

# The Mechanism of Delayed Ischemic Preconditioning in Alleviating Acute Ischemia/Reperfusion Renal Injury through Treg Mediated by Immature CD11c<sup>+</sup> Dendritic Cells

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## Keywords

Ischemia-reperfusion · Ischemic preconditioning · Dendritic cells · Acute kidney injury

## Abstract

**Introduction:** Renal ischemia-reperfusion injury (IRI) is one of the major causes of acute kidney injury, and its mechanism is complex involving multiple factors, while delayed ischemic preconditioning (DIPC) has a protective effect on the above process. In our previous study, we found that DIPC can exert its protection on renal IRI by inhibiting the maturation of dendritic cells (DCs), but the mechanism has not been clarified. This study aimed to investigate the protective mechanism of DIPC on renal IRI in mice through Treg mediated by immature DCs (imDCs). **Methods:** The IRI mice model, DIPC treatment, and conditional CD11c<sup>+</sup> DCs (CD11c-DTR) knock-out mice were used to perform our study. The maturation and differentiation of DCs and Treg cells in the kidney and spleen were analyzed by flow cytometry. HE staining was used to evaluate the pathology of the kidney tissue. The level of creatinine (Cr), oxidative stress factors (SOD, MDA), and inflammatory factors (TNF- $\alpha$ , IL-10, IL-4) were also measured. Then, imDCs were co-cultured with HK-2 cells, and apoptosis

was analyzed with flow cytometry and PI-Hoechst 33,342 fluorescence staining to assess the apoptosis rate of HK-2 cells under hypoxic-reoxygenated (H/R) conditions. **Results:** DIPC could decrease renal Cr levels, alleviate pathological renal damage, and reduce oxidative stress and inflammation caused by IRI. Moreover, DIPC could decrease the number of mature DCs (mDCs) and increase Treg lymphocyte infiltration in the kidney tissue, while the reduction of DCs reversed this process. In addition, our in vitro experiment found that in the H/R model, the apoptosis of HK-2 cells decreased which were co-cultured with imDCs. **Conclusion:** DIPC can regulate the differentiation of DCs into imDCs, thus affecting the differentiation level and distribution of Treg cells to exert its protective effect on renal IRI.

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## Introduction

Acute kidney injury (AKI) is a growing public global health problem associated with high morbidity, mortality, and health care costs [1, 2]. Studies have suggested that minor acute changes in the kidney can lead to short- and/or long-term complications [2]. Therefore, multidisci-

plinary collaborations are needed to establish early and better disease management.

Renal tissue is sensitive to the damage caused by ischemia and reperfusion, which is considered one of the main pathogenic factors of AKI. IRI can be seen after renal transplantation, cardiovascular surgery, and trauma. Besides inducing oxidative stress and apoptosis [3, 4], IRI can also induce microvascular injury, promote migration of inflammatory cells through the expression of adhesion factors on the surface of endothelial cells [5–7], and in turn promote the rise in serum Cr and increase inflammatory cell aggregation in renal tubular mesangial cells and inflammatory responses, leading to renal dysfunction [8, 9].

In 1986, Murry et al. [10] found that repeated short-term and low-intensity ischemic preconditioning (IPC) can improve myocardial cell injury caused by ischemia. Since then, this method has been widely used in the intervention of some diseases, especially for organs rich in blood oxygen supply and vulnerable to ischemia, hypoxia, and reperfusion injury, such as heart, brain, kidney, and liver [11–13]. IPC can reduce and resist the damage caused by long-term ischemia and hypoxia or IRI. Moreover, the protective effect of DIPC often appears 24–48 h after the first preconditioning, and its protective effect is stronger than the acute phases and lasts up to several days [11, 12]. DCs are considered the most potent class of antigen-presenting cells (APCs), which can efficiently absorb, process, and present antigens and activate initial T cells, promoting a primary immune system response. In our previous study, we found that DIPC protects against AKI after renal IR by inhibiting the maturation of DCs [14]. In the process of kidney disease, DCs act as the sentinel of immune surveillance, presenting antigens to T cells. These cells circulate through the blood to the kidneys and participate in local immune responses, thereby initiating an immune response [15, 16].

This study aims to investigate the protective mechanism of DIPC on renal injury induced by IR by focusing on CD11c<sup>+</sup> DCs, which demonstrates the specific regulatory mechanisms between DIPC and DCs in AKI and provides a new idea and theoretical basis for preventing and treating AKI disease with IRI characteristics.

## Materials and Methods

### *Mice*

The SPF-grade healthy male C57BL/6 mice (6–8 weeks old) were purchased from the Ningxia Medical University Animal Center. SPF-grade healthy male CD11c-DTR transgenic mice (DTR)

were purchased from Jackson Laboratories (USA). DTR mice were genotyped in the Ningxia Medical University Animal Facility, and 6–8-week-old DTR male mice were selected as the experimental group, while wild-type male mice of the same age were born in the same litter and were used as control group. Before starting the experiment, all mice were acclimatized to the environment for 1 week.

### *Bilateral IRI*

Animal experiments were performed in a sterile laminar flow animal operating room. Mice were anesthetized by intraperitoneal injection of 1% sodium pentobarbital solution at 6  $\mu$ L/g. The bilateral kidneys were then exposed, and the renal clips were separated with forceps; the clamping of bilateral renal clips and the clamping duration were selected according to the experimental grouping (e.g., the DIPC + IR group; i.e., both renal clips were surgically separated on day 1 and then clamped and preischemic for 15 min, followed by removal of the arterial clips and suturing of the incision). Then, the mice were reperused for 96 h. On day 5, both renal clips were separated again during the second operation, and with ischemia for 35 min, the arterial clips were removed, the skin was sutured, then the mice were sacrificed after 24 h of reperfusion.

All mice were placed on a thermostatic electric blanket during surgery and after anesthesia resuscitation; their anal temperature was monitored and maintained at 36.5°C–37.5°C. The specific surgical experimental grouping and surgical modeling flow are shown (Fig. 1a and 5a), and the proportion of knockout CD11c<sup>+</sup> cells is shown in online supplementary Figure 2 (for all online suppl. material, see [www.karger.com/doi/10.1159/000527172](http://www.karger.com/doi/10.1159/000527172)).

