Published in final edited form as:

*Nat Rev Nephrol*. 2016 May ; 12(5): 281–290. doi:10.1038/nrneph.2016.41.

# Role of TLRs and DAMPs in allograft inflammation and transplant outcomes

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## Abstract

Graft inflammation impairs the induction of solid organ transplant tolerance and enhances acute and chronic rejection. Elucidating the mechanisms by which inflammation is induced after organ transplantation could lead to novel therapeutics to improve transplant outcomes. In this Review we describe endogenous substances — damage-associated molecular patterns (DAMPs) — that are released after allograft reperfusion and induce inflammation. We also describe innate immune signalling pathways that are activated after solid organ transplantation, with a focus on Toll-like receptors (TLRs) and their signal adaptor, MYD88. Experimental and clinical studies have yielded a large body of evidence that TLRs and MYD88 are instrumental in initiating allograft inflammation and promoting the development of acute and chronic rejection. Ongoing clinical studies are testing TLR inhibition strategies in solid organ transplantation, although avoiding compromising host defence to pathogens is a key challenge. Further elucidation of the mechanisms by which sterile inflammation is induced, maintained and amplified within the allograft has the potential to lead to novel anti-inflammatory treatments that could improve outcomes for solid organ transplant recipients.

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Activation of the innate immune system after solid organ transplantation — a vital therapy for end-stage conditions — results in sterile inflammation, which enhances acute and

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Author contributions

All authors researched the data, discussed the content, wrote the article, and reviewed and edited the manuscript before submission.

Competing interests statement

The authors declare no competing interests.

chronic rejection and impairs transplant tolerance[1–5]. Our understanding of how the innate immune system contributes to the immune response after organ transplantation has increased over the past decade[1,6,7]; however, very little is known about the exact mechanisms by which inflammation is induced and maintained following organ transplantation.

In transplanted organs, sterile inflammation results from ischaemia–reperfusion injury (IRI), which occurs after the blood supply of an organ ceases and is then abruptly re-established. IRI is an obligatory component of the harvest and implantation of organs[8] that has important implications for the fate of the transplant. Indeed, increasing ischaemic time, and thus IRI severity, enhances the risk of acute and chronic allograft rejection[9]. In human kidney transplantation, increasing cold ischaemic time from 6 h to 30 h augmented the risk of graft failure by 40%[10]. Ischaemic time also affects the fate of transplanted human hearts[11–13]. IRI of a graft is exacerbated by brain death in deceased donors[14] and contributes to worse graft survival than with organs from living donors[15]. How brain death affects graft function requires further study, but experimental evidence shows that brain death activates a systemic immune response, with production of IFN-γ[16]. Mechanistically, the early induction of graft necrosis by IRI causes sterile inflammation by releasing ‘endogenous’ innate immune ligands known as damage-associated molecular patterns (DAMPs) (FIG. 1). DAMPs (BOX 1) are nuclear or cytosolic molecules that are typically hidden from the immune system inside cells. The signalling pathways triggered by DAMPs lead to migration of innate immune cells to kidney allografts, a process that precedes T-cell recruitment, which ultimately drives graft rejection[17].

Clinical and experimental evidence shows that inflammation has a critical effect on the fate of grafted organs. Thus, understanding how inflammation is initiated, amplified and resolved after solid organ transplantation could lead to new therapeutic paradigms and improve outcomes for recipients of solid organ transplants. In this Review, we discuss the contribution of sterile inflammation in solid organ transplantation, with a particular focus on DAMPs and their downstream signalling cascade triggered by Toll-like receptor (TLR) activation. Although pathogens induce inflammation and have a detrimental effect on transplant outcomes, we will not cover infectious inflammation as this subject has been reviewed elsewhere[3]. Likewise, the role of the complement system — another important component of the innate immune system — in organ transplantation has already been covered[18]. First, we discuss factors that are involved in activating and amplifying allograft inflammation. Then, we address specific innate immune signalling pathways that are activated after experimental organ transplantation with a focus on TLRs. We also consider clinical studies that have yielded evidence for the role of DAMPs and TLRs after solid organ transplantation. Lastly, we address future studies focused on developing novel therapeutics to dampen inflammation and improve outcomes for solid organ transplant recipients.

## Inflammation activators and amplifiers

After an organ is transplanted, it undergoes IRI, which leads to a degree of graft necrosis and the subsequent release of DAMPs, which initiate sterile inflammation (FIG. 1). An increasing number of organs are perfused *ex vivo* to increase the donor organ pool[19]. With this new *ex vivo* approach, the therapeutic window of opportunity to treat organs (such as

lungs or kidney) before implantation to reduce the deleterious effects of IRI may expand. Thus, identifying the key innate immune ligands that are released at the time of organ implantation could lead to novel therapies that, if applied locally to the organ before transplantation, could reduce graft inflammation, enable the dosages of systemic immunosuppressive medications to be decreased and improve patient outcomes.

Once released, DAMPs activate various graft cells such as endothelial cells and immune cells (macrophages and dendritic cells)[2]. Activation of graft-resident immune cells induces the production of chemokines, which recruit host immune cells such as neutrophils and macrophages into the graft. These host immune cells enhance inflammation and further promote the immune response to the graft (FIG. 1). Activation of the graft vasculature leads to the upregulation of adhesion molecules such as lymphocyte function-associated antigen 1 (LFA-1) and vascular cell adhesion protein 1 (VCAM-1) (REFS 20,21), which promote neutrophil attachment to the graft vasculature and facilitate neutrophil entry to the graft (FIG. 1). Importantly, host macrophages and dendritic cells that exit the graft after the initial inflammatory response following graft implantation traffic to the local draining lymph nodes where they initiate anti-donor adaptive immune responses[22,23] (FIG. 1).

