Published in final edited form as:

Shock. 2018 March ; 49(3): 269–276. doi:10.1097/SHK.0000000000000988.

The Protective Effect of a Short Peptide Derived from Cold-inducible RNA-binding Protein in Renal Ischemia-Reperfusion Injury

Joseph McGinn^{1,*}, Fangming Zhang^{2,*}, Monowar Aziz², Weng-Lang Yang^{1,2}, Jeffrey Nicaastro¹, Gene F. Coppa¹, and Ping Wang^{1,2}

¹Department of Surgery, Hofstra Northwell School of Medicine, Manhasset, NY

²Center for Immunology and Inflammation, The Feinstein Institute for Medical Research, Manhasset, NY

Abstract

Extracellular cold-inducible RNA-binding protein (CIRP) functions as damage-associated molecular pattern (DAMP) and has been demonstrated to be responsible in part for the damage occurring after renal ischemia-reperfusion (I/R). A short peptide derived from CIRP, named C23, binds to myeloid differentiation factor 2 (MD2), a Toll-like receptor 4 (TLR4) co-receptor. We hypothesize that C23 reduces renal ischemia-reperfusion (RIR) injury by blocking CIRP. We observed that pre-treatment with C23 significantly decreased the levels of recombinant mouse CIRP-induced TNF- α in a dose-dependent fashion in cultured macrophages. C57BL/6 mice were subjected to bilateral renal pedicle clamps for 35 min to induce ischemia, followed by reperfusion for 24 h and harvest of blood and renal tissue. C23 peptide (8 mg/kg) or vehicle was injected intraperitoneally at the beginning of reperfusion. Plasma TNF- α , IL-1 β , and IL-6 levels were decreased in C23-treated RIR mice as compared to vehicle-treated mice by 74%, 85% and 68%, respectively. Expressions of TNF- α and keratinocyte chemoattractant (KC) in the kidneys from C23-treated mice was decreased by 55% and 60%, respectively. Expression of kidney injury molecule-1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) in the kidney of C23-treated mice were significantly reduced by 46% and 55%, respectively. Renal tissue histological assessments revealed significant reduction in damage score by 44% in C23-treated mice. Finally, a survival study revealed a significant survival advantage with a 70% survival rate in C23 group versus 37 % in vehicle group. Thus, C23 has potential as a novel therapy for the patients suffering from I/R-induced renal injury.

Address for correspondence, proofs, and reprint requests to: Ping Wang, MD, Chief Scientific Officer, The Feinstein Institute for Medical Research, Professor and Vice Chairman for Research, Department of Surgery, Hofstra Northwell School of Medicine, 350 Community Drive, Manhasset, NY 11030, USA, Tel: (516) 562-3411, Fax: (516) 562-2396, pwang@northwell.edu.

*Co-first Authors

CONFLICT OF INTEREST

One of the authors (PW) is an inventor of the pending PCT application # WO2015048083 A1: "Peptides inhibiting cold-inducible RNA binding protein activity". TheraSource has an option to license this technology. Other authors report no financial conflicts of interest.

AUTHOR CONTRIBUTIONS

JM, WLY, PW designed the experiments. JM, FZ performed experiments. JM, FZ, MA performed statistical analysis. JM, MA wrote the paper. WLY, JN, GFC, PW reviewed data and manuscript. PW conceived the idea and supervised the whole project. All authors read and approved the final manuscript.

Keywords

CIRP; Renal Ischemia-Reperfusion; Kidneys; KIM-1; NGAL; C23 peptide

INTRODUCTION

Renal ischemia-reperfusion (RIR) injury is a common complication of cardiovascular surgery and sepsis-associated shock syndrome, and is a major contributor to acute kidney injury (AKI) (1). AKI occurs in 35% of all hospitalized patients and is associated with a 2–5 fold increase in mortality risk (2). Patients who survive AKI can develop chronic kidney disease and/or require temporary or sometimes permanent renal replacement therapy. AKI patients are two times more likely to be discharged to either a short- or long-term facility, compounding its burden on patients' health and financial resources (3). Currently our treatment of AKI is limited to prevention and support, however, recent advances in our understanding of the inflammatory component of this disease has provided possible avenues for future therapies.

Ischemia-reperfusion injury is characterized by restriction of blood supply to an organ followed by restoration of blood flow and re-oxygenation (4). This phenomenon exacerbates tissue damage by initiating an inflammatory cascade including reactive oxygen species (ROS), cytokines, chemokines, and leukocytes activation (4, 5). If ischemia continues, these cells die and release damage associated molecular patterns (DAMPs). After reconstitution of blood flow, DAMPs are recognized by pattern recognition receptors on cell surfaces, which in turn induce a sterile inflammatory response in the absence of exogenous pathogens (6, 7). Cells of the innate immune system, such as neutrophils and macrophages, are activated during this response and produce inflammatory cytokines and ROS that result in further damage to the kidney (4). Better understanding of the cellular pathophysiological mechanisms underlying kidney injury will result in the design of targeted therapies to prevent and treat the injury.

