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Targeted Donor Complement Blockade After Brain-Death Prevents Delayed Graft Function In A Non-Human Primate Model Of Kidney Transplantation.

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Abstract

Delayed graft function (DGF) in renal transplantation is associated with reduced graft survival and increased immunogenicity. The complement-driven inflammatory response after brain death (BD) and post-transplant reperfusion injury play significant roles in the pathogenesis of DGF. In a non-human primate model, we tested complement-blockade in BD donors to prevent DGF and improve graft survival. BD donors were maintained 20-hours, kidneys were procured and stored at 4°C for a 43–48 hours prior to implantation into ABO-compatible, non-sensitized, MHC-mismatched recipients. Animals were divided into three different donor-treatment groups: G1-Vehicle, G2-rhC1INH+heparin, and G3-heparin only. G2 donors showed significant reduction in classical complement pathway activation and decreased levels of TNFα and MCP-1. DGF was diagnosed in 4/6 (67%) G1-recipients, 3/3 (100%) of G3-recipients, and 0/6 (0%) of G2-recipients (p=0.008). In addition, G2-recipients showed superior renal function, reduced sC5b-9, and reduced urinary NGAL in the first week post-transplant. We observed no differences in incidence or severity of graft rejection between groups. Collectively, the data indicate that donor-management targeting

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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.

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 peri-transplant period, development of DGF is an important risk factor for acute cellular rejection (ACR), 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 neuro-hormonal changes known to trigger a systemic inflammatory response characterized by the release of pro-inflammatory cytokines from both innate and adaptive immune cells, including interleukin-1-beta (IL-1β), tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), and interleukin-17 (IL-17), chemokines and reactive oxygen species (ROS) and reactive nitrogen species including Nitric Oxide (NO). 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 transplantation 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 post-transplant kidney dysfunction and modulation of immune responses. We utilized a non-human primate (NHP) model of BD in older animals, prolonged cold ischemia, and transplantation into non-sensitized, fully-mismatched recipients to investigate the potential protective effect of this donor-management strategy to improve outcomes in kidney transplant 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 University of Wisconsin Primate Center (WNPRC) and Alpha Genesis Inc.(Yemassee, SC) . All animals were pre-screened negative for tuberculosis, Herpes B, SRV, SIV, and STLV-1. Each donor–recipient pair was ABO blood compatible, non-sensitized, and fully mismatched for major histocompatibility complex (MHC) class I and II alleles identified using microsatellite analysis as previously described (data not reported).[27] Animals were housed in accordance with NIH and USDA 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

Recombinant human C1 Inhibitor (rhC1INH) was provided by Pharming Technologies B.V. (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 in order to maintain hemodynamic stability and adequate oxygen delivery.[11,28] Briefly, donor animals were anesthesized, ventilated and monitored. A 16F Foley catheter was placed in the extradural space of the cranial fossa (i.e. the intra-cranial 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 IV fluid resuscitation and vasopressor support to achieve a stable mean arterial pressure and urinary output. Twenty hours after brain death 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) by intravenous bolus injection given at t= 180, 360, 540, 720, 900, and 1080 minutes. G2 donors received rhC1INH (500 U/kg/dose) treatment by intravenous bolus injection at the indicated time points in combination with continuous intravenous heparin infusion t=180 → t=1080 minutes titrated to a partial thromboplastin time (PTT) of 80–120 seconds. G3 donors received only continuous intravenous heparin infusion as described with dosing titrated to PTT 80–120 seconds (Figure 1a). Heparin was used in recipients of G2 to potentiate the activity of rhC1INH as described previously.[29–31]

All recipients underwent bilateral native nephrectomy at the time of transplant and heterotopic kidney transplantation was performed as described previously (Figure 1b).[32] Recipients were monitored for acid/base and electrolyte balance, serum BUN (blood urea nitrogen), creatinine, urinary output, proteinuria and behavioral abnormalities. No induction therapy was used, but maintenance therapy included mycophenolate-mofetil (MMF), tacrolimus, and prednisone. Tacrolimus levels were dose-adjusted bi-weekly 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 delayed graft function

DGF was defined as a failure of a fall in serum creatinine of at least 10% on 3 consecutive days in the first post-transplant week and/or serum creatinine at post-transplant day 7 >2.5 mg/dL.[1, 33]

2.4 Circulating Cytokines

Circulating levels of IL-6, IL-8, MCP-1, and TNFα in EDTA-plasma were measured with Biolegend LEGENDplex™ NHP Mix-and-Match Subpanel according to manufacturer recommended protocols.

2.5 Complement assessment

Blood was collected into Vacuettes® (Greiner Bio-One, Austria), serum tubes were allowed to clot 15 minutes prior to centrifugation for 10 minutes at 3000xg, 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). CP, AP, and LP activation were tested using the Wieslab Complement Kits (CP310, AP330, MP320, EuroDiagnostica, Sweden). Circulating levels of sC5b-9 (membrane attack complex, MAC) were measured using a commercially available sC5b-9 enzyme immunoassay (ELISA kit; Quidel, San Diego, CA). Measured values for each assay were normalized to serum albumin (VetTest Analyzer, Idexx, Westbrook, ME) 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 biopsies were collected from grafts prior to cold ischemia after the 20-hour BD period, as well as 60 minutes and also 7 days post-reperfusion, and finally at necropsy. Biopsies were also collected from the naïve native kidneys removed from recipients during the operation prior to graft reperfusion. Tissue was fixed in 10% formalin and embedded in paraffin or frozen in optimal cutting temperature (OCT) compound. 4μm slices were mounted onto slides and stained for histological assessment. Stains included hematoxylin and eosin (H/E), periodic acid–Schiff (PAS), Picro Sirius Red for estimation of fibrosis, as well as antibodies against CD68 (KP1, DAKO-Agilent), myeloperoxidase (MPO, Abcam), malondialdehyde (MDA, Abcam), and C4d, C3b, and C5b-9 (Ventana-Roche, AZ). The HIER method was used for antigen retrieval (BioGenex, San Ramon, CA). Slides for histopathology were interpreted by a renal pathologist. For immunohistochemistry, images were acquired from 6–12 random fields within each slide at appropriate magnification using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and processed using ImageJ software (NIH, Bethesda, MD) 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 (NGAL) measurement

Post-transplant urine was stored at −80˚C until analysis. Urinary 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 ± standard error of the mean (SEM) or standard deviation (SD). DGF incidence and resistive indices were analyzed by chi-square test. Comparisons of two groups were tested by two-tailed Student's T-test. Differences between 3 or more groups were tested by one-way ANOVA and Bonferroni's post-test correction or Kruskal-Wallis test and Dunn's post-test correction in data sets with non-normal distribution. Data sets with two independent variables were tested by two-way ANOVA followed by Bonferroni's correction. Differences between treatment groups were considered significant at $p<0.05$.

