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Rapamycin Ameliorates Renal Ischemia/Reperfusion Injury via Remodeling Immune Microenvironment

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Abbreviations mTOR: Mammalian Target of Rapamycin; IL-6: Interleukin-6; MCP-1: Monocyte Chemotactic Protein-1; TNF-α: Tumor Necrosis Factor-α; IL-1β: Interleukin-1β; IL-10: Interleukin-10; TGF-β: Transforming Growth Factor-β; IR: Ischemia-Reperfusion; Treg: Regulatory T Cells; DCs: Dendritic Cells; DCreg: Regulatory DCs; NKT: Natural Killer T; RBC: Red Blood Cell; PAMPs: Pathogen-Associated Molecular Patterns; Scr: Serum Creatinine; BUN: Blood Urea Nitrogen; PBS: Phosphate-Buffered Saline; HE: Hematoxylin and Eosin; dUTP: Digoxigenin-Deoxyuridine; TdT: Terminal Deoxynucleotidyl Transferase; ISEL: In Situ End-Labeling; ATP: Adenosine Triphosphate; TCR: T Cell Receptors; HIF: Hypoxia Induced Factor; WT: Wild Type; WBC: White Blood Cell

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Abstract

Background: Rapamycin plays a protective role in kidney Ischemia Reperfusion (IR) injury in early stage, but the mechanisms involved haven't been thoroughly revealed so far. We hypothesized this protective effect of rapamycin is relevant to the remodeling of immune microenvironment. With this purpose, we aim to investigate the change in proportion of Dendritic Cells (DCs), macrophages and Natural Killer T (NKT) cells in spleen, peripheral blood and IR induced kidney before and after rapamycin administration in a murine renal IR model. In addition, the effect of rapamycin on damage-promoting and damage-preventing cytokines in IR induced kidney was also investigated.

Materials and Methods: Balb/c mice were subjected to renal 30 min ischemia followed by 24h reperfusion. Rapamycin (2.5ml/kg) was administered by gavage daily, starting 1day before the operation. Renal function and histological changes were assessed. The proportion of NKT cells, macrophages and DCs in peripheral blood, spleen and kidney was detected by flow cytometry. The expression of pro-inflammatory cytokines Interleukin-6 (IL-6), Monocyte Chemotactic Protein-1(MCP-1), Tumor Necrosis Factor-α (TNF-α), Interleukin-1β (IL-1β) and anti-inflammatory cytokines Interleukin-10(IL-10), Transforming Growth Factor-β1 (TGF-β) were determined by RT-qPCR.

Results: Rapamycin significantly improved renal function and ameliorated histological injury and inhibit cellular apoptosis in IR-induced kidney tissue. The proportion of macrophages in spleen was decreased in rapamycin-treated group than in the sham and IR group. In contrast, the proportion of macrophages was raised in rapamycin group in comparison with the sham and IR group in the kidney. In spleen, rapamycin increased the proportion of DCs compared with the sham and IR group, but the proportion was decreased in peripheral blood and kidney. In rapamycin-treated group, the proportion of NKT cells in spleen was significantly decreased but increased in peripheral blood and kidney. In addition, rapamycin dramatically down-regulated the expression of IL-6, MCP-1, TNF-α and IL-1β and up-regulated IL-10 and TGF-β compared with IR group.

Conclusion: Rapamycin may protect kidney from IR injury through remodeling immune microenvironment-modulating the proportion of DCs macrophages and NKT cells in spleen, peripheral blood and kidney and the expression of inflammatory- related cytokines.

Background

Renal Ischemia-Reperfusion (IR) injury, a pro-inflammatory pathophysiological process occurred in kidney tissue, remains one of the major causes of acute kidney injury. It is also an unavoidable impairment during renal transplantation, leading to the increased rates of delayed graft function and even allograft dysfunction [1,2].

