Complement-Dependent Inflammation and Injury in a Murine Model of Brain Dead Donor Hearts

Carl Atkinson, Juan C. Varela, Stephen Tomlinson

Rationale: Donor brain death (BD) is an unavoidable occurrence in heart transplantation and results in profound physiological derangements that render the heart more susceptible to ischemia/reperfusion injury in the recipient and likely has negative long-term consequences to allograft survival.

Objective: We developed a novel mouse model of BD and investigated the role of complement in BD-induced myocardial inflammation and injury.

Methods and Results: BD was induced by inflation of a balloon catheter in the cranial cavity. BD in wild-type mice resulted in a significant increase in serum concentrations of the complement activation product complement component (C)3a, and immunohistochemical analysis of heart sections demonstrated C3 deposition on the vascular endothelium and surrounding myocytes. Following induction of BD in complement (C3)-deficient mice, cardiac troponin levels, and histological evidence of injury were significantly reduced compared to wild-type mice. C3 deficiency was also associated with reduced myocardial leukocyte infiltration and reduced or absent expression of P-selectin, intercellular adhesion molecule-1, vascular cell adhesion molecule-1, tumor necrosis factor-α, and interferon-β.

Conclusions: These data indicate an important role for complement in BD-induced inflammation and injury and suggest that a complement inhibitory strategy applied to the donor (in addition to the recipient) may provide graft protection. (Circ Res. 2009;105:1094-1101.)

Key Words: complement • brain death • heart transplantation • inflammation • mouse model

Heart transplantation has become a highly successful treatment for end-stage heart disease. Advances in immunosuppression have led to dramatic improvements in first-year survival, in large part, because of better modulation of the alloimmune response. However, despite these improvements, primary graft failure, acute rejection, and cardiac allograft vasculopathy are still major limitations to short and long-term survival. The precise mechanisms involved in the development of primary graft failure and chronic rejection are not well understood. Recently, brain death and ischemia/reperfusion injury (IRI) have been implicated in graft endothelial activation and injury, which, in turn, have been linked to the pathogenesis of graft dysfunction and chronic rejection.1–3 Clinical studies of renal transplant recipients and, to a lesser extent, donor brain death and heart transplant recipients have highlighted the deleterious impact that donor brain death (BD) has on transplant outcome.4 Recipients receiving allografts from living donors experience fewer rejection episodes, are less susceptible to primary graft failure, and, as a consequence, survive significantly longer than patients receiving organs from cadaveric sources.2–5 Initially, it was proposed that these significant improvements were a result of reduced ischemic times, but recent evidence points toward the effects of BD as a stimulating factor in priming the donor organ and rendering it proinflammatory.2,6,7

BD results in the outpouring of catecholamines, which promotes intense vasoconstriction, leading to chaotic swings in blood pressure, hypothermia, coagulopathies, hormone depletion, and electrolyte abnormalities. The effects on blood pressure are 2-fold: an initial hypertensive phase is followed by hypotension, resulting in an increase in oxygen supply to the heart. However, the increase is still insufficient to cover the enhanced demand and results in transient global myocardial ischemia.8,9 The imbalance in myocardial oxygen demand and supply renders peripheral organs ischemic, which may activate the endothelium by processes similar to that associated with IRI. The mechanism(s) of donor organ activation has yet to be fully elucidated, but much of the available data from animal studies suggest that imbalances in hormone status, the release of catecholamines and the systemic upregulation of proinflammatory cytokines are responsible for promoting direct organ damage and inflammation. Upregulation of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, have been implicated in endothelial activation, along with the expression of adhesion molecules.10,11

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From the Department of Microbiology and Immunology, Darby Children’s Research Institute, Medical University of South Carolina, Charleston.

Correspondence to Stephen Tomlinson, Department of Microbiology and Immunology, Children’s Research Institute, Medical University of South Carolina, 173 Ashley Ave, PO Box 250504, Charleston, SC29425. E-mail tomlinss@musc.edu

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It is well documented that BD initiates peripheral organ ischemia and that the heart sustains a period of global myocardial ischemia caused by the intense physiological changes associated with BD. However, whether BD-associated organ injury and “activation” have a similar mechanistic process to IRI is not clear, and although complement has been shown to play a key role in myocardial IRI, its role in the development of donor organ damage and the propagation of a “proinflammatory state” has not been explored.

