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REVIEW

The role of complement and Toll-like receptors in organ transplantation

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Summary

The innate immune system not only participates in host defence but also contributes to the control of adaptive immune responses. Complement and Toll-like receptors (TLR) are key components of innate immunity. Emerging evidence suggests their activation is involved in all major aspects of transplantation. This paper reviews the current understanding of how the complement and TLR on impact transplant injury.

Introduction

Metazoan species use at least two recognition strategies to sense and defeat pathogens [1]. The pattern-recognition strategy is based on the recognition of conserved molecular patterns that are shared by large group of pathogens and not subject to antigenic variability. Moreover, the 'missing self' strategy is based on the detection of molecular markers specific for self and absent from the pathogens or any other foreign entity. Recognition of these markers (also known as self-associated molecular patterns) by soluble and membrane receptors is associated with inhibiting innate immune response [2]. The targets of pattern recognition are detected by pattern recognition receptors (PRRs), including Toll-like receptor (TLR) family and complement. The complement system also involves the 'missing self' strategy. Pathogens generally lack membrane complement regulatory proteins, which allows activation of complement, leading to cell lysis or phagocytosis [3,4].

In addition to contributing to the first line of defence against microbial infection, both complement and TLRs have been found to be essential for bridging the gap between innate and adaptive immunity [5,6]. In the field of transplantation, previous research on graft rejection has largely focused on T-cell-mediated immunity. However, mounting evidence indicates that innate immunity plays an important role in all the major aspects of transplantation. In this review, we will discuss recent advances in our understanding of how the complement and TLRs participate in organ transplant injury.

Complement

The complement system includes over 30 components, regulators, and receptors that interact in a sequential manner to participate in host defence [7,8]. There are three pathways of complement activation, all of which generate C3 convertases that cleave C3 to C3a and C3b. In turn, C3b and C5b lead to formation of the membrane attack complex (MAC; C5b-9), which results in activation of granulocytes, endothelia and epithelia or, at higher concentration, to cell lysis. C3a and C5a have traditionally been considered as anaphylatoxins that act on specific

receptors to produce local inflammatory responses. Recent data have shown they were also able to either enhance or suppress T-cell immunity [9–11]. In addition to being a central part of complement activation, complement C3 provides a vital link between innate and adaptive functions of the immune system [5,12]. Interest in the role of complement as a regulator of the alloimmune response has focused on C3.

Complement and ischaemia/reperfusion injury

It has been established that nonimmunological injury, such as brain death and ischaemia/reperfusion (I/R) are important factors affecting allograft survival. Early evidence emerged from a clinical trial by Land *et al.* in 1994 [13,14]. Intra-operative treatment of postischaemic reperfusion injury significantly reduced the incidence of acute rejection and improved the long-term graft outcome. Further evidence came from a large study of 27 096 kidney transplant recipients, which showed that prolonged cold ischaemia and its manifestation as delayed graft function are associated with lower short-term and long-term graft survival rates [15]. Complement is one of a number of inflammatory mediators that participate in I/R injury.

Complement activation after ischaemia and reperfusion causes vascular and parenchymal cell injury. The precise mechanism may vary from one organ to another. In the heart, gut and muscle, the main lesion of complement-mediated injury is small vessel thrombosis, which is associated with vessel wall inflammatory cell infiltration and direct endothelial membrane injury [16–18]. In contrast, renal postischaemic injury appears to cause primary damage of the renal tubules, those worst affected being in the hypoxia-sensitive region of the corticomedullary junction [19].

The mechanism of action of complement leading to I/R injury has already been investigated in detail. Although recruited neutrophils play a part in postischaemic renal failure, there was no demonstrable role of leucocyte production of C3 [20,21]. MAC appears to play a major role [22]. The anaphylatoxin C5a has also been documented to contribute to the pathogenesis of ischaemic renal failure [23]. The products of C3 cleavage seem to have little direct importance for tubular injury. Rather, the main function of C3 activation is to drive the formation of C5b-9.