## DAMPs released after organ transplantation

**HMGB1.—**High mobility group box 1 (HMGB1), a nuclear protein that regulates gene transcription, is one of the best-characterized of the intracellular DAMPs that induce inflammation in transplanted organs and affect the rate of development of acute allograft rejection. In non-transplanted murine models of hepatic and renal IRI, HMGB1 levels are increased in the damaged organs and pharmacological blockade of HMGB1 attenuates inflammation[24–27]. These results, together with findings from other studies which indicate that HMGB1 induces inflammation either by activating TLR4 (REF. 28) or the receptor for advanced glycation end-products (RAGE)[29], have led researchers to examine the function of HMGB1 in experimental organ transplantation. A 2014 study found that inhibition of necroptosis, a form of regulated cell necrosis, reduced *Hmgb1* expression in cardiac isografts and administration of recombinant HMGB1 increased the expression of the proinflammatory cytokine IL-17 in the grafts[30]. Consistent with these findings, other studies have shown that hypothermic preservation of cardiac allografts increases *Hmgb1* expression after graft implantation, and IRI leads to release of HMGB1 from cardiac allografts[31]. Importantly, pharmacological blockade of HMGB1 activity extended cardiac allograft survival by 1 week, with reduced graft inflammation, in a full MHC-mismatched murine cardiac-transplant model[32]. In agreement with this latter study, another study of cardiac transplantation in mice found that inhibition of RAGE extended the median survival of cardiac allografts to almost 3 weeks, compared to just 1 week for allografts transplanted under control conditions[33]. Of note, RAGE is not critical for the induction of renal injury in non-transplant models of IRI in mice[34] and a definitive role for RAGE in the inflammatory response to renal transplantation is yet to be determined. Additionally, in a rat-to-mouse cardiac xenograft model, expression of HMGB1 was upregulated in the xenograft and inhibition of HMGB1 function via an anti-HMGB1 monoclonal antibody increased xenograft survival by about 1 week, with evidence of reduced graft inflammation[35].

The above findings indicate that HMGB1 participates in early allograft rejection and accelerates acute allograft rejection. Findings from a 2014 study suggest that HMGB1 also has a role in chronic allograft rejection, the leading cause of graft loss for which no effective new therapies are available. In an MHC class II mismatched murine model of chronic cardiac allograft rejection, levels of HMGB1 were elevated in the graft 2 months after transplantation[36]. Furthermore, administration of an anti-HMGB1 monoclonal antibody every 4 days during the first month after transplantation reduced the degree of graft vasculopathy by 50% and was accompanied with reduced graft levels of IL-17A, IFN-γ and inflammatory macrophages[36].

Repetitive minor ischaemic insults, either to the organ of interest or to remote tissues, including muscle, might elicit a protective response within the organ through a process called ischaemic preconditioning[37]. In a mouse model of kidney IRI, prior administration of recombinant HMGB1 provided significant protection against kidney dysfunction, histological damage and inflammation[38]. Further mechanistic analyses revealed that HMGB1 preconditioning elicited upregulation of Siglec-G, a negative regulator of TLR4 signalling[14]. Findings from studies in experimental infection models suggest that innate immune cells can exhibit a form of memory — they can enhance immune responses after repeat stimulation through a phenomenon known as ‘trained immunity’ (REF. 39). Whether this phenomenon occurs in organ transplantation has not yet been determined but will need to be investigated if repeated administration of DAMPs is to be considered as a ischaemic-preconditioning strategy in patients undergoing organ transplantation.

Overall, these studies indicate that HMGB1 is released after organ implantation, contributes to graft inflammation and accelerates graft rejection. However, transplant survival in the various models was typically only extended from 1 week to 3 weeks after transplantation with HMGB1 inhibition[32], indicating that HMGB1 is not the only contributor to graft inflammation.

**Hyaluronan.—**Hyaluronan (also called hyaluronic acid) is a glycosaminoglycan produced by mesenchymal cells that has been implicated in a variety of experimental models of sterile inflammation, most notably in bleomycin-induced lung injury[40–42] and in murine non-transplant IRI models[43,44]. In quiescent, noninflammatory states, hyaluronan exists in a large molecular weight form, which undergoes degradation upon inflammation. Hyaluronan fragments can activate TLR2 and TLR4 to induce inflammation[45], and the large form of hyaluronan is thought to be immunoregulatory[46]. However, a 2014 study found that overexpression of the enzyme that fragments hyaluronan in murine skin does not lead to skin inflammation, but rather promotes the migration of dendritic cells out of the skin, which reduces subsequent delayed-type hypersensitivity responses in the skin[47]. This study indicates that hyaluronan fragments could be important to control immune-cell emigration before immune activation. Whether this is also the case in internal organs requires further study.

