

# Indoleamine 2,3-Dioxygenase (IDO) Regulates Th17/Treg Immunity in Experimental IgA Nephropathy

(IgA nephropathy / IDO / Th17 / Treg / cytokine)

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**Abstract.** IgA nephropathy (IgAN) is the most common glomerulonephritis worldwide. Current studies have shown that the Th17/Treg immune balance may be involved in the occurrence of IgAN, but the exact mechanism is still unclear. Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyses degradation of tryptophan (Trp) through the kynurenine (Kyn) pathway; it can control inflammation and immune response by inducing Trp starvation. IDO may be a key molecule in regulating the Th17/Treg immune balance. However, it is not clear whether IDO is involved in the IgAN disease occurrence by regulating the Th17/Treg immune balance. In this study, an IgAN mouse model was established. The mice were intraperitoneally inoculated with IDO inhibitor 1-MT or agonist ISS-ODN to observe whether the IDO signalling pathway participates in the occurrence and development of IgAN by regulating the Th17/Treg immune balance. The results showed that IDO inhibitor 1-MT significantly increased renal in-

jury and glomerular IgA accumulation and up-regulated Th17/Treg and Th17-related cytokine expression in IgAN mice, while ISS-ODN significantly decreased renal injury and glomerular IgA accumulation, down-regulated Th17/Treg expression and inhibited Th17-related cytokine expression in IgAN mice. In conclusion, IDO was involved in the occurrence and progress of IgAN by regulating the Th17/Treg balance.

## Introduction

IgA nephropathy (IgAN) is the most common glomerulonephritis (GN) worldwide; its incidence in adults is about 25/100,000 (Rodrigues et al., 2017). The prevalence of IgAN seems to vary geographically, with East Asia and Pacific Rim Asian countries having the highest incidence (Du et al., 2017; Alexander et al., 2018). About 20 % to 30 % of IgAN patients develop into chronic renal failure within 20 years, which is an important cause of end-stage renal disease (ESRD) (Du et al., 2017).

Indoleamine 2,3-dioxygenase (IDO) is an enzyme that catalyses degradation of tryptophan (Trp) through the kynurenine (Kyn) pathway. It can control inflammation and immune response by inducing Trp starvation, and play a key role in the host defence against a variety of pathogens (Grohmann et al., 2003; Liu H. et al., 2009; Liu Y. et al., 2018). Macrophages, epithelial cells and dendritic cells (DC) can be induced to express IDO by pro-inflammatory cytokines (such as IFN- $\gamma$ ) or Toll-like receptor ligands (such as lipopolysaccharide). IDO can promote binding of costimulatory molecules (CD80, CD86) on antigen-presenting cells to cytotoxic T-lymphocyte antigen 4 (CTLA4) on the surface of T cells and negatively regulate activation of cytotoxic T cells (Liu et al., 2009). IDO can affect the immune response through two non-exclusive mechanisms: 1) formation of local "Trp amino acid deprivation", inhibiting proliferation of immune cells; 2) production of Trp metabolites with specific immunoregulatory or cytotoxic functions that inhibit T-cell activation and regulate differentiation of immature T cells into regulatory T (Treg)

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Abbreviations: AhR – aromatic hydrocarbon receptor, CCl<sub>4</sub> – carbon tetrachloride, CD200 – cluster of differentiation 200, CTLA4 – cytotoxic T-lymphocyte antigen 4, DC – dendritic cells, ELISA – enzyme-linked immunosorbent assay, ESRD – end-stage renal disease, GPCR – glucocorticoid-induced tumour necrosis factor receptor, GN – glomerulonephritis, HE – haematoxylin eosin, IDO – indoleamine 2,3-dioxygenase, IgAN – IgA nephropathy, IL – interleukin, IOD – integrated optical density, ISS-ODN – immunostimulatory oligodeoxynucleotide, Kyn – kynurenine, MT – methyl-tryptophan, PDC – plasmacytoid dendritic cells, Th – T helper, TLR9 – Toll-like receptor 9, Trp – tryptophan.

cells. Kyn regulates the immune response by activating transcription factor aromatic hydrocarbon receptor (AhR) in the absence of Trp starvation. IDO can also promote differentiation of Treg cells by inhibiting differentiation of Th17 cells. IDO is a crucial determinant in inducing Tregs by modulation of inflammatory responses in the IDO-KO mouse model of gingivitis (Tyagi et al., 2017).