Previous study indicated that the lectin pathway and classical pathway triggers complement-mediated I/R injury in the heart, intestine and skeletal muscle [24–27]. In contrast, complement activation after renal I/R occurs via the alternative pathway. Renal I/R injury proceeds in the absence of both C4 and Ig, indicating that neither the classical nor the lectin pathways are involved [22,28]. Thurman *et al.* [29] found that factor B-deficient mice strongly resisted renal I/R injury, confirming the alternative pathway is the main driver of complement activation after renal ischaemia and reperfusion.

While most of the circulating C3 is produced by hepatic synthesis, smaller amounts are generated at extrahepatic sites [30]. Local sources include epithelial cells, endothelial cells, macrophages and neutrophils [31–34]. In the postischaemic kidney, tubular epithelium is the most relevant site [22,35]. Although it is known that C3 mRNA is increased in ischaemic kidney [36], the relative contribution of local and systemic C3 in the pathogenesis was not clarified until very recently. Using a kidney transplant model, Farrar *et al.* [37] demonstrated that intrarenal synthesis of C3 is governed by the duration of ischaemia and the reperfusion time. Transplanted ischaemic C3-positive C57BL/6 kidney in syngeneic C3-positive or C3-negative recipients developed widespread tissue damage and acute renal failure. In contrast, ischaemic C3-negative grafts exhibited only mild injury even when transplanted into C3-positive recipients. Therefore, these data clearly show that local synthesis of C3 is essential for complement-mediated injury of renal I/R, while circulating C3 is dispensable in this model.

Under normal circumstances, the complement system is tightly controlled by membrane-bound and fluid-phase regulatory proteins. Several inhibitors are present within the mouse kidney, such as CD55 (DAF), CD59 and, in rodents, complement receptor 1-related protein y (Crry). Deficiency of CD55 or/and CD59 results in complement activation in the peritubular capillaries after I/R injury leading to severe damage [38,39]. However, in normal mice complement activation after I/R occurs along the tubular basement membrane. And Crry, one of the major inhibitors of C3 activation, is the main complement regulatory protein expressed on mouse tubular epithelial cells [40]. Most recently, Thurman *et al.* [41] have shown that Crry shifted away from the basolateral membrane and into the cytoplasm after 24 min of ischaemia, which subsequently resulted in extensive activation of complement on the tubules. Furthermore, mice expressing lower levels of Crry ($Crry^{+/-}$ mice) were more sensitive to ischaemic injury. Therefore, a factor contributing to alternative pathway of complement activation after I/R injury appeared to be altered expression of Crry within the tubular epithelial cells.

Complement and allograft rejection

Evidence that complement is a controller of adaptive immunity can be traced back at least three decades. The early observation by Nussenzweig *et al.* that B lymphocytes bound complement C3 suggested that the

complement system might be involved in adaptive immune responses [42]. Subsequently, Pepys [43] found that antibody responses to thymus-dependent and thymus-independent antigens were deficient in C3-depleted mice. Recently, accumulating experimental and clinical data support the notion that complement components are important regulators of T-cell function. As there have been many excellent reviews on the role of complement in the B-cell response [44], here we are concentrating on its importance as an effector of T-cell immunity.

Impaired T-cell responses in C3-deficient or -depleted mice were reported in several disease models including infection, asthma and autoimmune disease [45–47]. For kidney transplantation, it seems that local synthesis of complement is essential for regulating allograft rejection. Rejecting allograft undergoes upregulation of C3 mRNA expression, implying that local production of C3 is involved in alloimmune responses [48–50]. In a mouse kidney transplant model, most B10.Br recipients could not reject C3-deficient C57BL/6 donor kidneys within 100 days, whereas WT C57BL/6 grafts were rejected within 14 days [51]. This finding has been confirmed recently in another recipient strain (BALB/c) (T Lin, CA Farrar, W Zhou, SH Sacks, unpublished data). In humans kidney transplant, it has been shown donor C3 is able to affect the long-term graft survival [52]. Human C3 exists as two main allotypes, F (fast) and S (slow). C3 allotypes of 662 pairs of adult kidney donors and recipients were determined and then the relationship between C3 polymorphism status and grafts outcome data analysed. The results showed that graft survival and function was significantly better with a C3F/F or C3F/S donor allotype than a C3S/S allotype.