**Methods**

**BD Model**

Male C57BL/6 WT and complement component 3-null (C3−/−) mice were anesthetized with 10 mg/kg ketamine and 6 mg/kg xylazine by IP injection. BD was induced by a similar basic procedure to that outlined for rats by Pratschke et al12 but with modification for use in mice. A burr hole sufficient to insert a 2F Fogarty catheter was drilled into left parasagittal parietal bone. A 2F Fogarty was inflated, and mice were placed in a supine position for the rest of the experiment. Body temperature was monitored by rectal thermometer, and body temperature was maintained using a heated operating table (Harvard Apparatus, Boston, Mass) at >36°C. Mouse mean arterial blood pressure (MAP) and heart rate were monitored continuously using a Physiosax blood pressure monitor (Columbus Instruments, Ohio). Mice were intubated with a blunt tipped cannula and ventilated with a rodent ventilator with oxygen (Harvard Apparatus, Mass; respiration rate, 100/min; tidal volume, 8 mL/kg). The catheter was inflated with saline over a 20-minute period until spontaneous respiration ceased, determined by turning the ventilator off for 30 seconds. BD was further confirmed by the absence of corneal and pedal reflexes. The total volume of saline required to induce apnea was 80±100 µL. Inflation of the catheter induced a characteristic Cushing’s Response, during which time the MAP increased, followed later by a recovery of normotensive responses. If we were unable to maintain a MAP of 70 mm Hg for a sustained period, experiments were stopped, and the animal was removed from the study. Timing of the follow-up period began at the point of apnea detection. Confirmation of a brain dead state was carried out every 30 minutes by performing apnea tests and testing corneal and pedal reflexes. No further anesthesia was administered to brain dead animals. Sham controls underwent the same procedure with catheter inserted but without balloon inflation. Supplementary anesthesia was administered as required to sham controls if pedal or corneal reflexes became apparent. After 1 or 3 hours, the heart was removed and dissected into three transverse sections and serum collected. Two sections were immediately frozen in liquid nitrogen, and one was immersed in formalin for histological analysis. All procedures were approved by the Medical University of South Carolina on Animal Research, in accordance with the NIH Guide for Care and Use of Laboratory Animals.

**Histological Analysis**

Formalin-fixed sections of heart from controls and brain dead animals were stained with hematoxylin/eosin stains for histological analysis. Sections were assessed for evidence of myocardial damage using the criteria previously described.13 Specifically, features of interstitial and subendocardial hemorrhage and necrosis, contraction band necrosis, paradiscal contraction band lesions, and endothelial swelling were assessed in each heart. The presence of 1 or more of these features was considered indicative of cardiac damage and deemed positive for this study.

**Serum Analysis**

Serum analysis for cardiac troponin (cTnl) (Life Diagnostics), IL-1β, and TNF-α (R&D Systems) was performed by ELISA according to the recommendations of the manufacturers. Complement activation was assessed by ELISA for C3a (BD Biosciences).

**Immunohistochemistry**

The presence of complement (C3d; Dakocytomation), IgM (Cappell), neutrophils (GR1, BD Pharmingen), macrophages (mac-3, BD Pharmingen), P-selectin, E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 (BD Pharmingen) was assessed by immunohistochemistry. For C3d and IgM, analysis was performed on paraffin-processed sections with sections receiving microwave antigen retrieval in citrate buffer pH 6.0 before immunohistochemistry analysis. All antibodies were visualized with a standard avidin–biotin detection system (Vector Laboratories) as previously described.14 For further quantification of neutrophils we performed the naphthol AS-D chloroacetate esterase staining technique using a kit from Sigma Aldrich (St Louis, Mo) according to the instructions of the manufacturer. Neutrophil and macrophage numbers were quantified in 5 random high-power fields of each heart section and quantified by 2 independent investigators.

