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Complement Blockade in Recipients Prevents Delayed Graft Function and Delays Antibody-mediated Rejection in a Nonhuman Primate Model of Kidney Transplantation

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Background. Complement activation in kidney transplantation is implicated in the pathogenesis of delayed graft function (DGF). This study evaluated the therapeutic efficacy of high-dose recombinant human C1 esterase inhibitor (rhC1INH) to prevent DGF in a nonhuman primate model of kidney transplantation after brain death and prolonged cold ischemia.

Methods. Brain death donors underwent 20 h of conventional management. Procured kidneys were stored on ice for 44–48 h, then transplanted into ABO-compatible major histocompatibility complex-mismatched recipients. Recipients were treated with vehicle (n = 5) or rhC1INH 500 U/kg plus heparin 40 U/kg (n = 8) before reperfusion, 12 h, and 24 h posttransplant. Recipients were followed up for 120 d.

Results. Of vehicle-treated recipients, 80% (4 of 5) developed DGF versus 12.5% (1 of 8) rhC1INH-treated recipients (P = 0.015). rhC1INH-treated recipients had faster creatinine recovery, superior urinary output, and reduced urinary neutrophil gelatinase-associated lipocalin and tissue inhibitor of metalloproteinases 2-insulin-like growth factor-binding protein 7 throughout the first week, indicating reduced allograft injury. Treated recipients presented lower postperfusion plasma interleukin (IL)-6, IL-8, tumor necrosis factor-alpha, and IL-18, lower day 4 monocyte chemoattractant protein 1, and trended toward lower C5. Treated recipients exhibited less C3b/C5b-9 deposition on day 7 biopsies. rhC1INH-treated animals also trended toward prolonged mediated rejection-free survival.

Conclusions. Our results recommend high-dose C1INH complement blockade in transplant recipients as an effective strategy to reduce kidney injury and inflammation, prevent DGF, delay antibody-mediated rejection development, and improve transplant outcomes.

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INTRODUCTION

Delayed graft function (DGF), commonly defined as the need for dialysis within the first week posttransplantation, occurs in up to 40% of kidney transplant recipients.\textsuperscript{1-4} DGF is associated with increased incidence of acute cellular rejection (ACR) and antibody-mediated rejection (AMR) and reduced graft survival rates.\textsuperscript{2,5-11} DGF imparts a significant financial burden through increased intensive care unit admissions, prolonged hospitalization, posttransplant dialysis treatments, and repeat transplants, increasing the average cost by 10% per patient.\textsuperscript{15} Despite the high prevalence and costs of DGF, preventative therapeutic regimens remain elusive, and although short-term outcomes in kidney transplantation have improved significantly, long-term outcomes remain suboptimal with >20% of transplants going to prior allograft recipients.\textsuperscript{5,13} Additionally, about 20% of deceased donor kidneys are discarded, in part due to prolonged cold ischemia time (CIT) and advanced donor age, both of which are associated with increased risk of developing DGF.\textsuperscript{14-17} As demand continues to climb in an environment of scarce resources, it is increasingly imperative to develop DGF prevention strategies to improve long-term graft survival rates, reduce the incidence of AMR, and potentially expand the donor pool to include kidneys that are currently being discarded.

DGF results from acute kidney injury (AKI) suffered during ischemia-reperfusion injury (IRI) in transplantation. Increased inflammatory response, complement activation, and cytokine release are all implicated in the pathogenesis of DGF.\textsuperscript{18-21} The complement system, consisting of classical pathways (CPs), mannose-binding lectin (MBL), and alternative pathways (APs), is an essential component of the innate immune system.\textsuperscript{18-20} Complement-mediated injury starts in the donor with increased mRNA expression of complement components and upregulation of renal tubular anaphylatoxin receptors C3aR and C5aR.\textsuperscript{22,23} Although the liver produces the majority of complement proteins, up to 10%-16% of systemically circulating C3, a precursor of C5 convertase, is produced by renal proximal tubular cells.\textsuperscript{19,22,24-30} These factors foster local complement deposition, leukocyte migration and infiltration, and oxidative damage, exacerbating injury at reperfusion and increasing the risk of DGF and rejection.\textsuperscript{19,25,31-42}