How local complement regulates the anti-donor T-cell response is at present unclear. A recent study highlighted the role of dendritic cell (DC) synthesized C3 [53]. Compared with C3 sufficient DCs, C3 deficient DCs displayed reduced surface expression of major histocompatibility complex (MHC)-II and B7.2. Furthermore, C3 deficient DCs elicited impaired alloreactive T-cell responses *in vitro* and *in vivo*, favouring the polarization of $CD4^+$ T cells toward Th2 phenotype. Priming mice with C3 deficient DCs led to delayed skin allograft rejection compared with C3 sufficient DCs. It therefore seems DC synthesis of C3 is crucial for alloreactive T-cell responses. However, it may not be the sole explanation. As distinct from the belief that T -cell priming is exclusively a local lymph node event [54], recent evidence suggested that allorecognition may occur in the graft itself [55]. Moreover, *in vitro* experimentation has shown that proximal tubular epithelial cells (PTEC) were able to stimulate antigen-experienced alloreactive T cells. The response was enhanced when C3 deposited on PTEC [56]. Given the fact that the tubular epithelium is the main source of intrarenal C3 [48], these data imply that C3-producing parenchymal cells may focus the attention of alloreactive T cells. Another possibility is the effect of local complement mediated inflammation. It is now well recognized that DCs are essential both in the induction of antigen-specific immune responses and in the maintenance of tolerance [57]. Inflammation controls the balance between induction of immunity and tolerance [58]. As C3a and C5a are important proinflammatory factors, absence of C3 could reduce the local inflammatory responses, and consequently impair DC maturation and migration. Taken together, it is clear that local synthesis of C3 plays a vital role in acute kidney allograft rejection. However, the relative contribution of parenchyma and DCs is obscure. A transplant model in which C3 is only produced by DCs or parenchymal cells is needed to address this question.

Conversely, several works showed that C3 is associated with inducing tolerance. Induction of antigen-specific tolerance after intraocular injection is dependent on the ligation of iC3b to complement receptor type 3 on antigen-presenting cells (APCs) [59]. *In vitro* experiments showed that CD46, a regulatory protein controlling C3 activation, is able to induce $CD4^+$ T cells to a T-regulatory phenotype [60]. In transplantation, studies of the immune mechanism of rat liver transplant tolerance suggested that C3 might be linked to tolerogenic function. Fujino *et al.* [61] demonstrated that the C3 gene is up-regulated in peripheral blood lymphocytes from Lewis rat liver transplant recipients that had been induced to accept PVG livers. Pan *et al.* [62] documented that spontaneously tolerant DA to PVG rat liver transplant recipients had increased level of C3 protein in the serum. Of note in both studies, only late-stage expression of C3 gene (100 days after transplant) or protein (60 days) was measured. Cordoba *et al.* [63] used microarray analysis to determine the early changes in gene expression in the spleen of liver transplant recipients. Twenty-four hours after transplantation, C3 gene was up-regulated in the tolerant recipient compared with the rejecting one. These observations suggest variation of regulatory mechanism in different organs.

As indicated earlier, intrarenal production of C3 was essential for graft rejection, but which of the three main activation pathways of complement trigger this response is unclear. This is important because selective inhibition might allow limited therapeutic blockade without disrupting all the complement pathways vital to host defence. One study assessed the role of the classical and lectin pathways by investigating the common component C4 in mouse kidney transplant rejection [64]. In three donor-recipient strain combinations, allograft survival was independent of the presence of C4 in either the donor kidney or recipient mouse. In addition, tubular deposition of C3 to C9 occurred regardless of the absence or presence of

C4. These data suggest that complement activation and renal allograft rejection are independent of the classical and lectin pathways in these models, implying the alternative pathway is the main trigger for complement-mediated rejection.