Immunosuppressive therapy is necessary for patients receiving organ transplantation to prevent allo-rejection mediated by immune cells. Meanwhile, certain immunosuppressive drugs also have an important impact on allograft IR injury. Rapamycin, the inhibitor of the Mammalian Target of Rapamycin (mTOR) complex, is a kind of potent immune suppressor widely used in clinic. In recent years, great scale of researches focused on the effect of rapamycin on ischemic acute kidney injury. However, it has been difficult to reach a consensus. Many studies indicated that rapamycin inhibited apoptosis by preventing phosphorylation of pro-apoptotic protein such as p53 and activation of mitochondrial cell death pathway [3]. In addition, rapamycin enabled to enrich CD4+CD25+Foxp3+ Regulatory T Cells (Treg) to exert anti-inflammatory effects during IR process [4]. There was also evidence that rapamycin played a damage-promoting role during IR injury through such mechanisms as increase of NF-κB activity [5], pro-apoptosis [6], promotion of renal oxidative/nitrosative stress and aggravation of tissue infiltration of leukocytes [7].

With in-depth understanding of immune cells, some of them were found to exert immune regulatory effects, therefore preventing ischemia reperfusion injury by alleviating inflammatory response. Dendritic Cells (DCs) are acknowledged as the professional antigen-presenting cells where

antigens are processed and presented to T cell with MHC restriction. Recently a novel subtype of DCs has been identified with regulatory function for low expression of co-stimulatory molecules such as CD80/CD86. The Regulatory DCs (DCreg) were thought to cause T cell anergy and induce regulatory T cells [8]. NKT cells have been reported to regulate autoimmune disease and allogeneic immune response in according animal models [9,10]. Notably, a recent research demonstrated a significant protective role for Natural Killer T (NKT) cells in acute kidney injury caused by ischemia reperfusion [11]. Macrophages have long been considered as innate immune cells participating non-specific immune defense by recognizing Pathogen-Associated Molecular Patterns (PAMPs) expressed by various kinds of pathogens. However, M2 macrophages appear to have immune suppressive abilities by contributing to tissue repair and promoting Th2 responses [12]. In previous study, we found that rapamycin was capable of ameliorating renal ischemia-reperfusion injury in early stage and recruiting NKT cells to IR-induced kidney. The recruitment of renoprotective NKT cells may account for the protective role of rapamycin [13]. However, we still wonder if rapamycin could modulate other innate immune cells in IR-induced kidney such as DCs and macrophages mentioned before and hope that a clear and comprehensive relationship could be revealed between its regulatory effect on innate immune system and its protective role played in renal IR injury in the present study.

Based on that mTOR pathway is one of the key pathways which mediates cellular survival and energy metabolism, we hypothesized that rapamycin, the mTOR inhibitor, could modulate multiple innate immune cells and molecules during renal IR injury, which may have a close relationship with its renal protective effects.

Materials and Methods

Materials

Rapamycin oral solution (Cat. No. H20051081, East China Pharmaceutical, Co Ltd., Hangzhou, China) was diluted in normal saline (1:10) for gastric gavage.

Renal IR injury model

Male BALB/c mice (weighing 20-25g), were obtained from Shanghai Slac Lab Animal, Co., Ltd, and bred in an experimental animal room of SPF grade. All animal procedures were performed according to the guidelines of the Care and Use of the Laboratory Animal Ethical Commission of Fudan University. Mice were randomly divided into three groups (n = 5): (1) Sham group; (2) IR group: IR injury with 0.5 ml normal saline gavage; (3) Rapa group: IR injury with 0.5ml diluted Rapamycin suspension gavage at 24h, 1h before operation, as well as 12h after operation [14]. For the renal IR model, the mice were anaesthetized with pentobarbital at a dosage of 0.1g/kg body weight intraperitoneally. Core body temperature was maintained at 37ºC. The abdominal cavity was exposed via a midline incision, both kidneys were exposed and the renal pedicles were carefully isolated. Bilateral renal occlusion for 30 min was performed using non-traumatic vascular clamps. Occlusion was confirmed by observing blanching of the entire kidney surface. After removing the renal clips, the kidneys were observed for a further 5 min to ensure color change indicating blood reperfusion. Afterwards, 0.5ml saline at 37ºC was injected into the abdomen and the incision was sutured in two layers [15].

Animals were ethically sacrificed at 24h after IR injury and the whole blood drawn from the heart was centrifuged at 4ºC, 3000rpm, for 25min to obtain the serum sample. The level of Serum Creatinine (Scr) and Blood Urea Nitrogen (BUN) was measured by the automatic biochemistry analyzer (Hitachi 7060, Hitachi Ltd and Tokyo, Japan). One kidney was harvested and transversally cut at the midline. One half was fixed with 10% buffered formalin for histological assessment, while the rest was frozen at -80º for western blot and Real-Time Quantitative PCR (RT-qPCR). The other kidney and the spleen were kept in Phosphate-Buffered Saline (PBS) in preparation for flow cytometry analysis.

