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Toll-like receptors (TLRs) in transplantation

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1. ABSTRACT

TLRs have been extensively studied over the past decade for their ability to recognize microbial molecular patterns and activate innate immune cells to fight infections. They have also been described to provide a link between innate and adaptive immunity, as TLR signals also enhance the antigen presenting capacity of innate immune cells to T cells. In recent years, a contribution of TLR pathways to immune responses elicited by ischemia/reperfusion injury (IRI), allografts and xenografts has been uncovered, although the ligands that bind TLRs in these settings remain to be revealed. Such research has the potential to identify novel therapeutic targets that may facilitate allograft acceptance. In this review, we will summarize the results published to date on the role of TLRs in experimental and clinical transplantation.

2. INTRODUCTION

The Toll gene was first identified in drosophila in 1985, where it was initially shown to control embryonic dorsal-ventral polarity (1, 2) and later also found to encode for a transmembrane protein (3) capable, when engaged, to induce production of the anti-fungal agent drosomycin (4). A human homolog was described in 1997 (5). Since then, TLRs have been found to be highly conserved throughout vertebrate evolution.

TLRs are single-pass transmembrane proteins composed on an extracellular domain containing leucine-rich repeats responsible for recognition of distinct molecular patterns and an intracellular domain that resembles the IL-1R cytoplasmic tail (Toll/IL-1R or TIR domain) and is necessary for signaling (6). To date, 13

members have been identified in mice and 11 in humans. TLRs were first described as recognizing distinct microbial-associated molecular patterns expressed by bacteria, viruses or fungi, such as lipoproteins (TLR2), viral double-stranded RNA (TLR3), lipopolysaccharide (LPS) (TLR4), flagellin (TLR5), viral single-stranded RNA (TLR7) or unmethylated CpG oligonucleotides (TLR9). In addition to these exogenous ligands, an increasing number of putative endogenous ligands generated by cell stress or apoptosis have been reported to also bind and activate TLRs. These include fragments of extracellular matrices (fibronectin, heparin sulfate, hyaluronan), fibrinogen, surfactant protein-A, beta-defensin, heat shock proteins, and the high-mobility group box 1 protein (7-14). Most of these putative ligands appear to bind TLR2 and/or TLR4, although the effect of hyaluronan, for instance, was TIR-containing adaptor protein (TIRAP)-dependent but independent of TLR2, TLR4 or myeloid differentiation factor (MyD88) (14). Importantly, it has been difficult to absolutely exclude a contribution by known or unknown microbial contaminants in the ability of these stress/damage-induced molecular patterns to activate TLRs.

TLRs are expressed on cells of hematopoietic origin and their function has been best studied on antigen-presenting cells (APCs), but are also expressed on non-hematopoietically-derived cells such as epithelial cells and endothelial cells. Engagement of TLRs on innate immune cells results in cell signaling that depends on recruitment of different TIR domain-containing adaptor molecules (6). All known TLRs except for TLR3 utilize the adaptor protein MyD88. TLR2 and TLR 4 can signal via TIRAP in a MyD88-dependent manner and TLR4 and TLR3 can utilize TIR-containing adaptor protein inducing interferon (IFN)-beta (Trif) in a MyD88-independent manner. All TLRs activate the transcription factor NF-kappaB, whereas recruitment of Trif also induces activation of the transcription factor interferon regulatory factor (IRF)-3. TLR binding in macrophages and dendritic cells (DCs) promotes activation of innate immune responses, including enhancement of phagocytosis, upregulation of MHC and costimulatory molecules and production of chemokines and inflammatory cytokines such as IL-12, IL-6 and type I IFNs (15). These events in turn result in augmented antigen presentation to T cells and increased adaptive immune responses, such that TLRs provide a bridge between innate and adaptive immunity. Engagement of TLRs in conventional T cells was shown to promote T cell survival (16) and provide signals that costimulate T cells during TCR engagement (17), whereas ligation of TLRs in regulatory T cells (Tregs) has been reported to either enhance or inhibit their suppressive capacity (18-22). TLR binding on endothelial cells and pericytes appears to enhance vascular leakage (23, 24) whereas their engagement on epithelial cells may have anti-inflammatory or pro-inflammatory consequences, depending of the tissue targeted and the apical versus basolateral positioning of the TLRs (25, 26). All these cell types play important roles in defining immune responses to transplanted organs, predicting significant contributions of TLR signals in controlling transplant rejection and tolerance.

