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Cold Storage Followed by Transplantation Induces Immunoproteasome in Rat Kidney Allografts: Inhibition of Immunoproteasome Does Not Improve Function

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Corresponding Author:	Nirmala Parajuli University of Arkansas for Medical Sciences Little Rock, AR UNITED STATES
Corresponding Author E-Mail:	nparajuli@uams.edu
Other Authors:	Dinesh Bhattarai
	Seong-ok Lee
	Neelam Joshi
	Se-Ran Jun
	Sorena Lo
	Li Jiang
	Neriman Gokden
Order of Authors (with Contributor Roles):	Dinesh Bhattarai (Methodology; Writing – original draft; Writing – review & editing)
	Seong-ok Lee (Methodology; Writing – original draft; Writing – review & editing)
	Neelam Joshi (Methodology)
	Se-Ran Jun (Formal analysis; Writing – review & editing)
	Sorena Lo (Methodology; Writing – review & editing)
	Li Jiang (Methodology; Writing – review & editing)
	Neriman Gokden (Formal analysis; Visualization)
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Abstract:	Background It is a major clinical challenge to ensure the long-term function of transplanted kidneys. Specifically, the injury associated with cold storage of kidneys compromises the long-term function of the grafts after transplantation. Therefore, the molecular mechanisms underlying cold-storage–related kidney injury are attractive therapeutic targets to prevent injury and improve long-term graft function. Previously, we found that constitutive proteasome function was compromised in rat kidneys after cold storage followed by transplantation. Here, we evaluated the role of the immunoproteasome (iproteasome), a proteasome variant, during cold storage (CS) followed by transplantation. Methods

Established in vivo rat kidney transplant model with or without CS containing vehicle or iproteasome inhibitor (ONX 0914) was used in this study. The iproteasome function was performed using rat kidney homogenates and fluorescent-based peptide substrate specific to β5i subunit. Western blotting and quantitative RT-PCR were used to assess the subunit expression/level of the iproteasome (β5i) subunit.

Results

We demonstrated a decrease in the abundance of the β5i subunit of the iproteasome in kidneys during CS, but β5i levels increased in kidneys after CS and transplant. Despite the increase in β5i levels and its peptidase activity within kidneys, inhibiting β5i during CS did not improve graft function after transplantation.

Summary

These results suggest that the pharmacological inhibition of immunoproteasome function during CS does not improve graft function or outcome. In light of these findings, future studies targeting immunoproteasomes during both CS and transplantation may define the role of immunoproteasomes on short- and long-term kidney transplant outcomes.

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Key point #2: as follow-up to "**Key Points:** Please state the 2-3 key points of the article. The responses included here will be included with your final published paper. The key	Cold storage plus transplant increases iproeasome levels/assembly in renal allografts; IFN-g is a potential inducer of the iproteasome

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Cold Storage Followed by Transplantation Induces Immunoproteasome in Rat Kidney Allografts: Inhibition of Immunoproteasome Does Not Improve Function

Dinesh Bhattarai[1], Seong-ok Lee[1], Neelam Joshi[1], Se-Ran Jun[2], Sorena Lo[1], Li Jiang[1], and Neriman Gokden[3], Nirmala Parajuli[1,4,*]

[1]Department of Pharmacology and Toxicology, [2]Department of Biomedical Informatics, [3]Department of Pathology, and [4]Division of Nephrology, University of Arkansas for Medical Sciences, Little Rock, AR, USA.

*Correspondence: Nirmala Parajuli (nparajuli@uams.edu)

Keywords: kidney transplantation; cold storage; immunoproteasome, interferon-gamma

Key Points

- Cold storage increases the severity of graft dysfunction in a time dependent manner, and prolonged cold storage decreases animal survival
- Cold storage plus transplant increases iproeasome levels/assembly in renal allografts; IFN-g is a potential inducer of the iproteasome
- Inhibiting iproteasome ex vivo during renal cold storage did not confer graft protection after transplantation

Abstract

Background: It is a major clinical challenge to ensure the long-term function of transplanted kidneys. Specifically, the injury associated with cold storage of kidneys compromises the long-term function of the grafts after transplantation. Therefore, the molecular mechanisms underlying cold-storage–related kidney injury are attractive therapeutic targets to prevent injury and improve long-term graft function. Previously, we found that constitutive proteasome function was compromised in rat kidneys after cold storage followed by transplantation. Here, we evaluated the role of the immunoproteasome (*i*proteasome), a proteasome variant, during cold storage (CS) followed by transplantation.

Methods: Established in vivo rat kidney transplant model with or without CS containing vehicle or iproteasome inhibitor (ONX 0914) was used in this study. The *i*proteasome function was performed using rat kidney homogenates and fluorescent-based peptide substrate specific to β5i subunit. Western blotting and quantitative RT-PCR were used to assess the subunit expression/level of the *i*proteasome (β5i) subunit.

Results: We demonstrated a decrease in the abundance of the β5i subunit of the *i*proteasome in kidneys during CS, but β5i levels increased in kidneys after CS and transplant. Despite the increase in β5i levels and its peptidase activity within kidneys, inhibiting β5i during CS did not improve graft function after transplantation.

Summary: These results suggest that the pharmacological inhibition of immunoproteasome function during CS does not improve graft function or outcome. In light of these findings, future studies targeting immunoproteasomes during both CS and transplantation may define the role of immunoproteasomes on short- and long-term kidney transplant outcomes.

Introduction

It continues to be a challenge to optimize long-term graft function, especially when the transplanted kidneys come from deceased donors (up to 70%) [1-7]. These donor kidneys are flushed with and stored in cold storage (CS) solutions (static or machine perfusion) [8-10], until the recipient is identified. While it is well-known that CS is detrimental to graft function after transplantation[11-15], the mechanisms of CS-mediated renal injury are largely unknown.

Ischemia-reperfusion injury (IRI) is an unavoidable consequence of CS and transplantation, and the IRI is known to induce numerous pathophysiological pathways leading to cell stress and organ damage[16]. Following stress, the ubiquitin proteasome system (UPS) and mitochondria play a critical role in maintaining protein homeostasis and energy metabolism. The constitutive proteasome, which is part of the UPS, has 3 to 7 protease active sites (β-catalytic subunits - β1, β2, and β5) and it selectively degrades modified or damaged proteins to small peptides [17-19]. Similarly, the mitochondrial electron transport chain produces energy (ATP), and reactive oxygen species (ROS) are a byproduct. Previously, we showed that CS-induced mitochondrial oxidative stress and impaired mitochondrial function contributed to kidney injury during preservation in both rat and pig models [20-22]. Our follow-up study with a clinically relevant model of CS followed by transplantation (CS+Tx) demonstrated decreased peptidase activity of the β5 subunit of the constitutive proteasome, increased mitochondrial dysfunction, and severe renal damage/dysfunction after CS+Tx compared to transplantation without CS [23-25]. These results clearly indicate that extended CS negatively affects kidney function in our experimental model of CS+Tx.