As well as activating TLR2, TLR4 and their downstream cytoplasmic signal adaptor MYD88[40], hyaluronan can activate CD44 in T cells in the lung[41]. Levels of hyaluronan are elevated in human kidney allografts undergoing acute rejection[48], in minor mismatched skin

grafts undergoing rejection and in human lung transplants with evidence of chronic rejection[49], compared to levels in nonrejecting grafts. Fragmented, but not full length hyaluronan, acts via TLR2, TLR4 and MYD88 to activate dendritic cells to prime alloreactive T cells *in vitro* [49]. These findings are compatible with the reported presence of hyaluronan in fibrotic lung tissue from human lung transplant recipients[50]. Interestingly, in an orthotopic lung allograft model in which transplant tolerance was induced by combined anti-CD154 and CTLA4 immunoglobulin treatment, fragmented, but not full length hyaluronan, abrogated transplant tolerance via a TLR2, TLR4 and MYD88-dependent pathway[50]. Fragmented hyaluronan is therefore likely to contribute to inflammation and acute graft rejection and might also impair the tolerance of some organ allografts. Investigation of the role of fragmented hyaluronan in chronic allograft rejection requires tools to efficiently deplete hyaluronan or hyaluronan fragments in murine models.

**Other DAMPs.—**Other factors that contribute to sterile inflammation, such as uric acid[51] and mitochondrial DNA (which is involved in inflammation after trauma[52]), are not known to be causally involved in inflammation after solid organ transplantation. Some intracellular proteins, such as members of the heat shock protein-70 family, are thought to trigger inflammation; yet, when examined in experimental murine skin transplant models, *Hsp1b* deletion in the donor or *Hsp1a* deletion in both the donor and the recipient, did not affect the timing of allograft rejection[53,54]. Using proteomic approaches, the protein haptoglobin, which is known for its haeme binding and antioxidant properties[55], was found to be upregulated in both syngeneic and allogeneic skin grafts in mice[56]. Purified haptoglobin activates dendritic cells via MYD88 to enhance the priming of alloreactive T cells[56]. In a minor histocompatibility mismatched skin transplant model in which MYD88 is required for graft rejection[57], donor-derived haptoglobin accelerated acute graft rejection[56]. In a full MHC mismatched cardiac transplant model, recipient-derived haptoglobin, but not donor-derived haptoglobin, impaired the ability of CTLA4 immunoglobulin to induce indefinite cardiac allograft survival[58]. Haptoglobin amplified intragraft inflammation by stimulating the accumulation of dendritic cells in the graft, by increasing the expression of the proinflammatory cytokine IL-6 and of the neutrophil attracting chemokine CXCL2 (also known as MIP2), and by reducing the levels of the immunosuppressive cytokine IL-10 within the first 2 weeks of transplantation. Finally, haptoglobin was present in biopsy samples from human cardiac transplants that exhibited moderate cellular rejection, providing initial clinical translation of the experimental findings[58]. These studies indicate that discovery approaches such as proteomics can be powerful tools to identify novel DAMPs after organ transplantation.

Haptoglobin is probably not involved in the initiation of IRI-induced inflammation in the graft after transplantation. Therefore, we speculate that known (and possibly unknown) DAMPs, such as HMGB1, are released upon IRI and activate innate immune signalling pathways within the graft, which heighten the local inflammatory milieu and subsequently lead to the local release of proinflammatory cytokines such as IL-6 or IL-1. These inflammatory mediators then enter the circulation to induce the production of inflammatory amplifiers such as haptoglobin by the recipient (FIG. 1). These amplifiers tip the balance

from graft acceptance to graft rejection by increasing inflammation, which leads to an increase in subsequent anti-donor T-cell responses.

## Innate immunity in experimental grafts

When responding to an inflammatory insult, cellular innate immune recognition of pathogens occurs via cell surface receptors, endosomal receptors and cytosolic receptors[59]. Innate immune receptors can be categorized as inflammasome complexes[60], TLRs and retinoic acid-inducible gene I-like receptors (RLRs)[61,62]. RLRs are yet to be examined in transplanted organs; data on the inflammasome and TLRs in transplantation are described below.

### The inflammasome

The inflammasome is a group of intracellular multimeric protein complexes that are assembled in the cytoplasm in response to a priming signal, typically from DAMPs or PAMPs (BOX 1) via TLRs or other innate immune receptors[60]. The inflammasome complexes function as molecular scaffolds that recruit and activate caspase precursors, in particular procaspase-1 (REF. 60), via the adaptor protein PYCARD (also known as ASC). Inflammasome-mediated activation of caspase-1 leads to the production of IL-1β and IL-18.

Evidence for inflammasome activation after organ transplantation is beginning to emerge[63]. Specifically, in a murine cardiac allograft model, PYCARD and IL-1β were upregulated after transplantation in allografts, but not in syngeneic grafts[63]. A 2015 study identified PYCARD expression in rejecting human cardiac allograft specimens[64]. The mechanistic role of inflammasome activation in organ transplantation awaits further studies.

### Toll-like receptor-mediated signalling

TLRs (BOX 1) are the best-characterized innate immune receptors in organ transplantation. Over 10 TLRs have been recognized across all mammalian species examined[65]. TLR expression and function has been documented in many cell types, in particular in innate immune cells such as monocytes, macrophages, dendritic cells, neutrophils and natural killer cells[66]. Lymphocytes, including the recently identified class of innate lymphoid cells, also express TLRs[67]. Expression of TLRs on endothelial and epithelial cells has also been well characterized and is of great relevance to tissue responses to transplantation[44,68,69]. Animal models and human studies have demonstrated that TLRs are upregulated in the grafted organ following IRI and during rejection[44]. This increase in TLR levels is attributable to increased TLR expression by bone-marrow-derived cells, but also by cells within the parenchyma of the transplanted organ, as shown by experiments using bone-marrow chimeras[44,68]. Other forms of organ injury that can occur following transplantation, including sterile injury caused by toxins and ischaemia, as well as non-sterile injury resulting from infection[69], can also result in TLR upregulation and activation[70].

