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ORIGINAL ARTICLE

Generation of regulatory dendritic cells after treatment with paeoniflorin

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Abstract Regulatory dendritic cells are a potential therapeutic tool for assessing a variety of immune overreaction diseases. Paeoniflorin, a bioactive glucoside extracted from the Chinese herb white paeony root, has been shown to be effective at inhibiting the maturation and immunostimulatory function of murine bone marrow-derived dendritic cells. However, whether paeoniflorin can program conventional dendritic cells toward regulatory dendritic cells and the underlying mechanism remain unknown. Here, our study demonstrates that paeoniflorin can induce the production of regulatory dendritic cells from human peripheral blood monocyte-derived immature dendritic cells in the absence or presence of lipopolysaccharide (LPS) but not from mature dendritic cells, thereby demonstrating the potential of paeoniflorin as a specific immunosuppressive drug with fewer complications and side effects. These regulatory dendritic cells treated with paeoniflorin exhibited high CD11b/c and low CD80, CD86 and CD40 expression levels as well as enhanced abilities to capture antigen and promote the proliferation of $CD4^+CD25^+$ T cells and reduced abilities to migrate and promote the proliferation of $CD4^+$ T cells, which is associated with the upregulation of endogenous transforming growth factor (TGF)-β-mediated indoleamine 2,3-dioxygenase (IDO) expression. Collectively, paeoniflorin could program immature dendritic cells (imDCs) and imDCs stimulated with LPS toward a regulatory DC fate by upregulating the endogenous TGF-β-mediated IDO expression level, thereby demonstrating its potential as a specific immunosuppressive drug.

Keywords Paeoniflorin · Regulatory dendritic cells · TGF-β · IDO · Immunomodulation

Abbreviations

ANOVA	One-way analysis of variance
CD	Cluster of differentiation
DCs	Dendritic cells
DCregs	Regulatory dendritic cells
FCS	Fetal calf serum
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte–macrophage colony-stimulating factor
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IL	Interleukin
imDCs	Immature dendritic cells
IDO	Indoleamine 2,3-dioxygenase
LPS	Lipopolysaccharide
mDCs	Mature dendritic cells
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PI	Propidium iodide
TGF	Transforming growth factor
Tregs	Regulatory T cells
Pae	Paeoniflorin
1-MT	1-Methytryptophan

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Introduction

As heterogeneous antigen-presenting cells, dendritic cells (DCs) play a key role in inducing an immune response or tolerance. Furthermore, their dichotomous function and remarkable plasticity render them an attractive therapeutic tool for immune regulation. DCs can be divided into immature DCs (imDCs) and mature DCs (mDCs) depending on their maturation status. ImDCs have been increasingly recognized for mainly producing anti-inflammatory cytokines and for expressing low levels of co-stimulatory molecules, which induce immune tolerance. mDCs, which induce strong adaptive immunity, are the complete opposite of imDCs. Recently, the subsets of regulatory DCs (DCregs) involved in negatively regulating the immune response have received more attention. The generation of DCregs, which include main characteristics such as high cluster of differentiation (CD)11b expression with fewer co-stimulatory molecules, high expression of interleukin (IL)-10, transforming growth factor (TGF)-β or indoleamine 2,3-dioxygenase (IDO), an inhibitory effect on T cell proliferation, a stimulative effect on regulatory T cells (Tregs) and resistance to pro-inflammatory cytokine-induced maturation, has been proposed as a therapeutic strategy for excessive immune response-related diseases, such as post-transplant graft versus host disease [1–7].

The generation of DCregs can be accomplished using many manipulations, including pharmacological agents, such as immunosuppressive drugs [8, 9], physiological mediators, including anti-inflammatory cytokines such as TGF-β and stroma cells [10–12] and the genetic engineering of molecules, such as co-stimulatory molecules and cytokines [13, 14]. Recently, pharmacological treatments have gained interest as generators of DCregs for potential clinical applications [15, 16]. Although immunological tolerance can be acquired after treatment with immunosuppressive drugs, serious complications and side effects often occur because of their nonspecificity [17]. Therefore, exploring specific immunosuppressive drugs for generating DCregs has attracted much more attention.

White paeony root, a family member of *Paeonia lactiflora* Pall., has been used as an anti-inflammatory and immunomodulatory agent in traditional Chinese medicine for many years [18–20]. Paeoniflorin is a bioactive glucoside that is extracted from white paeony root and has been approved by the State of Food and Drug Administration of China to treat rheumatoid arthritis. Animal studies have shown that paeoniflorin can effectively treat immune overreaction diseases, such as allergic contact dermatitis and rheumatoid arthritis, by lowering the overall immune response. The mechanisms for lowering immune activation and response are closely related and involve increasing the production of anti-inflammatory cytokines, such as IL-10 and TGF-β, decreasing the production of inflammatory cytokines, such as tumor necrosis factor-α and IL-1β, reducing the proliferation of lymphocytes and decreasing the amount of antibodies produced by B lymphocytes [21–25]. Recently, paeoniflorin has been shown to be able to inhibit the maturation and immunostimulatory function of murine bone marrow-derived DCs via the high secretion of IL-10 and TGF-β and the reduction in IL-12 and co-stimulatory molecules [26]. However, whether paeoniflorin can program DCs toward a fate of becoming DCregs, a new subtype of DCs with an immune regulatory function and the underlying mechanism remain unclear. Here, our study investigates whether paeoniflorin can induce the production of DCregs from human monocyte-derived DCs.

Materials and methods

Cell culture

Peripheral blood mononuclear cells (PBMCs) were separated from the peripheral venous blood of healthy donors using a Ficoll density gradient. The donors provided informed consent for the experimental study, which was reviewed and approved by the ethics committee of Tianjin Medical University and was in accordance with the 1964 Helsinki Declaration. $CD14^+$ monocytes were isolated from the PBMCs, and approximately 90 % purity was achieved using $CD14^+$ magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Nordrhein-Westfalen, Germany). For DC differentiation, $CD14^+$ monocytes were cultured in 24-well plates at a concentration of 1×10^6 cells/mL in RPMI 1640 medium (Hyclone Thermo Scientific, Waltham, MA, USA) containing 20 % fetal calf serum (FCS) (Biological Industries, Kibbutz Beit Haemek, Israel), 60 ng/mL granulocyte–macrophage colony-stimulating factor (GM-CSF) (R&D Systems, Minneapolis, MN, USA) and 30 ng/mL IL-4 (R&D Systems, Minneapolis, MN, USA) for 5–7 days to obtain imDCs Then, 100 ng/mL of lipopolysaccharide (LPS) (Sigma-Aldrich, St. Louis, MO, USA) was added, and the cells were cultured for another 1–3 days to become mDCs. T lymphocytes were collected from the supernatant after the adherence of PBMCs to the plastic. $CD3^+$ T lymphocytes were separated from the lymphocytes using a $CD3^+$ T cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Nordrhein-Westfalen, Germany).

