Gain-of-function of IDO in DCs inhibits T cell immunity by metabolically regulating surface molecules and cytokines

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Received September 15, 2022; Accepted February 24, 2023

DOI: 10.3892/etm.2023.11933

Abstract. Both tolerogenicity and immunogenicity of dendritic cells (DCs) are regulated by their intracellular metabolism. As a rate-limiting enzyme of tryptophan (Trp) metabolism, indoleamine 2,3-dioxygenase (IDO) is involved in regulating the functions of numerous cell types, including DCs, a subset of which has a high capacity for producing IDO to control over-activated inflammation. To identify the mechanisms of IDO in DCs, stable DC lines with both gain- and reduction-of-function of IDO were established using a recombinant DNA technique. Although the IDO variation did not affect DC survival and migration, it altered Trp metabolism and other features of DCs analyzed by high-performance liquid chromatography and flow cytometry. On the surface of the DCs, IDO inhibited co-stimulatory CD86 but promoted co-inhibitory programmed cell death ligand 1 expression, and suppressed the antigen uptake, which ultimately led to the compromised ability of DCs to activate T cells. Furthermore, IDO also suppressed IL-12 secretion but enhanced that of IL-10 in DCs, which eventually induced T cells into tolerogenic phenotypes by inhibiting the differentiation of Th1 but promoting that of regulatory T cells. Collectively, the findings of the present study demonstrated that IDO is a key molecule for tolerogenic DC induction by metabolically regulating surface molecule and cytokine expression. This conclusion may lead to the targeted development of therapeutic drugs for autoimmune diseases.

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Key words: dendritic cells, gene recombination, indoleamine 2,3-dioxygenase, tryptophan metabolism, T cell immunity

Introduction

Indoleamine 2,3-dioxygenase (IDO) is the first and rate-limiting metabolic enzyme catabolizing tryptophan (Trp), and the kynurenine (Kyn) synthesis pathway (KP) is the metabolic pathway responsible for the majority of Trp catabolism (1). Previous studies have suggested that KP generates several bioactive catabolites with immunosuppressive properties (2), such as Kyn, 3-hydroxykynurenine, kynurenic acid, 3-hydroxyanthranilic acid (3-HAA), xanthurenic acid and quinolinic acid (QA), collectively termed Kyns (3). Of note, IDO is widely expressed at low levels under normal conditions and induced mainly at the site of inflammation (4). Moreover, the inflammation-activated Trp metabolism can cause changes in the systemic and intra- and extracellular Kyn/Trp ratios, which in turn create an environment of high local or systemic Kyn and low Trp content. The Kyn/Trp ratio is an indicator of Trp degradation and reflects the activity of IDO under different conditions (5). It can directly affect metabolic and immune signaling pathways. The functions of adjacent cells (e.g., T cells) are also changed by increasing local or systematic environments in Kyn and decreasing in Trp (6). For example, Trp depletion enables the production of anti-inflammatory cytokines by IDO-expressing dendritic cells (DCs) and macrophages, promotes the recruitment of regulatory T cells (Tregs), and prevents T cell activation and proliferation. In addition, the increase of Kyn can cause the differentiation of T cells toward Tregs and induce the apoptosis of effector T cells (7).

Furthermore, numerous studies have demonstrated that IDO is expressed in numerous cell types, including endothelial cells, vascular smooth muscle cells, macrophages, leucocytes, DCs, multiple types of cancer cells, and maternal-fetal interface cells (8). Of note, DCs are known to express the highest levels of IDO among immune cells (9). However, the

ability of DCs to produce IDO does not seem to be equally distributed among the different subsets. For example, CD8α or CD103-positive DCs express more IDO and establish immune tolerance compared to CD8α or CD103-negative DCs. Similarly, plasmacytoid DCs (pDCs) have a strong ability to produce IDO and mediate immunosuppression in specific settings (9,10). Conversely, DCs expressing low levels of IDO inhibit Treg development and T cell apoptosis (11). In brief, DCs are heterogeneous and exhibit both tolerogenicity and immunogenicity, due to the difference in IDO expression in DC subsets (12). Furthermore, IDO expression induces a stable, regulatory phenotype in DCs, which are designated as tolerogenic DCs (tolDCs), promoting the spread of regulatory functions to cells other than DCs (13).

Of note, the fate of the immunogenicity or DC tolerance, which are demonstrated by their extracellular functions are ultimately determined by their intracellular metabolism (14). For example, active oxidative phosphorylation in mitochondria is associated with immature DCs or tolDCs, while increased pathogen-sensitive glycolysis can promote the functions of immature DCs (15). As the rate-limiting metabolic enzyme of Trp catabolism, IDO is preferentially expressed in DCs that can mediate T(h) cell apoptosis for immune suppression (9). However, the exact roles of IDO in the immunological functions of DCs remain to be determined. In the present study, the endogenous expression of IDO was genetically altered in myeloid DCs to determine its variation on the overall functions of DCs. To this end, IDO-overexpressing DCs (IDO^{oe}DCs) and IDO-knockdown DCs (IDO^{kd}DCs) were successfully constructed using the recombinant DNA technique. It was further evaluated how IDO in DCs influenced Trp-Kyn metabolism, phenotype, function and related regulatory mechanism to provide a theoretical basis for the application of modified DC vaccines to the treatment of autoimmune diseases.

Materials and methods

Mice and cells. A total of 18 healthy male C57BL/6 mice (age, 6-8 weeks; weight, 18±5 g) and a total of 6 healthy male Kunming mice (age, 6-8 weeks; weight, 20±5 g) were purchased from BoYuan Laboratory Animal Ltd (Hefei, China), bred and maintained in a specific pathogen-free environment (temperature, 25±2˚C; humidity, 55±5%; 12 h light-dark cycles; and freely access to food and water) in the animal facility of Anhui Normal University (Wuhu, China). All animal health and behavior were monitored every 1 or 2 days. A total of 24 healthy male mice (age, 8-10 weeks; weight, 20±5 g) were anesthetized with pentobarbital sodium by intraperitoneal injection at a dose of 40 mg/kg (0.036 g/ml), and then painlessly sacrificed by cervical dislocation when they met the following criteria: rapid drop in body temperature and difficulty breathing. Absence of heartbeat and pupil dilation for 5 min was used to confirm death. The total experimental period was about four months. The experimental procedures were performed in accordance with the conditions specified and approved by the Animal Experimentation Ethics Committee of Anhui Normal University.

DC2.4 cells are an immature DC cell line derived from C57BL/6 mouse bone marrow progenitor cells cultured under GM-CSF conditions (16) and kindly provided by Professor Dong Yongjun of Tsinghua University (Beijing, China).

Cell cultures. The cells were cultured using standard methods as previously described (17) with slight modifications. Briefly, spleen cells were separated from Kunming mice, and the immature murine DC line, DC2.4, and its transduced derivatives were cultured in RPMI1640 medium supplemented with 100 U/ml penicillin, 100 mg/l streptomycin, 2 mmol/l L-glutamine, 50 μM 2-ME and 10% FBS.

