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Immunosuppressive effect of artemisinin and hydroxychloroquine combination therapy on IgA nephropathy via regulating the differentiation of CD4+ T cell subsets in rats

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ABSTRACT

Immunoglobulin A nephropathy (IgAN) is an autoimmune kidney disease with complex pathogenesis leading to end-stage renal damage. The crucial pathological characteristic in IgAN is IgA immune complexes deposition accompany with mesangial cell proliferation and mesangial matrix expansion. Artemisinin (ART) is isolated from traditional Chinese medicine *Artemisia annua* L. Hydroxychloroquine (HCQ) is a classical antimalarial drug used to treat autoimmune diseases. Both of them possess immunosuppressive, immunomodulatory and anti-inflammatory features. The aim of this study was to investigate the pharmacological effects of ART combined with HCQ (AH) and explore the underlying mechanisms in IgAN. In vivo, our results showed that AH could significantly improve kidney dysfunction, decrease mesangial matrix expansion as well as immune complexes in mesangial area visualized by H&E and PAS staining. The depositions of IgA immune complexes and complement 3 (C3) were obviously reduced after AH treatment by immunofluorescence. Interestingly, the morphology of kidney and spleen was significantly swelled but reverted by AH in IgAN rats. Further mechanistic study showed that the higher proportions of the Th2 and Th17 cells were reduced but the lower differentiation of Th1 and Treg cells subsets were promoted by AH. Taken together, this study demonstrated that there was an immunosuppressive effect of AH therapy on IgAN rats via regulating the differentiation of CD4+ T cell subsets, which provided an alternative approach for IgAN treatment.

1. Introduction

Immunoglobulin A nephropathy (IgAN), accompanied with complex pathogenesis, is an autoimmune kidney disease which leads to end-stage renal damage. Approximately 30–40% of the IgAN cases progress towards renal impairment and eventually ends up with renal failure after 20–30 years [1]. IgA immune complexes deposition in the mesangial area accompanied with mesangial cells proliferation and mesangial matrix expansion are primary inducers of IgAN, which resulted in hematuria, proteinuria, hypertension and impaired renal function [2–4]. Particularly, hematuria-induced by IgA immune complexes deposition is one of the most important clinical manifestation in IgAN. Thus, IgA immune complexes which has been well known for a key target leading to IgAN plays a crucial role in initiation of the development and evolution of the renal disorder. As a consequence, reducing the deposition of IgA immune complexes has been widely concerned in the treatment of IgAN [5].

Pioneer studies have implicated the IgA immune complex was produced by activated B cells which were regulated via T cells in IgAN patients [6]. Furthermore, the more T cells were activated in peripheral blood and immune organs, the more IgA was generated from B lymphocytes [7]. Excessive IgA immune complexes deposited in the

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mesangial area leading podocytes and tubulointerstitial damage ultimately incited glomerular injury [8]. Hence, T cell immune regulation is worthy of further study on IgAN, which will be helpful to find available methods to delay the progression of IgAN. Meanwhile, it is imperative to identify effective drugs which possesses of immunosuppressive or immunomodulatory properties and has potential therapeutic value for IgAN.

Artemisinin (ART) is an effective constituent originally extracted from traditional Chinese medicine *Artemisia annua* L. Multiple studies have displayed that there are many biological effects of ART, such as antimalarial, immunomodulatory, antitumor, anti-hypoglycemic activity and so on [9,10]. Furthermore, the evidence showed that ART exerts renoprotection effect through inhibiting the proliferation and activation of B cells, as well as reduces the generation of plasma B cells in MRL/lpr mice [11]. Besides, ART can suppress the development of Th17 to attenuate colonic injury and effectively block contact hypersensitivity response in vitro and in vivo [12,13]. Above all, these discoveries suggest that ART has the potential therapeutic effect in modulating T cells-mediated immune disorders on IgAN.

Hydroxychloroquine (HCQ), a classical antimalarial drug with immunosuppressive, immunomodulatory and anti-inflammatory properties, is clinically applied in the therapy of autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [14,15]. The plentiful benefits of HCQ for renoprotection are currently widely recognized, including reducing damage of kidney, lowering the risk of developing nephritis and improving therapy outcome [16]. Besides, it is reported that HCQ has a good therapeutic effect on IgAN patients by immune modulatory [17]. Nevertheless, HCQ has some adverse reactions in clinical applications with a high dose, containing toxic retinopathy and toxic hepatitis [18,19]. Therefore, it is quite imperative to find other drugs which play a synergistic role in immunoregulation with HCQ on the treatment of IgAN.

Above all, the goal of this study was to investigate whether the combination of ART and HCQ may have a synergistic immunosuppressive or immunomodulatory effect and demonstrate if AH could exert stronger therapeutic effect than ART or HCQ as a standalone application on the treatment of IgAN. On the other hand, our previous study has demonstrated an immunosuppressive effect of AH therapy on lupus nephritis through up-regulating KLF15 expression and curbing NF-κB activation [20]. Based on the study about biological activities of HCQ and ART, it is likely that combination of HCQ and ART may possess a considerable potential in IgAN field to be utilized. In order to examine the hypothesis and determine the underlying mechanism, we designed experiments to illustrate and compare the efficacy of treatment with ART, HCQ alone, as well as AH with three different doses in IgAN rats.

2. Materials and methods

2.1. Chemicals and reagents

Artemisinin (ART, Y160501) was provided by Sichuan Tongrentai pharmaceutical Co. LTD (Sichuan, China); Hydroxychloroquine (HCQ, 20180105) was obtained from Jiangsu Shenhua pharmaceutical Co. LTD (Jiangsu, China); dexamethasone (DXMS, 160401) was offered from Guangdong south China pharmaceutical group Co. LTD (Guangdong, China); Lipopolysaccharide (LPS, 017M4112V, sigma) was purchased from Sigma-Aldrich (St. Louis, MO, USA); Bovine serum albumin (BSA, 9048-46-8, MRC®) was from Jiangsu Enmo Asai Biotechnology Co. LTD (Jiangsu, China). The ELISA kits for IgA (88-50490-22) and IgG (88-50480-22, invitrogen) were provided by Thermo Fisher Scientific (Waltham, MA, USA) and the kits of Rat Interferon γ (IFN-γ, U19011785), Rat Interleukin 4 (IL-4, U02011786) and Rat Interleukin 17 (IL-17, T11011787) were supplied by Cusabio Biotech Co. LTD (Wuhan, China). The antibody against IgA (sc-373823) was from Santa Cruz Biotechnology (Dallas, TX, USA). Rabbit anti Complement 3 (C3, bs-2934R) was from Beijing Biosynthesis Biotechnology Co. LTD (Beijing, China). The secondary antibodies against IgG Fab2 Alexa Fluor 488 (4408S, 4412S) were purchased from Cell Signaling Technology (CST; Danvers, MA, USA). Anti-rat $CD4^+$ FITC (85-11-0040-82), Anti-rat Foxp3 PE (85-12-5773-80) and Anti-rat IL-17A PE-Cyanine7 (85-25-7177-80) were purchased from eBioscience (Shanghai, China), PE-Mouse Anti-rat IL-4 (555082) and Alexa Fluor®647 Mouse Anti-rat IFN-γ (562213) were from BD Bioscience (Gurgaon, Haryana, India).