The immunoproteasome (*i*proteasome) is a proteasome variant normally found in immune cell compartments [26, 27]. In response to inflammation, constitutive proteasome subunits (β1, β2, and β5) are exchanged for *i*proteasome subunits (β1i/LMP2, β2i/LMP10, and β5i/LMP7) in most non-immune cells [28, 29]. ROS activate the *i*proteasome, which, unlike the constitutive

proteasome, is resistant to oxidative stress and can function without ATP [26, 27, 30]. Recent studies indicate that inhibiting the *i*proteasome with ONX 0914—a specific, reversible inhibitor of the β5i subunit [31]—protects against cardiac and neuronal ischemia–reperfusion injury [32, 33]. It is not known whether CS mediates activation of *i*proteasome in kidney grafts, and if so, what role β5i plays in CS-mediated renal damage and dysfunction. Here, we hypothesized that CS+Tx increases β5i peptidase activity in the *i*proteasome in renal grafts; we predicted that the addition of ONX 0914 during CS would blunt β5i function, leading to improved graft function after transplantation. Here, we used rat models of kidney transplantation and evaluated *i*proteasome activity in the kidney grafts. Similarly, we evaluated inhibition of β5i activity during renal CS and evaluate the effects on allograft function after transplantation.

Materials and Methods

Animals

Lewis or Fischer rats were obtained from Charles River Laboratory. All animal experiments were performed according to the animal use protocol approved by the Institutional Animal Care and Use Committee, and in compliance with institutional/NIH guidelines.

Rat Surgery

Rat kidney transplant surgery was performed as described previously [23-25, 32-36]. Postoperatively, rats were given saline solution (5-10 ml, subcutaneously [SC]) to replace lost fluids and buprenorphine (2 mg/kg, SC) to alleviate pain.

Cold storage followed by transplant (CS+Tx) surgery: For the syngeneic model, Lewis rats were used as donors and recipients. For the allogeneic model, Fischer rats were used as donors, and Lewis rats were used as recipients. The right and left kidneys from the donor rat were flushed with and stored in University of Wisconsin (UW) solution, referred to here as CS solution, at 4°C

for 4 h or 18 h. For *i*proteasome inhibition studies, the donor kidney was flushed with and stored in CS solution containing ONX 0914 (β5i inhibitor, 100 nM) or DMSO (vehicle control). In the recipient rat, the native left kidney was removed, and the donor left kidney (from 4- or 18-h CS groups) was orthotopically implanted with end-to-end anastomosis (CS+Tx or CS+ONX 0914+Tx group, n=4). For 1-day surgery groups and 7-day survival surgery groups (both syngeneic), the native right kidney was removed at the time of the transplant so that renal function/rat survival depended entirely on the transplanted left kidney. For the 9-day surgery group (allogeneic), the native right kidney was removed on day 7 post-surgery, and the rats survived for an additional 2 days to assess the function of the transplanted (left) kidney.

Autotransplant (ATx) surgery: ATx surgery was included in these studies so that the effect of CS could be isolated from the effect of transplant surgery alone. ATx (n=4) was performed as described for the CS+Tx group above, except that the left kidney was removed, flushed with saline, and immediately transplanted back into the same rat without CS exposure; a right nephrectomy followed immediately.

Sham surgery: Rats underwent the same procedure for right nephrectomy but without renal transplantation (n=4). The right kidney was saved immediately as an untreated control kidney. The left kidney remained in the rat, which lived for the same number of days as CS+Tx or ATx animals.

Tissue Sample Collection

For all groups, kidneys and blood were collected under anesthesia 1 or 9 days post-surgery, and animals were euthanized. Kidneys were immediately processed with formalin for histology or flash-frozen and saved at -80°C. Isolated blood was collected in a tube with (plasma) or without heparin (serum), as appropriate. The blood was then centrifuged (5,000 *g*) at 4°C for 5 min, and the supernatant was saved at -80°C. For survival surgery, blood from the

same rats was collected 1, 3, and 7 days after surgery in a tube containing heparin and used for renal function assays.

In Vitro Cell Model

Normal rat kidney proximal tubular cell cultures (NRK-52E, ATCC) were maintained in warm growth medium (DMEM plus 5% FCS and 1% penicillin/streptomycin). NRK cells at 70% confluence were treated with growth medium containing IFN-γ (10 ng/ml, R&D Systems) or TNF-α (10 ng/ml, R&D Systems) at 37°C for 1 day [35, 36]. Control NRK cells were treated with vehicle only. After treatment, NRK cells were washed 2 times with cold PBS (4°C) and used to prepare RIPA lysates [23, 35, 36].

Renal Function and Serum Chemistry

Blood chemistry was determined in heparinized blood (arterial) using an iSTAT™ hand-held clinical chemistry analyzer and CHEM8+ cartridges as described by the manufacturer (Vetscan®, Abaxis, USA) [23-25, 32-36].

Histology and Immunohistochemistry

Paraffin sections from sham, CS+Tx, and CS+ONX +Tx kidneys (9 days post-surgery) were assessed for tubular injury using periodic acid-Schiff reaction as described previously [23]. Masson's Trichome staining was performed to assess renal fibrosis. Histological sections were evaluated in a blinded fashion. All parameters culminating in a tubular injury score were in the scale of 0-5 as described previously [23]. KIM-1 (Novus Bio, 1:800) and NGAL (Novus Bio, 1:500) immunohistochemistry on renal sections were performed as described previously[37].

Immunoproteasome Function

Peptidase activity of the β5i subunit of the immunoproteasome was measured in renal tissue homogenate by hydrolysis of the fluorogenic peptide substrate [23], specific for β5i/PSMB8 Acetyl-Ala-Asn-Trp-AMC (100 μM) (South Bay Bio) in the presence or absence of ONX 0914 (10 μM) (Cayman Chemical). Fluorescence was measured (excitation, 380 nm; emission, 460 nm) with a BioTek Synergy H1 plate reader (Agilent) [23].

SDS-PAGE/Native PAGE and Immunoblotting

For SDS-PAGE, renal extracts from whole-kidney homogenates were prepared with RIPA lysis buffer (Pierce) [38, 39]. For native PAGE, Renal extracts from whole-kidney homogenates were prepared with 0.9% digitonin lysis buffer as described in [40]. Renal extracts (20-30 μg) were resolved with a Bis-Tris (4%–12%) gel under denaturing (SDS-PAGE) or non-denaturing condition (native PAGE[41]) and then transferred to a PVDF membrane. After transfer, the membrane was incubated with 1X Red Alert Western Blot Stain (Millipore) for 10 min at room temperature. The membrane was quickly washed with ddH_2O to visualize protein bands, and images were taken with a FluorchemTM 8900. Western blot analysis was performed with antibodies for β5i (1:1000; LSBio). β-actin (1:1000; Sigma) or GAPDH (1:1000; Signalway) were used as loading control. Probed membranes were washed, incubated with horseradish peroxidase-conjugated secondary goat anti-mouse or goat anti-rabbit antibodies (1:30,000; Seracare KPL), and assayed for enhanced chemiluminescence (Thermo Fisher Scientific). Densitometry was performed with AlphaEase FC software (Alpha Innotech). For all SDS-PAGE western blot analyses, a densitometry ratio of target protein to the corresponding loading control was considered for statistical evaluation. For all native gel western blot analyses, a densitometry ratio of β5i to the corresponding whole-lane density of Red Alert™ stain was considered for statistical evaluation [35].

Quantitative Real-Time PCR

Total RNA was isolated from kidney tissue with an RNeasy Kit (Qiagen), and quantitative real-time PCR was carried out with Superscript III (Invitrogen) reverse-transcribed mRNA, the SYBR Green PCR Kit (Thermo Fisher Scientific), and the PCR primers (**Table 1**) [35]. The PCR reaction involved 45 cycles under the following conditions: 95°C for 10 s and 60°C for 20 s, and 75°C for 10 s. Amplification of the target gene was normalized to amplification of TATA box binding protein (*Tbp*) and to the levels of an appropriate control using the delta Ct ($2^{-\Delta\Delta Ct}$) method [35, 39].

