IL-2/Anti-IL-2 Complex Attenuates Renal Ischemia-Reperfusion Injury through Expansion of Regulatory T Cells

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ABSTRACT

Regulatory T cells (Tregs) can suppress immunologic damage in renal ischemia-reperfusion injury (IRI), but the isolation and ex vivo expansion of these cells for clinical application remains challenging. Here, we investigated whether the IL-2/anti-IL-2 complex (IL-2C), a mediator of Treg expansion, can attenuate renal IRI in mice. IL-2C administered before bilateral renal IRI induced Treg expansion in both spleen and kidney, improved renal function, and attenuated histologic renal injury and apoptosis after IRI. Furthermore, IL-2C administration reduced the expression of inflammatory cytokines and attenuated the infiltration of neutrophils and macrophages in renal tissue. Depletion of Tregs with anti-CD25 antibodies abrogated the beneficial effects of IL-2C. However, IL-2C-mediated renal protection was not dependent on either IL-10 or TGF-β. Notably, IL-2C administered after IRI also enhanced Treg expansion in spleen and kidney, increased tubular cell proliferation, improved renal function, and reduced renal fibrosis. In conclusion, these results indicate that IL-2C-induced Treg expansion attenuates acute renal damage and improves renal recovery in vivo, suggesting that IL-2C may be a therapeutic strategy for renal IRI.

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AKI is associated with high morbidity and mortality, and patients with AKI are at high risk for progression to CKD.1,2 Renal ischemia-reperfusion injury (IRI) is one of the major causes of AKI, and is an important cause of delayed graft function after kidney transplantation. However, clinical management of AKI including IRI remains largely supportive.

Inflammation is shown to be mainly involved in the pathogenesis of renal IRI, and renal IRI is now regarded as an acute inflammatory process. Both innate and adaptive immune cells participate in renal IRI.3,4 Foxp3+CD4+ regulatory T cells (Tregs) play a critical role in suppression of both adaptive and innate immune responses.5 Tregs have also been reported to attenuate renal IRI.6,7 However, clinical application of Tregs is practically hard, because isolation and expansion of rare Tregs are not easy, and there is also a risk for contamination.

Recently, a particular form of IL-2 mAbs (JES6-1) was reported to prevent interaction of IL-2 with IL-2 receptor β-chain without affecting binding to IL-2 receptor α-chain (CD25); thus, complex (IL-2C) of IL-2 and JES6-1 is reported to expand Tregs preferentially up to 4-fold without a significant effect on natural killer cells and “memory phenotype” CD8+ T cells.8,9 IL-2C treatment suppressed islet allograft rejection10 by inducing Tregs without significant side effects. Furthermore, the IL-2C treatment showed its therapeutic potential in adriamycin nephropathy, a form of CKD.11 However, there has been no study for the effect of the IL-2C on AKI. Here, we investigated whether the IL-2C can attenuate renal IRI by inducing Tregs using murine models.

First, we measured Tregs after renal IRI. We administered IL-2C or PBS for 3 consecutive days from 5 days before IRI,
Figure 1. Expansion of Tregs in both spleen and kidney after renal IRI by IL-2C treatment. IL-2C treatment significantly increases proportions of Foxp3^+CD4^+ Tregs among CD4^+ T cells in both spleen (A and B) and kidney (A and E), compared with the PBS control. Depletion of Tregs by PC61 treatment (IL-2C/PC-61) abrogates expansion of Tregs by IL-2C treatment. Absolute count of Foxp3^+CD4^+ Tregs is significantly increased in both spleen (C) and kidney (F) by IL-2C treatment. Foxp3^-CD4^+ T cells are also increased after IL-2C
because a previous study showed that Treg expansion reached a peak on day 5 after IL-2C treatment. IL-2C induced significant expansion of Foxp3+CD4+ Tregs in both spleen and kidney (IL-2C versus PBS, \( P < 0.001 \) on day 1 and \( P = 0.001 \) on day 5 for spleen, Figure 1, A and B; \( P < 0.001 \) on both day 1 and day 5 for kidney, Figure 1, A and E). Absolute numbers of Foxp3+CD4+ Tregs were also increased by 3- to 5-fold in the IL-2C group (IL-2C versus PBS, \( P = 0.001 \) on day 1 and \( P < 0.001 \) on day 5 for spleen, Figure 1C; \( P < 0.001 \) on day 1 and \( P = 0.01 \) on day 5 for kidney, Figure 1F). Foxp3+ CD4+ T cells were also increased after IL-2C treatment in kidney, but not in spleen (Figure 1, D and G); however, the balance between Tregs and non-Tregs was still skewed toward Tregs, because expansion of Tregs by IL-2C was more vigorous (Figure 1E). There was no significant increase in either memory CD8+ or natural killer cells after IL-2C (Figure 1, H and I). When anti-CD25 antibodies (PC61) were administered shortly after injection of IL-2C, expansion of Tregs in both spleen and kidney was completely abrogated (Figure 1, B and D). When renal functions were assessed after IRI, renal functions were significantly lower in the IL-2C group than in the PBS group (\( P = 0.001 \) for creatinine on day 1, \( P < 0.05 \) for creatinine on day 3, Figure 2). As expected based on its ability to abolish IL-2C–mediated T expanson, PC61 treatment abrogated beneficial effects of IL-2C on renal function after IRI. Both serum BUN and creatinine were significantly higher in the PC61 group than those in the IL-2C group (\( P = 0.014 \) for BUN, Figure 2A; \( P = 0.02 \) for creatinine, Figure 2B). Abrogation of beneficial effects of IL-2C by PC61 supports that expansion of Tregs was the main mechanism of beneficial effects of IL-2C on renal IRI.