Decay-accelerating factor (DAF; CD55) is a glycosylphosphatidylinositol-anchored membrane inhibitor of complement whose function is to dissociate C3 and C5 convertases in both the classical and alternative pathways [65,66]. Transgenic pigs expressing human decay accelerating factor have been widely used as donors in various nonhuman primate transplant models [67–69]. A recent intriguing study suggested that DAF also has the potential to regulate alloimmune T-cell responses [70]. Transplantation of $DAF^{-/-}$ females with $DAF^{-/-}$ male skin grafts led to increased frequency of anti-HY CD4 and CD8 T cells, when compared with control $DAF^{+/+}$ females engrafted with $DAF^{+/+}$ male skin. Similarly, in a heart allograft model, alloreactive T cells in recipient mice primed much higher frequencies to allogeneic $DAF^{-/-}$ grafts than that to $DAF^{+/+}$ transplants. Moreover, the absence of DAF on antigen-presenting cells also enhanced the T-cell proliferation and augmented the induced frequency of effector cells. These effects were largely dependent on local complement activation. Thus, hypo-reactivity of T cells can result from impaired complement activation, while T-cell hyper-responsiveness is a feature of overactivity of complement.

Toll-like receptors

The TLR family is one of the best characterized classes of PRRs in mammalian species. To date, 11 family members have been identified in the mammalian system [71]. TLRs 1, 2, 4, 5 and 6 are expressed on the cell surface and seem to be important for the recognition of bacterial products. In contrast, TLRs 3, 7, 8 and 9 are contained within intracellular compartments and specialize in viral recognition by detecting nucleic acids [3].

Toll-like receptors are expressed in a variety of cell types including antigen presenting cells, epithelial cells and endothelial cells, as well as in leucocytes like neutrophils, mast cells, basophils and eosinophils [3]. Activation of TLRs in these subsets contributes differently to host defences, such as up-regulation of selectins and chemokines, leucocyte recruitment and activation, and naïve T-cell priming [3,72,73].

TLR and Ischaemia/reperfusion injury

Toll-like receptor represent the host sentinel system responsive to infections by recognizing bacterial/viral-specific pathogen-associated molecular patterns. In addition, studies have shown that TLRs can be activated by endogenous ligands such as heat shock protein, heparan sulfate, surfactant and fibrinogen [74–77]. In noninfectious settings, such as I/R injury, endogenous ligands from damaged/stressed cells have the capability to active the TLRs. Activation of TLRs-bearing cells triggers the release of proinflammatory cytokines and chemokines and recruitment of macrophages, neutrophils and T cells, leading to a full-scale I/R injury.

Experimental data have shown the selective functional usage of TLRs during I/R injury in different organs. A murine model of myocardial I/R injury showed that TLR4-deficient mice had smaller infarctions and exhibited less inflammation after myocardial reperfusion injury [78]. Whereas, TLR-2 has been shown to involve in cardiac remodelling after myocardial infarction [79]. From studies in knockout mice, Zhai *et al.* [80] found that TLR4, but not TLR2, was required in initiating the I/R injury cascade, as reflected by liver function, pathology and local induction of proinflammatory cytokines/chemokines. The I/R-induced TLR4 activation was mediated by interferon (IFN) regulatory factor 3, but not myeloid differentiation factor 88 (MyD88). As TLR4 is expressed on both hepatocytes and nonparenchymal cells (NPC), Tsung *et al.* [81] examined the contribution of these cell types to the outcome of liver I/R injury. Chimeric mice were produced by adoptive transfer of donor bone marrow cells into irradiated recipient animals using combinations of TLR4 wild type (WT) and TLR4-/- mice. TLR4 WT mice that underwent adoptive transfer with TLR4-/- bone marrow cells were protected from liver I/R compared with WT/WT mice. In contrast, serum ALT levels in TLR4-/- mice transferred with TLR4 WT bone marrow cells remained comparable with those of WT/WT controls. These results suggested that TLR4 expressed on NPC plays a crucial role in the induction of liver I/R injury. In renal I/R injury, however, the key player seems to be TLR2 which is mainly expressed by tubular cells. Leemans *et al.* [82] found that TLR2 plays a proinflammatory role *in vivo* after renal I/R injury, as manifested by reduced cytokine and chemokine production as well as reduced leucocyte infiltration in TLR2-/- mice when compared with TLR2 WT animals. Using chimeric mice, they demonstrated that TLR2 expressed on the renal parenchyma plays a primary role in the early induction of inflammation and I/R injury. These results provide valuable information for designing organ-specific therapeutic strategies to ameliorate I/R injury in the clinic.