Statistical Analysis

Data are presented as the mean ± standard error of the mean (SEM) (GraphPad Prism). Data (n=4 per group) were analyzed with a 1-way ANOVA with Tukey's post hoc test or Mann-Whitney U test, 2-way ANOVA (to compare treatment and time effects) and an unpaired Student's t-test was used when comparing differences between the means of 2 groups at a 95% confidence level. Differences with $p<0.05$ were considered statistically significant.

Results

CS+Tx upregulates the iproteasome

We previously reported that proteasome (constitutive) function was decreased in transplanted rat kidneys 1 day after surgery when the kidneys had been subjected to 18-h CS (CS+Tx group) [23]. To determine whether the function of *i*proteasome is dysregulated, we evaluated β5i subunit levels and function under the same conditions (**Fig. 1**). CS+Tx increased both the level of *i*proteasome β5i subunit (**Fig. 1A**) and its function (**Fig. 1B**) at 1 day post-surgery compared to the ATx and sham controls.

IFN-γ upregulates the iproteasome in rat kidney proximal tubular cells

We demonstrated that 18-h CS+Tx (syngeneic) increased IFN-γ in rat kidneys [35, 36]. Here, we observed increased TNF-α expression in rat kidney grafts (syngeneic) after 18-h CS+Tx (**Fig. 2**). To determine whether these cytokines (IFN-γ and TNF-α) have the potential to induce the *i*proteasome in rat kidneys, NRK cells were treated with recombinant IFN-γ or TNF-α. Immunoblot assays of renal lysates demonstrated that IFN-γ (**Fig. 3A**), but not TNF-α (**Fig. 3B**), increased β5i subunit levels in NRK cells.

CS+Tx increases renal dysfunction and mortality

Previously, we showed that renal CS (4-h or 18-h) exacerbated organ dysfunction after transplantation (1 day post-surgery)[24, 25]. To further assay survival, 4 groups (sham, ATx, syngeneic 4-h CS+Tx, and syngeneic 18-h CS+Tx; in which native kidneys were removed and rats lived with transplanted donor or sham kidney only) were assigned to a 7-day survival and renal function study (**Fig. 4**). As expected, serum creatinine (SCr) and blood urea nitrogen (BUN) values (1 day post-transplant) were significantly more elevated in the 18-h CS+Tx animals than in the 4-h CS+Tx animals, indicating a severe loss of graft function reciprocal to the duration of CS (**Fig. 4A** and **B**). While SCr and BUN values in the ATx and 4-h CS+Tx groups returned to sham levels at 3 days post-transplant, these values continued to increase in the 18-h CS+Tx group (**Fig. 4A** and **B**). The sham, ATx, and 4-h CS+Tx animals all survived for up to 7 days post-surgery (**Fig. 4C**). Most importantly from a clinical perspective, animals in the 18-h CS+Tx group (syngeneic) did not survive longer than 3 days after transplant (**Fig. 4C**).

iproteasome is induced persistently in kidney allografts 9 days post-surgery

Next, we attempted to evaluate if CS contributes to a persistent increase of *i*proteasome in renal allografts, and we evaluated β5i in kidneys in our allogeneic transplant model (Fischer donor and Lewis recipient) with various CS times (4 h or 18 h). β5i levels increased significantly in rat kidneys in both 4-h CS+Tx and 18-h CS+Tx groups 9 days post-surgery compared to the sham group (**Fig. 5**).

Renal iproteasome composition is dysregulated during CS and after transplantation

The *i*proteasome is a multimeric enzyme complex (i26S/i30S) and is composed of a catalytic core (i20S) with one or two regulatory complexes [42]. β5i is a key subunit of the catalytic core of the *i*proteasome[43-45]. To examine the abundance of *i*proteasome composition (i26S/30S and i20S) under various CS and transplant conditions, we performed western blotting of kidney extracts (allogenic model) under non-denaturing conditions. Western blot with anti-β5i antibody revealed a significant decrease in the abundance of the i26S/30S *i*proteasome subunits after 4-h or 18-h CS, but the i20S catalytic core decreased only after 18-h CS (**Fig. 6A**). Conversely, both 4-h CS+Tx and 18-h CS+Tx increased i26S/30S levels in kidney allografts at 9 days post-surgery, but i20S levels remained unchanged (**Fig. 6B**).

Inhibiting β5i subunit during CS does not protect renal injury after CS+Tx

Finally, we investigated whether the CS-mediated induction of the *i*proteasome negatively affects renal function. Specifically, we tested whether pharmacologically inhibiting β5i with ONX 0914 during CS could mitigate renal injury/dysfunction. In our survival studies, the mortality rate was high for rats in the syngeneic 18-h CS+Tx group at 7 days post-surgery (**Fig. 4**). Therefore, we opted to use the allogeneic rat transplant model with 4-h CS to test the therapeutic effect of ONX 0914. Treating kidneys ex vivo with ONX 0914 did not improve renal function or tubular

injury/fibrosis after CS+Tx (**Fig. 7**). In parallel, we tested several other blood chemistry parameters with the iSTAT CHEM 8+ cartridge. These parameters were not affected at 4-h CS+Tx, and ONX 0914 did not affect blood chemistry, except that it decreased plasma chloride levels (**Fig. 8**). Overall, these results suggest that pharmacological inhibition of β5i during renal CS does not offer protection against renal injury after transplantation.

Discussion

The donor organs are vulnerable to damage caused by cold ischemia. We reported previously that CS reduced mitochondrial respiration and increased ROS, a byproduct of respiration in donor kidneys [20-22], suggesting that the CS induced cold-ischemic stress. Such stress impairs several pathways, including the constitutive proteasome [26]. The constitutive proteasome is susceptible to stress, but its variant, the immunoproteasome (*i*proteasome), is resistant to stress, including ROS [26, 27, 30]. Here, we showed that CS mediated a massive induction of β5i, a catalytic subunit of the *i*proteasome, in kidney grafts (both syngeneic and allogenic), suggesting abnormal or aberrant activation of the *i*proteasome. Although the *i*proteasome composition (i20S and i26/30S) was upregulated in transplanted kidneys that were exposed to CS, it was intriguing that some *i*proteasome complexes (i20S and i26/30S) were downregulated during CS. Given that kidney CS increased mitochondrial dysfunction and ROS [20-22], we postulate that ROS upregulated the *i*proteasome upon transplantation. Future studies are needed to establish the link between mitochondrial dysfunction (ROS) and β5i induction following CS and CS+Tx.

The *i*proteasome is resistant to stress and plays a key role in processing the antigenic peptides and stimulating adaptive immune system[46]. We previously observed CS-mediated induction of several inflammatory pathways, including IFN-γ and complement, and infiltration of CD68-positive macrophages in kidney grafts after CS+Tx [34-36]. Here, we report a substantial

upregulation of the *i*proteasome in both syngeneic and allogeneic models in response to CS (4-18 h), and this induction was sustained in transplanted kidneys for up to 9 days post-surgery. We further showed that exogenous IFN-γ, but not TNF-α, induced the *i*proteasome in proximal tubular cells, suggesting that CS+Tx induced the *i*proteasome (β5i subunit) via IFN-γ. IFN-γ is a potent inducer of *i*proteasome [47]. However, future studies should dissect the role of IFN-γ signaling in upregulating *i*proteasome function during CS+Tx. An important function of the *i*proteasome is to process antigenic peptides within antigen-presenting cells (e.g., macrophages and dendritic cells) and to present these peptides to adaptive immune cells (e.g., T cells) through the major histocompatibility complex [48]. Although CS increased the number of macrophages within transplanted kidneys [34], a limitation of our study was that it lacked an assessment of *i*proteasome levels within the infiltrated macrophage compartment. Similarly, future studies could investigate a link between *i*proteasome and the adaptive immune system (e.g., T cells) because activation of the adaptive immune system is a key mechanism of transplant rejection [49].

