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REVIEW

Innate immunity and organ transplantation: focus on lung transplantation

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Summary

Ischemia reperfusion injury (IRI) that occurs with solid organ transplantation activates the innate immune system to induce inflammation. This leads to enhanced acute allograft rejection, impaired transplant tolerance and accelerated progression of chronic rejection. In this review, we discuss the innate immune signaling pathways that have been shown to play a role in organ transplantation. In particular, we focus on Toll-like receptor signaling pathways and how they have influenced outcomes after organ transplantation both experimentally and from clinical studies. Furthermore, we describe the substances that trigger the innate immune system after transplantation and several of the key cellular mediators of inflammation. We specifically point out unique aspects of activation of the innate immune system after lung transplantation. Finally, we discuss the areas that should be investigated in the future to more clearly understand the influence of the innate immune system after organ transplantation.

Introduction

The immune system evolved to provide protection from inflammatory insults, such as infections, and can be broadly divided into innate and adaptive systems. The former is typically the "first line of defense" against pathogen invasion, and responds rapidly to the presence of microorganisms that have breached epithelial barriers. This rapid response consists of alterations in sentinel cells, for example dendritic cells (DCs) or macrophages, which upregulate activating costimulatory molecules (e.g., CD40/CD86), chemokine receptors (e.g., CCR7), and produce inflammatory cytokines, such as IL-12. Such maneuvers not only restrain pathogen invasion further, but also allow these cells to traffic to the draining lymph nodes where they communicate with cells of the adaptive immune system. The adaptive immune system then initiates an antigen-specific response to eliminate the pathogen with generation of memory cells, which provide enhanced protection upon re-encounter with the pathogen.

The role of the adaptive immune system has long been recognized as a critical response to organ transplantation. For example, there are experimental data that T cells, which are typically considered components of the adaptive system, are required for acute allograft rejection [1]. Clinically, several therapeutics, for example, anti-CD25 monoclonal antibodies, target T cells after transplantation. Both experimental and clinical studies have highlighted the contribution of humoral responses to allograft rejection [2,3]. Sensitized transplant recipients, who harbor preformed antibodies, as well as patients who develop alloantibodies de novo after transplantation are at high risk for graft rejection. The importance of the innate immune system has only recently been appreciated. In contrast with the adaptive immune system, which is characteristically generated within days after transplantation, the innate system is activated within minutes after graft implantation with the initial reperfusion injury. In this review, we describe some of the key innate immune pathways that have been implicated after organ transplantation,

the areas that we are yet to understand and the potential therapeutic implications.

Ischemia reperfusion injury activates the innate immune system

Ischemia reperfusion injury (IRI) is an inflammatory insult that is initiated by ischemia to an organ, and exacerbated by subsequent reoxygenation after reperfusion [4]. It can occur in an organ-specific fashion, for example, during acute coronary syndromes or can be multiorgan (e.g., during septic shock or circulatory failure caused by cardiac arrest). In organ transplantation, it is surgically induced with the donor procurement, cold preservation, and subsequent implantation with resulting reperfusion. Increasing IRI during organ transplantation leads to early primary graft failure.

Toll-like receptors (TLRs) are innate immune receptors that are expressed on both hematopoietic and parenchymal cells [5]. Within the immune system, TLRs are expressed by cells of both the innate and adaptive immune compartments. Cells of the innate system that express TLRs include DCs, macrophages, and neutrophils. TLR activation on these cells triggers an inflammatory response alerting the immune system to the presence of a noxious stimulus. Of the known 12 TLRs, all but one (TLR3) signals via a transducer termed MyD88, to induce inflammation [6]. TLR3 utilizes Trif for its signal transduction and this receptor is typically activated by double-stranded RNA contained within viruses [7]. TLR4 is unique in that it can signal via either MyD88 or Trif [6].

Several studies have now examined the importance of TLRs in different experimental models of IRI. In liver IRI models, TLR4, but not TLR2 deficient mice are protected from injury [8,9]. Furthermore, liver inflammation during liver IRI occurs in a MyD88-independent fashion [8,9]. TLR4 is a receptor that is activated by components of gram-negative bacteria, such as lipopolysaccharide (LPS). However, TLRs, including TLR4, have been reported to be activated by endogenous substances that are released during cell necrosis. For example, HMGB1, a nuclear protein that is released during cell necrosis, has been shown to contribute to liver IRI by activating TLR4 (Fig. 1) [10,11]. However, this does not exclude the possibility that other TLR4 activators exist during liver IRI and it is conceptually possible that during liver IRI, translocation of gut micro-flora, which contain LPS, enter the portal circulation and act as a source of TLR4 stimulation.