Cold-inducible RNA-binding protein (CIRP), a 172-amino acid protein, is a member of the family of cold shock protein that responds to cold stress (8). CIRP is expressed at low levels in various tissues and serves as an RNA chaperone during protein translation (9). We have recently demonstrated that extracellular CIRP functions as a novel DAMP that triggers pro-inflammatory responses in hemorrhagic shock and sepsis via the Toll-like receptor 4 (TLR4)-mediated pathway (10). Injection of recombinant murine CIRP (rmCIRP) into healthy mice produces a sterile systemic inflammatory response evidenced by increased organ injury and inflammatory markers (10). In a RIR model, CIRP knockout (*Cirbp*^{-/-}) mice have shown to have less kidney inflammation and reduced kidney injury markers compared to wild-type (WT) mice (11). Therefore, targeting CIRP could be a novel therapeutic approach to the treatment of RIR-induced kidney injuries.

By using surface plasmon resonance (SPR), we screened a large number of human CIRP derived 15-mer peptides, and identified a peptide GRGFSRGGGDRGYGG to exert highest affinity for TLR4-*myeloid differentiation factor 2* (MD2) complex and named it C23 peptide (10). Additionally, using computational modeling we confirmed the predicted binding of

C23 to the MD2 pocket further supporting C23's capability of interfering with CIRP's interaction with the TLR4/MD2 complex.

In the current study we hypothesized that C23 could serve as a CIRP antagonist to attenuate the renal injury in a murine model of RIR. We found the C23 treatment to be significantly effective in mitigating systemic and local inflammation, kidney biomarkers of injury, cellular apoptosis and tissue damage, and finally yielding an overall survival benefit. Thus, identification of C23's promising therapeutic potential in RIR will help advance the preclinical studies to the next level.

MATERIALS AND METHODS

In vitro Treatment of C23

A human peripheral blood monocyte THP-1 cell line was purchased from *American Type Culture Collection* (ATCC) and cultured in RPMI medium with 2- β -Mercaptoethanol to a final concentration of 0.05 mM and with 10% FBS. THP-1 monocytes (0.8×10^6 cells/ml) were seeded in a 48-well plate and differentiated into macrophages using *phorbol* 12-myristate 13-acetate (PMA) at 20 ng/ml over 48 h at 37°C. Differentiated THP-1 cells were then either left untreated or pre-treated with C23 peptide at various doses (0, 25, 50, 100, 250, 450 ng/ml) for 1 h, followed by the stimulation with rmCIRP at 300 ng/ml for 4 h. After rmCIRP treatment, the supernatant was collected for measurement of TNF- α using enzyme-linked immunosorbent assay (ELISA).

Animal Model of Renal Ischemia and Reperfusion

Adult male C57BL/6 mice (20–25 g), purchased from Taconic (Albany, New York), were housed in a temperature-controlled room on a 12 h light/dark cycle and fed a standard laboratory diet. Animals were randomly assigned to the sham, vehicle control and treatment groups. The renal IR operation was performed on mice during between 8 A.M. and 2 P.M. RIR was induced in mice as described previously (11). Briefly, the animals were anesthetized with 3.5% inhalational isoflurane while on a heating pad set to 38°C, then their abdomens prepped with 70% isopropyl alcohol. A midline incision was performed, and the bowel was displaced to reveal the bilateral renal hila. Microvascular clips were applied to each renal pedicle for 35 min. After removal of the clips, 8 mg/kg BW of C23 peptide (GRGFSRGGGDRGYGG synthesized from GenScript, Piscataway, NJ) in 100 μ L was injected intraperitoneally to serve as the treatment group while normal saline was injected *i.p.* for the vehicle group. This dose of C23 proved to be efficacious in a small unpublished pilot study on hemorrhagic shock within our lab. The abdomen was closed with a running 4–0 silk suture, and a 1 mL bolus of normal saline was given subcutaneously as resuscitation fluid. Reperfusion was allowed for 24 h, followed by the collection of blood and renal tissue samples. Sham animals did not undergo laparotomy. All experiments were performed in accordance with the guidelines for the use of experimental animals by the National Institutes of Health and were approved by the Institutional Animal Care and Use Committee of the Feinstein Institute for Medical Research.

Assessment of Cytokines

Blood samples were centrifuged at 6,000 g for 10 min to collect plasma and then either analyzed the cytokines immediately or stored at -80°C . Serum TNF- α , IL-6, and IL-1 β were determined with an ELISA kit, specific to mouse TNF- α , IL-6, and IL-1 β (BD Biosciences, San Diego, CA).

Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from kidney tissues using a Trizol reagent (Invitrogen, Carlsbad, CA, USA) and was reverse-transcribed into cDNA using reverse transcriptase enzyme (Applied Biosystems, Foster City, CA). A polymerase chain reaction (PCR) assay was carried out in 20 μl of a final volume containing 0.08 μmol of each forward and reverse primer, cDNA, and 10 μl SYBR Green PCR Master Mix (Applied Biosystems). Amplification was conducted in an Applied Biosystems Step One Plus real-time PCR machine under the thermal profile of 50°C for 2 min, 95°C for 10 min followed by 45 cycles of 95°C for 15 seconds and 60°C for 1 min. The level of mouse β -actin mRNA was used for normalization. Relative expression of mRNA was expressed as the fold change in comparison with the sham tissues. The primers used for quantitative real-time PCR are: The primer sequences are: TNF- α (NM_013693.2) Forward: AGACCCTCACACTC AGATCATCTTC, Reverse: TTGCTACGA CGTGGGCTACA; KC (NM_008176) Forward: GCTGGGATTAC CTCAAGAA, Reverse: ACAGGTGCCATCAGAGCAGT; NGAL (NM_005564.4) Forward: CTCAGAACTTGATCCCTGCC, Reverse: TCCTTGAGGCCAGAGACTT; KIM-1 (NM_134248.2) Forward: TGCTGCTACTGCTCCTTGTG, Reverse: GGGCCACTGGTAC TCATTCT; β -actin (NM_007393) Forward: CGTGAAAAGATGACCCAGATCA, Reverse: TGGTACGACCAGAGGCATACAG.

Kidney Histology

Renal tissue was fixed in 10% formalin and embedded in paraffin. Tissue was sectioned into 5- μm slices and stained with hematoxylin and eosin (H&E). Using light microscopy at 200 $\times$ magnification, the level of injury of the outer medulla was assessed in a blinded fashion and grouped into the following qualitative categories: tubular cell necrosis, tubular distension, and cast formation. Scores for each quality ranged from 0 (0% injury), 1 (<25%), 2 (25–50%), 3(50%–75%), 4 (>75%), for a maximal total score of 12 when combining them. Scores were averaged for each sample over 5 randomly selected fields (12).

TUNEL Assay

The presence of apoptotic cells was measured by *in situ* labeling of DNA fragmentation using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay kit (Roche Diagnostics, Indianapolis, IN). Slides were counterstained with 49,6-diamidino-29-phenylindole dihydrochloride (DAPI) and visualized under a fluorescent microscope (company source). The Nikon Elements computer software (source) objectively quantified the number of TUNEL-positive cells of randomly selected 4 sections for each sample.

Survival Study

Mice were subjected to our aforementioned RIR procedure, however, we used 30 min of ischemia time for survival as our original model was lethal at day one. Immediately after the RIR operation, we administered C23 i.p. (8 mg/kg) at the time of reperfusion while the vehicle received normal saline. The animals were then followed daily for 7 days.

Statistical Analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM) and compared by one-way analysis of variance (ANOVA) and Student-Newman-Keuls (SNK) test for multiple group comparisons. The Kaplan-Meier method was used for analyzing the survival data and comparisons between groups were done using the log-rank test. Significance was considered if $P < 0.05$ between the experimental groups. Our group sizes were comparable to our previous work with renal ischemia reperfusion (11, 13) and ultimately were justified by statistical significance.

RESULTS

C23 inhibits TNF- α production in macrophages stimulated with CIRP

We first examined the anti-inflammatory effect of C23 in an *in vitro* model using THP-1 cells. As shown in Fig. 1, we found a significant increase in TNF- α production compared to its basal level with the exposure of THP-1 cells to rmCIRP. However, pre-treatment of THP-1 cells to various doses of C23 significantly decreased rmCIRP-induced TNF- α production as compared to no C23-pretreated sample (Fig. 1). We found a dose-dependent reduction in TNF- α production with increasing concentrations of C23. Exposure with C23 at a low dose (25 ng/ml) 1 h before rmCIRP yields a 26% reduction in TNF- α production, while the percentage reduction was more dramatic (69%) with a high dose of C23 (450 ng/ml) pre-treatment compared to rmCIRP alone (Fig. 1).

Systemic inflammation is attenuated in C23-treated mice following RIR

The next step in translating this promising small peptide into a potential treatment was to use C23 in an animal model for RIR, followed by the assessment of systemic inflammatory cytokines such as TNF- α , IL-6 and IL-1 β . In the plasma the average level of TNF- α was markedly elevated in the vehicle-treated mice with RIR compared to sham mice. By contrast, this level was significantly reduced by 74% in the C23-treated animals compared to vehicle (Fig. 2A). The IL-6 level also was significantly elevated in the vehicle-treated group, while the C23 treatment arm had a significant decrease of 85% as compared to vehicle (Fig. 2B). Similarly, the IL-1 β was significantly reduced by 68% in the C23-treated animals compared to vehicle (Fig. 2C).

Kidney inflammation is reduced in C23-treated RIR mice

We next assessed for evidence of decreased inflammation in local inflamed tissue from the C23-treated RIR animals. The averaged gene expression of TNF- α in the vehicle group was 4.8-fold higher than the sham group, while the treatment with C23 reduced the TNF- α gene expression by 54% compared to vehicle-treated RIR animals (Fig 3A). Furthermore, the

expression of KC was reduced by 60% in the C23 treatment group relative to the vehicle-treated RIR mice (Fig. 3B).

