


BRIEF COMMUNICATION

The effects of brain death and ischemia on tolerance induction are organ-specific

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We have previously shown that 12 days of high-dose calcineurin inhibition induced tolerance in MHC inbred miniature swine receiving MHC-mismatched lung, kidney, or co-transplanted heart/kidney allografts. However, if lung grafts were procured from donation after brain death (DBD), and transplanted alone, they were rejected within 19-45 days. Here, we investigated whether donor brain death with or without allograft ischemia would also prevent tolerance induction in kidney or heart/kidney recipients. Four kidney recipients treated with 12 days of calcineurin inhibition received organs from donors rendered brain dead for 4 hours. Six heart/kidney recipients also treated with calcineurin inhibition received organs from donors rendered brain dead for 4 hours, 8 hours, or 4 hours with 4 additional hours of cold storage. In contrast to lung allograft recipients, all isolated kidney or heart/kidney recipients that received organs from DBD donors achieved long-term survival (>100 days) without histologic evidence of rejection. Proinflammatory cytokine gene expression was upregulated in lungs and hearts, but not kidney allografts, after brain death. These data suggest that the deleterious effects of brain death and ischemia on tolerance induction are organ-specific, which has implications for the application of tolerance to clinical transplantation.

KEYWORDS

animal models: porcine, heart transplantation/cardiology, kidney transplantation/nephrology, lung transplantation/pulmonology, rejection, tolerance: experimental, translational research/science

Abbreviations: ACR, acute cellular rejection; CAV, cardiac allograft vasculopathy; CML, cell-mediated lympholysis; Cr, chromium; GAPDH, glyceraldehyde-3-phosphate-dehydrogenase; IFN γ , interferon- γ ; KIM-1, kidney injury molecule-1; MLR, mixed-lymphocyte reaction; PBL, peripheral blood leukocyte; PCR, polymerase chain reaction; POD, postoperative day; PSL, percent specific lysis; SLA, swine lymphocyte antigen; TNF α , tumor necrosis factor- α ; TOLS, Treg rich organized lymphoid structures; Tregs, T regulatory cells.

1 | INTRODUCTION

Clinical studies have demonstrated that long-term tolerance can be achieved in human recipients of kidney allografts.¹ However, kidney allografts transplanted into these patients were procured from living-related donors and transplanted with short ischemic times. Thus the donor organs had not been exposed to the highly inflammatory milieu consequent to brain death, and nor had they been exposed to prolonged ischemia. Given the deleterious effects of brain death and ischemia on organ function and allograft survival,² it is necessary to understand how brain death and ischemia affect tolerance induction before extending tolerance protocols proven successful in recipients of living-donor organs to recipients of cadaveric-donor organs.

Our laboratory has previously studied the effects of brain death in a lung transplantation model using MHC-inbred miniature swine. We showed that a 12-day course of high-dose tacrolimus induced long-term tolerance in recipients of fully MHC-mismatched lung allografts procured from healthy, non-brain-dead donors. However, when lung allografts were procured from donors rendered brain dead and mechanically ventilated for 4 hours before organ procurement, they were all rejected within 45 days.³ These data confirm, in a preclinical model, that the deleterious effects of brain death in the donor have the potential to render an otherwise successful tolerance protocol ineffective.

In separate tolerance studies using miniature swine, it has been shown that a 12-day course of high-dose tacrolimus could also induce tolerance in recipients of fully MHC-mismatched kidney allografts,⁴ but not of heart allografts. In fact, when isolated heart allografts were transplanted across the same full MHC barrier with the same 12-day course of tacrolimus, they were all rejected within 40 days.⁵ However, when heart and kidney allografts from the same MHC-mismatched donor were *co-transplanted* under a 12-day course of tacrolimus, recipients uniformly became tolerant of both organs.⁵ These studies demonstrate that the ability of a particular tolerance protocol to induce long-term unresponsiveness is organ-specific.⁶

Given the striking organ-specific differences we have observed in the ability of tolerance-induction protocols to achieve immune unresponsiveness, we asked whether the effects of brain death and ischemia on tolerance induction would also differ depending on the organ transplanted. Here we show that in contrast to lung allograft recipients, donor brain death and prolonged organ ischemia did not prevent tolerance induction in isolated kidney or heart plus kidney allograft recipients.