To investigate the effect of paeoniflorin (Fig. 1) (Bellancom Chemistry, Beijing, China) on DC differentiation, paeoniflorin was added to the cell culture medium on day 0

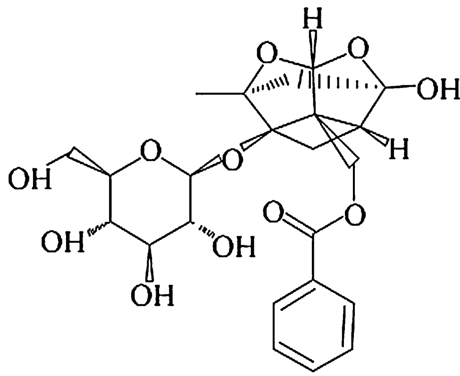

Fig. 1 Chemical structure of paeoniflorin. Molecular formula: C23H28O11, molecular weight: 480.47, purity: ≥98 %

of imDCs with or without LPS stimulation and mDCs. ImDCs, imDCs with LPS and mDCs were used as negative controls.

Apoptosis assay

To detect apoptosis in an early stage or later stage, imDCs and imDCs + LPS were treated with or without paeoniflorin at 10, 30 or 50 μg/mL, respectively. Four days later, the cells were suspended in 200 μL of phosphate-buffered saline (PBS) and incubated at room temperature for 20 min with fluorescein isothiocyanate (FITC) Annexin V and propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA).

Antigen uptake assay

After treatment with 10, 30 and 50 μg/mL paeoniflorin for 4 days, imDCs with or without LPS stimulation and mDCs at 1×10^7/mL were suspended with 100 μL of 10 % FCS RPMI-1640 medium containing 100 ng/μL fluorescein ovalbumin (AnaSpec, Fremont, CA, USA) and incubated at 37 °C for 40 min. Then, the cells were collected, washed with PBS and analyzed using a flow cytometer (FACSVerse, BD Biosciences, San Diego, CA, USA).

Transwell assay

imDCs with or without LPS stimulation and mDCs at a density of 5×10^5/mL were treated with or without paeoniflorin at concentrations of 10, 30 or 50 μg/mL for 4 days and then seeded onto the upper compartments of transwell chambers with an 8-μm pore (Millipore, Billerica, MA, USA), with the lower compartments containing RPMI-1640 and 20 % FCS. After incubation for 12 h, the cells in the lower compartment that had penetrated through the chamber were counted using a hemocytometer.

Mixed lymphocyte reaction

imDCs with or without LPS stimulation and mDCs at a density of 5×10^5/mL were cultured for 4 days in the absence or presence of paeoniflorin at 10, 30 and 50 μg/mL. DCs acted as stimulator cells (1×10^4/mL) and were co-cultured with responder lymphocytes (1×10^5/mL) at a ratio of 1:10 in 24-well plates for 5 days. At the end of the culture period, the cells were suspended in 100 μL of PBS and double-stained with anti-CD4$^+$ FITC and 7-AAD (BioLegend, San Diego, CA, USA). Then, the number of CD4$^+$ and 7-AAD$^-$ cells was counted using a flow cytometer (FACSVerse, BD Biosciences, San Diego, CA, USA). In some experiments, anti-TGF-β antibody (200 ng/mL) (BioLegend, San Diego, CA, USA) or 1-methytryptophan (1-MT) (200 μM/L) (Sigma-Aldrich, St. Louis, MO, USA) was added to the DCs 1 h before exposure to 30 μg/mL paeoniflorin.

To generate the Tregs, T cells were co-cultured with imDCs with or without LPS in the absence or presence of paeoniflorin at 30 μg/mL at a 1:10 ratio for 2 days. Then, the cells were suspended in 300 μL of PBS and double-stained with anti-CD4 FITC and anti-CD25 PE antibodies (BioLegend, San Diego, CA, USA). The number of CD4 and CD25 double-positive T cells was analyzed by flow cytometry to detect proliferation.

Flow cytometric analysis

To analyze the phenotype of the DCs and the surface marker expression levels, imDCs and imDCs + LPS were treated with or without paeoniflorin at 30 μg/mL. Approximately 4 days later, images of the test cells were taken using an inverted microscope. Then, the cells were suspended in 100 μL of PBS and incubated at 4 °C for 20–30 min with optimal concentrations of various human monoclonal antibodies conjugated with FITC or P-phycoerythrin. The following antibodies were used for staining: anti-CD11c, anti-CD11b, anti-human leukocyte antigen (HLA)-DR, anti-CD80, anti-CD86, anti-CD40 and isotype control antibodies (BioLegend, San Diego, CA, USA). The data were collected with a flow cytometer and analyzed with FlowJo software (TreeStar, Ashland, OR, USA).

Enzyme-linked immunosorbent assays

Five-day-old imDCs were treated with or without paeoniflorin for 4 days. Culture supernatants of the DCs were collected. The DCs were then co-cultured with lymphocytes for another five days, and the culture supernatants were collected. The levels of TGF-β, IL-10 and IL-12p70 produced by the DCs in the absence or presence of lymphocytes were determined by enzyme-linked

immunosorbent assays according to the manufacturer's instructions (Dakewe Bioengineering, Shenzhen, China).

Quantitative real-time PCR

Total RNA was isolated from the DCs after day 4 of paeoniflorin treatment using the acid guanidinium phenol–chloroform method. Two micrograms of RNA was used for cDNA synthesis using a reverse transcriptase kit (Invitrogen Life Technologies, Carlsbad, CA, USA). The relative expression level of *IDO* was determined by quantitative real-time PCR. The relative expressions of *IDO* mRNA were determined and normalized to the expression of the internal housekeeping gene *GAPDH*. The primers used for PCR amplification were as follows: *IDO*, forward: 5′-GCCCTTCAAGTGTTTCACCAA-3′ and reverse 5′-CCTTTCCAGCCAGA CAAATATATG-3′; *GADPH*, forward: 5′-TGCACCACCAACTGCTTAGC-3′ and reverse 5′-GGCATGGACTGTGGTCATGAG-3′. PCR amplification was performed for 40 cycles that consisted of the following three steps using an Applied Biosystems 7500 Fast Real-Time PCR System (Carlsbad, CA, USA): 10 s at 95 °C for denaturation, 30 s at 60 °C for primer annealing and 30 s at 60 °C for extension.

Western blot analysis

After day 4 of the treatment with 30 μg/mL paeoniflorin, the total protein of the DCs was extracted. In some experiments, anti-TGF-β antibody (200 ng/mL) was added to the DCs 1 h before exposure to paeoniflorin. Then, 50 μg of protein per sample was subjected to 10 % sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and the proteins were transferred onto polyvinylidene fluoride membranes (Solarbio, Beijing, China). Then, the membranes with blotted proteins were blocked followed by probing with anti-IDO (1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA) and anti-β-actin (1:1000 dilution, Cell Signaling Technology, Beverly, MA, USA) antibodies at 4 °C overnight. The membranes were washed and incubated at room temperature for 2 h with diluted secondary horseradish peroxidase (HRP)-marked antibodies (1:2500 dilution). Immunoreactive protein bands were detected using an enhanced chemiluminescence detection kit (Millipore, Billerica, MA, USA).

