Donor Brain Death Exacerbates Complement-Dependent Ischemia/Reperfusion Injury in Transplanted Hearts

Carl Atkinson, PhD*; Bernhard Floerchinger, MD, PhD*; Fei Qiao, MD*; Sarah Casey, MSc; Tucker Williamson, MSc; Ellen Moseley, BSc; Serban Stoica, MD, FRCS; Martin Goddard, MD, FRCPATH; Xupeng Ge, MD, PhD; Stefan G. Tullius, MD, PhD; Stephen Tomlinson, PhD

Background—Brain death (BD) can immunologically prime the donor organ and is thought to lead to exacerbated ischemia/reperfusion injury after transplantation. Using a newly developed mouse model of BD, we investigated the effect of donor BD on posttransplantation cardiac ischemia/reperfusion injury. We further investigated the therapeutic effect of a targeted complement inhibitor in recipients of BD donor hearts and addressed the clinical relevance of these studies by analyzing human heart biopsies from BD and normal donors.

Methods and Results—Hearts from living or BD donor C57BL/6 mice were transplanted into C57BL/6 or BALB/c recipients. Recipient mice were treated with the complement inhibitor CR2-Crry or vehicle control (n=6). Isografts were analyzed 48 hours after transplantation for injury, inflammation, and complement deposition, and allografts were monitored for graft survival. Human cardiac biopsies were analyzed for complement deposition and inflammatory cell infiltration. In the murine model, donor BD exacerbated ischemia/reperfusion injury and graft rejection, as demonstrated by increased myocardial injury, serum cardiac troponin, cellular infiltration, complement deposition, inflammatory chemokine and cytokine levels, and decreased graft survival. CR2-Crry treatment of recipients significantly reduced all measured outcomes in grafts from both BD and living donors compared with controls. Analysis of human samples documented the relevance of our experimental findings and revealed exacerbated complement deposition and inflammation in grafts from BD donors compared with grafts from living donors.

Conclusions—BD exacerbates posttransplantation cardiac ischemia/reperfusion injury in mice and humans and decreases survival of mouse allografts. Furthermore, targeted complement inhibition in recipient mice ameliorates BD-exacerbated ischemia/reperfusion injury. (Circulation. 2013;127:1290-1299.)

Key Words: inflammation ▪ ischemia ▪ reperfusion injury ▪ transplantation

Cardiac transplantation is an accepted therapy for end-stage cardiac disease. Although improvements have been made in early graft survival rates, largely as a result of the introduction of potent immunosuppressive regimens, primary graft failure and chronic rejection still present significant challenges to graft survival.1,2 The majority of organs used for transplantation are acquired from brain-dead (BD) donors. Evidence from clinical and experimental studies indicates that BD is a significant contributing factor to inferior graft function and is detrimental to the outcome of kidney, heart, lung, and liver transplants.3,4

BD evokes a potent Cushing response, which leads to hemodynamic fluctuations, global organ ischemia, hyperthermia, coagulopathies, hormone depletion, and electrolyte abnormalities.4,6 In addition to these physiological and hormonal imbalances, recent studies have demonstrated that components of the immune system activate donor organs, rendering them proinflammatory before implantation.5-9

One effector arm of the immune system that has attracted some attention in BD-induced inflammation is the complement system. Complement plays an important role in the pathogenesis of many inflammatory disease conditions when excessively or inappropriately activated.10 Biological effector functions of
complement are mediated through a variety of cleavage fragments that directly or indirectly promote inflammatory cell migration, activation, cytokine release, cell lysis, and adaptive immune cell modulation. In kidney transplantation, BD is associated with increased levels of complement activation. Complement activation in the BD donor has also been shown to correlate with a poorer patient outcome after renal transplantation. In a rat model of BD, treating the BD donor with the systemic complement inhibitor sCR1 before and 1 hour after BD induction resulted in improved renal transplantation outcomes. Finally, in developing a mouse model of BD, we demonstrated that deficiency of C3, a central protein throughout all complement activation pathways operate, is protective against BD-associated cardiac injury and inflammation.