Solid organ transplantation implies a surgical cutaneous incision possibly causing low grade penetration of recipient commensal bacteria, surgical stress that can induce intestinal bacterial translocation, and re-anastomosis of an organ which itself may carry donor commensal bacteria as in the case of lung or intestinal transplantation. In addition, transplanted organs undergo ischemia- and reperfusion-mediated damage, possibly inducing expression of endogenous TLR ligands (27) . As such, donor and recipient TLRs may be engaged after transplantation both by microbial molecular patterns and stress/damage-associated endogenous ligands. In this review we will summarize current knowledge regarding the role of TLR signals in transplant responses *in vivo*.

3. TLRS IN TRANSPLANTATION

3.1. Role of TLRs in IRI

The dependency of adaptive immune responses on early innate immunity has lead to the hypothesis that allograft rejection may be initiated by injury sustained during the transplant process. This injury could arise during tissue harvest, cold-storage and transport and/or implantation and reperfusion of the organ.

Ischemia induces the expression of endothelial adhesion molecules, and the production of inflammatory chemokines and cytokines. There is increasing evidence that TLRs are involved in triggering of these events. Early studies by Tsoulfas and colleagues (28) demonstrated that reperfusion of transplanted syngeneic rat liver resulted in increased circulating LPS and elevated mRNA levels of hepatic CD14 and LPS-binding protein (LBP), proteins involved in LPS-mediated signaling. Subsequent observations of improved function and reduced histological damage and pro-inflammatory responses in TLR4-deficient livers following isogenic orthotopic liver transplantation, support a direct role for TLR4 in induction of early IRI (29).

TLR4, IRF-3 and type I IFNs have been shown to play critical roles in a warm liver IRI model (30-32). Unexpectedly, a critical role for T cells (33, 34), namely the $CXCR3^{+}CD4^{+}$ pre-activated T cell subset, has been identified in promoting IRI. As the ligand for CXCR3, IFN-gamma-inducible protein 10 (CXCL10), is produced in a TLR-4/IRF-3 dependent manner following IRI (32), these data collectively suggest that a complex series of events downstream of TLR-4 and type I IFNs mediate IRI.

TLRs have also been implicated in kidney and heart IRI. Ischemia markedly enhanced synthesis of TLR2 and TLR4 mRNA in the distal renal tubular epithelium, the thin limb of Henle's loop, and the collecting ducts, leading Wolfs and colleagues (35) to speculate that TLRs potentially represent a mechanism of increased immune-surveillance during inflammation at the site in which ascending bacteria enter the kidney tissue. More recently, Shigeoka and colleagues (27) reported that mice lacking expression of TLR2, MyD88 and/or Trif were protected against sublethal renal ischemia. Likewise, TLR4-deficient mice were reported to sustain smaller infarctions and

exhibit less inflammation after myocardial ischemia-reperfusion injury (36).

The ligands that activate the TLR system during IRI have not been identified. Current data suggest that both pathogen-, as well as stress/damage-associated molecular patterns, may elicit IRI via the TLR signaling pathway. Alternatively, endogenous ligands may function independently of TLR engagement, but synergistically with microbial TLR agonists to induce IRI. Uric acid is an example of a stress/damage-dependent molecule that signals in a MyD88-dependent manner, but via the IL-1R rather than via TLR engagement (37).