In experimental models of cardiac IRI, both TLRs 2 and 4 have been implicated in inducing injury and subsequent inflammation [12,13]. This has been shown in mice deficient in either TLR that have been subjected to coronary ligation and subsequent reperfusion, a warm IRI model that may mimic clinical myocardial infarctions. In a heterotopic heart transplant model, in which the donor graft is subjected to cold preservation, a scenario that may mimic clinical organ procurement and implantation, both TLR4 and MyD88 have been found to contribute to the inflammatory response, in particular, the production of systemic IL-6 and MCP-1 after donor implantation [14,15]. Interestingly, both vascular endothelial cells and myocardial cells express TLRs. One study found that TLR2 expression on both hematopoietic and parenchymal cells contribute to endothelial dysfunction after warm cardiac IRI [16]. Another study indicated that TLR activation directly on cardiomyocytes *in vitro* induced inflammatory responses [17].

In renal IRI, TLRs 2 and 4 via MyD88 signaling have been found to be important for inflammation [18,19]. In particular, one study found that expression of TLR4 on kidney tubular epithelial cells mediates damage after renal IRI. Interestingly, this study associated this finding with an accumulation of several putative endogenous activators of the innate immune system, including HMGB1, heat shock protein (HSP) 70, a cellular chaperone and biglycan, a component of the extracellular matrix [19]. A follow-up study provided data that HMGB1 contributes to renal IRI [20]. All these endogenous activators have been shown to stimulate TLR4. A recent study employed an inhibitor of TLR2 to show that this treatment improved

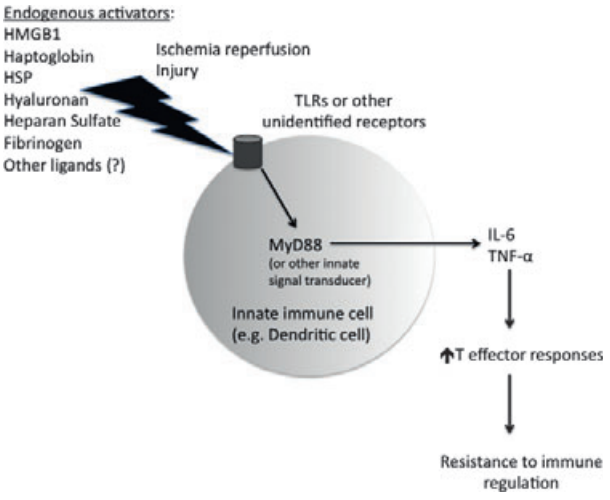

Figure 1 Innate immune activation after organ transplantation Ischemia reperfusion injury leads to the release of endogenous innate immune activators. Some of these activators are known to activate TLRs (e.g., TLR4) and some, such as haptoglobin, are not yet known to activate TLRs. Most endogenous ligands are known to signal via MyD88. In some organs, (e.g., liver) IRI is independent of MyD88, but dependent on IRF3. Innate immune activation leads to induction of proinflammatory cytokines that augment alloreactive T cell responses and renders these cells resistant to immune regulation.

renal function in an experimental renal IRI model [21]. We discuss lung IRI in a section on innate immunity and lung transplantation below.

The above experimental studies indicate that IRI may lead to the release of endogenous innate immune activators that stimulate TLRs (Fig. 1). This induction of inflammation may influence immune responses after organ transplantation.

TLR activation and acute allograft rejection

Several studies over the last 10 years have implicated a role for TLRs in acute graft rejection. Our laboratory reported that MyD88 signaling was critical for skin graft rejection across a minor mismatched barrier (HY antigen)[22]. In this model, MyD88 signaling within the donor and recipient contributed to graft rejection, DC maturation, and priming of graft reactive $CD8^+$ T cells [22]. When we tested the role of MyD88 in MHC-disparate skin transplant models, we no longer found that MyD88 was critical for graft rejection, although it still influenced DC maturation responses after transplantation [23]. A report found similar findings, but in this study, the investigators found that the alternate TLR signal adaptor downstream of TLR3 and 4, Trif, contributed to the tempo of acute allograft rejection [24]. Specifically, Trif and MyD88 signaling within the transplant maximally contributed to the tempo of acute skin graft rejection [24].