Statistical analysis

All data were analyzed by one-way analysis of variance (ANOVA) using SigmaPlot software (SPSS 16.0, Chicago, IL, USA). The statistical significance was defined as $p \leq 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). The data are expressed as the mean ± SD. The results are representative of three independent experiments.

Results

Effect of paeoniflorin on DC apoptosis

Paeoniflorin at concentrations of 10 and 30 μg/mL did not induce the apoptosis of imDCs and imDCs with LPS stimulation when compared to untreated controls. Following drug treatment, the apoptotic rate of imDCs was less than 10 % and that of imDCs with LPS stimulation was slightly more than 10 %. Although the apoptotic rate of DCs treated with combined LPS and paeoniflorin at 50 μg/mL was higher than that of the control cells, these small increases (11.18 ± 0.48 vs 8.48 ± 0.18, $p < 0.01$) have no biological significance (Fig. 2).

Effect of paeoniflorin on the functioning of DCs at different maturation stages

To investigate the effects of paeoniflorin on the ability of DCs at different maturation stages to uptake antigen, migrate and stimulate the proliferation of allogeneic T cells, imDCs, without or with LPS stimulation, and mDCs were used. An antigen uptake assay using FITC-ovalbumin was established to examine the endocytic ability of DCs. The results showed that the ability to uptake ovalbumin was much lower in the imDCs treated with paeoniflorin at 10 and 30 μg/mL than in the control imDCs, contributing to the inhibition of the antigen-presenting process (829.67 ± 298.03 vs 3514.00 ± 1544.53, $p < 0.01$ and 1844.00 ± 488.77 vs 3514.00 ± 1544.53, $p < 0.05$, respectively). The level of ovalbumin uptake in the group of imDCs simultaneously treated with LPS and 30 μg/mL paeoniflorin was much higher than that observed in the control group of imDCs with LPS, contributing to the differentiation into DCregs (6758.00 ± 1633.00 vs 903.67 ± 72.83, $p < 0.001$) (Fig. 3a). An inhibitory effect on the endocytic ability of mDCs was not observed.

The transwell assay was established using chambers with Millipore filters. The migration of imDCs decreased significantly, whereas the migration of imDCs stimulated with LPS and mDCs did not change except for when treated with 50 μg/mL paeoniflorin. As the drug dose increased, 10, 30 and 50 μg/mL, the inhibitory effect on the migration of imDCs increased (62 ± 1, 52 ± 3, 33 ± 3 vs 91 ± 2, respectively, $p < 0.001$) (Fig. 3b). Furthermore, paeoniflorin inhibited the stimulatory effect of the imDCs treated with LPS on the proliferation of allogeneic T cells in a dose-independent manner (10 μg/mL paeoniflorin, $p < 0.01$, 30 and 50 μg/mL paeoniflorin, $p < 0.001$);

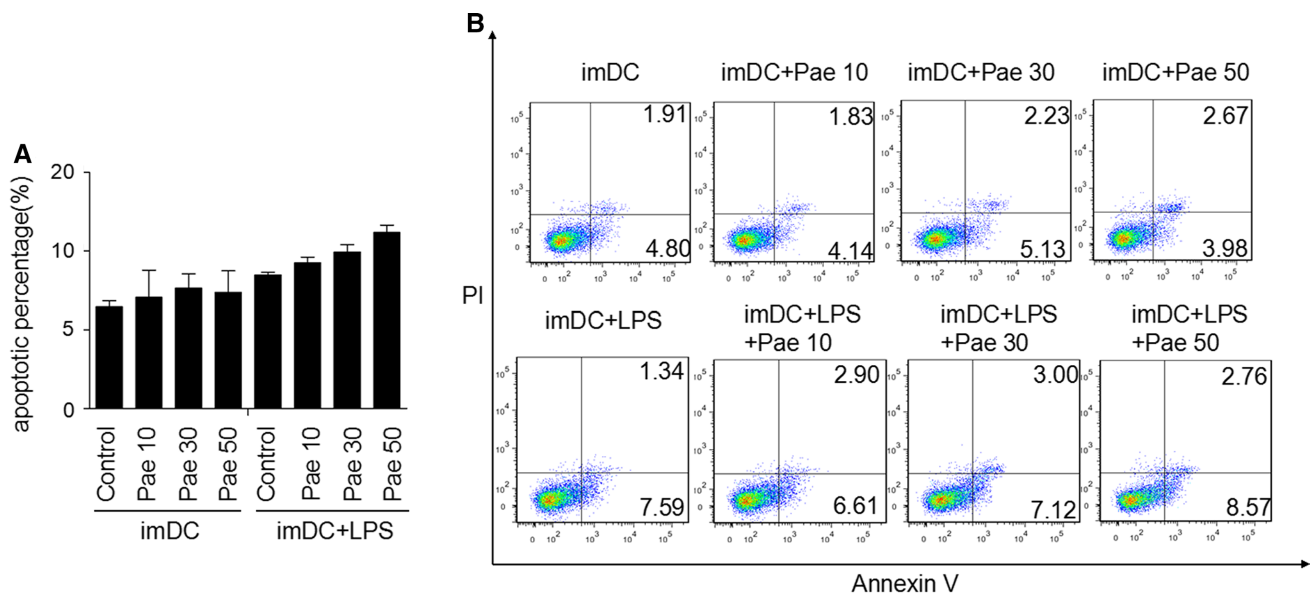

Fig. 2 Effect of paeoniflorin on DC apoptosis. Monocyte-derived imDCs and imDCs + LPS were treated with or without paeoniflorin at 10, 30 or 50 μg/mL. Four days later, the cells were incubated with FITC Annexin V and PI. **a** The statistical analysis of apoptotic percentage. The data are shown as the mean ± SD and are representative of three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. **b** The representative apoptosis *scatter plot* (gating on dendritic cells). *Pae* paeoniflorin, *drug unit* μg/mL

however, no inhibitory effects on the imDCs or mDCs were observed (Fig. 3c).