**RNA Extraction and Real-Time RT-PCR**

Total RNA was extracted from hearts using guanidine isothiocyanate and phenol-chloroform by standard methods.14 Analysis was performed using a My IQ Real-Time detection system (Bio-Rad) using previously described intron-spanning primers specific for ICAM-1 (forward, 5′-GGCTGGATGTGTCTCTA-3′; reverse, 5′-TCG-AGAGCCAGGAACAGG-3′); VCAM-1 (forward, 5′-CCCAACACAGGAGATGT-3′; reverse, 5′-CAGGATTTTGGGAGCTGTGTA-3′); E-selectin (forward, 5′-AGCTACCATGGAAACACGAC-3′; reverse, 5′-CGCAAGTTCTCCACGCTTGT-3′); P-selectin (forward, 5′-ATGCCCTGGCATGGCACACT-3′; reverse, 5′-CTT-CATCGCACTGAAGTGG-3′); TNF (forward, 5′-ATGCCCTGGCATGGACACT-3′; reverse, 5′-CTTCACTCGCACTGAAGTGG-3′); and IL-1β (forward, 5′-AGCTACCATGGAAACACGAC-3′; reverse, 5′-CGCAAGTTCTCCACGCTTGT-3′). All reactions were performed in triplicate, and the GAPDH gene was used as an internal control.

**Statistical Analysis**

All data are presented as means±SD. All data were subjected to statistical analysis using Statview Statistical Analysis Software 9 version 5 (SAS Institute Inc). Statistical analyses of collected data were interpreted by paired Student’s t test for comparison of 2 groups or ANOVA for the analysis of 3 or more groups with Newman–Keuls for post hoc analysis. A probability value of less than 0.05 was considered significant.

An expanded Methods section is available in the Online Data Supplement at http://circres.ahajournals.org.

**Results**

**Physiological Characteristics of BD Model**

Inflation of the intracranial catheter and induction of progressive BD was associated with a sharp chaotic swing to...
hypertension followed by a period of hypotension before the animal recovers to a normotensive state. Similar responses have been shown to occur in a rat model of progressive BD. There was no difference in MAP between WT and C3^{-/-} mice before or following BD (Figure 1). Furthermore, analysis of blood pH, O2, and PO2 demonstrated no significant differences in lung function (Online Table I). Thus, any differences between WT and C3^{-/-} mice in the inflammatory response to BD is unlikely to be caused by physiological differences in the animals response to BD induction. Animals were maintained for a maximum period of 3 hours post-BD induction, in large part, because of the significant technical difficulties in prolonged maintenance of mice in a normotensive state post-BD.

**Complement Activation**

To assess whether BD and the resultant physiological derangements lead to activation of the complement system, we analyzed serum and heart tissue for complement activation products. There was no significant increase in C3a levels at 1 hour after surgery in brain dead mice or sham-operated controls. However, by 3 hours, there was a significant increase in C3a levels in brain dead mice (Figure 2A). Complement deposition within the heart following BD was determined by C3d immunohistochemistry. C3d was deposited in hearts from 6 of 10 mice at 1 hour post-BD and in 10 of 10 mice by 3 hours post-BD. There was a low level of nonspecific staining for C3d in areas surrounding myocytes (as evidenced in hearts from C3^{-/-} mice), but there was a significant increase in the incidence and intensity of C3d staining in hearts from brain dead animals. Complement was deposited within capillaries, arterioles, and in the area surrounding myocytes (Figure 2B). There was also an increase in incidence and intensity of C3d staining in hearts from brain dead animals ventilated for 3 hours compared to animals supported for 1 hour (data not shown).

Complement activation has been shown to play a central role in the injury that ensues following ischemia and reperfusion. Recent studies have demonstrated that preexisting IgM antibodies that recognize so called “ischemic
antigens” play a central role in the activation of complement following ischemia and reperfusion. To investigate whether IgM antibodies are associated with complement activation following BD, we analyzed heart sections from WT and C3−/− brain dead animals for IgM immunoreactivity. IgM binding was seen in both WT and C3−/− mice. Positive staining for IgM (brown) can be seen on the endothelium of intramyocardial vessels. Some diffuse staining can be seen on myocytes in both groups. Representative images, n = 4 for each group.