C1 esterase inhibitor (C1INH) is a serine protease inhibitor that blocks components of the complement, contact, fibrinolytic, and coagulation systems.\textsuperscript{43} Currently approved by Food and Drug Administration for use in hereditary angioedema, C1INH products have garnered interest in the transplant community as a mitigator of IRI because of its anti-inflammatory properties.\textsuperscript{44} In humans, C1INH blocks the classical complement and MBL pathways by inactivation of C1s, C1r, and MASP2.\textsuperscript{43} This effect is potentiated by heparin, commonly used in organ procurement and transplantation, increasing the activity of C1INH up to 11-fold.\textsuperscript{44-46}

We previously demonstrated that donor pretreatment with recombinant human C1INH (rhC1INH) plus heparin can prevent DGF in a nonhuman primate (NHP) model of brain death (BD), prolonged CIT, and kidney transplantation; however, ACR and AMR incidences were unaffected.\textsuperscript{47} In this study, we tested perioperative and postoperative rhC1INH treatment in recipients to determine the impact on DGF and acute rejection within the first 120 d. We assessed markers of inflammation, complement activity, kidney function, and rejection to provide a comprehensive assessment and potential mechanism of rhC1INH-therapy in kidney transplantation.

MATERIALS AND METHODS

Animals

Rhesus macaques obtained from the Wisconsin National Primate Research Center and Alpha Genesis Inc. (Yemassee, SC) were housed in accordance with National Institutes of Health (NIH) and US Department of Agriculture animal welfare guidelines; all protocols were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison. All animals were prescreened for tuberculosis, herpes B, Simian type D retrovirus, Simian immunodeficiency virus, and Simian immunodeficiency virus. Each donor-recipient pair was ABO-compatible, nonsensitized, and fully mismatched for major histocompatibility complex class I and II alleles identified with microsatellite analysis as previously described.\textsuperscript{48} Recipient animals were divided into treatment groups: vehicle ($n = 5$) and rhC1INH ($n = 8$).

Experimental Design

Pharming Technologies B.V. (Leiden, The Netherlands) provided the rhC1INH (half-life of 2.5 h\textsuperscript{49}).

BD was induced and donors were maintained 20 h in an intensive care unit environment following previously published guidelines (Figure 1A).\textsuperscript{50} Briefly, animals were anesthetized and placed on a ventilator. A 16F Foley catheter was inserted into the extradural space and inflated until hemodynamic and neurologic signs of brain stem herniation were observed. Animals were managed for 20 h with standard donor management, including fluid resuscitation and vasopressor support (Table S1, SDC, http://links.lww.com/TP/C188). Donors subsequently underwent aortic cannulation, retrograde infusion of University of Wisconsin (UW) preservation solution (Organ Recovery Systems, IL) supplemented with heparin (5 U/mL), and bilateral nephrectomy. Recovered kidneys were stored in UW solution on ice for 44–48 h. Recipients underwent bilateral native nephrectomy and heterotopic kidney allotransplantation (Figure 1B).