2.2. Animals

Ninety SPF male Sprague-Dawley rats (Certificate No. SCXK 2016-0041) weighing 180–220 g were provided by The Experimental Animal Center of Southern Medical University. All animal experimental procedures were approved by the Animal Ethics Committee of Guangzhou University of Chinese Medicine and carried out in accordance with the guidelines of the European Community and the National Institute of Health of the USA. All rats were provided standard chow and tap water ad libitum and housed at 23 ± 2 °C. After one week of acclimatization, rats were stochastically divided into two groups, including control (n = 10) and IgAN model groups (n = 80). The modeling rats were orally administered with BSA (600 mg/kg) for 12 weeks, once every other day and subcutaneously injected with 0.6 mL of castor oil (containing 0.1 mL of CCL4) for 12 weeks, once a week. At the 6th, 8th, and 10th week, 0.05 mg of LPS (in saline) was performed into each rat through tail intravenous injection. Starting from the 7th week, the number of erythrocytes in the urine were collected every two weeks to make sure whether the model was successful. Subsequently, the model rats were randomly divided into seven groups: Model, DXMS (0.0 78 mg/kg), ART (33.33 mg/kg), HCQ (33.33 mg/kg), AH low dose (16.65 mg/kg), AH middle dose (33.33 mg/kg) and AH high dose (66.66 mg/kg) (ART: HCQ = 1:3). Saline (10 mL/kg) was orally administered to the control and model groups. The entire experiment lasted for 16 weeks.

2.3. Measurement of urinary erythrocyte

On the 7th, 9th, 11th, 13th, 15th and 16th week, 24 h urine was collected when all rats were restricted from food but provided free access to water. The urine (10 mL) was centrifuged at 4000 rpm for 5 min. The precipitate was blended in 200 μL urine and urinary erythrocyte was observed under the 400× microscope. Each urine sample was counted for 8 times in high power fields (HP), and the average number of urine erythrocyte was calculated.

2.4. Blood sampling and renal tissue removal

At the end of the experiment, the blood and renal tissue samples were collected by euthanizing of all the animals, as previously described [21]. Blood samples were separated by centrifugation at 3500 rpm at 4 °C for 15 min to obtain the serum for biochemical analysis. Renal tissues were divided into two parts. One part was fixed in 10% neutral formalin phosphate buffer for H&E and PAS staining, and the other was quickly frozen in liquid nitrogen and stored at −80 °C for immunofluorescence detection as a backup.

2.5. Detection of serum biochemical indexes

Serum contents of creatinine (CRE), albumin (ALB), total cholesterol (CHO) and glutamic-pyruvic transaminase (ALT) were detected by the kits of CRE (20180602), ALB (20180605), CHO (20180604) and ALT (20180605) were from Nanjing Jiancheng Bioengineering Institute, respectively.

2.6. H&E staining of renal tissue

The H&E staining was performed according to our previous protocol. Briefly, the kidney tissues were fixed with 10% formalin and made by dehydration, transparency, waxing and embedding. Then longitudinally cut into 4-μm-thick sections. The sections were dehydrated by gradient ethanol hydration and stained with hematoxylin-eosin (H&E). After that, ammonia was used for return blue in color and dyed with eosin. Finally, the slices were sealed with the neutral gum. The photomicrographs were captured under a 400× magnification light microscope (OLYMPUS BX53, Shanghai, China) to observe the histopathological changes of renal tissues.

2.7. Periodic Acid-Schiff staining of renal tissue

The previous two-step procedures were similar to H&E staining. Next the slices were performed with periodate for 10 min and rinsed with 70% ethyl alcohol, then stained with Schiff dye solution for 1 h in 37 °C incubator. After counterstained with hematoxylin, the 1% of alcohol hydrochloride was used for differentiation. Finally, the slices were sealed with the neutral gum and photomicrographs were captured under a 400× magnification optical microscope (OLYMPUS BX53, Shanghai, China) to observe the histopathological changes of renal tissues.

2.8. Immunofluorescence

The kidney tissues were embedded with Optimum Cutting Temperature (OTC) compound and sliced at a thickness of 6 μm, then fixed in acetone for 15 min at 4 °C and washed with PBS. Next, the permeabilization of 0.5% Triton-X-100 was applied to slices for 20 min and sealed with 5% goat serum for 30 min at room temperature. In addition, the primary antibody IgA (Santa, 1:100) and Complement 3 (Biosynthesis, 1:200) were dropped to the samples and incubated at 4 °C overnight in the dark. The samples were incubated with secondary antibody against IgG Fab2 Alexa Fluor 488 (CST, 1:200) at 37 °C for 1 h. Lastly, fluorescent quenching was used to seal samples and immunofluorescence images were observed under a 200× laser confocal (LSM800, ZEISS, Germany).

2.9. ELISA

ELISA kits were used to measure inflammation cytokines IFN-γ, IL-4 and IL-17 of renal tissue lysates. The assays were detected according to the manufactures' recommended protocol. Briefly, all reagents and worked standards were prepared, then added 100 μL of samples and standards to each well of 96-well plate. The plate was incubated at 37 °C for 2 h. Besides, inflammatory cytokines were detected by the addition of Biotin-antibody and HRP-avidin for 1 h at 37 °C. Reaction was performed with TMB substrate and protected it from light. Finally, stop solution was added to each well to measure the intensity of the color which would turn from blue to yellow. Absorbance at 450 nm was detected within 5 min.

2.10. Collection of peripheral blood T cells

At the end of the experiment, the blood was gathered by euthanizing all the animals, as previously described. Blood was removed from the abdominal aorta and collected to EDTA anticoagulation tube. Red blood cell lysis buffer (BOSTER) was added to 500 μL anticoagulation blood for 2 min and centrifuged by 300 rpm for 5 min at 4 °C. The blood red cells were removed and lymphocyte were resuspended in 4 mL of 1× phosphate-buffered saline (PBS) to wash residual red blood cell lysis buffer, then centrifuged at 300 *g* for 5 min. Supernatant was discarded and lymphocytes were cultured in 1640 medium and adjusted the concentration to 1×10^7/mL.