### *Assessment of Kidney Function and Histology after Renal IRI*

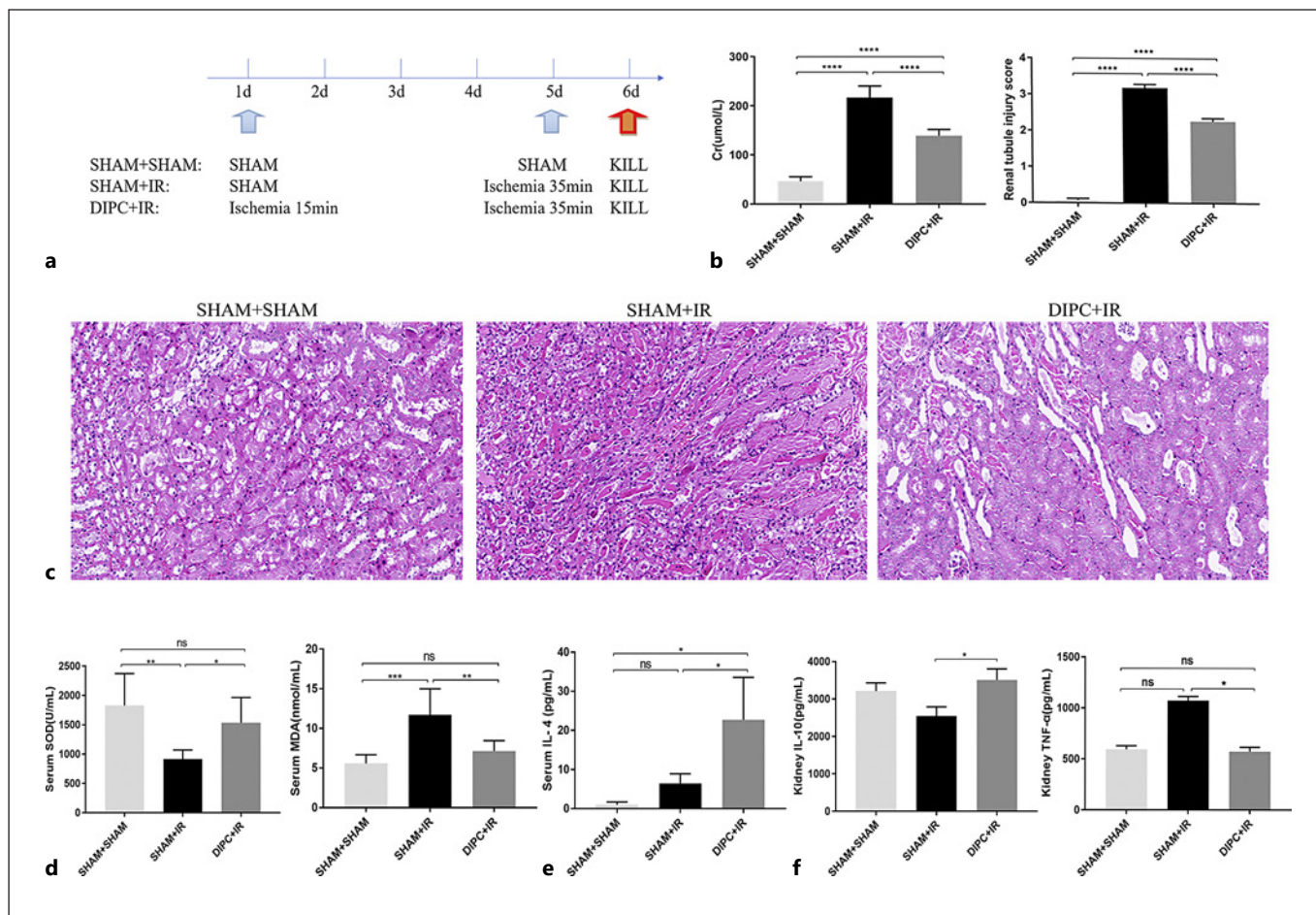
Plasma Cr was determined 24 h after renal IRI using a colorimetric assay according to the manufacturer's protocol (BioAssay Systems). After 24 h of kidney fixation, HE staining was performed. The tubular injury was scored using the Jablonski scale based on loss of tubular brush border, tubular dilatation, tubular pattern formation, tubular necrosis, and neutrophil infiltration. Ten nonoverlapping views of the dermal medullary junction at 200 $\times$  were randomly selected: no injury = 0, injury <25% = 1, 26–50% injury = 2, 51–70% injury = 3, and injury >75% = 4. Two pathologists did all assessments under double-blind conditions.

### *Treatment of Single-Cell Suspension of Spleen, Kidney, and Bone Marrow*

The mice were euthanized, and the tissues were collected. The kidney was immersed in 1 mL of collagenase I (Sigma) and digested for 1 h at 37°C in a thermostatic shaker, and subsequent treatment was the same as the spleen. The spleen and bone marrow were gently ground in RPMI 1640 medium containing 5% fetal bovine serum, filtered on a 200-mesh filter, and then centrifuged at 350 g for 5 min. The cell suspension was obtained after dissolving red blood cells, washing, and resuspension.

### *Flow Cytometry*

Cells were obtained on FACSCelesta or C6 flow cytometer (BD) and analyzed with FlowJo software. Cells were first pre-incubated (10 min) with anti-CD16/CD32 (eBioscience) Fc receptors to block nonspecific staining, then stained with directly conjugated primary antibodies for 45 min in 5% fetal bovine serum/phosphate-buffered saline (PBS) at predetermined optimal concentrations, washed, centrifuged, and then resuspended. The following



**Fig. 1.** Effect of DIPC on ischemia-reperfusion injury in mice. **a** Grouping and surgical preparation procedure of WT mice (SHAM, i.e., both sides of the kidney were separated without clamping the ischemia, and the incision was closed after 15 min or 35 min, respectively). **b** Serum creatinine levels and statistical plots of renal tubular injury scores in pathological sections. **c** Renal pathology of HE staining,  $\times 200$ . **d-f** Levels of oxidative stress factor SOD, MDA, and inflammatory factor IL-4, IL-10, TNF- $\alpha$ , in serum and kidney; ns,  $p > 0.05$ ,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  $****p < 0.0001$ .

