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Targeted donor complement blockade after brain death prevents delayed graft function in a nonhuman primate model of kidney transplantation

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Delayed graft function (DGF) in renal transplant is associated with reduced graft survival and increased immunogenicity. The complement-driven inflammatory response after brain death (BD) and posttransplant reperfusion injury play significant roles in the pathogenesis of DGF. In a nonhuman primate model, we tested complement-blockade in BD donors to prevent DGF and improve graft survival. BD donors were maintained for 20 hours; kidneys were procured and stored at 4°C for 43-48 hours prior to implantation into ABO-compatible, nonsensitized, MHC-mismatched recipients. Animals were divided into 3 donor-treatment groups: G1 - vehicle, G2 - rhC1INH+heparin, and G3 - heparin. G2 donors showed significant reduction in classical complement pathway activation and decreased levels of tumor necrosis factor α and monocyte chemoattractant protein 1. DGF was diagnosed in 4/6 (67%) G1 recipients, 3/3 (100%) G3 recipients, and 0/6 (0%) G2 recipients ($P = .008$). In addition, G2 recipients showed superior renal function, reduced sC5b-9, and reduced urinary neutrophil gelatinase-associated lipocalin in the first week posttransplant. We observed no differences in incidence or severity of graft rejection between groups. Collectively, the data indicate that donor-management targeting complement activation prevents the development of DGF. Our results suggest a pivotal role for complement activation in BD-induced renal injury and postulate complement blockade as a promising strategy for the prevention of DGF after transplantation.

Abbreviations: AKI, acute kidney injury; AMR, antibody-mediated rejection; AP, alternative (complement) pathway; BD, brain death; C1INH, C1 inhibitor; CP, classic (complement) pathway; DGF, delayed graft function; H/E, hematoxylin and eosin; IL, interleukin; IRI, ischemia-reperfusion injury; IV, intravenous; LP, mannose-binding lectin (complement) pathway; MCP-1, monocyte chemoattractant protein 1; NGAL, neutrophil gelatinase-associated lipocalin; NHP, nonhuman primate; rhC1INH, recombinant human C1 esterase inhibitor; TNF α , tumor necrosis factor alpha.

Juan S. Danobeitia and Tiffany J. Zens contributed equally to this manuscript and should both be considered first author for the purpose of publication.

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KEYWORDS

animal models: nonhuman primate, complement biology, delayed graft function (DGF), donors and donation: donation after brain death (DBD), immunosuppression/immune modulation, ischemia reperfusion injury (IRI), kidney transplantation/nephrology, translational research/science

1 | INTRODUCTION

Delayed graft function (DGF) manifests as a consequence of ischemia–reperfusion injury (IRI) and is characterized by acute kidney injury (AKI) within 7 days of transplant, requiring life-sustaining dialysis.¹ The incidence of DGF in kidney transplants from brain dead (BD) donors is approximately 26% in the United States, and this rate can reach as high as 37% in kidneys from older donors and those subjected to extended cold ischemia >36 hours.^{2–4} In addition to complications related to AKI in the peritransplant period, development of DGF is an important risk factor for acute cellular rejection, antibody-mediated rejection (AMR), and reduced graft survival.^{2,5–9} Inflammatory injury secondary to IRI is also key to mechanisms leading to DGF and subsequent graft rejection.⁵ During BD, the donor experiences neurohormonal changes known to trigger a systemic inflammatory response characterized by the release of proinflammatory cytokines from both innate and adaptive immune cells, including interleukin (IL)-1 β , tumor necrosis factor alpha (TNF α), interferon gamma, and IL-17; chemokines; and reactive oxygen and nitrogen species. This inflammatory response promotes recruitment of activated immune cells affecting vascular tone and exacerbating the degree of injury while enhancing graft immunogenicity.^{10–12}

Activation of complement, whether through the classic (CP), mannose-binding lectin (LP), or alternative (AP) pathway, has gained special attention due to its role in the pathogenesis of renal IRI, transplant rejection, and acute tubular injury.^{13–17} Recent reports suggest that systemic complement activation reduces renal allograft quality starting at the time of BD and progressing through cold storage and reperfusion.^{18–21}

Recombinant human C1 esterase inhibitor (rhC1INH) is a serine protease inhibitor that inactivates proteases of the complement, contact, fibrinolytic, and coagulation systems. It acts as a major regulator by inhibiting the CP and LP of complement activation and preventing amplification of the inflammatory response.^{22,23} In renal IRI and kidney transplant models, C1 inhibition has shown protective effects on vessel/organ integrity and reduced IRI and progression to AMR after renal transplantation.^{16,24–26} The objective of this study was to determine the impact of rhC1INH as a donor treatment strategy in BD conditions for the prevention of early posttransplant kidney dysfunction and modulation of immune responses. We used a nonhuman primate (NHP) model of BD in older animals, prolonged cold ischemia, and transplant into nonsensitized, fully mismatched recipients.

2 | MATERIALS AND METHODS

2.1 | Animals and animal care

Rhesus macaques were used in this study (Table 1). Donor animals (n = 8, aged 15–22 years) and transplant recipients (n = 15, aged 3–7 years) were obtained from the Wisconsin National Primate Research Center and Alpha Genesis, Inc (Yemassee, SC). All animals were prescreened negative for tuberculosis, herpesvirus B, simian type D retrovirus, simian immunodeficiency virus, and simian T cell leukemia virus type 1. Each donor–recipient pair was ABO blood compatible, nonsensitized, and fully mismatched for MHC class I and II alleles identified using microsatellite analysis as previously described (data not reported).²⁷ Animals were housed in accordance with National Institutes of Health and U.S. Department of Agriculture animal welfare guidelines; all protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin–Madison.

2.2 | Study drug and experimental design

rhC1INH was provided by Pharming Technologies BV (Leiden, the Netherlands).

Donor animals were randomly assigned to treatment groups (Figure 1A). Donors were maintained for a 20-hour period. BD donor management was performed following previously published guidelines to maintain hemodynamic stability and adequate oxygen delivery.^{11,28} Briefly, donor animals were anesthetized, ventilated, and monitored. A 16F Foley catheter was placed in the extradural space of the cranial fossa (i.e., the intracranial space) and gradually inflated until hemodynamic and neurologic signs of brain stem herniation were documented. Animals were monitored and received standard donor management based on intravenous (IV) fluid resuscitation and vasopressor support to achieve a stable mean arterial pressure and urinary output. Twenty hours after BD induction, both kidneys were recovered after cannulation and retrograde infusion of UW preservation solution (Organ Recovery Systems, IL) supplemented with heparin (5 U/mL). Recovered kidneys were preserved in UW solution at 4°C for a 43–48 hours prior to implantation into the recipient. G1 donors received vehicle treatment (0.9% normal saline) via IV bolus injection given at t = 180, 360, 540, 720, 900, and 1080 minutes. G2 donors received rhC1INH (500 U/kg/dose) treatment via IV bolus injection at the indicated time points in combination with continuous IV heparin