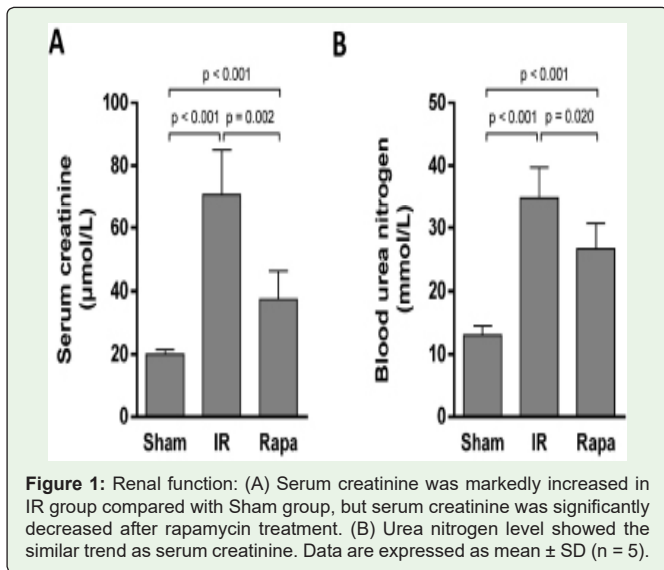### Single cell suspension preparation and flow cytometry

Spleen and kidney tissue were harvested from mice at given time points and crushed in mesh bags to obtain single cell suspensions, in which Red Blood Cells (RBCs) were lysed with hypotonic erythrocyte lysis buffer (Cat. No. RT122-02, TIANGEN Biotech, Beijing, China). Thereafter all single cells were re-suspended in staining buffer (Cat. No.550825, BD Bioscience, San Diego, CA, USA). The following monoclonal antibodies (1mg/ml; BD Phar Mingen) were used to identify NKT cells: FITC-labeled anti-mouse CD³ (Cat. No.555274, Clone: 17A2) and APC-labeled anti-mouse NK1.1 (Cat. No. 550627, Clone: PK136). FITC-labeled anti-mouse CD45 (Cat. No. 553079, Clone: 30-F11) and PE-labeled anti-mouse CD11b (Cat. No. 553311, Clone: M1/70) were used to identify dendritic cells. FITC-labeled anti-mouse CD45 (Cat. No. 553079, Clone: 30-F11) and APC-labeled F4/80 (Cat. No. 563899, Clone: F6/12) were used to identify macrophages. Flow cytometry data were acquired using BD FACS Aria II (BD Biosciences) and analyzed with FlowJo software 6.0 (Tree Star Inc., Ashland, OR, USA).

RT-qPCR

Total RNA was extracted from renal tissues with Trizol reagent (Cat. No.15596-026, Invitrogen, Carlsbad, USA). One μg of total RNA

Table 1: Primers used in the study.

Gene	Gen Bank accession No.	Length	Primer sequence (5′–3′)
GAPDH	NM_008084.3	1,444 bp	Upper: AGTTCAACGGCACAGTCAAG Lower: TACTCAGCACCAGCATCACC
IL-1β	NM_008361.4	1,348 bp	Upper: TGGGCTGGACTGTTTCTAATG Lower: GGTTTCTTGTGACCCTGAGC
IL-6	NM_001314054.1	1,083 bp	Upper: ACTTCCATCCAGTTGCCTTC Lower: CATTTCCACGATTTCCCAGA
TNF-α	NM_013693.3	1,653 bp	Upper: AAGGGAGAGTGGTCAGGTTG Lower: TCTGTGAGGAAGGCTGTGC
IL-10	NM_010548.2	1,306 bp	Upper: GCCTTATCGGAAATGATCCA Lower: GAGGGTCTTCAGCTTCTCACC
TGF-β	NM_011577.2	2,191 bp	Upper: ATTCCTGGCGTTACCTTGG Lower: AGCCCTGTATTCCGTCTCCT
MCP-1	NM_011333.3	806 bp	Upper: CATCCACGTGTTGGCTCA Lower: GATCATCTTGCTGGTGAATGAGT