Myocardial Damage

Serum cTnI is used as a marker of cardiac injury, and clinical studies have shown that raised cTnI levels are associated with poor posttransplant outcome. In sham-operated WT and C3−/− mice, levels of cTnI were slightly elevated compared to normal controls, but the difference was not significant (data not shown). In brain dead WT mice, cTnI levels were significantly higher than in either WT or C3−/− controls (P = 0.03) (Figure 3A). There was no significant increase in cTnI levels in C3−/− brain dead animals when compared to all other groups.

Heart sections were also assessed for morphological evidence of cardiac damage as denoted by the presence of coagulative necrosis, contraction band necrosis and swelling of the endothelial cells lining intramyocardial vessels. There was no histological evidence of myocyte damage in any sham-operated animals (either WT or C3−/−). However, in the brain dead groups, 45% of WT animals displayed at least 1 feature associated with cardiac damage compared to only 13% of C3−/− animals (P = 0.004). The most common histological feature seen was endothelial swelling, defined by the presence of enlarged and prominent endothelial nuclei (Figure 3B). To confirm that these morphological features were indeed attributable to endothelial activation, we performed immunohistochemistry studies investigating the expression of endothelial adhesion molecules.

Adhesion Molecule Expression

Numerous studies have highlighted the importance of adhesion molecule expression in donor organ priming post-BD. We, therefore, investigated the association of complement activation with the expression of P-selectin, E-selectin, ICAM-1, and VCAM-1 following BD. Adhesion molecule expression following BD in WT and C3−/− mice was determined by immunohistochemistry and scored on a scale of 0 to 3 as previously described. P-selectin and ICAM-1
expression was evident on endothelial cells as early as 1 hour, with no expression seen for E-selectin or VCAM-1. At 3 hours post-BD, P-selectin and ICAM-1 expression persisted, and VCAM-1 expression could now be detected, but staining for E-selectin remained negative (Figure 4 and Online Table II). In C3\textsuperscript{-/-} mice, there was no detectable expression of any adhesion molecule at 1 hour post-BD. At 3 hours post-BD, only P-selectin and ICAM-1 expression was detected but at a lower frequency and intensity than that seen in WT animals.

We further analyzed the effects of complement on the expression of adhesion molecule transcripts by quantitative real-time PCR. A significant increase in all mRNA levels was noted in WT and C3\textsuperscript{-/-} brain dead animals compared to sham controls. The expression E-selectin and VCAM was significantly increased in WT versus C3\textsuperscript{-/-} animals ($P<0.02$) (Figure 5A). Transcript expression of P-selectin and ICAM mRNA was not statistically different between the groups, even though protein expression of these two adhesion molecules appeared to be increased.

### Prolinflammatory Status

To further investigate whether complement plays a role in the propagation of a proinflammatory state, we quantified serum protein and tissue mRNA levels of TNF-\(\alpha\) and IL-1\(\beta\) by ELISA and quantitative real-time PCR. TNF-\(\alpha\) and IL-1\(\beta\) have both been implicated in the development of endothelial activation and adhesion molecule expression. There was no significant difference in serum protein levels of TNF\(\alpha\) or IL-1\(\beta\) in either WT or C3\textsuperscript{-/-} mice after BD compared to sham controls (data not shown). However, quantitative real-time PCR analysis revealed significantly reduced tissue transcription of both TNF-\(\alpha\) and IL-1\(\beta\) in C3\textsuperscript{-/-} mice compared to WT mice at 3 hours post-BD ($P<0.02$) (Figure 5B).