TABLE 1 Experimental design, kidney function, and survival data

Donor ID	Donor age (y)	Donor treatment group	rhC1-INH dose	Recovered kidney	Cold ischemia time (h)	Recipient ID	Age (y)	Weight (kg)	Graft survival (d)	Meet DGF criteria	Peak creatinine level (mg/dL)
Group 1											
Rhas84	20.8	Vehicle	N/A	Right kidney	44.5	R11051	4.3	8.3	3	Yes	14.8
				Left kidney	47.9	R11107	4.2	4.7	90	No	6.7
Rh2602	16.6	Vehicle	N/A	Left kidney	46.7	Rh2612	4.0	4.0	39	Yes	10.9
Rh2030	21.2	Vehicle	N/A	Right kidney	44.1	Rh2636	5.6	5.4	29	Yes	15.7
				Left kidney	47.2	R13020	3.6	5.9	10	Yes	18.4
Average	19.5 ± 1.8	N/A	N/A	N/A	46.1 ± 1.7	N/A	4.3 ± 0.7	5.7 ± 1.6	34.2 ± 34.4	4/5 (80%)	13.3 ± 4.6
Group 2											
Rhat64	21.2	rhC1INH + heparin	500 U/kg per time point	Right kidney	45.0	R13009	3.8	7.3	26	No	4.3
				Left kidney	47.8	R12063	4.3	5.4	26	No	3.8
Rh2241	18.8	rhC1INH + heparin	500 U/kg per time point	Left kidney	45.2	Rh2622	5.9	9.7	67	No	7.3
				Right kidney	46.8	Rh2625	4.8	7.1	32	No	13.0
Rhav29	20.1	rhC1INH + heparin	500 U/kg per time point	Right kidney	44.0	Rh2616	6.2	8.6	90	No	8.4
				Left kidney	46.0	Rh2623	5.1	8.0	46	No	14.8
Average	20.0 ± 1.2	N/A	N/A	N/A	45.8 ± 1.4	N/A	5.0 ± 0.9	7.4 ± 1.4	47.8 ± 25.8	0/6 (0%)	8.6 ± 4.5
Group 3											
Rh1966	20.7	Heparin	N/A	Left kidney	45.7	Rh2615	6.1	13.9	4	Yes	15.9
R01070	15.7	Heparin	N/A	Right kidney	45.2	Rh2627	6.0	11.4	5	Yes	20.0
				Left kidney	47.5	Rh2629	6.1	11.1	5	Yes	18.7
Average	18.2 ± 3.5	N/A	N/A	N/A	46.1 ± 1.2	N/A	6.0 ± 0.05	12.1 ± 1.5	4.6 ± 0.5	3/3 (100%)	18.2 ± 2.1
P-value	NS	N/A	N/A	N/A	NS	N/A	NS	P < .05 ^a	NS	P = .0045	P < .05 ^b

Note: Individual values presented along with group averages ± SD, significance calculated by 1-way ANOVA and Bonferroni's posttest correction.

Abbreviations: DGF, delayed graft failure; N/A, not applicable; NS, nonsignificant.

^aG1 vs G3.

^bG2 vs G3.

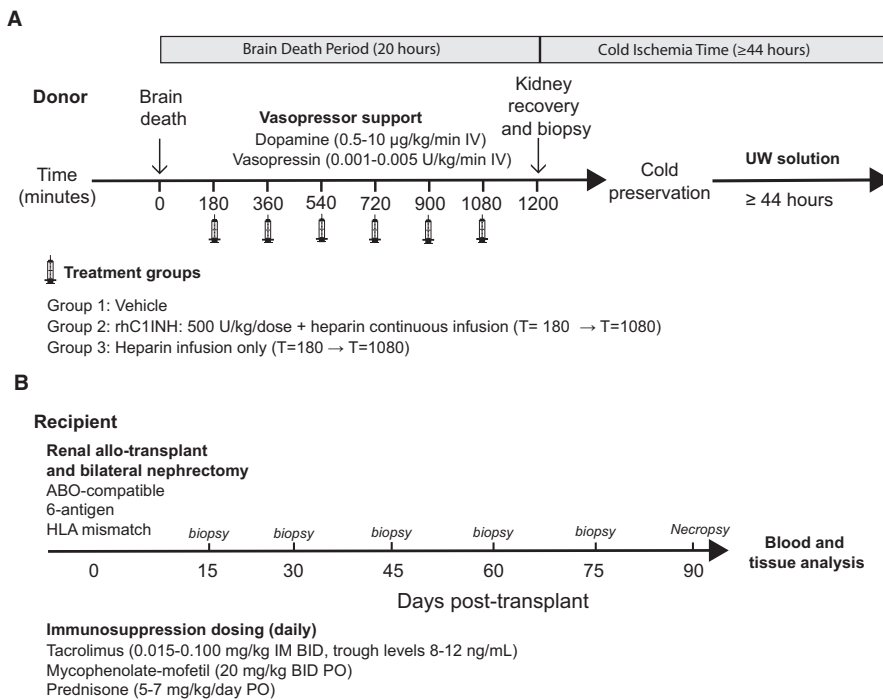


FIGURE 1 Experimental design

infusion $t = 180 \rightarrow t = 1080$ minutes titrated to a partial thromboplastin time of 80-120 seconds. G3 donors received only continuous IV heparin infusion as described with dosing titrated to a partial thromboplastin time of 80-120 seconds. Heparin was used to potentiate the activity of rhC1INH as described previously.²⁹⁻³¹

All recipients underwent bilateral native nephrectomy concurrent with heterotopic kidney transplant performed as described previously (Figure 1B).³² Recipients were monitored for acid-base and electrolyte balance, serum blood urea nitrogen, serum creatinine, urinary output, proteinuria, and behavioral abnormalities. No induction therapy was used, but maintenance therapy included mycophenolate mofetil, tacrolimus, and prednisone. Tacrolimus levels were dose-adjusted biweekly to maintain 8-12 ng/mL trough levels. Criteria for termination of the study were defined as (1) survival for 90 days or (2) progressive acute kidney failure and severe azotemia not responsive to medical management.

2.3 | Definition of DGF

DGF was defined as a failure of a fall in serum creatinine of at least 10% on 3 consecutive days in the first posttransplant week and/or serum creatinine >2.5 mg/dL at posttransplant day 7.^{1,33}

2.4 | Circulating cytokines

Circulating levels of IL-6, IL-8, monocyte chemoattractant protein (MCP)-1, and TNF α in EDTA-plasma were measured with LEGENDplex NHP Mix-and-Match Subpanel (Biolegend, San Diego, CA, USA) according to manufacturer-recommended protocols.

2.5 | Complement assessment

Blood was collected into Vacuettes (Greiner Bio-One, Kremsmünster, Austria); serum tubes were allowed to clot for 15 minutes before centrifugation for 10 minutes at 3 000 xg, then serum and K₃-EDTA-plasma were aliquoted and stored at -80°C . Each assay was tested for cross-reactivity with rhesus macaque and to establish a linear range. Assays were performed according to manufacturer instructions. C1 inhibitor (C1INH) was measured in rhesus serum using the C1INH ELISA Pair (Sino Biological SEK10995-5, Beijing, China). CP, AP, and LP activation were tested by using the Wieslab Complement Kits (CP310, AP330, MP320, EuroDiagnostica, Malmö, Sweden). Circulating levels of sC5b-9 (membrane attack complex) were measured by using a commercially available sC5b-9 enzyme immunoassay (ELISA kit; Quidel, San Diego, CA, USA). Measured values for each assay were normalized to serum albumin (VetTest Analyzer, Idexx, Westbrook, ME, USA) to account for hemodilution observed over the course of BD.