As detailed in the previous section, numerous endogenous TLR ligands, such as HMGB1 and hyaluronan, are upregulated after organ transplantation[44,57,68,71–74]. Ligand binding to TLRs, often accompanied by hetero-dimerization of several TLR types, triggers a cascade of intracellular signalling events that culminate in the production of proinflammatory cytokines

and chemokines[66]. Although TLRs signal through common downstream effectors, they also exhibit specificity as part of their downstream pathway is specific to individual TLRs and depends on the cell type involved. With the exception of TLR3, TLR signalling requires the recruitment of MYD88. TLR3 activation is mediated by the recruitment of TIR domain-containing adaptor molecule 1 (TICAM-1, also known as TRIF), whereas TLR4 signalling triggers both MYD88 and TICAM-1 pathways[65]. Signalling via the MYD88 pathway leads to the translocation of nuclear factor-κB from the cytosol to the nucleus, inducing the production of proinflammatory cytokines such as IL-6 and TNF, and chemokines, including CCL2 (also known as MCP-1) (REF. 66). Activation of the TLR/MYD88 pathway also initiates the transcription of two key inflammatory cytokine precursors, pro-IL-1β and pro-IL-18, and the assembly of the inflammasome. Signalling via the TICAM-1 pathway triggers production of type 1 interferons IFNα and IFNβ[66].

In mice kidney IRI results in rapid upregulation of *Tlr4* mRNA and TLR4, in tubular epithelial and endothelial cells in particular, and to a lesser extent in infiltrating leukocytes[44]. Deficiency of either *Tlr4* or *MYD88* affords substantial protection against kidney dysfunction, histological damage and inflammation following murine kidney IRI[44]. However, these results partly contrast with findings from another study showing that *Tlr4* is critical for the induction of renal IRI in mice whereas *MYD88* and *Ticam-1* are not[75]. Differences in the degree of cross-clamping used in these studies could explain the discrepant findings with regard to the requirement of *MYD88* for renal IRI[44,75]. Furthermore, the potential synergy between *Ticam-1* and *MYD88* has yet to be tested in double *Ticam-1*/*MYD88*-deficient mouse models of renal IRI.

The innate inflammatory response is necessary for the development of acute allograft rejection in a cardiac allograft model[76] and, as mentioned above, TLRs are required for renal IRI. Thus, the impairment of TLR signalling could prevent acute rejection after transplantation. Deficiency of *MYD88* in mice with minor HLA mismatches in a skin transplantation model was associated with reduced acute rejection and impaired dendritic cell maturation in draining lymph nodes[52]. In addition, loss of *MYD88* promoted the induction of tolerance in skin allografts when combined with co-stimulatory blockade[72,77], and downregulation of *MYD88* and *Ticam-1* through small interfering RNAs enhanced the survival of fully MHC-mismatched cardiac allografts to 40 days and indefinitely when combined with rapamycin[78]. Without co-stimulatory blockade, however, complete *MYD88* deficiency only delayed acute rejection in a fully MHC-mismatched heart allograft model[71] and did not prevent the rejection of fully HLA-mismatched skin grafts[71]. Finally, as mentioned above, *MYD88* and *Ticam-1* contribute to graft levels of IL-6, TNF and CCL2 3 h after reperfusion in syngeneic heart transplant models[79]. These results indicate that inhibiting TLR signalling synergizes with immune suppression to extend allograft survival.

In a life-sustaining, fully MHC-mismatched, murine kidney allograft model, *MYD88* deficiency (in both donor and recipient strains) led to superior graft survival compared to wild-type allografts (100 days versus 40 days, respectively)[73]. At 14 days after transplantation, the time at which acute rejection usually occurs, *MYD88*-deficient allografts showed markedly less evidence of acute rejection and graft inflammation than did wild-type allografts. *MYD88*-deficient allografts exhibited normal kidney function and little evidence

of chronic rejection 100 days after transplantation, whereas wild-type allografts developed marked albuminuria, elevated serum creatinine, prominent glomerulosclerosis, tubulointerstitial inflammation and fibrosis[73]. These findings support those of an earlier study performed in a murine kidney allograft model in which deficiency of *Tlr2*, *Tlr4* or *MYD88* in the recipient reduced chronic allograft damage and the number of macrophages and dendritic cells that infiltrated the allograft[80]. To investigate the possibility that *MYD88* deficiency leads to donor-specific tolerance, third-party skin transplants were conducted on mice with long-term surviving skin allografts[73]. Wild-type survivors rejected skin grafts from matched donors and third-party donors, whereas *MYD88*-deficient survivors rejected third-party skin grafts but accepted skin grafts from donor-strain mice, confirming that these mice had achieved donor-specific tolerance[73]. Transplant tolerance was mediated at least in part by $CD25^+$ regulatory T cells, as administration of a blocking anti-CD25 antibody abolished this tolerance[73]. Thus, the weight of experimental evidence clearly indicates that MYD88 is an important inducer of acute and chronic allograft rejection and prevents the induction of transplant tolerance.