Effect of paeoniflorin on DC cytokine secretion

Because no effect on the mDCs was observed, only the imDCs and the imDCs stimulated with LPS were selected to investigate the effect of paeoniflorin on the production of cytokines, including TGF-β, IL-10 and IL-12, from the DCs co-cultured with or without allogeneic lymphocytes. In the absence of lymphocytes, TGF-β, at 564.73 ± 3.52 pg/mL, was only detected in the group of imDCs simultaneously treated with LPS and 30 μg/mL paeoniflorin. After mixing with lymphocytes, the TGF-β levels in the imDC groups treated with paeoniflorin at 30 and 50 μg/mL increased to 109.77 ± 6.43 and 121.66 ± 2.48 pg/mL, respectively, in the absence of LPS and 578.38 ± 30.09 and 118.69 ± 7.44 pg/mL, respectively, in the presence of LPS. No secretion of TGF-β was observed in the group of DCs treated with 10 μg/mL paeoniflorin. In general, the drug dose determines its effect. When the drug dose is different, the effect is different, especially for drugs with immune regulatory function. The strongly increased TGF-β in the group of imDCs treated with 30 μg/mL paeoniflorin indicates that paeoniflorin at this dose is expected to become a specific immunosuppressive drug with less side effects and toxicity. Moreover, these results suggest that paeoniflorin can promote the strong secretion of TGF-β and that the increased TGF-β in the mixed lymphocyte reactions was produced by the DCs because no secreted TGF-β was detected in the supernatant of the lymphocytes (Fig. 4a). Before and after mixing with lymphocytes, the expression of IL-10 also increased, whereas the expression level of IL-10 from the imDCs with LPS and paeoniflorin mildly decreased compared with that of the control DCs ($p < 0.001$ when treated with paeoniflorin at 10 and 30 μg/mL in the absence of lymphocytes, and $p < 0.05$ when treated with paeoniflorin at 10 μg/mL in the presence of lymphocytes) (Fig. 4b). Additionally, the expression of IL-12 did not change (Fig. 4c).

Morphology and phenotypic characteristics of DCs with paeoniflorin treatment

To determine whether the DCs treated with paeoniflorin differentiated into the DCregs subtype, we analyzed the morphology and phenotypic characteristics of the generated DCs. Based on the above-mentioned results, 30 μg/mL paeoniflorin was selected as the optimal drug dose. As shown in Fig. 5a, no obvious change in the DC morphology was observed in the presence or absence of paeoniflorin. Regarding the expression of surface molecules, the mean fluorescence intensity (MFI) of CD11c-positive cells was higher for both the paeoniflorin-treated and paeoniflorin plus LPS-treated imDCs than that of the control imDCs and imDCs + LPS (914.67 ± 149.03, 843.33 ± 7.02 vs 517.33 ± 100.67, 593.00 ± 6.93, $p < 0.001$, $p < 0.01$, respectively). Additionally, the CD11b-positive

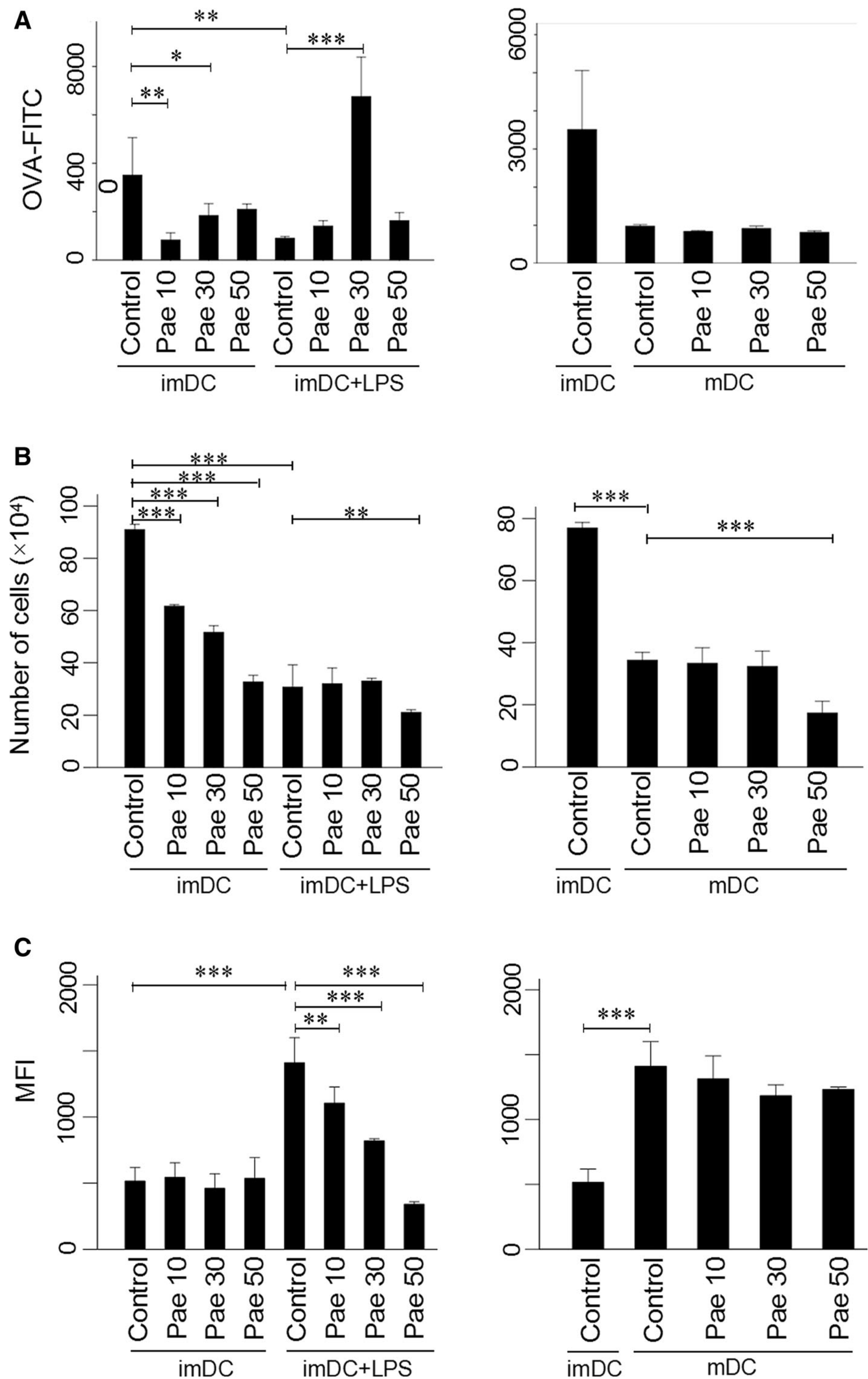

Fig. 3 Effect of paeoniflorin on the immune functions of DCs at different maturation stages. **a** OVA antigen uptake analysis of DCs. **b** Paeoniflorin inhibited the migration of imDCs. **c** Paeoniflorin inhibited the stimulative effect of DCs on the proliferation of CD4^{+} T cells. The data are shown as the mean ± SD and are representative of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. *OVA* ovalbumin, *Pae* paeoniflorin, *MFI* mean fluorescence intensity, *drug unit* μg/mL

ratio was also much higher for both the paeoniflorin-treated and paeoniflorin plus LPS-treated imDCs than that of the control imDCs and imDCs + LPS (16.35 ± 0.25 vs 13.35 ± 1.05, $p < 0.001$; 29.20 ± 0.50 vs 10.26 ± 0.54, $p < 0.001$, respectively). Conversely, the positive ratios of the co-stimulatory molecules, including CD80 and CD40, were all decreased in the imDCs treated with paeoniflorin and LPS compared with those in the control group (19.97 ± 1.85 vs 25.29 ± 3.68, $p < 0.05$; 62.78 ± 3.13 vs 80.79 ± 6.28, $p < 0.01$, respectively). The CD86-positive ratio was lower for both the paeoniflorin-treated and paeoniflorin plus LPS-treated imDCs than for the control imDCs and imDCs + LPS (6.25 ± 0.30, 65.20 ± 1.60 vs 11.80 ± 0.50, 79.75 ± 2.55; $p < 0.01$, $p < 0.001$, respectively).