Currently, kidneys from living donors produce better outcomes than kidneys from deceased donors [50, 51], so decreasing CS-associated injury would make progress toward improving outcomes of transplants with organs from deceased donors. It has been difficult to develop new therapies to support kidney transplants because few studies have investigated the CS-related mechanisms of organ damage. Accordingly, our goal was to evaluate CS-related immunopathogenic mechanisms of injury in kidney grafts after CS+Tx. We identified the upregulated *i*proteasome as a potential therapeutic target and subsequently tested ONX 0914—a specific, reversible inhibitor of the β5i subunit [31] of the *i*proteasome—in the context of CS to mitigate injury and improve kidney function after transplantation. However, this inhibitor, when used during CS alone, did not prevent acute tubular injury/graft function following transplantation. On the other hand, systemic administration of ONX 0914 post-transplant in rats

ameliorated chronic antibody-mediated rejection [52]. Future studies designed to target β5i both during CS and after transplantation are warranted to conclude the role of β5i on acute and chronic kidney injury following transplantation.

In summary, we showed that CS increases the severity of graft dysfunction in a time dependent manner (0-h<4-h<18-h), and further showed that prolonged CS (18-h) decreases overall animal survival. These data further indicate a need for therapeutic intervention during renal CS. Our data also demonstrated that CS alone decreases the abundance of *i*proteasome (β5i subunit); in contrast, reperfusion (transplantation) increases *i*proteasome levels/assembly in renal allografts, which correlates with allograft dysfunction after transplantation. Mechanistically, we showed that IFN-γ is a potential inducer of the *i*proteasome (β5i). Finally, inhibiting the *i*proteasome (β5i) during CS alone did not confer renal protection after transplantation. Future studies designed to inhibit the *i*proteasome during CS—as well as after transplantation—should be tested to define the role of the *i*proteasome during CS+Tx.

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References

1. Bottomley MJ, Harden PN: Update on the long-term complications of renal transplantation. *Br Med Bull,* 106**:** 117-134, 2013 10.1093/bmb/ldt012
2. Mohan S, Foley K, Chiles MC, Dube GK, Patzer RE, Pastan SO, et al.: The weekend effect alters the procurement and discard rates of deceased donor kidneys in the United States. *Kidney international,* 90**:** 157-163, 2016 10.1016/j.kint.2016.03.007
3. Stewart DE, Garcia VC, Rosendale JD, Klassen DK, Carrico BJ: Diagnosing the Decades-Long Rise in the Deceased Donor Kidney Discard Rate in the United States. *Transplantation,* 101**:** 575-587, 2017 10.1097/TP.0000000000001539
4. von der Lippe N, Waldum B, Brekke FB, Amro AA, Reisaeter AV, Os I: From dialysis to transplantation: a 5-year longitudinal study on self-reported quality of life. *BMC Nephrol,* 15**:** 191, 2014 10.1186/1471-2369-15-191
5. USRDS: End Stage Renal Disease: Chapter 7. Transplantation. 2023
6. USRDS: End Stage Renal Diseases; Chapter 1. Incidence, Prevalence, Patient Characteristics, and Treatment Modalities,
7. USRDS: End Stage Renal Disease: Chapter 6. Mortality. 2023
8. Wahlberg JA, Southard JH, Belzer FO: Development of a cold storage solution for pancreas preservation. *Cryobiology,* 23**:** 477-482, 1986 10.1016/0011-2240(86)90056-8
9. Chen Y, Shi J, Xia TC, Xu R, He X, Xia Y: Preservation Solutions for Kidney Transplantation: History, Advances and Mechanisms. *Cell transplantation,* 28**:** 1472-1489, 2019 10.1177/0963689719872699
10. Hoeger S, Lueg G, Tsagogiorgas C, Schneider M, Theisinger S, Theisinger B, et al.: UW is superior compared with HTK after prolonged preservation of renal grafts. *J Surg Res,* 170**:** e149-157, 2011 10.1016/j.jss.2011.05.020
11. UNOS data on kidney graft and patient survial: https://optn.transplant.hrsa.gov/data/view-data-reports/national-data/. 2019 Ed., 2019
12. Bos EM, Leuvenink HG, van GH, Ploeg RJ: Kidney grafts from brain dead donors: Inferior quality or opportunity for improvement? *Kidney international,* 72**:** 797-805, 2007 5002400 [pii];10.1038/sj.ki.5002400 [doi]
13. Debout A, Foucher Y, Trebern-Launay K, Legendre C, Kreis H, Mourad G, et al.: Each additional hour of cold ischemia time significantly increases the risk of graft failure and mortality following renal transplantation. *Kidney international*, 2014 ki2014304 [pii];10.1038/ki.2014.304 [doi]
14. Hoeger S, Petrov K, Reisenbuechler A, Fontana J, Selhorst J, Hanusch C, et al.: The additional detrimental effects of cold preservation on transplantation-associated injury in kidneys from living and brain-dead donor rats. *Transplantation,* 87**:** 52-58, 2009 10.1097/TP.0b013e318191b2ca
15. Salahudeen AK, Haider N, May W: Cold ischemia and the reduced long-term survival of cadaveric renal allografts. *Kidney international,* 65**:** 713-718, 2004 10.1111/j.1523-1755.2004.00416.x
16. Hariharan S, Israni AK, Danovitch G: Long-Term Survival after Kidney Transplantation. *The New England journal of medicine,* 385**:** 729-743, 2021 10.1056/NEJMra2014530
17. Ciechanover A, Schwartz AL: The ubiquitin-dependent proteolytic pathway: specificity of recognition of the proteolytic substrates. *Revis Biol Celular,* 20**:** 217-234, 1989
18. Schrader EK, Harstad KG, Matouschek A: Targeting proteins for degradation. *Nat ChemBiol,* 5**:** 815-822, 2009 nchembio.250 [pii];10.1038/nchembio.250 [doi]
19. Jung T, Grune T: Structure of the proteasome. *Progress in molecular biology and translational science,* 109**:** 1-39, 2012 10.1016/b978-0-12-397863-9.00001-8