Generation of IDO-encoded lentivirus. The complete mouse *IDO* sequence was cloned into the EcoRI and NheI restriction sites of pLJM-enhanced green fluorescent protein (EGFP) vector (pLJM1-EGFP#19319) (Fig. S1), whereas short hairpin knockdown construct for IDO was synthesized and cloned into a lentiviral vector pLKO.1 (pLKO.1-TRC cloning vector#10878) (Fig. S2). Following transformation into *E. coli* cells, these two recombinant constructs were amplified, and positive clones identified by reverse transcription-quantitative PCR (RT-qPCR; Fig. S3A and B), before the correct DNA inserts were confirmed again by sequencing (Fig. S4A). Furthermore, the confirmed *IDO*-modifying constructs and two packaging plasmids [psPAX2 (psPAX2#12260) and pCMV-VSV-G (pCMV-VSV-G#8454)] were co-transfected into 70-80% confluent 293FT human embryonic kidney cells in the presence of Hieff Trans® Liposomal Transfection Reagent (cat. no. 40802ES03; Shanghai Yeasen Biotechnology Co., Ltd.) at 37˚C for 48 h to produce competent first-generation lentiviral particles, which were harvested following transfection. Cell fragments in the lentivirus containing supernatants were removed by centrifugation (2,000 x g; 10 min; 4˚C) and filtered through a 0.45 mm cellulose acetate filter for DC infection. The pLKO.1-TRC cloning vector#10878, pLJM1-EGFP#19319, psPAX2#12260, and pCMV-VSV-G#8454 were kindly supplied by Professor Ye Shoudong from Anhui University as gifts (Hefei, China). The shRNA sequences were synthesized by Sangon Biotech Co., Ltd., as presented in Table I.

Lentiviral infection of DCs. For lentiviral transfection, $1x10^5$ DC2.4 cells were cultured in 24-well plates. The next day, once cells had reached 60-70% cell confluence, the first-generation lentiviral solution encoding *IDO or shIDO* was added on to the cultured DC2.4 at a multiplicity of infection (MOI) of 6 with lentiviral: cell solution of 1:1, and co-continued at 37˚C (5% CO_2) for 24 h in the presence of polybrene (5 μg/ml, cat. no. HB-PB-05, Hanbio Biotechnology Co., Ltd.), before the medium was replaced with 2 ml fresh complete RPMI1640 medium. After 72 h, puromycin [cat. no. 60209ES60; Yeasen Biotechnology (Shanghai) Co., Ltd.] was added to screen the infected DC2.4 cells at 2 μg/ml the following day. The overexpressed IDO or knockdown DC2.4 was assessed by RT-qPCR for mRNA expression, and by flow cytometry to determine intracellular protein expression.

Total RNA extraction and RT-qPCR analysis. As previously described (18), total RNA was extracted from gene-modified DCs and reverse-transcribed into complementary DNA (NovoScript® Plus All-in-one 1st Strand

Table I. Sequences of shRNAs.

Gene	Sequence (5'-3')
shRNA-IDO-F	CCGGCCTCGCAATAGTAGATACTTACTCGAGTAAGTATCTACTATTGCGAGGTTTTTG
shRNA-IDO-R	AATTCAAAAACCTCGCAATAGTAGATACTTACTCGAGTAAGTATCTACTATTGCGAGG
shRNA-Scramble-F	CCGGAATTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAATTTTTTTG
shRNA-Scramble-R	AATTCAAAAAAATTCTCCGAACGTGTCACGTCTCGAGACGTGACACGTTCGGAGAATT

F, forward; IDO, indoleamine 2,3-dioxygenase; R, reverse; shRNA, short hairpin RNA.

cDNA Synthesis SuperMix; Suzhou Novoprotein) according to the manufacturer's instructions. RT-qPCR with SYBR Green detection (NovoStart® SYBR qPCR SuperMix Plus; Suzhou Novoprotein) was performed using a qPCR detection system (cat. no. CFX96; Bio-Rad Laboratories, Inc. or LightCycler® 96; Roche Diagnostics GmbH) to quantify RNA expression. All data are expressed relative to β-actin, which served as the internal control. Data analysis was performed using LightCycler® 96 SW 1.1 Software (Roche Diagnostics GmbH) or CFX Manager Software (Bio-Rad Laboratories, Inc.). The $2^{-\Delta\Delta Cq}$ method was used to determine relative expression. The primers used are listed in Table II.

Table II. Primer sequences used for quantitative PCR.

Gene product	Sequence (5'-3')
IDO-forward	GAGAGTACATGCCTCCAGCC
IDO-reverse	CTCTTCCGACTTGTCGCCAT
β-actin-forward	TCATCACTATTGGCAACGAGC
β-actin-reverse	AACAGTCCGCCTAGAAGCAC

IDO, indoleamine 2,3-dioxygenase.

Trp and Kyn levels. The total activity of IDO was evaluated by measuring the levels of Trp (μmol/l; Shanghai Macklin Biochemical Co., Ltd.) and kyn (μmol/l; Shanghai Macklin Biochemical Co., Ltd.), whose concentrations in the supernatants of gene-modified DCs were measured by high-performance liquid chromatography (HPLC; LC-20A HPLC; Shimadzu Corporation), as previously described (19). The mobile phase was 15 mM acetic acid-sodium acetate buffer (pH 4) containing 8% acetonitrile (v/v). The UV monitoring wavelengths of Trp and Kyn were 280 and 360 nm, respectively. Kyn/Trp was calculated by relating the concentrations of Kyn to those of Trp, which allows for estimating IDO activity.

Determination of cell viability by annexin V/propidium iodide (PI) staining. As previously described (20), $1x10^6$ $vector^{ctrl}$DCs (control group) and IDO^{kd}DCs (IDO-knockdown DCs group) were treated with lipopolysaccharide (LPS) overnight, trypsinized and washed with PBS three times. Next, centrifugation was performed at 670 x g for 5 min. The cells were suspended in 500 μl standard buffer and then stained with Annexin V-FITC and PI (Annexin V-FITC/PI Apoptosis Detection Kit; 7Sea Biotech Co., Ltd.) for 15 min at room temperature. The fluorescence intensity of FITC and PI was measured quantitatively for every test using a BD Canto II flow cytometer or BD FACS Melody flow cytometer (BD Biosciences). Flow Jo software (Flow Jo version 10.6.2) was used to analyze the data. Flow cytometry was performed to detect the effect of IDO on the viability of DCs using four quadrants: PI-positive and Annexin V-FITC-negative necrotic cells; PI and Annexin V-FITC-positive late apoptotic cells; PI and Annexin V-FITC-negative normal living cells; and Annexin V-FITC-positive and PI-negative early apoptotic cells (21).

Assessment of DC migration in vivo. As previously described (22), $10x10^6$ $vector^{ctrl}$DCs or IDO^{kd}DCs were labelled with carboxyfluorescein succinimidyl ester (CFSE) at 37˚C for 10 min and treated with LPS overnight, before they were injected into the footpads of 6 healthy male C57BL/6 mice ($vector^{ctrl}$DCs, age, 8-10 weeks, n=3; IDO^{kd}DCs, age, 8-10 weeks, n=3). After 24 h, mice were anesthetized with 40 mg/kg (0.036 g/ml) sodium pentobarbital and then sacrificed. Single-cell suspensions were prepared from popliteal lymph nodes, and CFSE-positive cells of were detected by flow cytometry.

Immunophenotypic analysis by flow cytometry. Flow cytometry of the DC immunophenotype and intracellular expression of IDO was performed. As previously described (23), gene-modified DCs were stained with different combinations of monoclonal antibodies against PD-L1 (cat. no. 10F.9G2-BV421; BioLegend), CD86 (cat. no. GL1-FITC; eBioscience; Thermo Fisher Scientific, Inc.) and CD40 (cat. no. FGK45-PE; BioLegend) with the addition of PI (1 $\mu g\ ml^{-1}$) for phenotyping. Alternatively, these cells were stained intracellularly using rat purified anti-mouse IDO antibody (cat. no., mIDO-48; dilution, 1:200; BioLegend) and goat anti-rat IgG (H+L) cross-adsorbed secondary antibody, Alexa Fluor™ 488 (cat. no. A-11006; dilution, 1:500; Invitrogen; Thermo Fisher Scientific, Inc.). Analysis of the expression levels of targeted proteins was performed using a BD Canto II flow cytometer or BD FACS Melody flow cytometer (BD Biosciences). The data was analyzed using Flow Jo software (Flow Jo version 10.6.2).

