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Kidney-intrinsic factors determine the severity of ischemia/reperfusion injury in a mouse model of delayed graft function

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Abstract

Delayed graft function due to transplant ischemia/reperfusion injury adversely affects up to 50% of deceased-donor kidney transplant recipients. However, key factors contributing to the severity of ischemia/reperfusion injury remain unclear. Here, using a clinically relevant mouse model of delayed graft function, we demonstrated that donor genetic background and kidney-intrinsic MyD88/Trif-dependent innate immunity were key determinants of delayed graft function. Functional deterioration of kidney grafts directly corresponded with the duration of cold ischemia

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AUTHOR CONTRIBUTIONS

LQ, XQL, JW, XYY, CL, SH, FZ, and DP performed the experiments. LQ and ZJZ designed the study, analyzed and interpreted the results, and wrote and edited the manuscript. YSK and LL reviewed the histologic slides. DP and ZZ assisted with MRI experiments. YSK, ZZ, EBT and DF reviewed and revised the manuscript. MMA and ZJZ conceived and supervised the study.

DISCLOSURE

All the authors declared no competing interests.

SUPPLEMENTARY MATERIAL

SupplementaryFile(PDF)

time. The graft dysfunction became irreversible after cold ischemia time exceeded six hours. When cold ischemia time reached four hours, kidney grafts displayed histological features reflective of delayed graft function seen in clinical kidney transplantation. Notably, kidneys of B6 mice exhibited significantly more severe histological and functional impairment than kidneys of C3H or BALB/c mice, regardless of recipient strains or alloreactivities. Furthermore, allografts of B6 mice also showed an upregulation of IL-6, neutrophil gelatinase-associated lipocalin, and endoplasmic reticulum stress genes, as well as an increased influx of host neutrophils and memory CD8 T-cells. In contrast, donor MyD88/Trif deficiency inhibited neutrophil influx and decreased the expression of IL-6 and endoplasmic reticulum stress genes, along with improved graft function and prolonged allograft survival. Thus, kidney-intrinsic factors involving genetic characteristics and innate immunity serve as critical determinants of the severity of delayed graft function. This preclinical murine model allows for further investigations of the mechanisms underlying delayed graft function.

Keywords

delayed graft function; donor factors; innate immunity; ischemia/reperfusion injury; kidney transplantation; mouse model

DGF, commonly defined as the need for dialysis within the first postoperative week,[1] affects up to 50% of deceased-donor kidney transplant (KTx) recipients and results in increased health expenditures[2] and high risks of allograft rejection, failure, and death.[2–4] The discrepancy between kidney demand and supply has led to the heightened utilization of marginal or expanded criteria donor kidneys prone to DGF.[5,6] However, no US Food and Drug Administration-approved treatments for DGF are available to date.[7] DGF is a manifestation of transplant ischemia/reperfusion injury (IRI) with multiple etiologies, including donor age, prolonged warm/cold ischemic time (WIT/CIT), dialysis vintage, and human leukocyte antigen compatibility.[7,8] However, key determinants of DGF severity remain elusive, hindering the development of effective treatments.

Both donor and recipient factors have been implicated in the development of DGF, but most interventions tested in clinical trials are performed in recipients after KTx. These interventions include treatments with erythropoietin,[9–11] eculizumab,[12] carnitine,[13] C1 esterase inhibitor,[14] and remote ischemic conditioning,[15] with attempts being made to decrease hypoxia, inhibit oxidant stress and complement activation, or blunt the immune responses. However, these clinical trials carried out in recipients have achieved underwhelming success. In contrast, a randomized clinical trial published in the *New England Journal of Medicine* in 2015 reported that, despite the indeterminate mechanisms, mild hypothermia intervention in kidney donors significantly reduced the DGF rate from 59% to 30% in deceased-donor KTx recipients.[16] This evidence highlights the critical role of donor-derived kidney-intrinsic factors in DGF as well as the importance of identifying key determinants of DGF susceptibility, because targeting these factors in the immediate posttransplant period could significantly reduce DGF.[17,18]

Various animal models have been used to investigate mechanisms underlying kidney IRI and DGF. However, most published findings originate from acute kidney injury (AKI) models

induced by renal vessel clamping, which may be not relevant to clinical transplantation.[7,19–21] Recently, several modified mouse models of KTx to study transplant IRI, either through prolonging CIT or WIT, have been reported by several laboratories, including ours.[22–25] For instance, Nydam *et al.*[23] found that caspase inhibition during cold storage improved renal graft function using mouse syngeneic KTx models with a 0.5-hour CIT. However, to our knowledge, there has been no comprehensive study on relative contributions of donor versus recipient factors and IRI versus alloreactivities on the susceptibility and severity of DGF in a clinically relevant model.

To investigate the impact of IRI on post-transplant renal function, we previously reported a preclinical mouse model of KTx, in which donor kidneys were preserved in cold UW solution for a prolonged CIT and then transplanted into binephrectomized recipients, mimicking clinic scenarios in recipients with end-stage renal dysfunction.[22] Here, we performed a comprehensive histologic and functional analysis to further characterize this model as a surrogate of DGF to determine relative contributions of kidney-intrinsic factors versus recipient factors versus immunologic compatibility in the onset of DGF, and to examine whether donor-derived innate immunity plays a role in driving transplant IRI and DGF in kidney allografts.

RESULTS

Prolonged CIT induced early deterioration of graft function in mouse kidney transplant resembling clinical DGF

To determine the influences of CIT on renal graft function and survival, a serial of syngeneic KTx (isografts) with 0.5-, 4-, 6-, or 24-hour CIT were performed using B6 mice (Figure 1a). A CIT of 6 hours or more caused irreversible damage in B6 kidneys, as most recipients succumbed to renal failure within 3 days post-KTx, whereas approximately 75% of recipients of 4-hour CIT grafts recovered and survived for over 100 days (Figure 1b). Prolonged CIT significantly elevated levels of blood urea nitrogen (BUN) and blood creatinine (Cr) at postoperative day (POD) 1, indicating that renal graft function deteriorated in a CIT-dependent manner (Figure 1c). Moreover, increased Cr levels strongly correlated with levels of blood potassium (K^+), sodium (Na^+), and calcium (Ca^{2+}) (Figure 1d, Supplementary Figure S1) in a manner reminiscent of electrolyte abnormalities seen in patients with clinical DGF or AKI due to compromised renal function.[26,27] Histologically, substantial acute tubular necrosis, as characterized by swelling of tubular epithelial cells (ECs), loss of EC brush border, and accumulation of debris in tubular lumina,[28] was observed in the medulla of 4-hour CIT grafts but not in 0.5-hour CIT grafts, concomitant with inferior regenerative capacity as determined by Ki67 (a proliferation marker) staining at POD2 (Figure 1e). To evaluate blood flow and the oxygenation status of kidney grafts with prolonged CIT, we conducted a magnetic resonance imaging (MRI) evaluation in recipients at POD1 and were able to consistently detect differences between the 4-hour and 0.5- hour CIT grafts (Figure 1f). The representative shown data qualitatively indicate reduced perfusion (decrease in DCE- MRI-derived IAUC60 maps) and decreased oxygenation values (higher R2* kidney values) in the first group (4-hour CIT) as compared with the second one (0.5-hour). This preliminary imaging evaluation suggests and potentially confirms the

expected impairment in blood perfusion and the progression of the hypoxic environment in grafts with intensive IRI resembling clinical DGF.[29] Regarding the functional recovery pattern post-KTx, recipients of 0.5-hour CIT grafts returned to the baseline Cr level as early as at POD2, whereas recipients of 4-hour CIT grafts required 2 weeks to return to nearly baseline Cr level with a relatively high BUN amount (Figure 2a). Taken together, CIT strongly correlated with the severity of DGF and mouse KTx with 4-hour CIT proved to be an appropriate preclinical model for DGF investigation.