20. Mitchell T, Rotaru D, Saba H, Smith RA, Murphy MP, MacMillan-Crow LA: The mitochondria-targeted antioxidant mitoquinone protects against cold storage injury of renal tubular cells and rat kidneys. *J Pharmacol Exp Ther,* 336**:** 682-692, 2011 10.1124/jpet.110.176743
21. Mitchell T, Saba H, Laakman J, Parajuli N, MacMillan-Crow LA: Role of mitochondrial-derived oxidants in renal tubular cell cold-storage injury. *Free Radic Biol Med,* 49**:** 1273-1282, 2010 10.1016/j.freeradbiomed.2010.07.012
22. Parajuli N, Campbell LH, Marine A, Brockbank KG, Macmillan-Crow LA: MitoQ blunts mitochondrial and renal damage during cold preservation of porcine kidneys. *PLoS One,* 7**:** e48590, 2012 10.1371/journal.pone.0048590
23. Lo S, MacMillan-Crow LA, Parajuli N: Renal cold storage followed by transplantation impairs proteasome function and mitochondrial protein homeostasis. *American journal of physiology Renal physiology,* 316**:** F42-F53, 2019 10.1152/ajprenal.00316.2018
24. Parajuli N, Shrum S, Tobacyk J, Harb A, Arthur JM, MacMillan-Crow LA: Renal cold storage followed by transplantation impairs expression of key mitochondrial fission and fusion proteins. *PLoS One,* 12**:** e0185542, 2017 10.1371/journal.pone.0185542
25. Shrum S, MacMillan-Crow LA, Parajuli N: Cold Storage Exacerbates Renal and Mitochondrial Dysfunction Following Transplantation. *J Kidney,* 2, 2016
26. Aiken CT, Kaake RM, Wang X, Huang L: Oxidative stress-mediated regulation of proteasome complexes. *Mol Cell Proteomics,* 10**:** R110.006924, 2011 10.1074/mcp.M110.006924
27. Kimura H, Caturegli P, Takahashi M, Suzuki K: New Insights into the Function of the Immunoproteasome in Immune and Nonimmune Cells. *J Immunol Res,* 2015**:** 541984, 2015 10.1155/2015/541984 [doi]
28. Basler M, Mundt S, Bitzer A, Schmidt C, Groettrup M: The immunoproteasome: a novel drug target for autoimmune diseases. *Clin Exp Rheumatol,* 33**:** S74-79, 2015
29. Kaur G, Batra S: Emerging role of immunoproteasomes in pathophysiology. *Immunol Cell Biol,* 94**:** 812-820, 2016 10.1038/icb.2016.50
30. Seifert U, Bialy LP, Ebstein F, Bech-Otschir D, Voigt A, Schroter F, et al.: Immunoproteasomes preserve protein homeostasis upon interferon-induced oxidative stress. *Cell,* 142**:** 613-624, 2010 10.1016/j.cell.2010.07.036
31. Muchamuel T, Basler M, Aujay MA, Suzuki E, Kalim KW, Lauer C, et al.: A selective inhibitor of the immunoproteasome subunit LMP7 blocks cytokine production and attenuates progression of experimental arthritis. *Nature medicine,* 15**:** 781-787, 2009 nm.1978 [pii];10.1038/nm.1978 [doi]
32. Chen X, Zhang X, Wang Y, Lei H, Su H, Zeng J, et al.: Inhibition of immunoproteasome reduces infarction volume and attenuates inflammatory reaction in a rat model of ischemic stroke. *Cell Death Dis,* 6**:** e1626, 2015 10.1038/cddis.2014.586
33. Sula Karreci E, Fan H, Uehara M, Mihali AB, Singh PK, Kurdi AT, et al.: Brief treatment with a highly selective immunoproteasome inhibitor promotes long-term cardiac allograft acceptance in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 113**:** E8425-E8432, 2016 10.1073/pnas.1618548114
34. Lo S, Byrum SD, Tackett AJ, Parajuli N: Cold Storage Increases Albumin and Advanced Glycation-End Product-Albumin Levels in Kidney Transplants. *Transplantation Direct,* 5**:** e454, 2019 10.1097/txd.0000000000000897
35. Lo S, Jiang L, Stacks S, Lin H, Parajuli N: Aberrant activation of the complement system in renal grafts is mediated by cold storage. *American journal of physiology Renal physiology,* 320**:** F1174-F1190, 2021 10.1152/ajprenal.00670.2020
36. McGraw M, Miller D, Lo S, Parajuli N: Cold Storage Followed by Transplantation Induces Interferon-Gamma and STAT-1 in Kidney Grafts. *International journal of molecular sciences,* 24, 2023 10.3390/ijms24065468

37. Parajuli N, MacMillan-Crow LA: Role of reduced manganese superoxide dismutase in ischemia-reperfusion injury: a possible trigger for autophagy and mitochondrial biogenesis? *American journal of physiology Renal physiology,* 304**:** F257-267, 2013 10.1152/ajprenal.00435.2012
38. Parajuli N, Doppler W: Precision-cut slice cultures of tumors from MMTV-neu mice for the study of the ex vivo response to cytokines and cytotoxic drugs. *In Vitro Cell Dev Biol Anim,* 45**:** 442-450, 2009 10.1007/s11626-009-9212-7
39. Parajuli N, Muller-Holzner E, Bock G, Werner ER, Villunger A, Doppler W: Infiltrating CD11b+CD11c+ cells have the potential to mediate inducible nitric oxide synthase-dependent cell death in mammary carcinomas of HER-2/neu transgenic mice. *Int J Cancer,* 126**:** 896-908, 2010 10.1002/ijc.24805
40. Lo S, MacMillan-Crow LA, Parajuli N: Renal cold storage followed by transplantation impairs proteasome function and mitochondrial protein homeostasis. *American Journal of Physiology-Renal Physiology,* 316**:** F42-F53, 2019
41. Damman J, Nijboer WN, Schuurs TA, Leuvenink HG, Morariu AM, Tullius SG, et al.: Local renal complement C3 induction by donor brain death is associated with reduced renal allograft function after transplantation. *Nephrology Dialysis Transplantation,* 26**:** 2345-2354, 2011
42. Ferrington DA, Gregerson DS: Immunoproteasomes: structure, function, and antigen presentation. *Progress in molecular biology and translational science,* 109**:** 75-112, 2012 10.1016/B978-0-12-397863-9.00003-1
43. Akiyama K, Kagawa S, Tamura T, Shimbara N, Takashina M, Kristensen P, et al.: Replacement of proteasome subunits X and Y by LMP7 and LMP2 induced by interferon-gamma for acquirement of the functional diversity responsible for antigen processing. *FEBS letters,* 343**:** 85-88, 1994 10.1016/0014-5793(94)80612-8
44. Griffin TA, Nandi D, Cruz M, Fehling HJ, Kaer LV, Monaco JJ, et al.: Immunoproteasome assembly: cooperative incorporation of interferon gamma (IFN-gamma)-inducible subunits. *The Journal of experimental medicine,* 187**:** 97-104, 1998 10.1084/jem.187.1.97
45. Monaco JJ, McDevitt HO: H-2-linked low-molecular weight polypeptide antigens assemble into an unusual macromolecular complex. *Nature,* 309**:** 797-799, 1984 10.1038/309797a0
46. Murata S, Takahama Y, Kasahara M, Tanaka K: The immunoproteasome and thymoproteasome: functions, evolution and human disease. *Nature immunology,* 19**:** 923-931, 2018 10.1038/s41590-018-0186-z
47. Aki M, Shimbara N, Takashina M, Akiyama K, Kagawa S, Tamura T, et al.: Interferon-gamma induces different subunit organizations and functional diversity of proteasomes. *Journal of biochemistry,* 115**:** 257-269, 1994 10.1093/oxfordjournals.jbchem.a124327
48. Neefjes J, Jongsma ML, Paul P, Bakke O: Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature reviews Immunology,* 11**:** 823-836, 2011 10.1038/nri3084
49. Li Q, Lan P: Activation of immune signals during organ transplantation. *Signal transduction and targeted therapy,* 8**:** 110, 2023 10.1038/s41392-023-01377-9
50. Poggio ED, Augustine JJ, Arrigain S, Brennan DC, Schold JD: Long-term kidney transplant graft survival-Making progress when most needed. *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons,* 21**:** 2824-2832, 2021 10.1111/ajt.16463
51. Nemati E, Einollahi B, Lesan Pezeshki M, Porfarziani V, Fattahi MR: Does kidney transplantation with deceased or living donor affect graft survival? *Nephro-urology monthly,* 6**:** e12182, 2014 10.5812/numonthly.12182
52. Li J, Basler M, Alvarez G, Brunner T, Kirk CJ, Groettrup M: Immunoproteasome inhibition prevents chronic antibody-mediated allograft rejection in renal transplantation. *Kidney international,* 93**:** 670-680, 2018 10.1016/j.kint.2017.09.023