Analysis of the uptake ability of DCs. The uptake ability of DCs was examined as previously described (22) with slight modifications. Briefly, $0.2x10^6$ gene modified DCs were

stimulated with LPS (1 μg/ml) and cultured with fluorescent chicken ovalbumin (OVA-FITC; 50 μg/ml; Beijing Boshi Technology Co., Ltd.) in 500 ml complete RPMI1640 medium for 4 h at 37˚C. Following washing with phosphate-buffered saline, OVA-FITC by vector^{ctrl}DC or IDO^{kd}DC phagocytosis was detected by flow cytometry. DCs incubated with OVA-FITC at 4˚C were used as the negative control. Flow Jo software (Flow Jo version 10.6.2) was used to analyze the data.

ELISA assay. As previously described (24), IL-12, IL-6, interferon gamma (IFN-γ), and IL-10 levels in cell culture supernatant were determined using ELISA kits (R&D Systems, Inc.) according to the manufacturer's instructions. Briefly, vector^{ctrl}DCs or IDO^{kd}DCs were stimulated with LPS (1 μg/ml) and incubated at 37˚C for 24 h with CD4$^+$T cells, before the supernatants were collected, and their cytokine contents were measured using the ELISA kits.

T cell proliferation assay. T-cell proliferation was performed using standard methods as previously described (18) with slight modifications.

T-cell proliferation in situ (Ki67 assay): Healthy male C57BL/6 mice were sensitized via an intraperitoneal administration of 500 μg OVA protein once per week for 2 consecutive weeks. Three days after being last sensitized, 1x10^6 vector^{ctrl}DCs, or IDO^{kd}DCs pulsed with OVA protein (100 μg/ml) and stimulated with LPS (1 μg/ml) were intravenously injected into OVA sensitized mice (vector^{ctrl}DC control group, 8-10-week-old, n=3; IDO^{kd}DC-treated group, 8-10-week-old, n=3). After 72 h, mice were anesthetized with sodium pentobarbital at a dose of 40 mg/kg (0.036 g/ml), and then sacrificed. Splenic cells were separated and incubated with FACS antibodies against CD3e (cat. no. 145-2C11-PE-Cy7; eBioscience; Thermo Fisher Scientific, Inc.), CD4 (cat. no. GK1.5-PE; Invitrogen; Thermo Fisher Scientific, Inc.) and CD8 (cat. no. 1953-6-7-APC; BioLegend) for 30 min on ice. Next, cells were fixed and permeabilized in commercial solutions (cat. no. 51-2090KZ, BDBD Biosciences), and punched with Perm Buffer III (cat. no. 558050, BD Biosciences). Next, cells were stained intracellularly with Ki67 (cat. no. SoLA15-FITC; BioLegend) on ice for 30 min. Cell analysis was performed using a flow cytometer.

CD4$^+$T cell proliferation in vitro (CFSE assay): The proliferation of T cells *in vitro* was examined using standard methods, as previously described (25) with slight modifications. Briefly, male Kunming mice (age, 8-10 weeks; n=6) were anesthetized using sodium pentobarbital at a dose of 40 mg/kg (0.036 g/ml) and then sacrificed. Spleens of mice were extracted to make a single cell suspension. T cells were stained with FACS antibodies against CD3 and CD4 for 30 min on ice. Subsequently, CD4$^+$T cells >99% purity were FACS-sorted and labeled with CFSE (cat. no. C34554; CellTrace™ CFSE Cell Proliferation Kit; Invitrogen; Thermo Fisher Scientific, Inc.) for 10 min at 37˚C, and washed three times. Gene-modified DCs of C57BL/6 origin were treated with LPS for 16 h, followed by 10 g/ml mitomycin C (MMC) for 1 h, before they were co-cultured with the CFSE stained CD4$^+$T cells at a ratio of 1:10 in a 96-well plate. Three days later, the CFSE dilution of CD4$^+$T cells was examined using flow cytometry. The proliferated CD4$^+$T cells were calculated by the number of acquired CFSE^{low} CD4$^+$T cells x added BD calibrate APC beads/acquired bead number.

T cell differentiation assays in vivo. T cell differentiation assays *in vivo* were performed as previously as described (25,26) with slight modifications. A total of 1x10^6 OVA-pulsed LPS-treated gene-modified DCs were intravenously injected into 6 healthy male OVA-sensitized C57/BL mice, which were divided into two groups: The vector^{ctrl}DC-ctrl group (age, 8-10 weeks; n=3) and the IDO^{kd}DC-treated group (age, 8-10 weeks; n=3). After 72 h, mice were anesthetized with sodium pentobarbital at a dose of 40 mg/kg (0.036 g/ml), and then sacrificed. Spleens of mice were extracted to make a single cell suspension. The single cell suspension was obtained and cultured for 4-6 h in the presence of phorbol 12-myristate 13-acetate (PMA), ionomycin and brefeldin A. Next, cells were stained with CD3 and CD4 followed by fixation and permeabilization, and stained intracellularly with anti-mouse IL-17A (cat. no. TC11-18H10-APC; BD Pharmingen; BD Biosciences), IL-10 (cat. no. JES5-16E3-APC; BD Pharmingen; BD Biosciences), IL-4 (cat. no. 11B11-APC; BD Pharmingen; BD Biosciences), and IFN-γ (cat. no. XMG1.2; BD Pharmingen; BD Biosciences) mAb. Flow cytometry was performed using a flow cytometer. Flow Jo software (Flow Jo version 10.6.2) was used to analyze the data.

Statistical analysis. Data are expressed as the mean ± SEM; all experiments were repeated at least 3 times with similar results. Statistical differences were performed using an unpaired Student's t-test. Prism 6.01 (GraphPad Software, Inc.) was used for the statistical analysis of all data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Successful construction of stable DC lines with altered IDO expression and Trp-Kyn metabolism. To identify the role of IDO in DC functions, the intracellular expression of this rate-limiting enzyme was genetically altered in the Kyn pathway of DCs by both gain- and reduction-of-function approaches. The CD region of the *ido* gene was amplified and cloned into an overexpressing lentiviral vector, pLJM-EGFP. Similarly, a short hairpin knockdown construct for IDO was created by synthesizing *shIDO* and cloning it into a lentiviral vector, pLKO.1. The transfection efficiency of the EGFP-containing construct was evaluated under a fluorescence microscope (Fig. S4B). Finally, stable cell lines of IDO^{oe}DCs and IDO^{kd}DCs were established by infecting the DCs with the aforementioned competent lentiviral particles. Finally, their expression of the IDO gene, protein and IDO enzyme activity were examined by RT-qPCR, flow cytometry and HPLC, respectively.