Treatment with C23 reduced structural kidney damage following RIR

We prepared kidney tissue sections from each experimental group, stained with hematoxylin and eosin and examined them under light microscopy using a semi-quantitative scoring system in a randomized blinded fashion. As shown in Fig. 4A and B, the sections from sham mice revealed normal renal architecture and received an averaged injury score of 0.3. The averaged injury score for the vehicle-treated animals was 9.3 while the score for C23-treated mice with RIR was 5.3, representing a significant reduction by 43.5% in kidney injury as evidenced by the representative images shown in Fig. 4A.

Expression of AKI-specific injury biomarkers are reduced in C23-treated RIR mice

We next examined the renal tissue for markers specific for acute kidney injury using RT-PCR for NGAL and KIM-1. The averaged NGAL gene expression for the vehicle-treated group was 260-fold higher than the sham-operated mice while the expression of NGAL was significantly reduced by 55.4% in C23-treated mice compared to vehicle-treated mice with RIR (Fig. 5A). Similarly, the KIM-1 gene expression was 311-fold higher in the vehicle-treated animals than in the sham mice, while this was found to be significantly reduced by 46.3% in the C23-treated mice as compared to vehicle-treated group with RIR (Fig. 5B).

Treatment with C23 reduces cellular apoptosis in the kidney following RIR

To determine whether C23 treatment had an effect on apoptosis, we performed TUNEL assay on the renal tissues of our experimental groups. At 24 h after RIR, vehicle-treated kidney tissue sections showed prominent areas of TUNEL staining as compared to that of sham animals. However, as evidenced in Fig. 6A and B, there were fewer TUNEL-positive cells on average amongst the kidneys isolated from C23-treated mice following RIR. Using a computer-based software for counting the TUNEL-positive cells, we found the sham sections to have a few TUNEL-positive cells per section, while the kidney sections from vehicle-treated mice showed an average 78 TUNEL-positive cells per section. Strikingly, the C23 treatment group on the other hand had an average of 42 TUNEL-positive cells per section representing a 46 % decrease in TUNEL-positivity compared to the vehicle-treated animals after RIR (Fig. 6A and B).

C23 treatment improves survival in mice after RIR

In order to demonstrate if there was a long-term benefit of C23 treatment in RIR, we treated mice to our RIR model followed by either *i.p.* injection of normal saline or C23. At the end of 8 days following RIR, we noticed the overall survival rate of the vehicle-treated mice was 37%. On the other hand, the animals treated with C23 showed a significant improvement in their survival rate to reach 70% (Fig. 7).

DISCUSSION

DAMPs are endogenous nuclear or cytoplasmic biomolecules that elicit a sterile inflammation when released outside the cell following tissue injury (6). CIRP is a highly

conserved ubiquitous protein to facilitate RNA stabilization (8). However, when released extracellularly, CIRP acts as a DAMP during hemorrhagic shock and in a number of ischemia-reperfusion injury models (10, 11, 14, 15). In the current study, we are not able to definitely state what cells release extracellular CIRP after renal IR as we measured the levels and expression in whole kidney tissue lysate, which might contain bundle of cell-types such as tubular epithelial cells, endothelial cells and immune-competent cells. Here, although the mechanism of CIRP release into the extracellular milieu is not precisely known, one possible explanation of its release could be from damaged cells in the kidneys which spill over its intracellular contents including CIRP during renal I/R. The direct pro-inflammatory role of CIRP has been demonstrated in both *in vitro* and *in vivo* models, where it induces the production of TNF- α to further exaggerate inflammation (10). In RIR, the expression of CIRP is increased in both the kidneys and systemic circulation (11). Furthermore, *Cirbp*^{-/-} mice in a RIR model have revealed less injury than in wild-type mice as evidenced by a reduction in inflammatory parameters and kidney injury (11). In order to exploit the potential therapeutic value in blocking CIRP-induced injury after RIR, here we used a short peptide derived from the parent human CIRP protein and tested the efficacy of this molecule in RIR in terms of reducing pro-inflammatory milieu and renal injury.