Th17 cells are a subset of T helper cells that produce interleukin 17 (IL-17) and play a role in inflammation and tissue damage. There are some evidences that Th17 cells are associated with nephritis, asthma and other autoimmune diseases (Han et al., 2015; Sigdel et al., 2016; Zou et al., 2018). It was found that infiltrating Th17 cells secrete IL-17, which binds to its receptor and promotes CCL20 production in local kidney cells (Kaneko et al., 2018). CCL20 is a small cytokine that attracts lymphocytes, neutrophils, monocytes and dendritic cells to epithelial cells. The binding of CCL20 to its receptor CCR6 induces recruitment of inflammatory leukocytes (neutrophils, lymphocytes, etc.) and eventually leads to immune-mediated renal injury.

Some animal models have been established according to the known pathogenesis of IgAN, which can be roughly divided into immune-induced, secondary and spontaneous types. Secondary IgAN models limit their application due to the impact of primary disease. Serum IgA levels fluctuate greatly in spontaneous IgAN mice, and the incidence of IgA nephropathy is not high. However, the animal IgAN model induced by a xenogeneic protein is more similar to human IgAN in terms of clinical indicators and pathological changes (Imai et al., 1985; Amore et al., 1997). A modified IgAN mouse model induced by xenogeneic proteins was established in this study (Meng et al., 2014). We increased the dosage of BSA, used castor oil as the adjuvant, and reduced the dosage of CCl<sub>4</sub> to reduce its toxicity and alleviate liver injury.

In view of the relationship between the IDO-AhR signal axis and differentiation of IL-17 and Treg, we studied whether the IDO-AhR signal axis participated in the IgAN occurrence through differentiation of Th17 and Treg cells.

## Material and Methods

### *Experimental animals*

A total of 24 healthy female BALB/c mice weighting  $20 \pm 2$  g (6 weeks old) were purchased from Shanghai Slac Laboratory Animal Co., Ltd. They were kept at 25 °C and 70% humidity with a standard solid mixed food, and they were free to eat and drink. The mice were placed in a comfortable and quiet room for one week before the initiation of the experimental procedure. They were randomly divided into the control group, IgAN group, IgAN+ IDO inhibitor (1-methyl-tryptophan (1-MT); MedChem Express, Monmouth Junction, NJ) group, and IgAN+ IDO agonist (immunostimulatory

oligodeoxynucleotide; ISS-ODN; Shanghai Biosune Biotechnology Co., Ltd., Shanghai, China) group. There were six mice in each group. The mice in the control group were not treated. The mice in the IgAN group were given BSA (Roche; Indiana Polis, IN) every other day (800 mg/kg body weight), they were subcutaneously injected with 0.1 ml carbon tetrachloride (CCl<sub>4</sub>, 20%) and castor oil (80%) every week and with intraperitoneal injection of 0.08 ml once every two weeks, LPS (50 µg) was injected twice at the 6<sup>th</sup> week and the 8<sup>th</sup> week, respectively. In the IgAN+1-MT group, 1-MT was injected intraperitoneally (1 mg/day) from the 8<sup>th</sup> week in addition to the treatment of the IgAN group. In the IgAN+ISS-ODN group, ISS-ODN was injected intraperitoneally (1 µg/day) from the 8<sup>th</sup> week in addition to the treatment of the IgAN group. All animals were sacrificed after 11 weeks of continuous treatment.

This study was audited and approved by the Animal Ethics Committee of Hunan Provincial People's Hospital, the First Affiliated Hospital of Hunan Normal University. All experimental procedures and animal care were carried out under the guidance of the Ethics Committee in order to minimize the suffering of animals.

### *Observation of clinical indicators*

At the time point of detection, all mice were placed in metabolic cages to collect 24-h urine samples, and ACR (urinary albumin/creatinine) was detected by an ACR urine analyser (SHCHEER, Shanghai, China). The number of red blood cells in urine was counted using a blood cell counting chamber.

