 *g* for 5 min. The supernatant was removed, and an appropriate amount of medium was added to make a single-cell suspension. The cell density was adjusted to 2×10^5 cells/well, and 2 mL/well was inoculated into a 6-well plate and incubated at 37 °C with 5% CO_2. After co-culture for 24 h, the supernatant (PBMC) was discarded, and CCK-8 solutions with a volume of 110 µL were added to each sample and incubated for 2 h at 37 °C and 5% CO_2. At 450 nm wavelength, the optical density (OD) value of each hole was detected with an enzyme labeler.

Flow Cytometry Detection for Cell Apoptosis

Cell apoptosis was detected using flow cytometry by the Annexin V-APC double staining apoptosis detection kit (Item No. KGA1030, Jiangsu KeyGEN BioTECH Corp., Ltd., China). After co-culture, the supernatant (PBMC) was discarded. The cells were washed with 1× PBS and suspended in 100 μL binding buffer. Add 5 μL Annexin V-FITC and 5 μL PI, and incubate at room temperature in the dark for 10 to 15 min. Finally, 500 μL of binding buffer was added to the sample, and the apoptosis rate was detected by flow cytometry (Cytoflex, Beckman, USA) within 1 h.

Enzyme-linked Immunosorbent Assay (ELISA)

The contents of TNF-α, IL-1β, IL-6, IL-8, and INF-γ in the cell supernatant were detected by ELISA kits according to the instructions. In brief, except for blank wells, the sample wells were incubated with horseradish peroxidase (HRP)-labeled antibodies for 60 min at 37 °C, and then 50 μL of each substrate A and B were added and incubated at 37 °C in the dark for 15 min. Finally, the OD values of each well were determined at a wavelength of 450 nm. Mouse TNF-α ELISA KIT (Item No. ZC-390240), Mouse IL-1β ELISA KIT (Item No. ZC-37974), Mouse IL-6 ELISA KIT (Item No. ZC-37988), Mouse IL-8 ELISA KIT (Item No. ZC-37953), Mouse IFN-γ ELISA KIT (Item No. ZC-37905), and Mouse IDO ELISA KIT (Item No. ZC-39078) were purchased from Shanghai ZCIBIO Technology Co., Ltd. (China).

Western Blot

The proteins associated with the PI3K/Akt/mTOR signaling pathway in bladder cancer were detected by Western blot. The cell culture supernatant was collected, and the PBMC cells were obtained by centrifugation, quantified by bicinchoninic acid assay (BCA) protein concentration detection kit, and separated by polyacrylamide gel electrophoresis. After electrophoresis, the membrane was transferred to polyvinylidene fluoride (PVDF) membrane. After sealing 5% skim milk powder, add the primary antibodies, refrigerate at 4 °C overnight, wash the film with TBST after rewarming, add the secondary antibody and incubate for 2 h. The images were collected by gel imager, and the results were analyzed by Quantity One software. Antibody information: Goat Anti-Rabbit IgG (H + L) HRP, No. S0001, 1:5000, Affinity, China; β-actin, No. AC026, 1:50000, Abclonal, China; AKT, No. A17909, 1:2000, Abclonal, China; mTOR, No. A2445, 1:1000, Abclonal, China; p-AKT, No. AP0098, 1:1000, Abclonal, China; PI3K, No. AF6241, 1:2000, Affinity, China; p-mTOR, No. AP0094, 1:1000, Abclonal, China; p-PI3K, No. AF3241, 1:2000, Affinity, China.

Flow Cytometry Detection for T cell Proportion

The cell culture supernatant was collected, and the PBMC cells were obtained by centrifugation. Setting groups 1: GC cells +PBMC co-culture group (MFC to PBMC ratio 1:1, incubation for 24 h), GC cells +PBMC co-culture +IDO inhibitor group (1-MT, 5 mmol/L, incubation for 24 h). Groups 2: T cell group, T cell+oe-IDO group, and T cell +oe-IDO + LY294002 (PI3K pathway inhibitor) group. The cell density was adjusted to 3×10^5/well, 2 mL/well was inoculated in a 6-well plate, and incubated at 37 °C and 5% CO_2. 24 h later, according to the group, the suspension was centrifuged 250 *g* for 5 min to obtain cell precipitation. 100 μL PBS suspension cells were added to each tube labeled with CD4, CD8, and CD25 antibodies (FITC anti-mouse CD4, No. 116003, Biolegend, USA; APC anti-mouse CD8a, No. 162305, Biolegend, USA; PE anti-mouse/rat/human FOXP3, No. 320007, Biolegend, USA; PerCP anti-mouse CD25, No. 102027, Biolegend, USA), incubated at 4 °C for 30 min in dark light, centrifuged at 300 *g* for 5 min, and the supernatant was discarded. 1 × True Nuclear Fixation Concentrate 500 μL was added to each tube and incubated at room temperature for 50 min. Centrifuge at 350 *g* for 5 min, then discard the supernatant. Each tube was washed twice with 250 mL 1 × True Nuclear Perm, centrifuged at 350 *g* for 5 min, 100 μL 1× True Nuclear Perm resuspension cells were added to each tube with the FOXP3 antibody label, incubated at 4 °C for 30 min, centrifuged at 350 *g* for 5 min, and the supernatant was discarded. Each tube was washed with 250 μL 1× True Nuclear Perm, centrifuged at 350 *g* for 5 min, the supernatant was discarded, and 300 μL 1× True Nuclear Perm resuspension cells were detected and analyzed by computer.