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.

Over a 20-hour BD period, donor animals received six bolus intravenous administrations of either vehicle (Group 1, G1) or rhC1INH with continuous heparin infusion (Group 2, G2), or continuous heparin infusion only (Group 3, G3) starting at 3 hours after induction of BD, until 2 hours prior to organ recovery (Figure 1a, Table 1). To confirm the BD state, we monitored the hemodynamics of BD donors throughout this 20-hour period 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 as well as 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 prior to 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 post-induction showed significantly higher levels when compared to 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 (Figures 3c) showed wide variation and was not statistically different between groups. Activation of AP (Figures 3d) was significantly lower in G3 compared to G1 at both 720 ($p<0.05$) and 1200 minutes ($p<0.01$). We investigated complement activation in donors by analyzing C3b/C5b-9 deposition in kidney biopsies obtained immediately after the 20-hour BD period. Immunofluorescence analysis of G2 donor kidneys revealed minimal C5b-9 and

C3b deposition compared to increased staining of both markers in G1 and G3 kidneys prior to 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 TNFα and MCP-1 in BD donors.

We assessed levels of interleukin-6 (IL-6), TNFα, interleukin-8 (IL-8), and monocyte chemoattractant protein 1 (MCP-1) as pro-inflammatory cytokines and chemokines implicated in the acute inflammatory response to tissue injury and trauma during the BD period. We documented significantly lower levels of TNFα and MCP-1 in G2 donors compared to 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, non-sensitized, MHC fully-mismatched recipients who underwent bilateral nephrectomy of their native kidneys and received post-transplant maintenance immunosuppression (Figure 1b). We obtained biopsies from the grafts at 60 minutes and day 4 post-transplant and analyzed these for innate immune cell infiltration, complement deposition, and oxidative damage, and compared these to biopsies collected from recipient native kidneys. Due to concern for the well-being of the animals, biopsies were limited to only 2–3 recipients per group. In biopsies collected 60 minutes post-reperfusion, 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). Day 4 post-transplant, biopsies from recipients of G2 donor kidneys seemed to display lower levels of C3b/C5b-9 deposition compared to 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 post-transplant and found that values at 60 minutes were again equivalent for all experimental groups, while day 4 levels demonstrated significantly less sC5b-9 in recipients of G2 grafts compared to recipients of G1 and G3 grafts (Figure 6c, $p<0.05$). 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 post-transplant 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 ($p=0.0081$) (Table 1). Post-transplant 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 post-transplant ($p<0.05$, Figure 7a) and lower peak serum creatinine vs. G3 ($p<0.05$, Table 2) indicating superior renal function. In addition, G2 kidney recipients presented lower levels of urinary neutrophil gelatinase-associated lipocalin (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 post-transplant 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 post-transplant day 1 compared to 0/4 kidneys from G2 donors ($p=0.006$, Figure 7). Further, kidneys from G2 donors exhibited significantly longer graft survival compared to those from G1 and G3 donors ($p<0.05$, 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 post-transplant 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 stables for 20 hours prior to organ procurement, 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 last 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. While the mechanisms leading to complement 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 al.* showed that treatment of brain-dead 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.[25] 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 5–11 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 al.* recently demonstrated utilization of a heparin conjugate during hypothermic machine perfusion to reduce cold preservation injury and improve organ function shortly after reperfusion.[52] In a sheep model of IRI, Cheung Soo Shin, *et al.* observed that heparin therapy significantly attenuated neutrophil infiltration within the interstitium but did not affect the degree of renal damage or renal function as compared to animals that did not received treatment.[53] Our findings here show that recipients of kidneys from donors treated with a high dose of heparin alone (G3) suffer similar if not worse tubular injury through complement deposition when compared to controls (G1). In addition, we did not observe differences in neutrophil or monocyte infiltration of the grafts between the three different groups at the time of organ recovery or during transplantation (Figure S1).

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 TNFα and MCP-1, potent pro-inflammatory mediators known to enhance innate immune cell trafficking and amplify inflammatory response.[54] While 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 post-transplant, 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 post-transplant renal function in recipients of rhC1INH-treated donors by day 4 post-transplant. 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 transplantation. 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 non-marginal donors.

Transplant recipients who experience DGF are at increased risk of graft rejection and reduced graft survival.[4] We utilized 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 post-transplant period. We documented the expected onset of ACR 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). While 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 post-transplant 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 is limited. Jordan, *et al.* recently published the results of a phase I/II trial showing that patients receiving C1INH required fewer dialysis sessions in weeks 2–4 post-transplant and had superior renal function 12 months after surgery; this effect was most significant in those receiving low quality grafts.[61] These encouraging results support complement blockade in the peri-transplant period as a valid and attractive approach to protect kidneys from IRI, prevent dysfunction and improve long-term renal function after transplantation. Our unique strategy of using rhC1INH at the level of the donor for the prevention of post-transplant DGF could be coupled to a recipient treatment-regimen which may produce a synergistic effect that could constitute a valuable strategy for prevention of DGF and also potentially reduce immunogenicity in the graft.