TLRs and allograft rejection

Research into the role of TLRs in transplant rejection is at an early stage. As the interaction between DCs and

T cells is central to transplant rejection, the focus of experimental study in this area has been directed at DCs. TLR activation on DCs initiates a signalling pathway via their signal adaptor protein, inducing translocation of NF-κB and ultimately leading to DC maturation. The maturation is associated with increased expression of co-stimulatory molecules and secretion of proinflammatory cytokines [73,83]. Subsequently, DCs migrate to the draining lymph nodes and initiate an immune response by activating naïve T cells. This migration is mediated by TLR-induced down-regulation of receptors for inflammatory chemokines and upregulation of lymphoid chemokine receptors, especially CCR7 [84,85].

In the setting of transplantation, one unsolved issue is what ligands are crucial for TLRs to initiate alloreactive responses. The evidence that heat shock protein is upregulated during allograft rejection raised the possibility that TLRs may be involved in alloimmune responses [86]. MyD88 is an adaptor protein shared by all TLRs except TLR 3. Using a skin transplantation model, Goldstein *et al.* [87] documented that minor mismatched (HY-mismatched) allograft rejection does not occurred in MyD88$^{-/-}$ mice. In the absence of MyD88, male MyD88$^{-/-}$ male skin grafts transplanted to female MyD88$^{-/-}$ recipients survived more than 100 days, whereas the WT littermate rejected their grafts by day 25 after transplantation. Further experiments confirmed that the abrogation of graft rejection in the absence of MyD88 resulted from lack of DC maturation, leading to attenuate the generation of anti-donor specific T cells and impaired Th1 immunity. This is the first study that provided key evidence that TLRs are able to control adaptive immunity in rejection of minor MHC-mismatched tissue grafts.

To determine whether MyD88 plays a similar role in rejection of major MHC-mismatched allografts, the same group studied the rejection of skin and cardiac grafts in mice [88]. They showed when MyD88 was absent from the recipient alone or from both recipient and donor, the allografts were rejected without significant delay compared with WT controls. The number of matured DCs in the draining lymph node was reduced. In addition, the ability of DCs to prime naïve T cells and Th1 immune responses were significantly diminished, although Th2 immunity remained untouched.

Why MyD88 is crucial for the rejection of minor, but not fully mismatched skin graft is not clear. There are other innate immune receptors that are TLR-independent, such as mannose and complement receptors, DC-SIGN and scavenger receptors [89]. Recent studies have shown that the interaction of DCs with innate leucocytes, such as NK cells, NKT cells and $\gamma\delta$ T cells, represents one of the major control mechanisms for immunity that is independent of TLR ligands [90]. Thus, one possible explanation is that major MHC-mismatched transplants would lead to stronger TLR-independent immune responses. However, the reduction of mature DCs in the draining lymph node and the attenuation of Th1 immunity seems no significant difference when transplant MyD88$^{-/-}$ donor skin graft to minor or major mismatched recipient. A strong possibility is that the untouched Th2 immune response contributed to allograft rejection. This is in line with previous work showing that the Th2 immune response alone is sufficient to reject MHC-mismatched allografts [91,92]. Alternatively, allograft rejection may involve MyD88-independent signalling. Trif is an adaptor protein that mediates a MyD88-independent pathway through TLR3 and TLR4 [93,94]. Recently, Trif was identified as a crucial regulator of TLR4-dependent DC responses [95]. Simultaneous deletions of both MyD88 and Trif in mice result in prolonged skin graft survival, notably across a complete MHC and minor antigen barrier. Prolonged survival of skin grafts resulted from a reduced number of donor cells in draining lymphnodes and, subsequently, with delayed infiltration of recipient T cells into the grafted tissue [96].