Genetic attributes of donor kidneys, but not recipients, determined the severity of IRI and DGF

To examine genetic influences in mouse KTx with prolonged CIT, functional changes in B6 and BALB/c isografts with 4-hour CIT were compared. As shown in Figure 2a, BALB/c isografts had lower Cr and BUN levels at days 1 and 2 post- transplant and quicker functional recovery, suggesting higher IRI tolerance compared with that of B6 isografts. To determine the relative impact of donor versus recipient on the severity of IRI and DGF, renal graft function at POD1 was compared in the following KTx groups: BALB/c → B6, B6 → B6, B6 → BALB/c, and BALB/c → BALB/c. Strikingly, a significant elevation of blood Cr and BUN was seen in recipients of B6 kidneys relative to the recipients of BALB/c kidneys, regardless of recipient strain or donor-recipient MHC mismatch (Figure 2b). Correspondingly, BALB/c recipients of B6 allografts (B6 → BALB/c) had significantly shorter survival than did B6 recipients of BALB/c allografts (BALB/c → B6) (Figure 2c).

To further investigate the role of kidney factors in the susceptibility to DGF, we transplanted MHC fully mismatched kidneys from either B6 or BALB/c mice with 4-hour CIT into C3H recipients. The data confirmed that recipients of B6 allografts showed significantly higher Cr and BUN levels, electrolyte imbalance, and increased mortality in the acute phase as compared with recipients of BALB/c allografts (Figure 3a–c). Additional comparisons between the B6 → C3H versus C3H → B6 groups also revealed that C3H kidneys had superior function versus B6 kidneys after KTx (data not shown). Collectively, these results suggest that kidney-intrinsic factors involving genetic differences, rather than recipient factors or donor-recipient MHC mismatch, are dominant factors controlling DGF severity.

Distinctive immune cell populations in renal allografts in the early phase post-KTx with prolonged CIT

To determine early cellular events in renal allografts suffering severe IRI, kidneys from B6 mice with CD45.2 background were implanted into BALB/c CD45.1 congenic recipients after either 0.5- or 4-hour CIT. Multi-color flow cytometry analysis was performed to analyze immune cell phenotypes in renal allografts at POD1. As shown in Figure 4a and b, 4-hour CIT significantly augmented the influx of recipient-derived leukocytes as compared with 0.5-hour CIT. Over 70% of kidney-resident and over 90% of recipient-derived leukocytes, respectively, were $CD11b^+$ myeloid cells. Interestingly, these myeloid cells, including macrophages (Macs), dendritic cells (DCs), monocytes (Monos), and neutrophils, exhibited distinct phenotypes (Figure 4c and d), including $CD11b^{int}$ $F4/80^{hi}$ $CD11c^+$ $MHCII^+$ population (kidney- resident Macs/DCs), $CD11b^{hi}$ $F4/80^{int}$ $CD11c^{low}$ $MHCIr^-$ $Ly6C^+$population (recipient-derived Monos/Macs), and $CD11b^{hi}$ $F4/80^{low}$ $CD11c^-$ MHCII+ Ly6G

$^{+}$ population (recipient-derived neutrophils). Particularly, prolonged CIT to 4 hours significantly increased recipient-derived neutrophils and Monos/Macs.

Kidney-intrinsic factors affected the influx of myeloid cells and effector memory CD8 T-cells in renal allografts post-KTx with prolonged CIT

The influence of kidney-intrinsic factors on early cellular infiltrations was tested in B6 and BALB/c renal allografts (C3H as recipients) with 4-hour CIT at POD1. Consistent with the histologic impairment (Figure 5a, Supplementary Figure S2), B6 allografts had a higher frequency of Monos/Macs and neutrophils in comparison with BALB/c allografts post-KTx as quantified by both immunohistochemistry and flow cytometry analysis (Figure 5a–c and e). It is known that endogenous memory CD8 T-cells mediate cardiac allograft IRI and rejection.[30,31] We observed that B6 allografts had more effector memory CD8 T-cell infiltrates in comparison with BALB/c allografts (Figure 5d and e). No significant difference was observed for the memory CD4 T-cell population. These results demonstrate that donor genetic background alters the early infiltration of CD8 memory T-cells and inflammatory myeloid cells.

Upregulation of ER stress pathways was associated with increased susceptibility of DGF

Activation of endoplasmic reticulum (ER) stress pathways is associated with kidney IRI and other diseases involving sterile inflammation.[32–36] In human kidneys, differential expression of ER stress genes including glucose-regulated protein 78 kD *(GRP78)*, spliced X-box binding protein 1 *(XBP1S)*, and C/EBP homologous protein *(CHOP)* were associated with the severity of AKI,[32] and *CHOP* deficiency could reduce tubular EC apoptosis and attenuate AKI.[32,33] Moreover, our previous study in an arthritis model showed that inositol-requiring enzyme 1 α (IRE1α) was also a critical downstream of Toll-like receptor (TLR).[36] We, therefore, tested the key ER stress genes as well as inflammatory genes to determine whether the ER stress pathway was implicated in DGF. The results (Figure 6) showed that compared with BALB/c allografts, B6 allografts had significantly increased gene expression of interleukin-6 (IL-6)[37] and neutrophil gelatmase-associated lipocalin[38] at day 1 post-transplant. More interestingly, B6 allografts displayed upregulated gene expression of *GRP78, IRElα, CHOP,* and *XBP1S,* which are key components reflecting the activation of ER stress,[39] suggesting that the *IRElα/XBPlS* axis may be associated with DGF susceptibility and severity.

Kidney-intrinsic MyD88/Trif signaling pathway was a key determinant of DGF susceptibility

MyD88/Trif pathways are critical downstream adapter proteins of the TLR signaling crucial to innate immunity.[40] The activation of TLR pathways also leads to ER stress.[41] Our previous work has proven the important role of host MyD88 in renal allograft rejection,[42] yet roles of kidney-intrinsic MyD88/Trif pathways in IRI and DGF are still not clear. We hypothesized that the kidney-intrinsic innate immunity could be a key determinant of DGF. To test this, MyD88 knockout (KO) or MyD88 and Trif double knockout (DKO) mice were used as kidney donors in mouse models of KTx with 4-hour CIT preservation (Supplementary Figure S3, Figure 7a–f). We first showed that genetic ablation of MyD88 in B6 donors, but not BALB/c donors significantly reduced Cr levels post-transplant at POD1 and POD2, compared with wild-type (WT) kidneys, suggesting that kidney-intrinsic innate

immunity plays a role in the increased susceptibility to transplant IRI observed in B6 kidneys (Supplementary Figure S3). Moreover, compared with recipients of WT kidneys at POD1 and POD2 (Figure 7a), recipients of MyD88/Trif DKO kidney allografts showed improved graft function that was consistent with histologic findings (Figure 7e). Strikingly, MyD88/Trif DKO in the donor induced indefinite renal allograft survival and preserved intact renal allograft architecture after 100 days post-transplantation (Figure 7f). Further analysis of graft-infiltrating immune cells showed that the DKO allografts had significantly decreased the influx of recipient neutrophils at POD1 with lower mRNA levels of IL-6 and the ER stress gene, *XBP1S* (Figure 7b–e), indicating that MyD88/Trif signaling may play a role in mediating ER stress and exacerbating graft IRI.