The significance of our study resides in the novel approach of donor pre-treatment targeting complement inhibition with rhC1INH and heparin as a strategy to prevent DGF in kidney transplantation recipients in a clinically relevant model of BD in older donors, prolonged cold ischemia, and allo-transplantation 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 to 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 to kidney transplantation. While our focus has been on kidney transplantation, the positive impacts may encompass other transplantable organs as well. Further investigations into the mechanism of action of donor pre-treatment 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations:

ACR	Acute cellular rejection
AKI	Acute kidney injury
AMR	Antibody-mediated rejection
ANOVA	Analysis of variance
AP	Alternative (complement) pathway
BD	Brain death
BUN	blood urea nitrogen
C1INH	C1 inhibitor
CP	Classic (complement) pathway
DGF	Delayed graft function
ELISA	enzyme-linked immunosorbent assay
HAE	hereditary angioedema
H/E	Hematoxylin and Eosin
IFNγ	interferon gamma
IL-1β	interleukin-1-beta
IL-17	interleukin-17
IL-6	interleukin-6
IL-8	interleukin-8
IRI	ischemia-reperfusion injury
LP	mannose-binding lectin (complement) pathway
MAC	membrane attack complex
MAP	Mean arterial pressure
MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MMF	mycophenolate-mofetil

NGAL	neutrophil gelatinase-associated lipocalin
NHP	Non-human primate
NO	Nitric oxide
PTT	partial thromboplastin time
rhC1INH	recombinant human C1 esterase inhibitor
ROS	reactive oxygen species
SD	standard deviation
SEM	standard error of the mean
SRV	Simian type D retrovirus
SIV	Simian immunodeficiency virus
STLV-1	Simian T-cell leukemia virus type 1
TNFα	Tumor necrosis factor alpha

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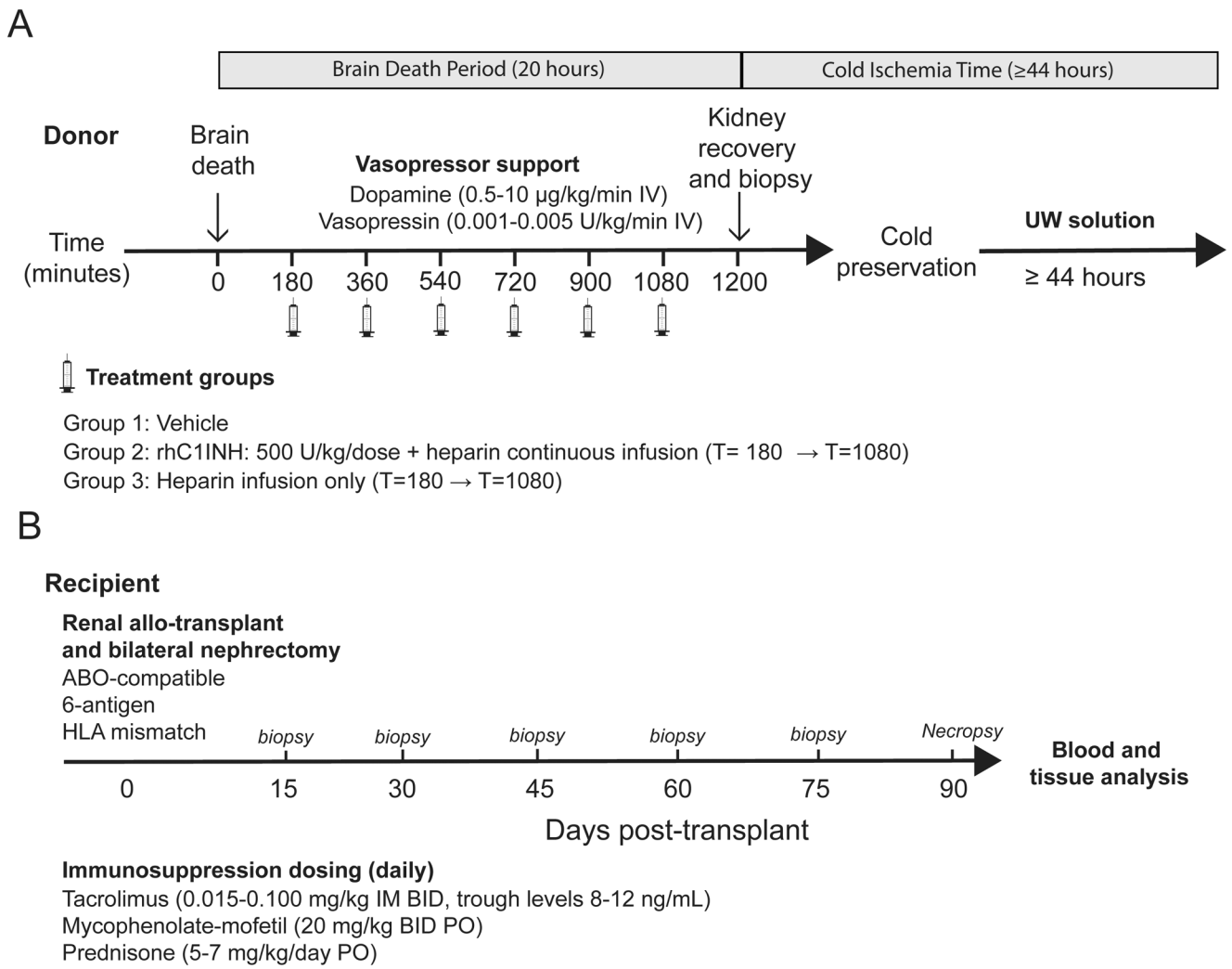


Figure 1. Experimental design.