The gateway of the innate immune response by which renal inflammation occurs in RIR is the activation of TLRs (16). During IR injury, renal tubular epithelial cells increase expression of TLR4, a major receptor responsible for the inflammatory injury of RIR (17, 18). TLR4 not only recognizes exogenous products of microorganisms but also recognizes DAMPs. Activation of TLR4 by DAMPs induces an inflammatory response characterized by the release of chemokines and cytokines that attract innate immune cells (4, 19). We previously have shown that CIRP functions as a DAMP by binding to MD2-TLR4 complex (10, 20). In quest of synthesizing a CIRP antagonist, we have previously determined the region of CIRP that binds to MD2 by synthesizing 32 oligopeptides covering the entire human CIRP sequence and used SPR to find the peptide with the highest binding affinity for MD2. We set up an *in vitro* model to assess TNF- α as a read-out for determining the efficacy of these CIRP-derived oligopeptides. Among them we chose a novel short peptide which exhibited highest binding affinity to TLR4/MD2 complex and inhibition of CIRP-induced TNF- α production by macrophages and named it C23. The dose-dependent response in TNF- α production further supports our previously hypothesized mechanism of CIRP signal transduction via MD2-TLR4 and demonstrates its potential as a therapeutic antagonist in RIR as well as other inflammatory conditions. While performing our *in vitro* study to elucidate the novel anti-inflammatory role of C23, aside from stimulating the cultured macrophages with recombinant murine CIRP, in a separate pilot *in vitro* experiment we also exposed the macrophages with lipopolysaccharides (LPS) or high mobility group box 1 (HMGB1) protein and found that C23 had no ability to reduce TNF- α production in those LPS- or HMGB1-treated macrophages (data not shown). This observation clearly indicates that C23 is solely a CIRP antagonist to attenuate CIRP-induced inflammation. After assessing the *in vitro* anti-inflammatory role of C23, we next focused on the pre-clinical study in which C23 was administrated into the RIR animals followed by the assessments of different disease parameters.

The production of inflammatory cytokines is a critical step in the initiation and propagation of maladaptive, harmful inflammation that occurs after kidney ischemia. The activation of TLR4 leads to the production of cytokines such as IL-6, IL-1 β , and TNF- α (10, 19). These cytokines have been shown to play critical roles in promoting inflammatory damage by recognizing their putative receptors that augment downstream signal transduction pathways to produce secondary inflammatory mediators (17, 21, 22). The resulting post-ischemia inflammatory milieu causes the local activation and leakage of endothelial cells causing local edema, enhanced local vasoconstriction, leukocyte activation and adherence, and coagulation activation, all of which lead to the local compromise of microcirculation and regional ischemia most commonly of the part of the kidney most susceptible to ischemia, the outer medulla, thus compounding the damage caused by the initial ischemic event. In mice treated with C23, the levels of circulating IL-6, IL-1 β , and TNF- α were significantly reduced as compared to the vehicles. Of note, the reduction of serum IL-1 β highlights the involvement of inflammasome pathway that C23 could be acting upon via impairing TLR4-mediated signaling. Recently, rmCIRP demonstrated induction of IL-1 β release via nuclear factor (NF)- κ B and caspase-1-mediated inflammasome pathway in murine lung endothelial cells (23). Consistent with the serum results, the gene expression of TNF- α and KC in kidney tissue of mice treated with C23 were also significantly reduced as compared with vehicle. Therefore, antagonizing CIRP effects with C23 limits pro-inflammatory mediators in the circulation as well as local tissue.

To assess for renal injury we used NGAL and KIM-1. NGAL is an iron-transporting protein produced by neutrophils and the epithelial cells of colon, trachea, lung, and kidney in humans and rodents (24). NGAL has recently earned interest as an injury marker more sensitive and specific for kidney injury than conventional AKI markers (24). KIM-1 is a phosphatidylserine receptor expressed in kidney epithelial cells which recognizes apoptotic cells and is unique for its lack of expression on myeloid cells (25). Expression of KIM-1 is absent in normal kidneys and appears to only be induced by stress to kidney epithelial cells (4). Similar to NGAL, KIM-1 has consistently demonstrated in preclinical and clinical studies to be a timely, sensitive, and specific marker for kidney injury (24, 25). The mice treated with C23 had significantly lower gene expression of both KIM-1 and NGAL when compared to the vehicle group, a strong indication of reduced damage in the treatment group. There is no evidence that the expression of either NGAL or KIM-1 is directly induced by a TLR4 dependent pathway, therefore, the reduction in these markers seen in C23-treated mice is likely the result of reduction in RIR-induced maladaptive inflammation harmful to kidney parenchyma.

Our histological findings revealed less architectural tissue damage in the C23-treated group than vehicle as evidenced by less tubal dilation, cast formation, less tubular cell necrosis. The histopathological evidence, used as the gold standard for AKI in humans and animals, echoed the aforementioned reduction in damage evidenced by the relative fall the AKI-specific injury biomarkers in the C23-treated mice. Similarly, apoptosis is a major pathological hallmark found in RIR. Once renal tubular epithelial cells die they detach from their basement membranes and physically occlude the nephron lumen causing further compromise of kidney function (3). Previous studies have shown that inhibition of apoptosis protects mice in RIR models. Daemen *et al.* demonstrated that the administration of the

antiapoptotic agents prevented the early onset of not only renal apoptosis, but also inflammation and tissue injury (26). A more recent study used apoptosis inhibitor in a murine RIR model with resultant increased clearance of tubular debris, less inflammation, and lower mortality (27). Likewise, mice treated with C23 in our study have significantly less TUNEL-positive cells than vehicle as well as a survival benefit. Additionally, we have previously discovered that CIRP induces pyroptosis via induction of the inflammasome pathway in lung endothelial cells (23). As stated earlier, IL-1 β , which was previously shown to be released along with induction of capase-1, inflammasome, and pyroptosis, was significantly decreased in the C23 treatment group compared to the vehicle-treated RIR animals in our study (23).