Immediately before arterial reperfusion of the graft, recipients received an IV bolus of vehicle (3.33 mL/kg normal saline) in the control group or rhC1INH (500 U/kg) plus heparin (40 U/kg) for the experimental group. The same dose was repeated at 12 and 24 h posttransplant. Recipients were followed up for 120 d. A heparin-alone group was not used because of detrimental outcomes when donors received heparin alone in our previous study.\textsuperscript{47} Immunosuppressive induction therapy was not administered. Maintenance immunosuppression included prednisone, mycophenolate mofetil, and tacrolimus guided by biweekly trough measurements to maintain 8–12 ng/mL. Basic metabolic panel (including creatinine) and blood gases were measured by i-STAT 1 Analyzer (Abaxis, Union City, CA). Termination criteria included (1) survival for 120 d or (2) severe azotemia, acute renal failure, or progressive AMR not responsive to medical management.
Definitions: DGF, AMR, ACR

DGF was defined as a failure of serum creatinine to fall by least 10% on 3 consecutive d in the first week posttransplant or serum creatinine at posttransplant day 7 >2.5 mg/dL.4,51 AMR and ACR were diagnosed using the 2017 Banff classification.52 AMR diagnosis required 2 of 3 criteria: (a) clinical acute increase in serum creatinine or oliguria/anuria, (b) histopathology on hematoxylin and eosin staining with C4d-positive immunohistochemistry, or (c) serological evidence of increased donor-specific antibody median fluorescence intensity shift ≥1.5.

Resistive Index

Intrarenal resistive indices (RIs) of kidney transplants [RI = (peak systolic velocity−end diastolic velocity)/(peak systolic velocity)] were measured using ultrasonography on postoperative day 1. RI >0.7 was considered elevated.

Urinary Marker Evaluation

Collected urine was stored at −80°C. Urinary neutrophil gelatinase-associated lipocalin (NGAL) levels were quantified using the NHP NGAL ELISA kit (Bioporto, Copenhagen, Denmark) according to manufacturer’s recommendations. Urinary tissue inhibitor of metalloproteinases 2 (TIMP2s) and insulin-like growth factor-binding protein 7 (IGFBP7) were measured with LEGENDplex NHP Mix-and-Match Subpanel (Biolegend, San Diego, CA) according to manufacturer’s recommendations.

Complement and Cytokine Assessments

Blood was collected into Vacuette (Greiner Bio-One, Austria). Serum tubes were allowed 15 min to clot before centrifugation with EDTA-plasma tubes for 10 min at 3000g, and aliquots were stored at −80°C. Circulating serum C1INH was measured via Sino Biological human C1 inhibitor/SerpinG1 ELISA kit (KIT10995). Activation of the complement CP, MBL, and AP pathways was tested using the Wieslab Complement Kits (CP310, LP320, and AP330, EuroDiagnostica, Sweden). Circulating sC5b-9 was measured using sC5b-9 ELISA (Quidel, San Diego, CA). C5 and C5α levels were measured using C5 ELISA (Abnova, Taipei City, Taiwan) and C5α ELISA (Novus, Abingdon, United Kingdom). All assays were performed according to manufacturer’s recommendations. Circulating interleukin (IL)-6, monocyte chemoattractant protein (MCP)-1, IL-8, tumor necrosis factor (TNF)α, and IL-18 were measured with LEGENDplex NHP Mix-and-Match Subpanel (Biolegend, San Diego, CA) according to manufacturer’s recommendations. Assay results for serum and plasma proteins were normalized to albumin, measured by VetTest Analyzer (Idexx, Westbrook, ME), to account for hemodilution.

Kidney Biopsy Histopathology and Immunohistochemistry

Recipient native kidney samples, ultrasound-guided core biopsies of the allografts, and necropsy specimens were fixed in 10% formalin or frozen in optimal cutting temperature compound. Slides cut at 4 µm thickness were stained with hematoxylin and eosin, periodic acid-Schiff, anti-CD68 (KP1, DAKO-Agilent), anti-MPO (MPO, Abcam), malondialdehyde (MDA, Abcam), C4d, C3b, and C5b-9 (Ventana-Roche, AZ). Diagnostic histopathology was blindly interpreted by 2 renal pathologists and graded according to the Banff 2017 criteria.52 Images for quantification were acquired from 6 to 12 random fields as available within each slide at appropriate magnification using an Olympus BX51 microscope (Olympus, Tokyo, Japan) and processed using ImageJ software (NIH, Bethesda, MD). Cell counts or area fraction measurements were collected.
using color separation, automatic threshold, and particle analysis algorithms.