Establishment of GC Mouse Xenograft Model with MFC Cells

Twelve C57BL/6 mice were used in the tumor formation experiment of C57BL/6 mice to detect the tumor growth effect of GC in vivo. The sample size was calculated according to G*prower 3.1.9.7. At 80% efficacy, the total sample size required 12 mice to obtain a significant difference of $p = 0.05$. MFC cells were washed on a super-clean workbench with PBS and digested by 0.25% trypsin, then blown and mixed with a pipette gun and placed in a centrifuge with the centrifuge speed set at 1000 r/min and centrifuge time at 5 min. After centrifugation, the cell density was adjusted to 5×10^5/mL. Twelve C57BL/6 mice were disinfected with 75% ethanol in the right armpit and inoculated with 0.2 mL of MFC cell suspension. 5–6 days

after the establishment of the mouse model, nodular nodules in the right axillary inoculation site of the mice could be seen by the naked eye, and the nodules in the mice reached 5 mm × 5 mm on the 7th day after the inoculation of MFC cells. In the MFC + IDO inhibitor (1-MT) group, IDO inhibitor was intraperitoneally injected at 100 mg/kg once every 7 days for 14 days. 21 days later, the C57BL/6 mice were sacrificed by the cervical detachment method. The tumor was taken out, the weight of the tumor was measured, photographs were taken, and tumor samples were collected for detection. This study was approved by the Experimental Ethics Committee of West China Hospital of Sichuan University and met the provisions of national experimental animal welfare ethics (Ethics number: 20231030001).

Immunohistochemistry

The tumor samples were sectioned at a thickness of 2 mm and mounted on a coated slide. After incubation at 60 °C for 30 min, heat-induced antigen repair was performed. The tissues were dewaxed and rehydrated by soaking in xylene and a series of ethanol in different concentrations. The endogenous peroxidase activity was blocked by H_2O_2, and goat serum sealer was added to the mixture at room temperature for 20 min. Ki-67, $CD4^+$, $CD8^+$, and $CD25^+$ antibodies were added at 4 °C overnight. Wash with PBS for 3 times, 5 min each time; drop the second antibody, 37 °C for 30 min; wash with PBS for 3 times, 5 min each time. The antigen complex was shown by binding goat serum with the Envision Flex Kit. Image-Pro Plus software was used to digitally quantify the percentage of stained area in total patch area and staining intensity.

Statistical Analysis

SPSS 26.0 software was used for statistical analysis, and the data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to evaluate the differences between multiple groups. A difference of $p < 0.05$ was considered statistically significant.

Results

IDO Promoted Cell Viability and Inhibited the Apoptosis of GC Cells

The effect of IDO on the proliferation of GC cell lines (MFC and NCl-N87 cells) was detected by CCK-8 assay. Compared with the control group, the cell viability of MFC and NCl-N87 cells was decreased after adding IDO inhibitor (1-MT), and the cell viability of MFC + PBMC and NCl-N87 + PBMC co-cultured GC cells was increased ($P < 0.01$). The cell viability of MFC and NCl-N87 cells was decreased after co-culture with PBMC + 1-MT ($P < 0.01$). These results indicate that IDO promotes the viability of GC cells, as shown in Fig. 1a, c. The effect of IDO on the apoptosis of GC cell lines (MFC and NCl-N87 cells) was detected by flow cytometry. Compared with the control group, the apoptosis rate of MFC and NCl-N87 cells increased after 1-MT was added ($P < 0.01$), and the apoptosis rate of MFC + PBMC and NCl-N87 + PBMC decreased ($P < 0.05$). Compared with MFC + PBMC or NCl-N87 + PBMC group, the apoptosis rate increased after adding 1-MT ($P < 0.01$). These results indicate that IDO inhibits apoptosis of MFC and NCl-N87 cells, as shown in Fig. 1b, d. In brief, IDO promoted cell viability and inhibited the apoptosis of GC cells.

IDO Promoted the Immune-inflammatory Response in MFC Cells

To investigate the effect of IDO on immune-inflammatory response of MFC cells, the levels of TNF-α, IL-1β, IL-6, IL-8, INF-γ, and IDO in cell culture supernatant were detected by ELISA. As shown in Fig. 2a–e, the results showed that compared with the control group, the levels of TNF-α, IL-1β, IL-6, IL-8, and INF-γ in the cell culture supernatant decreased after 1-MT treatment ($P < 0.01$), the expression level of MFC + PBMC increased ($P < 0.05$). Compared with MFC + PBMC, the contents of TNF-α, IL-6, IL-8, INF-γ, and IL-1β decreased after 1-MT was added ($P < 0.05$). In addition, co-cultured with 1-MT decreased the levels of IDO compared with the MFC + PBMC group ($P < 0.01$), as shown in Fig. 2f. These results suggested that IDO promoted the immune-inflammatory response in GC cells.

IDO Regulated T lymphocyte Differentiation Through Inhibiting the PI3K/Akt/mTOR Pathway

To investigate the molecular mechanism by which IDO regulates T cell differentiation, Western blot and flow cytometry were performed. Western blot analysis showed that compared with the control group, the expression levels of p-AKT, p-mTOR and p-PI3K in cell culture supernatant after 1-MT treatment increased, and the differences were statistically significant ($P < 0.05$). Compared with MFC + PBMC, the expression levels of p-mTOR and p-PI3K increased after 1-MT addition, and the differences were statistically significant ($P < 0.05$). These results suggested that IDO inhibited the PI3K/Akt/mTOR in GC cells, as shown in Fig. 3a–d. The proportions of $CD4^+/CD8^+$ and $CD4^+CD25^+Foxp3^+$Treg cells in PBMC cells were detected by flow cytometry. Compared with MFC + PBMC cells, the proportions of $CD4^+/CD8^+$ cells in PBMC cells were increased after 1-MT was added ($P < 0.05$) (Fig. 3e).

