Superagonistic CD28 protects against renal ischemia injury induced fibrosis through a regulatory T-cell expansion dependent mechanism

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Background
Acute kidney injury (AKI) affects almost 5% of all hospitalizations, with high morbidity and mortality [1]. The long-term prognosis of AKI is extremely poor. A 10-year follow-up study of AKI patients found that 19 to 31% of AKI patients eventually progressed to chronic kidney disease (CKD) or end stage renal disease (ESRD), and 12.5% of the patients required long-term dialysis [2–4]. The mechanisms driving the AKI to CKD/ESRD transition remain unclear. Inflammatory immune response plays an important role in AKI pathogenesis and has recently attracted attention [5–8]. CD4+ T cells differentiate into various effector T-helper (Th) cells upon antigen exposure and cytokines in the post-ischemic milieu. Th1 cells promote acute kidney injury whereas Th2 cells contribute to tissue repair. Th17 cells identified by interleukin-17(IL-17) secretion, have been verified to persist in the kidney for up to 5 weeks following initial acute kidney injury [9]. Regulatory T cells (Tregs), another Th cells subset, promote recovery from renal ischemic injury in an interleukin-10 (IL-10) dependent pathway [10].

Renal ischemia-reperfusion injury (IRI) is regarded as acute inflammatory disease, whereas immune cells play crucial roles during the entire process of AKI, extending across initiation, maintenance, and recovery phases [11]. Dendritic cells and macrophages demonstrate a paranoid effect when differentiating into various phenotypes. Treg-mediated negative immune-regulation was proven in various experimental models wherein Tregs were stimulated by different reagents such as interleukin-2(IL-2)/anti-IL-2 [12], IL-17A [13] and superagonistic CD28 (CD28sa), which strongly supports the protective role of Tregs against over-activation of inflammation [14].

In our previous study, we proved that CD28sa pretreatment as a pre-ischemia intervention against IRI, needs about 6 days to work at full capacity in order to stimulate an adequate amount of Tregs [14]. We were then interested in the role of Treg expansion during the AKI to CKD transition. Therefore, we designed the present study to investigate the renal chronic outcome at as long as 28 days after IRI injury following CD28sa pretreatment.

Methods
Experimental animals and surgical protocol
Six to eight-week-old male C57BL/6 J mice (weight, 20–25 g) were purchased from Silaike (China), 8–10 for each subgroup. Animals were housed in temperature- and humidity-controlled cages, with free access to water and rodent food on a 12-h light/dark cycle. Experiments were completed at Zhongshan Hospital Fudan University. The animal use protocols were approved by the Institutional Animal Care and Use Committee of Fudan University and Zhongshan Hospital. They also strictly adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All surgeries were performed under intraperitoneal 1% sodium pentobarbital anesthesia (3.0–4.5 ml/kg), intrarectal temperature of mice was maintained at 35.0 °C–36.0 °C with a heating pad during the surgeries. A midline abdominal
incision was made, and the mice were subjected to bilateral renal pedicle clamping for 35 min using non-traumatic microvascular clips. After removal of the clamps, blood restoration in the kidneys was confirmed visually. To maintain body fluid balance, all mice were supplemented with 0.5 ml of saline administered subcutaneously. A sham operation was performed in a similar manner, except for renal pedicle clamping. After surgery, mice were transferred to the recovery cages, one cage for each. Parameters like vital signs, time to wake up from anesthesia were monitored. Food intake was daily monitored, and animals were weighed once every other day. Mice were sacrificed at 1, 3, 7, 14, and 28 days after the procedure, and serum and kidney tissues were collected for various analyses. One percent pentobarbital sodium (4 ml/kg) were injected intraperitoneally, 15 min later, mice were sacrificed by cervical dislocation. The early mortality rate (mice died before the indicated time point) of mice subject to IR, ranged from 10 to 30%.

Protocols of CD28sa administration and Treg depletion

Superagonistic CD28 was purchased from AbD Serotec (USA). LEAF-purified anti-mouse CD25 antibody was purchased from Biolegend (USA). For the CD28sa-IR group, a bolus injection of 4–4.5 mg/kg CD28sa in 1 ml phosphate buffer (PBS) was intraperitoneally administered to mice 6 days (day 1) before IR. The ischemia preconditioning (IPC) was induced 6 days before IR for 18-min pre-ischemia. The sham operation group and PBS group were used as controls. In order to deplete Tregs, anti-CD25 antibodies (PC61, Biolegend, USA) were administered intraperitoneally to mice at a dose of 8–9 mg/kg on days 3 and 5 following the 35-min ischemia on day 7, for in vivo depletion of Tregs. IR, ischemia reperfusion; IPC, ischemia preconditioning; CD28sa, CD28 superagonists. Scr, serum creatinine; FCM, flow cytometry.