The above studies demonstrate that complement is activated in the BD donor and indicate that BD-induced complement activation negatively affects posttransplantation outcome, at least with regard to renal transplantation. These findings therefore raise the possibility that therapeutic manipulation of the complement system may be a viable treatment rationale to ameliorate the detrimental impact of BD on donor organs. Nevertheless, the complement system has important physiological roles, not least in host immunity to infection, and systemic complement inhibition would not be without risk, especially in an already immunosuppressed transplant recipient. In the sCR1 study mentioned above, the authors bypassed the potential risk of systemic inhibition in the recipient by treating the organ (kidney) donor. However, donor management remains a controversial topic of debate because of organ-specific and systemic effects. Interventions that improve kidney organ quality may not similarly improve cardiac or other organ outcome. For example, complement has important regenerative functions in the liver, and inhibition of complement in the donor may affect the repair and regenerative capacity of the liver after transplantation. In addition, C3a and C5a anaphylatoxins can have distinct opposing hemodynamic roles, with increased C3a activation causing hypertension and C5a causing hypotension, and infusion of complement inhibitors directly to the donor may further exacerbate the already complicated donor hemodynamic management.

With these issues in mind, we used our mouse model of BD to demonstrate that hearts from BD donors fared worse than hearts from living donors in terms of posttransplantation injury, inflammation, and allograft survival. We also investigated the therapeutic effect of treating the recipient with CR2-Crry, a targeted complement inhibitor comprising a CR2-targeting domain linked to the complement inhibitor Crry. A benefit of CR2-targeted complement inhibition, as we have previously shown, is that complement is inhibited locally at sites of complement activation but not systemically, and complement-dependent host defense mechanisms are not disrupted.

Methods

Male C57BL/6, BALB/c, and C57BL/6 pan-green fluorescent protein (GFP) mice (the last kindly provided by Dr M. Okabe, Osaka, Japan), 8 to 12 weeks old and between 20 and 30 g, were used for all studies. All animal procedures were performed according to approved experimental protocols in accordance with institutional animal care guidelines of the Medical University of South Carolina and Harvard Medical School.

Isograft studies comprised 5 experimental donor groups for the therapeutic studies (n=6): group 1, living donors; group 2, living donors with CR2-Crry–treated recipients; group 3, BD sham operated; group 4, BD donors; and group 5, BD donors with CR2-Crry–treated recipients. Untreated naive C57BL/6 mice were used as recipients, and a sixth group consisting of sham-operated recipient mice was included (sham controls). For allograft studies, C57BL/6 donor hearts were implanted into BALB/c recipients. Four experimental groups were included (n=4–6): group 1, living donors; group 2, BD sham operated; group 3, BD donors; and group 4, BD donors with CR2-Crry–treated recipients. To investigate the origin of inflammatory cells in grafts after transplantation, BD donor hearts from pan-GFP C57BL/6 mice were analyzed at time 0 before transplantation and at 6 and 48 hours after transplantation into C57BL/6 recipients. Recipients in the CR2-Cry treatment groups received a single intraperitoneal injection of 0.25 mg CR2-Cry. CR2-Cry was prepared and purified as previously described. Cardiac grafts and serum were harvested 48 hours after transplantation.

BD Induction

Donor animals were placed in the supine position. Blood pressure was monitored continuously via a 30-gauge cannula inserted into the left carotid artery and connected to a monitoring transducer system (Biopac Systems Inc, Santa Barbara, CA). After tracheotomy, a ventilation cannula was inserted, and animals were connected to a rodent ventilator (Harvard Apparatus Inc, Holliston, MA) with a respiration rate of 120 breaths per minute and a tidal volume of 300 to 400 μL (12–16 μL/g). Animals were subsequently placed in a prone position, and the parietal bone was exposed via a midline skin incision. A 4F balloon catheter was inserted via the paramedian borehole and inflated over a 10-minute period, achieving irreversible pontine ischemia by brainstem compression. In sham-operated controls, the balloon catheter was left in place without inflation during the entire observation period. BD was verified by an initial blood pressure peak (Cushing reflex), transient spontaneous muscular fasciculation of the rear limbs during brainstem compression, and subsequent absence of spinal reflexes. Afterward, reflex testing was performed at 10-minute intervals. In cases when persisting spinal reflexes were present for >10 minutes, animals were excluded from the study. Donors experiencing prolonged hypotensive periods <50 mm Hg for >20 minutes were also excluded. Figure 1 shows mean arterial pressure (MAP) values. During the entire 3-hour observation period, body temperature was maintained at 36°C to 37°C by a heating pad. Volume resuscitation was ensured with saline 0.9%.