Table 1. Primer sequences for qRT-PCR (RealTimePrimers.com)

Tnfa:	Forward, 5′-*CCCATTACTCTGACCCCTTT-3′*	Reverse, 5′-*TGAGCATCGTAGTTGTTGGA*-3′
***Il18*:**	Forward, 5'-CAGACCACTTTGGCAGACTT-3'	Reverse, 5'-ATCCTTCCATCCTTCACAG-3'
Tbp:	Forward, 5′-CGATAACCCAGAAAGTCGAA-3′	Reverse, 5′-AGATGGGAATTCCAGGAGTC-3′

Figure Legends

Figure 1. Cold storage increases *i*proteasome (β5i) level and function in rat kidneys after transplantation. Lewis rat kidneys were flushed with and stored in cold storage (CS) solution (4°C) for 18 h followed by transplantation to a recipient Lewis rat (n=4/group). Autotransplant (ATx, transplant with no CS) rats were used as transplant control and Sham (right nephrectomy, control kidney) rats were used as healthy controls. (**A**) Immunoblot of β5i (LMP7) proteins in rat kidney homogenates from untreated control, cold storage (CS), sham, autotransplantation (ATx), and cold storage plus transplant (CS+Tx) groups (n=4/group). β -actin served as loading control. Representative blots from 3 independent experiments shown. Graphs show densitometry (mean ± SEM) normalized to β-actin (n=4/group). (**B**) Immunoproteasome function (β5i peptidase activity) assayed in renal homogenates using a peptide substrate (Ac-ANW-AMC). Data are the mean ± SEM (bar graph, n=4/group). Differences between group means were compared with Student's t-test (2-group comparison) or ANOVA (3-group comparison). *p < 0.05 was considered statistically significant.

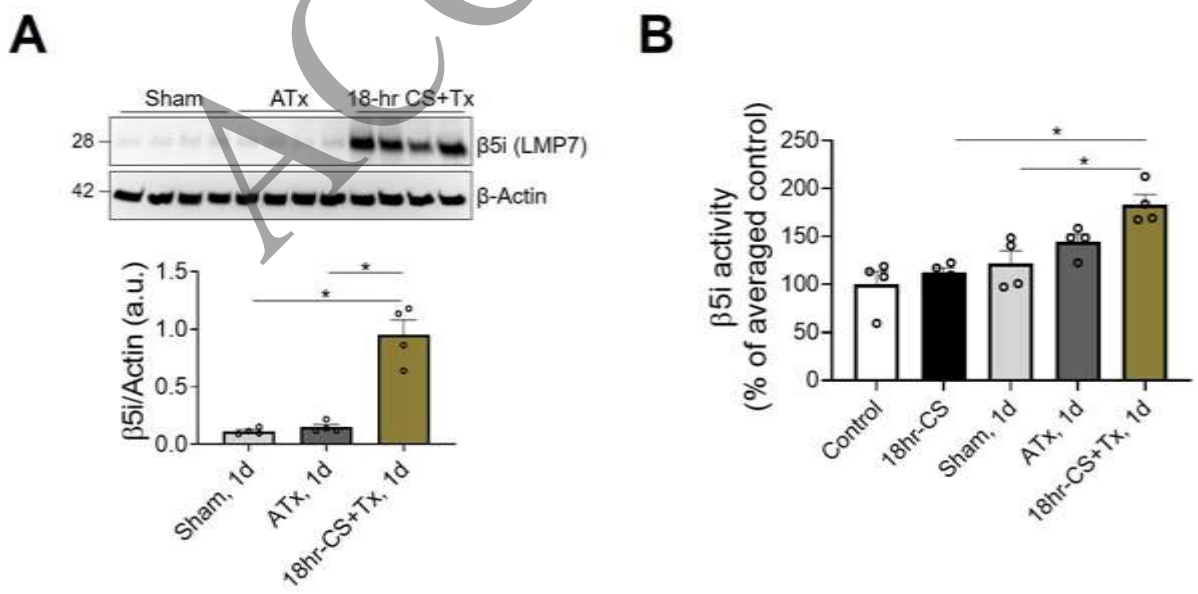

Figure 2. Cold storage injury increases mRNA abundance of tumor necrosis factor-alpha and interleukin-18 in rat kidneys after transplantation. Quantitative real-time PCR (SYBR Green) was performed with rat renal mRNA extracts to assay expression of TNF-α and IL-18 genes after kidney CS or CS+Tx (1 day post surgery). Sham kidneys were used as a control. Data are the mean ± SEM (bar graphs, n=3/group). Differences between group means were compared with ANOVA (3 groups comparison). *p < 0.05 was considered statistically significant.

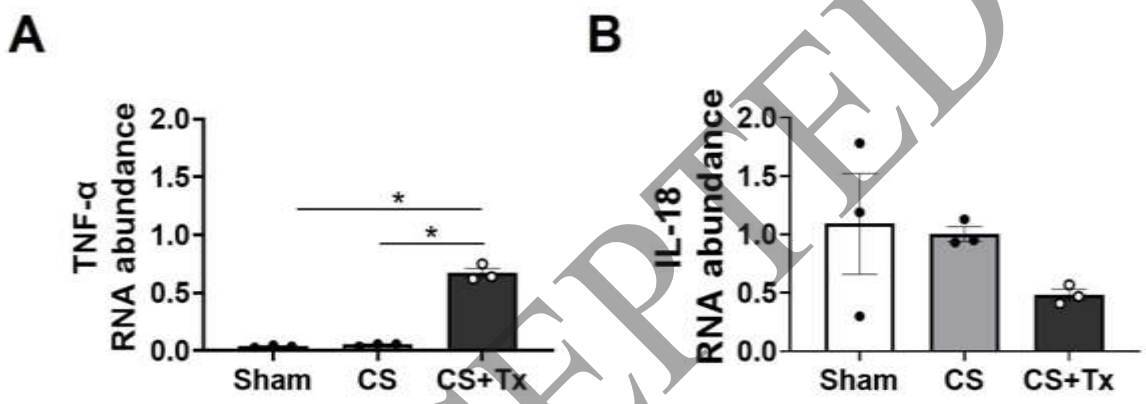

Figure 3. IFN-γ increases *i*proteasome (β5i) level in rat proximal tubular cells. Rat proximal tubular (NRK) cells were treated with IFN-γ (10 ng/ml) or TNF-α (10 ng/ml) and solubilized with RIPA lysis buffer. Representative immunoblots (n=3) showing (**A**) *i*proteasome (β5i protein) and (**B**) constitutive proteasome (β5 protein) levels in NRK cells after treatment with IFN-γ or TNF-α. β-actin served as loading control. Data are the mean ± SEM (dot plots, n=3). Differences between group means were compared with ANOVA (3-group comparison). *p < 0.05 was considered statistically significant.