## Inflammation in clinical transplantation

### Early inflammatory events

**DAMP-mediated signalling.—**HMGB1 is upregulated in human cadaveric kidney transplants[74]. Moreover, a follow-up analysis in human kidney transplant recipients showed that urinary HMGB1 levels were elevated as early as 3 h after transplantation[81]. In addition, the presence of HMGB1 in the peripheral blood of kidney transplant recipients was associated with an upregulation of TLR2 and TLR4 in peripheral blood leukocytes, which suggests that HMGB1 induces systemic TLR activation[81]. Similar observations have been made in liver transplant recipients[82]. Exposure of human tubular cells to HMGB1 *in vitro* induces the release of inflammatory cytokines (including IL-6, IL-1β, TNF and CCL2), suggesting that HMGB1 promotes inflammation in human organ transplantation[74] (TABLE 1).

Transcriptomic analysis of bronchoalveolar lavage fluid and lung tissue biopsy samples demonstrated that activation of innate immune pathways occurs in lung transplantation[83,84]. Specifically, gene expression analysis of bronchoalveolar lavage fluid before and after reperfusion in lung transplant recipients showed upregulation of the inflammasome (including NLRP3, IL-1β and caspase-1), inflammatory chemokines and cytokines (CXCL1, CXCL2, CCL7 and IL-6) and TLRs (TLR2, TLR4, TLR6 and TRL9) — key mediators of early graft dysfunction[83]. Of note, the gene expression analysis conducted in these studies does not detect post-translational modifications of proteins such as caspase-1 and pro-caspase-1, which are important inflammatory events. These studies also demonstrated that longer ventilation times were associated with a stronger inflammatory profile, including a higher expression of TLRs and inflammatory cytokines[83,84], indicating that mechanical ventilation directly causes lung injury and inflammation, and impairs early graft function.

**TLR-mediated signalling.—**Interestingly, in lung transplant recipients expression of TLRs strongly correlated with that of inflammatory cytokines, providing evidence that TLRs

are the main mediators of graft inflammation after human lung transplantation[83] (TABLE 1). The importance of TLR4 signalling in IRI after organ transplantation has been further illustrated by genetic studies of specific single nucleotide polymorphisms (SNPs) in the *Tlr4* allele. Presence of the *Tlr4* loss-of-function alleles Asp299Gly or Thr399Ile was associated with lower rates of acute allograft rejection after kidney, lung and liver transplantation, and with lower intragraft levels of proinflammatory cytokines such as TNF and CCL2 (REFS 74,85,86). In addition, TLR gene-expression profiling of biopsy samples from kidney transplant recipients showed increased expression of *TLR1*, *TLR2*, *TLR4*, *TLR7* and *TLR8* in patients undergoing acute rejection, and *TLR4* expression within the graft correlated with cellular infiltration and graft damage, according to the 2007 Banff classification[87], indicating that TLRs, and TLR4 in particular, are important for graft inflammation after kidney transplantation[74]. However, in a 2015 study, *TLR* SNPs did not correlate with outcomes after kidney transplantation[88]. These discrepancies could stem from differences in cohort sizes, SNPs analysed and statistical methods. Nonetheless, given that loss-of-function of *Tlr4* was affected by the expression of inflammatory cytokines in the graft[74], we suggest that the weight of current evidence indicates a functional role of TLR4 in graft inflammation.

The mechanisms by which TLR4 is upregulated during IRI after organ transplantation remain unclear, although one can speculate that hypoxic and oxidative stress mediated by IRI or subsequent inflammation induced by DAMPs promote TLR4 upregulation in human epithelial and immune cells[89–91]. In support of a role for DAMPs in triggering TLR4 upregulation, release of RAGE after graft implantation is predictive of a poor short-term outcome after lung transplantation[92]. Specifically, plasma levels of RAGE 4 h after reperfusion correlate with a longer duration of mechanical ventilation and length of stay in the intensive care unit, independently of graft ischaemia time[92].

## Inflammation and long-term graft survival

**TLRs.—**Genetic analyses in 787 liver allografts identified three donor SNPs in the *Tlr4* allele that were associated with graft failure[93]. However, in a separate study, when liver transplant recipients were stratified on the basis of their hepatitis C virus infection status, the association between *Tlr4* polymorphisms and late graft failure was only found in patients with hepatitis C, suggesting that *Tlr4* polymorphisms synergize with hepatitis C virus infection to enhance graft rejection[86]. In lung transplantation, recipient *Tlr4* SNPs influence graft rejection. An analysis of 110 lung transplant recipients, including 20 patients with bronchiolitis obliterans syndrome (BOS), identified an association between polymorphisms in *Tlr4, Tlr2* and *Tlr9* and the development of BOS[94], which indicates that these TLR polymorphisms enhance the development of chronic rejection after lung transplantation.

In kidney transplantation, loss-of-function polymorphisms in donor *TLR4* are associated with a reduction in the risk of acute rejection[95]; however, these outcomes do not translate into improvements in long-term kidney allograft function. Indeed, allograft function 1–5 years after transplantation was reported as similar, regardless of donor polymorphism status[96]. Similarly, in a case-controlled study of kidney transplant recipients, graft SNPs in TLRs 1–8 were not associated with biopsy-proven graft rejection or mortality by 4 years

after transplantation, although a comparison of kidney transplant recipients and donors identified an association between *TLR* SNPs and end-stage renal disease[88].