### Cellular Infiltration

Complement activation products C3a and C5a, as well as adhesion molecule expression, play important roles in the recruitment of immune cells to sites of inflammation. Therefore, we investigated inflammatory cell infiltration in brain dead animals. Neutrophils were present in all heart samples, with cells localized to vascular areas and spread between myocytes (Figure 6A and 6B). Although neutrophils were present in low numbers, there was a significant increase in neutrophil numbers in WT mice at both 1 hour (data not shown) and 3 hours (Figure 6C and 6D) post-BD compared to sham mice, as demonstrated by immunohistochemistry staining and enzyme histochemistry techniques. In contrast, the number of infiltrating neutrophils in hearts from C3\textsuperscript{-/-} brain dead mice was not significantly different to the number in C3\textsuperscript{-/-} and WT sham mice (Figure 6C and 6D).

### Discussion

BD results in physiological derangements which impact the inflammatory nature of the graft, and predisposes the graft to immunologic recognition and subsequent graft rejection.\textsuperscript{18–20} Here, we report on the development of a mouse model of BD,
Complement deficient mice had a significant out-pouring of catecholamines, and these molecules have been shown to induce direct damage to heart myocytes in a brain dead rat donor heart.15 Following BD, physiological changes in blood pressure and the herniation of the brain stem result in an expanding research into inflammatory and immune mechanisms associated with BD. We used the mouse BD model and C3 deficient mice to investigate the role of complement in the propagation of donor organ inflammatory status.

BD resulted in complement activation as demonstrated by the deposition of complement in the heart and the presence of complement activation products in the serum, and complement deposition increased with time post-BD. The deposition of complement in the mouse heart following BD is in keeping with a previous study that showed increased C3 deposition on the surface of tubular epithelial cells in a brain dead rat donor kidney.15 Following BD, physiological changes in blood pressure and the herniation of the brain stem result in an out-pouring of catecholamines, and these molecules have been shown to induce direct damage to heart myocytes in a canine model of explosive BD.21 However, the pathology of explosive BD is thought to be quite different from the progressive BD studied here, and it is unclear whether myocyte damage in progressive BD is induced directly bycatecholamines, impaired coronary perfusion, or by inflammatory mechanisms. Our data support an important role for the latter in that cardiac injury was significantly reduced in C3−/− mice compared to WT mice following BD. Elevated cTnI levels are associated with myocyte damage and have been used as a predictive index of graft outcome posttransplantation.22 Complement deficient mice had a significant overall reduction in cTnI levels compared to WT mice following BD. Furthermore, C3 deficiency was associated with a reduction in histological evidence of cardiac injury. However, it is important to note that the most common feature of heart injury seen in both groups was endothelial swelling, and that actual myocyte necrosis was rare in WT mice and absent in C3−/− mice. Thus, serum cTnI measured at relatively early time points post-BD is associated with discreet injury and not direct myocardial necrosis, at least as could be determined by histological analysis. Similarly, in an earlier study using a canine model, cTnI levels were substantially elevated, whereas myocyte necrosis was rarely seen following progressive BD,21 and there is an absence of histological evidence of myocyte necrosis in rat models of BD.2

As noted, the most prominent feature of cardiac damage noted post-BD was endothelial swelling. This feature is indicative of endothelial activation, a feature that can in part be investigated by analyzing endothelial adhesion molecule expression. Previous studies by Takada et al10,11 demonstrated that P-selectin expression increased post-BD and that blockade of this adhesion molecule not only resulted in a reduction in the proinflammatory status of the donor organ, but also resulted in improved posttransplant survival in models of cardiac and renal transplantation. A deleterious role for expression of P-selectin in the graft is supported by a study showing that transplantation of P-selectin and ICAM-1 deficient mouse hearts into WT recipients results in a reduction of IRI and prolonged graft survival.23 With regard to the present study, the complement activation products C3a, C5a and the MAC have been variously shown to either directly or indirectly induce the expression of P-selectin, E-selectin and ICAM-1.24 Furthermore, we have previously demonstrated that C3 deficiency or inhibition of C3 significantly reduces P-selectin protein expression and transcription in a model of ischemic stroke in vivo.14 Here we demonstrated that the endothelial expression of adhesion molecules in the mouse heart is associated with BD, and that C3 deficiency reduces adhesion molecule expression. P-selectin and ICAM-1 protein expression was detected 1 hour following BD in WT mice but not in C3−/− mice. At 3 hours post-BD, expression of P-selectin and ICAM-1 was detected in C3−/− mice, but at significantly lower levels than in WT mice. VCAM protein expression was detected only in WT mice at either time point following BD. These findings indicate an important role for complement as an effector mechanism in the early expression of adhesion molecules following BD.