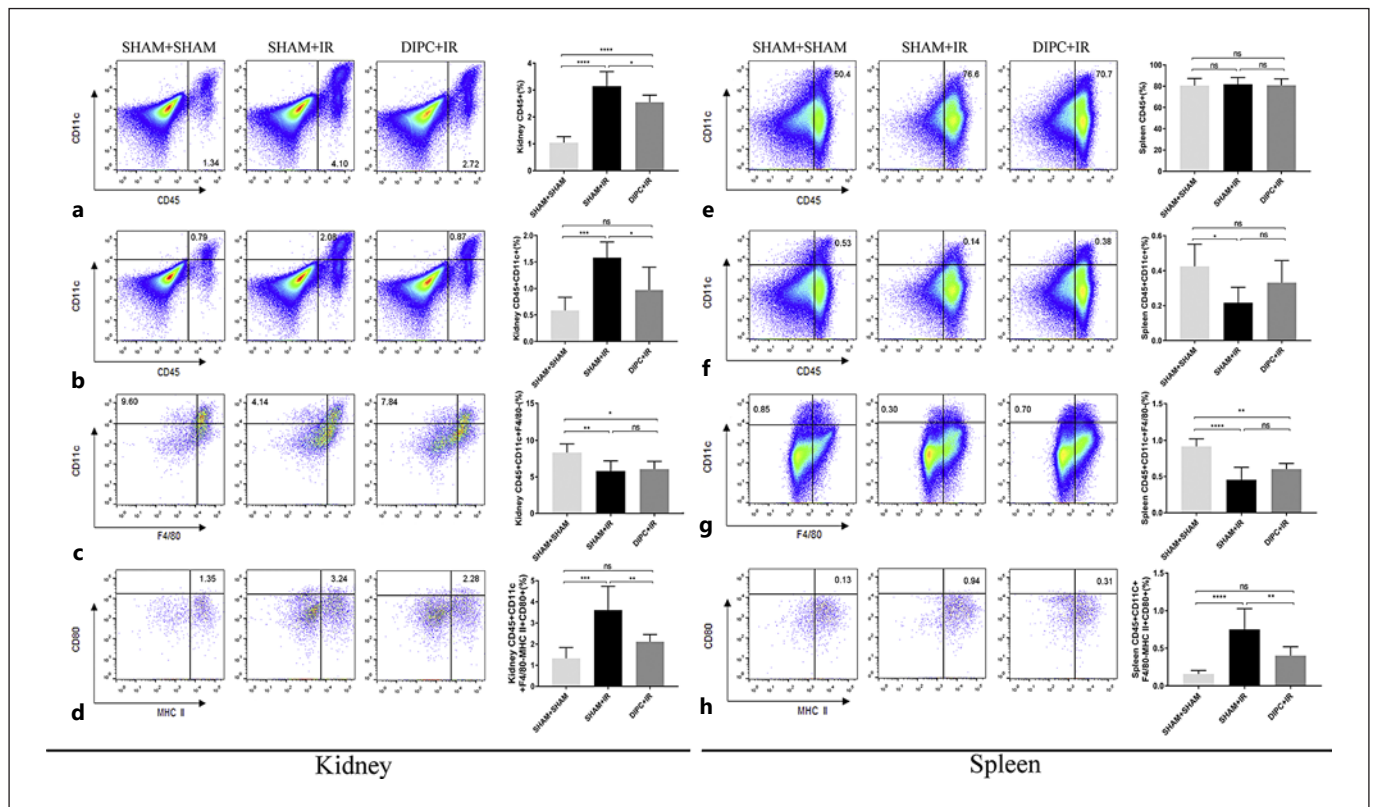
monoclonal primary anti-cellular antibodies were used: anti-mouse CD45 PE-Cyanine5.5, anti-mouse CD11c PE, anti-mouse F4/80 antigen eFluor 450, FITC anti-mouse MHC II, APC anti-mouse CD80 (B7-1), FITC anti-mouse CD4 (GK1.5), APC anti-mouse CD25 (PC61.5), PE anti-mouse/rat Foxp3 (FJK-16S), IL-17A monoclonal antibody, PE, IFN- $\gamma$  monoclonal antibody, PerCP-Cyanine5.5 were from eBioscience, USA, and PE anti-mouse IL-4 antibody was from BioLegend, USA.

#### Measurement of Oxidative Stress Levels and Inflammatory Factors in Mice

The supernatant of mouse serum and kidney tissue homogenate were retained for the determination of inflammatory factor indicators using CBA (BD) and ELISA (Thermo Fisher) and oxidative stress levels using SOD and MDA kits (Servicebio), respectively, following the manufacturer's instructions.

#### Cell Culture

Mouse bone marrow monocyte suspensions were prepared and resuspended in RPMI 1640 complete culture medium supplemented with recombinant granulocyte-macrophage colony-stimulating factor (concentration 40  $\mu\text{g/L}$ ) and rmIL-4 (concentration 10  $\mu\text{g/L}$ ) and inoculated into culture flasks. HK-2 cells were divided into 6 groups (HK-2 cells were cultured with or without mDCs/imDCs under normoxic and hypoxia/reoxygenation conditions, respectively). The imDCs were cultured until day 6, and the culture was continued with the addition of LPS stimulation 18 h before that to obtain mDCs. Under ordinary light microscopy, imDCs are round or oval, while mDCs have many long, tentacle-like protrusions that resemble dendrites. The molecular surface markers detected by flow cytometry showed that imDCs were low in CD80 and CD86, while mDCs were high in these molecules (online suppl. Fig. 3).



**Fig. 2.** Dendritic cells in the kidney and spleen. **a** Proportion of CD45<sup>+</sup> granulocytes to total renal cells. **b** Proportion of CD45<sup>+</sup>CD11c<sup>+</sup> cells to total renal cells. **c** Proportion of CD45<sup>+</sup>CD11c<sup>+</sup> F4/80<sup>-</sup> total DCs to CD45<sup>+</sup> granulocytes in the kidney. **d** Proportion of CD45<sup>+</sup> CD11c<sup>+</sup> F4/80<sup>-</sup>MHC II<sup>+</sup>CD80<sup>+</sup> mDCs to the proportion of total DCs in the kidney. **e-h** is the proportion of corresponding cells in the spleen (the typing method is the same as for the kidney); ns,  $p > 0.05$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

### Transwell Assay

HK-2 cells were inoculated into the lower layer of Transwell chambers, and the upper layer of co-cultured chambers were inoculated with imDCs or mDCs. All cells were usually cultured for 24 h. The cells in the normoxic culture group continued to be cultured without any treatment, and the hypoxic-reoxygenation group was reoxygenated for 1 h after 6 h of hypoxia at 1% O<sub>2</sub>, which resulted in the relevant cell models. The levels of apoptosis and necrosis of HK-2 cells were detected by flow cytometry and immunofluorescence.