### *Kidney pathological examination*

Kidney tissues were fixed with 4% paraformaldehyde and embedded in paraffin. They were cut into 4 µm tissue sections and stained with HE and Masson methods. The stained renal tissue sections were examined and analysed by pathologists under an optical microscope. The examination focused on glomerular abnormalities including segmental hyperplasia, mesangial matrix dilatation, capillary wall thickening, glomerular hypercytosis, hyaline degeneration, crescent formation, and fibrinoid necrosis. For immunofluorescence analysis, the kidney tissue was cut into frozen sections and fixed with acetone for 1 min. After fixation, samples were blocked with 5% normal goat serum diluted by PBS at room temperature for 1 h. They were washed with PBS three times and incubated with a goat anti-mouse IgA antibody (Santa Cruz; sc-3692, Dallas, TX) labelled by FITC at room temperature for 1 h. After washing with PBS for three times, they were mounted with anti-quenching tablets and observed under an immunofluorescence microscope. Five visual fields were randomly selected from each specimen and IOD values were counted and evaluated.

### *Isolation of leukocytes from kidney tissue*

The unilateral kidney of mice was completely cut by ophthalmic scissors into small pieces, which were incu-

bated with collagenase D (0.4 mg/ml) and DMEM containing 0.01 mg/ml DNase I and 10% FBS at 37 °C for 45 min. Samples were filtered with 70 µm and 40 µm nylon mesh, respectively, and washed with PBS for two times. A leukocyte-rich cell suspension was obtained by centrifugation using the Percoll density gradient (70% and 40%) method (Singer et al., 1995). The proportion of living cells detected by the trypan blue staining method was more than 95 %. The cells were subjected to flow cytometry analysis.

### Flow cytometry

The leukocytes isolated from kidney tissue were first incubated with CD3 (PE-Cy5; eBio, San Diego, CA) and CD4 antibodies (FITC; eBio) on ice for 30 min. To determine the IL-17 presence, the cells were re-suspended in RPMI 1640 containing 10% FBS and incubated with 50 ng/ml PMA and 1 µg/ml ionomycin at 37 °C in an atmosphere with 5% CO<sub>2</sub> for 5 h. The cells were collected, 3 µg/ml Brefeldin A was added, and the cells were incubated at 37 °C for 30 min. Cytofix/Cytoperm (eBioscience) was added and the cells were incubated on ice for 30 min. A rat anti-mouse IL-17 antibody (PE; eBioscience) was added and the cells were further incubated on ice for 30 min for IL-17 detection. To determine the Treg cell presence, the cells were incubated with CD4 (FITC, eBio., Franklin Lanes, NJ), and CD25 (APC, eBio.) on ice for 30 min, Fix/Perm buffer (eBio.) was then used for immobilization and permeabilization. Cells were then incubated with an anti-Foxp3 staining kit (PE, eBioscience) for 30 min. The cells were analysed using a flow cytometry system (Becton Dickinson FACSCalibur, Franklin Lanes, NJ) and CELL-Quest software (BD Biosciences).

### Enzyme-linked immunosorbent assay (ELISA)

The IL-17A, IL-21, IL-6, and CCL20 levels in the mouse kidney tissue were detected by an ELISA kit (USCN, Wuhan, China) in accordance to the kit instructions. The OD450 values were read by a microplate spectrophotometer.

### Statistical analysis

SPSS 17.0 software (SPSS; Chicago, IL) was used for all statistical analyses. The data are presented as the mean ± standard deviation. The comparison of mean values among multiple groups was analysed by multiple comparison tests, one-way analysis of variance (ANOVA) and LSD-t test. For all tests,  $P < 0.05$  was considered statistically significant.

## Results

### IDO signalling pathway affects ACR in IgAN mice

To determine the effect of IDO on renal injury in IgAN mice, ACR in different groups of mice was de-

tected by a urine test (Fig. 1). The results showed that 1-MT significantly increased renal injury in the IgAN mice ( $P < 0.01$ ), while ISS-ODN significantly decreased renal injury in the IgAN mice ( $P < 0.01$ ).