Although genetic association studies have yielded inconsistent results, transcriptional analysis of tissue biopsy samples from starndard criteria living-donor kidney grafts showed an ongoing injury response and TLR-mediated inflammation after transplantation[97,98]. Specifically, development of inflammation during the first year after kidney transplantation is associated with upregulation of TLR4 and TLR2 (REFS 97,98). Higher TLR expression was only observed in patients with both graft interstitial fibrosis and cellular infiltrates and not in patients with normal graft histology or fibrosis alone[97]. TLR4 expression is also increased in human kidney grafts undergoing chronic rejection compared to stable controls[99]. Consistent with these findings, the expression pattern of MYD88 and TLR4 in the peripheral blood leukocytes of human kidney transplant recipients enables distinction between patients with chronic rejection and operationally tolerant recipients[99]. In heart transplant recipients, expression of TLR4 on circulating monocytes associates with endothelial dysfunction within the allograft up to 3 years after transplantation, although evidence of chronic rejection was not reported in this study[100]. Although upregulation of the TLR pathway has been reported in these studies, it is often associated (and sometimes masked) by the overexpression of genes involved in the adaptive immune response and other components of innate immunity, which illustrates the molecular complexity of the immune response during graft rejection[97,101,102].

**DAMPs.—**DAMPs are present in chronically rejected human allografts[50,103]. Notably, lung transplant recipients with BOS have an accumulation of hyaluronan in the fibrous tissue of the airway lumen. These patients have a higher expression of hyaluronan synthase 1 in their lung tissue and higher levels of hyaluronan in their bronchoalveolar lavage fluid and plasma, as compared to grafted patients without BOS[50]. Extensive profiling of DAMPs in the bronchoalveolar lavage fluid of lung transplant recipients identified a specific biological pattern for BOS and for restrictive allograft syndrome. Specifically, levels of HMGB1 and S100 family proteins (including S100A8, S100A9, S100A8/A9, S100A12 and S100P), which can activate *Tlr4* and induce innate immune-cell recruitment and endothelial cell activation[104], were elevated in patients with restrictive allograft syndrome and BOS compared to control patients with stable graft function[103]. The production of these DAMPs was slightly increased in the restrictive allograft syndrome group compared to levels in the BOS group[103]. By contrast, expression of S100A8 in human kidney allografts was associated with a reduced incidence of chronic allograft vasculopathy[105]. This finding is consistent with experimental evidence showing that S100A8 contributes to resolution of inflammation after renal injury in non-transplant models of IRI[101] and reduces inflammation after allotransplantation[106,107]. Differences between lung and kidney transplants (for example lung transplants are exposed to the environment whereas kidney grafts are not), might explain the divergent results in regard to S100 proteins.

## Strategies to inhibit inflammation

**Inhibition of TLR signalling.—**Several antibodies and compounds that target TLR4 are in development or in clinical trials. Given that TLR4 is ubiquitously expressed and crucial

for providing protection against infection, strategies that aim to nonspecifically block the TLR4 signalling pathway might incur a considerable risk of infectious complications.

Genetic deletion of pivotal molecules in the innate immune response (individual TLRs or molecules essential for their downstream signalling, such as MYD88) is achievable in mice, particularly when performed in specific pathogen-free environments, and has provided mechanistic insights into the pathogenic roles of these factors, as discussed above. Such major interruptions of innate immune signalling pathways are also theoretically achievable in humans through the use of blocking antibodies or small molecular antagonists, but are likely to carry high risks of infection and cancer. Inhibiting the ligands that activate TLRs directly in the allograft before implantation is a conceptually attractive strategy to avoid compromising host defence in transplant recipients. This strategy has yet to be tested experimentally or clinically. Despite the above-mentioned concerns, several clinical studies that inhibit TLRs are in progress.

A phase II clinical trial is currently testing the therapeutic efficacy of OPN-305, a monoclonal antibody that blocks TLR2, to dampen inflammation and prevent early dysfunction of extended-criteria-donor organs (donor age >60 years)[108]. Another clinical trial will assess the safety and tolerability of an anti-TLR4 antibody in healthy individuals[109]. The results of these studies will undoubtedly provide interesting insights as to the therapeutic potential of blocking specific TLRs to reduce inflammation after organ transplantation.

**Stimulation of resolution pathways.—**Tissues express a large array of evolutionary conserved cellular pathways that control tissue damage and counterbalance the deleterious effects of IRI[110]. Acute inflammation triggers inflammation resolution pathways that are required for tissue repair, including those regulated by haemoxygenase 1, heat shock proteins and nuclear factor erythroid 2-related factor 2 (NFE2-related factor 2, also known as NRF2)[111,112]. An experimental study found that heat shock preconditioning reduces damage after renal IRI through the expansion of regulatory T cells[113]. Hypoxia-inducible factor 1α (Hif-1α) is an oxygen-sensitive transcription factor that regulates tissue metabolism and angiogenesis[114] and its induction reduces infarct size after myocardial infarction in mice[115]. Hif-1α is also critical for ischaemia preconditioning in murine models of myocardial IRI[116]. These results have given rise to strategies to precondition allografts using drugs that enhance the activity of Hif-1α or by inducing minor and remote ischaemic insults[117]. Such graft preconditioning should induce protective mechanisms that enable a greater degree of tolerance to tissue damage[118]. To date, the majority of the approaches being investigated in clinical trials to reduce inflammation have employed remote ischaemic preconditioning. In organ transplantation, this procedure consists of three 5-min cycles of left lower limb ischaemia, induced by an automated cuff inflator placed on the limb and inflated to 300 mm Hg, with an intervening 5 min of reperfusion during which the cuff is deflated[119]. However, this approach failed to demonstrate beneficial effects on early graft function or rates of acute rejection in human kidney transplantation[119]. Other experimental approaches that directly target stress responses in the damaged organ by enhancing Hif-1α activity via small molecules such as L-mimosine[120], or promoting HO1 activity by exposing the graft to haeme arginate[121] have shown beneficial effects and have provided an impetus

for several ongoing clinical studies[122–124]. Patient safety data are already available for haeme arginate and this agent is now under investigation in a randomized controlled clinical trial in recipients of deceased donor kidneys[122].