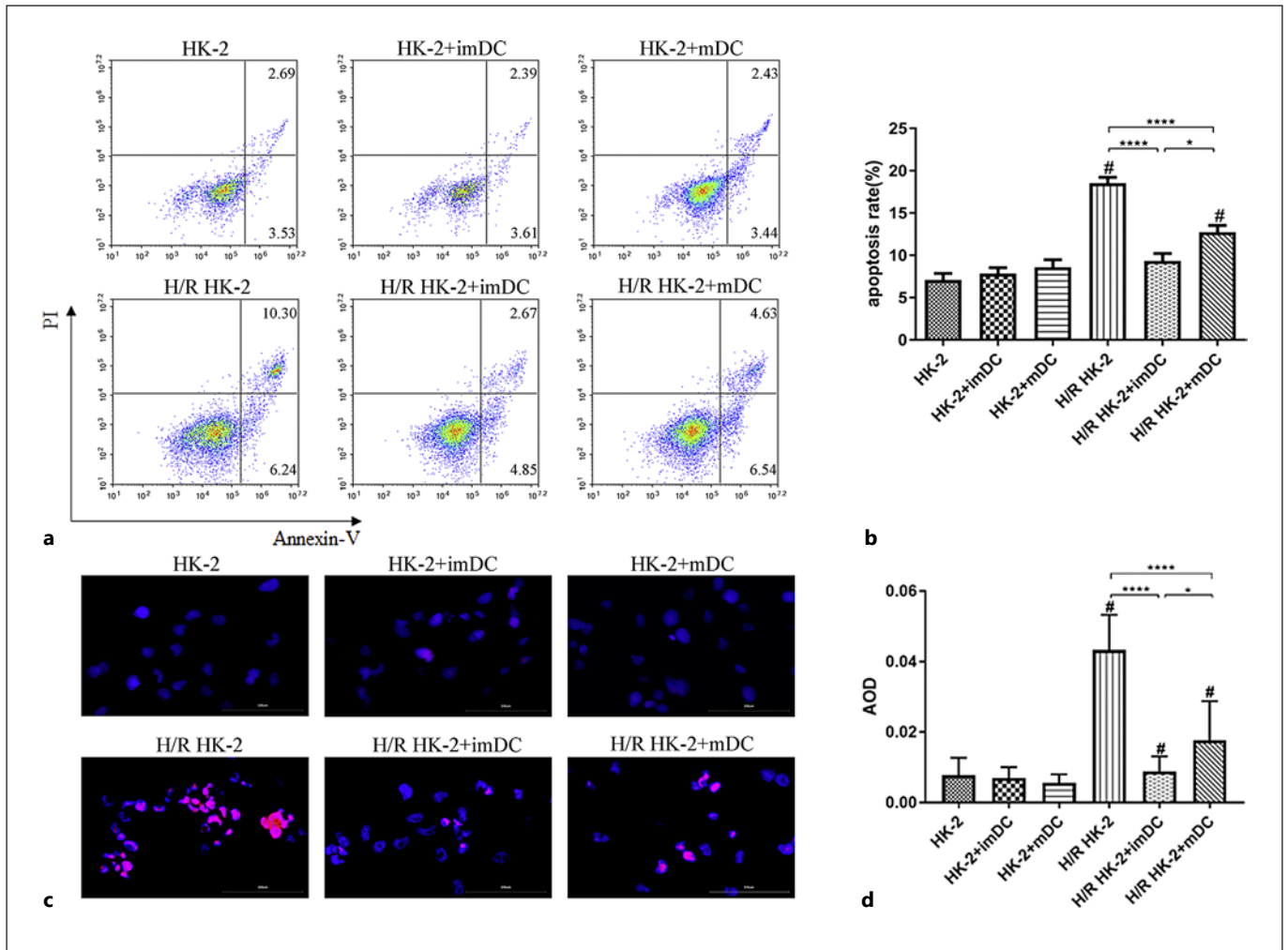
### Statistical Analysis

SPSS 25.0 (IBM Corporation, NY, USA) and GraphPad Prism 8.0 (GraphPad Software Inc., San Diego, CA, USA) were used for statistical analysis. The results were expressed as mean ± standard deviation. Data with normal distribution and homogeneous variances were compared by one-way ANOVA test. The Kruskal-Wallis nonparametric was used to compare data with abnormal distribution and/or variance unequal, and  $p < 0.05$  was statistically significant.

## Results

### DIPC Can Alleviate Renal Injury and Immune Inflammatory Caused by Ischemia-Reperfusion

First, we found that when mice were given 15 min renal ischemic preconditioning (DIPC + IR group), Cr levels decreased compared with the SHAM + IR group. Also, renal tubular dilatation was seen in the DIPC + IR group, along with reduced renal tubular injury score and tubular shape (Fig. 1a-c), which suggested that DIPC could alleviate pathological damage caused by renal IRI. Besides, the serum levels of SOD increased while MDA levels decreased in the DIPC + IR group compared to the SHAM + IR group (Fig. 1d), suggesting that DIPC could reduce the renal-induced systemic oxidative stress after IR. Next, serum inflammatory factor IL-4 was significantly elevated in the DIPC + IR group versus SHAM + IR group (Fig. 1e). Subsequently, we found that the proportion of



**Fig. 3.** Level of apoptosis and necrosis of HK-2 cells in co-culture with mDC or imDC. **a** Flow cytometry detection of apoptosis and necrosis ratio of HK-2 cells in each group, where Annexin-V<sup>+</sup>PI<sup>-</sup> is early apoptotic cells, Annexin-V<sup>+</sup>PI<sup>+</sup> is late apoptotic cells. **b** Flow statistics of early apoptosis and late apoptosis in each group. **c** PI-Hoechst 33342 fluorescence staining of the apoptotic and necrotic cells in each group graph, fluorescence microscopy ( $\times 40$ ). **d** Immunofluorescence AOD statistics of cell necrosis in each group; #  $p < 0.05$  compared with HK-2 group; ns,  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*\*\*  $p < 0.0001$ .

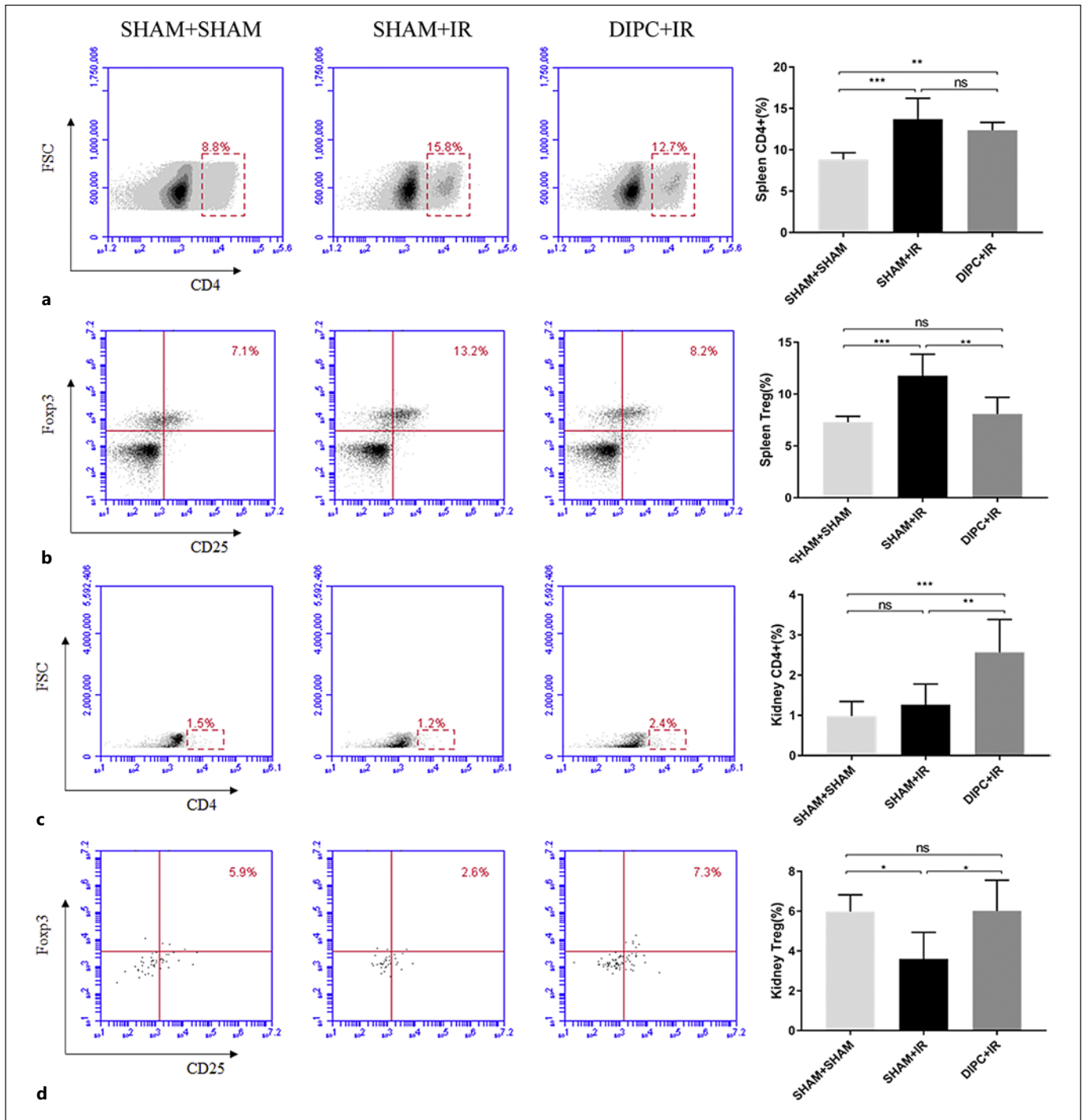
anti-inflammatory factor IL-10 increased after DIPC, while the pro-inflammatory factor TNF- $\alpha$  was reduced (Fig. 1f). Those results indicated the state of the immune mobilization.