Heart Transplantation

Cardiac grafts were placed in an infrarenal location via an abdominal midline incision by microsurgical technique. Graft function was assessed by manual palpation.

Histology

Tissue blocks were placed in 10% buffered formaldehyde solution for 48 hours before being embedded in paraffin. Heart sections were stained with hematoxylin and eosin and scored with a previously described histology scoring system on a scale of 0 to 3. The results are expressed as cumulative scores from 0 to 12. To further quantify evidence of cardiac damage, we measured cardiac enzyme troponin I activities as an index of cardiac damage (Life Diagnostics, West Chester, PA).

Complement Deposition

Paraffin-embedded heart tissue sections (4 μm) were stained for the presence of C3d by immunohistochemistry with an antibody directed against C3d (R&D Systems, Volcano, CA). Immunohistochemistry was scored semiquantitatively as described.

Immunohistochemistry

Neutrophils (GR1; BD Pharmingen, San Jose, CA) and macrophages (MAC-3; BD Pharmingen) were assessed by immunohistochemistry.
Human Samples

Twelve transmural Tru-Cut biopsies of the anterior free wall of the right ventricle of human donor hearts were procured from donors after transplantation treatment groups (CR2-Cry therapy or no therapy). General linear mixed models indicated that for both the isograft and allograft experiments, there were no significant differences in MAP values over time between the BD and BD+CR2-Cry groups. In the isograft experiment, the mean difference between group MAP values over time was 0.8 mm Hg (95% confidence interval, −9.1 to 10.6; P=0.87). In the allograft experiment, the mean difference between group MAP values over time was 0.7 mm Hg (95% confidence interval, −7.2 to 8.7; P=0.83).

ELISA Cytokine and Myeloperoxidase Analysis

Monocyte chemoattractant protein-1 (MCP-1), keratinocyte-derived chemokine, interleukin-1β, macrophage inflammatory protein-2, and myeloperoxidase were measured in heart grafts harvested at 48 hours after transplantation. Hearts were homogenized, and protein was extracted from tissues as described. Of chemokine/cytokine and myeloperoxidase were measured in heart grafts harvested at 48 hours after transplantation were stained with antibodies to either GR1 or MAC-3 and visualized with anti–rat 555. Sections were counterstained with TO-PRO-3 (Molecular Probes, Eugene, OR) and imaged with a Leica TCS-SP2 confocal microscope (Leica Microscope).

Statistics

GraphPad Prism version 5.0 for Mac OS X (GraphPad, San Diego, CA) and SAS version 9.2 (SAS Institute Inc, Cary, NC) were used for statistical analysis. Differences between various groups were compared by use of 1-way ANOVA with the Bonferroni multiple-comparisons test for post hoc analyses. Five specific pairwise comparisons were made to analyze the key questions of the effect of BD and CR2-Cry on transplantation outcome, specifically comparisons between living and BD, living and living+CR2-Cry, BD and BD+CR2-Cry, living and BD+CR2-Cry, and living and sham BD. For histological injury scores, the Kruskal-Wallis test was used, followed by the Dunn multiple-comparisons test for post hoc analyses. General linear mixed models were used to compare the MAP values between 2 BD groups over time, with separate models constructed for each experiment (isograft and allograft). In the models, MAP values were expressed as a function of several fixed effects: baseline MAP value (mm Hg), time (minutes), and BD group. Random mouse effects were also included to account for clustering of MAP values over time within individual mice. Autoregressive (type 1) error structures were selected on the basis of Akaike Information Criteria values of various error structures. SAS version 9.3 (PROC MIXED) was used to model repeated measurements over time. SAS Proc LIFETEST was used to conduct log-rank tests for comparing survival across the 4 groups.