2.11. Spleen cell collection

At the end of the experiment, the spleen tissues were obtained by euthanizing all the animals, as previously described. Half of the spleen tissues were grinded in incomplete 1640 medium and filtered through 300 mesh of nylon filter. The collected suspension was centrifuged at 300 *g* for 5 min, then discarded supernatant, added 3 mL of 1× red blood cell lysis buffer to separate the splenocytes. The splenocytes were washed with 2 mL of 1× PBS and centrifuged again to discard the remaining part of red blood cell lysis buffer. Lastly, the splenocytes were suspended in 1640 medium and the concentration was adjusted to 1×10^7/mL.

2.12. Preparation of inflammatory cytokines

The primary inflammatory cytokines of T cells were less secreted in blood and spleen, but PMA/Ionomycin and Monensin could activate T cells to yield more IFN-γ, IL-4 and IL-17. Thus, IFN-γ, IL-4 and IL-17 inflammatory cytokines were stimulated with PMA/Ionomycin mixture (70-CS1001, eBioscience) and Monensin Sodium (70-CS0004, eBioscience) for 4 h in 37 °C incubator. Subsequently, the stimulated inflammatory cytokines were detected by flow cytometry (FCM).

2.13. Flow cytometry

IFN-γ, IL-4, IL-17 and Foxp3 were analyzed by Flow cytometry (FCM). Firstly, we added 100 μL sample and 0.5 μL of FITC labeled Anti-rat CD4+ antibody (eBioscience) to flow tube, then incubated for 30 min in the dark. Next, the cells were washed twice with 1 mL of 1× PBS and centrifuged at 300 *g* for 5 min. Then the cells were permeabilized with BD cytofix/cytoperm Fixation/ permeabilization kit (MultiScienc) for 30 min and centrifuged. However, the Treg cells were permeabilized by Foxp3/Transcription Factor Fixation/ Permeabilization concentrate and Diluent (eBioscience). After permeabilization, we added 0.5 μL of PE-labeled Anti-rat IL-4 (BD), PE-labeled Anti-rat Foxp3 (eBioscience), PE-Cyanine7-labeled Anti-rat IL-17A (eBioscience) and Alexa Fluor®647 labeled Anti-rat IFN-γ (BD) to samples, incubated for 30 min in the dark and centrifuged, respectively. In the end, the cells were suspended with 300 μL of PBS and ran on FCM.

2.14. Statistical analysis

Data were expressed as the mean ± standard error. Statistical analysis was performed with SPSS 19.0 software for Windows (Chicago, IL, USA). Comparisons among groups were made by one-way ANOVA, followed by Duncan's test. Values of $P < 0.05$ were considered significant, and values of $P < 0.01$ were considered highly significant.

3. Results

3.1. AH decreased urinary erythrocyte and prevented renal impairment in IgAN rats

Hematuria is the most common clinical manifestation of IgAN among all renal abnormal indicators, such as proteinuria, hypertension, elevated serum creatinine, and so on. Urinary erythrocyte is a crucial biomarker of IgAN. As shown in Fig. 1A, the increase of urinary erythrocyte-induced by BSA accompanied with LPS was mitigated to some extent after ART, HCQ or AH treatment. Compared with ART or HCQ treatment, AH is more effective in inhibiting the generation of hematuria (CO: 2.6 ± 0.6; MO: 10.9 ± 2.2; ART: 4.5 ± 0.8; HCQ: 5.2 ± 1.4; AH: 3.3 ± 1.8). However, there was no statistical difference in the results of body weight change after modeling or treatment (Fig. 1B). In addition, the results of serum biochemical values were shown in Fig. 1C-E, the upregulated levels of CRE and CHO and the

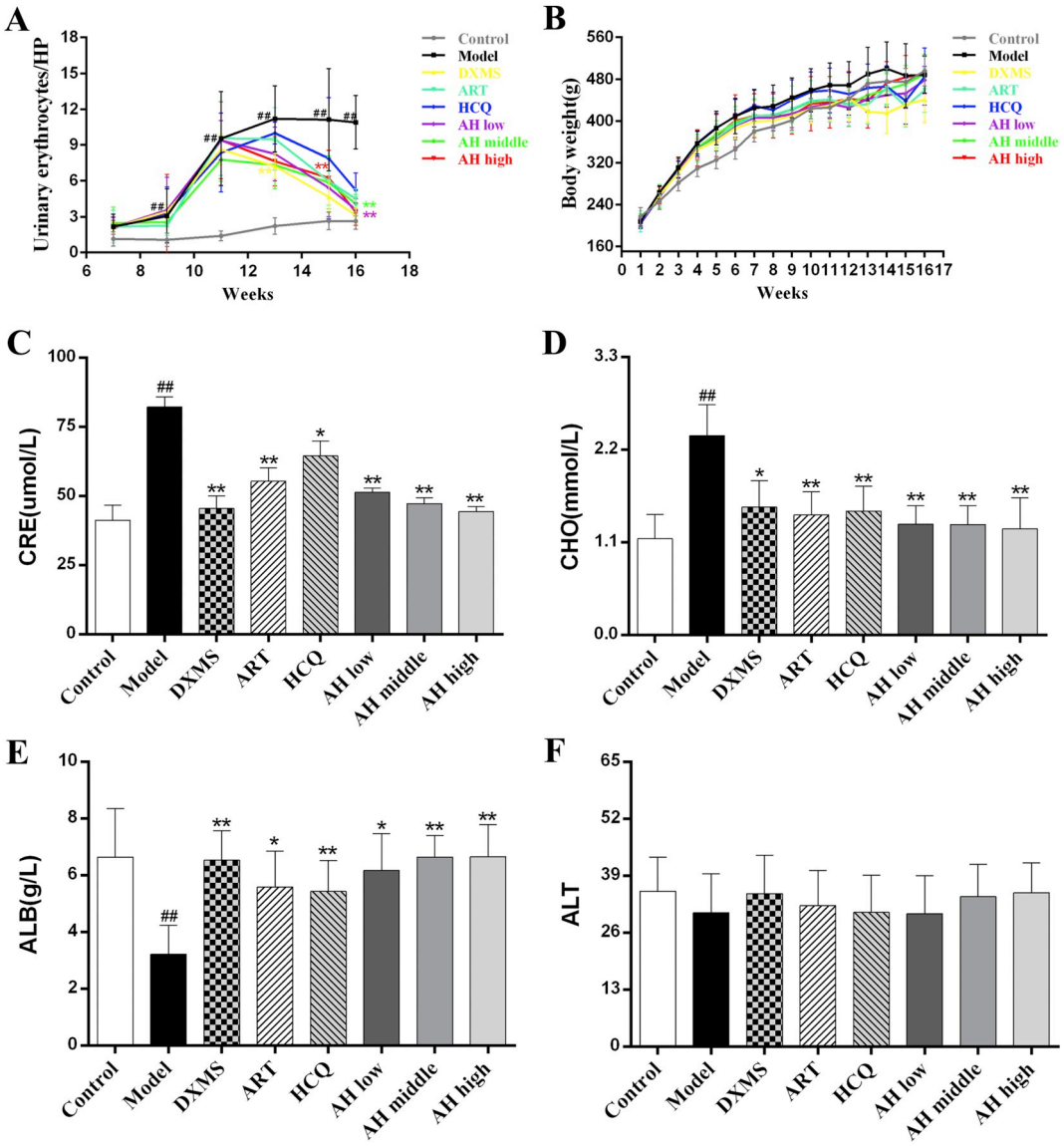

Fig. 1. AH decreased urinary erythrocyte and prevented renal impairment in IgAN rats. (A) Urinary erythrocyte in 7, 9 and 11 weeks of modeling and 13, 15 and 16 weeks of treating at high power field ($^{\#\#}P < 0.01$, compared with control group, $^{**}P < 0.01$, compared with model group, n = 10). (B) Body weight of rats in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16 weeks. (C–F) Levels of CRE, CHO, ALB and ALT in serum of rats ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{ns}P > 0.05$, n = 10).