Of note, we previously reported that we could find no appreciable difference in adhesion molecule expression in human heart samples from either cadaveric brain dead donors or living domino donors.1 However, as we previously hypothesized, the lack of difference in adhesion molecule expression in the human samples may be related to the proinflammatory intrathoracic milieu associated with cystic fibrosis in the domino donors and endothelial perturbations associated with cystic fibrosis25 (living donor hearts are obtained from cystic fibrosis patients undergoing combined heart-lung transplantation). Thus, in the absence of BD, adhesion molecule expression in domino donor hearts may be attributable to complement-independent mechanisms that are associated with an increased inflammatory burden. We also assessed adhesion molecule mRNA expression by quantitative real-time PCR. We observed significant increases in gene tran-
scription of P-selectin, E-selectin, ICAM-1 and VCAM-1 post-BD when compared to baseline controls, but unlike protein levels, only E-selectin and VCAM-1 transcription was significantly lower in C3<sup>-/-</sup> mice compared to WT. Constitutively expressed proteins can be readily mobilized from underneath the membrane (such as P-selectin from the Weibel-Palade bodies) in the initial stage. This first phase, which is called Type I activation, does not involve de novo protein synthesis. The second phase is transcriptionally regulated, a process that takes place 4 or more hours after activation.1,2,6 It is therefore likely that the lack of significant differences for certain adhesion molecules may be time dependent. Animals were only maintained for a period of 3 hours post-BD induction, due largely to the significant technical difficulties in prolonged maintenance of mice in a normotensive state post-BD.

Although we did not see a significant difference in transcription of all of the adhesion molecules analyzed, levels of TNF-α and IL-1β were significantly higher in WT compared to C3<sup>-/-</sup> mice following BD, indicating a role for complement in the expression of these cytokines. These proinflammatory cytokines are associated with graft injury; they are thought to promote intragraft inflammation and have been shown to have a deleterious effect on graft survival.2,6,7,27 Although TNF-α and IL-1β mRNA levels were elevated following BD, serum concentrations were not significantly different. This is perhaps not surprising because elevation of serum TNF-α and IL-1β occurs in models of explosive BD, but not in models of progressive BD.1,11

To determine whether the generation of complement activation products and the expression of adhesion molecules resulting from BD influenced the infiltration of inflammatory cells, we counted neutrophils and macrophages in heart sections isolated from brain dead WT and C3<sup>-/-</sup> mice following BD, indicating a role for complement in the proinflammatory status of the brain dead donor heart, as measured by adhesion molecule expression, cytokine expression, inflammatory cell infiltration, and cardiac injury. These data suggest that a therapeutic strategy to inhibit complement in the brain dead donor (as well as the recipient) may improve organ quality and graft function.

Sources of Funding

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Disclosures

None.