We acknowledge that our study has limitations in revealing the complete nature of C23's effects on CIRP-induced inflammation. We have previously demonstrated that CIRP is released from macrophages under hypoxic stress and have found increased levels of CIRP in kidney tissue after renal I/R. Because we measured the level and expression of CIRP in whole kidney tissue lysate, which contains varying cell-types such as tubular epithelial cells, endothelial cells and immune-competent cells, we cannot definitively state where exactly extracellular CIRP is released within the renal parenchyma after renal I/R. We therefore do not know exactly what cells are affected most by C23.

Another possible limitation is that our sham animals did not undergo laparotomy, however, based on our prior experience we noticed that sham mice with and without laparotomy have no statistically significant difference in the markers used in this study. Our shams also did not receive C23 but we will be studying this with our future studies using C23. However, we have our *in vitro* data showing no significant difference in the levels of TNF- α production by the macrophages while they were treated with C23 alone as compared to PBS-treated condition (Fig 1), indicating that C23 by itself did not elicit immune response.

In summary, we demonstrated C23's effectiveness with significantly increased survival in the C23-treated mice. This survival advantage was consistent with the associated decreases in inflammation, AKI-specific injury biomarker levels, tissue disruption, and apoptosis seen in the C23 treatment group as compared to the vehicle group. We therefore propose developing this novel therapy with further preclinical work defining C23's therapeutic window, optimal administration route and timing in hopes of eventually translating this approach into a renal I/R treatment for humans.

Acknowledgments

We thank Dr. Xiaoling Qiang for her technical assistance. This study was supported by the National Institutes of Health (NIH) grant R01HL076179 to PW.

ABBREVIATIONS

RIR	renal ischemia-reperfusion
CIRP	cold-inducible RNA-binding protein
DAMP	damage-associated molecular pattern

TLR4	Toll-like receptor 4
TNF	tumor necrosis factor
IL	interleukin
KC	keratinocyte chemoattractant
KIM-1	kidney injury molecule-1
NGAL	neutrophil gelatinase-associated lipocalin
AKI	acute kidney injury
MD2	myeloid differentiation factor 2
ELISA	enzyme-linked immunosorbent assay
H&E	hematoxylin and eosin
HMGB1	high mobility group box 1

References

1. Coca SG, Yusuf B, Shlipak MG, Garg AX, Parikh CR. Long-term risk of mortality and other adverse outcomes after acute kidney injury: a systematic review and meta-analysis. *Am J Kidney Dis.* 2009; 53(6):961–973. [PubMed: 19346042]
2. Kanbay M, Kasapoglu B, Perazella MA. Acute tubular necrosis and pre-renal acute kidney injury: utility of urine microscopy in their evaluation- a systematic review. *Int Urol Nephrol.* 2010; 42(2): 425–433. [PubMed: 19921458]
3. Liangos O, Wald R, O’Bell JW, Price L, Pereira BJ, Jaber BL. Epidemiology and outcomes of acute renal failure in hospitalized patients: a national survey. *Clin J Am Soc Nephrol.* 2006; 1(1):43–51. [PubMed: 17699189]
4. Bonventre JV, Yang L. Cellular pathophysiology of ischemic acute kidney injury. *J Clin Invest.* 2011; 121(11):4210–4221. [PubMed: 22045571]
5. Sharfuddin AA, Molitoris BA. Pathophysiology of ischemic acute kidney injury. *Nat Rev Nephrol.* 2011; 7(4):189–200. [PubMed: 21364518]
6. Bianchi ME. DAMPs, PAMPs and alarmins: all we need to know about danger. *J Leukoc Biol.* 2007; 81(1):1–5.
7. Ward PA. An endogenous factor mediates shock-induced injury. *Nat Med.* 2013; 19(11):1368–1369. [PubMed: 24202382]
8. Zhu X, Buhner C, Wellmann S. Cold-inducible proteins CIRP and RBM3, a unique couple with activities far beyond the cold. *Cell Mol Life Sci.* 2016; 73(20):3839–3859. [PubMed: 27147467]
9. Nishiyama H, Itoh K, Kaneko Y, Kishishita M, Yoshida O, Fujita J. A glycine-rich RNA-binding protein mediating cold-inducible suppression of mammalian cell growth. *J Cell Biol.* 1997; 137(4): 899–908. [PubMed: 9151692]
10. Qiang X, Yang WL, Wu R, Zhou M, Jacob A, Dong W, Kuncewitch M, Ji Y, Yang H, Wang H, et al. Cold-inducible RNA-binding protein (CIRP) triggers inflammatory responses in hemorrhagic shock and sepsis. *Nat Med.* 2013; 19(11):1489–1495. [PubMed: 24097189]
11. Cen C, Yang WL, Yen HT, Nicastro JM, Coppa GF, Wang P. Deficiency of cold-inducible ribonucleic acid-binding protein reduces renal injury after ischemia-reperfusion. *Surgery.* 2016; 160(2):473–483. [PubMed: 27267546]
12. Cen C, Aziz M, Yang WL, Nicastro JM, Coppa GF, Wang P. Osteopontin Blockade Attenuates Renal Injury After Ischemia Reperfusion by Inhibiting NK Cell Infiltration. *Shock.* 2017; 47(1): 52–60.