TLRs have been investigated in other experimental models of transplantation besides skin. We investigated the role of MyD88 in the heterotopic heart transplant model. We found that the absence of MyD88 in both donor and recipient was not critical for cardiac allograft rejection, although there was a small, but significant delay in the tempo of graft rejection as compared with wild type controls [23]. However, in a fully MHC-mismatched model of spontaneous kidney graft acceptance, absence of a negative regulator of TLR responses, Toll-IL-1R8, induced acute graft rejection [25]. In particular, absence of Toll-IL-1R8 enhanced IRI and DC maturation after kidney transplantation [25].

The role of TLRs in islet allograft rejection is controversial. The first experimental study in this area found that deletion of TLR4 in the donor islets was important for the tempo of graft rejection and recipient heme oxygenase contributed to the phenotype [26]. However, a later study did not find a role of either TLR4 or its adaptor proteins, MyD88 and Trif, within the donor in contributing to the tempo of acute islet allograft rejection [27]. A third study, which implanted islets within the portal vein did find a role for TLR4, but not TLR2 and 3 in the tempo of acute graft rejection. These findings raise the possibility that the route of islet transplantation (portal vein vs. under the kidney capsule) may be important for the contribution of TLRs in acute graft rejection. Another study provided evidence that recipient TLR4 expression contributed to islet allograft rejection and that rapamycin treatment of the TLR4-deficient recipients led to survival times greater than 90 days in 50% of recipients [28]. Together, these studies suggest that TLRs contribute to the tempo of acute islet allograft rejection; although future studies directly comparing different islet transplant techniques should be examined.

Intestinal allografts are exposed to commensal bacteria and the environment similar to lung transplants and in distinction to renal and cardiac grafts. One study found that recipient TLR4 contributed to the tempo of acute intestinal allograft rejection, and this was associated with reduced systemic inflammatory responses within the recipient [29]. It is possible that commensal bacteria are the source of innate immune activation in this model and it would be interesting to test this experimentally either with germ-free mice or mice that are treated with antibiotics to reduce the load of bacteria within the gut. In summary, TLRs either in the donor or the recipient contribute to a varying degree in experimental models of organ transplantation.

Experimental studies pointing to a role for TLRs in chronic vasculopathy are only just emerging. One study reported that TLR2/4 signaling via MyD88 enhanced the development of chronic rejection in an experimental kidney allograft model [30]. A clinical study in heart transplantation associated TLR4 gene expression with endothelial dysfunction [31]. Overall, the influence of TLRs on chronic rejection/vasculopathy is an under investigated area.

Several studies have investigated the role of the innate immune system in mediating graft-versus-host disease after allogeneic bone marrow transplantation. To evaluate whether the ability of host hematopoietic cells to respond to innate immune stimuli regulates graft-versus-host disease, Li and colleagues used bone marrow transplant recipients that were deficient in MyD88 and Trif in their hematopoietic compartment [32]. They found that graft-versus-host disease was not reduced in these recipients leading to the conclusion that activation of host antigen presenting cells possibly resulting from bacterial translocation from the gut is not critical to trigger graft-versus-host disease. One study, however, showed that TLR9 expression on nonhematopoietic host cells plays an important role in triggering acute graft-versus-host disease [33]. The authors suggested that bacterial breakdown products were enhancing alloimmune responses in their recipient animals following irradiation-induced myeloablation through activation of TLR9 on nonbone marrow-derived cells, such as intestinal epithelium. A recent study showed that

recipients of allogeneic bone marrow that carry a TLR4 mutation (C3H/HeJ) experience a higher rate of acute graft-versus-host disease than controls that respond normally to TLR4 stimulation (C3H/HeN) [34]. Host TLR4 mutants experience a higher infiltration of macrophages into the intestine and increased apoptosis of gut epithelium than wild type controls. Alternatively, one group reported that graft-versus-host disease was attenuated when the donor was not able to respond to TLR4 stimulation [35]. Thus, the role of innate immune activation following bone marrow transplantation is complex and experimental results may be impacted by variations in strain combinations as well as host conditioning regimens.