## Future considerations and conclusions

Identification of the optimal strategy for blocking and managing TLR-mediated inflammation is bound to be a challenging process. The critical function of TLRs in innate immunity[1], the dual role of TLR-mediated inflammation in providing host protection and inducing damage, the almost ubiquitous expression of TLRs, often underpinning cell-type-specific effects[66], and our increasing, but vastly incomplete understanding of the roles of TLRs in health and disease, highlight some of the challenges involved in the control of TLR-regulated inflammation after transplantation. Several aspects of TLR inhibition will need to be clarified if therapeutic strategies are to be developed: cell specific versus general effects of TLR inhibition; the potential for off-target or adverse effects, especially infection; the optimal mode of delivery, which is particularly relevant for intracellular TLRs such as TLR9; the duration and stability of TLR blockade; and the potential for urgent reversibility in the event of an infection. Despite the challenges, the important roles of TLRs in inflammation after organ transplantation are undisputed and inhibiting inappropriate or excessive inflammatory responses by targeting TLRs, TLR ligands or the products of TLR activation, are attractive strategies that could reduce graft inflammation after solid organ transplantation. Additionally, therapeutic modulation of other inflammatory pathways, such as those regulated by the inflammasome, will require future experimental investigation.

## Acknowledgements

D.R.G.'s work is supported by NIH/NIA grant R01AG028082, S.J.C.'s work is supported by NHMRC Project Grant funding and S.B.'s work is supported by the CENTAURE foundation, the IHU-Cesti project, the French National Research Agency, and the Nantes Metropole and the Pays de la Loire Region.

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**Key points**

- Sterile inflammation occurs in organs after their surgical removal and implantation into a recipient
- Inflammation that occurs after solid organ transplantation can precipitate acute allograft rejection, impede transplant tolerance and enhance the development of chronic allograft rejection
- Experimental and clinical studies have shown that several endogenous substances, also known as damage associated molecular patterns contribute to both acute and chronic allograft rejection
- Toll-like receptors, which are among the best characterized innate immune receptors, induce inflammation and impair outcomes after solid organ transplantation
- Clinical studies are investigating strategies to inhibit innate immune responses after organ transplantation; approaches to reduce inflammation without compromising host defence to pathogens would substantially improve outcomes for transplant recipients

**Box 1 |**

## Effectors of the sterile inflammation response

### PAMPs and DAMPs

Toll-like receptors (TLRs) and other innate immune receptors are collectively termed pattern recognition receptors (PRRs) as they recognize molecular patterns. When such patterns are expressed by pathogens, they are termed pathogen-associated molecular patterns (PAMPs). For example, lipopolysaccharide is a major component of the cell wall of Gram-negative bacteria and is the classic PAMP ligand for TLR4. When patterns are derived from endogenous sources following tissue damage, they are termed damage-associated molecular patterns (DAMPs). For example, HMGB1, a nuclear protein that binds DNA and modulates transcription in healthy cells, is released into the extracellular environment in response to cell damage and acts as a ligand for TLR4 and TLR9.

### Toll-like receptors

TLRs are one of several classes of innate immune receptors that serve primarily as sentinels of the immune system and respond rapidly to the presence of non-self molecules, tissue damage or tissue stress[125]. They are highly conserved, germ-line encoded, transmembrane PRRs that are capable of recognizing PAMPs or DAMPs[66]. TLRs are characterized by a ligand-binding N-terminal leucine-rich repeats signalling domain, a transmembrane domain and an intracytoplasmic tail consisting of a Toll/IL-1R homology domain[66]. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 span the plasma membrane and are ideally suited to respond to ligands present within the extracellular environment. TLR3 and TLRs 7–10 span endolysosomal membranes and are thereby well suited to respond to ligands composed of nucleic acids derived from invading organisms or damaged cells that have been processed within the endolysosome[66].

**Sterile inflammation**

Inflammation that occurs following the necrosis-mediated release of activators of inflammation in medical conditions such as ischaemia–reperfusion injury, crystalline-induced arthritis, acute lung injury and chronic inflammatory conditions, without an identifiable infectious precipitant.

**Bronchiolitis obliterans syndrome**

A manifestation of chronic allograft rejection characterized by a decline in pulmonary function that affects more than half of lung recipients 5 years after transplantation and accounts for a considerable proportion of lung allograft losses and recipient deaths.

**Operationally tolerant recipients**

Subgroup of patients who spontaneously tolerate their graft and maintain allograft function without the use of immunosuppressants for at least 1 year, in the absence of deleterious responses.

**Restrictive allograft syndrome**

Newly described phenotype of chronic lung allograft dysfunction characterized by a persistent decline in vital and total lung capacities and allograft parenchymal fibrosis.