First, it was demonstrated that IDO^{oe}DCs were successfully established via a lentiviral infection of DCs with recombinant pLJM-IDO plasmid, expressing more IDO transcripts than that of mock transduced DCs with control vector, Vector^{Ctrl}DCs (Fig. 1A). In addition, the successful overexpression of the *ido* gene in the IDO^{oe}DCs was further verified in protein expression (Fig. 1B). Conversely, following infection of lentivirus containing recombinant pLKO.1-shIDO

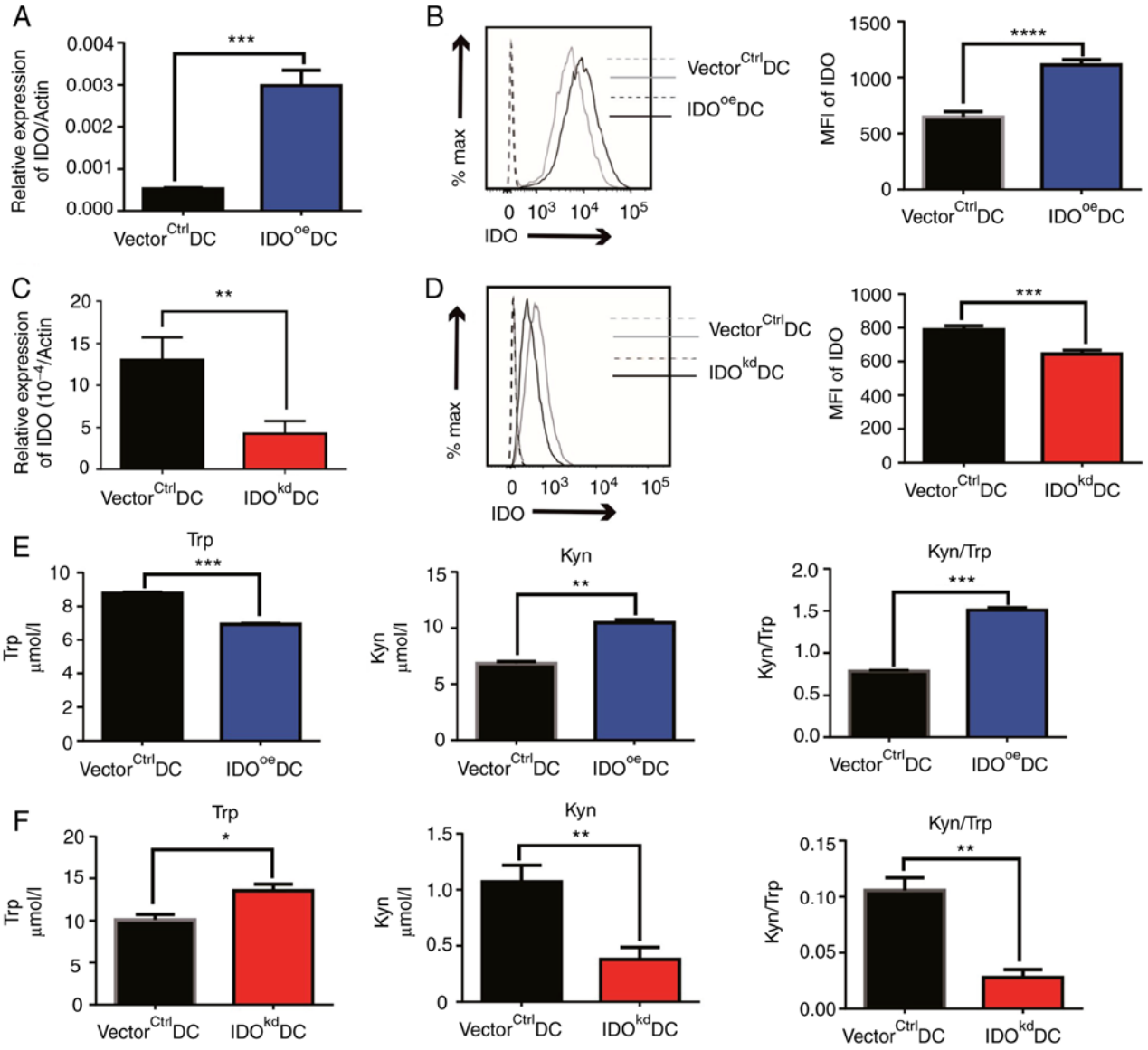

Figure 1. Effects of IDO on the Trp-Kyn metabolism of DCs *in vitro*. (A) DCs were infected with lentivirus containing pLJM1 or pLJM1-IDO plasmids to obtain Vector^{Ctrl}DCs or IDO^{oe}DCs, respectively. IDO expression of Vector^{Ctrl}DCs and IDO^{oe}DCs was detected by qPCR. (B) IDO protein expression on the two types of DCs in (A) was detected by flow cytometry (dotted line, unstained control; solid line, stained group). Representative histograms (left) and statistics (right) are shown. (C) DCs were infected with lentivirus containing pLKO1 or pLKO1-shIDO plasmids to obtain vector^{ctrl}DCs and IDO^{kd}DCs. IDO expression was detected by qPCR. (D) IDO protein expression on the two types of DCs in (C) was detected by flow cytometry. Representative histograms (left) and statistics (right) are shown. (E) Detection of Trp (left) and Kyn (center) concentrations, and the Kyn to Trp ratio (right) in the culture medium samples of Vector^{Ctrl}DCs and IDO^{oe}DCs cultured for 48 h by HPLC. (F) Detection of Trp (left) and Kyn (center) concentrations, and the Kyn to Trp ratio (right) in the culture medium samples of vector^{ctrl}DCs and IDO^{kd}DCs cultured for 48 h by HPLC. The results are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Each graph is representative of 2-3 independent experiments. DC, dendritic cell; HPLC, high-performance liquid chromatography; IDO, indoleamine 2,3-dioxygenase; Kyn, kynurenine; MFI, mean fluorescence intensity; qPCR, quantitative PCR; Trp, tryptophan; Vector^{Ctrl}DCs, DCs infected with control vector of pLJM1-EGFP; IDO^{oe}DCs, IDO-overexpressing DCs; vector^{ctrl}DCs, DCs infected with control vector of pLKO.1; IDO^{kd}DCs, IDO-knockdown DCs.

plasmid that can knock-down *ido* gene expression in the target cells, IDO^{kd}DCs exhibited a significantly compromised expression of the IDO transcript and protein than the DCs infected with virus containing control vector (vector^{ctrl}DCs; Fig. 1C and D). Furthermore, the activities of the IDO enzyme were also examined in the genetic variants by calculating the ratio of Kyn/Trp in the culture medium samples of DCs. HPLC detection demonstrated that Trp concentration was significantly lower in IDO^{oe}DCs than in Vector^{Ctrl}DCs. By contrast, the Kyn concentration and Kyn/Trp ratio were significantly higher in the IDO^{oe}DC group, as compared with Vector^{Ctrl}DCs (Figs. 1E and S5A). Of note, Trp concentration was significantly higher while Kyn concentration and Kyn/Trp ratio in IDO^{kd}DCs was significantly lower than that of vector^{ctrl}DCs (Figs. 1F and S5B), indicating a compromised activity in the Kyn pathway with a reduced IDO expression, which was not caused by the viability of DCs transduced with different vectors as the modified DCs had a similar survival rate (Fig. S6A). In combination, these data suggested that IDO expression was not only successfully modified, but its activity was also altered in terms of Trp-Kyn metabolism in DCs.