downregulated level of ALB in IgAN rats were reversed after ART, HCQ or AH treatment and among which, AH treatment had a most significant reversing trend.

We also wondered that whether carbon tetrachloride could cause liver damage in modeling. Fig. 1F revealed that there was no significant difference in liver, which illustrated that kidney injury was not caused by liver damage. All of these results exhibited that AH could effectively improve kidney function and lessen hematuria to alleviate kidney impairment in IgAN rats.

3.2. AH mitigated renal pathological damage and decreased the deposition of IgA immune complexes and C3 in IgAN rats

Next, we detected renal pathological damage. H&E staining of kidney tissues showed obvious proliferation of mesangial cells, extension of mesangial matrix and variable infiltration of inflammatory cells in rats that suffered from BSA and LPS, however, this effect was reversed after ART, HCQ, AH and DXMS administration (Fig. 2A), Additionally, IgAN is characterized by deposition of pathogenetic immune complexes (IgA, occasionally with IgG and C3) in the mesangium. PAS staining of kidney tissues suggested that immune complexes were deposited only in the mesangial areas rather than in podocytes or renal tubular epithelial cells, and the increase of deposition was significantly inhibited by AH and DXMS therapy (Fig. 2B). Similarly, as seen in Fig. 3A & Fig. 3B, AH notably inhibited the production of IgA and C3 in renal tissue by immunofluorescence. These results verified that AH greatly suppressed IgA immune complexes in IgAN rats and mitigated renal pathological damage, as did DXMS.

3.3. AH improved the morphology of kidney and spleen by regulating inflammatory cytokines of CD4+ T cells in IgAN rats

To further investigate the underlying mechanism that AH could reduce the increase of IgA deposition via regulating inflammatory cytokines of CD4+ T cells in IgAN rats. We visually observed an increase in the size of the kidney (Fig. 4A) and spleen (Fig. 4B) induced by BSA and LPS, however, the change was distinctly reversed after AH treatment in IgAN rats. Besides, as shown in Fig. 4C-I, the indexes which were the ratio of spleen, right or left kidney, thymus, liver, lung and heart weight to body weight were detected. Particularly, the indexes of

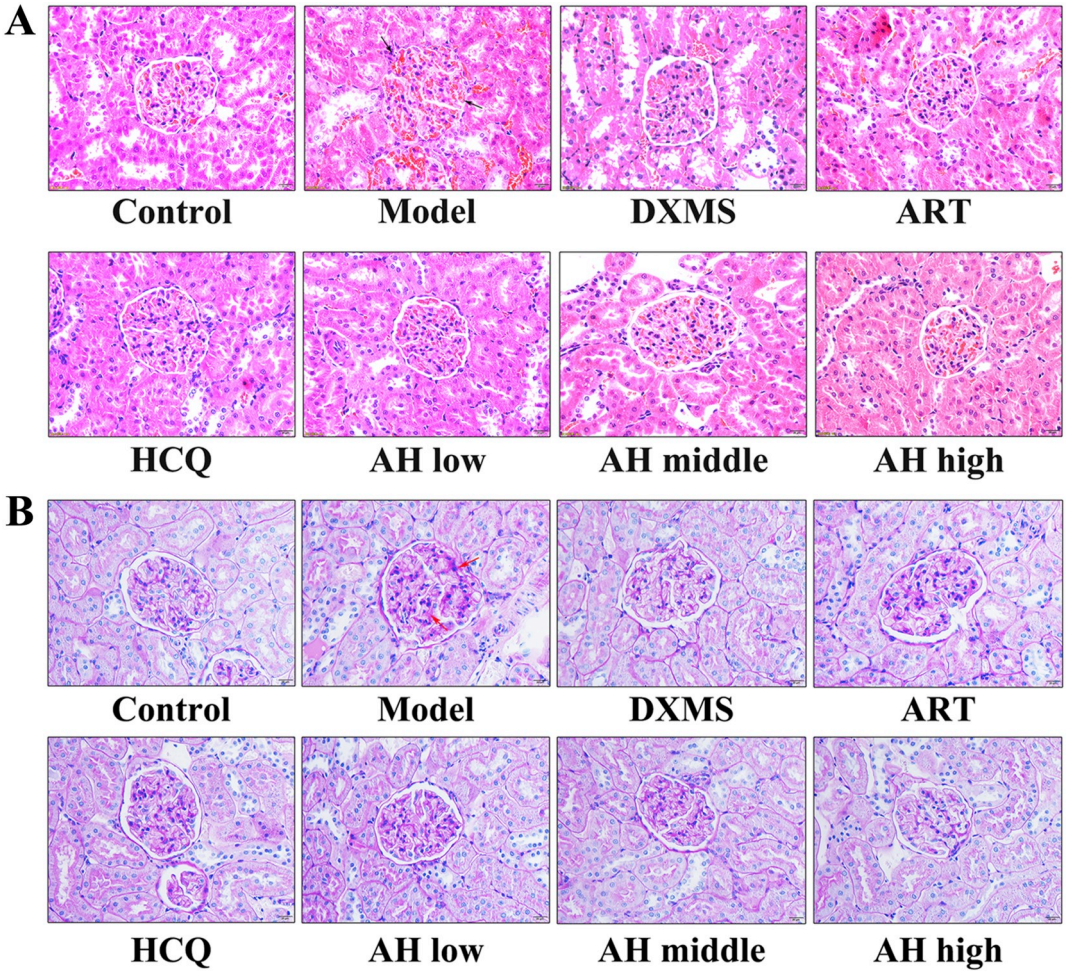

Fig. 2. AH mitigated renal pathological damage and decreased the production of immune complexes in IgAN rats. (A) Representative photomicrographs of H&E staining in glomerulus of renal tissues in IgAN rats (n = 10), 4 weeks after treatment, increased mesangial matrix and expanded mesangial area (black arrow) (original magnification ×400). (B) Representative photomicrographs of PAS staining in glomerulus of renal tissues in IgAN (n = 10) 4 weeks after treatment, deposition of immune complexes in mesangial area (aubergine region, red arrow) (original magnification ×400). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

spleen, kidney and thymus were significantly enlarged in modeling rats, but there was a conspicuously suppressive effect of AH and DXMS in IgAN rats. Undoubtedly, all of these results announced that AH could improve renal injury by inhibiting immune-inflammatory production in IgAN rats.