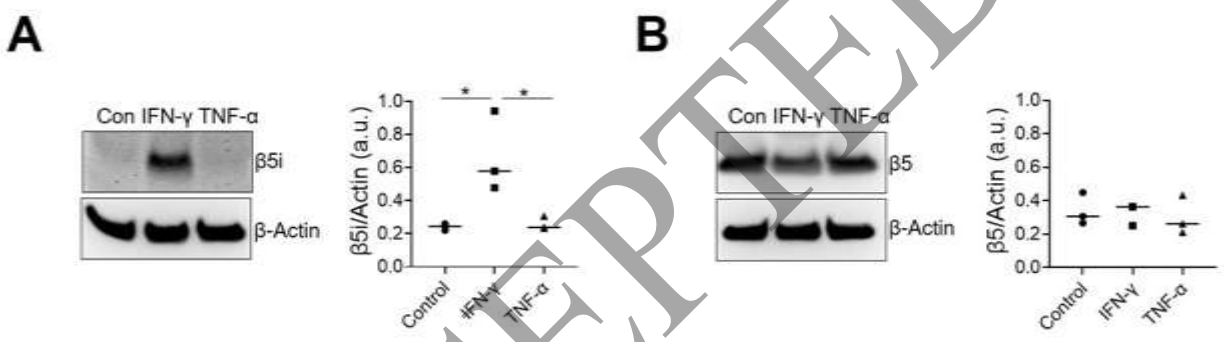

Figure 4. Prolonged cold storage of rat kidneys decreases organ function and rat survival after transplantation. Lewis rat kidneys were flushed with and stored in cold storage (CS) solution (4°C) for 4 h or 18 h followed by transplantation to a recipient Lewis rat (n=4/group). Autotransplant (ATx, transplant with no CS) and Sham rats were used as controls. Renal function was assayed in rat blood 1 and 3 days post-surgery by measuring (**A**) serum creatinine (SCr) and (**B**) blood urea nitrogen (BUN). Data are the mean ± SEM (dot plots, n=3/group). Differences between group means were compared with ANOVA (> 3-group comparison). *p < 0.05 was considered statistically significant. (**C**) Rat survival after surgery.

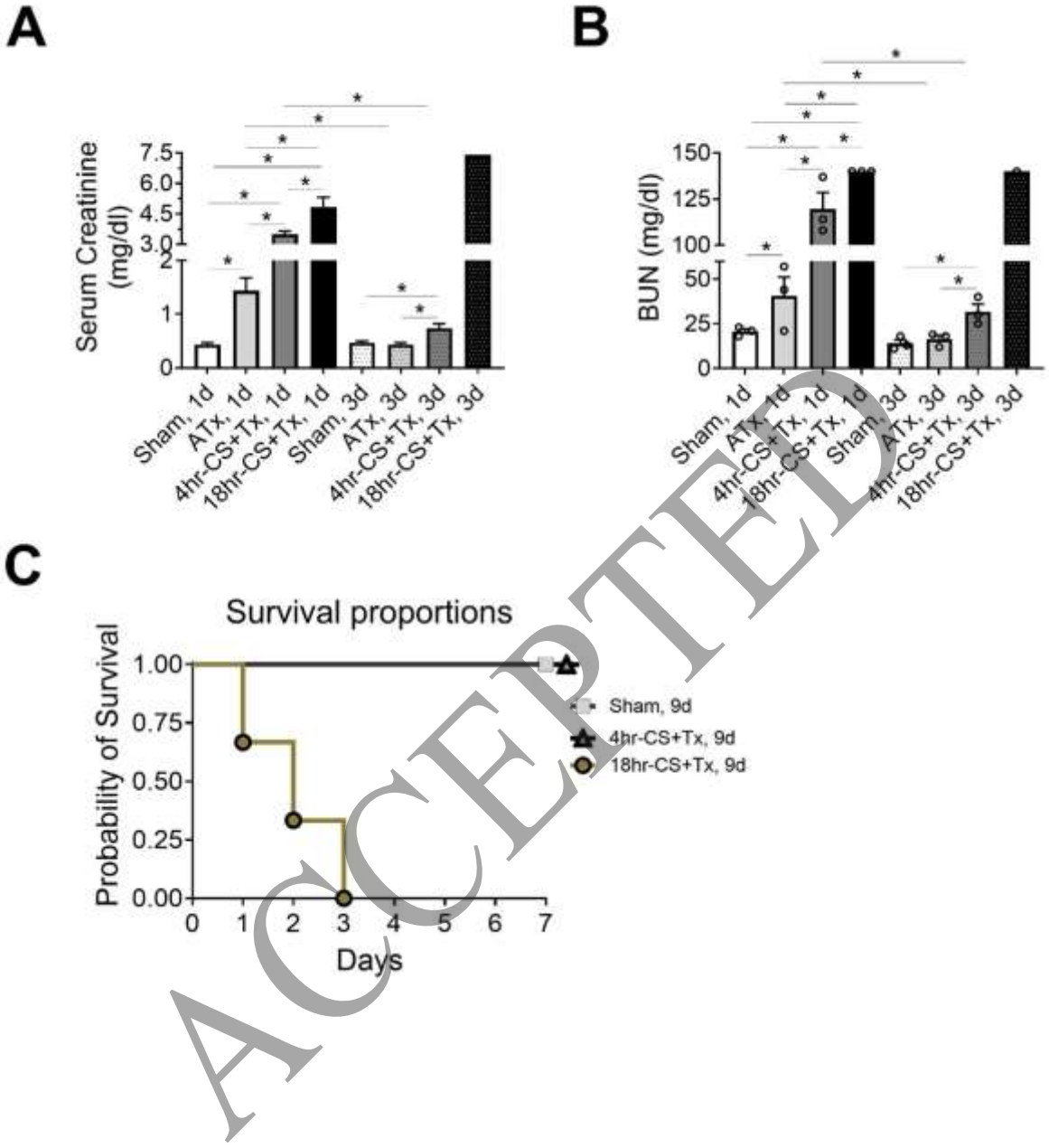

Figure 5. CS+Tx persistently increases immunoproteasome (β5i) level in rat kidney allografts. Fischer rat kidneys were flushed with and stored in cold storage (CS) solution (4°C) for 4 h or 18 h followed by transplantation to a recipient Lewis rat. Sham rats were used as a control. Immunoblot of β5i (LMP7) proteins in rat kidney homogenates from sham and cold storage and transplant (CS+Tx) groups (9 days post-surgery) (n = 4/group). GAPDH served as loading control. Representative blots from 3 independent experiments shown. Graphs show densitometry (mean ± SEM) normalized to GAPDH (n =4/group). *p < 0.05 was considered statistically significant.

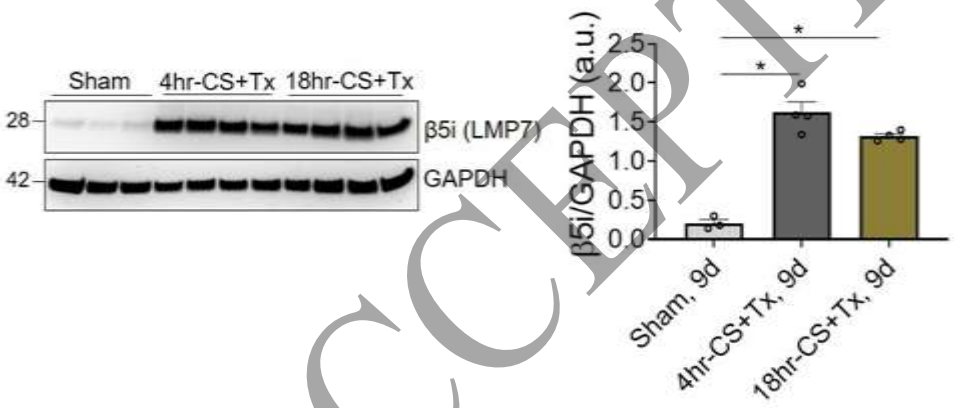

Figure 6. Abundance of *i*proteasome components changes during CS vs. CS+Tx. Fischer rat kidneys were flushed with and stored in cold storage (CS) solution (4°C) for 4 h or 18 h followed by transplantation to a recipient Lewis rat. Untreated (Control) or Sham rats were used as controls. (**A-B**) Non-denatured renal extracts (9 days post-surgery) prepared with digitonin lysis buffer were subjected to native gel electrophoresis followed by western blotting with anti-β5i antibody. Representative blots from 3 independent experiments shown. Graphs show densitometry (mean ± SEM) normalized to β-actin (n =4/group). *p < 0.05 was considered statistically significant.