*DIPC Inhibits the Aggregation of Inflammatory Cells in the Kidney and the Maturation and Differentiation of DCs*

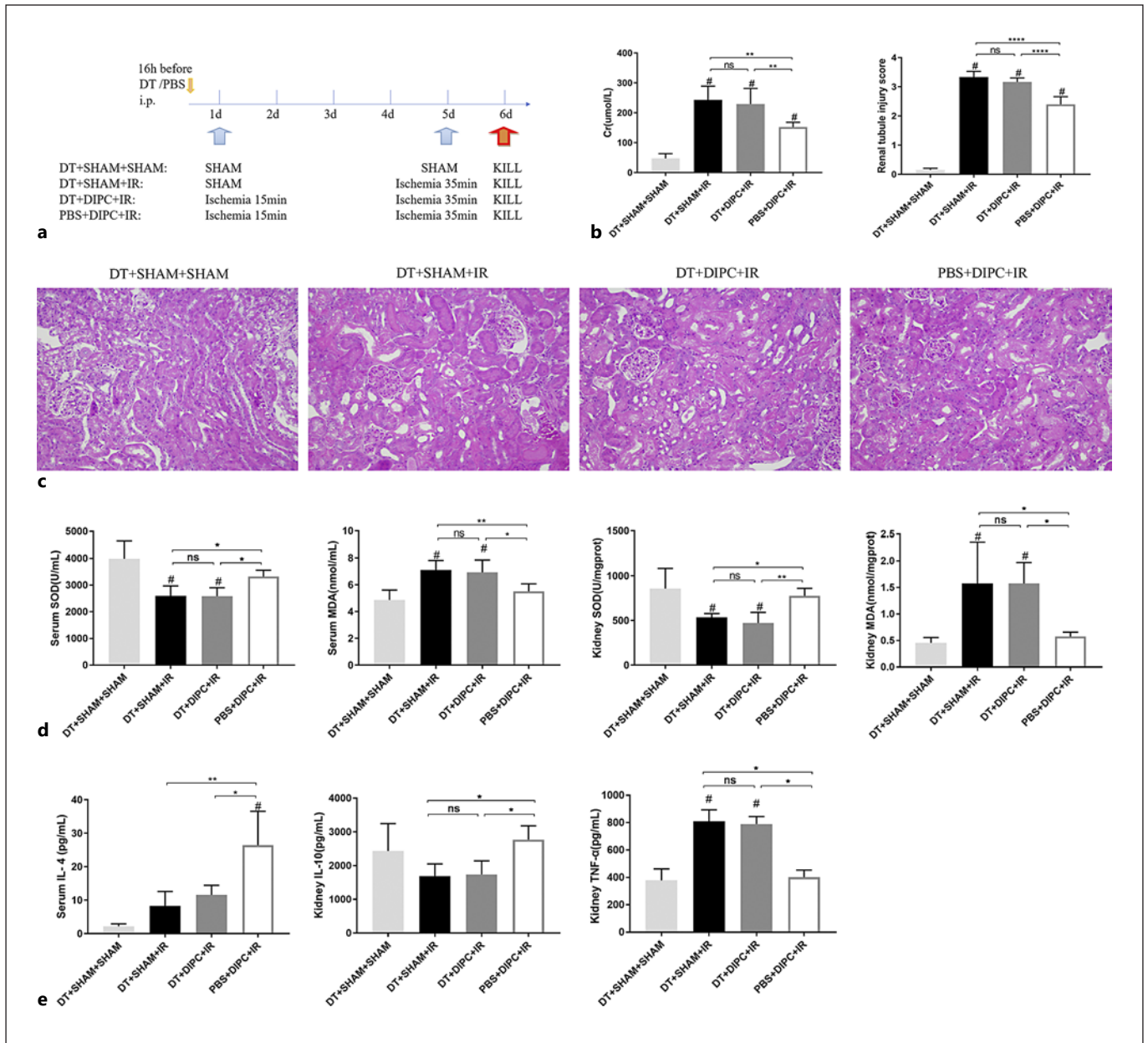
In this section, we found the proportion of leukocytes in the kidney was significantly higher during IRI (Fig. 2a), while the proportion of leukocytes was lower in the DIPC

+ IR group compared to mice receiving IRI only. This is another way to corroborate the effect of DIPC on attenuating renal IRI (Fig. 2b).

Further analysis revealed no significant difference in the total DCs between the two groups ( $p > 0.05$ , Fig. 2c), but the proportion of mDCs in the SHAM + IR group was significantly higher than that in the DIPC + IR group (Fig. 2d). Then, the spleen mDCs in the SHAM + IR group were significantly higher than in the sham-operated group, while mDCs were significantly reduced in the DIPC treated group (Fig. 2g, h). These results suggest that



**Fig. 4.** Changes in T-cell differentiation expression in the spleen and kidney. **a** Proportion of CD4<sup>+</sup> T cells to spleen cells. **b** Proportion of Treg cells to CD4<sup>+</sup> cells in the spleen. **c** Proportion of CD4<sup>+</sup> T cells in the kidney. **d** Proportion of Treg cells to CD4<sup>+</sup> cells in the kidney; ns,  $p > 0.05$ ; \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ .

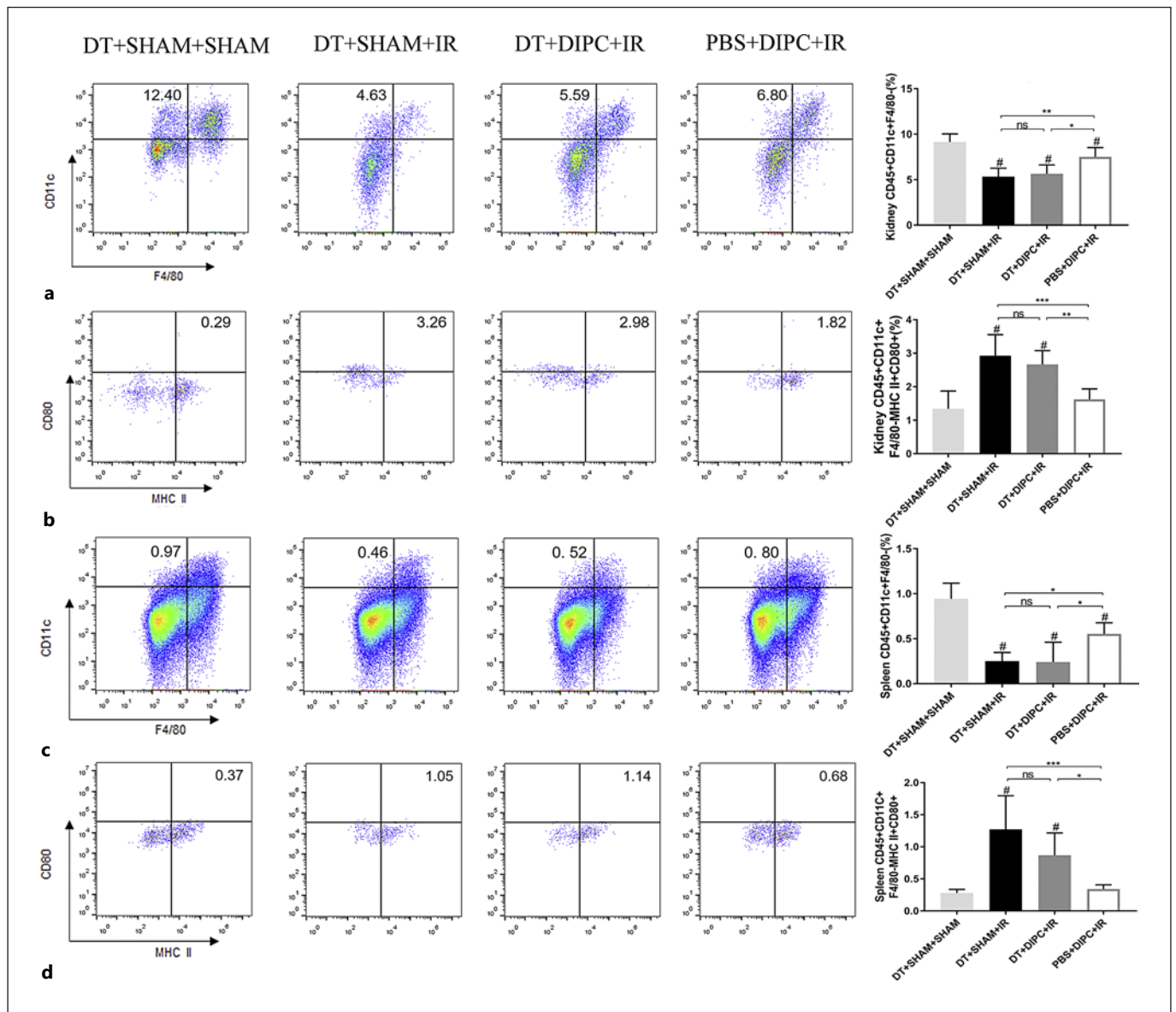


**Fig. 5.** Effect of DIPC on ischemia-reperfusion injury in DTR mice. **a** Grouping and surgical preparation procedure of DTR mice. **b** Serum creatinine levels and statistical plots of renal tubular injury scores in pathological sections. **c** Renal pathology of HE staining,  $\times 200$ . **d, e** Levels of oxidative stress factor SOD, MDA, and inflammatory factor IL-4, IL-10, TNF- $\alpha$  in serum and kidney.  $^{\#}p < 0.05$  compared with DT + SHAM + SHAM group; ns,  $p > 0.05$ ;  $^*p < 0.05$ ;  $^{**}p < 0.01$ .

the effect of DIPC on renal IRI may be achieved by inhibiting the differentiation of DC cells to mDCs or impeding mDCs migration to the kidney.

#### Protection of imDCs against HK-2 Cells under Hypoxic-Reoxygenated Conditions

In order to verify whether the protective effect of DIPC on the kidney is realized by affecting the differentiation of DC, we used a cell co-culture experiment with hypoxia-



**Fig. 6.** Dendritic cells in the kidney and spleen. **a** Proportion of CD45<sup>+</sup>CD11c<sup>+</sup> F4/80<sup>-</sup> total DCs to CD45<sup>+</sup> granulocytes in the kidney. **b** Proportion of CD45<sup>+</sup>CD11c<sup>+</sup> F4/80<sup>-</sup>MHC II<sup>+</sup>CD80<sup>+</sup> mDCs to the proportion of total DCs in the kidney. **c** Proportion of CD45<sup>+</sup>CD11c<sup>+</sup> F4/80<sup>-</sup> total DCs to CD45<sup>+</sup> granulocytes in the spleen. **d** Proportion of CD45<sup>+</sup> CD11c<sup>+</sup> F4/80<sup>-</sup>MHC II<sup>+</sup>CD80<sup>+</sup> mDCs to the proportion of total DCs in the spleen; #  $p < 0.05$  compared with DT + SHAM + SHAM group; ns,  $p > 0.05$ ; \*  $p < 0.05$ ; \*\*\*  $p < 0.001$ .