Interestingly, Th cell differentiation is a hallmark of ongoing immune reactions and Th cell subpopulations are defined by their cytokine profile. IFN-γ, IL-4 and IL-17 was respectively generated from Th1, Th2 and Th17 cells. In this study, we found that T cells were prominently activated in the kidney. In groups without AH treatment, CD4+ T cells showed increased Th2 subsets in response to IL-4, as well as Th17 subsets in response to IL-17, whereas Th1 responses upon IFN-γ stimulation was inhibited. The results of ELISA in Fig. 4J-L illustrated that the inflammatory cytokine IL-4 and IL-17 were upregulated and IFN-γ was downregulated in BSA and LPS-induced rats, compared to controls. However, AH significantly inhibited the upregulation of IL-4 (CO: 146.4 ± 47; MO: 416.8 ± 86; ART: 175.7 ± 46; HCQ: 214.4 ± 62; AH: 163.3 ± 30) and IL-17 (CO: 221.7 ± 51; MO: 559.7 ± 57; ART: 307.7 ± 78; HCQ: 290.5 ± 49; AH: 267.1 ± 74) secretions and prevented the downregulation of IFN-γ (CO: 1.7 ± 0.5; MO: 1.0 ± 0.2; ART: 1.4 ± 0.1; HCQ: 1.3 ± 0.1; AH: 1.6 ± 0.2) secretion in IgAN rats. All in all, our study revealed Th1 response often played a vital role in cell-mediated immune response. Owing to its immunosuppressive feature, AH was also critical for improving the morphology of kidney and spleen by regulating inflammatory cytokines of CD4+ T cells in IgAN rats.

3.4. The differentiations of Th2 and Th17 cells were inhibited while promoted of Th1 and Treg cells in peripheral blood and spleen after AH treatment in IgAN rats

To elucidate whether the local renal inflammation induced by BSA and LPS was also associated with systemic immune activation, we monitored the activation state of T cells in peripheral blood and spleen. Initially, we examined the presence of IL-4 (Th2), IFN-γ (Th1), IL-17 (Th17) and Foxp3 (Treg)-produced CD4+ T cells in peripheral blood in IgAN rats. Flow cytometry results shown in Fig. 5A exhibited a remarkable reduction of the secretions of IFN-γ and Foxp3 in IgAN rats, but the decrease of IFN-γ and Foxp3 were inhibited after AH treatment compared to controls (Fig. 5B & C), whereas the secretions of IL-4 and IL-17 were obviously downregulated by AH treated in peripheral blood in IgAN rats (Fig. 5D & E). Besides, the development of inflammatory T cell in spleen of immune organ in IgAN rats was assessed. As shown in Fig. 6A, intracellular cytokine staining showed that both the proportions of Th1 and Treg cells in the splenocytes were reduced in IgAN rats, while AH prevented the decreased secretions of Th1 and Treg cells, compared with the control group (Fig. 6B & C). Furthermore, the levels of IL-4 and IL-17 were enhanced obviously among total CD4+ T cells in

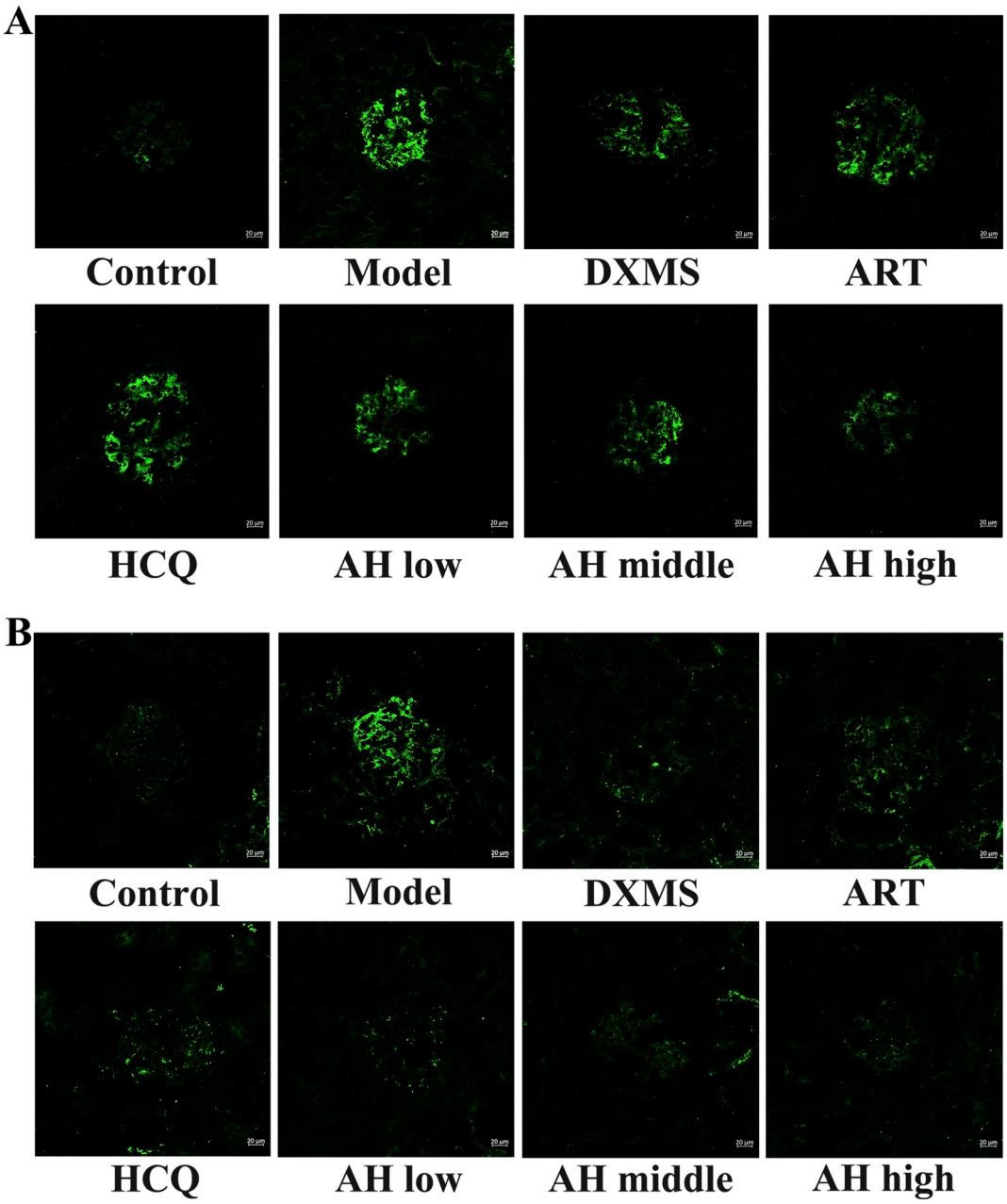

Fig. 3. AH reduced the depositions of IgA immune complexes and C3 to alleviate renal injure in IgAN rats. (A) 4 weeks after treatment, immunofluorescence results showed representative photomicrographs of IgA immune complexes in glomerulus of renal tissues in IgAN rats (n = 10). (B) Effect of AH treatment on immunofluorescence showed representative photomicrographs of C3 in glomerulus of renal tissues in IgAN rats (n = 10).