To further determine whether genetic background influences responses of renal tubular epithelial cells (RTECs) on stimulation of TLR signaling, we utilized a well-established *in vitro* primary RTEC culture system.[43] RTECs from B6 and BALB/c were isolated and cultured, respectively, and their phenotypes were characterized by their expression of aquaporin 1 (AQP1) and E-cadherin (E-cad) (Supplementary Figure S4). The RTECs were stimulated by the addition of lipopolysaccharides (LPSs) at various concentrations. Levels of cytokines (including tumor necrosis factor-α [TNF-α], IL-6, and IL-10) and kidney injury molecule-1 (KIM-1) in the supernatants were measured at 24 hours after LPS stimulation compared with untreated RTECs. As shown in Figure 8a, consistent with published studies, [36] levels of cytokines were increased by both B6 and BALB/c RTECs on LPS stimulation. Interestingly, we found that compared with B6 RTECs, BALB/ c RTECs produced significantly higher levels of cytokines (including TNF-a, IL-6, and IL-10) in a dose-dependent manner (Figure 8a). More importantly, expression of KIM-1, a known biomarker for renal cell injury,[44] was significantly increased by B6 RTECs as compared with that in BALB/c RTECs (Figure 8b). These results suggest that genetic background significantly influences cytokine production by RTECs in response to TLR signaling. The lower KIM-1 level detected in BALB/c RTECs in response to LPSs suggests that BALB/c kidneys may be more resistant to insult mediated by activation of TLR-dependent innate immunity.

DISCUSSION

To our knowledge, this is the first study to delineate distinct roles of donor versus recipient in DGF severity using a clinically relevant mouse model of DGF following transplant IRI. It has generated at least 3 important findings. First, similar to DGF seen in clinical settings, mouse KTx with prolonged CIT show histologic and functional deterioration that positively correlated with increased CIT, therefore providing a clinically relevant model for studying mechanisms and therapies of IRI and DGF. Second, kidney-intrinsic factors involving MHC background are key determinants of transplant IRI and DGF. Third, kidney-intrinsic MyD88/Trif pathway and ER stress activation are involved in regulating the severity of DGF.

Although the concept of genetic background-dependent sensitivity to kidney injury is recognized, less is known regarding distinct roles of kidney-intrinsic factors or extrinsic factors in driving the kidney injury. Understanding the mechanisms underlying kidney-intrinsic influences in DGF requires a clinically relevant model. The mouse model used in this study displays functional and histologic impairment characteristics that closely mimic

the clinical DGF. B6 kidneys displayed more severe graft injury regardless of alloreactivity and donor-recipient strain combinations, while BALB/c or C3H kidneys demonstrated higher resistance to IRI following transplantation. In addition, the worse IRI observed in B6 kidneys correlated with poorer long-term outcome observed in the kidney allografts from B6. Collectively, these findings suggest that inherent factors derived from donor kidneys are critical in determining the severity of DGF and that severe IRI adversely influencing long-term kidney allograft survival, likely by augmenting allograft rejection, though donor-recipient MHC mismatch plays a dominant role in driving the allograft rejection process. These results corroborate with clinical observations highlighting the importance of kidney factors in DGF.[45–47]

Unlike previous reports in heart transplant models,[48,49] over 30% of B6 recipients of BALB/c renal allografts survived over 100 days post-transplantation despite prolonged CIT (Figure 2c). We[42,50] and others[51] have previously reported that BALB/c recipients of B6 allografts develop more robust acute rejection and most recipients succumb to rejection in 4 weeks, whereas most of B6 recipients of BALB/c allografts typically survive beyond 100 days with characteristics of chronic rejection. Despite different dynamics, these allografts do develop histologic evidence of acute rejection (lymphocyte infiltration, tubulitis).[42,50] The kidneys are known to have enormous compensatory capacity, which may explain partially why some allografts survived prolonged time with chronic rejection. Moreover, differences reported in survival patterns can be also attributed to the difference in surgical techniques.[52]

Strain differences have been previously reported mostly in the context of host adaptive immune response.[53,54] Our study is unique in identifying an important role of kidney-intrinsic innate immunity and stress response in transplant-associated IRI. Macs and DCs are the most abundant leukocytes in kidneys and are key early mediators of innate immunity through phenotype switching.[55,56] While the precise cellular mechanism remains to be investigated, it is reasonable to speculate that B6 kidneys might be predisposed toward inflammatory M1 responses and BALB/c kidneys toward antiinflammatory M2 responses through interactions between TLR pathways and ER stress. However, this hypothesis warrants further investigation.

ER stress occurs when unfolded or misfolded proteins are abnormally accumulated in the ER lumen and leads to a cascade of adaptive responses determining cell fate.[57] Several pathophysiological stimuli such as hypoxia and glucose scarcity activate the ER stress pathway.[58] We observed an upregulation of ER stress-associated genes correlated with exacerbated injury in B6 kidney grafts, suggesting increased ER stress response in the donor kidneys contributed to severity of DGF. However, specific cell types that undergo ER stress were not determined in this study. ER stress has been implicated in renal tubular ECs,[32,33] podocytes,[34,59] and renal-resident Macs[35] during AKI and in various models; it is reasonable to speculate that these cells are also experiencing ER stress as a result of transplant-associated IRI. Given that DGF is characterized by acute tubular necrosis,[60] we suspect that RTECs may be the main cell types experiencing strong aberrant ER stress response in our model.

We and others have previously demonstrated that TLR-MyD88 pathway interacts with ER stress pathway.[36,41] We now demonstrate that kidney-intrinsic TLR signaling pathways contribute to the high susceptibility to IRI. Abrogating MyD88/Trif (downstream of TLR) in kidney donors significantly downregulated the expression of ER stress-associated genes and mitigated DGF. Results from the *in vitro* study confirmed that genetic background significantly affected TLR signaling-mediated production of cytokines. Surprisingly, LPS stimulation mediated increased levels of TNF-α, IL-6, and IL-10 by BALB/c RTECs compared with B6 RTECs. These cytokines are known to have dual roles, either detrimental or protective to cells, depending on cell types and disease settings. IL-6 plays important roles in both inflammatory process and tissue repair.[37,61,62] Increased IL-6 expression in kidney has been associated with severe kidney IRI,[63,64] consistent with our *in vivo* finding in B6 kidneys. However, IL-6 expression was higher in BALB/c RTECs than B6 RTECs in response to LPSs, which seems contradictory to the *in vivo* findings. We postulate that the different results may be attributed at least in part to the increased infiltrating Macs in kidney allografts from B6 as compared with those from BALB/c, as kidney-infiltrating Macs are a major source of IL-6 production during the early stage of iri.[63,65,66] Interestingly, several reports suggest that stimulation of IL-6 trans-signaling ameliorate AKI via an antioxidative stress mechanism and accelerate tubular regeneration.[37,67,68] We hypothesize that IL-6 secretion by RTECs might exert a protective effect during IRI. While precise implications of the in *vitro* findings demand further investigation, the lower KIM-1 level detected in BALB/c RTECs in response to LPSs suggests that BALB/c kidneys may be more resistant to insult mediated by activation of TLR-dependent innate immunity.

These findings are particularly clinically relevant, as mice and humans share significant similarities in the TLR sequence.[69–71] Specifically, the similarity between mouse and human TLR4 amino acid sequences is 62% in the extracellular domain, 70% in the transmembrane domain, and 83% in the cytoplasmic domain, as reviewed by Vaure and Liu.[69] It has been reported that the gut microbiome plays a role in solid organ transplant outcomes in mice,[72] likely by altering the TLR signaling. In this study, all animals were housed in the same barrier facility and their breeding generations were comparable. Therefore, the influence of the microbiome and breeding has been minimized.