Figure 1: Renal function: (A) Serum creatinine was markedly increased in IR group compared with Sham group, but serum creatinine was significantly decreased after rapamycin treatment. (B) Urea nitrogen level showed the similar trend as serum creatinine. Data are expressed as mean ± SD (n = 5).

was reverse transcribed into cDNA using a RevertAid™ First Strand cDNA Synthesis Kit (Cat. No. K1622, Fermentas, Glen Burnie, USA). Real-time quantitative PCR (QPCR) was performed using the SYBR Premix Ex Taq Kit (Cat. No.DRR420A, Takara Bio Inc., Otsu, Japan) in an ABI Prism 7900HT system (Applied Biosystems, Foster City, CA, USA). Thermocycler conditions included 2-minute incubation at 50°C, then 95°C for 10 minutes; this was followed by a 2-step PCR program, as follows: 95°C for 15 seconds and 60°C for 60 seconds for 40 cycles. GAPDH was used as an internal control to normalize differences in the amount of total RNA in each sample. Primers are listed in (Table 1).
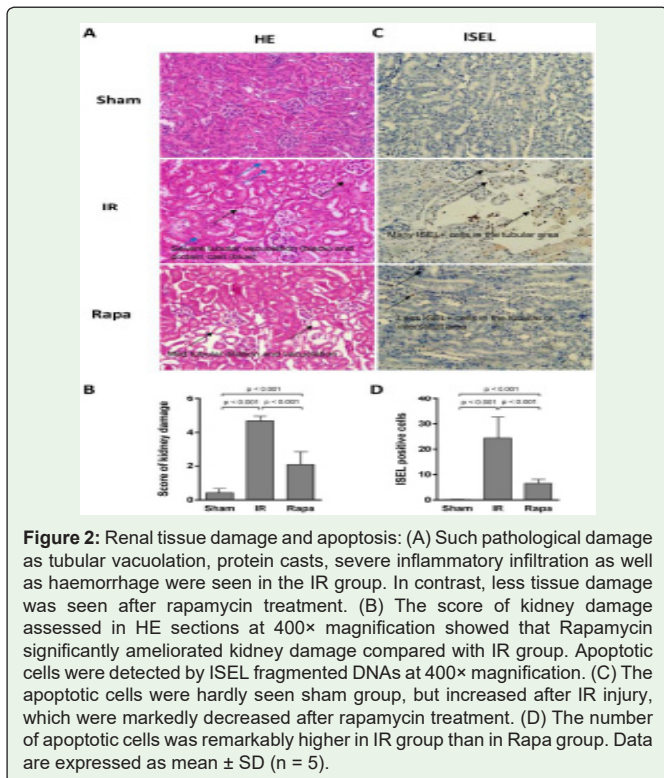

Figure 2: Renal tissue damage and apoptosis: (A) Such pathological damage as tubular vacuolation, protein casts, severe inflammatory infiltration as well as haemorrhage were seen in the IR group. In contrast, less tissue damage was seen after rapamycin treatment. (B) The score of kidney damage assessed in HE sections at 400× magnification showed that Rapamycin significantly ameliorated kidney damage compared with IR group. Apoptotic cells were detected by ISEL fragmented DNAs at 400× magnification. (C) The apoptotic cells were hardly seen sham group, but increased after IR injury, which were markedly decreased after rapamycin treatment. (D) The number of apoptotic cells was remarkably higher in IR group than in Rapa group. Data are expressed as mean ± SD (n = 5).
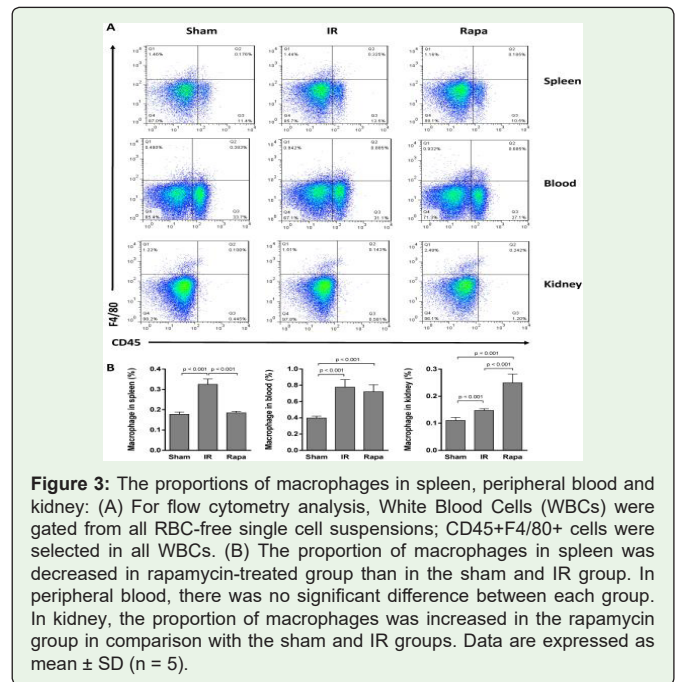