IgAN rats compared to the control, but which were declined after AH treatment in the spleen (Fig. 6D & E). To sum up, AH treatment significantly suppressed the differentiations of Th2 and Th17 cells and facilitated the productions of Th1 and Treg cells in peripheral blood and spleen. These findings demonstrated that the development of IgAN was influenced by AH which has an immunosuppressive effect on T cells differentiation in IgAN rats, and AH treatment could inhibit this progress of IgAN to alleviate the renal injury.

4. Discussion

In this study, we have demonstrated that AH treatment could prevent the production of urinary erythrocyte and improve kidney dysfunction in IgAN rats. In addition, T cell activation was accompanied by enhanced B cell activity in the kidney and spleen, and the productions of IgA and C3 were greatly inhibited by AH in the glomerular mesangium. Additionally, Treg cells play a key role in repression of effector T cell activity, so do Th1 cells. Thus, we have found that after AH treatment, differentiations of Th2 and Th17 cells were declined, Th1 and Treg cells were advanced in peripheral blood and spleen, which revealed that AH could regulate the differentiation of CD4+ T Cell subsets to mitigate the renal injury in IgAN rats.

Evidence from most studies showed a great correlation between T cells and B cells. Th cells were emerged during ongoing immune responses, which act by coordinating effector function of B cells [22]. The cooperation between Th and B cells was occurred during the initiation and fine-tuning of T cell dependent antibody responses [23]. Moreover, B cells with immunosuppressive and/or regulatory functions play an important role in modulating anti-tumor immune responses [24], which are able to released IgA aberrantly or excessively by abnormal

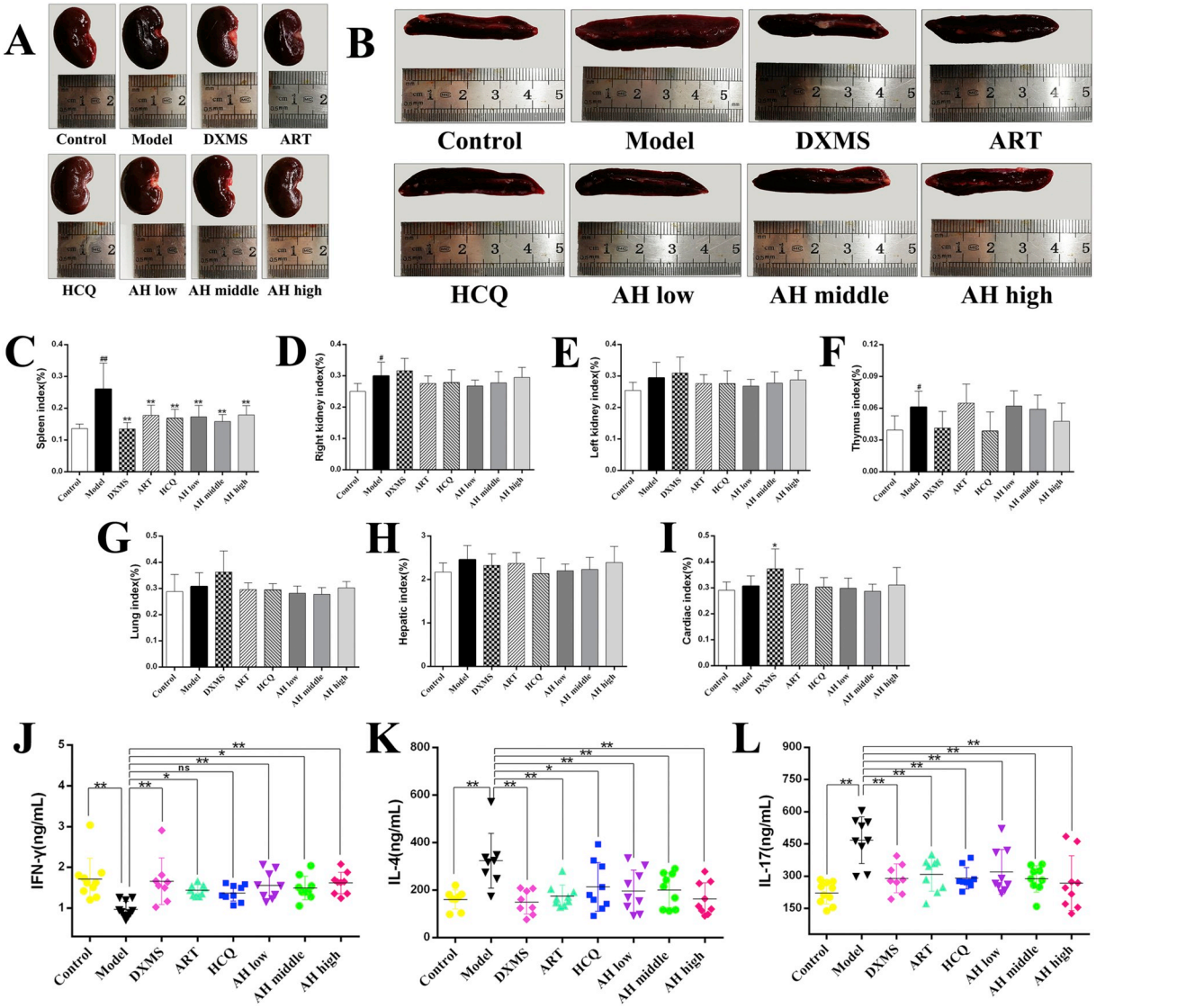

Fig. 4. AH improved the kidney and spleen morphologies by regulating inflammatory cytokines of CD4+ T cells in IgAN rats. (A) Representative photographs of kidney morphology of IgAN rats (n = 10). (B) Representative photographs of spleen morphology of IgAN rats (n = 10). (C–I) Indexes of spleen, right kidney, left kidney, thymus, lung, liver and heart for body weight in all IgAN rats ($^{\#\#}P < 0.01$, compared with control group; $^{**}P < 0.01$, $^{*}P < 0.05$, compared with model group, n = 10). (J–L) 4 weeks after treatment, ELISA was performed to evaluate the effects of AH on inflammatory cytokines IFN-γ, IL-4 and IL-17 of Th cells ($^{\#\#}P < 0.01$, compared with control group; $^{**}P < 0.01$, $^{*}P < 0.05$, compared with model group, n = 10).

regulation of T cells [25–27]. Nevertheless, IgAN is the most prevalent primary chronic glomerulonephritis that is resulted from increasing fraction of circulatory IgA immune complexes and progress of renal failure worldwide [1]. Interestingly, our results exhibited that IgAN model rats showed a severe deposition of IgA or C3 in the glomerular mesangium which lead to renal injury by regulating the differentiation of CD4+ T Cell subsets in Rats.