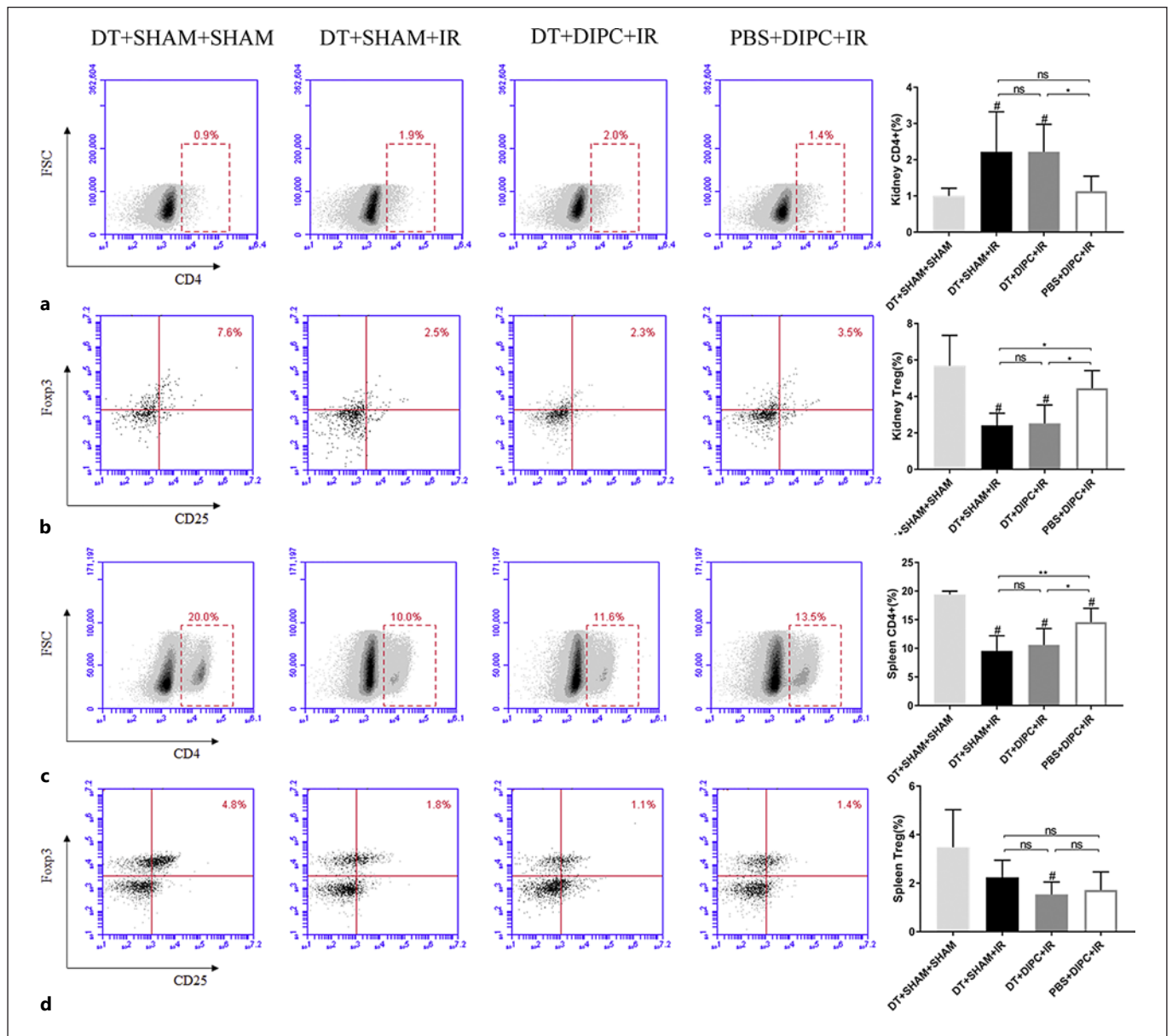
reoxygenation to verify the protective power of mDCs/imDCs, respectively. The results showed that the percentage of apoptosis and necrosis in HK-2 cells co-cultured with imDCs group after hypoxia-reoxygenation was significantly lower than that of HK-2 cells cultured alone and HK-2 cells co-cultured with mDCs after hypoxia-re-

oxygenation, indicating that imDCs had a significant protective effect (Fig. 3a–d).

#### *DIPC Increases the Differentiation of Treg Cells in the Kidney*

Further analysis showed no significant difference in the proportion of CD4<sup>+</sup> T cells, Th1, and Th17 cells in the





**Fig. 7.** Differentiation of T cells in the kidney and spleen. **a** Proportion of CD4<sup>+</sup> T cells to kidney cells. **b** Proportion of Treg cells to CD4<sup>+</sup> cells in the kidney. **c** Proportion of CD4<sup>+</sup> T cells in the spleen. **d** Proportion of Treg cells to CD4<sup>+</sup> cells in the spleen; #*p* < 0.05 compared with DT + SHAM + SHAM group; ns, *p* > 0.05; \**p* < 0.05; \*\**p* < 0.01.

DIPC + IR group compared with the SHAM + IR group in the spleen (online suppl. Fig. 1a, c). In contrast, we found a higher proportion of regulatory T cells (Tregs) in the spleen of the SHAM + IR group versus DIPC + IR group (Fig. 4a, b), and Th2 cells that IL-4 mainly secretes were increased in the DIPC + IR group (online suppl. Fig. 1b), which was also consistent with a previous study

(Fig. 1e). Then, we observed that Tregs were significantly higher in the DIPC + IR group than in the SHAM + IR group in the kidney (Fig. 4c, d). The above results might reflect a protective effect of DIPC on the kidney through imDCs explained by the stimulation of Treg cells' migration to the lesion site.

### *Elimination of CD11c<sup>+</sup> Cells Reduces the Protective Effect of DIPC on Kidney Function*

To explicate the experimental observations described above, the CD11c-DTR transgenic mice (DTR) were used. Results showed that when the target cells (CD11c<sup>+</sup> DCs) of DIPC were lost, the protective effect of DIPC disappeared, and there was no significant difference in serum Cr levels and renal tubular injury scores between the DT + DIPC + IR and DT + SHAM + SHAM groups ( $p > 0.05$ ). Yet, serum Cr levels and renal tubular injury scores were still significantly higher than those in the PBS + DIPC + IR group (Fig. 5b, c).

Further analysis of SOD and MDA levels revealed that there were no significant differences among its surgical model groups ( $p > 0.05$ ), while in serum and kidney tissue, SOD levels in the DT + DIPC + IR group were significantly lower than that in the PBS + DIPC + IR control group and were the same as in the DT + SHAM + IR group. Also, MDA content in both serum and kidney tissue was significantly decreased in the PBS + DIPC + IR group compared to the DT + DIPC + IR and DT + SHAM + IR group; yet, there was no significant difference in the DT-treated groups (Fig. 5d). Similarly, in detecting immune inflammatory factors, there were no difference in the levels of TNF- $\alpha$ , IL-10, and IL-4 in DT + DIPC + IR and DT + SHAM + SHAM groups ( $p > 0.05$ ) (Fig. 5e). All the above results suggest that the elimination of CD11c<sup>+</sup> cells reduce the effect of DIPC on oxidative stress and inflammatory response in the kidney.

### *Elimination of CD11c<sup>+</sup> Cells Reduces the Inhibition of Mature Differentiation of DCs in the Kidney and the Spleen by DIPC*

The flow cytometry results showed that in the kidney, the degree of leukocyte aggregation and the proportion of total DCs in the DT+DIPC+IR group had no significant difference with the DT + SHAM + IR group ( $p > 0.05$ , Fig. 6a). The proportion of mDCs was also not significantly different between DT + SHAM + IR and DT + DIPC + IR groups and was significantly higher than that in PBS + DIPC + IR ( $p > 0.05$ , Fig. 6b), indicating the protective effect of DIPC was reduced after intraperitoneal injection of DT and presumably which was caused by the decrease of imDCs. Similar results were found in the spleen ( $p > 0.05$ , Fig. 6c, d). The above results suggest that with the elimination of CD11c<sup>+</sup>DCs by DT and the disappearance of the DIPC target (such as the reduction of imDCs), the protective effect of DIPC on the kidney disappears.