2 | MATERIAL AND METHODS

2.1 | Animals

Transplant donors and recipients were selected from our herd of MHC inbred miniature swine (age 3-6 months, weight 15-50 kg).⁷ Swine leukocyte antigen (SLA)⁸⁸ (MHC class I^c/II^d) donor

organs were transplanted into SLA^{dd} (MHC class I^d/II^d) recipients to achieve a 2-haplotype, class I MHC mismatch, whereas SLA^{dd} (MHC class I^d/II^d) donor organs were transplanted into SLA^{cc} (MHC class I^c/II^c) recipients to achieve a 2-haplotype, full MHC mismatch. All animal care and procedures were approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee and conducted in compliance with the Guide for the Care and Use of Laboratory Animals.

2.2 | Experimental groups

Eight miniature swine received combined heart and kidney allografts from the same fully MHC disparate donors and were treated with a 12-day course of tacrolimus. Organs in Group 1 recipients were procured from donors rendered brain dead for 4 hours (n = 2). Organs in Group 2 were procured from donors rendered brain dead for 8 hours (n = 2). Organs in Group 3 were procured from donors rendered brain dead for 4 hours and then stored for 4 additional hours in University of Wisconsin solution on ice (n = 2). Group 4 animals received a combined heart/kidney transplant without brain death but were taken back to the operating room on postoperative day (POD) 2 to undergo renal artery clamping for 80 minutes to induce a period of warm ischemic injury in the kidney graft. Group 5 animals underwent bilateral native nephrectomies and then received isolated MHC class I disparate kidney allografts from donors rendered brain dead for 4 hours and a 12-day course of cyclosporine (n = 4). Recipients were followed for over 3 months and regarded as tolerant if, at POD 100, heart allografts showed strong contractions with no signs of rejection on biopsy and recipients had normal creatinine levels with no rejection on kidney biopsy.

2.3 | Operative procedures

2.3.1 | Donor brain death and cold ischemia

Under general anesthesia, brain death was induced by intracranial inflation of a 30 cc Foley catheter over a period of 1 minute³ which elicited a Cushing response. All donors were mechanically ventilated and similarly supported with crystalloid fluids and dopamine to maintain an adequate blood pressure for 4 or 8 hours following brain death. Organs in Group 1, 2, 4, and 5 were transplanted immediately after organ procurement. Organs in Group 3 were stored in University of Wisconsin solution on ice to prolong the cold ischemic times; 3 hours for kidneys and 4 hours for hearts (as hearts were implanted after the kidneys).

2.3.2 | Heart and kidney transplantation

Recipients underwent bilateral nephrectomy. The aorta and inferior vena cava were used for end-to-side arterial and venous anastomoses for both the heart and kidney, with the heart placed on the right and the kidney on the left in cases of heart/kidney co-transplantation.

A vesicoureteral anastomosis was performed as part of the kidney implantation.⁸

2.3.3 | Renal artery clamping

The laparotomy was re-opened and, after heparin administration (200 units/kg), the renal artery was clamped for 80 minutes with a bulldog clamp. Twenty-four hours after clamping, a kidney biopsy was performed to evaluate the degree of ischemic injury. Kidney-injury molecule-1 (KIM-1), a transmembrane protein that is specifically up-regulated in injured proximal tubular epithelial cells was used to assess the degree of ischemic renal injury.⁹

2.3.4 | Skin grafting

Split-thickness skin grafts were placed on the dorsum of long-term tolerant recipients in Group 4. Animals received fresh (self: SLA^{cc}) or frozen (donor: SLA^{dd}, third party: SLA^{ll}) skin grafts.

2.4 | Immunosuppression and rejection monitoring

Tacrolimus (Haorui Pharma-Chem Inc., Irvine, CA) was administered as a continuous infusion at a dose of 0.08-0.20 mg/kg (target trough level of 30-50 ng/ml) for 12 consecutive days, starting on the day of transplantation. Cyclosporine was given as a daily intravenous infusion over 1 hour (13 to 16 mg/kg/day with target levels 400 to 800 ng/mL) for 12 consecutive days, starting on the day of transplantation. Cardiac allograft rejection was defined by loss of a ventricular impulse on palpation and/or the lack of ventricular contraction on echocardiography. Renal allograft rejection was defined as sustained rise in serum creatinine to >10 mg/dL and/or uremia.