In particular, as a pivotal part in immune response, CD4+ T cells can influence the IgAN progress or development. Studies have shown that Treg cells maintained immune balance by handicapping the activation and proliferation of T cells. Foxp3 is a transcription factor that has been considered to be the most specific markers of the Treg cells [28,29]. Previous studies suggested that Treg cells were thought to be essential in patients with IgAN and autoimmune diseases [30,31]. In contrast, Th17 cells, secreting Th17 family cytokine IL-17A, is the potent inducer of tissue inflammation [32,33]. In this study, our results revealed that the expression of Foxp3 in Treg cells was reduced in IgAN rats, while the cytokine IL-17 in Th17 cells was elevated. In general, the appropriate balance between Treg and Th17 cells is crucial for the stability of immune homeostasis in IgAN.

In addition to the two subgroups of Treg and Th17, there are also Th1 and Th2 subpopulations in polarization of T cells, which has a direct or indirect effect on the induction of B cell proliferation and the promotion of secretion of various immunoglobulins [34,35]. Th1 cells mainly secrete IFN-γ, IL-2, etc., participating in cellular immunity and mediating the activation of cytotoxic T cells and macrophages; Th2 cells primarily secrete IL-4, IL-5, etc. [36]. Researches have shown that the principal cytokines generated by circulating T cells in IgAN were Th2 type and Th2 cytokines that could induce the overproduction of abnormally glycosylated IgA1 which were prone to deposit in mesangium [6,37]. In our study, flow cytometry multi-staining technique was used to detect the presence of IFN-γ and IL-4 in peripheral blood and spleen in IgAN rats used to observe the balance of Th1 and Th2. The results showed that the expression of IL-4 were significantly higher than the Th1 in the peripheral blood of the IgAN group. It was confirmed that the advantage of Th2 was obvious and affirmed the correlation between Th2 cells and the production of IgA and C3 in renal tissue of IgAN rats. These findings demonstrated that the development of IgAN was

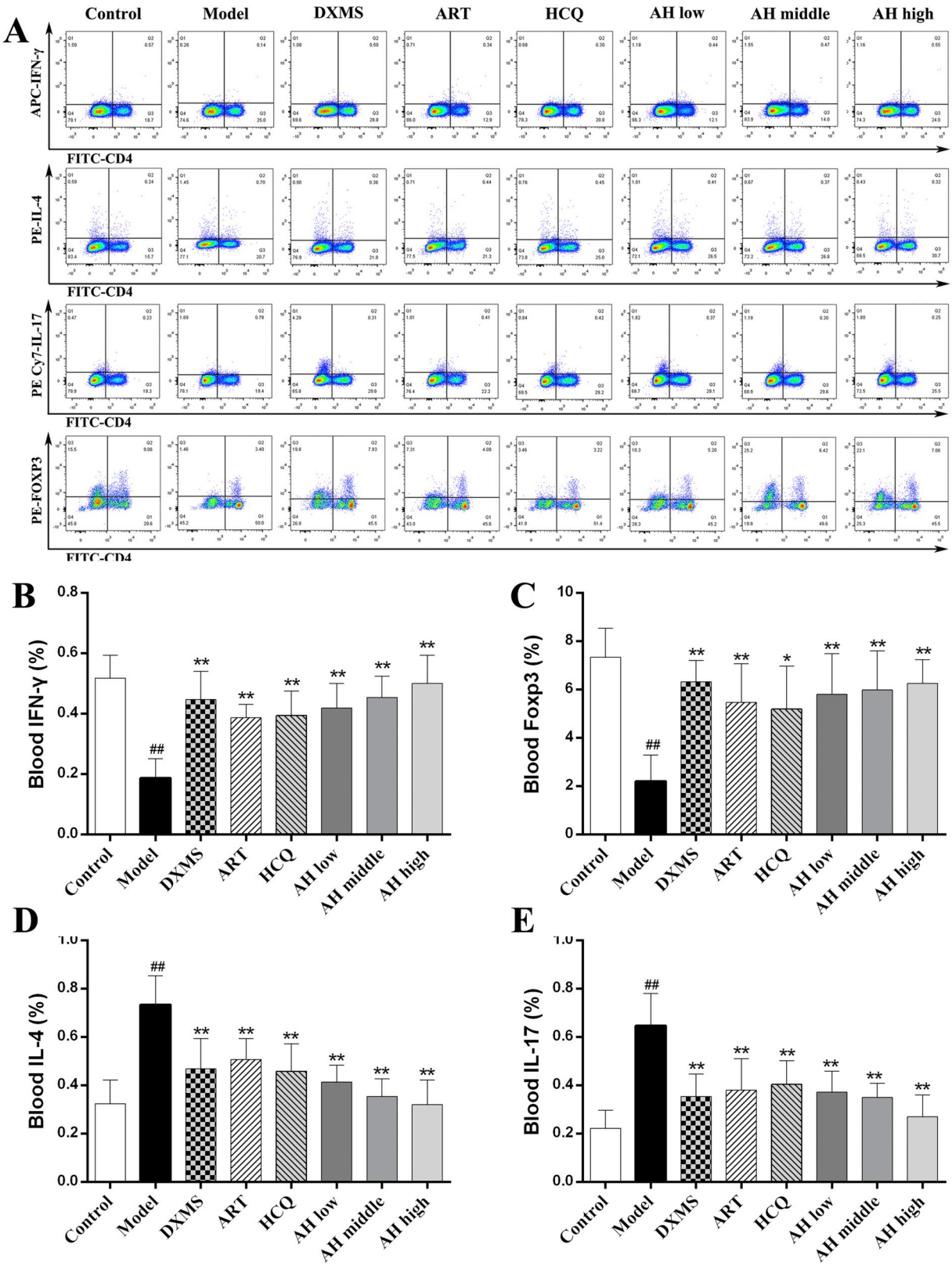

Fig. 5. Th2 cells and Th17 cells differentiation was inhibited while Th1 cells and Treg cells differentiation was promoted in peripheral blood after AH treatment in IgAN rats. (A) Flow cytometry of the surface expression of IFN-γ, IL-4, IL-17 and Foxp3 on the naive CD4+ T cells stimulated with antibody to CD4 (anti CD4) in peripheral blood. Numbers in quadrants or above bracketed lines indicate percent cells in each area. (B–E) Quantification of the results in A. Data are representative of eight independent experiments with similar results ($^{\#\#}P < 0.01$, compared with control group; $^{**}P < 0.01$, $^{*}P < 0.05$, compared with model group, n = 8).