Results

Experimental Studies

Brain Death

Irreversible pontine ischemia was achieved by balloon catheter inflation of 82±7 μL saline without significant differences between study groups. After induction of BD, animals were followed up for 3 hours. On completion of the BD procedure, hearts were harvested from donor animals and transplanted into mice that were randomized into 4 groups: CR2-Cry–treated and untreated groups for isograft transplantation and CR2-Cry–treated and untreated groups for allograft transplantation. To ensure that the effects of CR2-Cry on isograft and allograft transplantation groups were associated with therapy, not donor organ quality, we analyzed the MAP profiles from each individual donor. Figure 1 shows the MAP of BD donor mice that are grouped into their posttransplantation treatment groups. We performed a general linear mixed model analysis to determine whether there were any differences in the quality of donor organs between groups. Results of the general linear mixed models indicated that for both the isograft and allograft experiments, there were no significant differences in mean MAP values over time between the BD and BD+CR2-Cry groups. In the isograft experiment (Figure 1A), the mean difference between groups MAP values over time was 0.8 mm Hg (95% confidence interval, −9.1 to 10.6; P=0.87). In the allograft experiment (Figure 1B), the mean difference between groups MAP values over time was 0.7 mm Hg (95% confidence interval, −7.2 to 8.7; P=0.83; Figure 1A and 1B). Cold and warm ischemic times of the cardiac grafts were similar in all experimental groups.

BD Exacerbates Ischemia/Reperfusion Injury

Hearts procured 48 hours after transplantation from recipients that received either living or BD donor hearts exhibited key features associated with ischemia/reperfusion injury (IRI), was visualized by detection methods (DakoCytomation). The investigative protocol was approved by the local research committee.
including myocyte damage in the epicardium, endocardium, and myocardium. Transplanted hearts also showed evidence of inflammatory cell infiltration and endothelial activation denoted by endothelial swelling. However, histological scores of injury and inflammation were higher in grafts from recipients that received BD donor hearts (Figure 2A). In accordance with these histological observations, serum levels of cardiac troponin I, an index of cardiac cell damage, were also significantly higher in recipients receiving BD donor hearts compared with recipients receiving living donor hearts (Figure 2B).

**Treatment of Recipients With a Targeted Complement Inhibitor Protects Both Living and BD Donor Hearts From IRI**

Complement is known to play a role in IRI of multiple organs, and because the data above show that cardiac IRI is exacerbated in BD donor hearts, we investigated the effect of a targeted complement inhibitor on IRI after the transplantation of both living and BD donor hearts. Recipients were treated with 0.25 mg CR2-Crry immediately after reperfusion. CR2-Crry reduced cardiac injury profiles in hearts transplanted from both living and BD donors (Figure 2A and 2B). Of note, CR2-Crry treatment reduced injury in living and BD hearts to levels at or below those seen in living and sham BD transplanted hearts without inhibitor treatment.

**Donor BD Exacerbates Posttransplantation Complement Deposition in Grafts**

In a previous report, we demonstrated that complement is activated as a consequence of BD and subsequent animal management. We therefore investigated whether the exacerbated injury seen in BD donor hearts after transplantation is associated with increased C3 deposition. Immunohistochemistry demonstrated the presence of the complement activation product C3d in all grafts analyzed, with staining present on endothelial cells lining capillaries and in areas of myocyte damage (Figure 3A). Quantification of C3d deposition revealed significantly higher levels of C3d in grafts from BD donors compared with grafts from living donors. Furthermore, treatment of recipients with CR2-Crry significantly reduced C3d deposition in both living and BD donor hearts, with C3d levels in BD donor grafts reduced to that seen in living donor grafts from recipients that did not receive inhibitor (Figure 3). Of note, C3d levels in grafts from both living and BD donors correlated with histological injury scores.