IRI induced significant tubular injury in renal tissue 1 day after IRI. IL-2C treatment protected renal tissue injury, and tubular injury score was lower in the IL-2C group than in the PBS group (\( P = 0.003 \), Figure 3A). IL-2C attenuated apoptosis in renal tissue 1 day after IRI (\( P < 0.001 \), Figure 3B). When renal regeneration was assessed 3 and 10 days after IRI, the number of proliferating tubular cells was significantly higher in IL-2C group on day 3 (\( P = 0.02 \), Figure 3C).

We also assessed renal inflammation by means of immunohistochemical staining and tissue cytokine measurement. Neutrophils and macrophages are the predominant infiltrates in the injury phase of renal IRI, and Treg depletion significantly increased their infiltration. Immunohistochemical study demonstrated that IRI increased infiltration of both neutrophils and macrophages into renal tissues 1 day after renal IRI, and that IL-2C significantly attenuated the infiltration of these cells after IRI (\( P < 0.001 \) for neutrophils, Figure 3D; \( P = 0.03 \) for macrophages, Figure 3E). Increased infiltration of CD4+ T cells in the IL-2C group was attributed to the increased infiltration of both Foxp3+ and Foxp3- cells (Figure 1, F and G, and Supplemental Figure 1A). Foxp3+ CD4+ Tregs were mainly observed in the corticomedullary junction in the IL-2C group (Supplemental Figure 1C). However, there was no difference in infiltration of CD8+ T cells (Supplemental Figure 1B), and there was very low infiltration of B cells in both the PBS and IL-2C groups (data not shown). The role of the innate immune response in IRI has been well established. Depletion of neutrophils or macrophages showed a protective effect in IRI. Tregs can suppress innate immune cells directly as well as indirectly by suppressing T cells. Our data suggest that
expanded Tregs by IL-2C attenuated renal IRI mainly through suppression of innate immune responses. These results were in parallel with a recent report that demonstrated that depletion of Tregs resulted in infiltration of more innate immune cells, and higher expression of innate cytokines in the kidney without significant effect on T cells or B cells.  

When cytokines in renal tissue were measured 1 day after IRI, IRI increased IL-6 and CCL2. IL-2C treatment significantly decreased expression of both IL-6 and CCL2 (P=0.04 and P<0.05 for IL-6 and CCL2, respectively, Supplemental Figure 2, A and B). PC61 treatment abrogated beneficial effects of IL-2C on expression of both IL-6 and CCL2 (Supplemental Figure 2, A and B). There was a discrepancy among the previous studies regarding the levels of TNF-α and IFN-γ after IRI according to the experimental settings including methods of cytokine measurement, 19–21 Levels of these cytokines in this study were not different between IL-2C and the control groups, consistent with a previous study that used the multiplex-bead array (Supplemental Figure 2, C and D). 21 IL-10 was not increased by IL-2C (Supplemental Figure 2E). Taken together, IL-2C treatment attenuated renal inflammation by decreasing infiltration of innate immune cells and expression of IL-6/CCL2.