### Inhibition of IDO signalling pathway aggravated renal injury in IgAN mice and activation of IDO signalling pathway reduced renal injury in IgAN mice

The HE and Mason staining results showed that the glomerular mesangium and collagen fibres increased significantly in the IgAN mice compared to the control group. The IDO inhibitor 1-MT promoted mesangial proliferation and collagen fibrillogenesis, while ISS-ODN reduced mesangial proliferation and collagen fibrillogenesis (Fig. 2).

### Inhibition of IDO signalling pathway aggravated IgA accumulation in IgAN mice and activation of IDO signalling pathway reduced IgA accumulation

The results of indirect immunofluorescence showed that glomerular IgA increased in the IgAN mice compared with the control group. The IDO inhibitor 1-MT promoted glomerular IgA accumulation, while the agonist ISS-ODN reduced glomerular IgA accumulation (Fig. 3).

### Inhibition of IDO signalling pathway increased and activation of IDO decreased the Th17/ Treg ratio in the kidney tissue of IgAN mice

Flow cytometry showed that IDO inhibitor 1-MT increased the proportion of Th17 cells and decreased the proportion of Treg cells in the kidney tissue of IgAN mice, while ISS-ODN decreased the proportion of Th17 cells and increased the proportion of Treg cells in the kidney tissue of IgAN mice (Fig. 4).

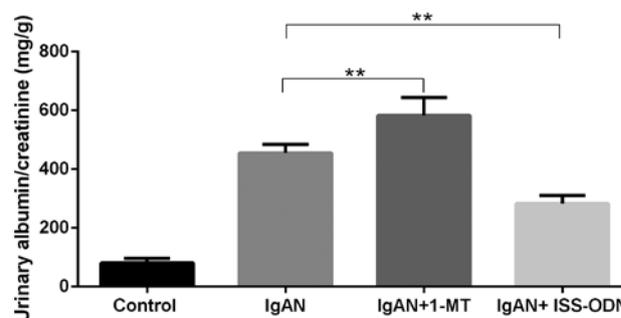


Fig. 1. Effect of IDO enzyme activity on ACR in IgAN mice  
\*\* $P < 0.01$  vs. control

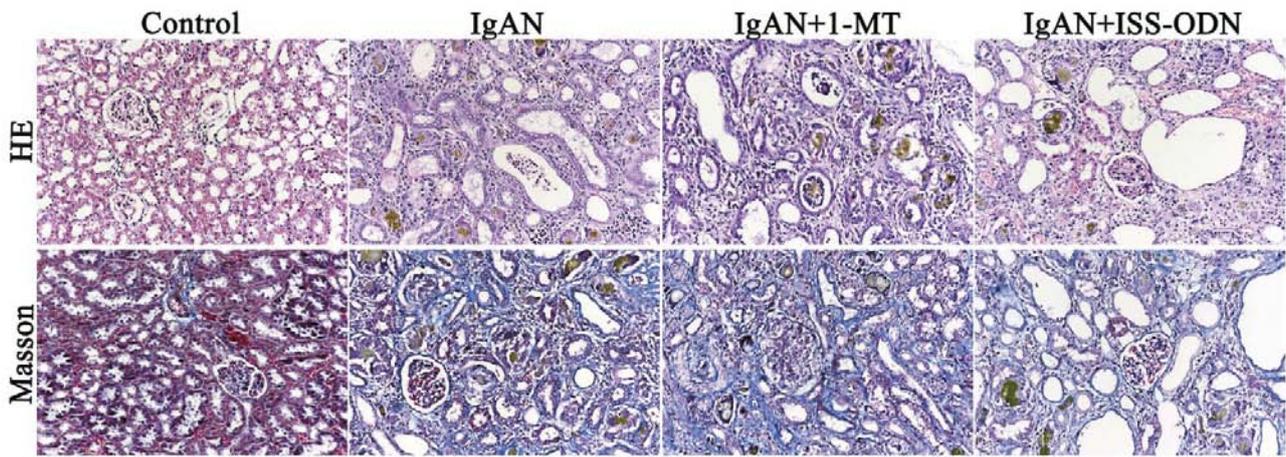


Fig. 2. Effect of IDO enzyme activity on renal injury in IgAN mice  
The glomerular mesangium and collagen fibres were increased significantly in IgAN mice.