2.6 | Histology and microscopic evaluation

At the discretion of the veterinary staff and attending surgeons, kidney core biopsy specimens were collected from grafts before cold ischemia after the 20-hour BD period, as well as 60 minutes and 7 days postreperfusion and finally at necropsy. Biopsy specimens were also collected from the naïve native kidneys removed from recipients during the operation and prior to graft reperfusion. Tissue was fixed in 10% formalin and embedded in paraffin or frozen in optimal cutting temperature (OCT) compound. 4- μm slices were mounted onto slides and stained for histological assessment. Stains included hematoxylin

and eosin (H/E), periodic acid–Schiff (PAS), and Picro Sirius Red for estimation of fibrosis, as well as antibodies against CD68 (KP1; DAKO-Agilent, Santa Clara, CA, USA), myeloperoxidase (MPO; Abcam, Cambridge, UK), malondialdehyde (MDA; Abcam), and C4d, C3b, and C5b-9 (Ventana-Roche, Tucson, AZ, USA). The HIER method was used for antigen retrieval (BioGenex, San Ramon, CA, USA). Slides for histopathology were interpreted by a renal pathologist. For immunohistochemistry, images were acquired from 6–12 random nonoverlapping fields within each slide at appropriate magnification using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and processed using ImageJ software (National Institutes of Health, Bethesda, MD, USA) according to internal laboratory protocols. Cell counts or area fraction measurements for each image were quantified using color-separation, automatic thresholding, and particle-analysis algorithms.

2.7 | Urinary neutrophil gelatinase-associated lipocalin measurement

Posttransplant urine was stored at -80°C until analysis. Urinary neutrophil gelatinase-associated lipocalin (NGAL) level was quantified using the NHP NGAL ELISA kit (Bioporto, Copenhagen, Denmark) according to the manufacturer protocol.

2.8 | Statistical analysis

Statistical analyses were performed using GraphPad Prism V5.04. All data are shown as mean \pm SEM or SD. DGF incidence and resistive indices were analyzed by χ^2 test. Comparisons of 2 groups were tested by 2-tailed Student's *t* test. Differences between 3 or more groups were tested by 1-way ANOVA and Bonferroni post-test correction or Kruskal-Wallis test and Dunn posttest correction in data sets with nonnormal distribution. Data sets with 2 independent variables were tested by 2-way ANOVA followed by Bonferroni correction. Differences between treatment groups were considered significant at $P < .05$.

3 | RESULTS

3.1 | rhC1INH treatment results in sustained elevation of circulating C1INH, inhibits complement activation, and reduces C3b/C5b-9 deposition in BD donors

During the 20-hour BD period, donor animals received 6 bolus IV administrations of either vehicle (G1) or rhC1INH with continuous heparin infusion (G2), or continuous heparin infusion only (G3) starting at 3 hours after induction of BD, until 2 hours before organ recovery (Figure 1A, Table 1). To confirm the BD state, we monitored the hemodynamics of BD donors throughout this 20-hour period

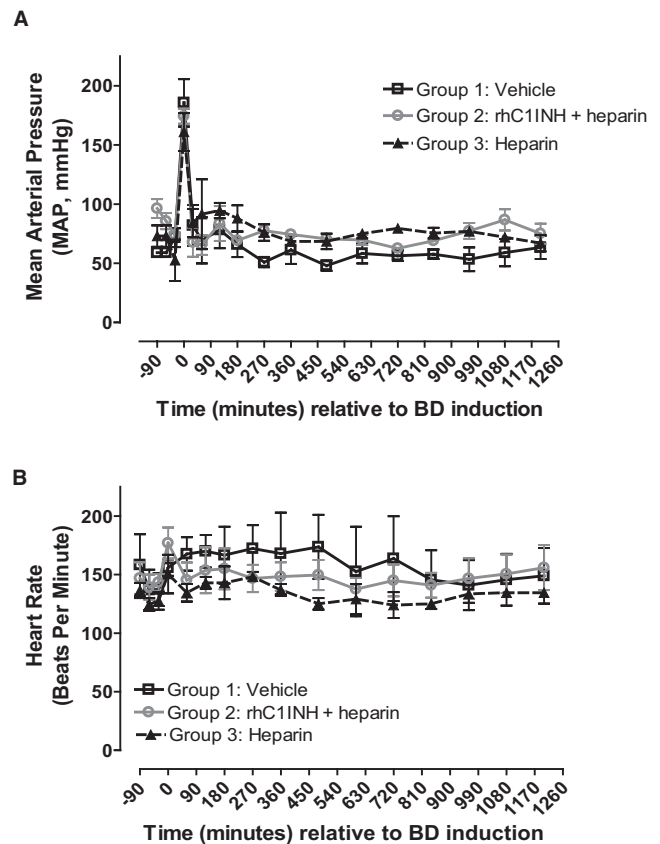


FIGURE 2 Hemodynamic assessment of brain-dead (BD) donors over the course of the experimental period. A, Mean arterial pressure (MAP) measured in mmHg by continuous invasive intra-arterial monitor. B, Heart rate in beats per minute

and observed the Cushing reflex as a result of increased intracranial pressure characterized by hypertensive response followed by hypotension, tachycardia, then bradycardia (Figure 2). We did not detect clinically significant differences for any parameters between groups at any of the time points investigated (Table 2).

We evaluated circulating levels of C1INH and the activity of the CP, AP, and LP of complement activation to confirm the therapeutic range of the drug after systemic delivery. Endogenous C1INH levels measured before the initiation of treatment (i.e., at -30 and $+30$ minutes) showed no difference between groups. G2 donors received rhC1INH as described, circulating C1INH measured at 720 and 1200 minutes postinduction showed significantly higher levels compared with donors in G1 and G3 (Figure 3A). G2 donors also showed significant suppression of the CP at 720 and 1200 minutes after BD, in contrast to the G1 and G3 donors (Figure 3B), in parallel to the increased circulating C1INH levels. Activation of LP (Figure 3C) showed wide variation and was not statistically different between groups. Activation of AP (Figure 3D) was significantly lower in G3 compared with G1 at both 720 ($P < .05$) and 1200 minutes ($P < .01$). We investigated complement activation in donors by analyzing C3b/C5b-9 deposition in kidney biopsy specimens obtained immediately after the 20-hour BD period. Immunofluorescence analysis of G2 donor kidneys revealed minimal C5b-9 and C3b deposition compared with

TABLE 2 Kidney donor vital signs and laboratory values at baseline and 20 hours after brain death