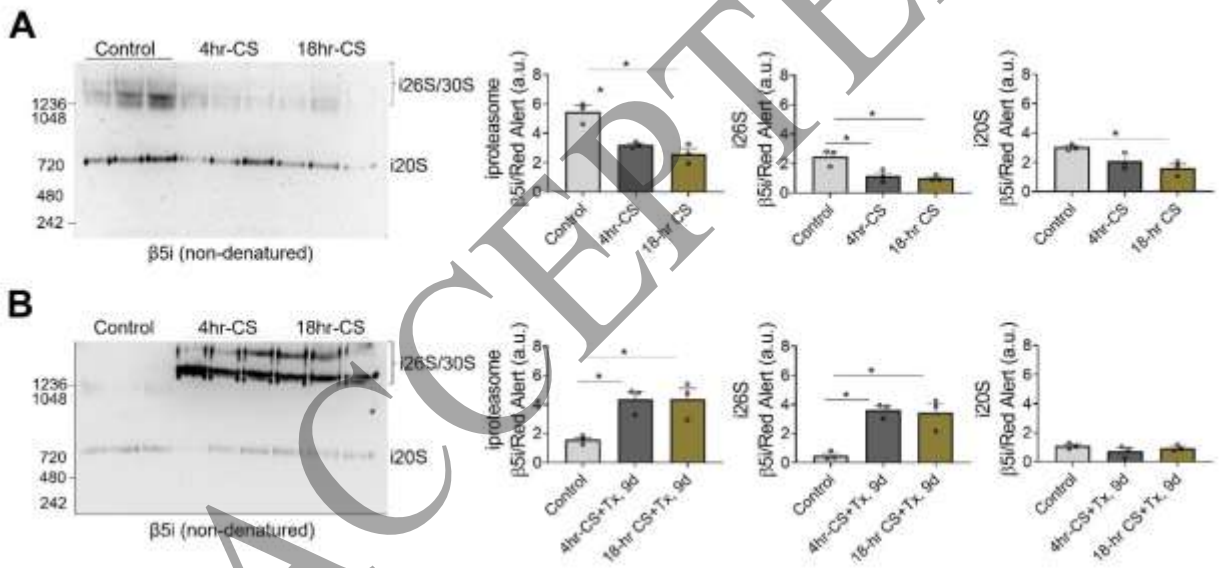

Figure 7. Treating rat kidneys with ONX 0914 in CS solution does not improve allograft function/injury after transplantation. Lewis rat kidneys were treated with the β5i inhibitor ONX 0914 (0 or 100 nM) in cold storage (CS) solution (4°C) for 4 h followed by transplantation to a recipient Fischer rat (n=4/group, 9-day post-surgery). Sham rats were used as a control. (**A-B**) Morphological study of rat kidney sections was performed using Periodic acid-Schiff (PAS) staining. (**A**) Representative images of Periodic acid-Schiff (PAS) stained renal sections (400 X magnification). (**B**) Tubular injury was assessed and scored in a blinded fashion using the PAS stained renal sections from A. Data are the mean ± SEM (bar graph, n=4/group). (**C-D**) Renal function was assayed in rat blood (9 days post-surgery) by measuring serum creatinine (SCr) (**C**) or blood urea nitrogen (BUN) (**D**). Data are the mean ± SEM (violin plots, n=4/group). Differences between group means were compared with Student's t-test (2-group comparison) or ANOVA (3-group comparison). *p < 0.05 was considered statistically significant. (**E**) & (**F**) Representative images of KIM1 and NGAL immunohistochemistry of renal sections (400 X magnification; n=4/group, respectively. (**G**) Representative images of Masson's trichome staining of renal sections (400 X magnifiction; n=4/group). O Medulla: Outer Medulla; I Medulla: Inner Medulla

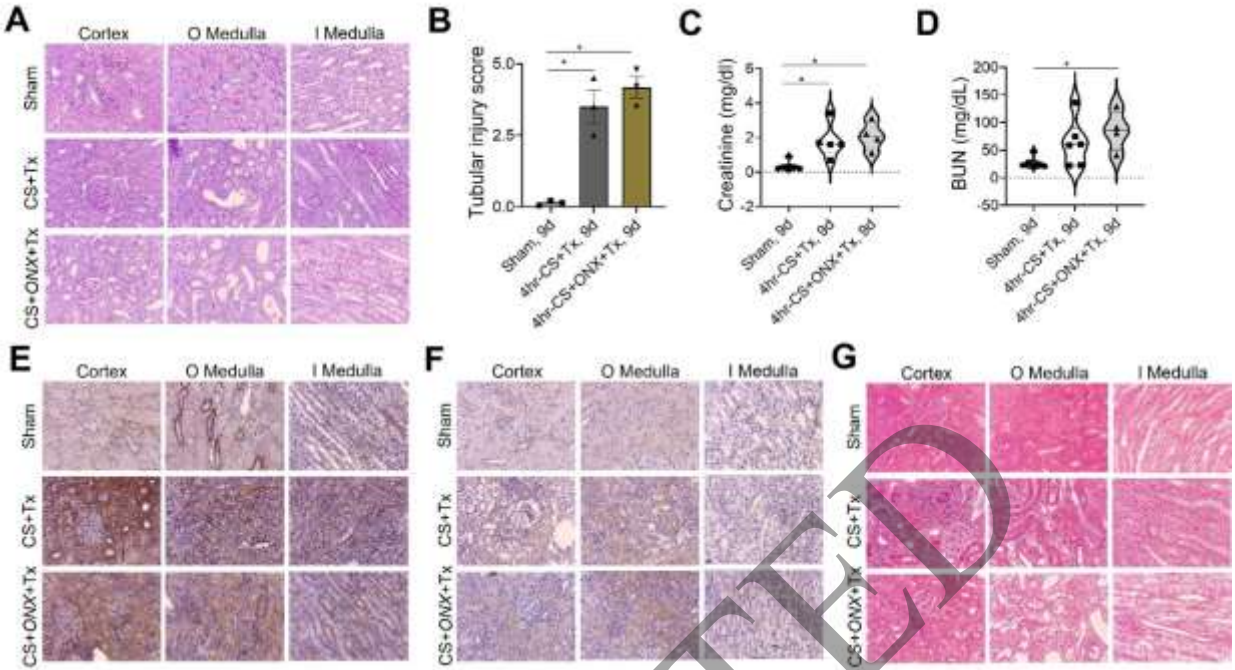

Figure 8. Effect of treating rat kidneys with ONX 0914 during CS on blood chemistry after CS+Tx. Lewis rat kidneys were treated with the β5i inhibitor ONX 0914 (0 or 100 nM) in cold storage (CS) solution (4°C) for 4 h followed by transplantation to a recipient Lewis rat (n=4/group). Sham rats were used as a control. (**A-I**) Blood chemistry measured with i-STAT Chem8^{+} cartridge and blood chemistry analyzer. Data are the mean ± SEM (bar graph, n=4). Differences between group means were compared with Student's t-test (2-group comparison) or ANOVA (3-group comparison). *$p < 0.05$ was considered statistically significant.