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

Running title: Atkinson et al.; Donor brain death exacerbates post-transplant IRI

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Abstract:

Background—Brain death (BD) can immunologically prime the donor organ and is thought to lead to exacerbated ischemia reperfusion injury (IRI) post-transplantation. Using a newly developed mouse model of BD, we investigated the effect of donor BD on post transplant cardiac IRI. 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 analysis of human heart biopsies from BD and domino (living) donors.

Methods and Results—Hearts from living or brain dead 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 post-transplant for injury, inflammation and complement deposition, and allografts monitored for graft survival. Human cardiac biopsies were analyzed for complement deposition and inflammatory cell infiltration. In the murine model, donor BD exacerbated IRI and graft rejection as demonstrated by increased myocardial injury, serum cardiac troponin, cellular infiltration, inflammatory chemokine and cytokine levels, complement deposition, and decreased graft survival. CR2-Crry treatment of recipients significantly reduced all measured outcomes in grafts from both BD and living donors compared to 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 to grafts from living donors.

Conclusions—BD exacerbates post-transplant cardiac IRI in mice and humans, and decreases survival of mouse allografts. Further, targeted complement inhibition in recipient mice ameliorates BD-exacerbated IRI.

Key words: Ischemia reperfusion injury, inflammation, transplantation.
Introduction

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

Brain death evokes a potent Cushing’s response that leads to hemodynamic fluctuations, global organ ischemia, hypothermia, coagulopathies, hormone depletion, and electrolyte abnormalities\textsuperscript{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 pro-inflammatory prior to implantation\textsuperscript{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\textsuperscript{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\textsuperscript{11-13}. In kidney transplantation, BD is associated with increased levels of complement activation\textsuperscript{14-18}. Complement activation in the BD donor has also been shown to correlate with a poorer patient outcome after renal transplantation\textsuperscript{19}. Using a rat model of BD, treatment of the BD donor with a systemic complement inhibitor, sCR1, prior to and 1 hour post BD induction, resulted in improved renal
transplant outcomes\textsuperscript{17}. Finally, in developing a mouse model of BD, we demonstrated that deficiency of C3, a central protein through which all complement activation pathways operate, is protective against BD-associated cardiac injury and inflammation\textsuperscript{20}.

The above studies demonstrate that complement is activated in the BD donor, and indicate that BD-induced complement activation negatively impacts post-transplant 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 referenced above, the authors bypass the potential risk of systemic inhibition in the recipient by treating the organ (kidney) donor. However, donor management remains a controversial topic of debate due to 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\textsuperscript{21-23}, and inhibition of complement in the donor may impact 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\textsuperscript{24}, 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 post-transplant 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-GFP mice (latter kindly gifted by Dr Okabe, Japan), 8–12 weeks old and between 20-30 grams, 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):

1. Living donor, 2. Living donor with CR2-Crry treated recipient, 3. BD sham operated, 4. BD donor, 5. BD donor with CR2-Crry treated recipient. Untreated naïve C57BL/6 mice were used as recipients, and a 6th group consisting 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): 1. Living donor, 2. BD sham operated, 4. BD donor, 5. BD donor with CR2-Crry treated recipient. To investigate the origin of inflammatory cells in grafts post transplantation, BD donor hearts from pan-GFP C57BL/6 mice were analysed at time 0 prior to transplantation, and at 6 and 48 hrs after transplantation into C57BL/6 recipients. Recipients in CR2-Crry treatment groups received a single intraperitoneal injection of 0.25 mg CR2-Crry. CR2-Crry was prepared and purified as previously described. Cardiac grafts and serum were harvested 48 hours post transplantation.
Brain death induction

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

Heart transplantation

Cardiac grafts were placed into 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 h before being embedded in paraffin. Heart sections were stained with H&E, and scored using a histology scoring system on a scale of 0-3 previously described\(^\text{26}\). 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 (4μm) were stained for the presence of C3d by immunohistochemistry using an Ab directed against C3d (R&D Systems, Volcano, CA). Immunohistochemistry was scored semiquantitatively as described\(^\text{27}\).

**Immunohistochemistry**

Neutrophils (GR1; BD Pharmingen, San Jose, CA) and macrophages (mac-3; BD Pharmingen) were assessed by immunohistochemistry and quantified by computerized image analysis (Zeiss Microsystems, USA). For cell fate studies, sections from GFP\(^+\) donor hearts at 0, 6 and 48 hrs post transplantation were stained with antibodies to either GR1 or MAC-3, and visualized using anti-rat 555. Sections were counterstained with TO-PRO-3 (Molecular Probes, OR, USA) and imaged using a Leica TCS-SP2 Confocal microscope (Leica Microscope, USA).