Figure 3: The proportions of macrophages in spleen, peripheral blood and kidney: (A) For flow cytometry analysis, White Blood Cells (WBCs) were gated from all RBC-free single cell suspensions; CD45+F4/80+ cells were selected in all WBCs. (B) The proportion of macrophages in spleen was decreased in rapamycin-treated group than in the sham and IR group. In peripheral blood, there was no significant difference between each group. In kidney, the proportion of macrophages was increased in the rapamycin group in comparison with the sham and IR groups. Data are expressed as mean ± SD (n = 5).

Histological assessment

Renal specimens were fixed in 10% neutral buffered formalin and paraffin-embedded. Deparaffinized sections (5-10 μm) were stained with Hematoxylin and Eosin (HE). The tissue sections were blind-labeled and reviewed by two renal pathologists. A histologic score system was used to estimate the renal damage, which was graded by the percentage of tubule injury: 0 (<1%); 1 (1–10%); 2 (11–20%); 3 (21–40%); 4 (41–60%); 5 (61–75%); 6 (>75%) [16]. The scores represented the severity of tubular injury (including loss of proximal tubule brush border, cell swelling or vacuolization, and cell necrosis): the score ranges of 1–2 represented mild injury, 3-4 represented moderate injury, and 5-6 represented severe injury [15,17-20].
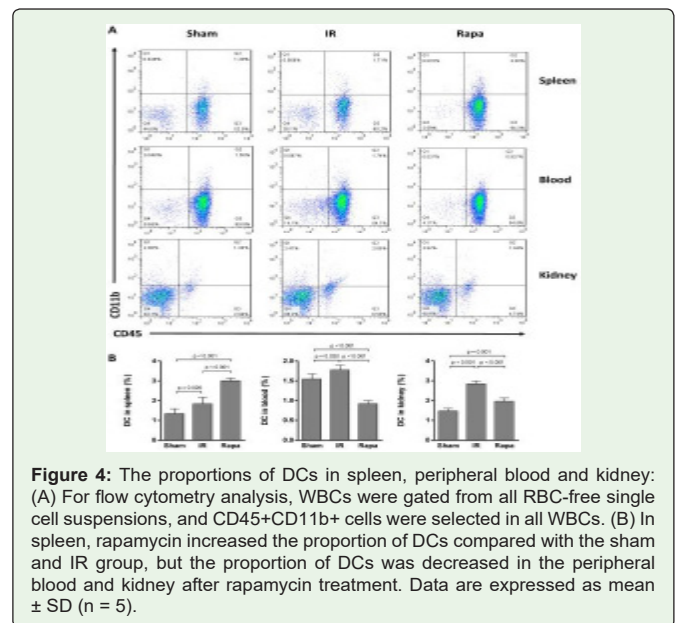