3.2. Function of TLRs in acute rejection of solid organ allografts

The role of TLR signals in acute allograft rejection was first described by Goldstein and colleagues using MyD88-deficient mice in a minor mismatch (male into female) skin transplantation model (38). The authors demonstrated that lack of donor and recipient MyD88 was sufficient to enable permanent skin allograft acceptance, although expression of MyD88 in either the donor or the recipient was sufficient to promote acute rejection. Importantly, a role for IL-1 or IL-18 that can also activate the MyD88 pathway was excluded, as mice deficient in interleukin (IL)-1beta-converting enzyme and caspase 1 (ICE-deficient) rejected skin allografts normally. Absence of rejection in MyD88-deficient mice was correlated with a reduction in the presence of mature DCs in draining lymph nodes (dLNs), although whether this was due to reduced migration of donor DCs and/or reduced maturation could not be distinguished. Nevertheless, these alterations correlated with diminished Th1 differentiation, as inferred from decreased IFN-gamma production upon allogeneic restimulation. Transfer of wildtype antigen presenting cells (APCs) was sufficient to restore rejection underscoring the importance of TLR signals for APC maturation and subsequent T cell activation and differentiation. In contrast, lack of TLR2 or TLR4 signaling was not sufficient to prevent allograft rejection (38, 39).

Unlike the minor mismatch model, MyD88 deficiency in both donor and recipient was not enough to prolong survival of fully allogeneic skin or cardiac allografts despite reduced numbers of mature DCs in dLNs and reduced Th1 differentiation (40). To determine if rejection of fully allogeneic grafts in MyD88-deficient mice was due to the remaining Trif-mediated TLR signaling, McKay and colleagues utilized mice with a combined deficiency in MyD88 and Trif (41). Ablation of both adaptors in donor animals indeed resulted in a modestly prolonged survival of fully allogeneic skin grafts in wildtype recipients despite competent MyD88 and Trif signaling by recipient cells. It remains to be determined whether ablation of both adaptors simultaneously in recipient and donor mice would result in permanent acceptance of fully allogeneic transplants. Of interest, prolongation of survival of MyD88/Trif-double deficient skin was correlated with reduced migration of donor DCs to dLNs, indicating that TLR signals control APC movement.

Collectively, these results emphasize the importance of donor and recipient TLR-mediated signals in priming allogeneic immune responses. Whether TLRs are engaged by endogenous or exogenous ligands in this setting remains to be established.

3.3. Role of TLRs in graft *versus* host disease (GVHD)

Like acute allograft rejection, GVHD is known to be driven by T cells although, in this case, of donor rather than of recipient origin. These donor T cells infiltrate target organs such as skin, liver, intestine and lung causing a sometimes fatal disease. A case for TLR-dependent signals in facilitating GVHD has been made. Whereas mature donor T cells cause GVHD in allogeneic recipients, they fail to cause GVHD in established mixed chimeric mice containing both recipient and donor hematopoietic cells despite efficiently eradicating recipient hematopoietic cells. Absence of GVHD in this setting has recently been ascribed to a failure of donor T cells to migrate into target organs (42). However, topical administration of the TLR7 synthetic ligand Imiquimod to one flank resulted in unilateral lymphocytic infiltration to the treated skin side, indicating that local inflammation can drive T cell migration into target tissues (42). Similarly, in a rat model of fully allogeneic bone marrow transplantation that does not normally mediate GVHD, inhalation of the TLR4 agonist LPS resulted in pulmonary pathology resembling lymphocytic bronchiolitis, further supporting the idea that local inflammatory factors may contribute to specific organ targeting in GVHD (43).

3.4. TLRs in acute xenograft rejection

The role of TLR signaling in promoting acute xenograft rejection has also been investigated. Similarly to results obtained in fully allogeneic transplant models, absence of MyD88 in recipient mice did not result in prolonged survival of fetal porcine islet-like cell clusters, although reduced graft IFN-gamma mRNA was observed (44). Porcine xenografts have also been shown to induce higher upregulation of TLR mRNA levels in murine macrophages than allografts (45). Although MyD88-deficient murine macrophages display reduced activation than wildtype counterparts upon exposure to porcine xenografts, this defect does not impact acute xenograft rejection (46). As for allografts, it remains possible that complete ablation of TLR signaling in both donor and recipient tissues may prevent acute xenograft rejection.