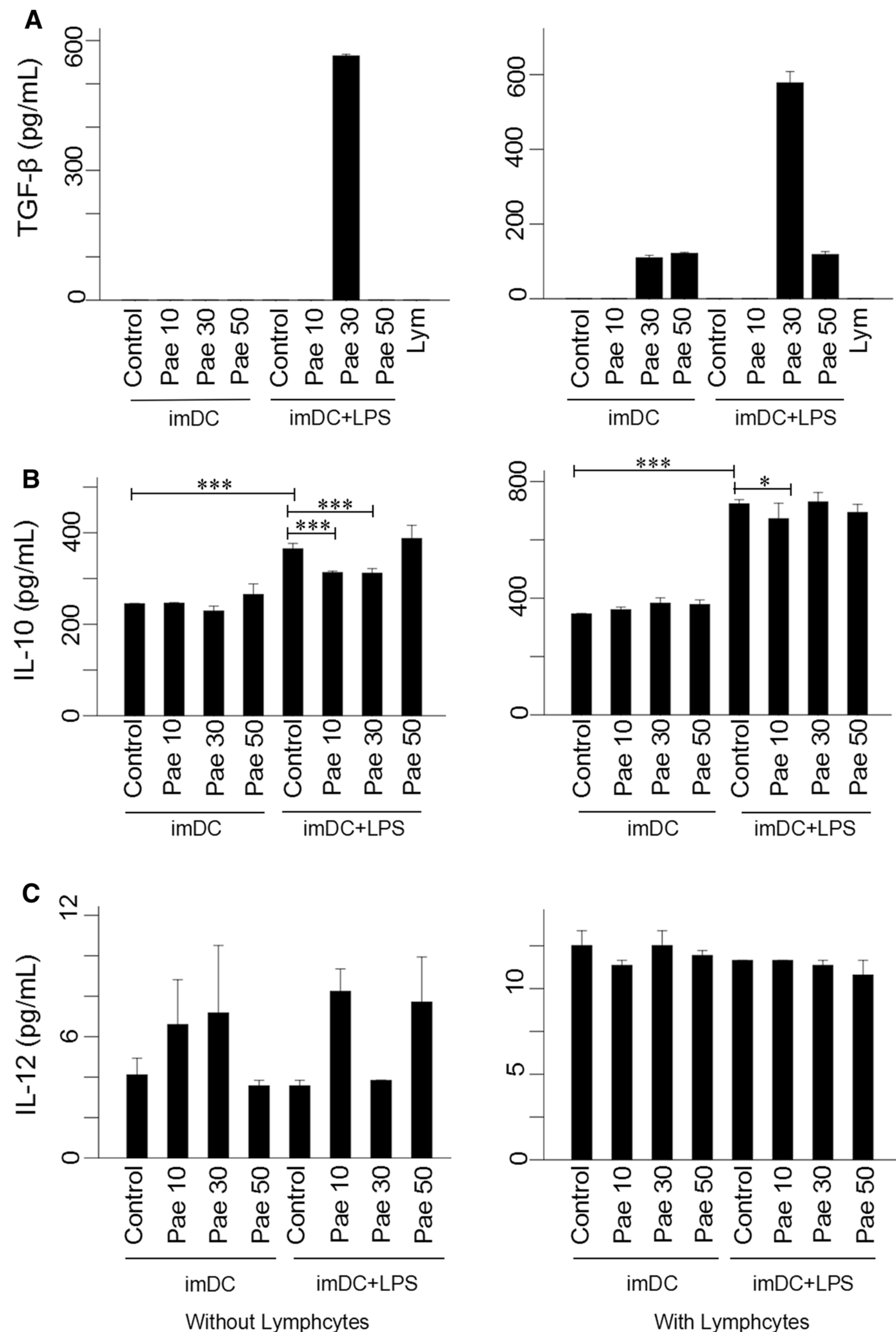

Fig. 4 Cytokine production of DCs co-cultured with or without allogeneic lymphocytes. Five-day-old monocyte-derived imDCs were treated with or without paeoniflorin for 4 days. The culture supernatant of the DCs was collected to detect the expression levels of **a** TGF-β, **b** IL-10 and **c** IL-12p70. *Left* without lymphocytes. *Right* with lymphocytes. The data are shown as the mean ± SD and are representative of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. *Pae* paeoniflorin, *drug unit* μg/mL

The HLA-DR-positive ratios were comparable among the four groups of DCs (Fig. 5b).

In the study of the proliferation of Tregs, we showed that the presence of LPS in the control imDCs decreased the positive percentage of Tregs. After the administration of paeoniflorin, co-culture of imDCs in the presence of LPS with T cells resulted in a striking enrichment of Tregs. Surprisingly, in the absence of LPS, imDCs treated with paeoniflorin had no effect on the positive percentage of Tregs (Fig. 6).

Mechanism of generating regulatory DCs with paeoniflorin treatment

As shown in Fig. 5a, paeoniflorin significantly promoted the endogenous production of TGF-β by the DCs. Here, we showed that the expression of *IDO* at both the protein and mRNA levels in the imDCs + LPS group exposed to 30 μg/mL paeoniflorin was almost two times greater than that of the imDCs + LPS control group (Fig. 7a, mRNA, 47.00 ± 3.66 vs 85.16 ± 2.83, $p < 0.01$, protein, 1.09 ± 0.09