In a mouse skin transplant model, absence of TLR4 had no effect on the survival of either major or minor histocompatibility-mismatched grafts [97]. However, clinical data support the possibility that TLR4 may participate in acute and chronic allograft rejection. Two human TLR4 polymorphisms, Asp299Gly and Thr399Ile, are associated with blunted responsiveness to lipopolysaccharide (LPS) [98]. Lung transplant patients with the 299 or 399 polymorphism exhibited reduced acute rejection compared with WT controls [99]. Another study investigated 238 renal transplant patients over a 95-month follow-up period. The same TLR4 polymorphism presented lower rate of acute rejection and reduced post-transplant atherosclerotic events [100]. Recently, Palmer *et al.* found that patients received donor kidneys heterozygous for the Asp299Gly or Thr399Ile alleles had a reduced acute rejection. There was no association with recipient TLR4 allele and rejection [101]. In addition, the polymorphisms of one of the important endogenous ligands, Hsp 70, has also been shown contribute to the development of acute rejection after renal transplantation [102]. Further investigations are needed to reconcile the conflicting data from animal experiments and clinical observations.

Transplantation using anti-CD154 monoclonal antibody (mAb) has successfully induced tolerance or prolonged allograft survival in different animal models [103,104]. Anti-CD154 mAb blocks the interaction between CD154 on T cells and CD40 on APCs, inhibiting naive alloreactive CD8$^+$ T-cell activation. The mechanism of promoting long-term graft survival by CD154

targeted therapy also involves the induction of $CD4^+$ Treg [105]. TLR activation, however, is capable of maturing APCs independently of CD40-CD154 interactions [106]. Administration of TLR2, TLR3, TLR4 or TLR9 agonist during treatment with anti-CD154 mAb abrogates skin allograft survival induced by costimulation blockade [107]. The underlying mechanism is by protecting alloreactive $CD8^+$ T cells from apoptosis, subsequently leading to alloreactive $CD8^+$ T-cell expansion and rapid rejection of the allograft [107]. This study suggested that activation of TLRs pretransplantation could prevent tolerance induction by costimulation blockade. However, are these TLRs signals capable of breaking costimulation blockade induced tolerance after transplantation? Zhai *et al.* [105] established a murine cardiac transplant model in which tolerance was induced by a single dose of anti-CD154 mAb at the time of transplantation. They demonstrated that $CD4^+$ Treg were responsible for maintaining unresponsiveness in this model. Following administration of LPS in concert with donor-type skin graft challenge in tolerant recipients, neither of the allografts was rejected, indicating that TLR4 activation was not capable of breaking Treg-mediated alloimmune tolerance in this model.

Concluding remarks

Renewed interest in the innate immune system has greatly expanded our understanding of how the complement and TLR control adaptive immunity. However, a number of important questions remain. Local synthesis of complement C3 plays a vital role in acute kidney allograft rejection. Distinguishing the relative contribution of different sources of local synthesis could provide crucial information for the potential design of therapy. Ligation of TLRs expressed in DCs has been considered as an important mechanism for DCs maturation. During allograft rejection, however, the ligands of TLRs are not yet known. Apart from the common MyD88-dependent pathways, each TLR seems to have its own signal pathway [108]. Their effects on rejection require further resolution. Finally, activation of innate immune system after transplantation is a complex process. It is likely there is a regulatory link between the complement and TLR system [109]. This topic provides a rich area for further investigation.

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