**Complement Inhibition Attenuates Inflammatory Cell Infiltration**

We next investigated whether the exacerbated complement activation seen in BD donor grafts was associated with increased infiltration of neutrophils and macrophages. Correlating with the histological determination of injury and C3d deposition, levels of myeloperoxidase were significantly elevated in grafts from BD donors compared with grafts from living donors. Furthermore, treatment of recipients with CR2-Crry significantly reduced myeloperoxidase levels in both living and BD donor hearts, with C3d levels in BD donor grafts reduced to that seen in living donor grafts from recipients that did not receive inhibitor (Figure 3A). Quantification revealed significantly higher numbers of both cell types were not significantly different between groups (not shown). Quantification revealed significantly higher numbers of both neutrophils and macrophages in BD donor grafts compared with living donor grafts, and CR2-Crry treatment reduced neutrophil and macrophage numbers in both living and BD donors (Figure 4A).

To further quantify specific inflammatory cell infiltration, we analyzed graft sections by immunohistochemistry for neutrophils (anti-GR1) and macrophages (anti–MAC-3). Neutrophils and macrophages were present in all groups, with infiltrating cells present in the epicardial, endocardial, and myocardial compartments between myocytes and localized in larger numbers at the epicardial surface. The distributions of both cell types were not significantly different between groups (not shown). Quantification revealed significantly higher numbers of both neutrophils and macrophages in BD donor grafts compared with living donor grafts, and CR2-Crry treatment reduced neutrophil and macrophage numbers in both living and BD donors (Figure 4B and 4C).

**Donor BD Increases Intrgraft Proinflammatory Cytokine Expression**

Previous studies in rat models have shown that progressive BD induction in donor animals results in increased levels of inflammatory cytokines in transplanted organs. Here, in this newly developed mouse model, we assayed

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**Figure 2.** Assessment of cardiac injury in recipients of living or brain dead (BD) donor hearts. When indicated, recipients were treated with CR2-Crry. A, Histological quantification of cardiac injury in grafts harvested 48 hours after transplantation, quantified by use of a 0 to 12 cumulative injury score. Pairwise comparisons between living and living+CR2-Crry (##P<0.01), living and BD (**P<0.05), and BD and BD+CR2-Crry (###P<0.001). Differences between living and BD+CR2-Crry and between living and BD sham were not significant. B, Serum cardiac troponin I levels in recipient mice 48 hours after transplantation. Pairwise comparisons between living and living+CR2-Crry (#P<0.05), living and BD (**P<0.001), and BD and BD+CR2-Crry (##P<0.001). Differences between living and BD+CR2-Crry and between living and BD sham were not significant. Results are expressed as mean±SE (n=6–12).
for chemokines involved in neutrophil recruitment/activation (macrophage inflammatory protein-2 and keratinocyte-derived chemokine), a chemokine involved in monocyte recruitment (MCP-1), and a cytokine associated with IRI in transplanted hearts (interleukin-1β). At 48 hours after transplantation, levels of macrophage inflammatory protein-2, interleukin-1β, MCP-1, and keratinocyte-derived chemokine were significantly elevated in grafts from all groups compared with control normal hearts. Levels of macrophage inflammatory protein-2, keratinocyte-derived chemokine, and interleukin-1β, but not MCP-1, were significantly higher in BD donor grafts than in living donor grafts (Figure 5).
CR2-Crry treatment of recipients receiving either living or BD donor grafts resulted in a significant reduction in all measured chemokine/cytokines, including MCP-1, compared with the respective grafts from untreated recipients. Furthermore, as with the other markers of injury and inflammation reported above, CR2-Crry treatment reduced chemokine/cytokine levels in BD grafts to levels at or below (in the case of MCP-1) those seen in living donor grafts.