We did not detect any difference regarding the renal function of the BALB/c MyD88 KO versus MyD88 WT at POD1, which is distinct from the report by Kwan *et al.*[73] that TLR4 KO in BALB/c donors reduced severity of injury versus in WT BALB/c at 2 weeks, suggesting that lack of TLR signaling could be beneficial to BALB/c donors in the rejection phase. Su *et al.*[74] showed that prolonged CIT increased recruitment of the memory CD4 and CD8 T-cells in mouse cardiac allografts with 8-hour CIT. However, we did not detect a significant difference in lymphocyte populations but myeloid populations in renal grafts, suggesting that the increased recruitment of the T-cells in grafts may be also timing-dependent or organ-specific.

Other kidney-intrinsic factors including anatomical differences, the size of kidneys, number of nephrons, and the microvasculature could also contribute to the difference between the strains. Previous studies in human and animal models proposed that lower nephron numbers are predisposed to primary hypertension and renal diseases including transplant rejection.

[75–78] A larger kidney mass may harbor more tubules and glomeruli and could contribute to a higher ischemic tolerance.[79] Adult C3H and BALB/c mice were shown to have larger kidney mass relative to B6 mice; however, no correlation was found between nephron number and body weight.[80] It is possible that susceptibilities to IRI may be influenced by nephron number or kidney mass, but this was not examined in the current study.

MRI was performed to evaluate blood perfusion and oxygenation of kidney isografts. Our preliminary data demonstrated a promising trend for the IAUC60 parameter suggesting reduced kidney perfusion following transplant with 4–hour CIT in both grafted and native kidneys, while the higher R2* cortical values reported here for the 4-hour CIT grafted kidneys compared with 0.5-hour CIT kidneys may reflect decreased oxygenation, as was similarly reported in other clinical reports.[29] Interestingly, native kidneys of the transplants with 4-hour CIT also displayed a reduced perfusion (IAUC60) compared with 0.5-hour CIT. While the underlying mechanism is not clear, we suspect that the extended CIT may have a systemic effect that influences the native kidney perfusion. Despite the lack of conclusive interpretation, these data demonstrate the potential of MRI to differentiate between groups and also to further strengthen the clinical relevance of our DGF mouse model.

In conclusion, our findings highlight the critical role of kidney-intrinsic factors, including MHC background and innate immunity in DGF and KTx outcomes, and underscore the necessity of developing individualized therapeutic strategies directed at modulating donor organs prior to transplant for improving transplant outcomes. In addition, while mouse models of KTx with prolonged CIT provide useful tools to investigate mechanisms and potential therapeutic interventions, careful considerations should be taken in choosing adequate strain combinations.

METHODS

Mice and KTx

All mice were used according to protocols approved by the Internal Animal Care and Use Committee of Northwestern University. The surgical procedure for the life-supporting KTx was modified based on the method previously described by our laboratory.[22,42,81,82]

Statistical analysis

GraphPad Prism (GraphPad Software, Inc., San Diego, CA) was used for data analysis. A P value of < 0.05 was considered to represent a statistically significant difference. Error bars throughout indicate SEM.

Comprehensive materials and methods including animals, KTx and sham surgery, histology, cellular and molecular analyses, blood biochemistry detection, functional MRI tests,[83,84] and statistical analysis are detailed in the Supplementary Methods.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Statement

Transplant ischemia/reperfusion injury (IRI)-mediated delayed graft function (DGF) adversely affects up to 50% of deceased-donor kidney transplant recipients. Factors contributing to susceptibility to IRI remain under-determined. Using a clinically relevant mouse model that strictly mimics the clinical DGF, this study highlights the critical role of kidney-intrinsic factors, including major histocompatibility complex (MHC) background and MyD88/Trif-dependent innate immunity in DGF and kidney transplant outcomes, and underscores the necessity of developing individualized therapeutic strategies directed at modulating donor organs prior to transplant for improving transplant outcomes in the clinic.

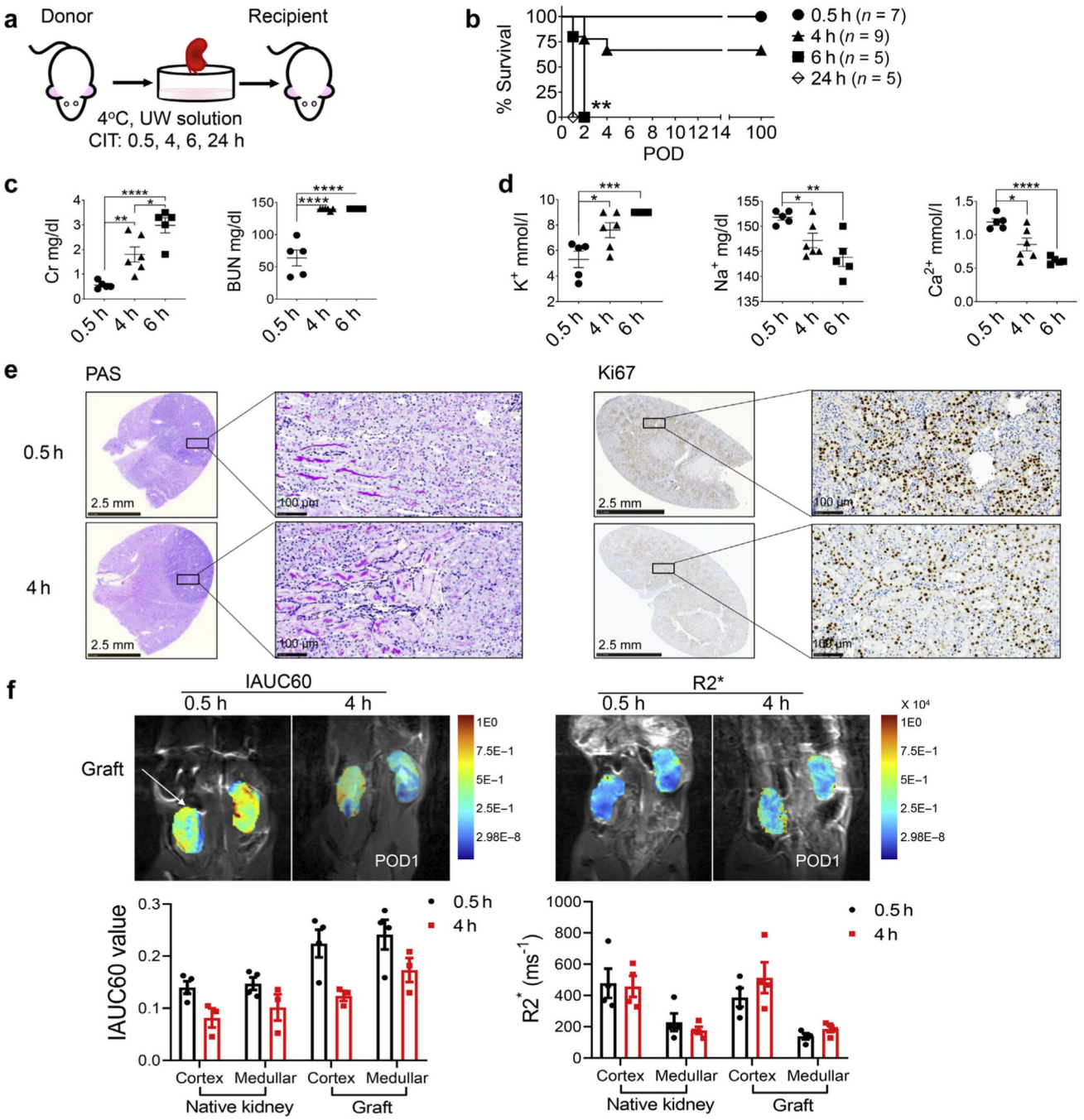

Figure 1 |. Prolonged cold ischemic time (CIT) to 4 hours in mouse kidney transplantation induced the functional decline in grafts reminiscent of clinical delayed graft function.