Parameter	T = 1200 minutes (20 hours)				P value	P value
	Group 1: vehicle	Group 2: rhC1INH + heparin	Group 3: heparin	Group 2: rhC1INH + heparin		
Vitals, mean ± SD						
Temperature, °F	98.7 ± 1.8	95.8 ± 1.7	97.9 ± 1.8	98.7 ± 1.4	98.6 ± 1.8	99.9 ± 0.2
Pulse, bpm	138.3 ± 24.7	139.3 ± 18.6	125.0 ± 14.1	156.7 ± 28.0	150.7 ± 33.6	134.0 ± 8.5
MAP, mm Hg	70.0 ± 6.2	78.3 ± 8.5	52.5 ± 24.7	64.0 ± 16.1	77.7 ± 6.4	59.5 ± 0.7
UOP, mL/kg/h	NA			3.3 ± 0.8	4.9 ± 3.9	12.2 ± 7.6
Metabolic panel, mean ± SD						
Na, mmol/L	147.3 ± 2.3	146.3 ± 1.5	148.5 ± 0.7	143.7 ± 2.9	150.0 ± 4.4	157.5 ± 0.7
K, mmol/L	3.5 ± 0.4	3.5 ± 1.3	3.3 ± 0.0	3.6 ± 0.5	2.9 ± 0.3	3.2 ± 0.1
Cl, mmol/L	111.3 ± 2.3	109.3 ± 1.2	111.0 ± 1.4	107.7 ± 2.9	112.0 ± 5.2	119.5 ± 2.1
CO ₂ , mmol/L	23.7 ± 0.6	26.0 ± 1.0	24.0 ± 2.8	22.3 ± 2.5	23.7 ± 3.1	23.0 ± 2.8
BUN, mg/dL	18.0 ± 1.7	14.7 ± 4.0	21.5 ± 0.7	16.7 ± 5.5	13.3 ± 8.6	8.5 ± 0.7
Cr, mg/dL	0.7 ± 0.2	0.4 ± 0.1	0.6 ± 0.0	0.8 ± 0.1	0.5 ± 0.2	0.5 ± 0.1
Glc, mg/dL	68.3 ± 5.5	84.3 ± 16.9	61.0 ± 8.5	117.0 ± 37.3	106.7 ± 41.7	135.5 ± 2.1
CBC, mean ± SD						
Hb, g/dL	12.6 ± 0.4	13.0 ± 1.2	11.7 ± 4.1	9.3 ± 2.0	10.6 ± 0.9	11.6 ± 1.9
Hct, %	37.0 ± 1.0	38.3 ± 3.5	34.5 ± 12.0	27.3 ± 5.9	31.0 ± 2.6	34.0 ± 5.7
ABG, mean ± SD						
pH	7.4 ± 0.0	7.3 ± 0.0	7.3 ± 0.1	7.3 ± 0.1	7.4 ± 0.1	7.3 ± 0.1
P _{CO₂} , mm Hg	49.7 ± 14.1	49.1 ± 6.8	49.3 ± 3.9	50.2 ± 11.8	35.7 ± 10.7	47.3 ± 11.7
P _{O₂} , mm Hg	478.7 ± 115.0	323.0 ± 138.3	401.5 ± 41.7	288.7 ± 169.4	258.3 ± 115.5	272.5 ± 40.3
HCO ₃ , mmol/L	26.0 ± 1.2	26.3 ± 1.1	25.4 ± 0.9	23.6 ± 3.1	27.7 ± 9.8	22.6 ± 2.5
Lac, mmol/L	0.5 ± 0.2	0.3 ± 0.0	0.4 ± 0.2	0.5 ± 0.2	0.8 ± 0.4	0.6 ± 0.0

Note: Data presented as average values ± SD. Vital signs were monitored throughout the BD period; no clinically significant differences were detected for any parameters between groups at any of the time points investigated. Body temperature was monitored in degrees Fahrenheit (°F).

Abbreviations: ABG, arterial blood gas; bpm, beats per minute; BUN, blood urea nitrogen; CBC, complete blood count; Cr, creatinine; Glc, glucose; Hb, hemoglobin; Hct, hematocrit; Lac, lactate; MAP, mean arterial pressure; UOP, urine output.

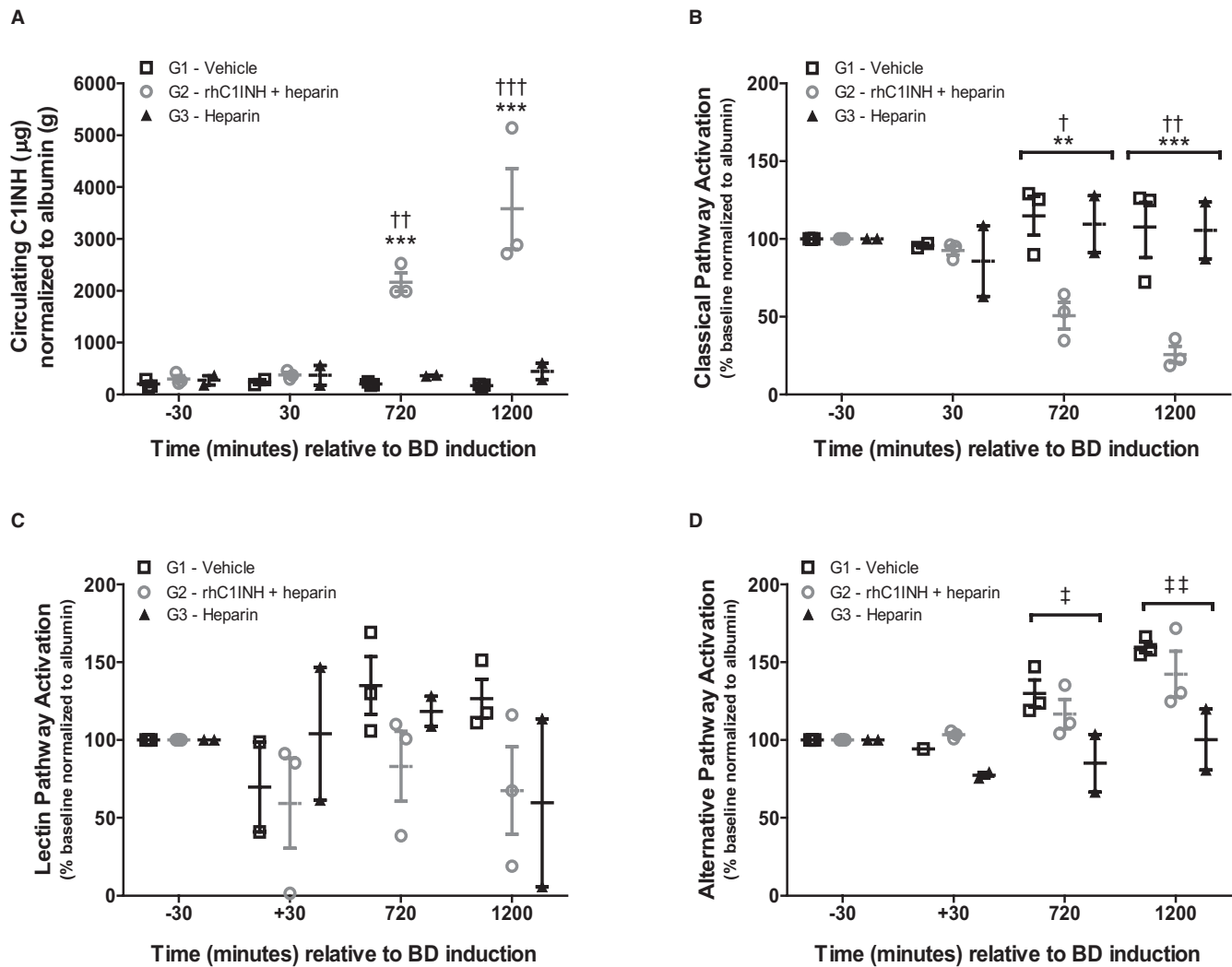


FIGURE 3 rhC1INH treatment results in sustained elevation of circulating C1INH and inhibits complement activation in the brain-dead (BD) donor. A, Levels of serum C1INH in G1 (vehicle, $n = 3$), G2 (rhC1INH + heparin, $n = 3$), and G3 (heparin, $n = 2$) donors at -30, 30, 720, and 1200 minutes relative to BD induction, 5 minutes after bolus injection of drug or vehicle where applicable. Data are presented as sample C1INH (μg) normalized to serum albumin (g) to compensate for dilution effects. B-D, Complement activation determined by the complement system screen assay of the (B) classic (complement) pathway, (C) mannose-binding lectin (complement) pathway, and (D) alternative (complement) pathway in G1, G2, and G3 donors. Data are expressed as percent activation normalized to albumin, relative to baseline (30 minutes before induction of BD). Data in A-D presented as mean \pm SEM values, significance calculated by 2-way ANOVA and Bonferroni's post-hoc correction (** $P < .01$ G1 vs G2; *** $P < .001$ G1 vs G2; $\dagger P < .05$ G2 vs G3; $\dagger\dagger P < .01$ G2 vs G3; $\dagger\dagger\dagger P < .001$ G2 vs G3; $\ddagger P < .05$ G1 vs G3; $\ddagger\dagger P < .01$ G1 vs G3)

increased staining of both markers in G1 and G3 kidneys before cold storage (Figure 4). Renal pathology did not differ between treatment groups based on evaluation of H/E and PAS stains (not shown).