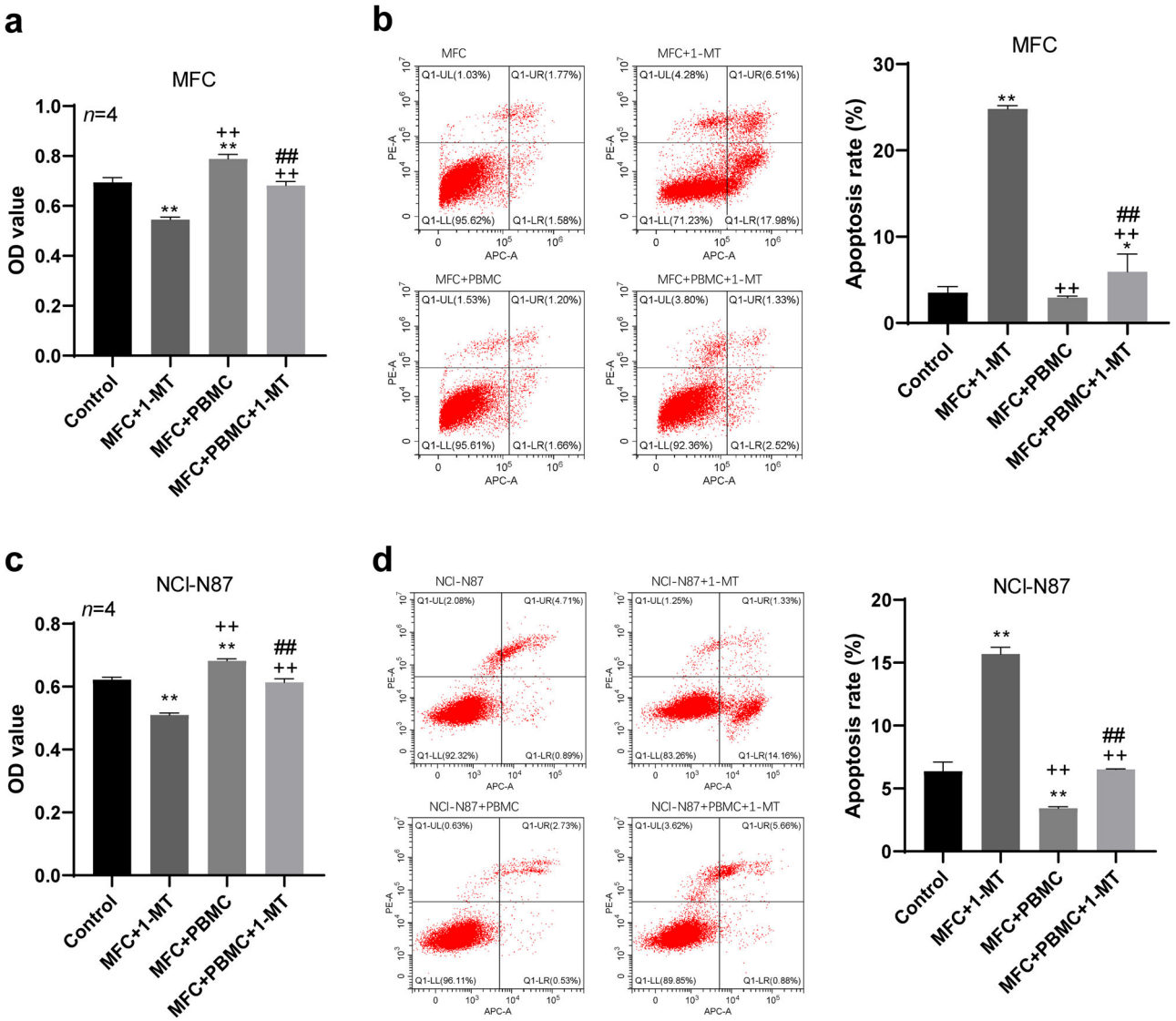

Fig. 1 IDO promoted cell viability and inhibited the apoptosis of gastric cancer (GC) cells. CCK-8 assay was used to detect MFC cells (**a**) and NCl-N87 cells (**c**) proliferation. Flow cytometry was used to detect MFC cells (**b**) and NCl-N87 cells (**d**) apoptosis. Data were expressed as mean ± SD ($n = 4$ each group). Compared with the Control group, $^{*}P < 0.05$, $^{**}P < 0.01$; Compared with MFC + 1-MT group, $^{+}P < 0.05$, $^{++}P < 0.01$; Compared with MFC + PBMC + 1-MT group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$

The proportion of $CD4^{+}CD25^{+}Foxp3^{+}$Treg cells was decreased ($P < 0.01$), as shown in Fig. 3f.

In addition, to confirm that IDO regulates T lymphocyte differentiation by inhibiting the PI3K/Akt/mTOR pathway, T cells transfected with overexpression of IDO were treated with the PI3K pathway inhibitor LY294002. As shown in Fig. 4a, compared with the normal T cells, over-expression of IDO significantly reduced the viability of T cells, which was further reduced by the addition of LY294002 ($P < 0.01$). Compared with the normal T cells, over-expression of IDO decreased the ratio of $CD4^{+}/CD8^{+}$ cells, and further decreased after LY294002 treatment, while over-expression of IDO increased the ratio of Treg cells, and further increased after LY294002 treatment ($P < 0.05$) (Fig. 4b). Western blot showed that over-expression of IDO decreased the expression of p-AKT, p-mTOR, and p-PI3K proteins compared with the normal T cells, and further decreased after LY294002 treatment (Fig. 4c–f). In summary, these results suggested that IDO suppressed T lymphocyte differentiation through inhibiting the PI3K/Akt/mTOR pathway.