3.5. Role of TLRs in chronic allograft rejection

Vascular lesions in organs undergoing chronic rejection resemble those in atherosclerosis. Because TLR signals have been strongly associated with development of atherosclerotic plaques (47, 48), it is hypothesized that the TLR pathway may also contribute to chronic allograft rejection. However, little is known of the role of TLR signaling in chronic rejection. In mice immunosuppressed with a combination of anti-CD4 and anti-CD8 monoclonal antibodies (mAbs) that resulted in prolonged cardiac allograft survival with grafts showing modest signs of chronic rejection, elevated levels of TLR4 mRNA were found when compared with syngeneic grafts (49). Similarly, in patients with evidence of allograft endothelial

dysfunction after cardiac transplantation, mRNA transcripts for TLR4, protein expression for TLR4 and CD80 on circulating monocytes and secretion of IL-12 and TNF, all target genes downstream of TLR signaling, were found to be at higher levels than in graft recipients devoid of endothelial dysfunction (49). Together, these results outline a correlation between the TLR4 signaling pathway and the development of chronic rejection, although causality remains to be demonstrated.

3.6. Effect of TLR signals on transplantation tolerance

As TLR signals were shown to promote allograft rejection, it became plausible that these pathways may also antagonize attempts at inducing transplantation tolerance. We have hypothesized that the reduced susceptibility of skin, intestine and lung allografts to transplant acceptance in experimental models as well as in patients may be due to increased introduction in the recipient of donor commensal bacteria from these colonized organs when compared with sterile organs such as heart or kidney, resulting in increased TLR signaling (50). This hypothesis is compatible with results obtained by Goldstein and colleagues as well as by our group showing that elimination of donor and recipient MyD88 in a fully allogeneic skin graft model results in the ability of costimulation-targeting therapies (anti-CD154+CTLA-4-Ig) to induce permanent allograft survival (50, 51). This was ascribed to increased susceptibility of T cells to Treg-mediated suppression (51). Conversely, our hypothesis also predicts that deliberate activation of TLR signals at the time of transplantation would prevent the induction of tolerance to sterile organs that are otherwise readily accepted. Consistent with this idea, we have shown that administration of the TLR9 agonist CpG, or of the TLR2 ligand Pam_3Cys, is sufficient to prevent acceptance of fully allogeneic cardiac allografts in mice treated with anti-CD154+donor-specific transfusion (DST) (50). Of interest, CpG-mediated rejection in this setting was correlated with reduced intra-graft expression of the chemokines CCL17 and CCL22 that can attract CCR4-expressing Tregs, and with decreased recruitment of FoxP3-expressing Tregs (50). Thus, in addition to promoting DC maturation and Th1 differentiation that are likely to facilitate allograft rejection, TLR signals appear to also control Treg migration and ultimately the intra-graft ratio of effector T cells to Tregs that may determine allograft fate.

In addition to preventing tolerance of sterile organs, TLR signaling in the form of administration of CpG, Poly I:C (a TLR3 agonist), LPS or Pam_3Cys can also prevent acceptance of skin transplants in fully allogeneic recipients treated with a regimen (anti-CD154+DST) strong enough to induce acceptance of this bacteria-colonized organ (52). In this model, anti-CD154/DST-mediated acceptance is due to deletion of alloreactive T cells. Injection of TLR agonists was shown to prevent apoptosis of graft-specific T cells (52), indicating another mechanism by which TLR signals can promote allograft rejection. More recently, the same group has shown that the pro-rejection effect of LPS in this model is TLR4- and MyD88-dependent, whereas that of Poly I:C is surprisingly TLR3-independent (53). Interestingly, rejection in both cases is due to secretion of type I IFN which is necessary and sufficient to prevent skin allograft acceptance, pointing to type I IFNs as potential therapeutic targets for achieving transplantation tolerance.