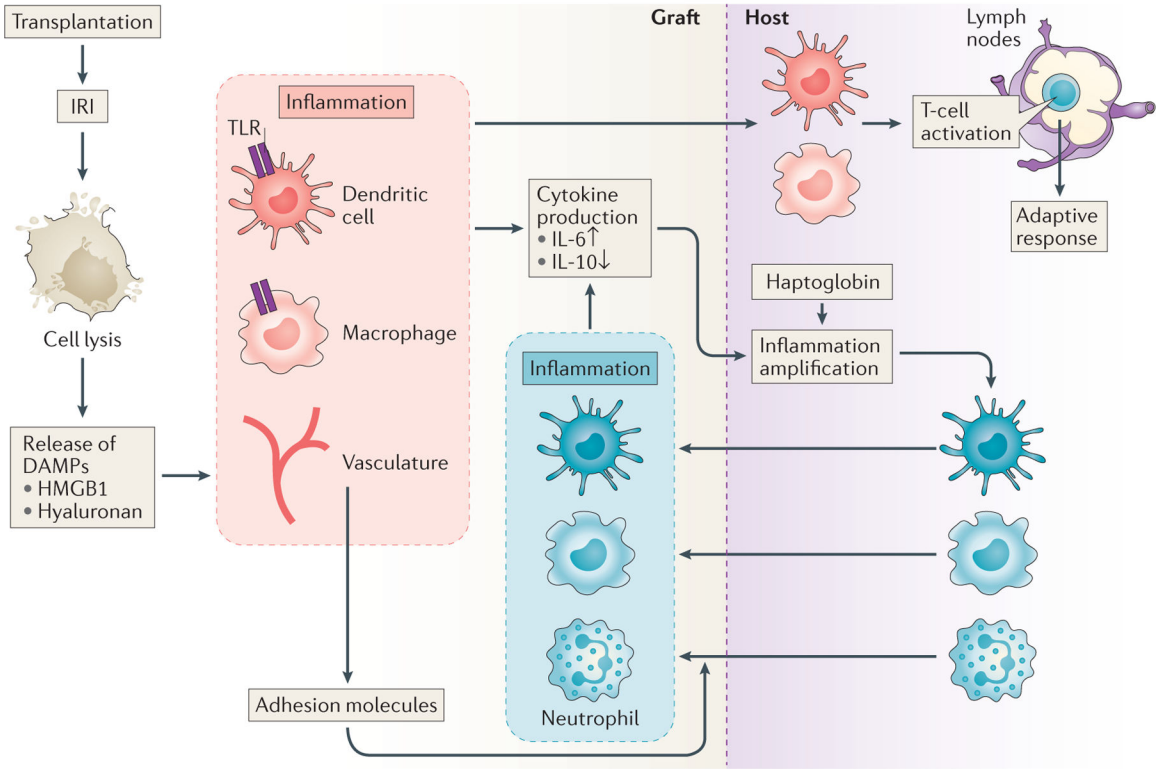


**Figure 1 |. Inflammation initiation and maintenance after organ transplantation.**

Ischaemia–reperfusion injury (IRI) induces a degree of graft cell necrosis and breakdown of the extracellular matrix. These processes lead to the release of damage-associated molecular patterns (DAMPs), which can heighten the intragraft inflammatory milieu by activating innate immune signalling pathways via the toll-like receptors (TLR). Graft cells (vascular endothelial cells, macrophages and dendritic cells) upregulate adhesion molecules (such as VCAM-1 and L-selectin) and co-stimulatory molecules (such as CD80). They also secrete inflammatory cytokines (such as IL-6 and TNF), which enhance immune cell recruitment into the graft and activate activate recipient cells to produce amplifiers of inflammation such as haptoglobin, which enter the circulation and amplify inflammation within the transplant. This increased inflammation further activates graft resident or infiltrated immune cells and induces their migration to the draining lymph nodes where they can activate naive anti-donor T cells to initiate adaptive anti-donor immune responses. The inflammatory reaction triggered by IRI can ultimately lead to graft rejection.

**Table 1 |**

DAMPs and TLRs in clinical solid organ transplantation

| Allograft | Evidence of involvement of DAMPs and TLRs |
|---|---|
| Kidney | • Transplant recipients show increased levels of HMGB1 and TLR4/2 in the blood 3 h after transplantation[74] |
| | • Recipients with loss-of function *Tlr4* alleles (Asp299Gly or Thr399Ile) have lower intragraft levels of proinflammatory cytokines (TNF and CCL2) and improved immediate graft function than patients with wild-type *Tlr4* alleles[74,81] |
| | • The long-term development of graft inflammation is associated with an upregulation of *TLR* expression in the graft and blood of transplant recipients[97,98] |
| | • S100 proteins enhance inflammation resolution after IRI[105,106] |
| Lung | • The expression of TLRs and other innate immune components (such as inflammasome and chemokines) is increased in BAL fluid and lung tissue biopsy samples after reperfusion[84] |
| | • Release of RAGE, a receptor for HMGB1, after graft implantation is predictive of a poor short-term outcome[92] |
| | • Release of DAMPs (HMGB1 and S100 protein family) occurs in patients with chronic lung allograft dysfunction[50,103] |
| Liver | • HMGB1 is a marker of liver injury early after transplantation[82] |
| | • Polymorphisms in the *Tlr4* allele are associated with higher rates of immediate graft function after liver transplantation[86] |
| Heart | • Expression of TLR4 on circulating monocytes associates with endothelial dysfunction within the allograft up to 3 years after transplantation without any evidence of chronic rejection[100] |

BAL, bronchoalveolar lavage; DAMP, damage-associated molecular pattern; TLR, Toll-like receptor.