In clinical transplantation, the first report to implicate TLR in acute allograft rejection was in lung transplantation [36]. Using a genetic approach, this study found that recipients with a hypo-responsive TLR4 gene exhibit an enhanced time until the first acute allograft rejection response up to 1 year after transplantation as compared with recipients with the wild type gene [36]. In a subsequent study, the investigators determined that this effect is maintained up to 3 years post transplantation [37]. Moreover, lung transplant recipients, who have a polymorphism in the CD14 promoter, which is associated with increased transcriptional activity and enhanced responses to innate immune stimuli, suffer early onset of acute and chronic graft rejection [38]. Further aspects of innate immune activation and lung transplantation are discussed below.

Similar findings for the TLR4 gene polymorphism were noted in kidney transplantation [39], although in this study, the authors reported that there were reduced atherosclerotic and acute allograft rejection events after transplantation in recipients with hypo-responsive TLR4 genes, but these recipients exhibited increased infectious episodes [39]. Another study found that TLR4 is expressed in donor kidneys and it is significantly upregulated in deceased rather than living related donor allografts [40]. The investigators of this study found that donors in whom TLR4 signaling is reduced exhibit increased immediate graft function compared with those with intact TLR4 signaling [40]. Clinical studies have also suggested that activation of the innate immune system through TLR4 contribute to acute rejection of hepatic grafts and chronic rejection of heart transplants [31,41].

TLRs and transplant tolerance

Although we reported that MyD88 signaling was dispensable for acute graft rejection of fully MHC-mismatched grafts, we subsequently initiated studies to determine if MyD88 signaling altered the induction of transplant tolerance [42]. In a fully MHC-mismatched skin allograft model, we found that, whereas wild-type mice rejected their allografts after treatment with peri-operative anti-CD154 and CTLA4 Ig, most MyD88-deficient recipients accepted their allografts indefinitely and that this acceptance was donor-specific [42]. Thus, our study establishes that MyD88 signaling impairs transplant tolerance. Another group reported similar findings concurrently to ours [43]. These experimental findings have been translated to clinical transplantation, as patients that exhibit operational tolerance of kidney transplants, exhibit reduced MyD88 expression within the peripheral blood mononuclear cells as compared with recipients that exhibit chronic rejection of their kidney transplants [44]. Further experimental support that TLR activation impairs transplant tolerance was provided by several studies that demonstrated that systemic administration of TLR activators (in the form of CpG, which activates TLR9 or LPS, which activates TLR4) abrogated transplant tolerance induction of cardiac or skin allografts [43,45,46].

In our study, we found that MyD88 signaling enhanced the production of IL-6 and TNF-α by DCs in transplant recipients treated with costimulatory blockade [42]. We subsequently found that recipients that were deficient in both IL-6 and TNF-α exhibited a similar phenotype to $MyD88^{-/-}$ recipients that were given a full MHC-mismatched skin graft and peri-operative costimulatory blockade: allograft acceptance in most recipients [47]. A subsequent study in a cardiac allograft model implicated IL-6 in transplant tolerance induction [48]. We found that IL-6 and TNF-α synergize to increase T effector cell proliferation to render these cells less prone to immune regulation by regulatory T cells (Fig. 1) [47].

Endogenous activators of the innate immune system and organ transplantation

The experimental and clinical studies described above indicate a role for TLRs and their signal adaptors in inducing inflammation after organ transplantation. The substances that activate the innate immune system after organ transplantation have remained elusive. Possible activators may be microbial or may be released by necrotic cells, i.e., endogenous activators [49]. As stated above, there is accumulating evidence that during inflammation endogenous activators of the innate immune system are released, whereas these substances remain hidden from the immune system in the quiescent state. HMGB1, HSP, hyaluronan, heparan sulfate, uric acid, and fibrinogen are examples of endogenous activators that have been implicated in a variety of models of sterile inflammation (Fig. 1) [50,51]. Recently, f-actin that is released from necrotic cells has been shown to bind to DNGR1 on $CD8^{+}$ DCs [52,53], but whether this induces inflammatory signals remains to be determined.