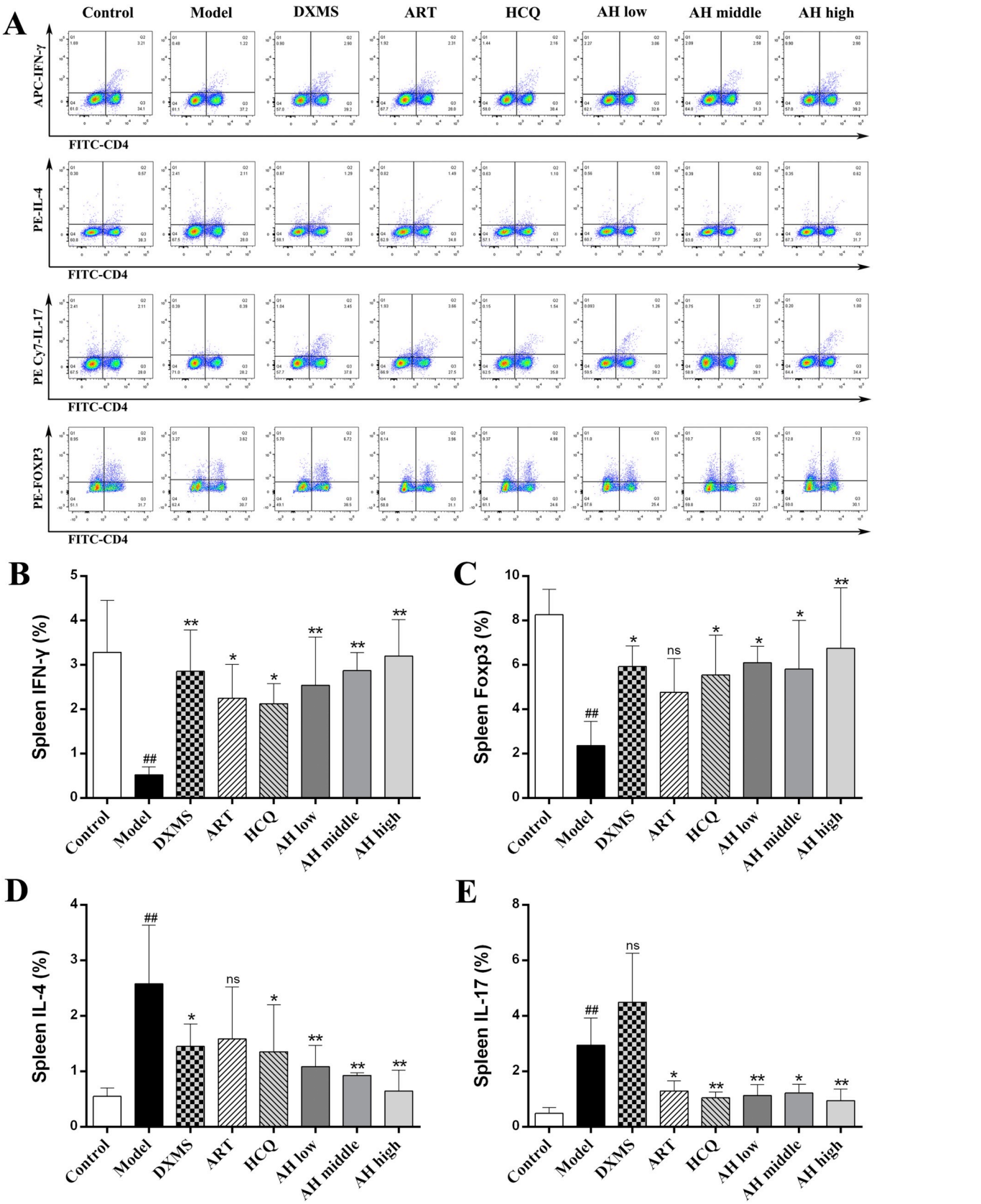

Fig. 6. Th2 cells and Th17 cells differentiation was inhibited while Th1 cells and Treg cells differentiation was promoted in the spleen after AH treatment in IgAN rats. (A) Flow cytometry of the surface expression of IFN-γ, IL-4, IL-17 and Foxp3 on the naive CD4 + T cells stimulated with antibody to CD4 (anti CD4) in spleen. Numbers in quadrants or above bracketed lines indicate percent cells in each area. (B–E) Quantification of the results in A. Data are representative of eight independent experiments with similar results ($^{\#\#}P < 0.01$, compared with control group; $^{**}P < 0.01$, $^{*}P < 0.05$, compared with model group, n = 8).

influenced by the T cells polarization and related with the immune imbalance.

ART is a sesquiterpene trioxane lactone from *Artemisia annua* L., which has been shown to affect immune responses. Studies have identified that ART as an effective therapeutic agent for treating immune-related diseases by effectively inducing the generation of Tregs and suppressing the development of Th17 [13,38,39]. In addition, HCQ is an antimalarial drug that has been used in the treatment of systemic lupus erythematosus and rheumatoid arthritis treatment for many years. Researches manifested that HCQ was used to evaluate the immunomodulatory activity of Th17 cytokines in healthy individuals and patients [40], and exerted similar effects on Tregs in several of autoimmune diseases [41,42]. However, HCQ is one of the systemic medications that may cause retinal toxicity. One case has reported that a patient who has been taking HCQ for a long time suffered from visual field defects contributing to therapy cessation, which illustrated that HCQ has side effects of retinal toxicity and enhanced renal impairment [18,43]. If renal damage occurs, the drug should be replaced or the dose should be reduced with close inspection of the visual function.

Unexpectedly, we originally used AH treatment to reduce the dose and side-effect of HCQ. Experiment results exhibited that AH combination had a stronger protective effect on renal injury via inhibiting the differentiation of Th cells in IgAN rats. Moreover, in our previous study, we have applied for a patent for the AH combination, which not only illustrate HCQ could significantly reduce the toxicity but also effectively increase the therapeutic functions for C-BSA induced chronic kidney disease in rats. Furthermore, in this study, we found that AH combination had significant regulation on differentiation of Th cells, suppressed the activation of B cells and diminished the deposition of IgA immune complexes to mitigate renal pathological damage in IgAN rats.

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Conflict of interest

The authors have no conflict of interest to declare.

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