### *Diminished Stimulatory Effect of DIPC on Treg Differentiation after CD11c<sup>+</sup> Cell Conditional Ablation*

In this study, no difference in the proportion of CD4<sup>+</sup> T cells was found in the kidney of DT + SHAM + IR and DT + DIPC + IR groups, yet which in DT + DIPC + IR groups were higher than in control mice (PBS + DIPC + IR group) (Fig. 7a). Similarly, DCs were eliminated after DT injection, and the adaptive injury stimulus given by DIPC had no DCs to receive, so the capacity of Tregs that their APCs could stimulate was decreased, and the proportion of Tregs in mice in the DT + DIPC + IR group was significantly lower than that in the PBS + DIPC + IR control group and was equal to that in the DT + SHAM + IR group (Fig. 7b), which is consistent with the results of Cao et al. who concluded that Tregs start to increase after 72h renal IRI [17]. Also, in the spleen, the higher proportion of CD4<sup>+</sup> T cells in the PBS + DIPC + IR group might be related to the stimulation of differentiation by DCs after the action of DIPC, while no statistical difference was found in the proportion of Tregs cells in the spleen between groups (Fig. 7c, d).

## **Discussion**

After renal IR, leukocytes can be activated by various cytokines (IL-1, IL-4, TNF- $\alpha$ , etc.) and inflammatory mediators such as ROS produced after IR, producing more ROS and arachidonic acid, enhance renal function injury [18, 19]. When renal IRI is reduced, oxidative stress levels in both serum and renal tissues decrease, as evidenced by an increase in SOD levels and a decrease in MDA levels (commonly oxidative stress indicators), which is consistent with our results on the protective effect of DIPC and consequently on the level of oxidative stress.

Renal cell apoptosis and necrosis caused by renal IRI may also induce the release of inflammatory factors. IL-4 can change the secretion and distribution of inflammatory factors as well as the migration and maturation of different immune cells, including DCs, Th1/2, and Treg cells [20–22]. TNF- $\alpha$  is a potent mediator of the inflammatory response, and the process of inflammatory response can result in an increase of TNF- $\alpha$  [23, 24]. Also, IL-10 has a pivotal regulatory role in inflammation, which is recognized as a major suppressor of the inflammatory response [25, 26]. Our results indicate that the protective effect of DIPC may be achieved through the regulation of immune inflammatory reaction.

DCs are the main source of intrarenal TNF- $\alpha$  production during the early “sterile” intrinsic immune response to renal IRI [27]. Under physiological homeostasis, DCs express MHC class I and II molecules at moderate levels and co-stimulatory molecules (e.g., CD80, CD86, CD40, etc.) at low levels [28–30]. When stimulated, DCs respond to “danger” signals and then differentiate and mature. The inducing mDCs can carry antigenic molecules captured in the kidney and migrate to secondary lymph nodes [28, 31], and then secrete inflammatory factors and chemokines such as TNF- $\alpha$ , IL-2, IL-6, IL-12 to promote T lymphocyte proliferation and activation, thus initiating adaptive immune response [31, 32]. Tolerogenic DCs, mainly obtained by stimulating imDCs *in vitro*, have multiple functions on the immune response. In addition to the autocrine IL-10 anti-inflammatory inhibition of antigen presentation, it was found that the combination of IL-10 with other cytokines (such as pleiotropic TGF- $\beta$ ) could enhance their respective anti-inflammatory functions and induce the production of powerful regulatory cells in the context of antigen specificity [33, 34]. Combined with the previously mentioned role of imDCs in phagocytosis and maintenance of autoimmune homeostasis [34], we speculated that imDCs may be converted into tolerogenic DCs during DIPC or release regulatory factors such as IL-10 and TGF- $\beta$  during co-culture, thus reducing apoptosis and necrosis cells. We also found that after DIPC treatment, although the level of mDC decreased, the overall level of DCs remained unchanged. The results implied that DIPC might protect the renal IRI by increasing imDCs, and then the cell experiments confirmed that. When HK-2 cells were co-cultured with imDCs after hypoxic-reoxygenated, the proportion of apoptosis and necrosis showed a lower level which is consistent with the validation of other experiments on the different roles of DCs [35–37]. Based on our data, we concluded that at least two ways might realize the protective effect of DIPC on the kidney. One was by inhibiting the maturation of DCs, reducing their transmission of antigenic information, and thus diminishing the ability to initiate an adaptive immune response. Another meaningful way is by promoting DCs differentiation into imDCs, which exert its protective function in many ways.

DCs can effectively stimulate and promote the proliferation of T lymphocytes and, depending on the nature of their maturation stimulus, presumably the cytokines they secrete [31, 32, 38], which then promote the differentiation of naïve T lymphocytes to specific T cells [31–33]. It has been verified that both early and late stages of AKI are characterized by infiltration of T lymphocytes. In contrast,

CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Tregs are anti-inflammatory lymphocytes that infiltrate into the kidney after 3 days in a mouse model of ischemia and promote repair after IRI [39, 40]. Some studies have confirmed that Treg has immunosuppressive and anti-inflammatory effects, protecting against ischemia-reperfusion AKI [39, 41]. In this study, we found that Treg cells were significantly increased in the kidney but less expressed in spleen in DIPC + IR group, which may indicate there exists a possible mechanism that the protective effect of DIPC may be realized by regulating the distribution of Tregs cells to injury site such as kidney that was caused by ischemia-reperfusion. Still, the imDCs stimulated by DIPC can migrate to the spleen and nearby lymph nodes to stimulate the transformation of the original T cells into Treg. Because the stimulation of DIPC is transient and weak, DCs will not continue to be stimulated and differentiated. As a result, DIPC might give the kidney a short period of ischemic pre-adaptation to mild injury stimulation allowing DCs to differentiate and stimulate T cells to differentiate to Tregs, thus producing a local protective effect during the second kidney IRI surgery. However, when DCs are eliminated (specifically imDC), DIPC loses its target cells, thereby weakening the function of inflammatory factor secretion and Treg differentiation, and losing its protective effect on the kidney.

To sum up, our data suggest that DIPC could exert anti-inflammatory and repair function and inhibit the differentiation and aggregation of other pro-inflammatory cells at the site of renal injury by affecting the differentiation of DCs and the generation of Tregs, thus exerting a protective role in renal IRI. This study provides a new perspective on the mechanism of DIPC against renal IRI and a possible theoretical avenue for treating AKI.

## Acknowledgments

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## Statement of Ethics

All animal studies (including the mice euthanasia procedure) were done in compliance with the Institutional Animal Care and Use Committee of Ningxia Medical University and guidelines and conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. This study protocol was reviewed and approved by Medical Research Ethics Review Committee of Ningxia Medical University General Hospital, approval No. (2018-050).

## Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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## Author Contributions

Wenjuan Yang, Ting Zhang, and Menghua Chen designed and revised the experiment. Wenjuan Yang wrote the manuscript. Wenjuan Yang, Tao Peng, Chunli Shi, and Ting Zhang performed the main experiment, and Chunli Shi and Fang Cui performed the cell sorting. Wenjuan Yang and Menghua Chen performed the cell images.

## Data Availability Statement

All data generated or analyzed during this study are included in this published article and its online supplementary material. Further inquiries can be directed to the corresponding author.

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