Donor Specific Antibody

Donor-specific alloantibody production was assessed by flow cytometric crossmatch of donor splenocytes with serially collected recipient EDTA-plasma samples. Donor cells incubated with recipient plasma were stained with fluorescein isothiocyanate-labeled anti-monkey IgG (NIH NHP Reagent Resource, Boston, MA), PerCP-Cy5.5 CD20 (BD Pharmingen, San Diego, CA), and APC CD3 (BD Pharmingen, San Diego, CA). Data were acquired on a Cytek DxP upgraded BD FACSCalibur and analyzed with FlowJo (Treestar Inc., USA).

Statistics

Statistical analyses were performed using GraphPad Prism V.8.2 (GraphPad, USA) and represented as mean±SEM. Chi-square test was used to analyze DGF incidence and intrarenal RI. Normality and variance were tested using the Shapiro-Wilk test and F-test, whereupon appropriate tests were used if equal variance. For histology comparisons involving native kidneys, differences between the groups were tested by 1-way ANOVA with Tukey’s correction. Kruskal-Wallis test or Mann-Whitney U test was used for nonnormally distributed data. Data sets were tested for outliers using the Grubbs test. Kaplan-Meier death-censored analysis was performed on AMR and ACR outcomes with significance determined data. Data sets were tested for outliers using the Grubbs test. Kaplan-Meier death-censored analysis was performed on AMR and ACR outcomes with significance determined.

RESULTS

RhC1INH Reduces Renal Injury and Incidence of DGF and Improves Urine Output and Resistive Index

DGF incidence was significantly reduced among rhC1INH-treated animals (12.5%, 1 of 8) compared with vehicle-treated animals (80.0%, 4 of 5) (Table 1; P=0.015). RhC1INH-treated animals exhibited faster serum creatinine recovery (Figure 2A; P=0.046) and urine output (Figure 2B; P=0.011) in the first week posttransplant, including significantly lower creatinine levels at days 6 and 7 (P=0.018 and P=0.031, respectively) and significantly higher urine output as early as day 2, proceeding through day 5 (P=0.020, P=0.002, P=0.026, P=0.026).

Urinary kidney injury marker NGAL was significantly lower in rhC1INH-treated animals during the first week (P=0.008) and daily at days 1, 3, and 5 (Figure 2C; P=0.034, P=0.007, P=0.008, respectively). TIMP2-IGFBP7 was also lower in rhC1INH recipients (P=0.019), with a significant difference on day 1 (Figure 2D; P=0.030). Day 1 RI measured by Doppler ultrasound revealed a significant elevation in 100% (4 of 4) vehicle animals versus only 14% (1 of 7) of rhC1INH animals (Figure 2E and F; P=0.002). Ultrasounds were unavailable for 1 vehicle-treated and 1 rhC1INH-treated animal.

rhC1INH Reduces Classical and MBL Complement Pathway Activity and C3b/C5b-9 Deposition but Does Not Impact Alternative Pathway Activity or C5/5a Levels

We assessed exogenous and endogenous serum C1INH levels, CP, MBL, and AP activity at baseline and 60 min postreperfusion (T60). Serum C1INH was significantly elevated in rhC1INH recipients at T60, demonstrating the presence of the drug, whereas vehicle recipients remained elevated in rhC1INH recipients at T60, demonstrating the presence of the drug.