3.2 | rhC1INH treatment limits systemic levels of $\text{TNF}\alpha$ and MCP-1 in BD donors

We assessed levels of IL-6, $\text{TNF}\alpha$, IL-8, and MCP-1 as proinflammatory cytokines and chemokines implicated in the acute inflammatory response to tissue injury and trauma during the BD period. We documented significantly lower levels of $\text{TNF}\alpha$ and MCP-1 in G2 donors compared with G1 and/or G3 donors (Figure 5A,B). No significant

differences were observed in the circulating levels of IL-6 or IL-8 between groups (Figure 5C,D).

3.3 | Donor rhC1INH treatment reduces circulating sC5-b9 in recipients

After prolonged cold preservation (43-48 hours), donor grafts were transplanted into ABO-compatible, nonsensitized, MHC fully mismatched recipients who underwent bilateral nephrectomy of their native kidneys and received posttransplant maintenance immunosuppression (Figure 1B). We obtained biopsies from the grafts at 60 minutes and day 4 posttransplant and analyzed these for innate

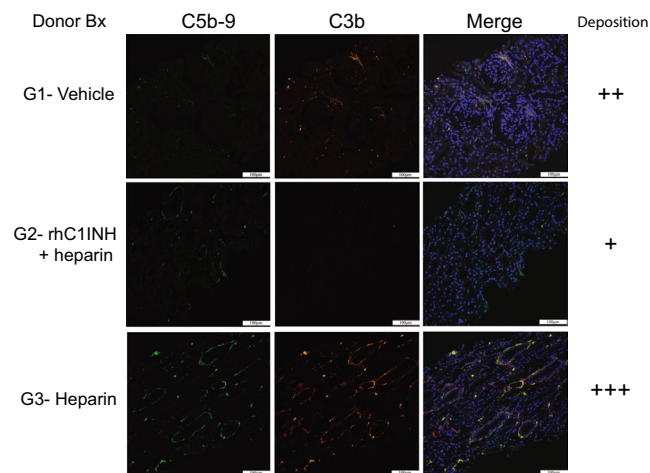


FIGURE 4 rhC1INH treatment reduces C3b/C5b-9 deposition in the brain-dead (BD) donor graft. A, B, Representative micrographs at 200 \times magnification depicting C5b-9 (green) and C3b (orange) deposition by immunofluorescent staining in kidney biopsies obtained from G1 (vehicle), G2 (rhC1INH + heparin), and G3 (heparin) donor grafts at the time of organ recovery; semiquantitative assessment of combined complement deposition

immune cell infiltration, complement deposition, and oxidative damage. We compared these with biopsy samples collected from recipient native kidneys. Due to concern for the well-being of the animals, biopsies were limited to only 2 or 3 recipients per group. In biopsy specimens collected 60 minutes postreperfusion, renal pathology evaluated by H/E and PAS did not differ between treatment groups, nor were differences observed in the level of complement deposition (C3b/C5b-9), immune cell infiltration (CD68⁺ macrophages and MPO⁺ neutrophils), or oxidative damage (MDA), although all were higher than was observed in naïve native kidneys (data not shown). On day 4 posttransplant, biopsy specimens from recipients of G2 donor kidneys seemed to display lower levels of C3b/C5b-9 deposition compared with recipients of G1 and G3 donors; however, the limited number of specimens lacked statistical power to establish significance of the results (Figure 6A,B). We further analyzed levels of soluble C5b-9 (sC5b-9) in circulation as a measure of complement activation at 60 minutes and 4 days posttransplant and found that values at 60 minutes were again equivalent for all experimental groups, whereas day 4 levels demonstrated significantly less sC5b-9 in recipients of G2 grafts compared with recipients of G1 and G3 grafts (Figure 6C, $P < .05$). Day 4 levels of oxidative damage and immune cell infiltration by immunohistochemistry were equivalent between experimental groups (Figure S1).

3.4 | Donor rhC1INH treatment improves posttransplant renal function, reduces injury and incidence of DGF, and improves graft survival

Recipients of kidneys from G1 and G3 donors exhibited significant kidney dysfunction within the first week after surgery. DGF was

diagnosed in 4/6 recipients of G1 kidneys and in 3/3 G3 graft recipients. None (0/6) of the recipients of G2 donor grafts met criteria for DGF ($P = .0081$, Table 1). Posttransplant serum creatinine was elevated in recipients of G1 and G3 donors; in contrast, recipients of G2 donor kidneys displayed lower serum creatinine at days 4-6 posttransplant ($P < .05$, Figure 7A) and lower peak serum creatinine vs G3 ($P < .05$, Table 2) indicating superior renal function. In addition, G2 kidney recipients presented lower levels of urinary NGAL than G1 kidney recipients, indicating reduced renal injury (Figure 7B). Recipients in G3 remained anuric until euthanasia criteria were met, preventing measurement of urinary NGAL.

To further investigate the impact of donor therapy on renal function, we performed daily ultrasounds to determine renal resistive indices in transplanted grafts. Resistive index measurement has been used to assess posttransplant renal function in the clinic; studies have shown a correlation between elevated indices and progression to DGF.³⁴ All imaged kidneys transplanted from G1 (4/4) and G3 (2/2) donors showed elevated indices on posttransplant day 1 compared with 0/4 kidneys from G2 donors ($P = .006$, Figure 8). Further, kidneys from G2 donors exhibited significantly longer graft survival compared with those from G1 and G3 donors ($P < .05$, Table 1).

Altogether, these results indicate that donor treatment with rhC1INH provided a protective effect in renal grafts subjected to BD and prolonged cold ischemia, demonstrable through improved renal function in the first week posttransplant and overall graft survival.

4 | DISCUSSION

We investigated the impact of targeted complement blockade in BD organ donors using rhC1INH in combination with heparin to prevent DGF and improve graft survival. For this purpose, we induced BD in older rhesus macaque donors and maintained them hemodynamically stable for 20 hours before organ procurement. We then subjected the kidneys to prolonged cold storage (44-48 hours) with the intent of generating a translational model of clinical DGF using the definitions proposed by Boom et al.^{1,33}

Complement activation has gained significant attention in the context of IRI and organ donation in the past decade.³⁵ Our approach targeting the complement-driven inflammatory response in older BD donors in the context of prolonged cold storage resulted in a significant reduction in the incidence of DGF in recipients along with superior renal function within the first 2 weeks after transplant as evinced by significantly lower serum creatinine and decreased urinary NGAL measurements. Our data indicate that C1 complement blockade and heparin treatment in the donor limits inflammation by reducing cytokine, CP activity and deposition of complement-proteins within the graft and that this has an ameliorating effect on complement-mediated tissue injury in transplant recipients.