2.5 | Histopathological examination

Scoring of acute rejection on cardiac allograft biopsies was based on the International Society for Heart and Lung Transplantation System.¹⁰ Acute rejection in kidney allograft biopsies was scored according to the Banff classification.¹¹ Some kidney samples were also stained with an anti-mouse/rat FoxP3 antibody (clone: FJK-16s, eBioscience, Inc., San Diego, CA).

2.6 | Cell-mediated lympholysis (CML) assay

Effector cells were incubated with target cells at effector/target ratios of 100:1, 50:1, 25:1, and 12.5:1. Two target cells were tested in each assay: ¹ Peripheral blood leukocytes (PBL) SLA matched to the donor (SLA^{dd}: class I^{dd} and class II^{dd}) and ² third-party PBLs. ⁵¹Cr release was determined on a gamma counter (Micromedics, Huntsville, AL). The results were expressed as a percentage of specific lysis and calculated as follows: Percentage of specific lysis = ((Experimental release [cpm] – Spontaneous release [cpm]) / (Maximum release [cpm] – Spontaneous release [cpm])) × 100.⁵

2.7 | Mixed lymphocyte reaction (MLR) assay

Cultures containing 4 × 10⁶ responder and 4 × 10⁶ irradiated (2500 cGy) stimulator peripheral blood mononuclear cells were incubated for 5 days, after which 1 uCi of [3H]-thymidine was added to each well. [3H]-thymidine incorporation was determined in triplicate samples by beta-scintillation counting. Absolute counts were compensated for background and then expressed as stimulation indices (SIs), calculated as SI = average cpm for a responder – stimulator pair per cpm of the same responder stimulated by an autologous stimulator.⁵

2.8 | Flow cytometry

The presence of anti-donor immunoglobulin (IgM and IgG) in the serum of experimental swine was examined by flow cytometry using a Becton Dickinson FACScalibur (Sunnyvale, CA) as described previously.⁵

2.9 | Immunofluorescence staining for KIM-1

Tissue from a kidney biopsy performed 24 hours after renal artery clamping was stained with the anti-pig-KIM-1 antibody as described previously.¹²

2.10 | Quantitative PCR

Kidney, lung, and heart biopsy specimens obtained before and after brain death were submerged in RNAlater (Qiagen, Valencia, CA) and snap-frozen in liquid nitrogen. RNA was isolated from tissue (Qiagen RNeasy Mini Kit) and used to derive first-strand complementary DNA (Invitrogen cDNA Synthesis Kit). Fold-change difference was calculated using the double-delta Ct method, and all samples were normalized to GAPDH. Quantitative polymerase chain reaction (qPCR) primers were synthesized based on previously published reports of swine-specific assays for IL-1, IL-6, tumor necrosis factor α (TNF-α), interferon γ (IFN-γ), and GAPDH.¹³

3 | RESULTS

3.1 | Donor brain death did not prevent tolerance induction in recipients of co-transplanted heart and kidney allografts or isolated kidney allografts

We have previously demonstrated that MHC-mismatched recipients of co-transplanted heart/kidney allografts from the same healthy, non-brain-dead donors all became tolerant of their allografts after a 12-day course of tacrolimus and survived long term.⁵ Table 1 shows that long-term, stable tolerance was still observed in uncomplicated recipients, which received fully MHC-disparate heart and kidney allografts from donors rendered brain dead for 4 hours (Group 1) or for 8 hours (Group 2) before organ

TABLE 1 Graft survival and histology of heart-kidney and isolated kidney recipients

Group	Organ	Protocol	Animal #	Graft survival	Histology at 3 months
1	Heart & Kidney	Brain death × 4 h	21690	>100 days	Heart: ISHLT OR Kidney: i1t2 + TOLS
			21505	16 days (died of PE)	n/a (i0t1 + TOLS at 16 days)
2	Heart & Kidney	Brain death × 8 h	22025	>100 days	Heart: ISHLT OR Kidney: i1t3 + TOLS
			22029	>100 days	Heart: ISHLT 1R Kidney: i0t2 + TOLS
3	Heart & Kidney	Brain death × 4 h Cold ischemia × 4 h	21740	>100 days	Heart: ISHLT OR Kidney: i1t2 + TOLS
			22026	>100 days	Heart: ISHLT OR Kidney: i1t2 no TOLS
4	Heart & Kidney	Warm renal ischemia × 80 min	21420	>100 days	Heart: ISHLT OR Kidney: i1t2 + TOLS
			21736	>100 days	Heart: ISHLT OR Kidney: i1t2 + TOLS
5	Kidney	Brain death × 4 h	18055	>100 days	i1t2 + TOLS
			18225	>100 days	i0t3 + TOLS
			18226	>100 days	i1t2 + TOLS
			18353	>100 days	i0t2 + TOLS

POD, postoperative day; ISHLT, International Society for Heart and Lung Transplantation; ACR, acute cellular rejection; PE, pulmonary embolism; TOLS, Treg-rich Organized Lymphoid Structure; i, t Banff lesion scores for interstitial inflammation and tubulitis, respectively.