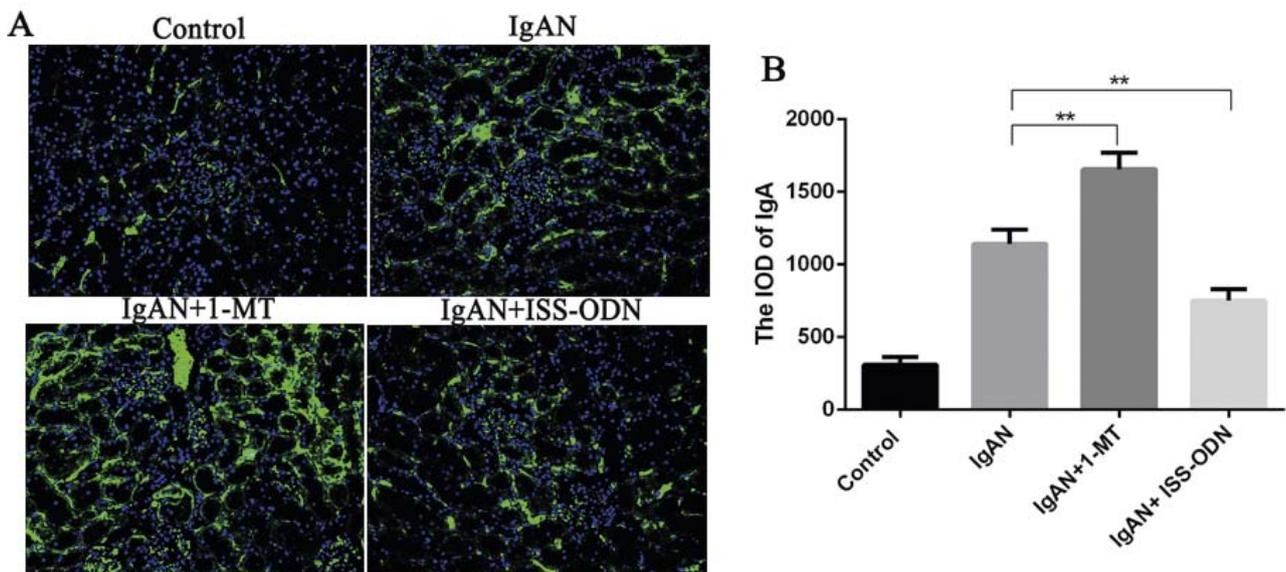


Fig. 3. Effect of IDO enzyme activity on IgA accumulation in the kidney of IgAN mice  
1-MT promoted while ISS-ODN inhibited the accumulation of IgA in the kidney.

A: Observation of glomerular IgA content in mice of different groups by indirect immunofluorescence; B: integrated optical density (IOD) values of different groups. \*\*P < 0.01 vs. control

*Inhibition of IDO signalling pathway promoted increase of Th17-related cytokines in the kidney tissue of IgAN mice, and activation of IDO signalling pathway inhibited increase of Th17-related cytokines in the kidney tissue of IgAN mice*

ELISA results of Th17-related cytokines in the kidney tissue of mice from the four experimental groups showed that 1-MT promoted expression of the Th17-related cytokines in the kidney tissue of IgAN mice, while ISS-ODN inhibited it (Fig. 5).