<table>
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<tr>
<th>Recipient characteristics and incidence of DGF</th>
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<tr>
<td>Recipient ID</td>
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<tr>
<td>Vehicle group</td>
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<td>rhC1INH group</td>
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<td>Rh2732</td>
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<td>Mean/SEM</td>
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Individual animals are listed as mean±SEM in bold. Significance for DGF incidence calculated by chi-square (P-value in italics).
<sup>a</sup>Serum creatinine <2.5 mg/dL by d 7.
<sup>b</sup>Failure for serum creatinine to drop at least 10% over 3 consecutive d.
near baseline (Figure 3A; \(P=0.016\)). CP and MBL pathway activities were both significantly higher in vehicle animals at T60 when compared with rhC1INH-treated animals who remained near or below baseline levels (Figure 3B, \(P=0.036\); Figure 3C, \(P=0.022\)). Although AP activity trended higher at T60 for both groups, no significant differences were observed between groups or time points (Figure 3D).

Plasma sC5b-9 increased 2.39-fold in the vehicle-treated group at T60 and 2.28-fold on day 4, significantly higher than the rhC1INH-treated group (Figure 3F; \(P<0.001\) and \(P=0.001\)). There was no significant difference in plasma C5 or C5a between groups or time points (Figure S1A and B, SDC, http://links.lww.com/TP/C188). Immunofluorescent staining of biopsies at 1 wk posttransplant revealed

**FIGURE 2.** RhC1INH reduces renal injury and incidence of DGF, improves urine output and resistive index. A, Daily serum creatinine levels (mg/dL) and B, urinary output (mL/d) for vehicle-treated \((n=5)\) vs rhC1INH-treated \((n=8)\) recipients in the first postoperative wk with the AUC measured for creatinine (mg/dL\(^2\)) are presented as mean ± SEM. Rh2714 and Rh2733 had an acute increase in creatinine after renal biopsy at d 6, followed by a significant drop in creatinine on d 8, creatinine values from d 6 and 7 were excluded for analysis. Urine kidney injury marker C, NGAL (ng/mL) is presented as individual values and AUC. Our historical control data found baseline NGAL levels to be below the detectable assay limit in naive samples. Calculated D, urinary TIMP2·IGFBP7 and AUC (ng\(^2\)/1000 for sample, (ng\(^2\)/1000)\(^2\) for AUC) are presented as mean ± SEM. E, Representative Doppler tracings of the renal arterial waveforms. Statistics: individual and AUC data for creatinine, urine output, and urinary NGAL were normally distributed with equal variance and parametric testing was used. Urinary TIMP2·IGFBP7 individual and AUC data were normally distributed, however, variance was not equal and nonparametric testing was used. F, RI significance was calculated by chi-square test (*\(P<0.05\); **\(P<0.01\)). AUC, area under the curve; DGF, delayed graft function; IGFBP7, insulin-like growth factor-binding protein 7; NGAL, neutrophil gelatinase-associated lipocalin; rhC1INH, recombinant human C1 esterase inhibitor; RI, resistive index; TIMP2, tissue inhibitor of metalloproteinases 2.
significantly less deposition of C3b/C5b-9 in rhC1INH-treated animals (Figure 3F and G; \(P=0.048\)).

**RhC1INH Reduces Inflammatory Cytokines**

Plasma cytokines were quantified at baseline, T60, and day 4 (Figure 4A–E). RhC1INH-treated animals presented significantly less IL-6, IL-8, TNFα, and IL-18 than vehicle-treated animals at T60 (\(P=0.030, P=0.030, P=0.005,\) and \(P=0.030,\) respectively) and significantly less MCP-1 and IL-18 on day 4 (\(P=0.030\) and \(P=0.011,\) respectively).

**RhC1INH Treatment Trends Toward Reduced Cellular Infiltration and Oxidative Damage in the First Posttransplant Week**

Immunohistochemistry in biopsies 1 wk posttransplant revealed no significant difference in macrophage (CD68+) or neutrophil (MPO+) infiltration between groups, though there was a trend toward increased macrophage infiltration compared with native biopsies and a significant increase in neutrophil infiltration between vehicle-treated graft recipients and native kidneys (Figure 5A; \(P=0.015\))—this difference was not observed between rhC1INH-treated and native kidneys. No significant difference was observed in MDA staining comparing oxidative damage in rhC1INH-treated to native kidneys or rhC1INH-treated to vehicle kidneys, but a significant increase was again observed comparing vehicle to native kidneys (Figure 5B; \(P=0.038\)).