**ELISA Cytokine and Myeloperoxidase Analysis (MPO)**

MCP-1, KC, IL-1β, MIP-2 and MPO were measured in heart grafts harvested at 48 hrs post-transplantation. Hearts were homogenized, and protein extracted from tissues as described\(^\text{26}\). Chemokine/cytokine and MPO levels were measured using ELISA kits (R&D Systems, USA, and Northwestern Life Science Specialties, USA) in accordance with the manufacturer’s recommendations.

**Human Samples**
Twelve-transmural tru cut biopsies of the anterior free wall of the right ventricle of human donor hearts were procured from donors following optimization, prior to implantation, 10 mins post reperfusion and 1 week post reperfusion, as previously described. Of the cardiac biopsies studied, 8 male recipients (mean age 49.3 years, range 17 – 62) were obtained from BD heart beating donors and 4 from living (domino) donors (mean age 50 years, range 36-61). All underwent transplantation between February 2000 and May 2001. Non-transplant control tissue came from 5 patients undergoing elective revascularization. The mean age of controls was 59.1±9.2 years, and none had congestive cardiac failure. Each biopsy was stained with antibodies to C4d (Abcam, UK) and C3d (DakoCytomation, UK), and for monocyte/macrophages (MAC387) and mature macrophages (CD68) (DakoCytomation, UK). Antibody binding was visualized by detection methods (DakoCytomation, UK). The investigative protocol was approved by the Local Research Committee.

Statistics
GraphPad Prism version 5.0 for Mac OS X (GraphPad, San Diego, CA) and SAS v9.2 (Cary, NC) were used for statistical analysis. Differences between various groups were compared using 1-way analysis of variance with Bonferroni multiple comparisons test for post hoc analyses. Five specific pairwise comparisons were made to analyze the key questions of the effect of brain death and CR2-Crry on transplant outcome. Specifically, comparisons between living vs. BD; living vs. living+CR2-Crry; BD vs. BD+CR2-Crry; living vs. BD+CR2-Crry; living vs. Sham BD. For histological injury scores, the Kruskal–Wallis test was used, followed by Dunn’s 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 (mmHg), time (minutes), and BD group; random mouse effects were also included to account for clustering of MAP values over time within individual mice. Auto-regressive (type 1) error structures were selected, based on Akaike Information Criteria (AIC) values of various error structures. SAS v9.3 (PROC MIXED) was used for the modeling of repeated measurements over time. SAS Proc LIFETEST was used to conduct logrank tests for comparing survival across the 4 groups (Cary, NC).

Results

Experimental studies:

Brain Death

Irreversible pontine ischemia was achieved by balloon catheter inflation of 82±27 μL saline without significant differences between study groups. After induction of BD, animals were followed for three hours. On completion of BD procedure, hearts were harvested from donor animals and transplanted into mice that were randomized into four groups: CR2-Crry treated and untreated groups for isograft transplantation, and CR2-Crry treated and untreated groups for allograft transplantation. To ensure that effects of CR2-Crry on isograft and allograft transplantation groups were associated with therapy, and not donor organ quality, we analysed the mean arterial pressure profiles from each individual donor. Figure 1 shows the mean arterial pressure of brain dead donor mice that are grouped into their post transplant treatment groups. We performed a general linear mixed model analysis to determine whether there were any differences in 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-Crry groups. In the
isograft experiment (Fig 1A), the mean difference between groups MAP values over time was
0.8 mmHg (95% confidence interval: -9.1 to 10.6, p=0.87). In the allograft experiment (Fig 1B),
the mean difference between groups MAP values over time was 0.7 mmHg (95% confidence
interval: -7.2 to 8.7, p=0.83) (Fig 1A & B). Cold and warm ischemic times of the cardiac grafts
were similar in all experimental groups.

**Brain death exacerbates ischemia reperfusion injury**

Hearts procured 48 hours post-transplant from recipients that received either living or BD donor
hearts exhibited key features associated with ischemia reperfusion injury (IRI), including
myocyte damage in the epicardium, endocardium, and myocardium. Transplanted hearts also
showed evidence of inflammatory cell infiltration, as well as endothelial activation denoted by
endothelial swelling. However, histological scores of injury and inflammation were higher in
grafts from recipients that received BD donor hearts (Fig 2A). In accordance with these
histological observations, serum levels cardiac troponin I, an index of cardiac cell damage, was
also significantly higher in recipients receiving BD donor hearts compared to recipients receiving
living donor hearts (Fig. 2B).