## Discussion

In the field of autoimmune diseases, the Th17/Treg immune balance has attracted attention, and studies have also shown that this immune balance is related to a variety of kidney diseases (Liu et al., 2011; Zhang et al., 2014; Li et al., 2016; Zhu et al., 2018). In the children with nephrotic syndrome, the number of Th17 cells increased, Treg cells decreased, and the expression of IL-17 in renal tissue was increased, which suggested that the immune balance of Th17/Treg was disturbed (Liu et al., 2011; Zhang et al., 2018). A recent study has shown that a Th17/Treg immune balance disorder also occurs in IgAN patients (Peng et al., 2013). In the IgAN patients, the decrease in the Treg cell counts causes dimin-

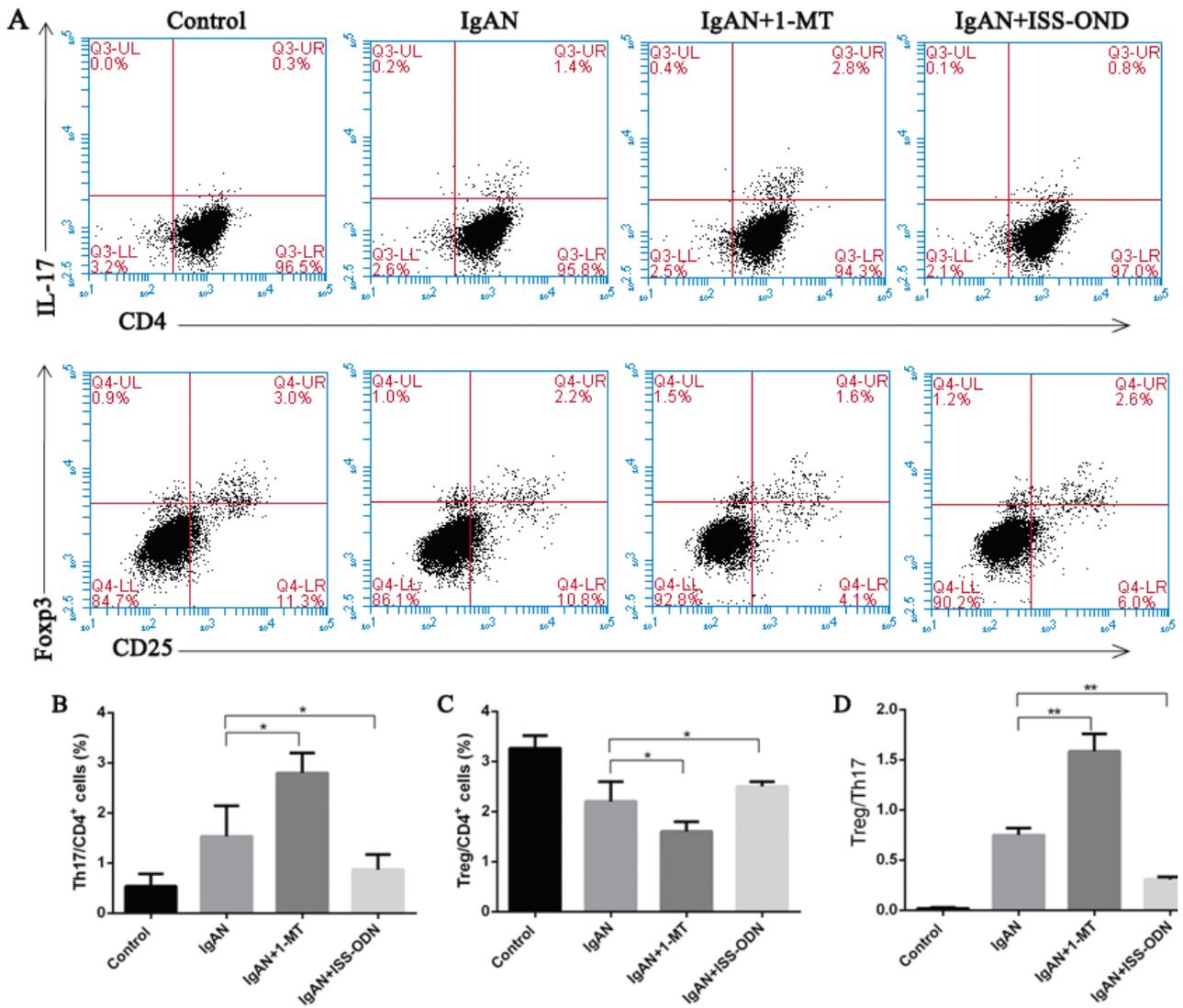


Fig. 4. Effect of IDO enzyme activity on Th17/Treg cells in the kidney of IgAN mice