IDO Regulated T-lymphocyte Differentiation to Promote Tumor Growth in MFC Cell Transplanted Tumor-bearing GC Mice

Mice pattern to establish 5–6 days later, the naked eye can see inoculated mice on the right side of axillary appeared

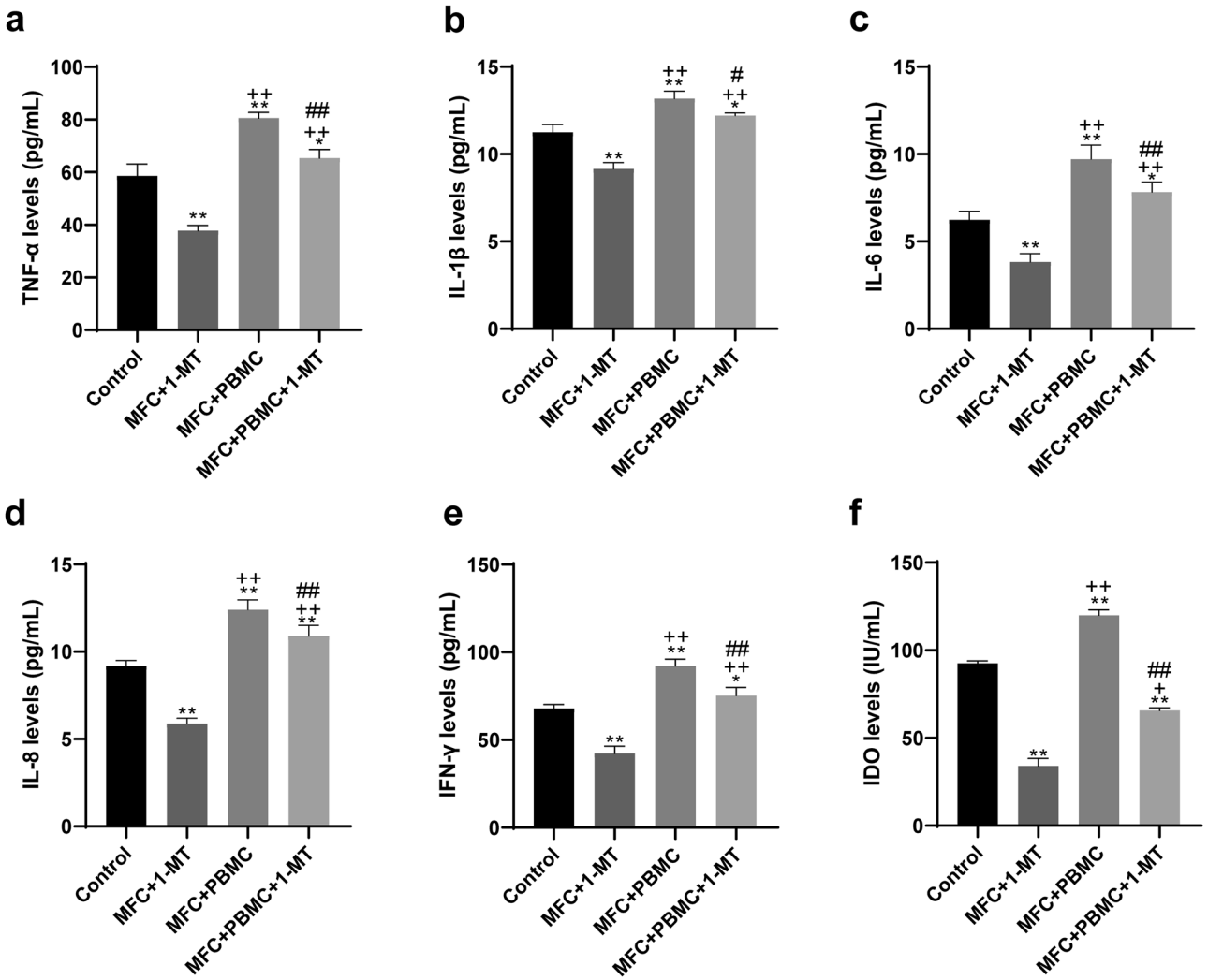

Fig. 2 IDO promoted the immune-inflammatory response in MFC cells. The inflammation factors levels of TNF-α (**a**), IL-1β (**b**), IL-6 (**c**), IL-8 (**d**), INF-γ (**e**), and IDO (**f**) in MFC cells co-cultured with PBMC cells were detected using ELISA. Data were expressed as mean ± SD ($n = 3$ each group). Compared with the Control group, $^{*}P < 0.05$, $^{**}P < 0.01$; Compared with MFC + 1-MT group, $^{+}P < 0.05$, $^{++}P < 0.01$; Compared with MFC + PBMC + 1-MT group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$

nodular plaque, in the seventh day of inoculation of MFC cells in mice nodules are reaching 5 mm × 5 mm. Tumor diameter was measured on days 7, 14, and 21 of vaccination. The results showed that compared with the control group (MFC), the body weight of mice injected with IDO inhibitor 1-MT increased ($P < 0.05$) 21 days later, as shown in Fig. 5a. The results of tumor diameter measurement showed that, compared with the control group, the tumor size and weight of mice added 1-MT were significantly reduced on the 14th and 21th days ($P < 0.01$), as shown in Fig. 5b–d. After 21 days, the C57BL/6 mice were sacrificed. The expression of Ki-67 was detected by immunohistochemistry in two groups of transplanted tumors. Compared with the MFC group, the expression levels of $CD4^{+}$ and $CD8^{+}$ were increased and $CD25^{+}$ were decreased after 1-MT treatment, with statistical significance ($P < 0.05$), as shown in Fig. 5e, f. At the same time, compared with the MFC group, the expression of Ki-67 decreased after 1-MT treatment ($P < 0.01$), as shown in Fig. 5e, g. In short, IDO promoted tumor growth in GC mice by inhibiting T-lymphocyte differentiation.