DC-derived IDO inhibits T cell proliferation both in vitro and in vivo. As professional APCs, the prime function of DCs is to stimulate T cells to activate adaptive immunity. We asked whether the proliferation of T cells was affected by the altered IDO-related Trp-Kyn metabolism in DCs. To this end, OVA protein (500 μg/mouse) was intraperitoneally injected into C57BL/6 mice once a week for 2 consecutive weeks. Next, $1x10^6$ gene-modified DCs were pulsed with 100 μg/ml OVA protein, stimulated with 1 μg/ml LPS and injected intravenously into the OVA-sensitized mice. Their trafficking of injected DCs was then examined, and the expression of Ki-67, a marker of proliferation, on T cells *in vivo* was detected by flow cytometry. While the migration of the DCs *in vivo* was not affected by IDO knockdown (Fig. S6B), it was found that the adoptive transfer of OVA-pulsed IDO-sufficient DCs (vector^{ctrl}DCs) can effectively stimulate the proliferation of CD4$^+$T and CD8$^+$T cells in the OVA-primed mice, demonstrating a successful establishment of the antigen-specific activation of T cells *in vivo* by DCs (Fig. 2A and B). However, when IDO^{kd}DCs with limited IDO amounts and activity in this working animal model was injected, the proportion and number of proliferating CD4$^+$Ki67$^+$T and CD8$^+$Ki67$^+$T cells in mice were significantly enhanced (Fig. 2A and B). To exclude the possibility of environmental interference from other cell lineages *in vivo*, CD4$^+$T cell proliferation was assessed using a CFSE dilution *in vitro*. IDO^{kd}DCs were co-cultured with CFSE-stained allogeneic CD4$^+$T cells sorted for 3 days using a flow sorting apparatus, it was then found that the proliferation of CD4$^+$T cells co-cultured with IDO^{kd}DCs was higher than that of T cells co-cultured with vector^{ctrl}DCs, regardless of LPS stimulation, as measured by the percentage of CFSE low population that entered the cell cycle (Fig. 2C). In combination, these data suggested that IDO deficiency with compromised enzymatic activity for cellular Trp-Kyn metabolism exerted great effects on the T cell stimulating function of DCs, both *in vivo* and *in vitro*.

IDO inhibits phagocytosis, downregulates CD86 and upregulates PD-L1 in DCs. The suppressive effect of DC-derived IDO on T cell proliferation prompted us to investigate the mechanism underlying the intracellular Trp metabolic abnormality due to the IDO variation. Since DCs stimulate proliferation by presenting phagocytosed antigen on their surface, in the presence of a series of co-receptors with either stimulatory or inhibitory functions, we wanted to examine the impact of IDO on both antigen phagocytosis and co-receptor expression in DCs. To this end, both vector^{ctrl}DCs and IDO^{kd}DCs were co-cultured with OVA-FITC for 4 h, before the OVA uptake capacity by the DCs was analyzed using flow cytometry. It was found that IDO^{kd}DCs had a stronger uptake capacity of OVA antigen compared with vector^{ctrl}DCs, implying that suppressed Trp metabolism due to reduced IDO inhibit the phagocytosis of DCs. The MFI of this is shown in a bar chart (Fig. 3A). Next, we studied the impact of altered IDO expression on the surface molecules of DCs. The co-stimulatory molecules of IDO^{oe}DCs and IDO^{kd}DCs were detected by flow cytometry, and it was found that, compared with Vector^{Ctrl}DCs, CD40 was unchanged, CD86 was downregulated and PD-L1 was upregulated in IDO^{oe}DCs. The MFI of this is shown in a bar chart (Fig. 3B). Conversely, the expression of CD86 on the surface of IDO^{kd}DCs was higher, PD-L1 was lower and no change was observed in CD40, when compared with vector^{ctrl}DCs (Fig. 3C). Collectively, these data suggested that IDO in DCs suppressed their antigen uptake, and inhibited stimulatory but promoted co-inhibitory molecules on DCs, which ultimately resulted in diminished T cell proliferation.

Diminished Kyn metabolism in IDO^{kd}DCs skews T cell differentiation toward proinflammatory phenotypes. The upregulation of PD-L1 on DCs by IDO suggests their tolerogenic potential. Consistently, as the first and rate-limiting enzyme in Trp metabolism, IDO plays an important role in immune tolerance (27,28), and Trp metabolism-generated metabolite Kyn promotes Treg differentiation, which may also affect Th17 and Th1/Th2 cell generation from T cells (29). Therefore, it was studied whether, in addition to T cell proliferation, T cell differentiation was also affected by IDO-related Trp-Kyn metabolism in DCs. To check this, OVA-pulsed vector^{ctrl}DCs or IDO^{kd}DCs were intravenously injected into C57BL/6 mice sensitized by an intraperitoneal administration of OVA antigen twice. After 72 h, the spleens of mice were extracted to make a single cell suspension, treated with PMA/ionomycin, and analyzed by flow cytometry for intracellular IFN-γ, IL-17A, IL-4, and IL-10, as indicators of Th1, Th17, Th2, and Treg subsets, respectively. It was found that, as compared to vector^{ctrl}DCs, the administration of IDO^{kd}DCs significantly up-regulated CD4$^+$IFN-γ$^+$Th1 cells, but down-regulated CD4$^+$IL-4$^+$Th2 and CD4$^+$IL-10$^+$Treg cells, whereas CD4$^+$IL-17A$^+$Th17 cells remained unchanged (Fig. 4A and B). Consistent with the above data *in vivo*, it was also confirmed that IDO had a similar effect on DC-stimulated T cell differentiation *in vitro*, as supernatants from the co-culture of IDO^{kd}DCs and CD4$^+$T cells expressed more IFN-γ and less IL-10 compared with the vector^{ctrl}DCs *in vitro* (Fig. 4C). In combination, these data suggested that the absence of IDO may impair the tolerogenic activities of DCs and consequently skew T-cell differentiation toward Th1 cells away from Tregs, possibly via the IDO-related Trp-Kyn metabolism.

IDO inhibits IL-12 but promotes IL-10 production from DCs. Since DC-mediated T cell differentiation is driven by a number of polarizing cytokines secreted by them, the impact of IDO on the production of these cytokines in DCs was investigated. Vector^{ctrl}DCs or IDO^{kd}DCs were treated with LPS (1 μg/ml) and incubated at 37˚C for 24 h; their cytokine production in the supernatants was then quantified using ELISA. It was found that IDO^{kd}DCs secreted more Th1 polarizing IL-12p70 (Fig. 5A) but less Treg polarizing IL-10 (Fig. 5B) than that of vector^{ctrl}DCs, whereas the production of Th17 polarizing cytokine IL-6 was similar between the two lentiviral-infected DCs (Fig. 5A). The differential impacts of IDO reduction on the production of the three cytokines suggested that IDO specifically affects Th1-polarizing IL-12 and Treg-polarizing IL-10 secretion from DCs, which fit in well with their downstream T cell differentiation profiles. These data demonstrated molecular evidence to account for the tolerogenic regulation of DC-mediated T cell differentiation by IDO.