Indefinite allograft acceptance induced by a combination of cyclosporine and serpin-1 in a rat model of cardiac allograft has been correlated with diminished intra-graft expression of TLR2, TLR4 and MyD88 48h after transplantation, thus further correlating allograft acceptance with, presumably, lack of TLR signaling (54).

The TLR pathway has also been invoked in the increased susceptibility of neonates to transplantation tolerance, as neonatal B cells have recently been shown to prevent TLR-mediated DC maturation via secretion of IL-10. Moreover, TLR-activated neonatal B cells reduced Th1, but not Th2, alloresponses *in vitro* and *in vivo* (55).

Collectively, these studies identify several distinct effector pathways by which TLR signals can oppose graft acceptance, i.e. promotion of Th1 differentiation, antagonism of alloreactive T cell deletion and inhibition of intra-graft Treg recruitment. As such, they point to the danger of infections in transplanted patients, as these may reduce the capacity of immunosuppressive drugs to prevent acute rejection. This is in keeping with experimental observations of viral and parasitic infections preventing transplantation tolerance in mice (56-58), as well as with observations of viral and bacterial infections precipitating acute rejection episodes in transplanted patients (59-64).

3.7. Human TLR expression and polymorphisms in transplantation

The importance of TLR signaling in human transplantation is also emerging. Induction of mRNA expression for various TLRs was identified in biopsies from transplanted lungs collected after reperfusion, with expression of TLR4 mRNA correlating with that of IL-8 (65). Interestingly, mRNA expression of the putative endogenous TLR ligand HSP70 was also significantly induced by reperfusion (65). Thus, ligands and receptors are present in human allografts.

Polymorphisms in TLRs have been described that correlate with the fate of transplanted organs in humans. First, recipients exhibiting TLR4 Asp299Gly and Thr399Ile, two polymorphisms associated with LPS hyporesponsiveness, were found to have reduced rates of acute rejection compare to control recipients within the first 6 months post-transplantation, whereas donor polymorphisms did not affect transplant outcome (66). Reduced incidence of acute rejection was confirmed by the same group when observations were extended to the first 3 years after transplantation (67). A trend towards reduced severity of bronchiolitis obliterans syndrome (BOS), the clinical manifestation of chronic lung allograft rejection, was also noted (67). More recently, the effects of polymorphism 159TT in CD14, a molecule that associates with TLR4 for LPS recognition, have been investigated in lung transplant recipients. This polymorphism results in

increased transcription of CD14 and was found to correlate with earlier onset of acute lung allograft rejection, BOS and graft loss and with increased levels of circulating soluble CD14, TNF and IFN-gamma, suggesting enhanced immune responses in these recipients (68). Collectively, these data suggest that productive activation of TLR4 may contribute to acute and perhaps chronic rejection of lung allografts.

Similar results have been obtained for renal allografts, although the contribution of donor *versus* recipient TLR4 polymorphisms differs between studies. In one report, recipient TLR4 polymorphisms correlated with reduced acute rejection and atherosclerotic events (69). As expected from decreased TLR4 signaling, these patients also experienced more frequent severe bacterial and opportunistic infections (69). Improved renal allograft survival in recipients bearing the TLR4 Asp299Gly was confirmed by Fekete and colleagues (70). Interestingly, a polymorphism in a putative endogenous TLR ligand induced by IRI (*HSPA1B 1267AA*) was also reported more frequently in long-term acceptors of renal allografts (70). In a subsequent study, only donor but not recipient TLR4 polymorphisms were associated with increased incidence of acute rejection (71). Despite the slight discrepancies, these results further support a contribution for TLR4 signaling in human allograft rejection.