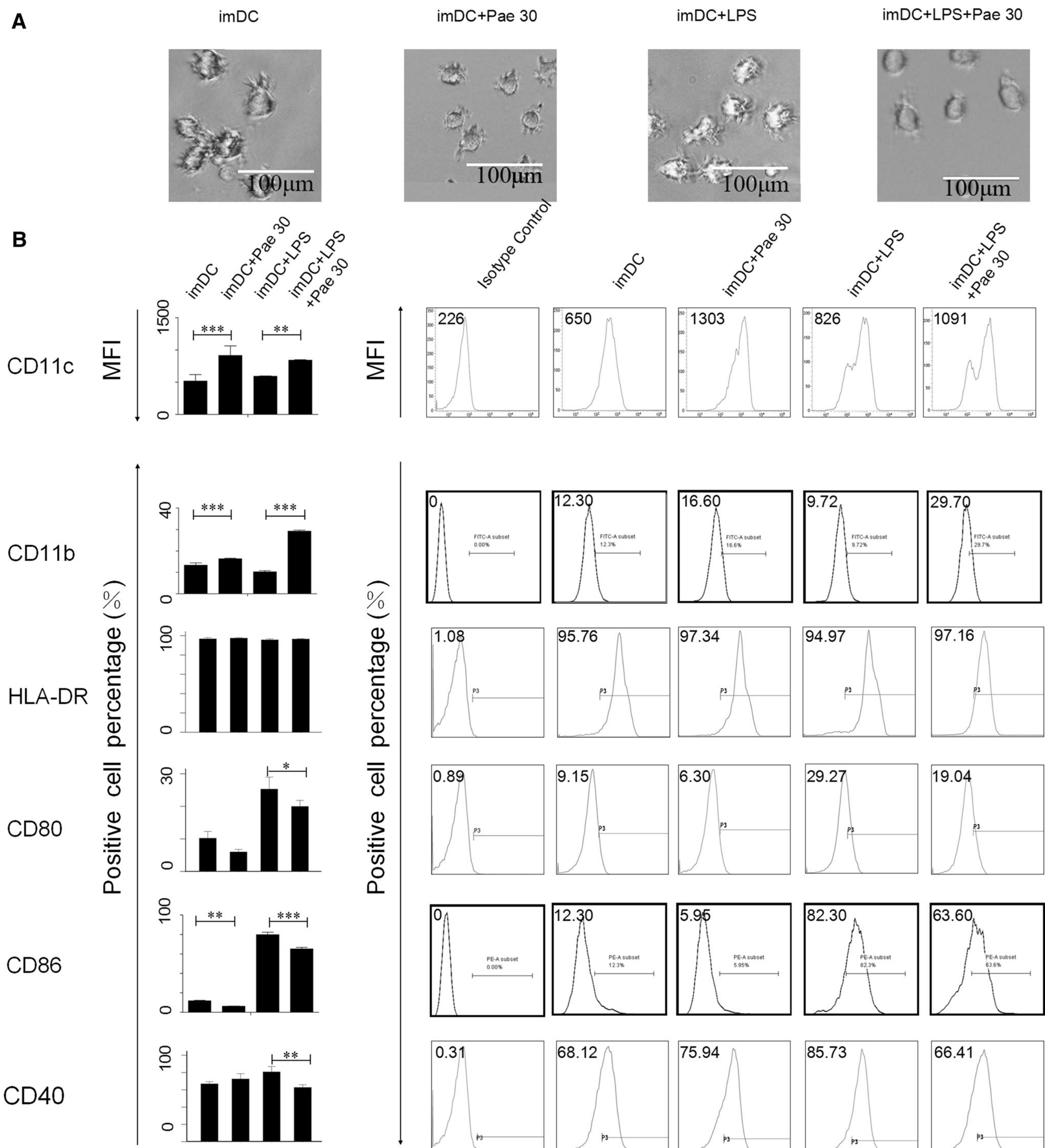

Fig. 5 Morphology and phenotypic characteristics of DCs treated with paeoniflorin. Monocyte-derived imDCs and imDCs + LPS were treated with or without paeoniflorin for 4 days. **a** Morphology of DCs. Images of the test cells were taken using an inverted microscope. **b** Phenotype analysis of DCs (gating on the DCs). The data are shown as the mean ± SD and are representative of three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. *Pae* paeoniflorin, *MFI* mean fluorescence intensity, *drug unit* μg/mL

vs 2.48 ± 0.19, $p < 0.01$). To elucidate the mechanism by which paeoniflorin induces conventional DCs to differentiate into the DCregs subtype, we investigated whether TGF-β and IDO were indeed the factors responsible for the inhibitory effects observed in the DCs. TGF-β-neutralizing antibody and the IDO-specific blocker 1-MT were added to the co-culture system 1 h before the administration of paeoniflorin. After 4 days of co-culture, the DCs were

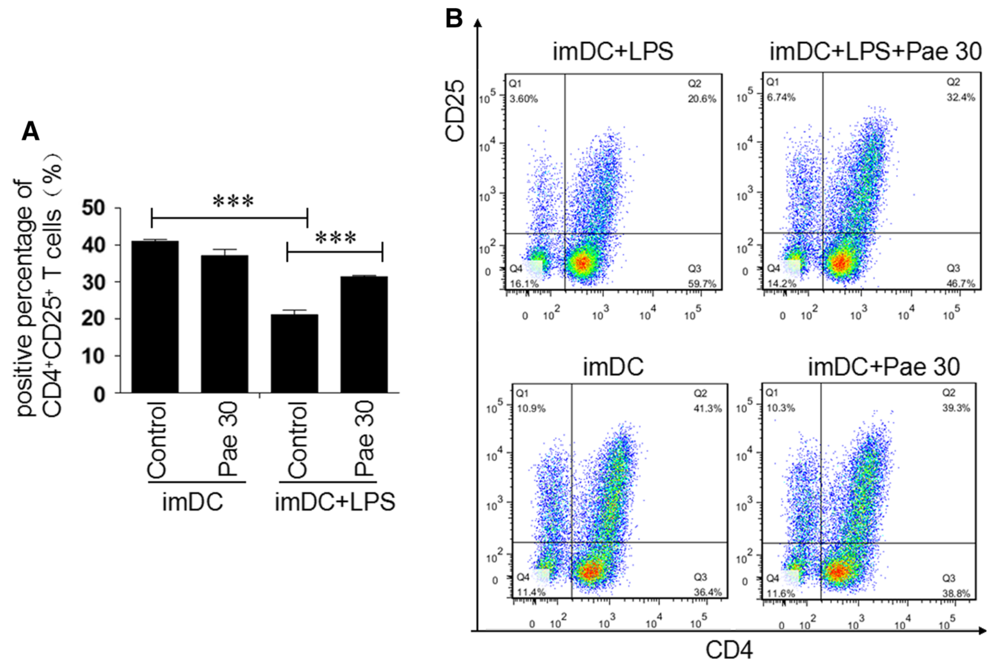

Fig. 6 Effect of paeoniflorin on the proliferation of Tregs. **a** The statistical analysis of the positive percentage of $CD4^{+}CD25^{+}$ T cells. The data are shown as the mean ± SD and are representative of three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$. **b** Representative scatter plot of $CD4^{+}CD25^{+}$ T cells (gating on the T cells). *Pae* paeoniflorin, *drug unit* μg/mL

collected to assess their regulatory function. We found that blocking TGF-β or the use of 1-MT led to the loss of the inhibitory effect of DCregs on T cell proliferation, indicating that TGF-β and IDO were absolutely necessary for maintaining the DCs in the tolerogenic state (Fig. 7b).

Finally, we investigated whether the previously observed paeoniflorin-mediated endogenous TGF-β production could induce IDO expression in DCs. For this experiment, cells in the imDCs and imDCs + LPS groups, previously treated without or with TGF-β-neutralizing antibody for 1 h, were treated with 30 μg/mL paeoniflorin for 4 days. Our results showed that the addition of the TGF-β-neutralizing antibody significantly inhibited paeoniflorin-induced IDO production (Fig. 7 C).