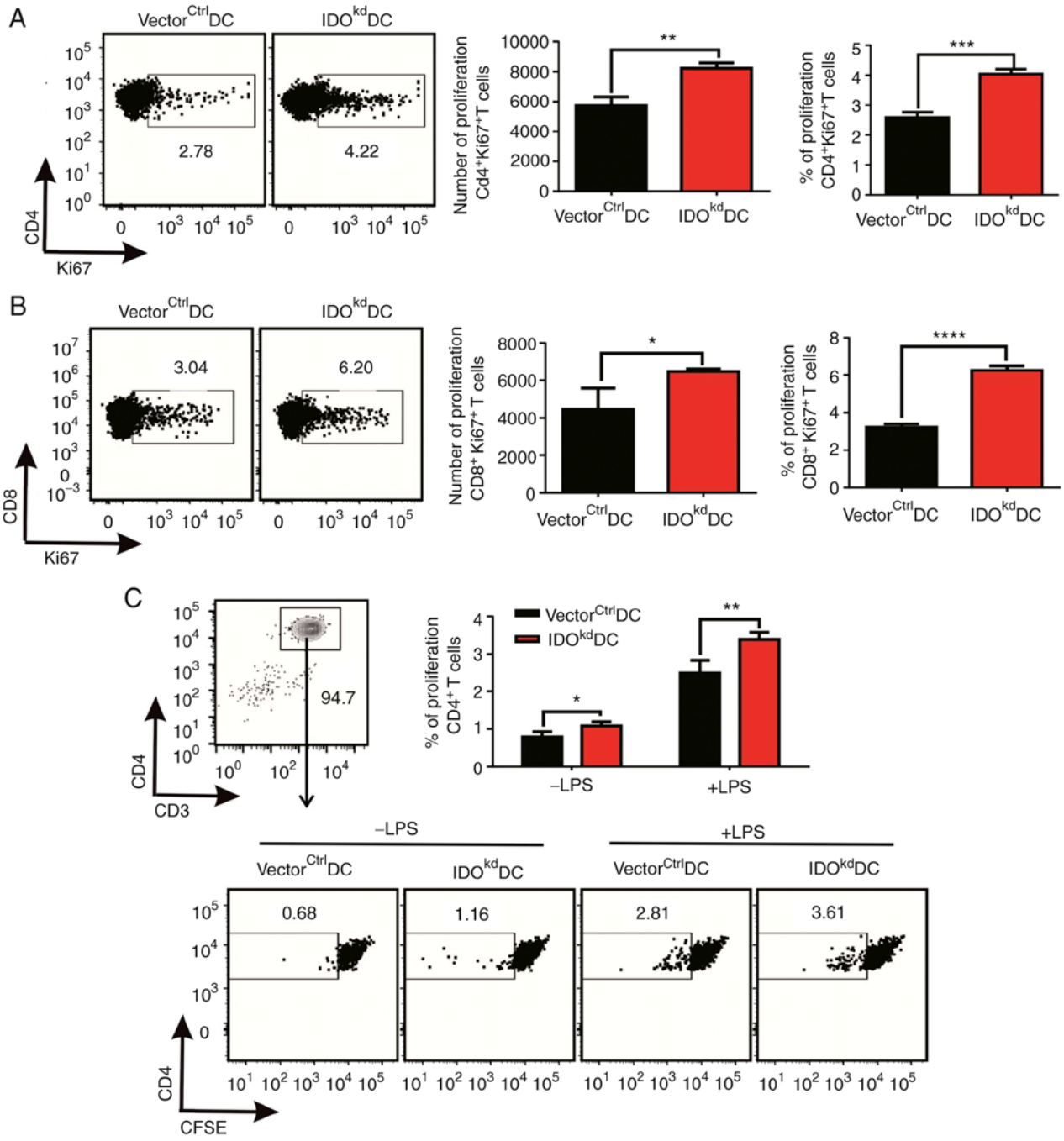

Figure 2. Effects of IDO on DC-stimulated T cell proliferation *in vivo* and *in vitro*. A total of 1x10^6 OVA-pulsed vector^{ctrl}DCs or IDO^{kd}DCs were intravenously injected into OVA-sensitized mice (n=3 mice per group). After 72 h, spleens of mice were extracted to make a single cell suspension, and flow cytometry was conducted to analyze the percentage and number of (A) CD4$^+$Ki67$^+$T cells and (B) CD8$^+$Ki67$^+$T cells. Statistics are shown on the right. (C) Effects of IDO on DC-stimulated CD4$^+$T cell proliferation in mixed lymphocyte reaction. (C) Single cell suspensions from the spleens of Kunming mice were made and stained with Anti-PE-cy7-CD3 and Anti-PE-CD4 monoclonal antibodies, before CD3$^+$CD4$^+$T cells were sorted using a flow sorting apparatus (left panel). DCs of C57/B6 mice origin were treated with LPS overnight, before treatment with mitomycin C at a final concentration of 10 μg/ml for 1 h. Afterwards, the DCs were co-cultured with CFSE-stained allogeneic CD4$^+$T cells sorted by flow cytometry at a ratio of 1:10 for 3 days, and flow cytometry was employed to analyze the percentage of CFSE$^+$ cells. (C) Representative scatter plot (bottom) with CD3$^+$CD4$^+$T cells gated to show the expression levels of CFSE and statistical analysis of multiple experiments (right). The results are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. CFSE, carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; LPS, lipopolysaccharides; OVA, ovalbumin; vector^{ctrl}DCs, DCs infected with control vector of pLKO.1; IDO^{kd}DCs, IDO-knockdown DCs; PE, phycoerythrin.

Discussion

Several studies have shown that, as the rate-limiting enzyme in cell metabolism, IDO plays key roles in DC biology (9,30). For example, Hwu *et al* (31) found that activated DCs can express IDO using Northern blot analysis of IDO mRNA and HPLC to detect production of functionally active IDO. Subsequently, Terness *et al* (32) proposed the term 'IDO-expressing dendritic cells'. Likewise, many scholars also used the term 'IDO-expressing dendritic cells' to study the role of IDO-expressing DCs in immune related diseases (33,34). IDO-expressing DCs could be