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Brain Death Model
Male C57BL/6 wildtype (Cat no. 000644) and C3-/- (Cat no.003641) mice were purchased from Jackson Laboratories, were anesthetized with 10 mg/kg ketamine and 6 mg/kg xylazine by i.p injection. Brain death was induced by a similar basic procedure to that outlined for rats by Pratschke et al., but with modification for use in mice. Briefly, a burr hole sufficient to insert a 2F Fogarty catheter was drilled into left parasagittal parietal bone. A 2F Fogarty was inserted and mice placed in a supine position for the rest of the experiment. Body temperature was monitored by rectal thermometer, and body temperature maintained using a heated operating table (Harvard Apparatus, Boston, USA) at >36°C. Mouse mean arterial blood pressure (MAP) and heart rate was monitored continuously using physiomax blood pressure monitor (Columbus Instruments, OH, USA). Mice were intubated with a blunt tipped cannula and ventilated with a rodent ventilator with oxygen (Harvard Apparatus, MA; respiration rate, 100/minutes; tidal volume, 8 mL/kg). The catheter was inflated with saline over a 20 minute period until spontaneous respiration ceased, determined by turning the ventilator off for 30 seconds. Brain death was further confirmed by the absence of corneal and pedal reflexes. The total volume of saline required to induce apnea was 80 µl ± 25 µl. Inflation of the catheter induced a characteristic Cushing’s Response, during which time the MAP increased, followed later by a recovery of normotensive responses. If we were unable to maintain a MAP of 70 mmHg for a sustained period, experiments were stopped and the animal removed from the study. Timing of the follow-up period began at the point of apnea detection. Confirmation of a brain dead state was carried out every 30 minutes by performing apnea tests and testing corneal and pedal reflexes. No further anesthesia was administered to brain dead animals. Sham controls underwent the same procedure with catheter inserted but without balloon inflation. Supplementary anesthesia was administered as required to sham controls if pedal or corneal reflexes became apparent. After 1 or 3 hours, the heart was removed and dissected into three transverse sections and serum collected. Two sections were immediately frozen in liquid nitrogen and one immersed in formalin for histological analysis. All procedures were approved by the Medical University of South Carolina on Animal Research, in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals.

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Serum Analysis
Serum analysis for cardiac troponin I (cTnl) (Life Diagnostics, USA), IL-1β and TNF-α (R&D Systems, USA), was performed by ELISA according to manufacturers recommendations. Complement activation was assessed by ELISA for C3a (BD Biosciences, USA) according to manufacturers recommendations.

Immunohistochemistry
The presence of complement (C3d, Dakocytomation, USA), IgM (Cappell, USA), neutrophils (GR1, BD Pharmingen, USA), macrophages (mac-3, BD Pharmingen, USA), P-selectin, E-selectin, I-CAM-1, and V-CAM-1 (BD Pharmingen, USA) was assessed by immunohistochemistry. For C3d and IgM analysis was performed on paraffin processed sections with sections receiving microwave antigen retrieval in citrate buffer pH6.0 prior to immunohistochemistry analysis. All antibodies were visualized with a standard avidin biotin detection system (Vector Laboratories, USA) as previously described \(^{14}\). Briefly, following antigen retrieval step, in necessary, sections were washed in 3 changes of Phosphate buffer saline, pH 7.6 (PBS). Sections were then incubated in 3% hydrogen peroxide (Sigma Aldrich, St Louis, USA) for 10 minutes to quench endogenous peroxidase activity. Sections were subsequently washed in running tap water and then equilibrated in PBS buffer. Sections were then blocked with protein blocking solution (Vector Laboratories, USA) for 20 minutes, prior to incubation with primary antibody for 1 hr at room temperature. Following primary slides were again washed in PBS and then incubated with either rat-anti-mouse or goat anti-rabbit biotinylated antibodies for 1 hr. Finally, sections were incubated with streptavidin complex (Vector Laboratories, USA) prior to incubation with 3, 3, dianinobenzidine, resulting in a brown reaction product. Sections were then counterstained with hematoxylin (Dako, USA). For further quantification of neutrophils we performed the naphthol AS-D chloroacetate esterase staining technique using a kit from Sigma Aldrich (St Louis, USA) according to the manufacturers instructions. Neutrophil and macrophage numbers were quantified in five random high power fields of each heart section, and quantified by two independent investigators.

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Statistical Analysis
All data are presented as Mean ± SD. All data were subjected to statistical analysis using Statview Statistical Analysis Software 9 version 5 (SAS Institute Inc.). Statistical analyses of collected data were interpreted by paired Student's t test for comparison of two groups or analysis of variance for the analysis of three or more groups with Newman-Keuls for posthoc analysis. A p value of less than 0.05 was considered significant.
Table 1. Comparison of blood $O_2$, $pO_2$ and pH in wt and C3-/- mice pre and post BD.

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<td>C3-/-</td>
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Table 2. Quantification of adhesion molecule immunostaining in wt and C3-/- mice 3 hours post BD, scored on a scale of 0-3 (scoring - = 0, + = 1, ++ = 2, +++ 3).

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