We recently identified haptoglobin as a novel protein activator of the innate immune system that contributes to the tempo of acute skin transplant rejection in mice [54]. We found that necrotic skin lysates trigger inflammatory responses by bone marrow-derived DCs (BMDCs) *in vivo* and that similar to our prior *in vivo* transplant models, this occurred via a MyD88-dependent process [54]. We also found that the process of skin transplantation increases the ability of skin lysates to induce inflammatory responses by DCs. Furthermore, we noted that allogeneic skin transplantation augments the inflammatory response of skin lysates to a superior degree than syngeneic lysates [54]. This indicates that skin transplantation increases the concentration of innate immune triggers and this is enhanced more in allogeneic than in syngeneic transplants.

We performed comparative proteomics between lysates from nontransplanted skin and transplanted skin and identified that haptoglobin is upregulated after transplantation. We independently confirmed that haptoglobin is upregulated by employing an ELISA-based assay [54]. Haptoglobin is a heme-binding protein that is a known acute phase protein with immune modulatory functions [55,56]. It is best characterized as binding to free heme, which would otherwise induce oxidative stress [56,57]. Its immune modulatory roles include reducing inflammatory responses induced by LPS *in vivo* and enhancing delayed type hypersensitivity responses *in vivo* in mice [58,59].

As we found that haptoglobin activates MyD88-dependent inflammatory responses in DCs, we examined the role of donor haptoglobin using a minor mismatched skin transplant model, in which we previously demonstrated that rejection occurs via MyD88 [22]. Specifically, male skin from either wild- type or haptoglobin-deficient donors was transplanted onto female wild-type recipients [54]. We found that graft survival was significantly delayed with haptoglobin-deficient skin grafts as compared to wild type grafts, although some haptoglobin-deficient grafts were rejected. Furthermore, donor haptoglobin expression enhances anti-donor T cell responses [54].

Thus, our recent study documents that haptoglobin is a novel activator of the innate immune system that accelerates the tempo of minor mismatched skin graft rejection. Additional studies will be needed to examine whether haptoglobin plays a role in other transplant models that are either more immunogenic or employ immediately vascularized organs.

Lung transplantation: unique aspects of innate immune activation

Lung allografts are exposed to environmental antigens that can be inhaled or aspirated. Thus, lung transplantation likely activates the innate immune system uniquely compared with other organ transplants. There is already substantial evidence that innate immune activation regulates alloimmune responses and negatively impacts outcomes after pulmonary transplantation. Severe lung IRI manifests itself as hypoxemia and development of radiographic lung infiltrates. A recent series from our institution demonstrated that IRI after lung transplantation adversely affects both short-term and long-term survival [60]. The hospital mortality for lung transplant recipients, who suffered from primary graft dysfunction, was significantly higher than that for patients, who did not have this complication. Furthermore, survival rates at 1, 5, and 10 years after transplantation were significantly higher for those lung recipients without primary graft dysfunction. Importantly, primary graft dysfunction was associated with lower rates of freedom from bronchiolitis obliterans syndrome, a manifestation of chronic rejection characterized by progressive obstructive ventilatory disorder secondary to fibrotic occlusion of the distal airways, at all time points examined. Thus, clinical lung IRI negatively impacts patient survival.

Several investigators have used pulmonary clamping models to model IRI observed after lung transplantation. While the caveat exists that lungs are subjected to warm rather than cold ischemia during these experiments, important insights have been gained. At least two groups have observed that lung IRI is markedly attenuated in TLR4-deficient mice [61,62]. Interestingly, this effect is independent of signaling through MyD88 and dependent on TLR4 expression on parenchymal rather than hematopoietic cells [62]. To this end, TLR4 engagement on pulmonary endothelial cells may play a role in cytoskeletal rearrangement and edema formation. The potential significance of these observations is substantiated by reports that levels of endogenous TLR ligands increase after reperfusion of human lungs [63]. Furthermore, levels of endogenous TLR ligands in the airways of human lung transplant recipients have been shown to correlate with the development of bronchiolitis obliterans [64].

Multiple reports point to a pivotal role for alveolar macrophages as orchestrators of innate immune responses within the lung [65,66]. IRI is markedly attenuated when alveolar macrophages are depleted, which is in large part because of reduced expression of proinflammatory cytokines and chemokines [66]. Interestingly, production of TNF-α by alveolar macrophages augments secretion of proinflammatory cytokines and chemokines by alveolar epithelial cells [67]. As alveolar macrophages express several pattern recognition receptors, they are likely to play a critical role in shaping immune responses to lung allografts in response with respiratory infections. Other cells

may also mediate innate immune responses that are relevant to lung transplantation. For example, natural killer T cell-mediated secretion of IL-17 has been shown to promote neutrophilic infiltration into lungs and exacerbate IRI [68].