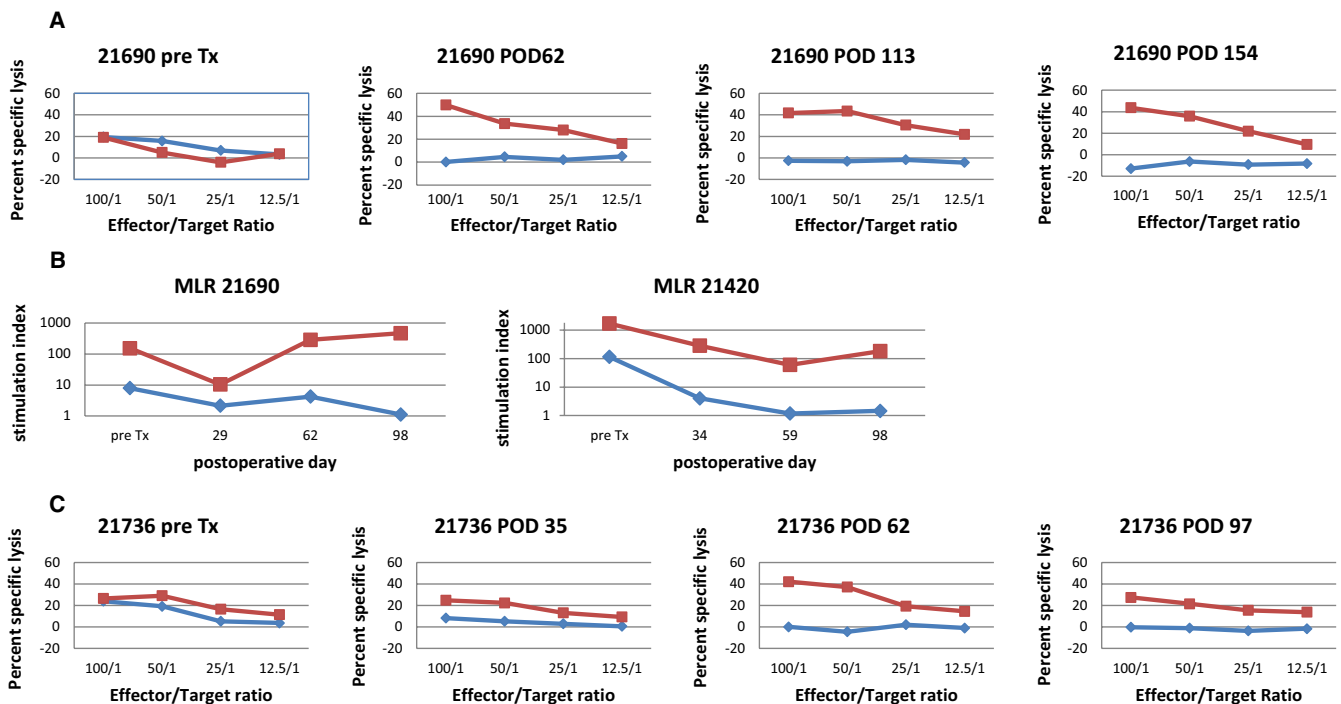


FIGURE 1 (A) Serial cell-mediated lympholysis (CML) assays using responder cells from animal #21690. Pretransplantation anti-donor responses (blue) were lost by POD 62 but strong responses against third party antigen (Yorkshire PBMC) (red) persisted. (B) Representative mixed-lymphocyte reaction (MLR) assays. In animal #21690 and animal #21420, pretransplantation anti-donor responses were lost by POD 30 (blue) but strong response against third-party antigen (Yorkshire PBMC) (red) persisted. (C) Serial CML assays using responder cells from animal #21736. Pretransplantation anti-donor responses were lost by POD35 (blue), but strong responses against third-party antigen (Yorkshire PBMC) (red) persisted