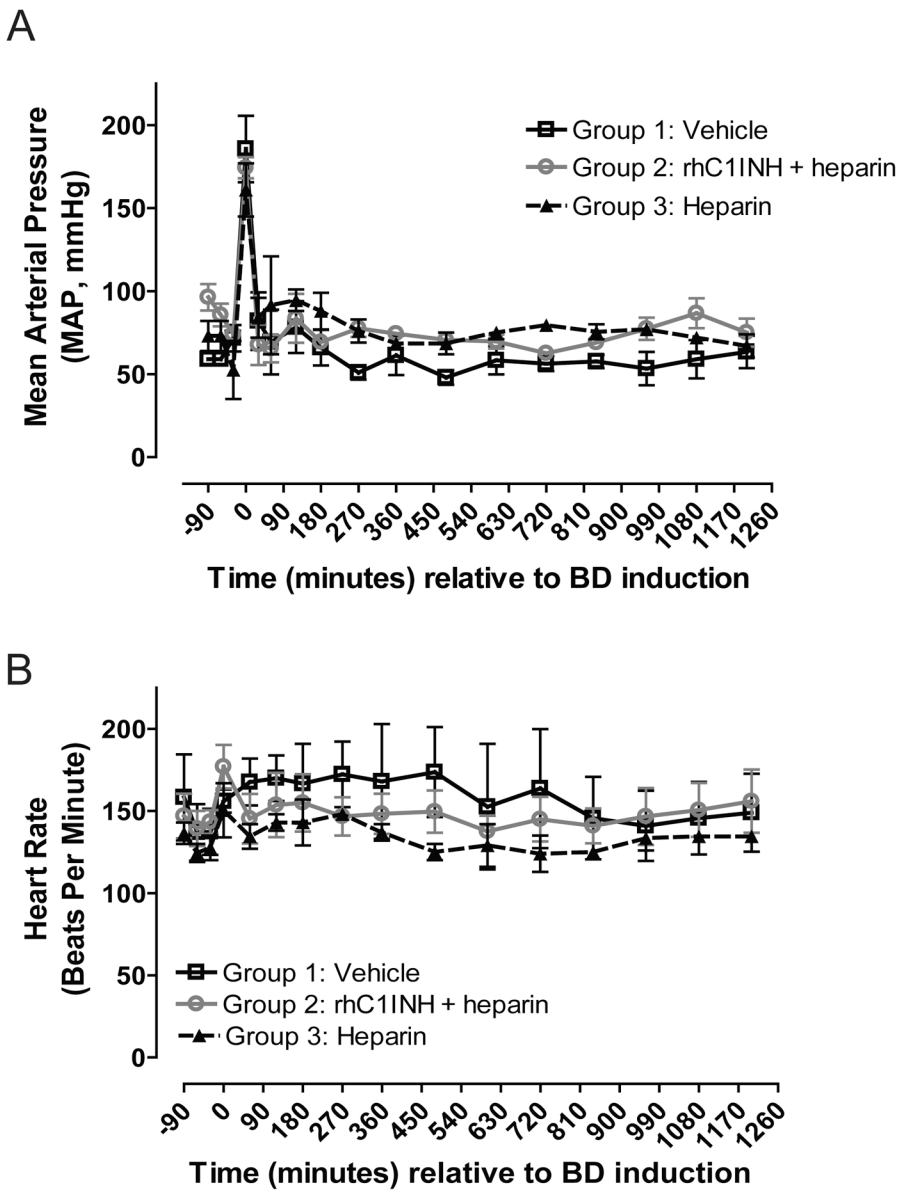


Figure 2.

Hemodynamic assessment of brain-dead 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.

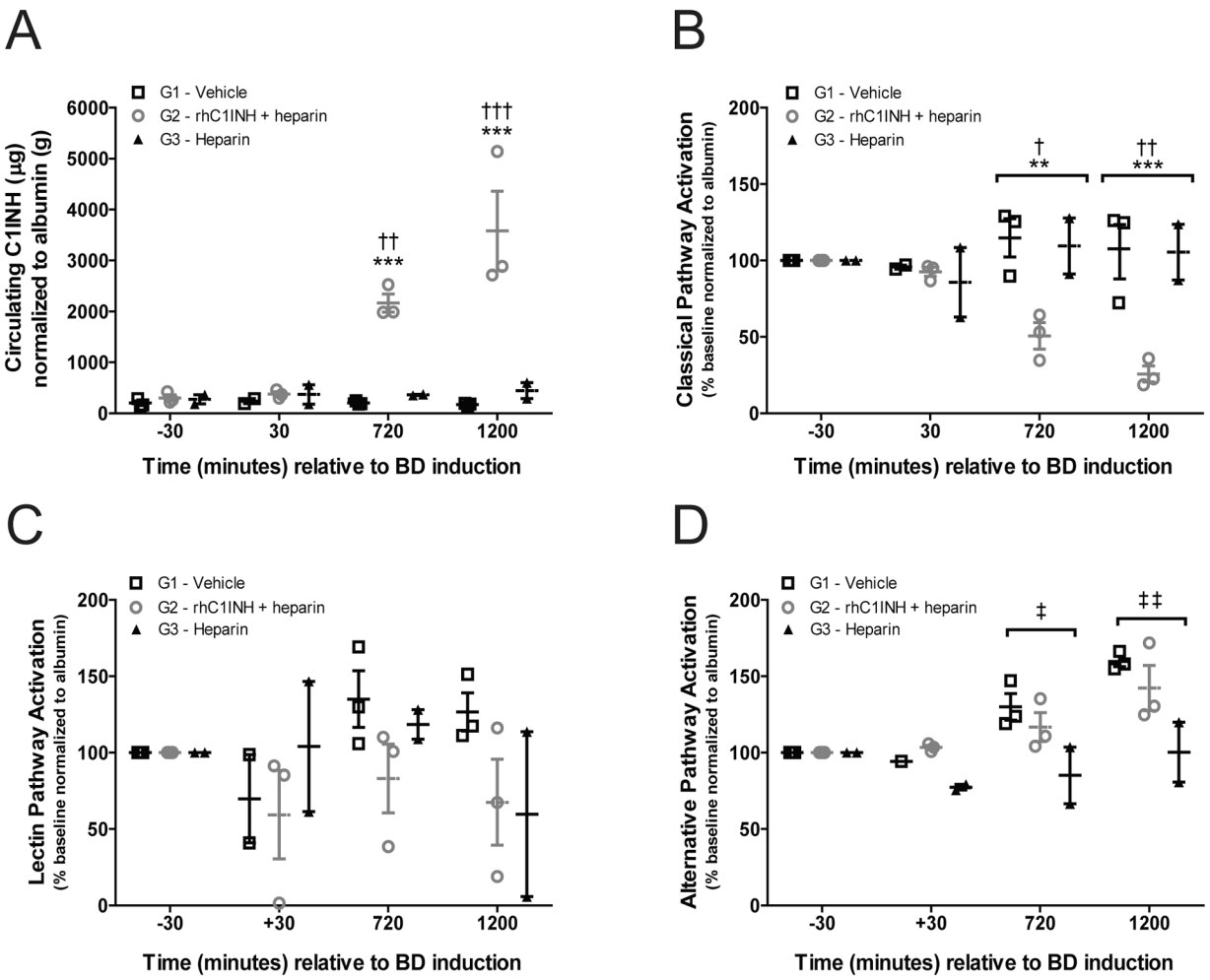


Figure 3. rhC1INH treatment results in sustained elevation of circulating C1INH and inhibits complement activation in the BD donor.

(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, five 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) CP, (c) LP, and (d) AP 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 values ±SEM, significance calculated by two-way ANOVA and Bonferroni's post-hoc correction (**p<0.01 G1 vs G2; ***p<0.001 G1 vs G2; †p<0.05 G2 vs G3; ††p<0.01 G2 vs G3; †††p<0.001 G2 vs G3).