A similar study has been conducted in liver allograft recipients transplanted for hepatitis C-induced liver failure, although allograft outcome results are seemingly opposite to those in lung and kidney allograft recipients. Hepatitis C virus is known to signal via TLR2 and TLR4. Although the TLR4 polymorphisms described above did not influence liver transplant outcome, patients homozygous for the TLR2 polymorphisms Arg753Gln all developed post-transplant cirrhosis, required re-transplantation and died (72). Like those in TLR4, this TLR2 polymorphism is known to result in defective intracellular signaling and impaired cytokine secretion in response to its ligands (72), but promoted impaired, rather than improved, graft survival. These results may be interpreted to reflect reduced anti-hepatitis virus immunity, rather than increased alloimmunity, and may therefore be consistent with the increased incidence of severe bacterial infections observed in patients harboring the TLR4 polymorphisms (69).

In bone marrow transplant recipients, TLR4 polymorphisms resulting in LPS hyporesponsiveness were also correlated with a trend towards reduced risk of acute GVHD, but increased risk for gram-negative bacteremia (73), although a more recent study found increased risk of severe acute GVHD when both donors and recipients expressed the TLR4 Thr399Ile mutation (74) leaving the role of TLR4 in GVHD unresolved.

4. SUMMARY AND PERSPECTIVE

In conclusion, TLR-mediated signals are now known to play a role in acute allograft rejection and can also actively prevent the induction of transplantation tolerance by costimulation-targeting therapies. Some evidence implicates TLR signals also in xenograft rejection, GVHD and the development of chronic rejection. Furthermore, genetic analyses of the effects of TLR polymorphisms on the outcome of transplanted grafts support their importance in the clinic. When mechanisms have been investigated, the pro-rejection effects of TLRs have been ascribed to prevention of deletion of alloreactive T cells, promotion of Th1 differentiation, reduced intra-graft migration of Tregs and secretion of type I IFNs that themselves trigger multiple immune consequences (75). The association of loss-of-function TLR polymorphisms with increased severity of bacterial infections in patients cautions against long-term targeting of TLR pathways as a therapeutic tool to prevent transplant rejection or facilitate transplantation tolerance. However, transient targeting of TLR pathways or of some of their target gene products such as type I IFNs may deserve consideration to promote graft acceptance.

Infections are common in the peri-operative period of transplant recipients as well as at later time points due to their immunocompromised state. The ability of infections to precipitate episodes of acute rejection in the clinic may therefore in some instances be dependent on TLR engagement by microbial patterns. Our current unpublished results in mouse models of skin and cardiac transplantation indicate that although several single TLR ligands can prevent the induction of transplantation tolerance, they fail to break established tolerance when administered to stable allograft recipients. It remains to be established whether combinations of TLR agonists with different signaling properties or of TLR signaling with non-TLR pathways activated by microbial patterns such as the Nod family or other microbial sensing receptors can reverse transplantation tolerance. Establishing such models would be highly relevant clinically and may lead to the development of new therapies that reduce alloreactivity during infectious episodes without compromising anti-microbial defenses.

Finally, the ligands that bind to TLRs during transplant responses remain to be identified. It is possible, as we have hypothesized, that the higher immunogenicity and resistance to graft acceptance elicited by organs colonized with commensal bacteria is due to the ability of translocated donor bacteria to enhance alloimmune responses via TLR-dependent and -independent mechanisms. Conversely, it may be endogenous TLR ligands induced by cellular damage, and not microbial patterns, that augment alloimmunity after transplantation and, of course, these hypotheses are not mutually exclusive.

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Abbreviations: TLR: Toll-like receptor; MyD88: myeloid differentiation factor 88; IL: interleukin; IFN: interferon; IRI: ischemia/reperfusion injury; APC: antigen-presenting cell; DC: dendritic cell; dLN: draining lymph node; GVHD: graft *versus* host disease; BOS: bronchiolitis obliterans syndrome.

Key Words: TLR, Transplantation, Allograft, Xenograft, GVHD, Acute Rejection, Chronic Rejection, Tolerance, Review

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