**Treatment of recipients with a targeted complement inhibitor protects both living and
brain dead donor hearts from IRI**

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

**Donor brain death exacerbates post transplant complement deposition in grafts**

In a previous report, we demonstrated that complement is activated as a consequence of BD and subsequent animal management\(^\text{20}\). 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 (Fig. 3A). Quantification of C3d deposition revealed significantly higher levels of C3d in grafts from BD donors compared to 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 (Fig. 3B). 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 MPO were significantly elevated in grafts from BD donors compared to grafts from living donors. Furthermore, treatment of recipients with CR2-Crry significantly reduced MPO levels in grafts from both living and BD donors (Fig. 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 distribution of both cell types was not significantly different between
groups (not shown). Quantification revealed significantly higher numbers of both neutrophils and
macrophages in BD donor grafts compared to living donor grafts, and CR2-Crry treatment
reduced neutrophil and macrophage numbers in both living and BD donor grafts (Fig 4B and C).

Donor brain death increases intragraft pro-inflammatory 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\(^4,29-31\). Here, in this
newly developed mouse model, we assayed for chemokines involved in neutrophil
recruitment/activation (MIP-2 and KC), a chemokine involved in monocyte recruitment (MCP-
1), and a cytokine associated with IRI in transplanted hearts (IL-1\(\beta\)). At 48 hours post-transplant,
levels of MIP-2, IL-1\(\beta\), MCP-1, and KC were significantly elevated in grafts from all groups
compared to control normal hearts. Levels of MIP-2, KC and IL-1 \(\beta\), but not MCP-1, were
significantly higher in BD donor grafts than living donor grafts (Fig. 5). CR2-Crry treatment of
recipients receiving either living or BD donor grafts resulted in a significant reduction of all
measured chemokine/cytokines, including MCP-1, compared to the respective grafts from
untreated recipients. Further, 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) that seen in living donor grafts.

Donor brain death 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 transplants.
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) as compared to living, sham operated, and BD CR2-Crry treated animals (14, 13 and 16 days, respectively) (Fig. 6). Logrank 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 when compared to 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 or BD + CR2-Crry.

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 prior to implantation and ten minutes post 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). Using 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, 5/8 were positive for C4d and 8/8 were positive for C3d. In pre-implantation biopsies from living donor hearts, 0/4 were positive for C4d and 1/4 were positive for C3d. Ten minutes post transplantation of BD donor hearts, 5/8 biopsies were positive for C4d and 8/8 were positive C3d, whereas for biopsies from living donors, again 0/4 were positive for C4d and 1/4 for C3d. In all samples that stained positive, both C4d and C3d was 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 pre and post-transplantation, biopsies procured at four time points (following donor optimization, prior to implantation, 10 mins post-reperfusion, and one week post reperfusion) were analyzed for the presence of monocytes/macrophages (MAC387) and mature tissue macrophages (CD68). MAC387 and CD68 positive cells were present within vessels, in perivascular spaces and 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 to living donors at all time points, except for CD68 positive cells prior to transplantation (Fig. 8A and B). Of note, the number of MAC387 and CD68 positive cells in grafts from BD donors remained elevated for at least a week post-transplant, whereas in living donor grafts the level of positive cells were not significantly different at 10 minutes post-reperfusion than in normal heart controls ($p > 0.05$). We did not have access to one-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 following BD\textsuperscript{20}. The above human data similarly shows increased infiltration of neutrophils and macrophages in the BD vs. living donor organ prior to transplantation, and also that these increases persist one week post 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 (following brain death, but prior to implantation), and at 6 and 48 hours post 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\textsuperscript{20}. We were, however, unable to detect any GFP+GR1+ or GFP+Mac-3+ cells in grafts at either time point post transplantation (\textbf{supplemental Figure 1}). These data indicate that while BD increases donor organ inflammatory cell burden prior to transplantation, the main source of neutrophils and macrophages post-transplantation are recipient derived.