**Donor BD Significantly Reduces Allograft Survival**

To further assess the impact of BD in transplantation and the therapeutic potential of targeted complement inhibition in a more clinically relevant setting, we performed allograft transplantations. Hearts from C57BL/6 living, sham, or BD donors were transplanted into BALB/c recipients. In concordance with the isograft data above, BD was associated with significantly poorer median graft survival (11 days) compared with living, sham operated, and BD CR2-Crry–treated animals (14, 13, and 16 days, respectively; Figure 6). Log-rank test suggested that the survival times were significantly different among groups ($P<0.0001$). All pairwise comparisons demonstrated that survival was significantly greater among each group compared with the BD group ($P<0.01$ for all), even after a Bonferroni adjustment for multiple comparisons. No significant difference was seen between living, sham BD, and BD+CR2-Crry animals.

**Clinical Studies: Complement Activation and Immune Cell Infiltration**

To address the clinical relevance of our findings, complement activation was assessed in human Tru-Cut biopsies of donor hearts before implantation and 10 minutes after reperfusion. We analyzed complement deposition in 8 biopsies from human hearts taken from BD donors and in 4 biopsies from human hearts taken from living (domino) donors (from cystic fibrosis patients undergoing combined heart-lung transplantation). With the use of accepted guidelines for histology scoring of C4d and C3d deposition, biopsies were deemed positive if 2+ immunostaining was noted. In biopsies taken from BD donors before implantation, 5 of 8 were positive for C4d and 8 of 8 were positive for C3d. In preimplantation biopsies from living donor hearts, 0 of 4 were positive for C4d and 1 of 4 was positive for C3d. Ten minutes after transplantation of BD donor hearts, 5 of 8 biopsies were positive for C4d and 8 of 8 were positive for C3d, whereas for biopsies from living donors, again 0 of 4 were positive for C4d and 1 of 4 was positive for C3d. In all samples that stained positive, both C4d and C3d were seen predominantly in capillaries and arterioles and, in some cases, the area surrounding myocytes. The staining pattern was similar to that which we recently described for C3d in hearts from BD mice. Representative images for C4d staining of human biopsy sections at the time of donor optimization are shown in Figure 7.
To investigate the inflammatory status of BD and living donor hearts before and after transplantation, biopsies procured at 4 time points (after donor optimization, before implantation, 10 minutes after reperfusion, and 1 week after reperfusion) were analyzed for the presence of monocytes/macrophages (MAC387) and mature tissue macrophages (CD68). MAC387- and CD68-positive cells were present within vessels and in perivascular spaces and were interspersed between myocytes in biopsies taken from both BD and living donors. However, the number of both MAC387- and CD68-positive cells was significantly higher in samples from BD donors compared with living donors at all time points, except for CD68-positive cells before transplantation (Figure 8A and 8B). Of note, the number of MAC387- and CD68-positive cells in grafts from BD donors remained elevated for at least a week after transplantation, whereas in living donor grafts, the levels of positive cells were not significantly different at 10 minutes after reperfusion compared with normal control hearts (P>0.05). We did not have access to 1-week data for the living donor group.

Origin of Neutrophils and Macrophages in Murine Grafts

We previously demonstrated a significant increase in the infiltration of neutrophils and macrophages in mouse donor hearts after BD.20 The above human data similarly show increased infiltration of neutrophils and macrophages in the BD versus living donor organ before transplantation and that these increases persist 1 week after transplantation. We therefore investigated whether donor-derived inflammatory cells persist in the BD donor organ after transplantation. Using a pan-GFP mouse as BD donor, we assessed GFP+ neutrophils and GFP+ macrophages in grafts at time 0 (after BD but before implantation) and at 6 and 48 hours after transplantation into syngeneic recipients. At time 0, neutrophils and macrophages were present within vessels, at the epicardial surface, and in the subvascular space, as previously described.20 However, we were unable to detect any GFP+GR1+ or GFP+Mac-3+ cells in grafts at either time point after transplantation (Figure I in the online-only Data Supplement). These data indicate that although BD increases donor organ inflammatory cell burden before transplantation, the main source of neutrophils and macrophages after transplantation is the recipient.