Figure 4: The proportions of DCs in spleen, peripheral blood and kidney: (A) For flow cytometry analysis, WBCs were gated from all RBC-free single cell suspensions, and CD45+CD11b+ cells were selected in all WBCs. (B) In spleen, rapamycin increased the proportion of DCs compared with the sham and IR group, but the proportion of DCs was decreased in the peripheral blood and kidney after rapamycin treatment. Data are expressed as mean ± SD (n = 5).
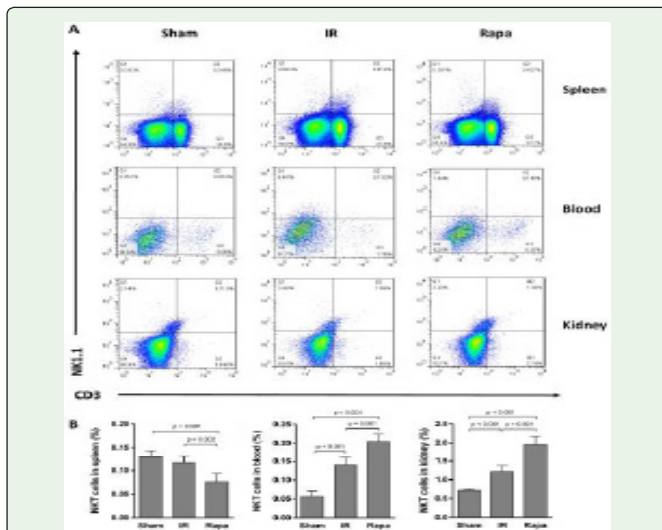
Figure 5: The proportions of NKT cells in spleen, peripheral blood and kidney: (A) For flow cytometry analysis, lymphocytes were gated from all RBC-free single cell suspensions, and CD3+NK1.1+ cells were selected in all lymphocytes. (B) The proportion of NKT cells in rapamycin-treated group was decreased in spleen but significantly increased in peripheral blood and IR induced kidney tissue compared with the IR and sham group. Data are expressed as mean ± SD (n = 5).
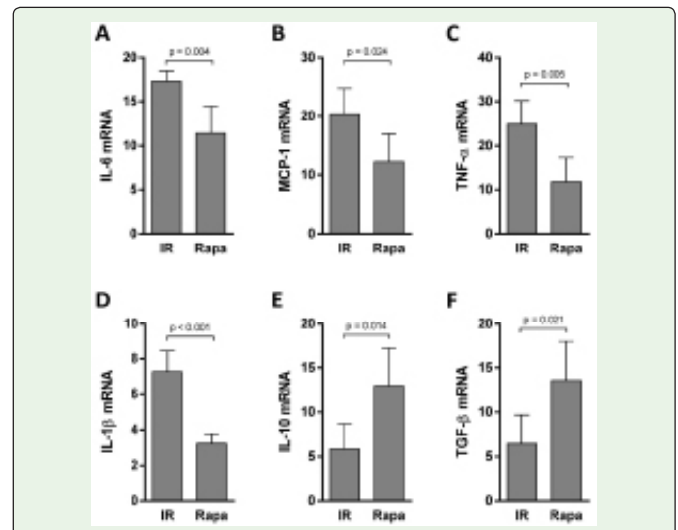

Figure 6: The expression of pro-inflammatory and anti-inflammatory cytokines in kidney tissue: (A-D) The expression of multiple pro-inflammatory and anti-inflammatory cytokines was analyzed by RT-qPCR. Rapamycin dramatically down-regulated the expression of IL-6, MCP-1, TNF-α and IL-1β which were increased in IR group. (E-F) In contrast, the expression of IL-10 and TGF-β was markedly up-regulated after rapamycin administration in comparison with the IR group. Data are expressed as mean ± SD (n = 5).

In situ end-labeling apoptotic cells

Five micrometer paraffin sections were used to label fragmented DNAs *in situ* with Digoxigenin-Deoxyuridine (dUTP) by Terminal Deoxynucleotidyl Transferase (TdT) using a TUNEL Apoptosis Detection Kit (Cat. No.17-141, Millipore, MA, USA). Briefly, sections were digested by 40 μg/ml proteinase K (Cat. No.539470-10MG,EMD Chemicals, NJ, USA) for 15 min at 37°C, incubated with TdT and digoxigenin-dUTP at 37°C for 60min and transferred to wash/stop buffer for 30min. After adding anti-digoxigenin-peroxidase complex for 30min, these sections were developed by DAB substrate. Apoptotic cells were examined at 400× magnification over 20 fields for semi-quantitation.

Statistical analysis

Statistical analysis (SPSS 18.0 software, SPSS Inc, Armonk, NY, USA) was performed with the two-tailed independent Student's t-test after the demonstration of homogeneity of variance with the F test or one-way ANOVA for more than two groups. The Scheffe test was used for post-hoc analysis. Values of P less than 0.05 were considered significant. All values were presented as mean ± SD.