Discussion

The occurrence and development of GC are closely related to the state of anti-tumor immune function of the body, and its occurrence is often accompanied by the decline of immune function or the suppression of immune function to different degrees, so that the tumor can escape the attack of the immune system [13]. Although surgery is still one of the main methods for the treatment of GC, immunotherapy is

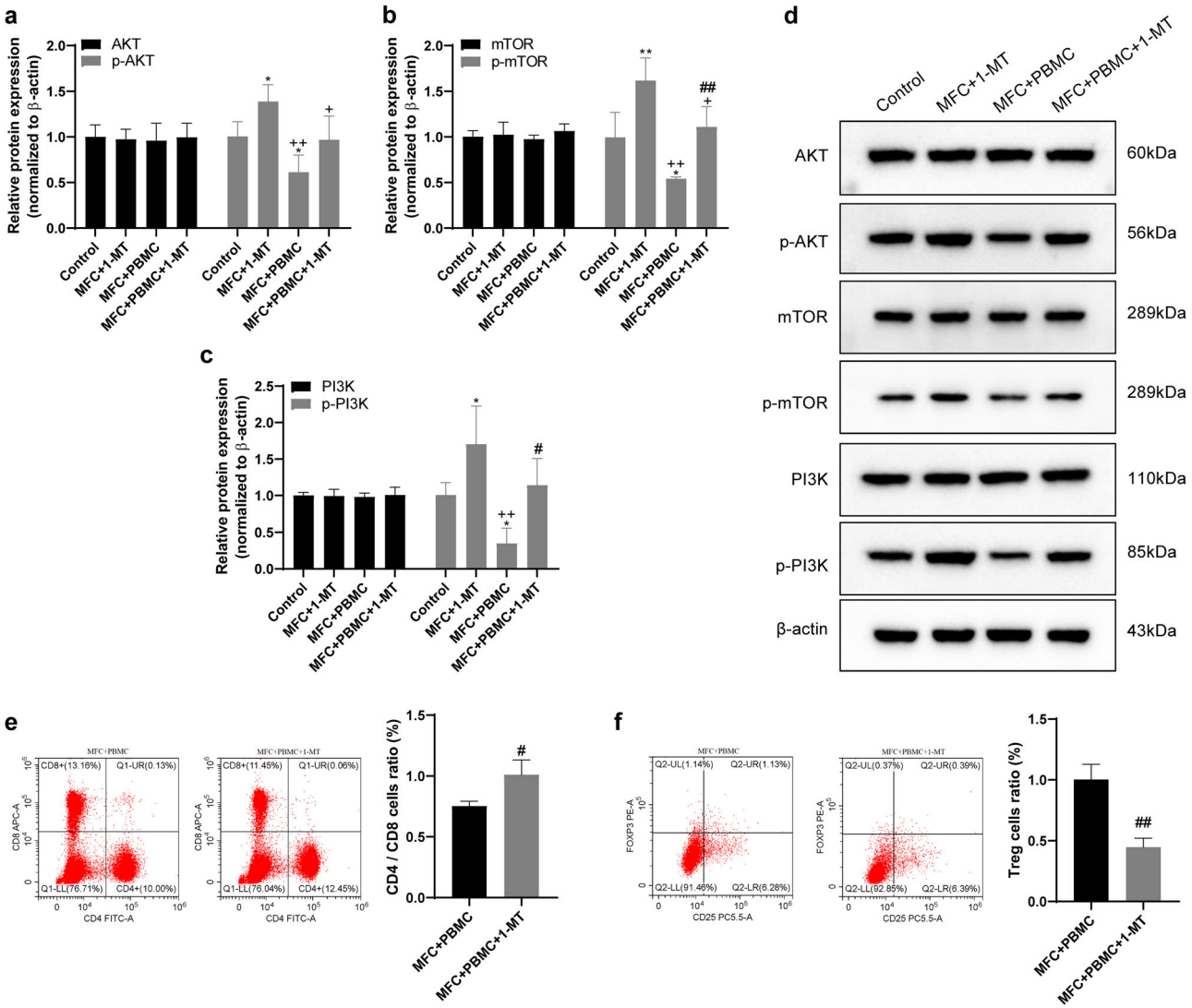

Fig. 3 IDO regulated T lymphocyte differentiation through inhibiting the PI3K/Akt/mTOR pathway. **a–d** Western blot was used to detect the AKT/mTOR/PI3K pathway related proteins (AKT, p-AKT, mTOR, p- mTOR, PI3K, p-PI3K) in MFC cells co-cultured with PBMC cells. Flow cytometry to detect the proportion of $CD4^+/CD8^+$ (**e**) and $CD4^+CD25^+Foxp3^+$Treg (**f**) cell in PBMC cells. Data were expressed as mean ± SD ($n = 3$ each group). Compared with the Control group, $^{*}P < 0.05$, $^{**}P < 0.01$; Compared with MFC + 1-MT group, $^{+}P < 0.05$, $^{++}P < 0.01$; Compared with MFC + PBMC + 1-MT group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$

playing an increasingly important role as an important part of adjuvant therapy for cancer. In tumor immunity, the change of tumor microenvironment can lead to local immune tolerance of tumor, and the abnormal expression of various molecules and cells plays an important role in the formation of local immune tolerance of tumor [14]. In recent years, studies on IDO and memory T cells have confirmed that the abnormal expression of IDO and memory T cells in the tumor microenvironment has very important significance for the immune tolerance, occurrence, and development of tumors.