procurement. Allografts in these tolerant recipients survived over 100 days without any histologic evidence of rejection on serial cardiac biopsies (Figure S1A). Serial CML assays (Figure 1A) and MLR (Figure 1B) assays demonstrated the loss of host anti-donor T cells responses by PODs 30-60, whereas serial FACS analysis revealed the absence of significant alloantibody levels at any time point (Figure S2). One animal (#21505) died on POD 16 from pulmonary embolism. Another animal (#22029) developed recurrent pneumonias with septicemia (Gram-positive cocci and Gram-negative rods), requiring intravenous antibiotic (vancomycin, piperacillin/tazobactam) treatment for several weeks. However, despite this prolonged inflammatory state, recipient #22029 continued to exhibit donor-specific unresponsiveness in assays in vitro with only minimal changes on cardiac biopsies (Table 1).

The kidney allografts in these recipients all maintained normal function, as evidenced by serial creatinine levels, despite the finding of a variable mononuclear cellular infiltrate classified as Banff borderline or T cell-mediated rejection I (TCMR I) (Figure S1B). The infiltrates often formed organized aggregates around arterioles and small arteries rich in FoxP3+ cells (Figure S1C) similar to the T_{reg} -rich organized lymphoid structures (TOLS) that we have observed in spontaneously accepted murine kidney allografts.¹⁴ TOLS were seen as early as 16 days (#21505) and were present in 10 of 11 allograft kidneys at 3 months (Table 1), but they were not observed in the heart allografts.

Donor brain death had no effect on recipients of isolated kidney allografts, as long-term stable tolerance was also observed in all cyclosporine-treated recipients receiving class I disparate kidney allografts from donors rendered brain dead for 4 hours (Table 1, Group 5). Although the protocol used in Group 5 varied from that in Groups 1-4, previous studies in MHC-inbred miniature swine indicate that the rejection response and survival of class I disparate kidney¹⁵ or heart allografts⁸ transplanted under the cover of cyclosporine mimic that of fully MHC-disparate kidneys⁴ or hearts⁵ transplanted under the cover of tacrolimus. Isolated cardiac allografts were not tested in this brain death study, as hearts from non-brain-dead donors were all rejected within 40 days.⁵

3.2 | Comparison of donor brain death effects in recipients of MHC disparate heart/kidney versus lung allografts

To demonstrate the organ-specific effects of brain death on tolerance induction, we compared the survival times of recipients who were co-transplanted with heart/kidney allografts from brain dead donors to recipients of brain-dead lung allografts (data published previously³). In this comparison, all brain-dead allografts were retrieved 4 hours after intracerebral injury and all allografts were transplanted across a full MHC barrier with the same 12-day course of tacrolimus. Figure 2 shows the clear organ-specific differences in the effects of brain death on tolerance; recipients of lungs from donations after brain death all rejected by postoperative day 45,

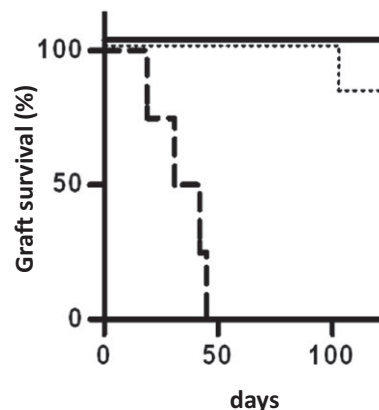


FIGURE 2 Donor brain death effects in recipients of heart/kidney versus lung allografts. Survival times of brain dead heart and kidney allograft recipients (solid line) are compared to the survival times of non-brain-dead lung allograft recipients (dotted line) and brain-dead lung allograft recipients (dashed line). The lung allograft data were published previously³

whereas recipients of kidneys or heart/kidney allografts from these donors survived long term.

3.3 | Organ-specific changes in tissue cytokine gene expression after brain death

To determine the inflammatory effects of brain death, we measured the change in cytokine gene expression of Toll-like receptor 2 (TLR2), TLR4, IL-1, IL-6, TNF α , and IFN- γ in donor organs. Kidney, lung, and heart tissue was collected from animals ($n = 4$ for kidney, $n = 3$ for lung, $n = 2$ for heart) before and 4 hours after induction of brain death. qPCR was performed and fold change in gene expression was calculated before normalizing to GAPDH. There was little change in RNA levels of cytokine gene expression in the kidneys (Figure 3A). In contrast, substantial increases in RNA for IL-1 and IL-6 were observed in 2 of 3 lungs and in 2 of 2 hearts after brain death (Figure 3B, C).