13. Matsuda A, Wu R, Jacob A, Komura H, Zhou M, Wang Z, Aziz MM, Wang P. Protective effect of milk fat globule-epidermal growth factor-factor VIII after renal ischemia-reperfusion injury in mice. *Crit Care Med.* 2011; 39(9):2039–2047. [PubMed: 21666453]
14. Godwin A, Yang WL, Sharma A, Khader A, Wang Z, Zhang F, Nicastro J, Coppa GF, Wang P. Blocking Cold-Inducible RNA-Binding Protein (CIRP) Protects Liver from Ischemia/Reperfusion Injury. *Shock.* 2015; 43(1):24–30. [PubMed: 25186836]
15. Zhou M, Yang WL, Ji Y, Qiang X, Wang P. Cold-inducible RNA-binding protein mediates neuroinflammation in cerebral ischemia. *Biochim Biophys Acta.* 2014; 1840(7):2253–2261. [PubMed: 24613680]
16. Leemans JC, Kors L, Anders HJ, Florquin S. Pattern recognition receptors and the inflammasome in kidney disease. *Nat Rev Nephrol.* 2014; 10(7):398–414. [PubMed: 24890433]
17. Jang HR, Ko GJ, Wasowska BA, Rabb H. The interaction between ischemia-reperfusion and immune responses in the kidney. *J Mol Med (Berl).* 2009; 87(9):859–864. [PubMed: 19562316]
18. Wolfs TG, Buurman WA, van Schadewijk A, de Vries B, Daemen MA, Hiemstra PS, van't Veer C. In vivo expression of Toll-like receptor 2 and 4 by renal epithelial cells: IFN-gamma and TNF-alpha mediated up-regulation during inflammation. *J Immunol.* 2002; 168(3):1286–1293. [PubMed: 11801667]
19. Brochu C, Cabrita MA, Melanson BD, Hamill JD, Lau R, Pratt MA, McKay Bruce C. NF-kappaB-dependent role for cold-inducible RNA binding protein in regulating interleukin 1beta. *PLoS One.* 2013; 8(2):e57426. [PubMed: 23437386]
20. Li Z, Fan EK, Liu J, Scott MJ, Li Y, Li S, Xie W, Billiar TR, Wilson MA, Jiang Y, et al. Cold-inducible RNA-binding protein through TLR4 signaling induces mitochondrial DNA fragmentation and regulates macrophage cell death after trauma. *Cell Death Dis.* 2017; 8(5):e2775. [PubMed: 28492546]
21. Kielar ML, John R, Bennett M, Richardson JA, Shelton JM, Chen L, Jeyarajah DR, Zhou XJ, Zhou H, Chiquett B, et al. Maladaptive role of IL-6 in ischemic acute renal failure. *J Am Soc Nephrol.* 2005; 16(11):3315–3325. [PubMed: 16192425]
22. Prieto-Moure B, Lloris-Carsi JM, Belda-Antoli M, Toledo-Pereyra LH, Cejalvo-Lapena D. Allopurinol Protective Effect of Renal Ischemia by Downregulating TNF-alpha, IL-1beta, and IL-6 Response. *J Invest Surg.* 2017; 30(3):143–151. [PubMed: 27690698]
23. Yang WL, Sharma A, Wang Z, Li Z, Fan J, Wang P. Cold-inducible RNA-binding protein causes endothelial dysfunction via activation of Nlrp3 inflammasome. *Sci Rep.* 2016; 6:26571. [PubMed: 27217302]
24. Haase M, Bellomo R, Haase-Fielitz A. Neutrophil gelatinase-associated lipocalin. *Current Opinion in Critical Care.* 2010; 16(6):526–532. [PubMed: 20871395]
25. Bonventre JV. Kidney injury molecule-1: a translational journey. *Trans Am Clin Climatol Assoc.* 2014; 125:293–299. [PubMed: 25125746]
26. Daemen MA, van't Veer C, Denecker G, Heemskerk VH, Wolfs TG, Clauss M, Vandenabeele P, Buurman WA. Inhibition of apoptosis induced by ischemia-reperfusion prevents inflammation. *J Clin Invest.* 1999; 104(5):541–549. [PubMed: 10487768]
27. Arai S, Kitada K, Yamazaki T, Takai R, Zhang X, Tsugawa Y, Sugisawa R, Matsumoto A, Mori M, Yoshihara Y, et al. Apoptosis inhibitor of macrophage protein enhances intraluminal debris clearance and ameliorates acute kidney injury in mice. *Nat Med.* 2016; 22(2):183–193. [PubMed: 26726878]