IDO is a cellular enzyme containing heme iron, mainly distributed in the thymus medulla and T lymphocyte region of secondary lymphoid organs, and scattered in some immune tolerance or immunity tissues, such as the thymus, gastrointestinal mucosa, epididymis, placenta, and anterior chamber, etc [15]. It plays a very important role in tumor immune escape [16, 17]. Under normal conditions, IDO is expressed at a low level. In the processes of inflammation, infection, tumor and pregnancy, the expression of IDO is significantly increased, and it plays an important role in metabolic immunomodulation by degrading tryptophan in local tissues, inducing host immune defense, inhibiting T lymphocyte immunity and anti-tumor immunity, inducing maternal fetal immune tolerance and graft immune tolerance [18]. Tumors can evade the surveillance of the immune system through a variety of ways, among which IDO and memory T cells are two important factors that have attracted

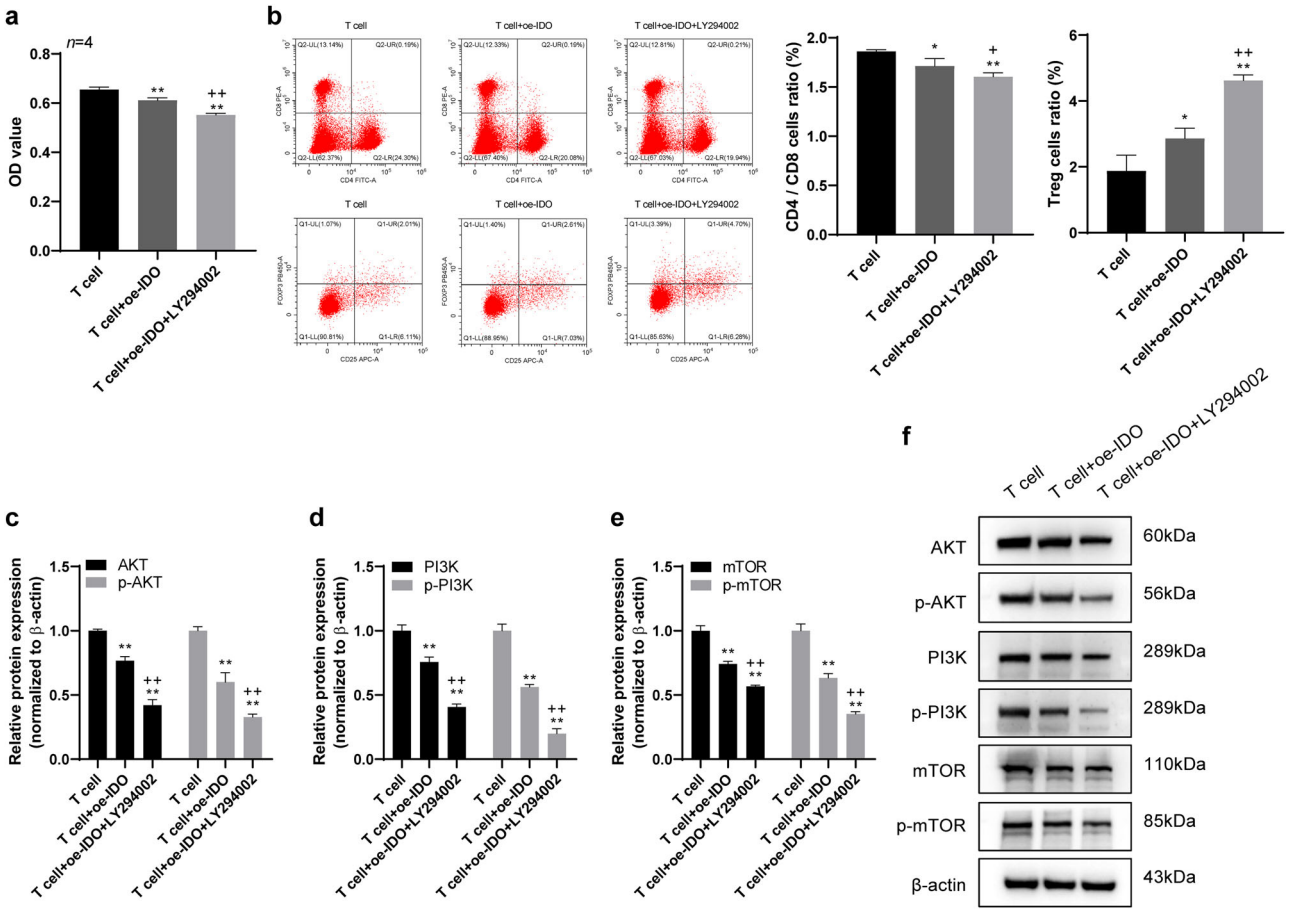

Fig. 4 Over-expression of IDO suppressed T cells differentiation by inhibiting the PI3K/Akt/mTOR pathway. T cells transfected with overexpression of IDO were treated with the PI3K pathway inhibitor LY294002. **a** CCK-8 assay was used to detect T cells viability. **b** Flow cytometry to detect the proportion of $CD4^+/CD8^+$ and $CD4^+CD25^+Foxp3^+$Treg cell in T cells. **c**–**f** Western blot was used to detect the AKT/mTOR/PI3K pathway related proteins (AKT, p-AKT, mTOR, p- mTOR, PI3K, p-PI3K) in T cells. Data were expressed as mean ± SD ($n = 3$ each group). Compared with the T cell group, $^*P < 0.05$, $^{**}P < 0.01$; Compared with T cell+oe-IDO group, $^+P < 0.05$, $^{++}P < 0.01$

much attention in the mechanism of tumor immune escape in recent years. Both of them play an important role in the occurrence and development of tumors, and Treg cells have a significant impact on the regulation of both.