(**a**) Schematic diagram of the experimental design showing mouse kidney transplantation with different prolonged CIT. Briefly, B6 kidneys were harvested and subjected to 0.5-, 4-, 6-, or 24-hour CIT by preservation in 4 °C UW solution prior to transplantation. Bilateral nephrectomy was performed so the kidney grafts served as the life-supporting organs. Blood creatinine (Cr), blood urea nitrogen (BUN), and electrolyte levels were measured post-transplantation at the selected time point. (**b**) Survival curve of B6 kidney isografts with 0.5-

($n = 7$), 4- ($n = 9$), 6- ($n = 5$), or 24-hour ($n = 5$) CIT. Grafts with 6-hour or longer CIT had very high mortality on postoperative day 2 (POD2) (**$P < 0.01$ vs. 4 hours; Mantel-Cox test). Levels of (**c**) blood Cr and BUN and (**d**) electrolytes including potassium (K^+), sodium (Na^+), and calcium (Ca^{2+}) on POD1 in B6 mice that received kidney isografts with 0.5-, 4-, and 6-hour CIT. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$; Student's *t* test. (**e**) Representative histologic images showing B6 kidney isografts with 4-hour CIT displayed more intensive acute tubular necrosis and decreased tubular proliferation than did the grafts with 0.5-hour CIT. Grafts were harvested on POD1 and POD2, preserved in formalin, and then stained with periodic acid-Schiff (PAS) or Ki67. Bars = 25 μm (full images) and 100 μm (insets). (**f**) Representative images and bar graphs of functional magnetic resonance imaging at POD1 to evaluate the blood perfusion and hypoxia of kidney isografts with different CIT ($n = 4$ per group). The mouse contralateral native kidney was kept *in situ* in the functional magnetic resonance imaging study groups.

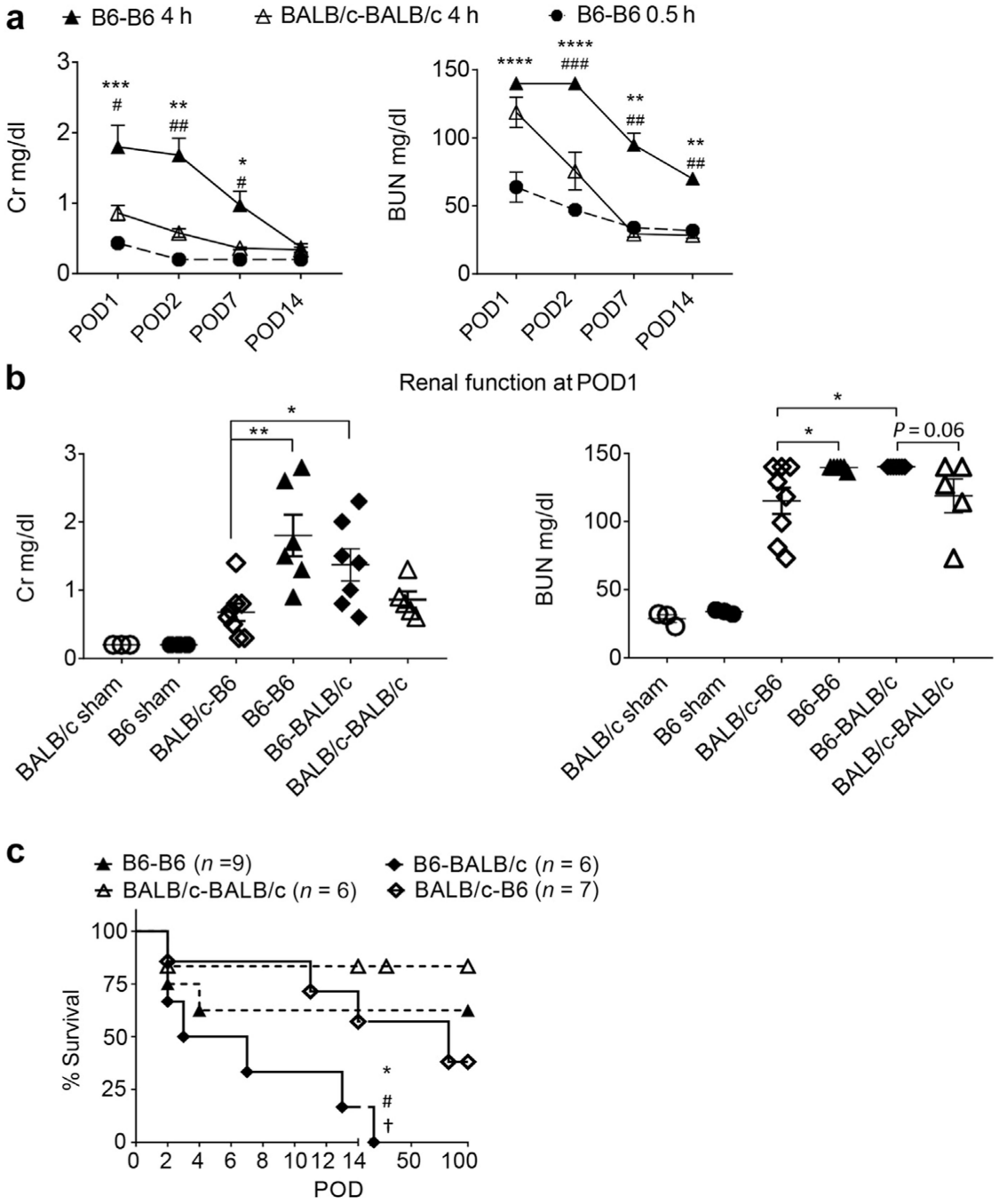

Figure 2 |. Kidney-intrinsic factors, rather than the recipient factors or donor-recipient major histocompatibility complex mismatch, more greatly contributed to the onset of delayed graft function in mice.

(**a**) Graphs showed the dynamics of graft functional recovery in B6 and BALB/c syngeneic kidney transplants with 0.5- or 4-hour cold ischemic time (CIT), as indicated by levels of blood creatinine (Cr) and blood urea nitrogen (BUN) measured on postoperative days (PODs) 1, 2, 7, and 14 ($n = 5–9$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.001$ when comparing B6 → B6 4-hour versus B6 → B6 0.5-hour groups. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ when comparing B6 → B6 4-hour versus BALB/c → BALB/c 4-hour

groups; Student's *t* test. (**b**) The Cr and BUN measurements on POD1 after allogeneic or syngeneic kidney transplantation with 4-hour CIT in the groups including BALB/c → B6 (n = 8), B6 → B6 (n = 6), B6 → BALB/c (n = 7), BALB/c → BALB/ c (*n* = 5). $*P < 0.05$, and $**P < 0.01$; Student's *t* test. (**c**) The survival curve of the B6 and BALB/c recipients after syngeneic or allogeneic kidney transplantation with 4-hour CIT. Data showed that B6 → BALB/c group had inferior survival in comparison with BALB/c → B6 ($*P < 0.05$), B6 → B6 ($^{\#}P < 0.05$), and BALB/c → BALB/c ($^{+}P < 0.05$); Mantel-Cox test.