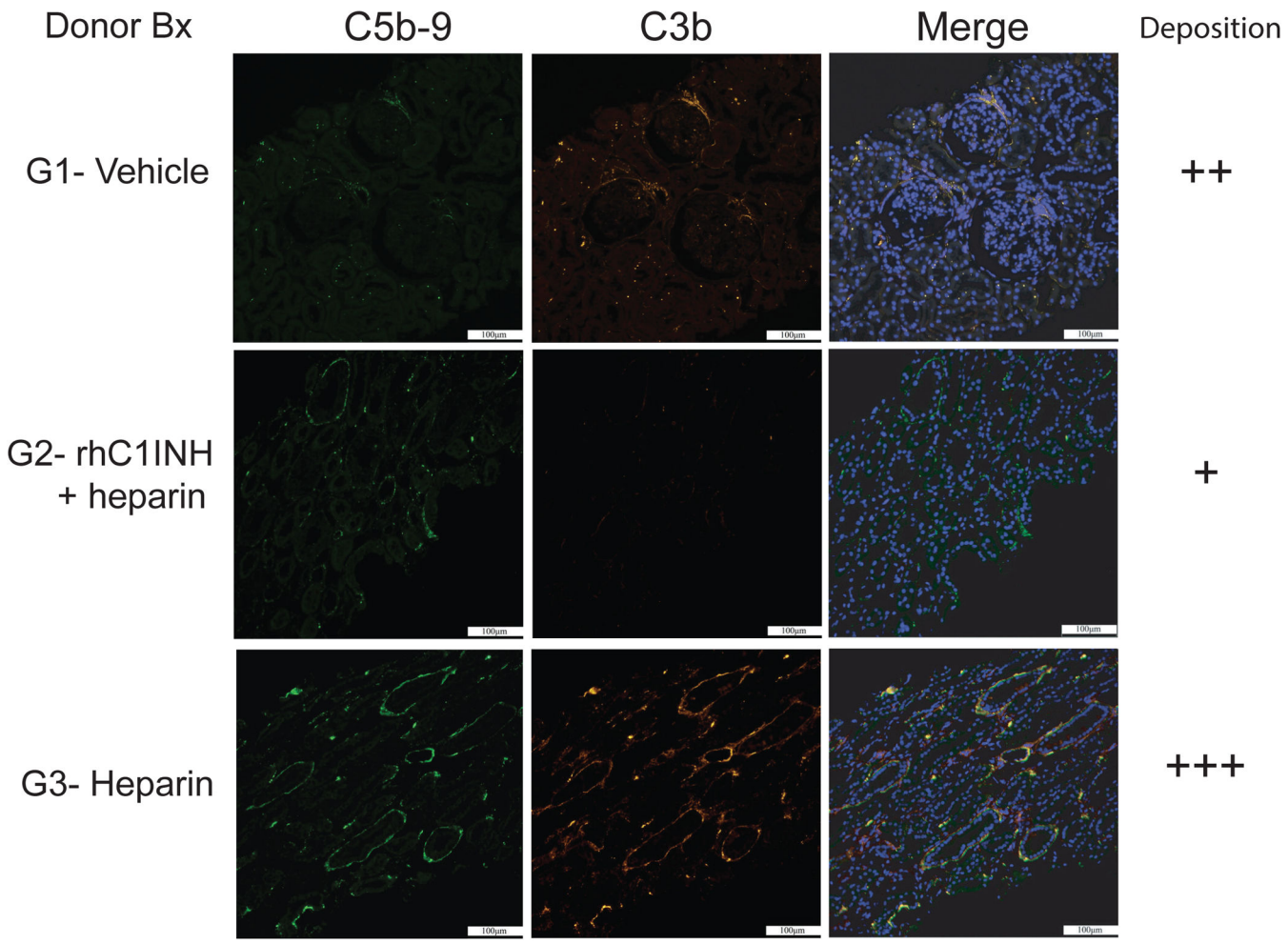


Figure 4. rhC1INH treatment reduces C3b/C5b-9 deposition in the BD donor graft.

(a-b) Representative micrographs at 200X magnification depicting C5b-9 (green) and C3b (orange) deposition by immune-fluorescent 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.