Examining the role of the innate immune system in lung allotransplantation has been hampered by a lack of experimental models. In 2007, our laboratory reported the development of an orthotopic vascularized lung transplantation model in the mouse, which mirrors primary graft dysfunction and acute rejection responses observed in humans [69]. This model has allowed us to perform studies investigating mechanisms of IRI and gain insight into how innate immune responses regulate alloimmunity.

As pulmonary IRI is characterized by neutrophilic infiltration, our study focused on examining the role of this innate cell population. We have demonstrated that following murine lung transplantation serum G-CSF concentrations rise, which results in rapid increases in neutrophil production in the bone marrow and in neutrophilic graft infiltration [70]. Clinical data have suggested that extended cold ischemic storage is associated with worse primary graft dysfunction and higher rates of bronchiolitis obliterans [71]. Notably, extended cold ischemic graft storage in the mouse model enhances G-CSF-mediated granulopoiesis and neutrophil graft infiltration, resulting in exacerbation of IRI [70]. Treatment of graft recipients with a neutrophil-specific depleting antibody resulted in significant amelioration of pulmonary IRI establishing that neutrophils play an important role in mediating this condition. Using intravital two-photon microscopy, we have identified that blood monocytes, an innate immune cell population, are important mediators of transendothelial migration of neutrophils within reperfused lung grafts [72]. In addition, neutrophils often cluster around graft-infiltrating monocytes within the graft tissue raising the possibility that monocytes provide chemotactic cues for neutrophils following their extravasation. Monocytes can differentiate into macrophages or dendritic cells. Our study has shown that monocyte-derived recipient dendritic cells can acquire intact donor MHC molecules and can contribute to $CD4^+$ direct allorecognition within lung allografts [73]. Interestingly, monocytes that are deficient in MyD88 do not efficiently differentiate toward dendritic cells in lung grafts providing further evidence that innate immune signals influence adaptive responses to lung transplants [74].

Unlike other solid organ transplants where alloimmune responses are initiated in graft-draining secondary lymphoid organs our group has shown that after pulmonary transplantation T cells are primed in lung grafts rather than mediastinal lymph nodes or spleen [75,76]. To this end, we have observed clustering of recipient T cells around graft-resident donor dendritic cells within 1 day of engraftment. Thus, early encounters between innate and adaptive immune cells within lung grafts are likely to shape the fate of pulmonary transplants. For example, we have reported that following prolonged cold ischemic storage G-CSF-mediated granulopoiesis abrogates immunosuppression-induced lung allograft acceptance [77]. Specifically, we observed that graft-infiltrating neutrophils interact with donor-derived dendritic cells within lung allografts shortly after reperfusion and promote their production of IL-12 in a cell contact-dependent fashion, which results in T cell differentiation toward T_h1 cells and graft rejection. Moreover, as lungs are exposed to the external environment, immune or stromal cells in the airways or graft parenchyma can be modulated by pathogens, such as respiratory viruses or bacteria. Similar to primary graft dysfunction, bacterial airway colonization or respiratory infections are associated with a higher risk of lung graft failure caused by chronic rejection again pointing to a close link between innate and adaptive immune responses in determining outcomes after lung transplantation in humans [78–80].

Conclusion and future directions

Our understanding of the role that the innate immune system plays after organ transplantation is emerging. Experimental studies indicate that IRI that occurs after organ transplantation activates TLRs to induce inflammation. Signaling via the TLR signal adaptor MyD88 impairs experimental transplant tolerance induction and humans with operational tolerance of kidney transplants exhibit lower expression of MyD88 than kidney transplant recipients with chronic rejection. The substances that activate the innate immune system after organ transplantation are only just being appreciated. Lung transplantation is unique in that grafts are exposed to environmental antigens and that immune recognition pathways that lead to rejection are distinct from those employed by other solid organs. Differential release of innate immune ligands from different organs after IRI is an unexplored area. Additional investigations are required to define the innate immune pathways that are common to all solid organ transplants and those that are unique to organs, such as lung, liver, heart, and kidney.

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