Results

Rapamycin attenuated renal dysfunction, ameliorated renal histologic damage and apoptosis Scr and BUN were markedly increased by IR injury compared with sham group. After rapamycin treatment, Scr and BUN level were significantly reduced compared with the IR group (Figure 1).

Histological assessment in each group was performed in HE stained sections. There were significant tubular changes including loss of brush border, dilation of renal tubules, as well as inflammatory infiltration and protein casts in the tubular area following IR injury compared with the sham group. In contrast, rapamycin treatment significantly ameliorated tubular lesions (Figure 2). The semi-quantitative assessment of histologic lesion showed a significantly lower score in the rapamycin group compared with the IR group post 24h reperfusion (Figure 2). Consistently, the number of apoptotic cells, detected by *In Situ* End-Labeling (ISEL) fragmented DNAs, in tubulointerstitial areas was significantly decreased by rapamycin compared with the IR group (Figure 2).

Rapamycin modulated macrophages, DCs and NKT cells in spleen, peripheral blood and IR induced kidney.

To investigate the modulatory effect of rapamycin on NKT cells, Dendritic cells and macrophages in IR injury, we respectively analyzed the proportions of each kind of cells in the peripheral blood, spleen and kidney. For macrophages, the proportions were decreased in rapamycin-treated group than in the sham and IR group in spleen. In peripheral blood, there was no significant difference between each group. In contrast, the proportion of macrophages was increased in the rapamycin group in comparison with the sham and IR groups in the kidney (Figure 3). The proportion of DCs, however, showed the opposite trend. In spleen, rapamycin increased the proportion of DCs compared with the sham and IR group, but the proportion was decreased in the peripheral blood and kidney (Figure 4). For NKT cells, the proportion in rapamycin-treated group was decreased in spleen but significantly increased in peripheral blood and IR induced kidney tissue compared with the IR and sham group (Figure 5).

Rapamycin modulated the expression of pro-inflammatory and anti-inflammatory cytokines in IR induced kidney.

After rapamycin administration, the expression of multiple pro-inflammatory and anti-inflammatory cytokines was analyzed by RT-qPCR. Rapamycin dramatically down-regulated the expression of IL-6, MCP-1, TNF-α and IL-1β compared with the IR group in which these pro-inflammatory cytokines was increased after suffering from IR injury (Figure 6). The expression of IL-10 and TGF-β was markedly

up-regulated after rapamycin administration in comparison with the IR group and sham group (Figure 6).

Discussion

In this study, we showed that rapamycin protected the murine kidney from IR injury in the early stage (24h post reperfusion) by inhibiting apoptosis in tubulointerstitial area and modulating the proportions of immune cells including DCs, NKT cells and macrophages in the spleen, peripheral blood and IR induced kidney tissue. In addition, rapamycin down-regulated the expression of such pro-inflammatory cytokinesas IL-6, MCP-1, TNF-α, IL-1β and up-regulated such anti-inflammatory cytokinesas TGF-β1 and IL-10 in the kidney.

Apoptosis plays an important role in IR injury. The hypoxia condition due to ischemia may exhaust the reserved Adenosine Triphosphate (ATP) and undermine intracellular homeostasis [21]. Moreover, energy scarcity may lead to mitochondria dysfunction, which further initiates the activation of apoptotic mediators [22]. In this study, we found that rapamycin inhibited cellular apoptosis in the tubulointerstitial area of IR-induced kidney tissue, demonstrating the protective effects on renal structure.