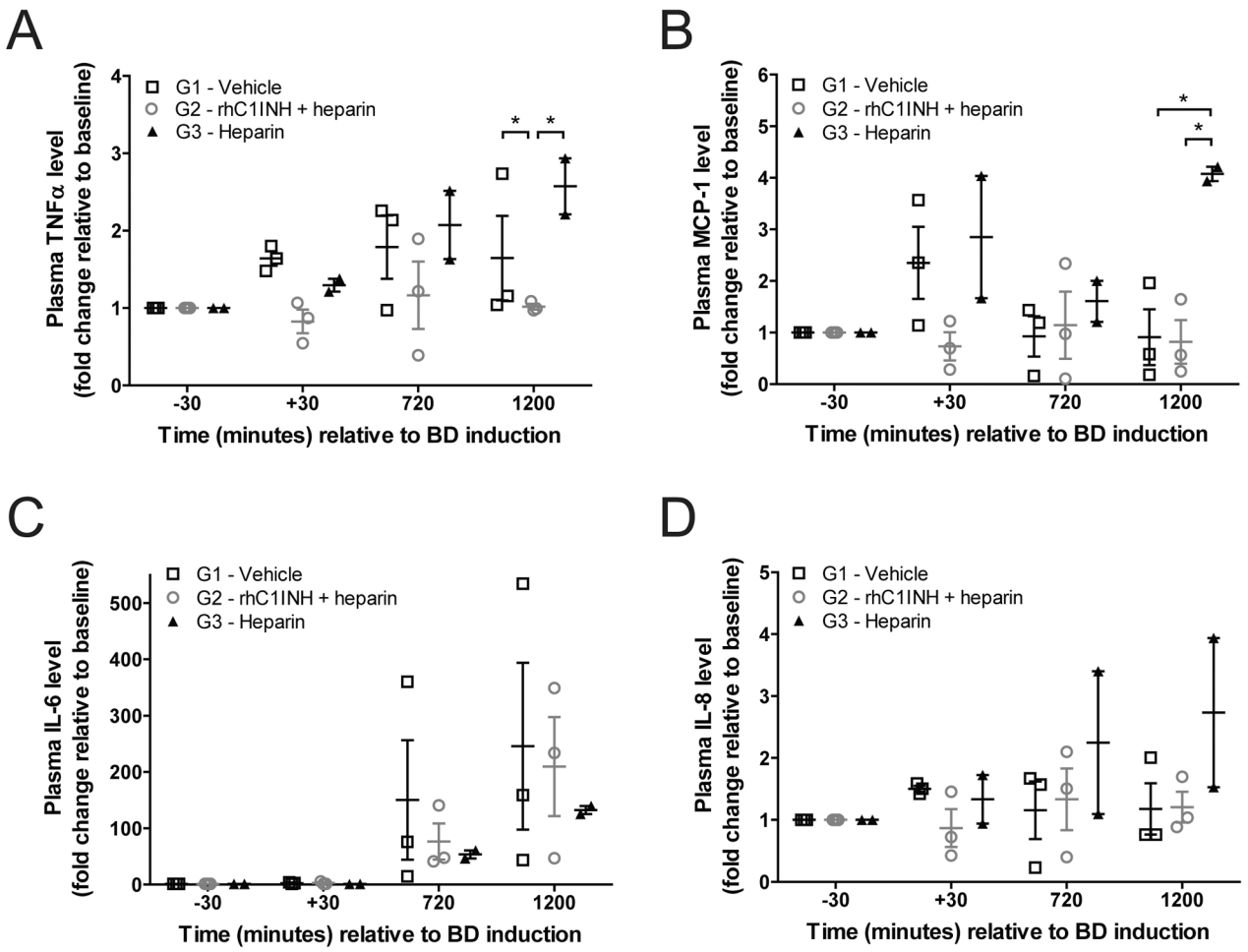


Figure 5. rhC1INH treatment limits systemic levels of TNFα and MCP-1 in BD donors.

Plasma levels of (a) IL-6, (b) IL-8, (c) TNFα, 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 one-way ANOVA and Bonferroni's post-hoc correction (*p<0.05).

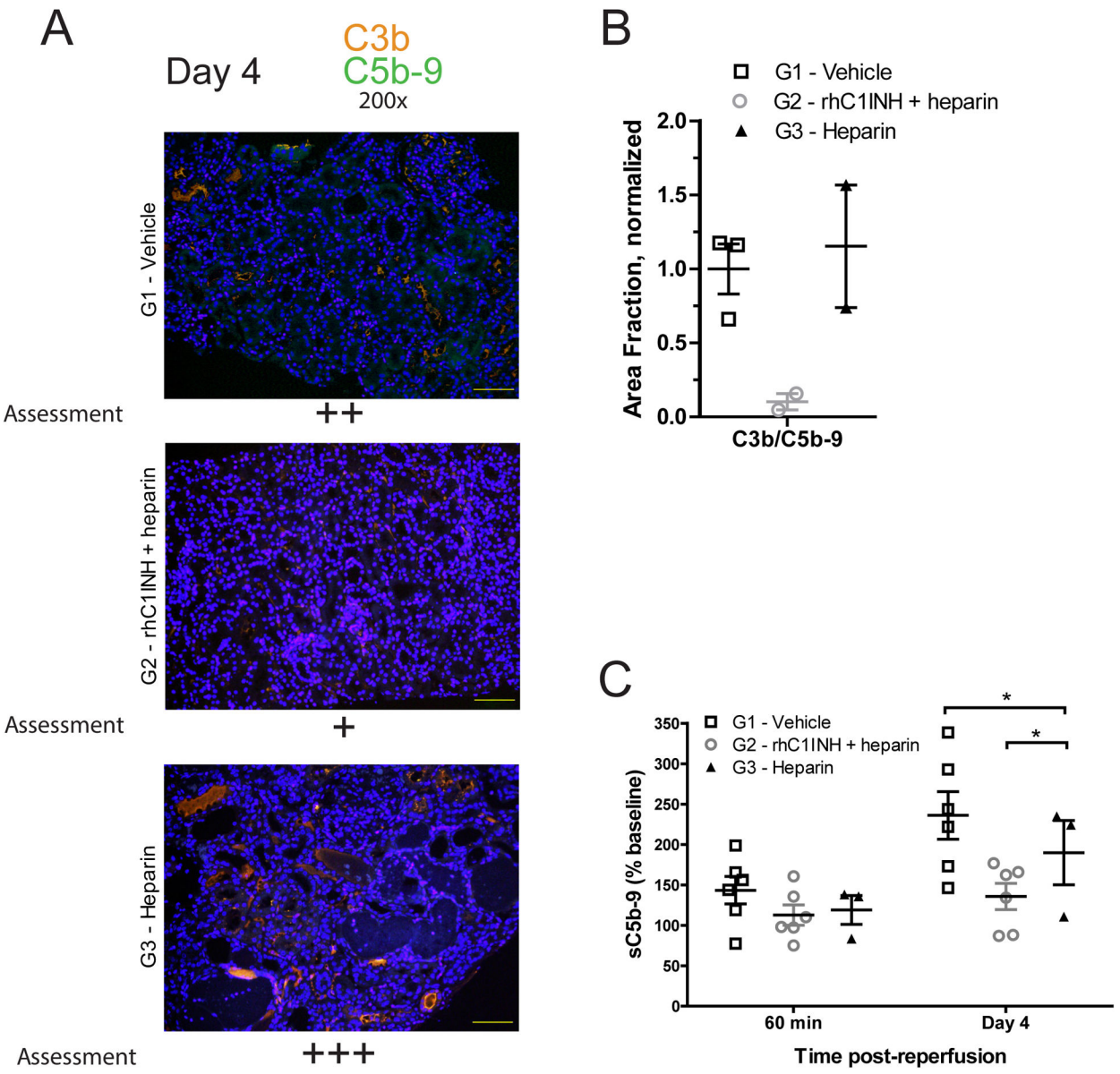


Figure 6. rhC1INH treatment in BD donors reduces C3b/C5b-9 deposition and circulating sC5-b9 in transplant recipients during the first post-operative week.

(a) Representative micrographs at 200X 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 post-transplant; semiquantitative assessment. (b) Quantitative assessment of C3b/C5b9 deposition at day 4 post-transplant - G1 (vehicle, n=3), G2 (rhC1INH+heparin, n=3), and G3 (heparin, n=2), one G3 biopsy was excluded by outlier test. Data expressed as area fraction normalized to G1-Vehicle average ±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 post-transplant, analyzed by ELISA, data expressed as percent value relative to baseline (pre-transplant). Significance is calculated by one-way ANOVA and Bonferroni's post-hoc correction (*$p<0.05$).