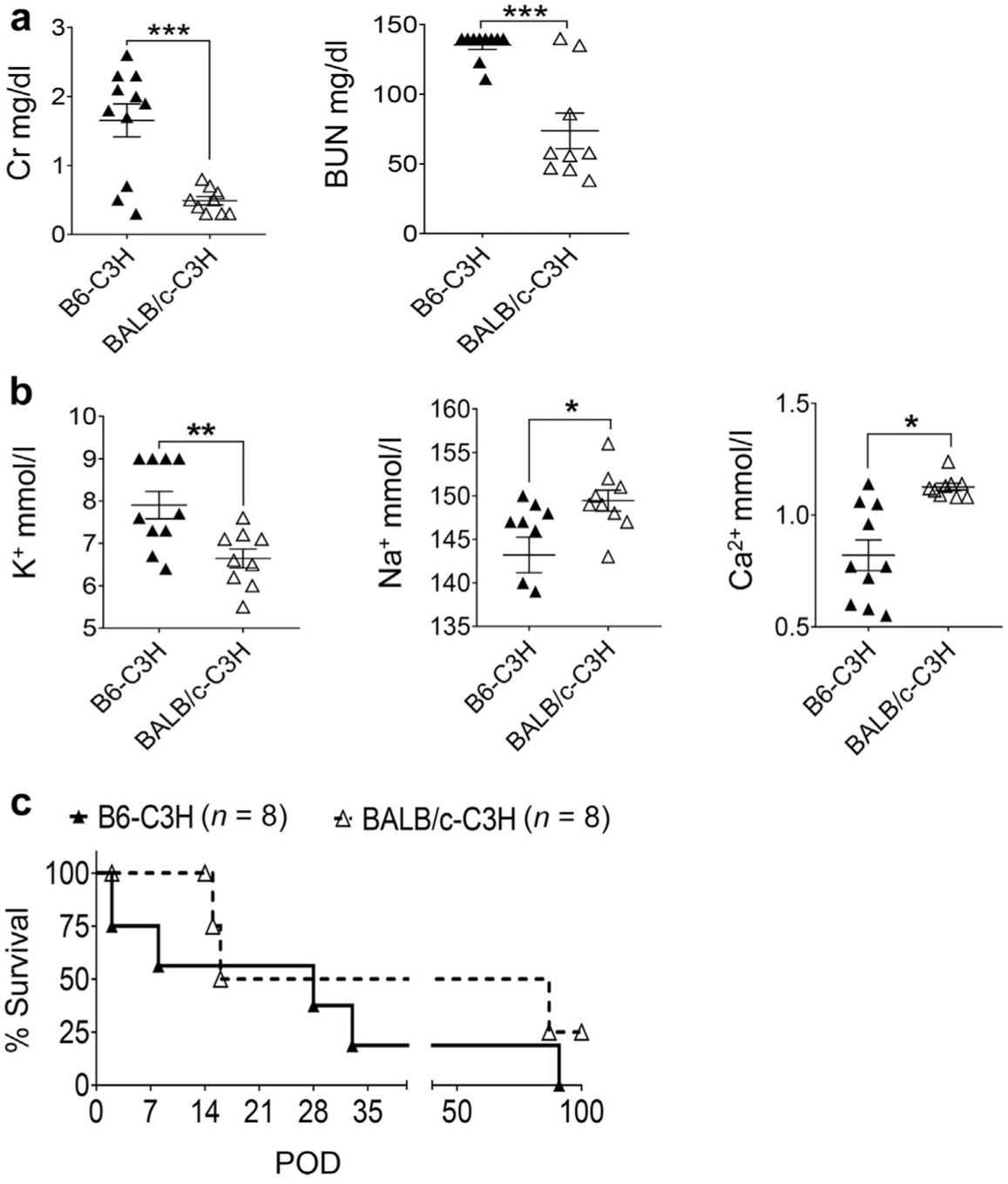

Figure 3 |. B6 renal allografts exhibited more intensive transplant ischemia/reperfusion injury in comparison with BALB/c allografts when transplanted into the same recipients.

B6 or BALB/c kidneys with 4-hour CIT were used as donors, and C3H mice were used as recipients. (**a**) Blood creatinine (Cr) and blood urea nitrogen (BUN) and (**b**) electrolyte levels including K^+, Na^+, and Ca^{2+} on postoperative day 1 (POD1) were compared ($n = 9$–11 in each group). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.01$, Student's t test. (**c**) The survival curve showed b6 allografts experienced inferior survival in the first postoperative week as

compared with BALB/c allografts, but long-term survival was comparable. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$; Mantel-Cox test.

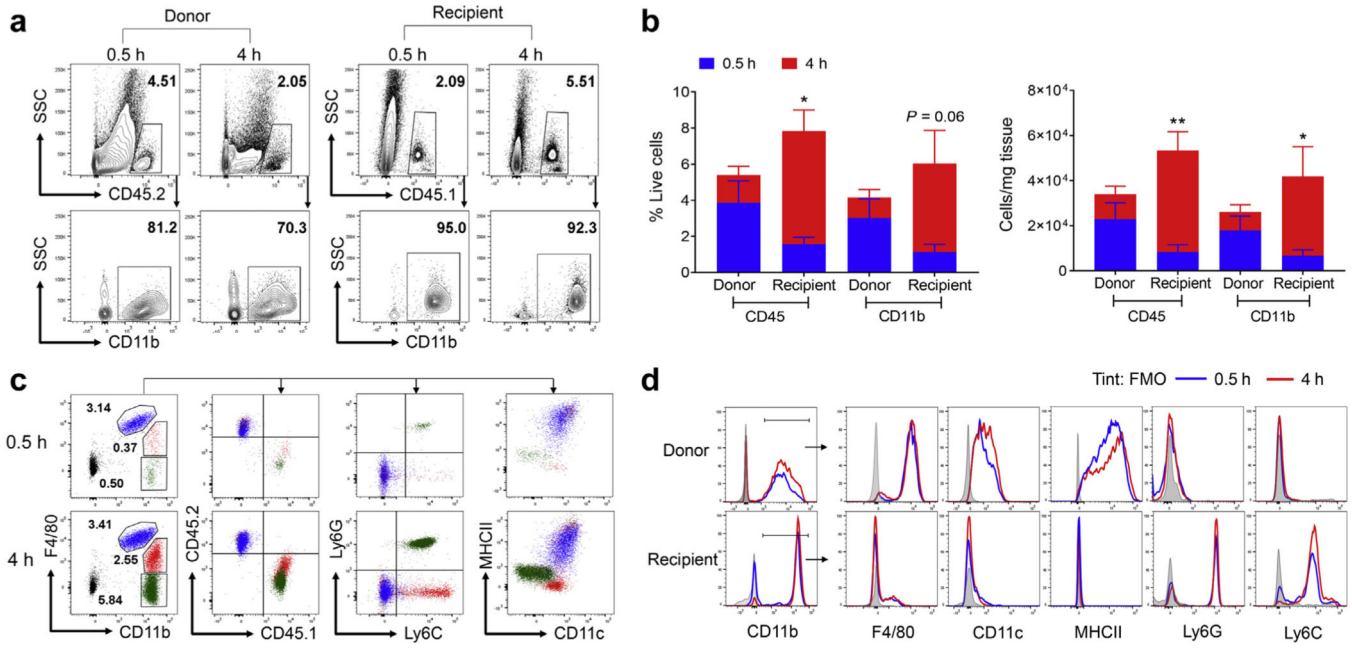

Figure 4 |. Intragraft immune cell compartments at the early phase of post-transplantation with prolonged cold ischemic time.