**DGF Prevention With RhC1INH Delays Antibody-mediated Rejection**

Recipients were followed for up to 120 d with serial biopsies for histopathology and immunohistochemistry to diagnose injury, rejection, and cellular infiltration; donor-specific antibody and clinical parameters were also used to diagnose AMR (Table S2, SDC, http://links.lww.com/TP/C188).

During the 120-d period, vehicle recipients averaged 44.7 d before AMR diagnosis, whereas rhC1INH-treated animals averaged 65.3 d; animals euthanized ≤14 d were excluded from the averages. The trends for AMR presentation were not significantly different between treatment groups (Figure 6A; \(P=0.226\)); however, a subanalysis comparing the DGF+ vehicle animals (\(n=4\)) and rhC1INH animal (\(n=1\)) against the DGF− vehicle animal (\(n=1\)) and rhC1INH-treated animals (Figure S2, SDC, http://links.lww.com/TP/C188).

**DISCUSSION**

Our NHP model of kidney transplantation after donor BD and prolonged CIT (44–48 h) presents the unique opportunity to evaluate factors contributing to DGF in the transplant environment and to test therapeutic regimens against those factors in isolation, without confounding factors that are mostly unavoidable in clinical trials. This includes complement and cytokine activation resulting from chronic kidney disease or donation after cardiocirculatory death, which are known to have a higher incidence of DGF than BD donation in clinical practice.\(^{1,3,7,47,50,53-56}\) Previously, we demonstrated that BD donor intervention with high-dose rhC1INH prevents DGF—the drug effect carrying over to untreated recipients.\(^9\) In this study of recipient treatment, 3 doses of rhC1INH at 500 U/kg magnified by combining with heparin, delivered over the first 24 h posttransplant, only 1 of 8 rhC1INH-treated recipients developed DGF.

Reduced allograft injury was evident through the enhanced renal recovery seen by superior serum creatinine levels, urine output, and RI; and through reduced urinary injury markers, NGAL and TIMP2·IGFBP7, in the first week posttransplant. Previous studies of swine kidney autotransplantation showed no significant increase of NGAL in the first week posttransplant after 60 min of warm ischemia plus 24-h CIT.\(^{37}\) While this may be species specific, other studies have indicated that donor urinary NGAL increases during BD, early evidence of injury that may carry over to recipients of those kidneys if left unmanaged in the donor.\(^{38,59}\) The elevation of urinary TIMP2 and IGFBP7, cell cycle arrest biomarkers produced by renal tubular cells, has been attributed to increased filtration, reduced absorption by renal tubules, and proximal tubular cell leakage in the setting of AKI.\(^{60}\) On average, TIMP2·IGFBP7 levels observed in our rhC1INH-treated recipients remained under the 0.3 ng\(^2/1000\) threshold predictive of AKI.\(^{61}\) In contrast, vehicle animals remained >0.3 ng\(^2/1000\) until day 7.

We investigated complement activity and the presence of key complement activation products, including anaphylatoxin C5a, C3b, and terminal complex C5b-9, as the primary agents precipitating AKI leading to DGF. Naesens et al showed that expression of upstream complement proteins significantly increased in renal biopsies from deceased human donors compared with living donors.\(^{31}\) C5a, involved in inflammatory response and priming of alloreactive T cells due to widespread C5aR expression in immune cells and renal tubules, is upregulated after IRI.\(^{18,19,23,35,62-65}\) In mice, C5αR blockade reduces inflammatory cytokines, cellular infiltration, and protects against IRI.\(^{19}\) In our study, circulating C5 and C5αa levels were not statistically different between groups at T60.