Discussion

Paeoniflorin, a bioactive glucoside extracted from the traditional Chinese medicine white paeony root, has been approved by the State of Food and Drug Administration of China for the treatment of rheumatoid arthritis. Animal studies have shown that paeoniflorin can effectively treat immune overreaction diseases by lowering the immune activation and response [21–25]. Recently, Liu's report showed that paeoniflorin was able to inhibit the maturation and immunostimulatory function of murine bone marrow-derived DCs via the high secretion of IL-10 and TGF-β and the reduction in IL-12 and co-stimulatory molecules [26]. The generation of DCregs has gained further interest regarding its potential clinical application for treating excessive immune response-related diseases, such as post-transplant graft versus host disease, autoimmune disease and allergies [3, 27–29]; however, whether paeoniflorin is capable of inducing the production of DCregs has not been reported.

An analysis of the DC phenotype showed that paeoniflorin-treated DCs with LPS stimulation exhibited high levels of CD11b/c molecules and low expression levels of co-stimulatory molecules. An investigation of the immune function showed that the endocytic ability, the expression levels of TGF-β and IDO and the ability to promote the proliferation of $CD4^{+}CD25^{+}$ Treg cells were greater in the imDCs with combined LPS and paeoniflorin treatment. The $CD4^{+}CD25^{+}$ Treg cell has been known not only to contribute to the maintenance of tolerance to self-antigens but also to limit alloreactive responses, which play an important role in the prevention of autoimmunity and post-transplant rejection [30, 31]. These results suggest that paeoniflorin is capable of inducing human monocyte-derived DCs to differentiate into DCregs, and paeoniflorin-conditioned DCs are able to maintain these tolerogenic characteristics even after stimulation with the heterogenetic antigen LPS. Moreover, paeoniflorin had no effect on the apoptosis of DCs, which indicated that the modulatory effects of paeoniflorin on DC immune function resulted from the differentiation-inducing effects of the drug.

It is well known that the DC activation status determines the outcome of the immune response, immune activation or tolerance. In the present study, the DCs with different activation statuses, including imDCs, imDCs stimulated

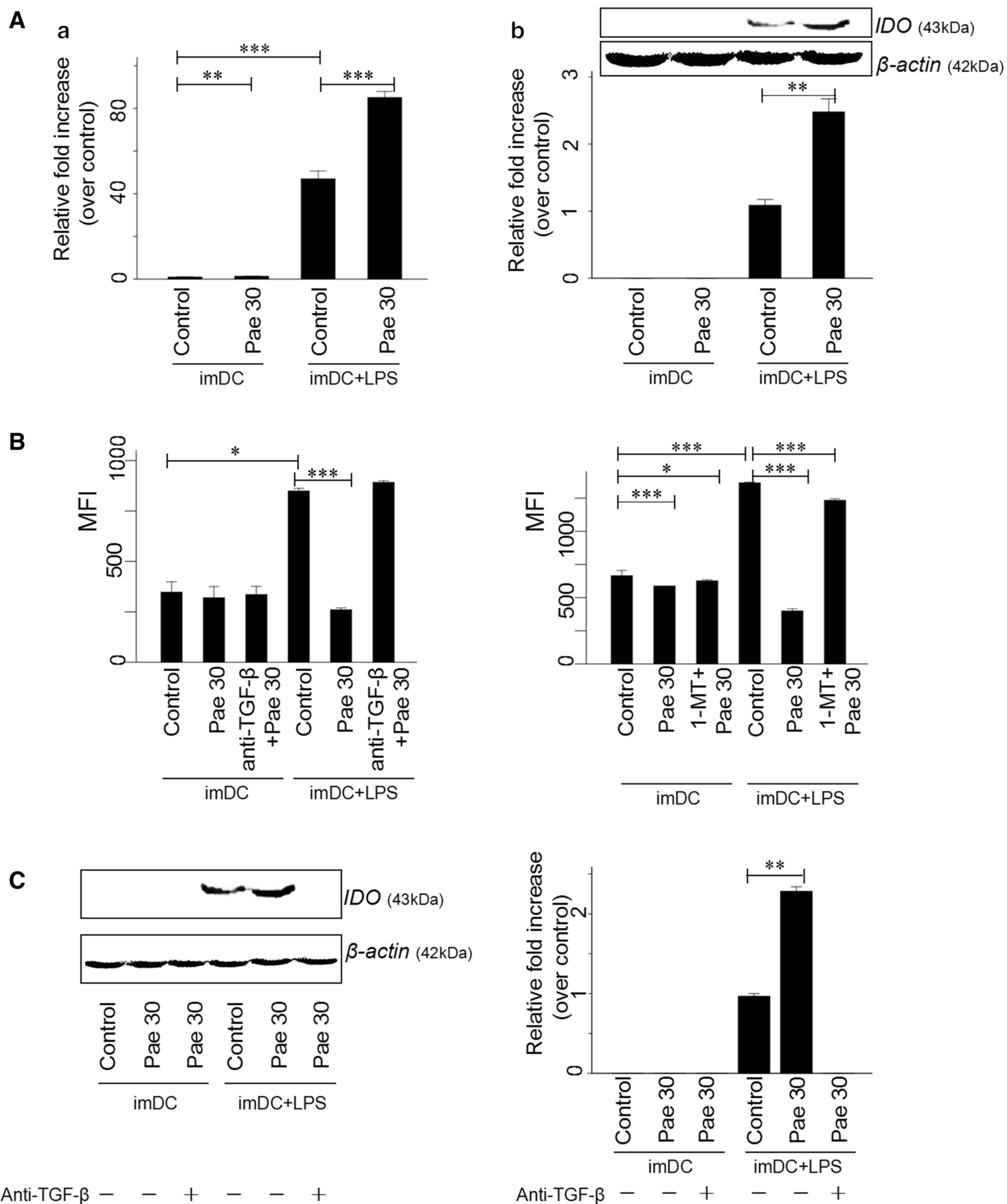

Fig. 7 Mechanism of generating regulatory DCs with paeoniflorin treatment. Monocyte-derived imDCs and imDCs in the presence of LPS were treated with or without paeoniflorin for 4 days. **a** Paeoniflorin promoted the expression of IDO. (*a*) mRNA level of *IDO*. (*b*) Protein level of IDO. **b** Anti-TGF-β antibody or 1-MT reversed the inhibitory effect of paeoniflorin on DC-mediated T cell proliferation. **c** Anti-TGF-β antibody inhibited the expression of IDO. The data are shown as the mean ± SD and are representative of three independent experiments. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. 1-MT, 1-methytryptophan; *Pae* paeoniflorin, *drug unit* μg/mL

with LPS and mDCs, were investigated. The immune functions, including capturing allogenic antigen capture, transmembrane migration and promotion of T cell proliferation in imDCs with or without LPS stimulation, but not mDCs, were affected by paeoniflorin. These findings emphasize that the condition responsible for the inhibitory effect of paeoniflorin on DC function is the degree of DC activation. Due to the different functions of DCs in different stages, paeoniflorin possesses different effects. ImDCs are primarily localized in the peripheral tissues.