Immunohistochemistry staining

The number of stain positive cells was analyzed using immunohistochemistry (IHC) as described previously [14]. Anti-Fibronectin rabbit antibody (Cell signaling technology, USA), anti-Collagen IV rabbit antibody (Abcam, USA), and anti-8-OHdg rabbit antibody (Abcam, USA) were used as primary antibodies according to the manufacturer’s instructions. Secondary antibodies (1:5000; Jackson ImmunoResearch) were also used according to the manufacturer’s instructions. All immunohistochemistry staining was analyzed as follows: under the microscope (200x or 400x), the sections were moved randomly to take 20–25 pictures per section, followed by analysis using Image Pro-Plus 6.0 Software (Media Cybernetics, USA) to identify the target cells.

Fluorescence-activated cell sorting (FACS) analyses

For detection of CD4+Foxp3+ Tregs, spleens, blood, and kidneys of C57BL/6 mice were subjected to flow cytometry analysis as described previously [14]. Anti-CD4- fluorescein isothiocyanate (FITC), anti-CD25-Phycocerythin (PE), anti-Foxp3-efluor450, anti-IL-17A-allophycocyanin (APC), anti-CD11c-PE, anti-MHCII-FITC antibodies were purchased from eBioscience (USA). FACS Attune Nxt (Life, Thermo) was used for the analysis.

Western blot analyses

Relative protein abundances were detected using western blotting as we previously described [6–8, 14]. The antibodies we used in this study were from CST, Abcam, BioLegend and eBioscience.

Histologic analyses

Renal tubulointerstitial fibrosis was detected using Masson’s trichrome staining or sirius red staining, the extent of renal fibrosis was shown as the percentage of blue-stained area or sirius red-stained area in renal cortex and outer medulla.
Renal lysate cytokines measurement
The mouse cytokines interleukin-1α (IL-1α/β), IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17A, Eotaxin, granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), interferon-γ (IFN-γ), keratinocyte chemoattractant, monocyte chemotactic protein-1 (MCP-1, MCAF), macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-1β (MIP-1β), RANTES, and tumor necrosis factor-α (TNF-α) were quantified using Mouse 23-plex Multi-Analyte Kit (Bio-Plex Suspension Array System; Bio-Rad, Hercules, CA, USA). The antibody array experiment was performed according to their established protocol by Wayen Biotechnology (Shanghai, China). The exact protocol was administered according to what had been reported before [15].

Statistical analysis
Data were shown as mean ± standard error. When comparing two samples, student’s t unpaired t test was used. When comparing more than two samples, Dunnet’s post hoc test for parametric data or the Kruskal-Wallis for nonparametric data was used. A P value less than 0.05 was considered to be statistically significant. All statistical analyses were done using SPSS software version 17.0.

Results
CD28sa administration alleviated IRI-induced renal inflammation
When renal function was assessed after IRI, significant improvement was found in the CD28sa group compared to that in the IR group (Fig. 2a). Due to its ability to systemically neutralize CD28sa induced Treg expansion,
PC61 abrogated the renoprotective effects of CD28sa on day 1 and day 7 post-IRI (Fig. 2a).

We measured the percentage of Tregs, dendritic cells, and Th17 cells in the kidney, peripheral blood, and spleens by flow cytometry at 24 h, 7 days, 14 days, and 28 days post-IRI in the mice model. Since we previously found Treg expansion reached a peak at 6 days after CD28sa treatment [14], we administered CD28sa or PBS at 6 days before IRI. CD28sa induced a significant increase in the percentage of Foxp3+CD4+ Tregs of CD4+ T cells from the spleen, blood, and kidney (Fig. 2b-d). When anti-CD25 antibody (PC61) was administered after injection of CD28sa on day 3 and 5, expansion of Tregs in the spleen, peripheral blood, and kidney was mostly abrogated in the short term (Fig. 2b-d).