Renal allografts with 0.5- or 4-hour cold ischemic times were harvested at postoperative day 1 and flow cytometry was performed for the analysis of intragraft immune cells. CD45.2 B6 mice were used as donors and BALB/c CD45.1 mice were used as recipients. (**a**) Representative dot plots show the percentages of intragraft immune cells and $CD11b^+$ myeloid cells derived from either donor or recipients in grafts (gated on total live singlets). (**b**) The absolute number of intragraft immune cells and $CD11b^+$ myeloid cells. (**c**) Representative dot plots showed the phenotypes of the intragraft myeloid cells (gate on live singlets). (**d**) The intensity of donor-resident and recipient-derived immune cells showed distinctive expression of CD11b, F4/80, CD11c, major histocompatibility complex II (MHCII), lymphocyte antigen 6 complex locus G (Ly6G), and Ly6C (gate from CD45.1- or CD45.2-positive live singlets). FMO, fluorescence minus 1; SSC, side scatter.

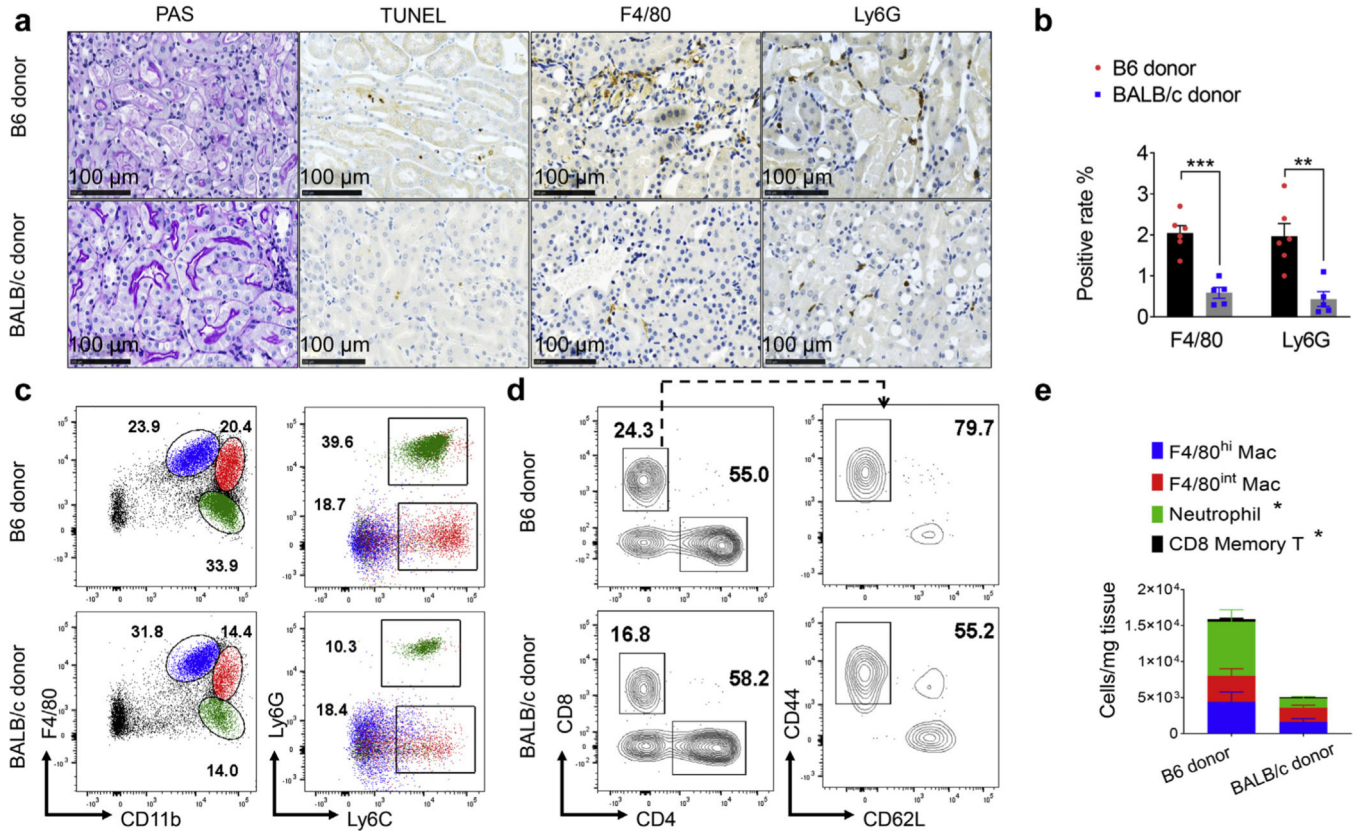

Figure 5 |. Kidney-intrinsic genetics affected the early myeloid cell and endogenous memory CD8 T-cell infiltration in renal allografts with prolonged cold ischemic time.

(**a**) Periodic acid-Schiff (PAS), terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL), F4/80, and lymphocyte antigen 6 complex locus G (Ly6G) staining in B6 and BALB/c allografts with 4-hour cold ischemic time on postoperative day 1 (C3H mice were used as recipients). Bars = 100 μm. (**b**) Bar graphs showing the positive rate of F4/80 and Ly6G in histologic slides (n = 5–6). $^{**}P < 0.05$ and $^{***}P < 0.001$; Student's t test. (**c**) Flow cytometry analysis of graft-infiltrating myeloid cells (gated on total $CD45^+$ live singlets) and (**d**) endogenous T-cells in B6 allografts and BALB/c allografts at postoperative day 1. (**e**) Bar graphs of cell counts. $^{*}P < 0.05$; Student's t test. Mac, macrophage.

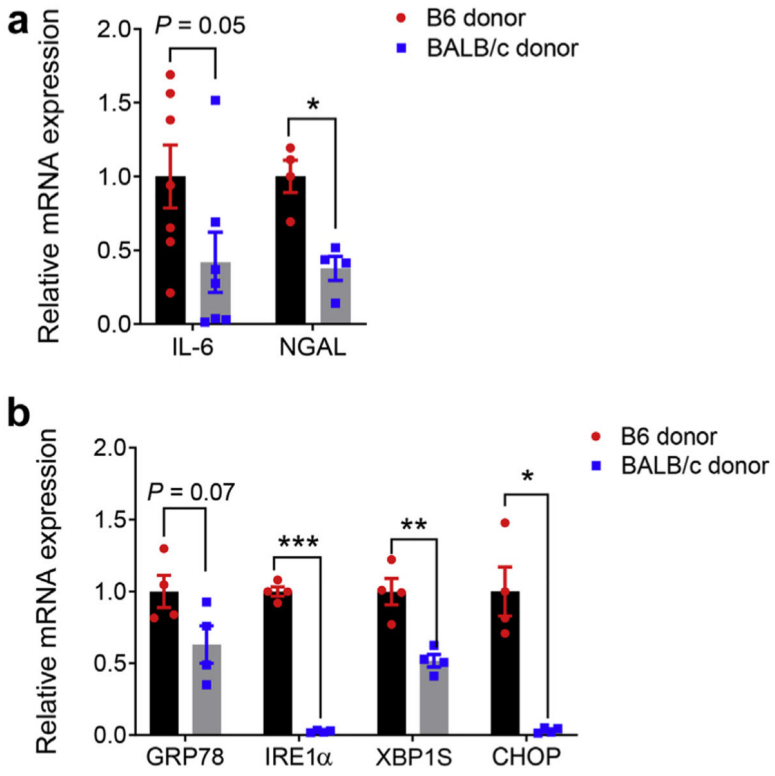

Figure 6 |. The upregulation of endoplasmic reticulum stress pathway genes was associated with increased susceptibility of delayed graft function.

B6 or BALB/c kidneys with 4-hour cold ischemic time were transplanted into C3H recipients. The expression of the indicated genes of B6 and BALB/c kidney allografts (n = 4–7) on postoperative day las determined by quantitative real-time polymerase chain reaction. *$P < 0.05$, **$P < 0.01$, and ****$P < 0.0001$; Student's t test. CHOP, C/EBP homologous protein; GRP78, glucose-regulated protein 78 kD; IL-6, interleukin-6; IREIα, inositol-requiring enzyme 1 α; NGAL, neutrophil gelatinase- associated lipocalin; XBP1S, spliced X-box binding protein 1.