Peak levels of C5a may have been missed due to its rapid breakdown by the serum enzyme carboxypeptidase B.\(^{34}\) In a study evaluating complement activator ISIS 3202, a complement activator, in cynomolgus monkeys, C5a levels peaked near 10 min, followed by a reduction almost to baseline within 30 min.\(^{66}\) We cannot here confirm the role of C5a in AKI-mediated DGF. Instead, our model presents a significant increase in circulating sC5b-9 and local TIMP2·IGFBP7 levels observed in our rhC1INH-treated recipients remained under the 0.3 ng\(^2/1000\) threshold predictive of AKI.\(^{61}\) In contrast, vehicle animals remained >0.3 ng\(^2/1000\) until day 7.

We investigated complement activity upstream, we are able to abrogate direct complement-mediated AKI, which is otherwise sufficient to culminate in DGF.

Our investigation of proinflammatory cytokines in the first week revealed significantly lower levels of IL-6, MCP-1, IL-8, TNFα, and IL-18 in rhC1INH-treated recipients. IL-8 and MCP-1 are especially important in directing innate immune cells to sites of injury, and all play a
role in immune cell activation and differentiation. IL-18 has gained interest as a novel biomarker of renal injury in serum and urine because of upregulation in the distal tubular epithelia during injury. IL-18 can recruit and prime neutrophils as well as stimulate the production of proinflammatory IL-6, IL-1β, interferon-γ, and MCP-1.
which in turn can promote T-cell differentiation into Th1 and Th17 cells, both of which promote later allograft rejection. The reduced levels of these markers at T60, and especially MCP-1 and IL-18 at day 4, the trend toward reduced macrophage and neutrophil infiltration, and reduced oxidative damage exhibited by MDA staining all further illustrate the reduction in kidney injury as a result of recipient treatment.

A number of studies have evaluated the use of complement inhibition in humans, baboons, pigs, and rodents for amelioration of IRI and improved transplant outcomes. In rodent models of IRI, AP activity
predominates, whereas CP and MBL activity predominate in NHP, swine, and humans. In 2 recent clinical trials by Schröppel et al, eculizumab, a C5-inhibitor that primarily inhibits the AP, was evaluated for the prevention of IRI but failed to prevent DGF. Harder et al determined through in vitro hemolytic studies that robust activation of the classical complement pathway is sufficient to overcome eculizumab therapy even amidst full abrogation of AP activity, and only through combination with direct upstream inactivation of C3 (significantly produced in the kidney itself) is suppression of CP and presumably MBL pathway-mediated tissue injury accomplished. Chun et al demonstrated in murine heart transplants that peritransplant C1INH administration or recipient C3 and MBL deficiencies, but not factor B (AP) deficiency, yielded a survival benefit against cold ischemic injury. Use of rhC1INH to block complement activity upstream of C3 without impacting AP activity may prove beneficial to protect immunosuppressed recipients against pathogens in the posttransplant period.

In our study, rhC1INH recipient treatment blocked peritransplant increases in CP and MBL activity. Sustained AP activity did not prevent reduction of sC5b-9 or local C3b/C5b-9 deposition, nor hamper prevention of DGF. Notably, Jordan et al evaluated the use of C1INH at 50 U/kg (used for the treatment of hereditary angioedema) to prevent DGF in human kidney transplant recipients, whereas this reduced the number of dialysis sessions, it did not prevent DGF. At their 3.5 y follow-up, the C1INH-treated recipients exhibited superior renal function and graft survival when compared with placebo group. When they evaluated the use of C1INH (20 U/kg/dose) in HLA sensitized patients to prevent AMR, they observed lower DGF incidence versus placebo but had similar rates of AMR despite reduced C1q+ HLA antibodies, which increase the risk of AMR.