C1 inhibitor plays a central role in the modulation of inflammation by upstream regulation of the complement, coagulation, and contact systems. Although the mechanisms leading to complement

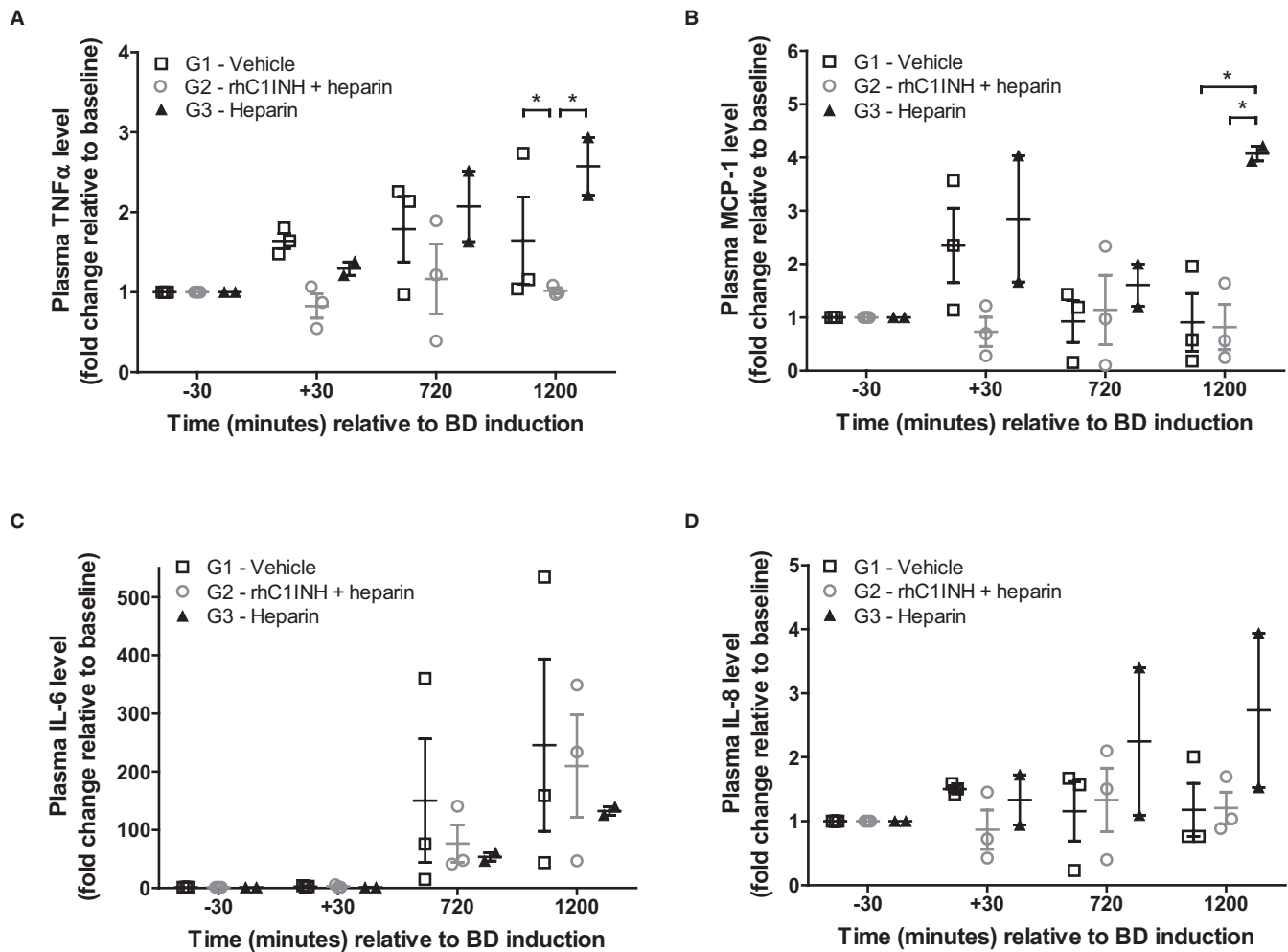


FIGURE 5 rhC1INH treatment limits systemic levels of tumor necrosis factor (TNF) α and monocyte chemoattractant protein 1 (MCP-1) in brain-dead (BD) donors. Plasma levels of (A) interleukin (IL)-6, (B) IL-8, (C) TNF α , and (D) MCP-1 in donors from G1 (vehicle, $n = 3$), G2 (rhC1INH + heparin, $n = 3$), and G3 (heparin, $n = 2$). Data expressed as fold change relative to baseline value; significance is calculated by 1-way ANOVA and Bonferroni's post-hoc correction ($*P < .05$)

activation during BD remain unclear, studies using knock-out technology and other complement-intervention strategies during IRI and BD have shown reduced tissue inflammation and improved renal function after reperfusion in multiple models.^{19,24-26,36-40} Poppelaars et al²⁵ showed that treatment of BD rats with rhC1INH resulted in reduced renal mRNA expression and serum levels of IL-6, improved renal function, and reduced renal injury prior to transplantation. These observations are supported further by multiple studies demonstrating the protective anti-inflammatory effect of C1 blockade in models of sepsis, as well as renal, neurological, myocardial, and intestinal IRI.^{16,41-46} Although rodent models of renal IRI indicate a predominant role for the alternative pathway in complement-mediated renal injury, recent reports suggest that CP and LP are critical in the pathogenesis of IRI, DGF, and acute rejection in large animal models and humans.^{35,37,47-50}

Interactions between C1INH and heparin have been reported to augment rhC1INH activity 5- to 11-fold and potentiate the inhibitory effect on C1-dependent activation of the complement cascade.³⁰ The synergistic effect between rhC1INH and heparin has

been previously shown to enhance inhibition of the CP, LP, and AP in human samples in a dose-dependent fashion.³¹ We exploited this interaction to maximize complement inhibition in donors in our model. Heparin is known to inhibit neutrophil adhesion, chemotaxis, and reactive oxygen species production.⁵¹ The use of heparin to ameliorate IRI remains controversial. Sedigh et al⁵² recently demonstrated the use of a heparin conjugate during hypothermic machine perfusion to reduce cold preservation injury and improve organ function shortly after reperfusion. In a sheep model of IRI, Shin et al⁵³ observed that heparin therapy significantly attenuated neutrophil infiltration within the interstitium but did not affect the degree of renal damage or renal function compared with animals that did not receive treatment. Our findings here show that recipients of kidneys from donors treated with a high dose of heparin alone (G3) experienced similar, if not worse, tubular injury through complement deposition compared with controls (G1). In addition, we did not observe differences in neutrophil or monocyte infiltration of the grafts between the 3 different groups at the time of organ recovery or during transplant (Figure S1).