The balance between Th17 and Tregs plays a key role in the maintenance of immune homeostasis in vivo. To illustrate whether the expansion effect of CD28sa could be connected to Th17 cells during IRI-induced inflammation and fibrosis, we checked the Th17 cell percentage at different time points after IRI. The percentage of IL-17A+CD4+ Th17 cells of the renal tissue indicated a remarkable decrease in the CD28sa-ischemia reperfusion (IR) group compared with the IR group at 24 h after IRI (Fig. 2e-f). Tregs are capable of inhibiting Th17 cells and other effector T cells. The percentage of CD11c+MHCII+ dendritic cells in the kidney increased significantly in the CD28sa-ischemia reperfusion (IR) group (Fig. 2g-h). These results suggested that CD28sa treatment inhibited Th17 cell accumulation and promoted Tregs and CD11c+MHCII+ dendritic cell accumulation in the early stage of post-IRI inflammation.

**CD28sa administration alleviated the IRI-induced renal fibrosis**

To further verify the protective effects of CD28sa against IRI-induced renal fibrosis, we checked the histological changes of mouse kidneys. Tubular cell vacuolization, cast formation, and loss of brush border was predominant at the cortico-medullary junction by 28 days after IRI. In contrast, mice treated with CD28sa presented mild renal morphologic abnormalities (Fig. 3a).

Subsequently, we assessed kidney fibrosis at 28 days post-injury. Pathological examination showed no renal fibrotic lesions in the PBS-treated group. Tubulointerstitial fibrosis was prominent in the IRI group, with excess collagen deposition evidenced by masson staining and Sirius red staining (Fig. 3b-c). Simultaneously, CD28sa-treated mice showed attenuated renal pathological damage and less collagen deposition (Fig. 3b-c). Western blot also showed that renal expression of collagen IV protein was reduced in the CD28sa-IR group compared with that in the IR group (Fig. 3d-e). As we previously reported, CD28sa mimicked the renoprotective effects of IPC on acute kidney ischemic injury. In this study, we have also observed that CD28sa mimicked the renoprotective effects of IPC on the long-term outcome. As shown in Fig. 3d-e, either IPC treatment or CD28sa treatment significantly attenuated renal protein expression of collagen IV at day 28 post IRI. Conversely, PC61 abolished all the beneficial effects conferred by CD28sa treatment.

**CD28sa attenuated the IRI-induced extracellular matrix deposition and oxidative stress**

Extracellular matrix (ECM) is a three-dimensional network of extracellular macromolecules such as collagen, enzymes, and glycoproteins that provide biochemical and structural support of surrounding cells. We examined the expression of fibronectin and collagen IV to fully indicate the ECM deposition of kidneys. Immunohistochemistry staining showed that fibronectin and collagen IV deposition induced by IR injury was significantly mitigated by CD28sa treatment (Fig. 4a-b).

Oxidative stress is known to have detrimental effects on T cells and natural killer (NK) cells both in chronic inflammatory conditions and in cancer [16]. Tregs have been reported to possess anti-oxidative capacity in the cancer microenvironment [17], therefore, we checked the expression of DNA associated oxidative stress marker, 8-Oxoguanine (8-OHdg) among different groups. Immunohistochemistry staining revealed that CD28sa treatment significantly downregulated 8-OHdg expression at day 28 post IRI (Fig. 4c), whereas depletion of Tregs by the anti-CD25 antibody PC61 significantly reversed the anti-oxidative effects secondary to CD28sa treatment. What’s more, CD28sa treatment showed similar anti-fibrotic and anti-oxidative effects with IPC treatment, as both CD28sa-IR group and IPC-IR group presented less fibronectin and Collagen IV deposition as well as less 8-OHdg expression, compared to the IR group (Fig. 4d-f).

Apoptosis and cell proliferation play a key role in tissue injury and repair [18]. At 28 days post injury, there was still an increased number of TUNEL positive cells in the kidneys of the IR group compared with those in the control group, which was attenuated by CD28sa treatment (Fig. 5a-b). Meanwhile, Ki67 staining of kidney specimens showed an increased number of proliferating cells in mice with CD28sa treatment (Fig. 5a,c). Similarly, these protective effects could be reversed by PC61 administration.