Discussion

Donor organ injury induced by BD and IRI is considered to be an important factor in delayed graft function and the
accelerated onset of graft rejection after transplantation. Here, we investigated the role of complement in BD-associated IRI of transplanted hearts. Using our recently described murine model of BD, we show that donor BD enhances complement activation and exacerbates IRI in cardiac isografts and reduces cardiac allograft survival. We show that these findings correlate with clinical data obtained from analysis of human cardiac biopsies and that, in contrast to BD donor human hearts, there is minimal or absent complement activation in living donor human hearts, both at the time of organ procurement and at 10 minutes after reperfusion. Furthermore, we demonstrate that a site-specific targeted complement inhibitor administered to recipient mice effectively protects cardiac grafts against BD-exacerbated IRI.

Previous studies in animal models have shown that BD is associated with cardiac injury that is thought to exacerbate IRI and rejection, and we have shown a role for complement in BD-induced cardiac injury in mice. In addition, previous studies in rat models of BD and kidney transplantation have demonstrated that BD induces systemic and local (renal) complement activation. It was also shown recently that treatment of BD donor rats with the systemic complement inhibitor sC1 improved renal graft function in the early posttransplantation period and diminished graft mRNA levels of some cytokines. Nevertheless, a key consideration for donor therapy is that any intervention should not affect the successful use of all potential organs. This is a potential limitation of complement inhibition in the donor because it is not known if all donor organs would benefit from such therapy (refer to the introduction). Here, we show that complement inhibition in the recipient immediately after donor organ implantation significantly ameliorates cardiac graft damage and returns inflammation and injury profiles in grafts from BD donors to those seen in grafts from living donors. In these studies, we used a targeted complement inhibitor, CR2-Crry. The CR2 moiety of this fusion protein binds to the C3 cleavage products iC3b and C3d that are deposited at sites of complement activation, which in the present model is the transplanted heart. We have previously shown that multiple doses of CR2-Crry have a minimal effect on serum complement activity and, unlike even a single dose of a systemic inhibitor, do not enhance host susceptibility to infection. This is an important consideration in a transplant recipient who will be heavily immunosuppressed. Although not examined in the context of BD, another strategy that has been investigated to reduce renal IRI after transplantation is perfusion of the donor kidney with a derivative of sC1 that contains a myristoylated peptide (APT070) and that targets to cell membranes, albeit nonspecifically. Pretreatment of rat kidneys with APT070 improved posttransplantation outcome. Nevertheless, although this approach is specific to the transplanted organ, further administration to the recipient is not possible without systemically inhibiting complement. This constraint does not apply to CR2-mediated site-specific targeting, and prolonged complement inhibition in the recipient may be beneficial because complement is also implicated in acute allograft rejection. Of note, a CR2-targeted inhibitor of the alternative complement pathway, TT30 (human CR2-iH), is currently in phase I clinical trials for paroxysmal nocturnal hemoglobinuria (http://www.clinicaltrials.gov/ct2/results?term=tt30).

The increased cardiac injury (histology scores and serum troponin) in murine grafts from BD versus living donors correlates directly with significantly increased numbers of infiltrating inflammatory cells, as determined by myeloperoxidase levels, immunohistochemistry, and graft chemokine levels. We demonstrate that BD is also associated with a significant elevation of infiltrating inflammatory cells in human cardiac grafts, which may contribute to poorer clinical outcome of grafts from BD donors. In the human samples, we show that macrophages are elevated at all time points in BD compared with living donor heart biopsies. Furthermore, this increase in cellular infiltrate in BD hearts persists 1 week after transplantation. One-week posttransplantation biopsies from the living donor group were not available, but cell numbers in living donor graft biopsies are not significantly higher than in control biopsies at 10 minutes after reperfusion. Our studies using pan-GFP donor mice show elevated numbers of neutrophils and macrophages in BD donor hearts before transplantation, and this finding is in keeping with our previous observations. However, although the increased numbers of neutrophils and macrophages seen in mouse BD donor organs persisted after transplantation, further analysis indicated that the infiltrating cells were of recipient origin, and we were unable to detect any donor-derived inflammatory cells within the grafts at 6 and 48 hours after transplantation. This was an unexpected finding. Although the absence of donor-derived neutrophils may be easier to explain given their relatively short life span, macrophages may reside in tissues for many weeks. It is possible that macrophages migrate out of the graft early after transplantation and act as antigen-presenting cells, which may accelerate graft recognition and rejection, a hypothesis supported by the increased tempo of rejection seen in recipients of BD donor hearts. Further studies to delineate the role of donor macrophages in allore cognition are warranted but fall beyond the scope of this study.