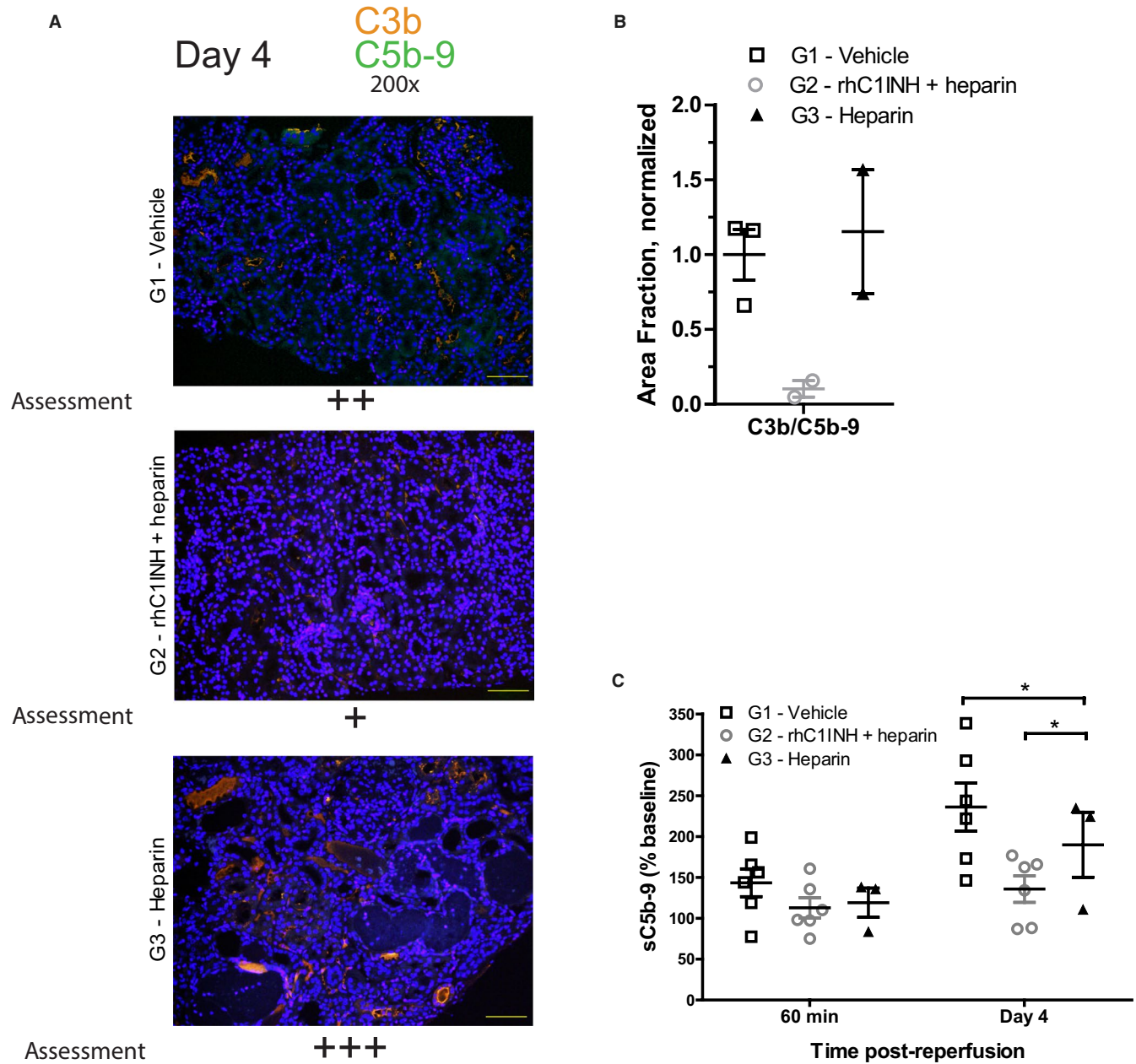
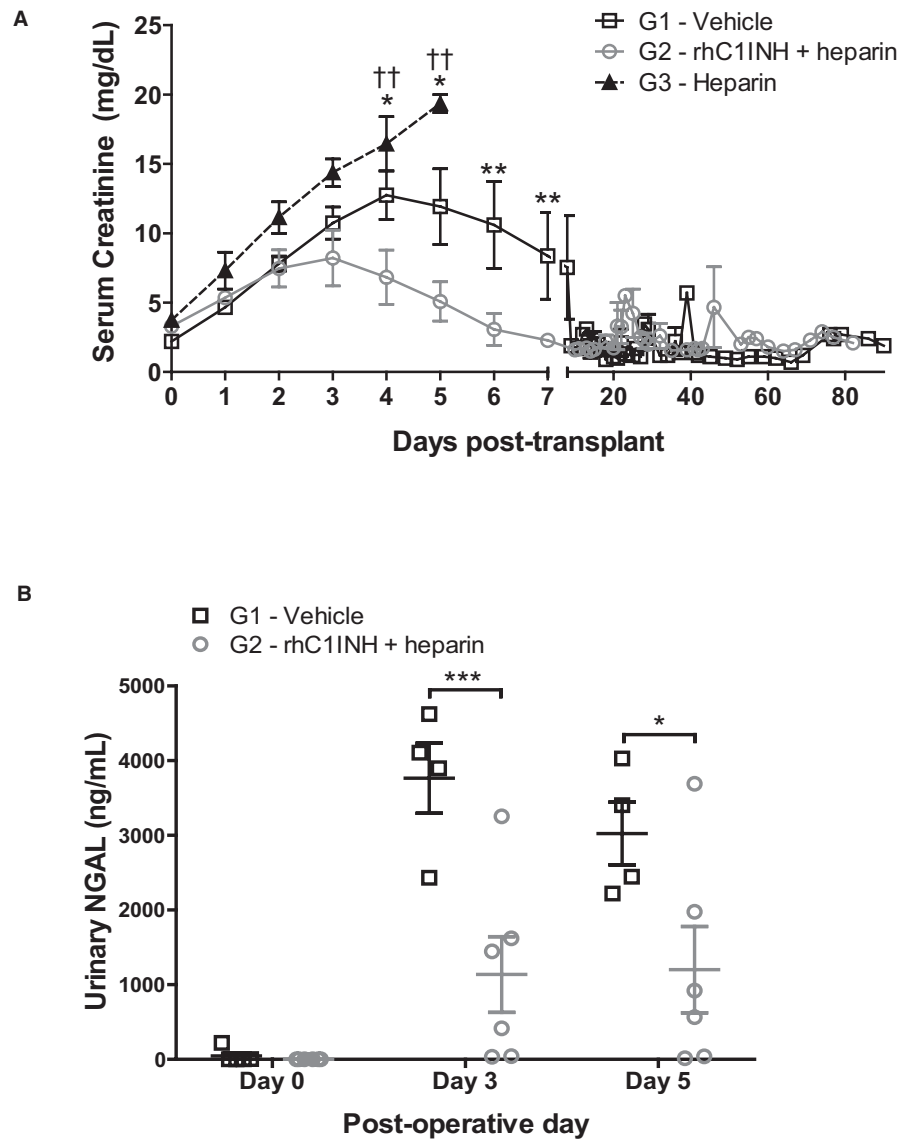


FIGURE 6 rhC1INH treatment in brain-dead (BD) donors reduces C3b/C5b-9 deposition and circulating sC5b-9 in transplant recipients during the first postoperative week. A, Representative micrographs at 200 \times magnification depicting C5b-9 (green) and C3b (orange) combined deposition by immune-fluorescent staining in kidney biopsies obtained from G1 (vehicle), G2 (rhC1INH + heparin), and G3 (heparin) grafts at day 4 posttransplant; semiquantitative assessment. B, Quantitative assessment of C3b/C5b-9 deposition at day 4 posttransplant—G1 (vehicle, $n = 3$), G2 (rhC1INH + heparin, $n = 3$), and G3 (heparin, $n = 2$)—G3 biopsy was excluded by outlier test; data expressed as area fraction normalized to G1-vehicle average \pm SEM. C, Serum levels of sC5b-9 in recipients of kidney grafts from donors in G1 (vehicle, $n = 6$), G2 (rhC1INH + heparin, $n = 6$), and G3 (heparin, $n = 3$) at 60 minutes and day 4 posttransplant, analyzed by ELISA, data expressed as percent value relative to baseline (pretransplant). Significance is calculated by 1-way ANOVA and Bonferroni post-hoc correction ($*P < .05$)