**CD28sa may decrease Th17 cells by inhibiting the expression of IL-6 and IL-17A**

To further investigate the mechanism of CD28sa-induced anti-inflammation and anti-fibrosis effects, we examined the serum levels of 23 cytokines in mice over time after IRI. The expression of several cytokines differed between the IR group and CD28sa-IR. IL-10, the most well-known Treg effector cytokine, was increased...
in the CD28sa-IR group compared with the IR group at 24 h and 7 days after IRI (Fig. 6a). IL-6, a pro-inflammatory cytokine, which may associate with downregulated Th17 cells, presented a decrease in the CD28sa-IR group at 24 h and 7 days after IRI (Fig. 6b). IL-17A, the typical effector cytokine of Th17 cells was decreased significantly in the CD28sa-IR group compared with that in the IR-group at 24 h and 7 days (Fig. 6c).

**Discussion**

In the present study, we demonstrated the following points: (i) We used the previously reported mouse IRI model and extended the observation window to 7, 14, and 28 days post IRI. A single injection of CD28sa was able to provide remarkable functional and histological protection to the kidneys with less extracellular matrix deposition; (ii) The immuno-inflammatory response of CD28sa pre-treated IRI mice was characterized by an increased percentage of Tregs and MHCIiCD11c+ dendritic cells, a decreased percentage of Th17 cells and increased secretion of the Treg effector cytokine, IL-10. (iii) CD28sa pretreatment also resulted in less renal cell apoptosis and less oxidative stress marked by less TUNEL and 8-OHdG positive cells. These results demonstrated that CD28sa pretreatment induced a systemic immune tolerance status characterized by expansion of Tregs and CD11c+MHCIi+ dendritic cells, resulting in reduced chronic kidney injury and better long-term prognosis.

CD25+Foxp3+ Tregs are important in the negative regulation of immune responses and immune tolerance in various injury models such as brain damage after ischemia stroke.
Sang-Kyung Jo's team reported that Tregs were likely to contribute to the repair process in IR injury as well as to the tolerance induction mechanism upon subsequent injury [21]. CD28sa amplifies T cell receptor (TCR) signals and is observed to have pronounced therapeutic effects in rodent models for infection-associated inflammation [22], solid-organ transplantation [23–25], rheumatoid arthritis [26] and ischemic organ injury [14, 19]. Moreover, CD28sa-activated Tregs were shown to switch to an IL-10-secreting phenotype and to accumulate at sites of injury [27]. Taken together, it is reasonable to hypothesize that CD28sa exerted a protective effect in the recovery phase of IR by mediating a tolerance induction mechanism. Although, the magnitude of increase in the systemic percentage of Tregs in our study was small from day 7 after IRI, the role of Tregs proved to be important because partial depletion of Tregs using the anti-CD25 antibody, PC61, prophylactically reversed the protective effect of CD28sa. This result was consistent with the protective mechanism of Tregs in the recovery phase of IR mice preconditioned with ischemia [21].

The Treg expansion effect of CD28sa depended largely on the MHCII expression of antigen presenting cells [28]. Therefore, we also tested the percentage of MHCII+CD11c+ dendritic cells (DCs) from day 7 after IRI injury in mice. Macrophages and DCs are both derived from monocyte/macrophage-lineage common precursor cells and share many surface markers including CD11c. It is thus difficult to distinguish them in an in-vivo system. FCM results showed that the percentage of CD11c+MHCII+ dendritic cells in kidneys was increased upon CD28sa pretreatment at 7 days after IR injury whereas this increment was abolished by partial Tregs depletion using PC61. This is consistent with a previous report demonstrating that CD11b+ cells might represent tolerogenic DCs that potentiate Treg activation [29]. We also observed that cytokine secretion changes in the kidneys post-IR. Among tissue cytokines, IL-6, a well-known inflammatory cytokine, was decreased...
significantly in CD28sa pre-treated ischemic kidneys at 24 h after IR.

CD8+ Tregs have been reported to inhibit effector T cell populations through oxidative phosphorylation in microvesicles [30]. Oxidative stress has also been reported to affect the functions of Tregs [31]. In this study, the effects of CD28sa on apoptosis and oxidative stress in the kidney was also tested at 28 days after IR injury. Prophylactic administration of CD28sa still resulted in less apoptotic kidney cells and ameliorated oxidative stress burden. Combined with the results from our previous study, the beneficial effects of CD28sa are presented in the short-term (within 7 days post IRI), as well as in the long-term (at 28 days post IRI).

It is well known that a severe AKI episode can lead to CKD. Further, sustained inflammation is associated with CKD, our observations in mouse IRI models have revealed a negative association between CD28sa triggered Treg expansion and CKD progression. Renal fibrosis development tended to be much slighter in the CD28sa-IR group than that in the solo IRI. On day 28 post IR, the latter showed a significantly greater Sirius-red-positive area than that in the former, along with prominent intrarenal inflammation, apoptosis, and oxidative stress.