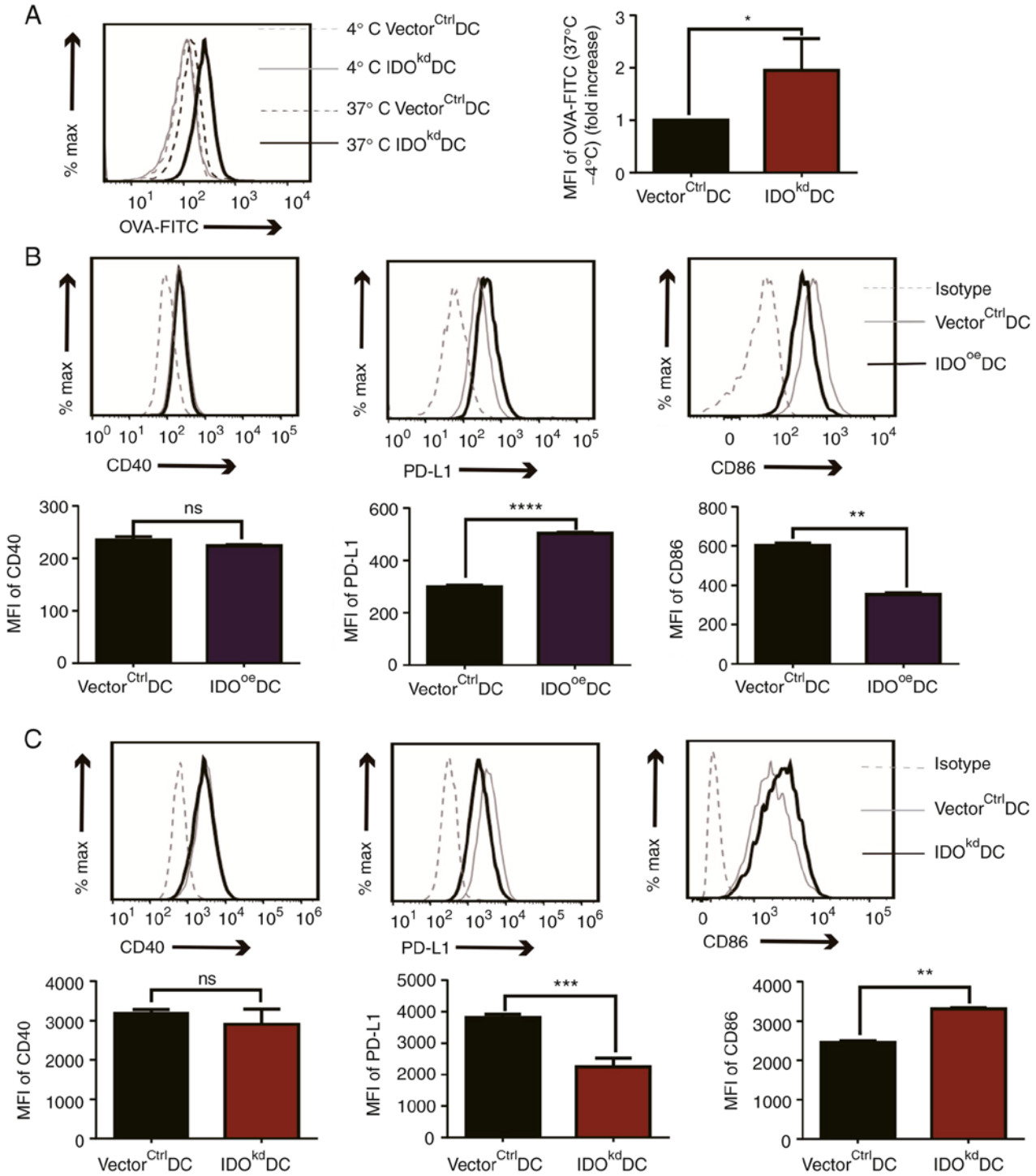

Figure 3. Effects of IDO on the phagocytosis and phenotype of DCs. (A) Phagocytosis of OVA-FITC by the genetically modified DCs was analyzed by FACS after co-c ulture at 37˚C for 4 h. The culture environment at 4˚C was used as a control. (B) Both Vector^{Ctrl}DCs and IDO^{oe}DCs were treated with LPS overnight, and the MFI of CD40, CD86 and PD-L1 on the surface of IDO^{oe}DCs was detected by flow cytometry. (C) Both vector^{ctrl}DCs and IDO^{kd}DCs were treated with LPS overnight, and the MFI of CD40, CD86 and PD-L1 on the surface of IDO^{kd}DCs was detected by flow cytometry. The results are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; LPS, lipopolysaccharides; MFI, mean fluorescence intensity; ns, not significant (P>0.05); OVA-FITC, fluorescein isothiocyanate-labeled ovalbumin; PD-L1, programmed cell death ligand 1; Vector^{Ctrl}DCs, DCs infected with control vector of pLJM1-EGFP; IDO^{oe}DCs, IDO-overexpressing DCs; vector^{ctrl}DCs, DCs infected with control vector of pLKO.1; IDO^{kd}DCs, IDO-knockdown DCs.

characterized phenotypically by co-expression of CD123 and CCR6, expression of major histocompatibility complex class II and costimulatory molecules, functionally by suppressing the T-cell response and promoting immune tolerance (35). In the present study, the immunomodulatory effect of IDO on the functions of DCs both *in vitro* and *in vivo* was systematically studied by successfully constructing stable DC lines using both gain-of-function and reduction-of-function methods for IDO with lentiviral infection. The present study provided evidence that IDO is a key molecule that

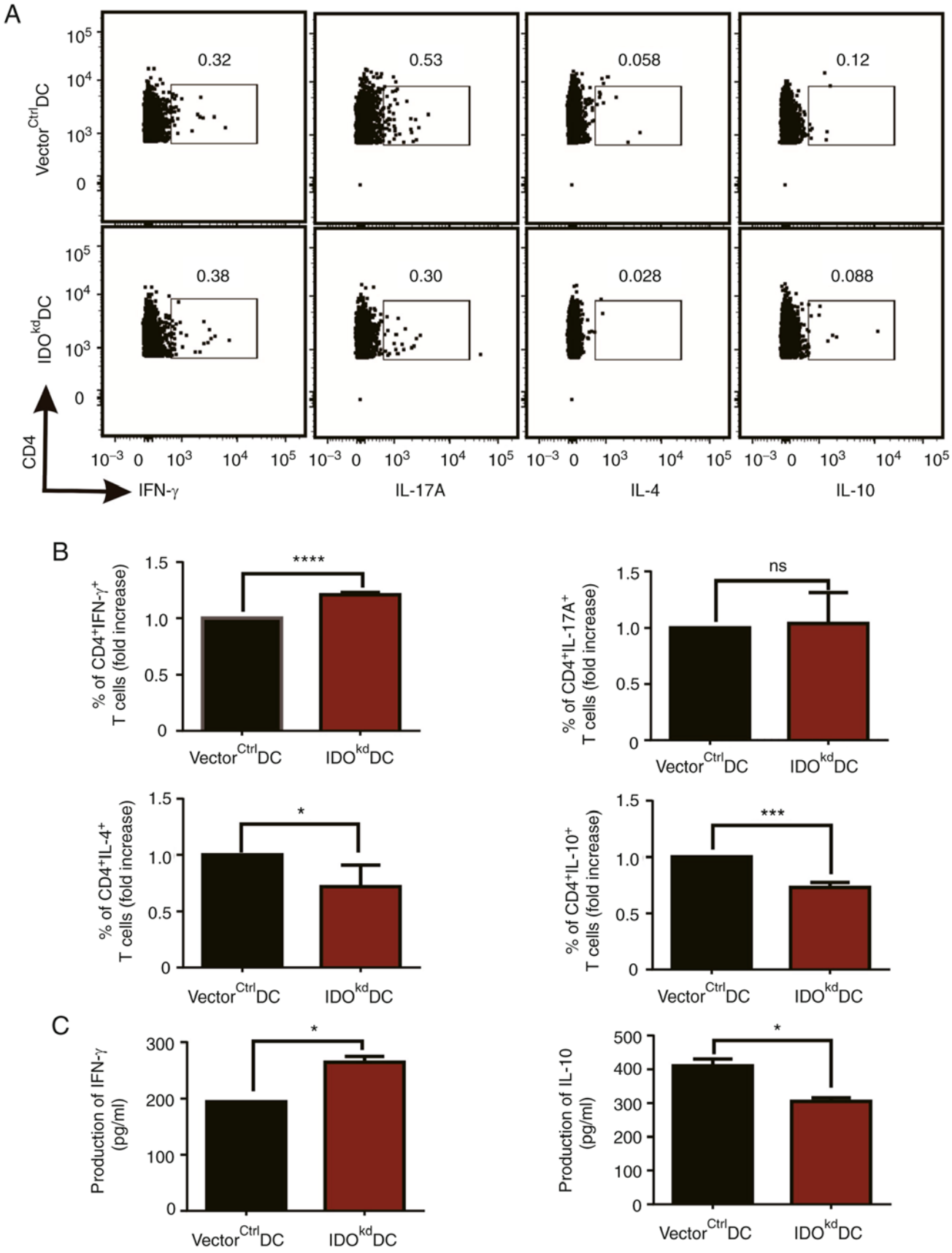

Figure 4. Effects of IDO on DC-stimulated T cell differentiation *in vivo* and *in vitro*. A total of $1x10^6$ OVA-pulsed vector^{ctrl}DCs and IDO^{kd}DCs were intravenously injected into OVA-sensitized mice (n=3 mice per group). After 72 h, the spleens of the mice were extracted to make a single cell suspension. The secretion of T cytokines was detected by flow cytometry. (A) Representative scatter plots and (B) statistics are shown. (C) Effects of IDO on DC-stimulated T cell differentiation *in vitro*. Sorted CD3$^+$CD4$^+$T cells from the spleens of Kunming mice were co-cultured with vector^{ctrl}DCs or IDO^{kd}DCs. The supernatants of CD4$^+$T cells co-cultured with vector^{ctrl}DCs or IDO^{kd}DCs were collected 3 days later, and the secretion of T cytokines was detected by ELISA. The results are presented as the mean ± SEM (n=3). *P<0.05, ***P<0.001, ****P<0.0001. DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; ns, not significant (P>0.05); OVA, ovalbumin; vector^{ctrl}DCs, DCs infected with control vector of pLKO.1; IDO^{kd}DCs, IDO-knockdown DCs.