In the present studies, we included 2 control groups: a living donor control and a sham BD control. Sham BD controls were anesthetized and ventilated and had a catheter placed for 3 hours without balloon inflation. Ventilatory support has been associated with increased neutrophil and macrophage infiltration into the lungs; therefore, we anticipated that sham BD animals would have a poorer prognosis after transplantation than living controls. Somewhat surprisingly, however, there was no significant difference in measured outcomes between sham BD and living controls in IRI or allograft survival.

Conclusions

Important findings of this study are that treatment of the recipient with a targeted complement inhibitor protects against cardiac graft damage and improves allograft survival and that posttransplantation IRI in grafts from BD donors is reduced to a level at or below that seen in grafts from living donors. This finding is significant because we further demonstrate a clinical correlation between complement activation and inflammation in hearts from BD versus living donors, and previous clinical studies have shown that recipients of living donor grafts have a reduced risk of primary graft failure and acute rejection, as well as a delayed onset of chronic rejection. The data suggest that complement inhibition may be used to ameliorate
BD-exacerbated IRI and that treatment of the recipient will mitigate potential complications of donor therapy such as time of administration after BD diagnosis and possible negative effects on other donor organs. Furthermore, a complement inhibitor targeted to the transplanted organ, as used in the present study, may also be given at multiple time points after transplantation with minimal effect on complement-dependent host immune or homeostatic functions.

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Disclosures
Dr Tomlinson is an inventor of licensed patents on CR2-targeted complement inhibitors. The other authors report no conflicts.

References
Clinical Perspective

The majority of organs used for transplantation are acquired from brain-dead donors, and evidence from clinical and experimental studies indicates that brain death is a significant contributing factor to ischemia/reperfusion injury and subsequent inferior graft function and is detrimental to the outcome of kidney, heart, lung, and liver transplantations. We investigated the role of complement in brain death–associated ischemia/reperfusion injury of transplanted hearts using our recently described murine model. We show that donor brain death enhances complement activation and exacerbates ischemia/reperfusion injury in transplanted cardiac grafts. We also show that treatment of recipient mice with a targeted complement inhibitor protects against cardiac graft damage and that posttransplantation ischemia/reperfusion injury in grafts from brain-dead donors is reduced to a level at or below that seen in grafts from living donors. Furthermore, we demonstrate a clinical correlation between complement activation and inflammation in hearts from brain-dead versus living donors, and previous clinical studies have shown that recipients of living donor grafts have a reduced risk of primary graft failure and acute rejection, as well as a delayed onset of chronic rejection. The data suggest that complement inhibition may be used to ameliorate brain death–exacerbated ischemia/reperfusion injury and that treating the recipient will mitigate potential complications of donor therapy. A complement inhibitor targeted to the transplanted organ, as used in the present study, may also be given at multiple time points after transplantation with minimal effect on complement-dependent host immune or homeostatic functions.
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**SUPPLEMENTAL MATERIAL**

**Supplemental Figure 1.** GFP Cell fate analysis. GFP hearts were analyzed at 0, 6 and 48 hrs post transplantation for the presence of donor derived neutrophils (Gr-1, red) and macrophages (MAC-3, red) using immunohistochemical (DAB, brown) and immunofluorescent techniques. Note the presence of neutrophils and macrophages in all samples at all time points with immunohistochemistry staining. Fate analysis demonstrated that dual positive GFP+ GR-1+ (yellow) or GFP + Mac-3+ (yellow) were only seen at time 0 (time of harvest from brain dead donor). Scale bar represents 30 μM.