\textbf{Discussion}

Donor organ injury induced by BD and IRI are considered to be important factors in delayed graft function and the accelerated onset of graft rejection following transplantation\textsuperscript{32-34}. 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 post-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\textsuperscript{32,35,36}, and we have shown a role for complement in BD induced cardiac injury in mice\textsuperscript{20}. In addition, previous studies in rat models of BD and kidney transplantation have demonstrated that BD induces systemic and local (renal) complement activation\textsuperscript{17}. It was also recently shown that treatment of BD donor rats with the systemic complement inhibitor, sCR1, improved renal graft function in the early post transplant period and diminished graft mRNA levels of some cytokines\textsuperscript{17}. Nevertheless, a key consideration
for donor therapy is that any intervention should not impact the successful utilization of all potential organs. This is a potential limitation of complement inhibition in the donor, since it is not known if all donor organs would benefit from such therapy (refer to introduction). Here we show that complement inhibition in the recipient immediately following donor organ implantation significantly ameliorates cardiac graft damage, and returns inflammation and injury profiles in grafts from BD donors to that seen in grafts from living donors. In these studies, we utilized 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 current model is the transplanted heart. We have previously shown that multiple doses of CR2-Crry have minimal effect on serum complement activity and, unlike even a single dose of a systemic inhibitor, does 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 sCR1 that contains a myristoylated peptide (APT070) and that targets to cell membranes, albeit nonspecifically. Pre-treatment of rat kidneys with APT070 improved post-transplant outcome.

Nevertheless, while 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 since complement is also implicated in acute allograft rejection. Of note, a CR2-targeted inhibitor of the alternative complement pathway, TT30 (human CR2-fH), is currently in phase I clinical trials for paroxysmal nocturnal hemoglobinuria (http://clinicaltrials.gov/ct2/results?term=tt30).
The increased cardiac injury (histology scores and serum troponin) in murine grafts from BD vs. living donors correlates directly with significantly increased numbers of infiltrating inflammatory cells, as determined by MPO 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 to living donor heart biopsies. Furthermore, this increase in cellular infiltrate in BD hearts persists one-week post transplantation. One-week post-transplant 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 post-reperfusion. Our studies using pan-GFP donor mice show elevated numbers of neutrophils and macrophages in BD donor hearts prior to transplantation, and this finding is in keeping with our previous observations. However, while the increased numbers of neutrophils and macrophages seen in mouse BD donor organs persisted after transplantation, further analysis indicated 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 hrs post transplantation. This was an unexpected finding. While 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 post 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 brain dead 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 current studies we included two control groups; a living donor control and a sham
BD control. Sham BD controls were anesthetized, ventilated, and catheter placed for 3 hours without balloon inflation. Ventilatory support has been associated with increased neutrophil and macrophage infiltration into the lungs, and we therefore anticipated that Sham BD animals would have a poorer prognosis post-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.

In summary, an important finding of this study is that treatment of the recipient with a targeted complement inhibitor protects against cardiac graft damage and improves allograft survival, and that post-transplant IRI in grafts from BD donors is reduced to a level at or below that seen in grafts from living donors. This is significant because we further demonstrate a clinical correlation between complement activation and inflammation in hearts from BD vs. 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 utilized to ameliorate BD exacerbated IRI, and treatment of the recipient will mitigate potential complications of donor therapy, such as time of administration post BD diagnosis and possible negative effects on other donor organs. Furthermore, a complement inhibitor targeted to the transplanted organ, as used in the current study, may also be given at multiple time points post transplantation with minimal effect on complement-dependent host immune or homeostatic functions.

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Conflict of Interest Disclosures: Stephen Tomlinson is inventor of licensed patents on CR2-targeted complement inhibitors. The other authors report no conflicts.

References:


**Figure Legends:**

**Figure 1.** Mean arterial pressures of brain dead heart beating donors over 3 hour period. Isograft (A) and allograft (B) recipients grouped based on their post transplant treatment groups (CR2-Crry therapy or no therapy). 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-Crry groups. In isograft experiment, the mean difference between groups MAP values over time was 0.8 mmHg (95% confidence interval: -9.1 to 10.6, p=0.87). In the allograft experiment, the mean difference between groups MAP values over time was 0.7 mmHg (95% confidence interval: -7.2 to 8.7, p=0.83).