**A:** Detection of Th17 and Treg cells in the kidney tissues of mice in different groups by flow cytometry; **B:** Th17 cell proportion in the kidney tissues of mice in different groups. 1-MT up-regulated the proportion of Th17 cells in the kidney tissue of IgAN mice, while ISS-ODN inhibited the proportion of Th17 cells in the kidney tissue of IgAN mice; **C:** Treg cell proportion in the kidney tissues of mice in different groups, 1-MT down-regulated the proportion of Treg cells in the kidney tissue of IgAN mice, while ISS-ODN up-regulated the proportion of Th17 cells in the kidney tissue of IgAN mice; **D:** Th17/Treg ratio in the kidney tissues of mice in different groups.

\* $P < 0.05$ ; \*\* $P < 0.01$

ished immunosuppressive regulation, which triggers over-immune response to pathogens, increase in production of inflammatory cytokines, and promotes IgA increase by antibody class switch, leading to the occurrence of IgAN (Lin et al., 2012). In this experimental study, we found that the proportion of Th17 significantly increased in IgAN mice, while the proportion of Treg cells decreased. Further, we demonstrated that these changes were affected by IDO activity.

IDO could induce tryptophan catabolism and cause tryptophan depletion, which inhibits T-cell immunity, promotes apoptosis in activated T cells, and thus contributes to immune tolerance (Grohmann et al., 2003; Mellor

and Munn, 2004). The IDO activity could also mediate binding of ligands Toll-like receptor 9 (TLR9), cluster of differentiation 200 (CD200) and glucocorticoid-induced tumour necrosis factor receptor (GITR) on plasmacytoid dendritic cells (PDC) with their corresponding receptors on T cells to induce production of specific Treg cells (Zoso et al., 2014). In addition, in animal models, it was found that the number of Treg cells in the spleen increased after intravenous injection of ISS-NO, while subcutaneous injection of IDO inhibitor 1-MT promoted transformation of Treg cells into Th17 cells (Satpute et al., 2009). Therefore, IDO may be a molecular switch regulating the Th17/Treg immune balance.