Cancer cells can enhance proliferation and angiogenesis by directly or indirectly interacting with tumor microenvironment components, thereby preventing apoptosis, avoiding hypoxia, and developing immune tolerance [19]. Tumor growth, immune escape, and tumor microenvironment are inextricably linked. Therefore, the characterization of cellular infiltration in the tumor microenvironment is critical for predicting the response to immune checkpoint blockade therapy and improving the success rate of therapy. In order to analyze the effects of IDO on GC cells and immune cells, this study used mouse GC cells (MFC and NCI-N87) and peripheral blood mononuclear cells (PBMC) to construct a co-culture model of GC cells and immune cells by mixed inoculation, according to the ratio of PBMC: GC was 1:1. The effects of IDO on the biological behavior of GC cells were further analyzed at the cellular level, and the proliferation and apoptosis of cells in each group were respectively detected in this study. The results showed that IDO significantly promoted the proliferation of GC cells and inhibited apoptosis. In addition, the proliferation ability of GC cells was further promoted after PBMC co-culture. However, after the addition of 1-MT, the proliferation of GC cells was inhibited and the apoptosis rate increased. A large number of studies have confirmed that IDO is increased in some solid tumor cells and malignant tumors of the blood system, accompanied by the suppression of local T lymphocyte proliferation, thus mediating tumor cells to escape the attack of the immune system [20–22]. Moreover, IDO inhibitor 1-MT can partially reverse this effect [23]. Combined with previous literature reports and the results of this study, it is suggested that the co-culture of immune cells and GC cells can promote the malignant biological behavior of GC cells, promote the proliferation and invasion of GC cells, and inhibit apoptosis, and 1-MT can reverse this effect.