A previous study reported that Tregs from IL-10 knockout mice have defects in protection of renal IRI. 6 In order to assess the role of IL-10 in IL-2C–mediated renal protection from IRI, we administered IL-2C in IL-10 knockout mice. IL-2C induced expansion of Tregs in IL-10 knockout mice as well as wild-type mice (Supplemental Figure 3A and B). Although renal injury was more prominent in IL-10 knockout mice, IL-2C attenuated renal impairment in IL-10 knockout mice as well as wild-type mice (Supplemental Figure 3C). These data suggested that IL-10 is dispensable in the IL-2C–mediated expansion of Tregs and protective effects for IRI. Several differences in experimental settings such as mouse strain, number of Tregs, number of effector T cells, and method of Treg potentiation might contribute to the apparent discrepancy. Although the suppressive activity of IL-10− Tregs on a per cell basis could be weaker, the much higher number of IL-10− Tregs in IL-10 knockout mice might be sufficient to control IRI in response to IL-2C, whereas the small number of adoptive-transferred IL-10− Tregs was insufficient to control IRI in the previous study. 6 Next, we investigated whether TGF-β plays the crucial role in IL-2C–mediated protection from IRI, and found that neutralizing anti-TGF-β treatment did not abrogate beneficial effects of IL-2C (PBS versus IL-2C/anti-TGF-β, P=0.004; IL-2C/anti-TGF-β versus IL-2C/isotype control, P=0.57, Supplemental Figure 3D). Overall, these data suggest that renal protection by IL-2C–induced Tregs might not be dependent on a single mechanism of suppression. Tregs can suppress target cells through various mechanisms including contact-mediated regulation as well as soluble factor-mediated regulation according to the environmental context. 22,23 Further studies are needed to elucidate the detailed mechanisms of IL-2C–mediated renal protection from IRI.

We performed additional experiments to determine the therapeutic potential of IL-2C during the recovery phase after renal IRI. As seen with the above prophylactic approach, IL-2C treatment after IRI also induced significant expansion of Foxp3+CD4+ Tregs in both spleen and kidney (Figure 4, A and B). Renal function in the IL-2C group was slightly improved on day 5 in the bilateral IRI model (P=0.01 for BUN, Figure 4C; P=0.002 for creatinine, Figure 4D). In addition, renal tubular cell proliferation significantly increased on day 5 (P=0.04, Figure 4E), and renal fibrosis also significantly decreased on day 28 after IL-2C treatment in the unilateral model (P=0.01 for Masson trichrome staining, Figure 4F; P<0.001 for type IV collagen; P=0.04 for fibronectin, Figure 4G), suggesting that IL-2C contributes to improving renal recovery.

In conclusion, IL-2C can attenuate acute renal damage, and improve renal recovery in IRI by expanding Tregs. Considering its convenience of manipulation and safety, IL-2C is promising for clinical application to renal IRI.

CONCISE METHODS

Experimental Animals and Renal IRI
Six- to eight-week-old male C57BL/6 mice (weight, 20–25 g) were purchased from Orient (Seongnam, Korea). Foxp3-GFP knock-in C57BL/6 mice were generously provided by Dr. A.Y. Rudensky (University of Washington, Seattle, WA) and IL-10 knockout C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). The animal use protocols were approved by the Institutional Animal Care and Use Committee of Seoul National University and Seoul National University Hospital. Mice were subjected to bilateral renal pedicle clamping for 28 minutes or unilateral clamping for 40 minutes. The animals were kept at a constant body temperature (37°C) using a warm pad. After the clamps were removed, the reperfusion of kidneys was observed for 1 minute. A sham operation was performed in a similar manner, except for clamping of the renal pedicles.

Protocols of IL-2C Administration and Treg Depletion
Recombinant murine IL-2 was purchased from ebioscience (San Diego, CA) and anti-mouse

**Figure 3.** Attenuation of renal tissue injury and infiltration of inflammatory cells after IRI by IL-2C treatment. (A and B) IL-2C treatment significantly attenuates histologic tubular injury that is assessed by tubular injury score (A), and renal apoptosis (B). (C) Proliferation of renal tubular cells is also significantly increased on day 3 by IL-2C treatment. (D and E) IL-2C treatment significantly suppressed infiltration of Ly6G+ neutrophil (D) and F4/80+ macrophages (E) into renal tissues. n=9–10 per group. *P<0.05 for IL-2C versus PBS. PCNA, proliferating cell nuclear antigen. Original magnification, ×200 in A, B, D, and E; ×40 in C.