3.4 | Cold ischemia in addition to brain death did not prevent the induction of tolerance of heart and kidney allografts

When the heart and kidneys were exposed to a 3- to 4-hour period of cold ischemia in addition to the 4-hour period of brain death, long-term stable tolerance was still achieved (Table 1, Group 3). Serial heart biopsies showed no rejection and in vitro assays revealed no anti-donor T or B cell responses after 3 months (Table 1). The only difference noted in this group was the cardiac allograft's greater susceptibility to ventricular arrhythmia after reperfusion.

3.5 | Warm ischemic renal injury induced by renal artery clamping did not prevent the induction of tolerance

Two additional heart/kidney transplants were performed using organs from healthy, non-brain-dead donors that were intentionally subjected

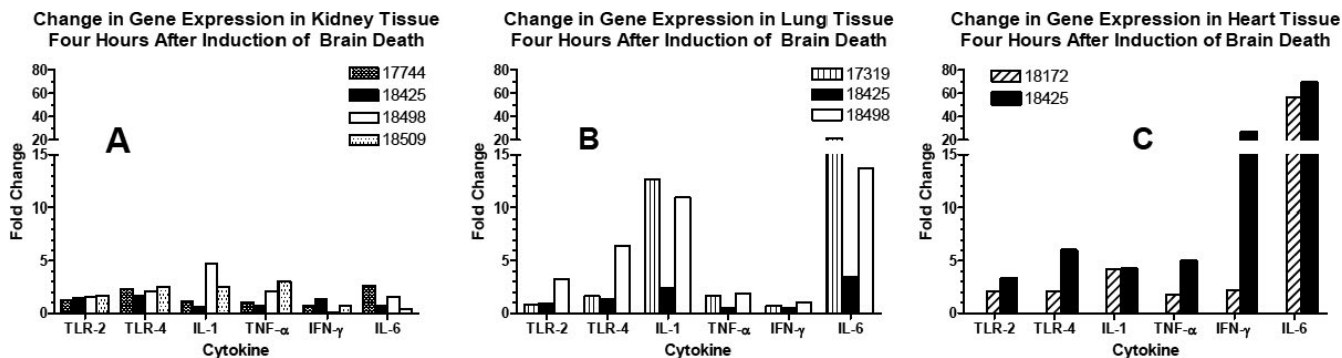


FIGURE 3 Change in tissue gene expression after brain death. qPCR was performed on RNA isolated from kidney (A), lung (B), and heart (C) tissue collected from animals before and 4 hours after induction of brain death. Fold change difference was calculated using the double delta Ct method, and all samples were normalized to GAPDH

to warm ischemia by returning the recipients to the operating room on POD 2 to undergo in situ donor renal artery clamping for 80 minutes (Table 1, Group 4). This resulted in a sharp increase in serum creatinine in both recipients, with levels peaking between 4 and 6 mg/dL 72 hours after the ischemic insult. However, creatinine levels gradually decreased to baseline levels over a period of 2 weeks. Kidney biopsies performed 24 hours after clamping and stained for KIM-1 confirmed that the renal tubule epithelium had sustained an ischemic injury (compare Figure S1D, E). Despite this extended period of warm ischemia with documented tubular injury, both recipients accepted the heart and kidney allografts long term and showed no anti-donor T cell (Figure 1B, C) or B cell responses. Serial biopsies showed no signs of cellular rejection in the heart (Figure S1F) and minimal infiltrate in the kidney (Table 1).

Donor-specific (SLA^{dd}) and third-party (SLA^{ll}) skin grafts were placed on the Group 4 recipients on PODs 97 (#21736) and 98 (#21420). The third-party skin grafts were rejected within 10 days; however, the donor-specific skin grafts survived to days 35 (#21420) and 62 (#21736). Of note, serial biopsies of the heart and kidney allografts in both recipients showed no evidence of rejection well after rejection of donor skin (over 60 days). This finding demonstrates that even after an ischemic insult, the tolerogenic kidney can induce and maintain a robust state of unresponsiveness.