Recent studies have proved that certain kinds of immune cells played an important role in IR injury, resulting in ameliorating or exaggerating injury. NKT cells, expressing both T Cell Receptors (TCR) and natural killer cell receptors [23], have been reported to actively participate in IR injury in multiple solid organs. In kidney, the study from Li et al. supported the essential role of NKT cells in the innate immune response of renal IR injury by mediating neutrophil infiltration and production of IFN-γ [24]. Consistent with this, Yang et al. reported that sulfatide-reactive NKT cells abrogated renal IR Injury. Modulation of cellular infiltration and cytokine expressions along with molecular changes such as hypoxia induced factor-1α (HIF-1α) and IL-10 were the main mechanisms for protection [11]. Importantly, our previous study showed that rapamycin could recruit NKT cells from spleen to the IR-induced kidney to ameliorate renal IR injury in the early stage and the recruitment was mediated by the interaction between CXCL9, CXCL10 and CXCR3 [13]. Dendritic cells, the most potent professional antigen-presenting cells, induce and regulate both innate and adaptive immune responses. As to its role in IR injury, Zhang et al. found in their research that hepatic warm IR injury was significantly lower in DC-deficient than in Wild Type (WT) mice with lower alanine aminotransferase level, reduced hepatic necrosis, and lower neutrophil infiltration, indicating that lack of both liver-resident and blood-borne DC ameliorated hepatic warm IR injury. Adoptive transfer of splenic or hepatic WT DC into DC-deficient or WT mice increased hepatic warm IR injury, suggesting injurious roles of DC infusion [25]. In renal IR injury, DC could regulate Th1-Th2 polarity in kidney IR injury via expressing sphingosine 1-phosphate receptor-3 [26]. In addition, Rama et al. reported that the ischemic environment promoted DCs maturation, which triggers a local increase in immunogenicity and activate T cells to draw an immune-inflammatory response to the target organ, therefore aggravating renal IR injury [27]. These studies suggested that resident DCs in IR-induced organs may be hazardous, thus down-regulating the proportion of DCs or attenuating local DCs maturation could contribute to alleviating tissue injury. This study suggested that down-regulated proportion of DC in IR induced kidney contributed to alleviating tissue injury.

In kidney IR model, macrophages increased progressively after reperfusion and remain high in the renal interstitium from 7 days after IR injury. It is noteworthy that macrophages have recently been proposed to aggravate tissue injury during the inflammatory phase while have a protective effect during the repair phase. Lee et al. found that macrophages expressed pro-inflammatory markers during the initial phase of IRI, whereas macrophages displayed an alternatively activated phenotype during the repair phase. When M1 macrophages were adoptively transferred early after injury, they switched to an M2 phenotype within the kidney during the later recovery phase [12]. These findings are consistent with several other published studies in which macrophage ablation resulted in deleterious outcomes [28, 29]. Together, these studies show that macrophages undergo a switch from a pro-inflammatory to a trophic phenotype that supports the transition from tubule injury to tubule repair in acute kidney injury [30].

In this study, it is demonstrated that after rapamycin treatment the proportion of tissue-protective NKT cells was markedly raised in IR induced kidney tissue. This study also showed that in accordance with the changed proportion in kidney of rapamycin-treated group, the proportion of NKT cells was decreased in spleen and increased in peripheral blood compared with the IR group, which implied the recruited NKT cells in the kidney may originate from spleen and peripheral blood. However, this point of view needs to be confirmed by further evidences. In contrast, the proportion of deleterious DCs showed the opposite trend, which was significantly reduced in kidney tissue in rapamycin group compared with IR group and sham group. Considering the fact that rapamycin may also attenuate the maturation of resident DCs [27], we thought it partially accounted for the decreased proportion of DCs in the kidney tissue after treatment. The number of macrophages was observed to be increased in kidney tissue in rapamycin-treated group. In terms of phenotype switch of macrophages mentioned above, the pro-inflammatory macrophages may play a major role in the early stage, which contributes to the worsened renal tubule injury. This seems to be contradictory to the overall protective effect of rapamycin in 24h follow-up after IR injury, of which the reason is yet to study. Taking these findings together into consideration, rapamycin may protect kidney against ischemia-reperfusion injury via modulating the distribution of macrophages, DCs and NKT cells in involved organs. In addition, Rapamycin rebalanced inflammatory factors in IR induced kidney by increasing the expression of anti-inflammatory cytokines and decreasing pro-inflammatory cytokines. We for the first time comprehensively studied the regulatory function of rapamycin on immune microenvironment and revealed that there existed relationship between the remodeling of immune microenvironment and its protective role in renal IR injury.

Conclusion

Our research demonstrated for the first time that rapamycin was able to protect kidney from IR injury in the early stage via modulating multiple innate immune cells *in situ* and molecules in the murine model. In the following study, additional researches should reveal the effects of rapamycin on specific subtypes of innate immune cells as well as the molecular pathways involved in the modulatory function of rapamycin.

Authors' Contributions

CZ and CY conceived of the study, and participated in the design

of the study and performed the statistical analysis. LW carried out the animal surgery. YZ performed the pathological and molecular studies. RR conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

Acknowledgement

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