**Fig. 5** CD28sa mitigated cell apoptosis and proliferation. **a.** TdT-mediated dNTP nick end labeling (TUNEL)-positive cells and Ki67-positive cells of the kidney sections (original magnification ×200, Bar = 100 μM). **b.** Quantification statistics of TUNEL-positive stained cells. **C.** Quantification of Ki67 positive stained nuclei. *P < 0.05 (n = 6)

**Fig. 6** Serum levels of cytokines from mice. **a.** Interleukin-10 expression at different time points after IR injury. **b.** Serum levels of interleukin-6 from different time points after IR injury. **c.** Interleukin-17A expression from different time points after IR injury. *P < 0.05 compared with the sham group (n = 6). *P < 0.05 compared with the IR group (n = 6). *P < 0.05 compared with the CD28sa-IR group (n = 6)
suggesting that pretreatment with CD28sa may mitigate renal fibrosis in the long term via upregulation of Tregs.

However, several limitations exist in this study worth mentioning. First, we solely investigated the percentage of Foxp3⁺ Tregs in the progression of CKD following IRI-induced AKI. The lack of a reliable biomarker which was predictive of uncontrolled T cells stimulation by CD28sa was the pivotal factor for the failure of clinical safety testing [32]. We didn’t analyze the expression of CD44 and CD62L in Tregs to distinguish between activated Tregs and resting Tregs. In the future study, we need to focus on related inhibitory signals such as PD-1, which could be used to predict the activation state of Tregs, serving as a potential biomarker to indicate the effects of Treg stimulatory biologic, CD28sa. Secondly, although we had identified the role of CD28sa induced Tregs expansion in retarding AKI to CKD transition, the pharmacodynamics and pharmackinetics of CD28sa remained unclear. Third, although we have observed the homeostasis between Tregs and Th17 induced by CD28sa, the exact molecular mechanism of Th17 suppression secondary to CD28sa treatment is still unclear. A complete overview of all T-helper cell reactions to IRI or to CD28sa should be required.

Conclusions

In summary, we provide evidence that CD28sa treatment negatively regulates immune responses in a way mediated by the Th 17 cells /Tregs balance, which might promote post-IRI recovery of the kidney. Identifying the mechanisms of CD28sa induced peripheral immune tolerance might be helpful to develop novel strategies for improving post-IRI prognosis.

Abbreviations

8-OHdG: 8-Oxoguanine; AKI: Acute kidney injury; APC: Allophycocyanin; CD: Cluster of differentiation; CD28sa: superagonistic CD28; CKD: Chronic kidney disease; DC: Dendritic cells; ECM: Extracellular matrix; ESRD: End stage renal disease; FACS: Fluorescence-activated cell sorting; FITC: Fluorescein isothiocyanate; Foxp3: Forkhead/winged helix transcription factor p3; G-CSF: Granulocyte-colony stimulating factor; G-MCSF: Granulocyte-macrophage colony stimulating factor; IHC: Immunohistochemistry; IL-1α: Interleukin-1 alpha; IL-1β: Interleukin-1 beta; IL-10: Interleukin-10; IL-12: Interleukin-12; IL-17: Interleukin-17; IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9: Interleukin-2; INF-α: Interferon-α; IR: Ischemia-reperfusion; IRI: Ischemia-reperfusion injury; MCP-1: Monocyte chemotactic protein-1; MHCII: Major histocompatibility complex class II; MIP-1α: Macrophage inflammatory protein-1α; MIP-1β: Macrophage inflammatory protein-1B; NK: Natural killer cells; PBS: Phosphate buffer; PE: Phycoerythrin; PAGE: polyacrylamide gel electrophoresis; RT: Room temperature; SDS: Sodium dodecyl sulfate; TCR: T cell receptor; Th: T-helper; TNF-α: Tumor necrosis factor-α; Tregs: Regulatory T cells

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Authors’ contributions

YF and XD: obtained funding and conceptualized the study. YL performed the experiments. XW acquired and analyzed the data. NX performed the histological examination of the kidney. YF and YL were the major contributors in writing the manuscript. YF, YL, NX, XW and XD contributed to data interpretation, discussion and manuscript preparation. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The animal use protocols were approved by the Institutional Animal Care and Use Committee of Fudan University and Zhongshan Hospital. They also strictly adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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