Remarkable in our model is the effect observed in the group treated with rhC1INH + heparin (G2) in which any possible deleterious effect of heparin is superceded by the enhanced effect of complement inhibition when combined with heparin. Our treatment with rhC1INH + heparin in BD donors led to a significant decrease in CP activity as well as a decrease in systemic release

of TNF α and MCP-1, potent proinflammatory mediators known to enhance innate immune cell trafficking and amplify inflammatory response.⁵⁴ Although we did not observe a reduction in the level of neutrophil and macrophage infiltration, we did note reduced tissue deposition of C3b/C5b-9 in renal grafts from treated donors both at organ recovery and during the first week posttransplant,

FIGURE 7 rhC1INH treatment in brain-dead (BD) donors improves graft function and reduces kidney injury and incidence of delayed graft failure in transplant recipients. A, Serum creatinine levels in recipients of kidney grafts from donors in G1 (vehicle, $n = 6$), G2 (rhC1INH + heparin, $n = 6$), and G3 (heparin, $n = 3$). Data are expressed as mean \pm SEM values, significance is calculated by 2-way ANOVA and Bonferroni post-hoc correction. B, Urinary NGAL measured at baseline, day 3, and day 5 posttransplant in recipients of kidney grafts from donors in G1 (vehicle, $n = 6$), G2 (rhC1INH + heparin, $n = 6$), and G3 (heparin, $n = 3$). Data are expressed as mean \pm SEM values, significance is calculated by Student t test. * $P < .05$, ** $P < .01$, *** $P < .001$, G1 vs G2; †† $P < .01$, G2 vs G3



although our observations lacked sufficient power to demonstrate a significant difference between groups. Nevertheless, these results correlate to observations in humans and animal models of inflammatory injury and C1INH administration.^{16,55,56} The formation and deposition of C5b-9 has been directly linked to tubular epithelial injury and characterized by tubular thinning, protein cast formation, and tubular dilation in IRI.⁵⁷ Selective blockade of the CP with rhC1INH has previously been shown to prevent acute tubular damage in a porcine model of renal warm IRI.^{16,58} Furthermore, circulating sC5b-9 has been proposed as a biomarker of tissue injury and AKI severity.⁵⁹ These data correlate to our observation of reduced circulating sC5b-9 coupled to the previously indicated superior renal function in recipients of rhC1INH-treated donors by day 4 posttransplant. As stated previously, the ischemic and inflammatory environment recreated by this model is likely much more severe than that of marginal grafts currently used for transplant. As such, our observations on the reduction of DGF and improved kidney function may translate in the form of an even larger advantage in standard clinical practice with nonmarginal donors.

Transplant recipients who experience DGF are at increased risk of graft rejection and reduced graft survival.⁴ We used a fully mismatched model of renal transplantation after BD to reduce the potential for immune-tolerant regulation providing accessory protection to the graft in the posttransplant period. We documented the expected onset of acute cellular rejection and AMR in grafts that survived the DGF period (data not shown); however, we noted a significant increase in graft survival in recipients of rhC1INH-treated donors (Table 1). Although donor treatment with rhC1INH + heparin did not abrogate development of graft rejection, it did reduce the inflammatory state of rhC1INH-treated donors and led to superior posttransplant renal function and reduced incidence of DGF in their recipients. This observation matches clinical studies demonstrating that the inflammatory state of BD donors and the development of DGF are both independently associated with progression to acute rejection.^{2,4,6-9,60}

However, clinical data on the protective effect of C1 inhibition in the context of IRI and DGF are limited. Jordan et al.⁶¹ recently published the results of a phase I/II trial showing that

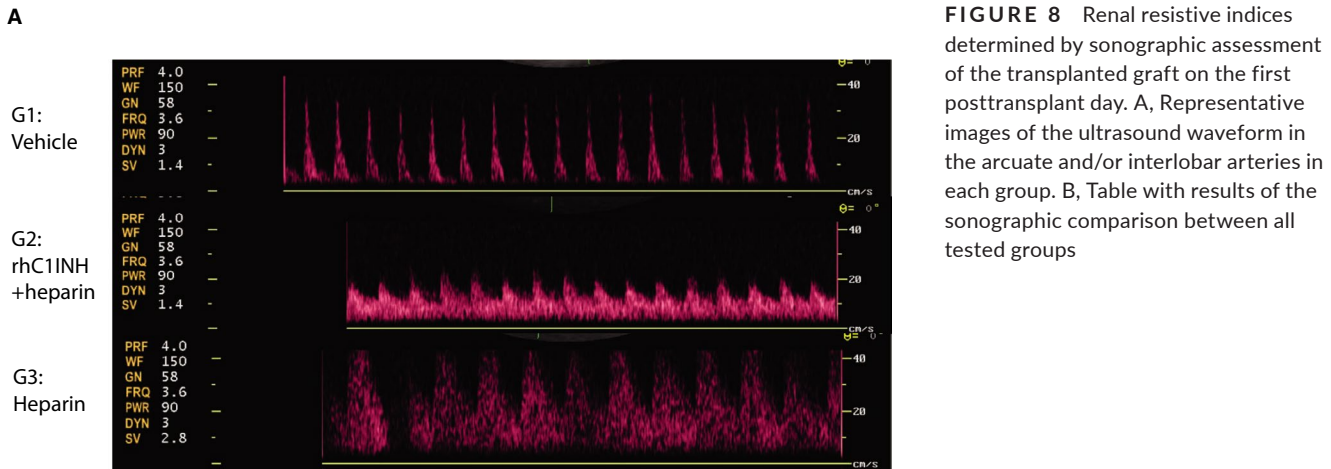


FIGURE 8 Renal resistive indices determined by sonographic assessment of the transplanted graft on the first posttransplant day. A, Representative images of the ultrasound waveform in the arcuate and/or interlobar arteries in each group. B, Table with results of the sonographic comparison between all tested groups

B
Ultrasound analysis of renal grafts on post-transplant day 1

Resistive Indices	G1: Vehicle	G2: rhC1INH +heparin	G3: Heparin	P value
High	4/4 (100%)	0/4 (0%)	0/2 (0%)	0.006
Normal	0/4 (0%)	4/4 (100%)	2/2 (100%)	

patients receiving C1INH required fewer dialysis sessions in weeks 2-4 posttransplant and had superior renal function 12 months after surgery; this effect was most significant in those receiving low-quality grafts. These encouraging results support complement blockade in the peritransplant period as a valid and attractive approach to protect kidneys from IRI, prevent dysfunction, and improve long-term renal function after transplant. Our unique strategy of using rhC1INH at the level of the donor for the prevention of posttransplant DGF could be coupled to a recipient treatment regimen, which may produce a synergistic effect that could constitute a valuable strategy for the prevention of DGF and potentially reduce immunogenicity in the graft.

The significance of our study resides in the novel approach of donor pretreatment targeting complement inhibition with rhC1INH and heparin as a strategy to prevent DGF in kidney transplant recipients in a clinically relevant model of BD in older donors, prolonged cold ischemia, and allo-transplant in NHP. Our results indicate that treatment with rhC1INH and heparin during BD limits systemic and local activation of the complement system and the inflammatory response, providing a protective effect in the host kidneys that translates into reduced risk of DGF and improved transplant outcomes. Successful clinical implementation of these findings could vastly increase the pool of acceptable donors, reduce DGF rates, improve graft life and patient survival, and decrease morbidity and cost of care associated with kidney transplantation. Although our focus has been on kidney transplant, the positive impacts may encompass other transplantable organs as well. Further investigations into the mechanism of action of donor pretreatment with rhC1INH and heparin, particularly in regard to other organs, as well as clinical trials on the effectiveness of targeting the complement system at the donor level, are warranted to further validate these results.

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DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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