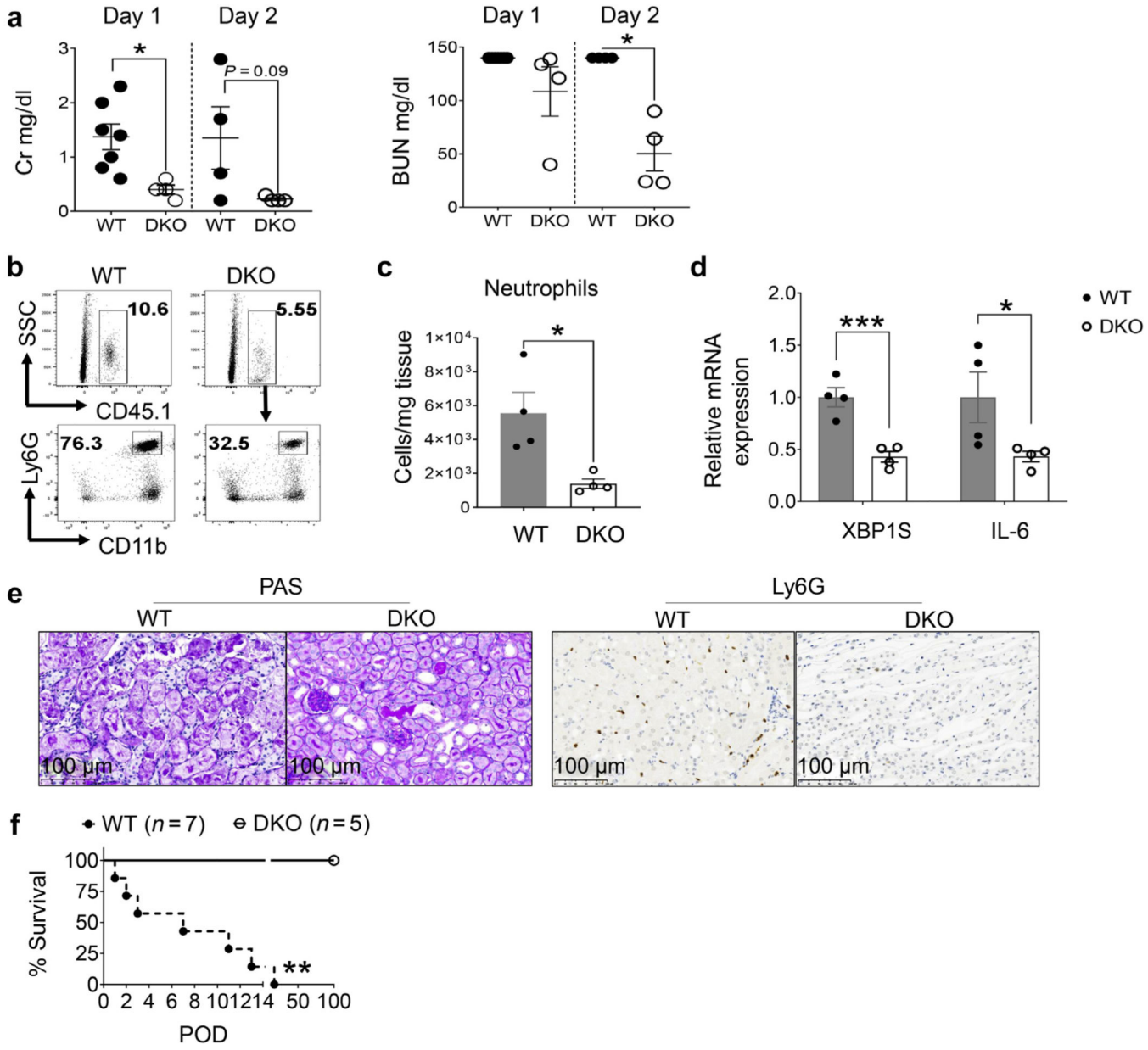

Figure 7 |. Deficiency of kidney-intrinsic MyD88/Trif signaling pathways reduced graft inflammation and endoplasmic reticulum stress gene activation, along with the improved graft function and survival.

Kidneys with 4-hour cold ischemic time from MyD88/Trif double knockout (DKO) mice and B6 wild-type (WT) controls were transplanted into BALB/c recipients (DKO → BALB/c vs. WT → BALB/c). (**a**) Blood creatinine (Cr) and blood urea nitrogen (BUN) levels were measured at postoperative days (PODs) 1 and 2. *$P < 0.05$; Student's *t* test for each selected time point. (**b**) Representative dot plots showing the percentages of the recipient-derived total leukocytes (gate on live singlets) and neutrophils. (**c**) The absolute neutrophil cell counts in WT and DKO renal allografts at POD1. *$P < 0.05$; Student's *t* test. (**d**) Spliced X-box binding protein 1 *(XBP1S)* and interleukin-6 *(IL-6)* gene expression in B6 WT and DKO renal allografts ($n = 4$ in each group) on POD1 as determined by

quantitative real-time polymerase chain reaction. *$P < 0.05$ and ***$P < 0.001$; Student's *t* test. (**e**) Periodic acid-Schiff (PAS) and lymphocyte antigen 6 complex locus G (Ly6G) staining in B6 WT control and DKO renal allografts with 4-hour cold ischemic time on the POD1. Bars = 100 mm. (**f**) The survival curve of the recipient mice in the allogeneic groups (DKO → BALB/c vs. WT/ BALB/c). **$P < 0.01$; Mantel-Cox test. SSC, side scatter.

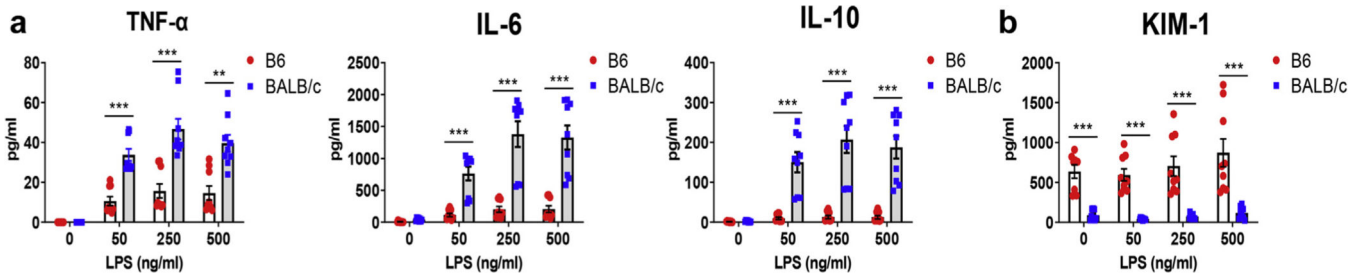

Figure 8 |. Differential production of cytokines and renal tubular cell injury marker kidney injury molecule-1 (KIM-1) in the primary cultured renal tubular epithelial cells (RTECs) from B6 and BALB/c mice.

RTECs from B6 and BALB/c mice were isolated and cultured for 7 days following a standard protocol as described in the Methods. TECs were stimulated with different doses of lipopolysaccharides (LPSs) for 24 hours. The levels of cytokines (**a**) including tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and IL-10 and the tubular cell injury marker (**b**) KIM-1 were analyzed by enzyme-linked immunosorbent assay. Error bars represent data from 3 independent experiments. Student's *t* test was used for the statistical analysis. $^{**}P < 0.01$, $^{***}P < 0.005$.