Their main function is antigen phagocytosis and migration to lymphoid tissues to present antigen. The imDCs treated with paeoniflorin showed a decreased ability to uptake ovalbumin and migrate, contributing to the inhibition of both the process of antigen-presenting and migration to lymphoid tissues. After exposure to inflammatory agents, such as LPS, DCs undergo a maturation process with reduced antigen phagocytosis and migration, as well as increased promotion of lymphocyte proliferation, which helps them to remain at lymphoid tissues to elicit an adaptive immunity. Therefore, the increased level of ovalbumin uptake in imDCs simultaneously treated with LPS contributes to the differentiation into DCregs, while no change of migration in imDCs treated with LPS contributes to them staying in lymphoid tissues to elicit adaptive immune response. The inhibitory effect of paeoniflorin on the proliferation of T cells and the stimulative effect on the proliferation of Tregs contribute to the differentiation into DCregs. Second, it is the specific properties of the Chinese medicine that determine the different effects of the drug on the different stages of DCs. These results have shown that paeoniflorin has the potential to become a specific immunosuppressive drug that only acts on imDCs and imDCs in the presence of an antigen trigger with few complications and side effects, which include broad immune suppression. These results are in contrast with those reported by Liu, who only observed the effect of paeoniflorin on imDCs stimulated by contact with the sensitizer 1-chloro-2,4-dinitrobenzene.

The drug dose often determines its effect. When the drug dose is different, the effect is different. As the drug dose increased, 10, 30 and 50 μg/mL, the inhibitory effect of paeoniflorin on the immune function of DCs increased. Paeoniflorin at 30 μg/mL was capable of promoting the strong secretion of TGF-β, a key cytokine in inducing immune tolerance, and this response was greatly superior to those of 10 and 50 μg/mL paeoniflorin. Moreover, 30 μg/mL paeoniflorin only acted on imDCs and imDCs in the presence of an antigen trigger, while 50 μg/mL paeoniflorin still acted on mDCs, eliciting the inhibition of the migration of mDCs, meaning paeoniflorin at the dose of 30 μg/mL is expected to be a specific immunosuppressive drug.

The underlying mechanisms responsible for the paeoniflorin-mediated production of DCregs have remained unclear. TGF-β is a potent regulatory cytokine that is considered to be a key factor in inducing immune tolerance [32]. TGF-β has been shown to contribute to the differentiation of DCregs in vitro and in vivo [33]. Interestingly, the autocrine secretion of TGF-β was strongly upregulated in the imDCs treated with both LPS and 30 μg/mL paeoniflorin. This secretion may contribute to the establishment of tolerance in response to allogenic antigens. Indeed, we found that treatment with an anti-TGF-β antibody that blocks TGF-β signaling completely reversed the inhibitory effect of DCs on the proliferation of T cells. IDO, a catabolic enzyme responsible for the degradation of tryptophan via the kynurenine pathway, also plays a key role in immune tolerance by inhibiting T cell activation and proliferation [34–36]. Investigators have shown that distinct subsets of DCs expressing IDO play a prominent role in IDO-mediated immune tolerance, thereby contributing to excessive immune response-related diseases, such as the long-term maintenance of allografts and the suppression of the development of arthritis and allergies [36–39]. Here, paeoniflorin at a concentration of 30 μg/mL was shown to strongly upregulate the level of IDO from imDCs in the presence of LPS. Furthermore, IDO was shown to be critically necessary for the paeoniflorin-mediated effects using 1-MT, a competitive inhibitor of IDO, which completely ablated the stimulative effect of the DCs on lymphocyte proliferation.

Finally, we investigated whether paeoniflorin can induce IDO expression by the endogenous production of TGF-β. TGF-β has been shown to initiate or reinforce the expression of IDO, which acts as a signaling protein involved in the long-term tolerance established by dendritic cells [39–42]. Our data indicated that paeoniflorin was largely responsible for the increased expression of IDO in the imDCs + LPS group. This effect could be blocked by treatment with the neutralizing anti-TGF-β antibody. These data suggest that IDO possesses a signal-transducing activity in DCs that induces the long-term expression of IDO itself and maintains the stable regulatory phenotype of DCs. Moreover, this effect is triggered by TGF-β and requires the autocrine production of TGF-β from DCs. These findings indicate the possible synergistic potential of TGF-β and IDO in the treatment of human pathologies sustained by the overreacting immune response.

In conclusion, paeoniflorin can induce the production of DCregs from imDCs and imDCs stimulated with LPS, but not mDCs. These paeoniflorin-treated DCregs were shown to possess a phenotype consisting of high CD11b/c and low CD80, CD86 and CD40 expression levels, as well as increased phagocytosis of antigen, a stimulative function on the proliferation of Tregs and an inhibitory function on migration. Furthermore, paeoniflorin induces IDO expression through a mechanism that involves autocrine stimulation with DC-secreted TGF-β (Fig. 8).

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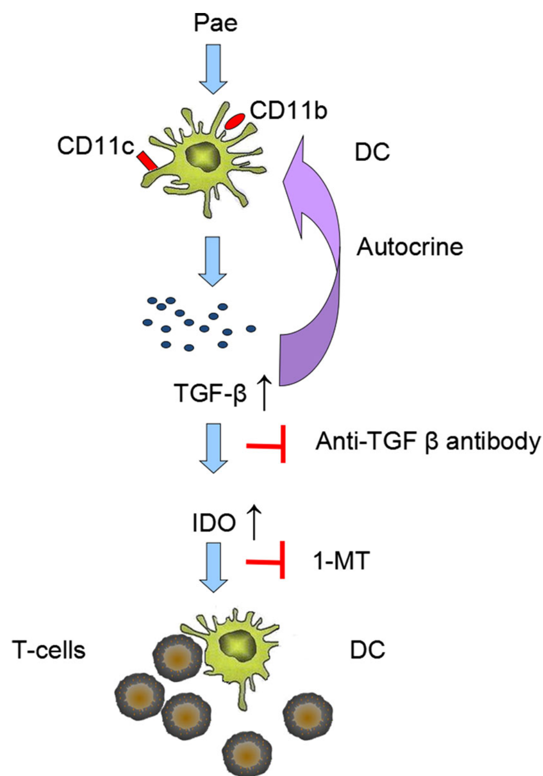

Fig. 8 Summary of paeoniflorin programing DCs toward the regulatory DC fate. Paeoniflorin programmed conventional DCs toward regulatory DCs with a high CD11b/c expression phenotype via the upregulation of autocrine TGF-β-mediated IDO expression. Anti-TGF-β antibody inhibited the expression of IDO in DCs, which specifically reversed the inhibition of paeoniflorin on DC-mediated T cell proliferation. *Pae* paeoniflorin, *DC* dendritic cells block

Compliance with ethical standards

Ethical approval This manuscript does not contain any financial/commercial conflicts of interests. The human participants provided informed consent for the experimental study, which has been reviewed and approved by the ethics committee of Tianjin Medical University and in accordance with the 1964 Helsinki Declaration.

Conflict of interest The authors declare no financial or commercial conflicts of interest.

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