4 | DISCUSSION

The recent successes achieved in inducing tolerance in human recipients of living-donor kidney allografts has prompted investigators to consider applying the same protocols to recipients of organs procured from brain-dead donors.* However, there is little experimental literature to guide this new initiative, as most small- and large-animal tolerance studies have used organs from healthy, non-brain-dead donors transplanted under optimal circumstances with very short

ischemic times. This concern is heightened by our previous report demonstrating that donor brain death can render ineffective a tolerance protocol that was otherwise successful in recipients receiving lungs from healthy, non-brain-dead donors (Figure 2).³

Previously documented organ-specific differences in tolerance induction^{4,5} led us to consider whether the effects of brain death and ischemia on tolerance induction would also differ depending on the organ transplanted. Here we demonstrate that, in contrast to lung allograft recipients, brain death and ischemia (cold and warm) did not prevent tolerance induction in isolated kidney or heart plus kidney recipients. These findings suggest that, like tolerance, the effects of donor brain death and ischemia vary with the organ transplanted.

The effect of donor brain death and organ ischemia on organ quality has been studied extensively; however, investigations into the effect of brain death and ischemia on tolerance induction are limited. Francuski et al¹⁶ showed that donor brain death affected the long-term function and histology of rat kidneys transplanted into recipients treated with 10 days of CyA and anti-CD4 mAb therapy, but it did not affect overall graft survival. The same group demonstrated that extended cold ischemia did not interfere with the induction of rat kidney allograft tolerance using anti-CD4 mAb treatment.¹⁷ These findings are in line with our results in porcine recipients of isolated kidney allograft. However, to our knowledge, comparable studies evaluating the effects of brain death on tolerance in heart or lung allograft recipients have not been performed.

Our finding that isolated kidney recipients and heart/kidney recipients were spared from the deleterious effects of brain death and ischemia (in contrast to lung allografts) suggests an organ-specific mechanism that has not been described previously. These results extend our earlier studies using non-brain-dead donors by showing that not only are kidney allografts able to confer tolerance on otherwise tolerance-resistant hearts,⁵ but also that they possess an intrinsic ability to counter the deleterious effects of brain death and ischemia on tolerance induction.

To investigate the mechanisms underlying these organ-specific differences, we measured the changes in gene expression of TLR2,

*Request for Proposals from the Immune Tolerance Network (ITN) entitled "Clinical Trials of Immune Tolerance in Transplantation using Deceased Donor Organs" at <http://www.immunetolerance.org/sites/files/2014%20RFP%20Deceased%20Donor%20Transplantation.pdf>.

TLR4, IL-1, IL-6, TNF- α , and IFN- γ , which occurred in kidney, heart, and lung tissue after 4 hours of brain death. Little change in RNA levels of cytokine gene expression was observed in kidney tissue but substantial increases in RNA for IL-1 and IL-6 were observed in lung and heart tissue after brain death. These organ-specific differences in gene expression following brain death are consistent with previous reports in the literature^{18,19}

These findings suggest that the differential effects observed after brain death observed in our study result from organ-specific differences in the upregulation of proinflammatory genes like IL-6 versus protective genes such as HO-1. This theory posits that following brain death, the balance of protective gene expression versus inflammatory gene expression in kidney allografts favors a protective milieu, allowing for the induction of tolerance, whereas in lung allografts, that balance is shifted toward a more proinflammatory state, which tilts the overall immune response from tolerance toward rejection. Recently, Zheng et al²⁰ demonstrated a population of donor-derived, nonclassical monocytes retained in donor lung grafts that may explain the proinflammatory nature of these allografts.

In summary, our results show that the deleterious effects of brain death and ischemia on tolerance induction differ depending on the organ transplanted, with kidney being more resistant to the effects of brain death and ischemia than lungs. Moreover, kidney allografts from donors after brain death can extend their protective effects to co-transplanted heart allografts from donors after brain death. These findings suggest that attempts to apply tolerance induction protocols to recipients of extra-renal organs from donors after brain death may require separating in time the organ implantation and the initiation of a tolerance protocol. Indeed, we have shown that delaying the induction of tolerance until the inflammatory state associated with brain death and ischemia/reperfusion (I/R) injury dissipates is both feasible and effective.²¹

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DISCLOSURES

The authors of this manuscript have no conflicts of interest to disclose as described by the *American Journal of Transplantation*.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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