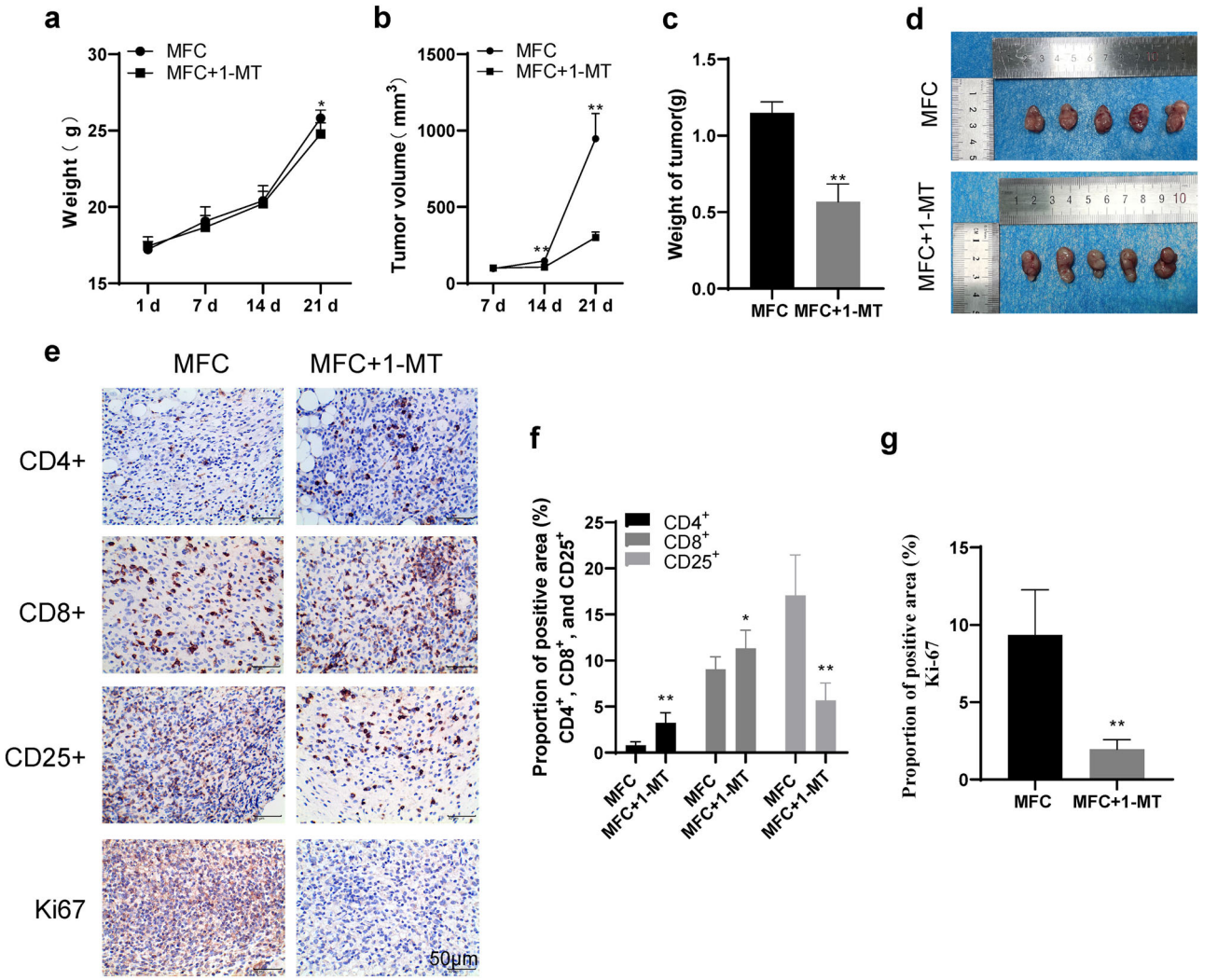

Fig. 5 IDO regulated T-lymphocyte differentiation to promote tumor growth in MFC cell transplanted tumor-bearing GC mice. **a** Body weight of mice. **b** Tumor volume. **c** Tumor weight. **d** Schematic diagram of the tumor. **e** Immunohistochemical detection of Ki-67, $CD4^+$, $CD8^+$, and $CD25^+$ in tumors and Schematic representation. **f** $CD4^+$, $CD8^+$, and $CD25^+$ positive area proportion. **g** Ki-67 positive area proportion. Data were expressed as mean ± SD ($n = 6$ each group). Compared with the MFC group, $^*P < 0.05$, $^{**}P < 0.01$

Tumor cells can attract antigen-presenting cells to gather around them, such as monocyte-derived macrophages and dendritic cells. Studies have found that IDO can be induced by lipopolysaccharide and cytokines in these cells. Tumor cells not only express IDO themselves, but also attract IDO-rich antigen-presenting cells [24]. Tumor-associated antigen-presenting cells gather around tumor tissues and secrete IDO to induce the immune tolerance of T cells to tumor antigen stimulation, thereby indirectly inhibiting the anti-tumor immune effect mediated by T cells, thereby forming the immune escape of tumor cells. In the tumor microenvironment, high expression of IDO can inhibit the immune response of local T cells. T lymphocytes mediate specific cellular immunity and can specifically recognize antigens, thus proliferating and differentiating into effector cells, eliminating non-self antigen substances or inducing immune tolerance, so as to maintain the normal physiological state of the body [25]. Chen et al. [26] found that inducing high expression of IDO in melanocytoma led to an increase in tryptophan metabolites. However, in vitro studies have shown that the proliferation of T cells is inhibited, the activity of T cells is reduced and even apoptosis occurs in the absence of tryptophan and/or kynurenine. In order to further understand whether T lymphocytes are related to IDO in GC cell immune response, this study co-cultured PBMC cells and MFC cells, and added 1-MT to observe the role of IDO in tumor immune response. The results showed that the cell activity of T lymphocytes increased in co-culture, but reversed after adding 1-MT. The reason may be that 1-MT as an inhibitor of IDO can weaken the inhibitory effect of IDO on the proliferation of T lymphocytes and increase the cytotoxic activity of T lymphocytes.

IDO is commonly overexpressed in malignant tumors of epithelial origin and plays a role in promoting tumor immune tolerance [27]. For example, IDO and kynurenine pathway metabolites promote the activation of PI3K-AKT signaling pathway in tumor colonic epithelium, thereby inhibiting cell apoptosis and promoting cancer cell proliferation [27]. In addition, gene silencing and overexpression experiments showed that IDO1 promoted cardiomyocyte hypertrophy by activating the protein synthesis pathway through the AKT-mTOR-S6K1 signaling axis [28]. Through the detection of PI3K/Akt/mTOR path-related expression proteins, it was found that the expression of p-AKT, p-mTOR and p-PI3K increased after the inhibition of IDO level. Meanwhile, it was found that the ratio of $CD4^+/CD8^+$ cells increased and the ratio of Treg cells decreased in the MFC + PBMC group after 1-MT treatment. In addition, our results suggest that IDO regulates the proliferation and secretion of T cells through inhibiting the PI3K/Akt/mTOR signaling pathway in GC. Further detection through animal experiments showed that compared with the blank control group, after 1-MT intervention, the expression levels of CD4+ and CD8+ were increased, as well as $CD25^+$ and ki-67 were decreased in cancer tissues. Our study suggested that IDO promoted tumor growth in GC mice in vivo by inhibiting T-lymphocyte differentiation. However, there are some limitations in this study. Only the expression of $CD4^+$, and $CD8^+$, $CD25^+$ was detected by immunohistochemistry in this study, and the proportions of $CD4^+$, and $CD8^+$, $CD25^+$ cells in peripheral blood of mice and control mice will be further analyzed in the future.

Conclusion

Co-culture of GC cells and immune cells can not only improve the proliferation of GC cells, but also inhibit cell apoptosis. IDO may regulate T lymphocyte differentiation and promote the growth of GC cells through PI3K/Akt/mTOR signaling pathway, which can be inhibited by IDO inhibitor 1-MT. This study lays the necessary theoretical and experimental foundation for further finding new drug targets and developing new immunotherapy drugs for GC.

Data Availability

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

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Competing interests The authors declare no competing interests.

Ethics approval and consent to participate This study was approved by the Experimental Ethics Committee of West China Hospital of Sichuan University and meted the provisions of national experimental animal welfare ethics (Ethics number: 20231030001).

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Abbreviations

GC	gastric cancer
IDO	indoleamine 2, 3-dioxygenase
1-MT	1-methyl-tryptophan
ELISA	enzyme-linked immunosorbent assay

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