Figure 4. Beneficial effects of IL-2C treatment on the recovery phase after renal IRI. (A and B) When IL-2C is administered after bilateral or unilateral IRI for 3 consecutive days, IL-2C significantly increases proportions of Foxp3+CD4+ Tregs in both spleen and kidney compared with the PBS. (C and D) IL-2C treatment significantly improves the levels of BUN and creatinine on day 5 after bilateral IRI. n=9–10 per group. (E–G) IL-2C treatment after unilateral IRI also increases renal tubular cell proliferation (PCNA) on day 5 (E), and reduces renal fibrosis on day 28 (F), which is associated with reduced expression of type IV collagen and fibronectin in kidney (G). n=5–8 per group. *P<0.05 for IL-2C versus PBS. PCNA, proliferating cell nuclear antigen.
IL-2 mAbs (JES6-1) were provided by C.D. Surh (The Scripps Institute, La Jolla, CA). IL-2C was mixed with anti-IL-2 (JES6-1) at a 1:5 ratio (1 μg of recombinant murine IL-2 and 5 μg of anti-IL-2), and incubated at 37°C for 30 minutes. IL-2C or PBS was intraperitoneally administered to mice for 3 consecutive days from 5 days before bilateral IRI. The sham operation group and the PBS group were used for controls. Blood, kidney tissues, and spleen were harvested 1, 3, 5, and 10 days after renal IRI. In order to deplete Tregs, anti-CD25 antibodies (PC61; Bio-XCell, West Lebanon, NH) were administered to mice at a dose of 0.3 mg/mouse on 2 consecutive days from 1 day before IRI. Depletion of Foxp3+CD4+ cells was confirmed by flow cytometric examination of spleen and kidney. In the TGF-β-blocking experiments, 150 μg of anti-TGF-β antibodies (AB-100-NA; R&D Systems, Minneapolis, MN) or rabbit IgG isotype control antibodies (eBioscience, San Diego, CA) were intraperitoneally administered to mice on day –4 and day 0 before bilateral renal IRI. Next, IL-2C was also administered for 3 consecutive days from 1 day after bilateral or unilateral IRI in order to investigate the effect of IL-2C on the recovery phase after renal IRI. Renal function and histology were assessed thereafter on days 5, 10, and 28.

FACS Analyses

For the detection of CD4+Foxp3+Tregs, spleens and kidneys of Foxp3-GFP knock-in C57BL/6 mice were used. Flow cytometric analysis of kidney cells was performed as previously described.24 Anti-CD4-APC, Anti-CD8-PE, and anti-CD45-PE antibodies were purchased from BD biosciences (San Jose, CA). Anti-CD3-APC, anti-CD44-APC, and anti-CD45-PE antibodies were purchased from eBioscience. FACS Canto (BD Biosciences) was used for the analysis.

Biochemical Tests and Measurement of Cytokines and Chemokines

Serum BUN and creatinine were measured using a 7070 Hitachi analyzer (Hitachi, Tokyo, Japan). Quantification of various cytokines and chemokines in kidney tissues was performed using a cytometric bead array according to the manufacturer’s protocol (BD Cytometric Bead Array, BD Biosciences).

Histologic Analyses

Tubular injury was semiquantitatively assessed in periodic acid–Schiff stained kidney sections as follows. Degree of tubular cell necrosis and loss of periodic acid–Schiff positive tubular brush borders as well as cast formation were graded from 1 to 4 as previously described.13 In immunohistochemical staining, we used rat anti-mouse F4/80 (eBioscience), Gr-1 (eBioscience), CD4 (Abcam, Cambridge, UK), CD8 (Abcam), and B220 (eBioscience) in order to detect macrophages, neutrophils, CD4+ T cells, CD8+ T cells, and B cells, respectively. Eight to 10 high-power fields were captured, and the mean number of positive cells in the field was calculated for quantification. Proliferating cell nuclear antigen staining was also performed. Proliferating cell nuclear antigen positive cells in the corticomedullary junction and outer medulla were measured by counting 8–10 high-power fields (×200) per section, and the mean number was calculated. In immunofluorescence staining, CD4 cells were stained by Alexa 594 rabbit anti-rat IgG (Invitrogen, Carlsbad, CA) as secondary antibodies in Foxp3-GFP knock-in C57BL/6 mice. Cell nuclei were counterstained with diamidino-2-phenylindole. Cellular infiltration in renal tissues was assessed using a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc. North America, Thornwood, NY). Renal fibrosis was assessed by Masson trichrome staining on day 28 after unilateral IRI, and the area of fibrosis was expressed as the percentage area of the blue-stained area in the renal cortex and outer medulla.

Western Blot Analyses

The whole cell extracts were loaded, and separated by SDS-PAGE under reducing conditions, as previously described.25 The proteins were detected by antibodies for type IV collagen, and fibronectin (Abcam), and were visualized using horseradish peroxidase–linked goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA), which was followed by enhanced chemiluminescence (Amersham, Arlington Heights, IL).

Statistical Analyses

All data are presented as the mean ± SE, and were analyzed by the t test. A P value <0.05 was considered statistically significant. All analyses were performed using SPSS statistical software (version 17.0; IBM Corporation, Armonk, NY).

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DISCLOSURES

C.D.S. and C.A. are shareholders of Nascent Biologics, Inc.

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IL-2C Attenuates Renal IRI

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Supplemental Figure 1

(A) Graph showing CD4^+ cells per HPF (x200) for Sham, PBS, and IL-2C treatments.

(B) Graph showing CD8^+ cells per HPF (x200) for Sham, PBS, and IL-2C treatments.

(C) Immunofluorescence images for Sham, PBS, and IL-2C treatments, comparing cell distribution and intensity across conditions.