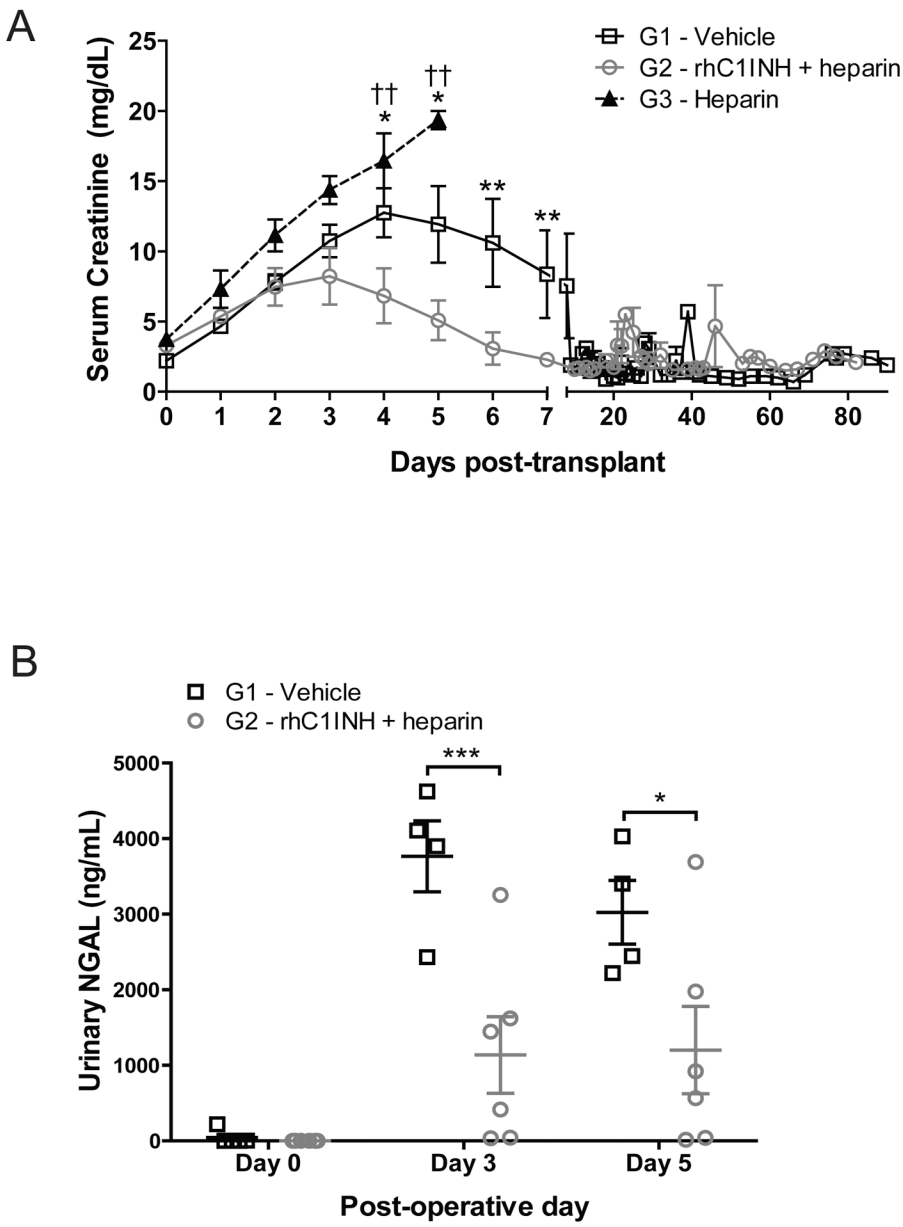
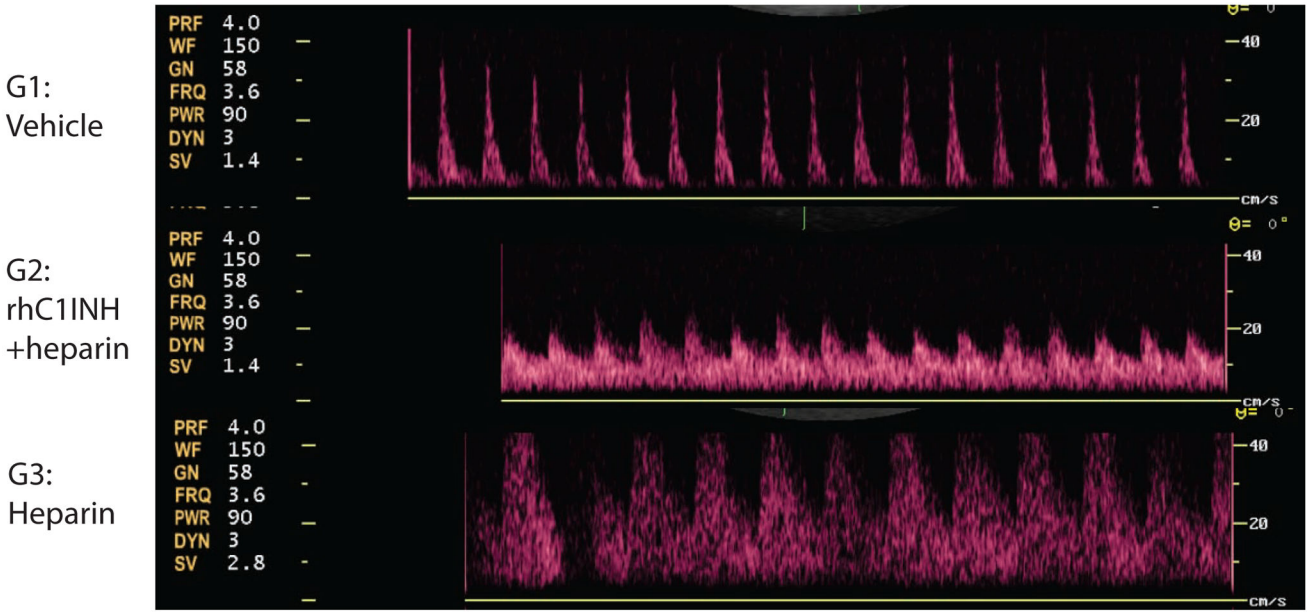


Figure 7. rhC1INH treatment in BD donors improves graft function and reduces kidney injury and incidence of DGF in transplant recipients.