**Figure 2.** Assessment of cardiac injury in recipients of living or brain dead donor hearts. Where
indicated, recipients were treated with CR2-Crry. A. Histological quantification of cardiac injury in grafts harvested 48 hrs post transplantation, quantified using 0-12 cumulative injury score. Pairwise comparisons between living vs. living+CR2-Crry (#p<0.05), living vs. BD (**p<0.05) and BD vs. BD+CR2-Crry (##p<0.05). Differences between living vs. BD+CR2-Crry and between living vs. BD Sham were not significant. B. Serum cardiac troponin I levels in recipient mice 48 hrs post transplantation. Pairwise comparisons between living vs. living+CR2-Crry and between living vs. BD Sham were not significant. Results expressed as Mean ± SE; (n=6-12).

**Figure 3.** C3d deposition in grafts isolated 48hrs post transplantation. A. Representative C3d immunostained images from recipients of living and BD donor cardiac grafts. C3d is localized to endothelial cells and myocytes. Representative of n=6. Original magnification x10. B. Semiquantitative analysis of C3d deposition in grafts from living and BD donors. Pairwise comparisons between living vs. living+CR2-Crry (#p<0.05), living vs. BD (**p<0.01) and BD vs. BD+CR2-Crry (##p<0.001). Differences between living vs. BD+CR2-Crry and living vs BD Sham were not significant. Results expressed as Mean ± SE; (n=6).

**Figure 4.** Effect of brain death and complement inhibition on inflammatory cell infiltration. A. Myeloperoxidase activity in cardiac grafts 48 hrs post transplantation. Pairwise comparisons between living vs. living+CR2-Crry (#p<0.05), living vs. BD (**p<0.001) and BD vs. BD+CR2-Crry (##p<0.001). Differences between living vs. BD+CR2-Crry and living vs BD Sham were not significant. Immunohistochemistry quantification of neutrophil (B) and macrophage (C)
infiltration as marked by GR-1 and MAC-3, respectively. Pairwise comparisons for neutrophil studies (B) between living vs. living+CR2-Crry (#p<0.05) and living vs. BD (**p<0.01). Differences between BD vs. BD+CR2-Crry, living vs. BD+CR2-Crry and Living vs. BD Sham were not significant. For macrophage studies (C), pairwise comparisons between living vs. living+CR2-Crry (#p<0.05), living vs. BD (**p<0.01) and BD vs. BD+CR2-Crry (##p<0.01). Differences between living vs. BD+CR2-Crry and living vs. BD Sham were not significant. Results expressed as Mean ± SE; (n=4-6).

**Figure 5.** Effect of brain death and complement inhibition on graft chemokine and cytokine expression. Cardiac grafts were harvested 48 hrs post transplantation. Cytokines in cardiac graft homogenates were measured by ELISA. Pairwise comparisons between living vs. living+CR2-Crry (#p<0.05), living vs. BD (**p<0.001, with exception of MCP-1, which was not significant) and BD vs. BD+CR2-Crry (##p<0.001). Differences between living vs. BD+CR2-Crry and living vs. BD Sham were not significant. Results expressed as mean ± SE; (n=4-6).

**Figure 6.** Effect of brain death and complement inhibition on allograft survival post transplantation. Hearts from C57BL/6 living, sham or BD donors were transplanted into BALB/c recipients treated with either CR2-Crry or PBS. Logrank tests comparing survival across the 4 groups demonstrated survival times were significantly different between groups (p<0.0001). All pairwise comparisons demonstrated that survival was significantly greater among each group when compared to 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 or BD+CR2-Crry. (n=4-6).
Figure 7. Immunohistochemistry localization of C4d on human cardiac biopsies obtained at donor optimization from living and brain dead donors. Note the capillary immunostaining of endothelial cells in the brain dead samples. Images are representative of n=3 for living, and n=8 for brain dead donors. Original magnification x400.

Figure 8. Immunohistochemistry localization of monocyte/macrophages (MAC387) and mature tissue macrophages (CD68) in human cardiac biopsies from living and BD donor organs. Results expressed as Mean ± SE; n=3-10. #p<0.05 living vs. BD.
Figure 1

A

B

Mean Arterial Pressure (mmHg)

Time Post BD Induction

Sham Control
BD
BD + CR2-Crry

Mean Arterial Pressure (mmHg)

Time Post BD Induction

Sham Control
BD
BD + CR2-Crry
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7

Living

Brain Dead
Figure 8
Donor Brain Death Exacerbates Complement-Dependent Ischemia Reperfusion Injury in Transplanted Hearts
Carl Atkinson, Bernhard Floerchinger, Fei Qiao, Sarah Casey, Tucker Williamson, Ellen Moseley, Serban Stoica, Martin Goddard, Xupeng Ge, Stefan G. Tullius and Stephen Tomlinson

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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.