Figure 1. C23 inhibits TNF- α production in macrophages stimulated with CIRP

A total of 0.8×10^6 differentiated THP-1 cells were treated with either PBS or C23 at 25, 50, 100, 250, or 450 ng/ml doses. After 1 h, cells were then stimulated with rmCIRP at 300 ng/ml for 4 h, followed by the assessment of TNF- α levels in the culture supernatant by ELISA. We used triplicate cell subcultures for each experimental arm and repeated our process in 3 separate experiments. Data are expressed as mean $\pm$ SEM and compared by one-way analysis of variance and Student-Newman-Keuls method. * $P < 0.05$ versus no rmCIRP; # $P < 0.05$ versus rmCIRP without C23. rmCIRP, recombinant murine cold-inducible RNA-binding protein; TNF- α , tumor necrosis factor- α ; ELISA, enzyme-linked immunosorbent assay.

Figure 2. Treatment with C23 attenuates systemic inflammation in the mice after RIR

Serum was collected 24 h after RIR and used to measure (A) TNF- α , (B) IL-6, (C) IL-1 β using ELISA. Data are expressed as mean $\pm$ SEM (sham: $n = 4$ mice; RIR + vehicle: $n = 5$ mice; RIR + vehicle: $n = 5$ mice) and compared by one-way analysis of variance and Student-Newman-Keuls method. * $P < 0.05$ versus sham; # $P < 0.05$ versus vehicle. RIR, renal ischemia-reperfusion; SEM, standard error of the mean; TNF- α , tumor necrosis factor- α ; ELISA, enzyme-linked immunosorbent assay.

Figure 3. Treatment with C23 attenuates the expression of TNF- α and KC in the mice after RIR Kidney tissue was collected 24 h after RIR and analyzed for (A) TNF- α and (B) KC gene expression by quantitative RT-PCR. Data are expressed as mean $\pm$ SEM (sham: n = 4 mice; RIR + vehicle: n = 5 mice; RIR + vehicle: n = 5 mice) and compared by one-way analysis of variance and Student-Newman-Keuls method. * P < 0.05 versus sham; # P < 0.05 versus vehicle. RIR, renal ischemia-reperfusion; SEM, standard error of the mean; TNF- α , tumor necrosis factor- α ; KC, keratinocyte chemoattractant; RT-PCR, real time polymerase chain reaction.

Figure 4. Treatment with C23 improves renal histology injury in the mice after RIR

(A) Kidney tissue was collected 24 h after RIR and analyzed for histological assessment (H&E) of outer medulla for each experimental group at 200× magnification. (B) Averaged semi-quantitative score of experimental groups. Data are expressed as mean ± SEM (sham: n = 4 mice; RIR + vehicle: n = 5 mice; RIR + vehicle: n = 5 mice) and compared by one-way analysis of variance and Student-Newman-Keuls method. * $P < 0.05$ versus sham; # $P < 0.05$ versus vehicle. RIR, renal ischemia- reperfusion; H&E, hematoxylin and eosin; SEM, standard error of the mean.

Figure 5. C23 treatment attenuates the expression of KIM-1 and NGAL in the kidneys of the mice after RIR

After 24 h of RIR, kidney tissue from each experimental group was harvested to assess mRNA expression of (A) NGAL and (B) KIM-1 by quantitative RT-PCR. Data are expressed as mean $\pm$ SEM (sham: n = 4 mice; RIR + vehicle: n = 5 mice; RIR + vehicle: n = 5 mice) and compared by one-way analysis of variance and Student-Newman-Keuls method.

* $P < 0.05$ versus sham; # $P < 0.05$ versus vehicle. RIR, renal ischemia- reperfusion; SEM, standard error of the mean; NGAL, neutrophil gelatinase-associated lipocalin; KIM-1, kidney injury molecule 1.

Figure 6. C23 treatment reduces apoptotic cells in the kidneys of the mice after RIR

Kidney tissue was harvested 24 h after RIR and the tissue sections from all experimental groups were subjected to TUNEL assay. (A) The sections were visualized under fluorescent microscope at 100 $\times$ magnifications. (B) Averaged quantification of TUNEL-positive cells per section per group by computer counting software. Data are expressed as mean $\pm$ SEM (sham: $n = 4$ mice; RIR + vehicle: $n = 5$ mice; RIR + vehicle: $n = 5$ mice) and compared by one-way analysis of variance and Student-Newman-Keuls method. * $P < 0.05$ versus sham; # $P < 0.05$ versus vehicle. RIR, renal ischemia- reperfusion; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling; SEM, standard error of the mean.

Figure 7. C23 treatment improves overall survival rate of the mice after RIR

Mice were subjected to 30 min of ischemia followed by reperfusion with intraperitoneal injection of either vehicle (normal saline) or C23 (8 mg/kg BW). A Kaplan-Meier survival curve generated from RIR + vehicle (n = 19 mice) and RIR + C23 (n = 20 mice) after an 8-day monitoring period is shown. * $P < 0.05$ versus vehicle. RIR, Renal Ischemia Reperfusion.