(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 values ±SEM, significance is calculated by two-way ANOVA and Bonferroni's post-hoc correction (*p<0.05, **p<0.01, G1 vs G2; ††p<0.01, G2 vs G3). (b) Urinary NGAL measured at baseline, day 3, and day 5 post-transplant 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 values ±SEM, significance is calculated by Student's T-test.

A



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%)	

Figure 8. Renal resistive indices determined by sonographic assessment of the transplanted graft on the first post-transplant 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.

Table 1.

Experimental design, kidney function and survival data

Individual values presented along with group averages ±SD, significance calculated by one-way ANOVA (*p<0.05 G1 vs G2; †p<0.05 G1 vs G3; ‡p<0.05 G2 vs G3).

Donor ID	Donor Age (years)	Donor Treatment group	rhC1-INH Dose	Recovered kidney	Cold ischemia time (hours)	Recipient ID	Age (years)	Weight (Kg)	Graft Survival (Days)	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	500U/kg per time point	Right kidney	45.0	R13009	3.8	7.3	26	No	4.3
		rhC1INH + heparin	500U/kg per time point	Left kidney	47.8	R12063	4.3	5.4	26	No	3.8
Rh2241	18.8	rhC1INH + heparin	500U/kg per time point	Left kidney	45.2	Rh2622	5.9	9.7	67	No	7.3
		rhC1INH + heparin	500U/kg per time point	Right kidney	46.8	Rh2625	4.8	7.1	32	No	13.0
Rhav29	20.1	rhC1INH + heparin	500U/kg per time point	Right kidney	44.0	Rh2616	6.2	8.6	90	No	8.4
		rhC1INH + heparin	500U/kg per time point	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
		Heparin	N/A	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** ‡

Donor ID	Donor Age (years)	Donor Treatment group	rhC1-INH Dose	Recovered kidney	Cold ischemia time (hours)	Recipient ID	Age (years)	Weight (Kg)	Graft Survival (Days)	Meet DGF criteria	Peak creatinine level (mg/dL)
P-value	**ns**	**N/A**	**N/A**	**N/A**	**ns**	**N/A**	**ns**	**† p < 0.05**	**ns**	**p= 0.0045**	**‡ p < 0.05**

*G1 vs G2,

†G1 vs G3

‡G2 vs G3

Table 2.

Kidney Donor Vital Signs and Laboratory Values at Baseline and 20 hours after Brain Death.

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 Farenheit (°F).

	Baseline				T=1200 minutes (20 hours)			
Parameter	**Group 1 Vehicle**	**Group 2 rhC1INH + heparin**	**Group 3: Heparin**	**P Value**	**Group 1 Vehicle**	**Group 2 rhC1INH + heparin**	**Group 3: Heparin**	**P Value**
Vitals, mean ± SD								
Temperature, ○F	98.7 ± 1.8	95.8 ± 1.7	97.9 ± 1.8	0.226	98.7 ± 1.4	98.6 ± 1.8	99.9 ± 0.2	0.614
Pulse, bpm	138.3 ± 24.7	139.3 ± 18.6	125.0 ± 14.1	0.726	156.7 ± 28.0	150.7 ± 33.6	134.0 ± 8.5	0.686
MAP, mmHg	70.0 ± 6.2	78.3 ± 8.5	52.5 ± 24.7	0.184	64.0 ± 16.1	77.7 ± 6.4	59.5 ± 0.7	0.234
UOP, mL/kg/h	NA				3.3 ± 0.8	4.9 ± 3.9	12.2 ± 7.6	0.149
Metabolic panel , mean ± SD								
Na, mmol/L	147.3 ± 2.3	146.3 ± 1.5	148.5 ± 0.7	0.465	143.7 ± 2.9	150.0 ± 4.4	157.5 ± 0.7	0.016
K, mmol/L	3.5 ± 0.4	3.5 ± 1.3	3.3 ± 0.0	0.945	3.6 ± 0.5	2.9 ± 0.3	3.2 ± 0.1	0.151
Cl, mmol/L	111.3 ± 2.3	109.3 ± 1.2	111.0 ± 1.4	0.406	107.7 ± 2.9	112.0 ± 5.2	119.5 ± 2.1	0.053
CO2, mmol/L	23.7 ± 0.6	26.0 ± 1.0	24.0 ± 2.8	0.211	22.3 ± 2.5	23.7 ± 3.1	23.0 ± 2.8	0.849
BUN, mg/dL	18.0 ± 1.7	14.7 ± 4.0	21.5 ± 0.7	0.107	16.7 ± 5.5	13.3 ± 8.6	8.5 ± 0.7	0.446
Cr, mg/dL	0.7 ± 0.2	0.4 ± 0.1	0.6 ± 0.0	0.064	0.8 ± 0.1	0.5 ± 0.2	0.5 ± 0.1	0.031
Glc, mg/dL	68.3 ± 5.5	84.3 ± 16.9	61.0 ± 8.5	0.167	117.0 ± 37.3	106.7 ± 41.7	135.5 ± 2.1	0.690
CBC , mean ± SD								
Hb, g/dL	12.6 ± 0.4	13.0 ± 1.2	11.7 ± 4.1	0.775	9.3 ± 2.0	10.6 ± 0.9	11.6 ± 1.9	0.382
Hct, %	37.0 ± 1.0	38.3 ± 3.5	34.5 ± 12.0	0.782	27.3 ± 5.9	31.0 ± 2.6	34.0 ± 5.7	0.374
ABG, mean ± SD								
pH	7.4 ± 0.0	7.3 ± 0.0	7.3 ± 0.1	0.468	7.3 ± 0.1	7.4 ± 0.1	7.3 ± 0.1	0.181
pCO2, mmHg	49.7 ± 14.1	49.1 ± 6.8	49.3 ± 3.9	0.997	50.2 ± 11.8	35.7 ± 10.7	47.3 ± 11.7	0.344
pO2, mmHg	478.7 ± 115.0	323.0 ± 138.3	401.5 ± 41.7	0.336	288.7 ± 169.4	258.3 ± 115.5	272.5 ± 40.3	0.961
HCO3, mmol/L	26.0 ± 1.2	26.3 ± 1.1	25.4 ± 0.9	0.675	23.6 ± 3.1	27.7 ± 9.8	22.6 ± 2.5	0.661
Lac, mmol/L	0.5 ± 0.2	0.3 ± 0.0	0.4 ± 0.2	0.639	0.5 ± 0.2	0.8 ± 0.4	0.6 ± 0.0	0.629

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.