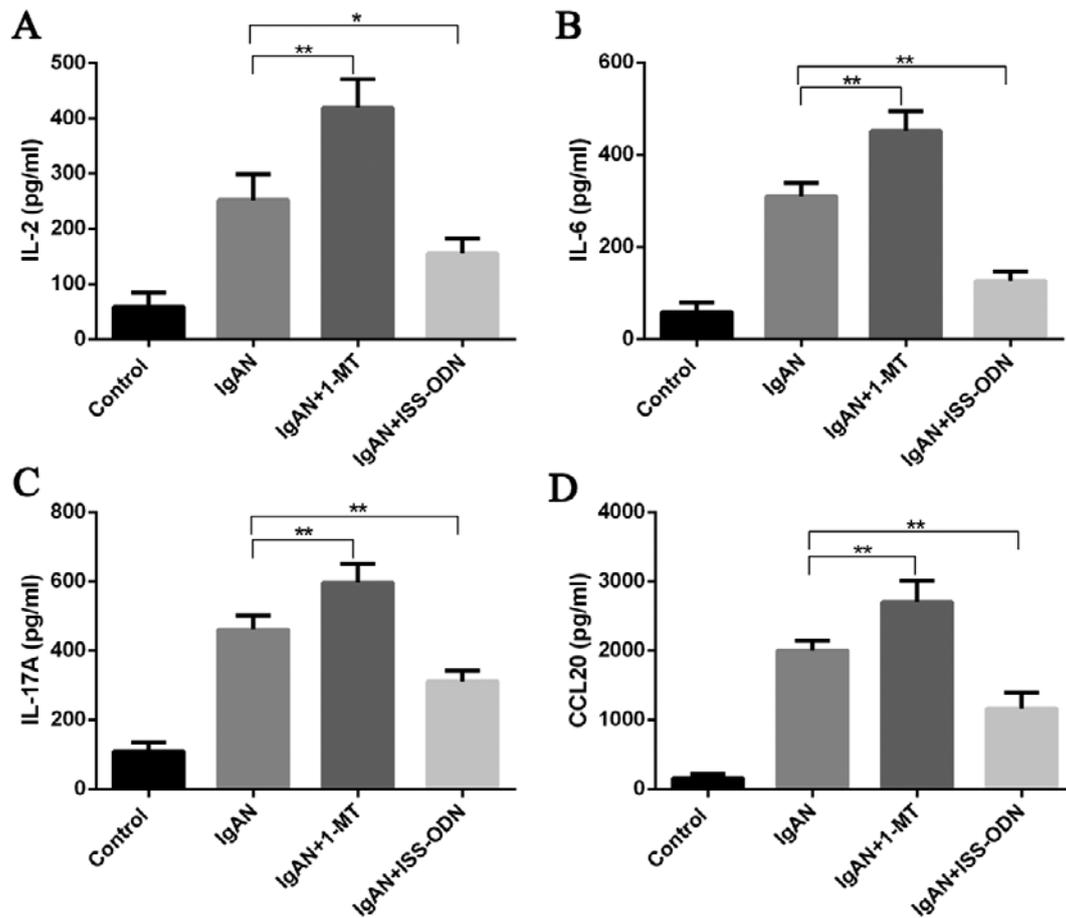


Fig. 5. Effect of IDO enzyme activity on the expression of Th17-related cytokines in the kidney tissue of IgAN mice. 1-MT up-regulated the levels of IL-2, IL-6, IL-17A, and CCL20 in the kidney tissue of IgAN mice, while ISS-ODN inhibited their levels in the kidney tissue of IgAN mice.

Local infiltration of renal tissue by T cells is the main feature of glomerulonephritis in IgAN patients. CD4<sup>+</sup> T-helper cells are thought to play a key regulatory role in the pathologic immune response leading to the IgAN disease. Th17 and Treg cells are important subsets of CD4<sup>+</sup> T-helper cells. Compared with IL-17<sup>-/-</sup> mice, wild-type mice may suffer from a more severe nephritis (Paust et al., 2009). The Th17/Treg ratio increased with the increase in proteinuria and decrease of the albumin level in patients with minimal change nephrotic syndrome (Fujigaki et al., 2017). Huang found that the proportion of CD4<sup>+</sup>CD25<sup>+</sup> cells in IgAN patients was significantly lower than that in healthy individuals (Huang et al., 2014). Therefore, the Th17/Treg immune balance may be directly involved in the occurrence of IgAN. In this study, we found that IDO inhibitor 1-MT significantly increased the number of Th17 cells, up-regulated expression of Th17-related cytokines (IL-17, IL-21 and IL-6), and down-regulated the number of Treg cells in the IgAN mice. In contrast, the levels of Th17 cells and related cytokines decreased, while the number of Treg cells increased significantly after the treatment with ISS-ODN, the IDO agonist. These results suggest that

IDO may be involved in the pathogenesis of IgAN by affecting the Th17/Treg immune balance.

In addition, we observed that the expression level of CCL20 was also affected by the IDO activity. The IDO inhibitor 1-MT significantly increased, while ISS-ODN significantly decreased, the CCL20 expression level. CCL20 is a member of the CC chemokine family and the only known chemokine that interacts with CCR6. Th17 can secrete CCL20 in both humans and mice, and CCL20 can promote migration of Th17 and Treg mediated by CCR6. Depletion of CCR6 in Th17 cells inhibits recruitment of Th17 and Tregs to the inflamed tissues (Lu et al., 2017). A study has shown that CCR6 deficiency can reduce the infiltration of Th17 cells in glomerulonephritis, suggesting that CCR6 mediates infiltration of Th17 cells in the kidney (Koga et al., 2016).

### Conclusions

Our study has found that reduction of the IDO enzyme activity in IgAN mice aggravates the disease, promotes infiltration of the kidney tissue by Th17 cells, and increases expression of related inflammatory factors. CCL20 acting through CCR6 may further increase the

accumulation of Th17 cells due to a positive feedback. IDO may thus be a key molecule in the development and progression of IgAN.

### Competing interests

The authors declare that they have no competing interests to disclose.

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