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FIGURE 5. rhC1INH-treatment trends toward reduced cellular infiltration and oxidative damage in the first posttransplant wk. Day 7 biopsies from vehicle (n=3), rhC1INH-treated (n=6), and native (n=5) were stained for (A) macrophages (measured by CD68) and neutrophils (measured by MPO), represented as percent positive cells for individual animals. B, Oxidative damage (measured by MDA) represented as individual values. Statistics: CD68 and MPO were normally distributed with equal variance and parametric tests were used. MDA exhibited was not normally distributed so nonparametric testing was used (*P<0.05). MDA, malondialdehyde; rhC1INH, recombinant human C1 esterase inhibitor.

FIGURE 6. DGF prevention with rhC1INH delays AMR. Kaplan-Meier survival analysis of (A) vehicle-treated recipients (n=5) averaged 44.7 d before AMR diagnosis, whereas rhC1INH-treated recipients (n=8) averaged 65.3 d (P=0.226). Two animals in the vehicle group and 3 in the rhC1INH group were death censored. B, Recipients who developed DGF (n=5) vs recipients without DGF (n=8). Significance calculated with log-rank test (P=0.008). Statistics: log-rank test was used to compare groups. AMR, antibody-mediated rejection; DGF, delayed graft function; rhC1INH, recombinant human C1 esterase inhibitor.
In our own preclinical studies, we failed to prevent DGF through donor intervention at doses as high as 200 U/kg (data not shown), which informed the strategy used in this study. Our efficacious use of 500 U/kg rhC1INH, 10 times the recommended dose used by Jordan et al, supplemented with heparin to further potentiate the activity of the inhibitor to obtain maximal blockade of complement-associated pathways and also protect against thrombus formation, provides a significant enhancement to the clinical regimen under investigation and holds the promise for establishing a best-practice treatment for all future transplant recipients. Further, our model uses HLA fully mismatched donor/recipient pairing to maximize the risk of de novo rejection.

Our recipient rhC1INH-treatment regimen did not prevent the development of ACR or AMR. However, we did observe that recipients with DGF experienced rapid onset of AMR compared with non-DGF recipients, which did observe that recipients with DGF experienced rapid onset of AMR compared with non-DGF recipients, which was limited from attaining significance because of sample size, but confirms previous findings that developing DGF leads to increased risk and acceleration of AMR development.1,5,11,13,91 We are encouraged that a more robust regimen to prevent DGF will lead to a significant difference in AMR-free survival given sufficient sample size. Indeed, even in the absence of transplantation, Cippà et al92 found that within 12 mo of renal IRI in mice, B-cell activation and immunoglobulin production are upregulated, leading to chronic kidney injury. They further noted the development of intrarenal ectopic lymphoid organs—we observed a similar phenomenon in some of our recipients (data not shown). By preventing renal injury and subsequent DGF through C1 complement blockade, we may reduce such a response and so delay and prevent rejection.

Our study has several limitations. Statistical power is impeded by small group sizes inherent to NHP models; observed trends may have attained significance with a larger sample size. In the interest of animal well-being, some samples were not collected, resulting in fewer comparable biopsies or blood samples at certain time points. Four serum samples at T60 in rhC1INH-treated recipients were hemolyzed and could not be used for C1INH or complement pathway evaluation. Individual animals euthanized before AMR development during the study period further limited sample size and histological comparison at 120 d. Rh2714 and Rh2733 had acute increases in creatinine after renal biopsies at day 6, followed by a significant drop in creatinine on day 8—these were excluded as resulting from procedural artifact and DGF status was already determined at this point.

In this study, we found that perioperative recipient treatment with rhC1INH at high doses prevents DGF, similar to our donor intervention study.89 Recipient treatment with rhC1INH reduced direct complement-mediated injury as well as systemic inflammation through reduced levels of key cytokines and chemokines. Preclinical studies are underway in our laboratory to investigate the potential synergistic effects of dual treatment of donors and recipients with rhC1INH. Further we are developing a model of donation after cardiocirculatory death donation to clarify differences from BD donation and effective treatment regimens.

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REFERENCES