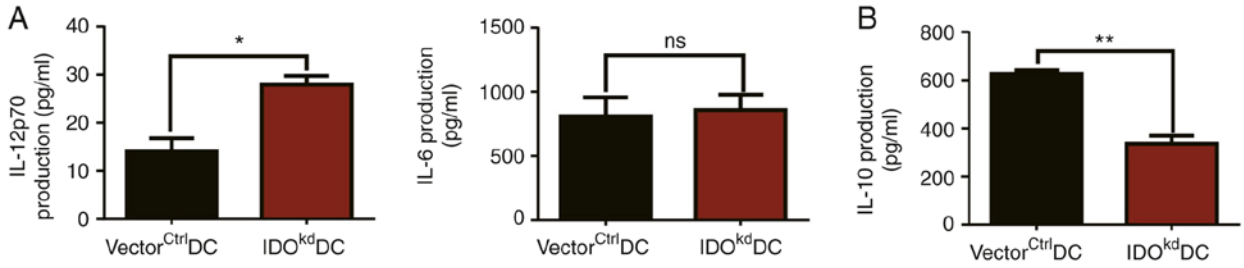

Figure 5. Effect of IDO on T cell polarization factors secreted by DCs. Both vector^{ctrl}DCs and IDO^{kd}DCs were incubated at 37˚C for 24 h, and the supernatants of vector^{ctrl}DCs and IDO^{kd}DCs medium were collected. Subsequently, the levels of (A) IL-12p70, IL-6 and (B) IL-10 were examined in these supernatants by ELISA. The results are presented as the mean ± SEM (n=3). *P<0.05, **P<0.01. DC, dendritic cell; IDO, indoleamine 2,3-dioxygenase; ns, not significant (P>0.05); vector^{ctrl}DCs, DCs infected with control vector of pLKO.1; IDO^{kd}DCs, IDO-knockdown DCs.

induces DC tolerance and inhibits T cell immunity by altering intracellular Trp metabolism to regulate surface molecule and cytokine expression in DCs. It was shown that IDO modulated Trp-Kyn metabolism in DCs, which led to the downregulation of CD86, upregulation of PD-L1, and suppression of antigen uptake to promote the transformation of DCs into tolDCs s; this ultimately resulted in a decrease in T cell proliferation. In addition, it was also identified that IDO inhibited the secretion of IL-12, while promoting the secretion of IL-10 from DCs for Treg differentiation. Collectively, these findings uncovered a key mechanism of IDO in DC tolerance by altering Trp metabolism, thus opening a door for the use of immunotherapy in autoimmune diseases with IDO-expressing DCs as tolerance-inducting vaccines.

Cellular metabolism has been identified as a key component in determining the fate of DC immunogenicity or tolerance (36). The significant finding in this study lies in the identification that IDO is primarily responsible for inducing DC tolerance via Trp metabolism. To date, several specific candidate markers of tolDCs have been identified. For example, co-stimulatory molecules such as CD80 and CD86 are considered to be representative indicators of tolDCs (12,37). Inhibitory molecules, such as programmed cell death ligand 1 (PD-L1), PD-L2, CD83, and C-C Motif Chemokine Ligand 22 are also considered to be markers of tolDCs (38,39). The present study found that IDO in IDO^{oe}DCs inhibited phagocytosis and CD86 expression and promoted PD-L1 expression, leading to the induction of tolDCs. A previous report showed that IDO exerted its effect on cell proliferation. For example, IDO in macrophages affects T cell proliferation (40). Of note, Trp starvation limited T cell proliferation by disrupting the T cell cycle mechanism (31), and Trp catabolites Kyn or 3-HAA inhibited T cell proliferation (41). Coincidentally, it was found in the present study that IDO deficiency was accompanied by a reduction in Trp consumption and Kyn production in IDO^{kd}DCs, which promoted T cell proliferation both *in vitro* and *in vivo*, suggesting that IDO likely inhibits T cell proliferation through a Trp metabolism-mediated regulation of surface molecules CD86 and PD-L1 on DCs. In addition, the examination of cytokines is important when examining the immunomodulatory activities of DCs (42). For example, anti-inflammatory cytokines, including IL-4 and IL-10, are also regarded as markers of tolDCs (38). Furthermore, tolDCs or semimature DCs at an early stage of maturation usually exhibit a decreased expression of IL-12 and increased expression of tolerogenic cytokines IL-10, IL-27 and TGF-β (43). These DC-derived cytokines, which are an essential link between innate and acquired immunity, are associated with T cell differentiation and other immune regulatory roles (44). For example, DC-secreted IL-10 promoted Treg development, while IL-12 induced the differentiation of CD4^{+}T cells into Th1 cells (45,46). In addition, TGF-β played a negative role on the function of several cells, as was reported in the cancer patients associated with natural killer (NK) cell inhibition (47). In the present study, it was demonstrated that IDO in IDO^{kd}DCs inhibited the secretion of IL-10 but promoted that of IL-12 in DCs, leading to the inhibition of naive T-cell differentiation toward Th1 cells and away from Tregs, suggesting that the absence of IDO impairs DC tolerance and consequently promotes T cell immunity. It should be noted that active Trp metabolism in DCs also affects the microenvironment and T cell responses. For example, Trp depletion resulted in preferential apoptosis in Th1 cells over Th2 cells due to the increased sensitivity of Th1 cells to Kyn metabolites (48). Trp metabolites bound to AHR to induce forkhead box P3 expression and promoted the generation and differentiation of Tregs involved in immunosuppression (49). In addition, Kyn promoted the differentiation of naive CD4^{+}T-cells into Tregs instead of Th17 cells (50). Based on these data, we assumed that IDO^{kd}DCs skews T cell differentiation toward Th1 cells rather than Treg cells by Trp metabolism and cytokine regulation. Overall, considering the formidable immunomodulatory effects of IDO on DCs and T cells, we propose that Trp metabolism-mediated regulation of surface molecule and cytokine expression in DCs is the important pathway through which IDO induces DC tolerance to inhibit T cell immunity.

In conclusion, the data presented in the present study demonstrated that IDO is a key molecule that induces the transformation of DCs into tolDCs and inhibits T cell immunity by altering Trp metabolism, regulating surface molecules and cytokines in DCs. This conclusion can provide a theoretical basis of therapeutic drugs for the prevention and treatment of autoimmune diseases. To date, only a few clinical trials on inflammatory and autoimmune disorders have been performed. Therefore, as tolDCs of metabolic regulation, the genetic modification of IDO in DCs, such as the IDO^{oe}DCs in the present study, could be used for vaccine inoculation in disease models in the future, offering more possibilities for the application of tolerogenic vaccine in clinical trials.

Acknowledgements

Not applicable.

Funding

The present study was financially supported by National Natural Science Foundation of China Major Research Plan Project (grant no. 91742101), Anhui International Science and Technology Collaborative Project, China (grant no. 1604b0602017), Natural Science Foundation of Anhui Province, China (grant no. 1608085MH160), Molecular Enzymology and Molecular Detection Excellent Innovation Team of Universities in Anhui Province (grant no. 2022AH010012), Outstanding Innovative Research Team for Molecular Enzymology and Detection in Anhui Provincial Universities (grant no. 2022AH010012), and The Undergraduate Research Fund of Wannan Medical College (grant no. 18103010160).

Availability of data and materials

All the data generated or analyzed during this study are included in this published article.

Authors' contributions

FW and LL confirm the authenticity of all the raw data. FW and LL conducted the experiments and analyzed the data. FW wrote the first draft of the manuscript. LZ, JW, ML, WZ and CZ helped perform the experiments. YX designed the study and wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The study protocol was reviewed and approved by Anhui Normal University Animal Experimentation Ethics Committee (approval number AHNU-ET2022015; Wuhu, China). All animal experiments were carried out in accordance with the guidelines for the care and use of laboratory animals. All laboratory procedures were used to reduce the pain of the mice.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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