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Transplant Immunology 16 (2006) 25–31
Transplant Immunology
www.elsevier.com/locate/trim

Toll-like receptors and graft rejection

Guangwei Liu [a], You Wu [a], Shouliang Gong [b], Yong Zhao [a,*]

[a] Transplantation Biology Research Division, State Key Laboratory of Biomembrane and Membrane Biotechnology, Institute of Zoology, Chinese Academy of Sciences, Beisihuan Xi Road 25, Beijing 100080, China
[b] Ministry of Health Radiobiology Research Unit, School of Public Health, Jilin University, Changchun, China

Received 31 January 2006; accepted 9 March 2006

Abstract

Innate immunity plays a role in fighting against invading microorganisms. Emerging evidence suggests that in addition to responding to pathogen-associated molecular patterns of microorganisms, Toll-like receptors (TLRs) can be activated by endogenous ligands expressed by mammalian cells. Clinical and laboratory studies have shown that TLRs may participate in organ graft rejection and transplant immune tolerance, which are briefly reviewed in the present manuscript.

Keywords: Toll-like receptors; Organ transplantation; Graft rejection; Innate immunity

1. Introduction

With the development and application of immunosuppressive strategies, organ transplantation has become a common and effective therapy for patients with end-stage organ failure. Our general understanding of transplantation immunology is based on the concept that the immune system is able to distinguish self and non-self signals. Triggering immune responses relies on the specific recognition of pathogens or antigens. Until recently, we have believed that this function is mainly achieved by the adaptive immune system in vertebrates. This classical concept was challenged by Medzhitov and Janeway, who proposed that innate immunity might participate in decoding the patterns of self and non-self signals [1,2]. With the discovery of Toll-like receptors (TLRs) and their ligands endogenously expressed by mammalian cells, the role of TLRs has begun to be investigated in organ transplantation rejection and immune tolerance. This review will focus on studies related to both TLRs and organ transplantation rejection.

2. TLRs and their ligands

TLRs are a group of recently discovered innate pathogen recognition receptors (PRRs) which have been shown to be critical for recognizing conserved motifs on invading pathogens [3,4]. To date, 11 TLRs have been discovered, most of which recognize specific pathogen associated molecular patterns (PAMPs) [5,6]. Recent research has focused on identifying the PAMPs that are recognized by the eleven members of the TLR family. TLR4, in a receptor complex which includes CD14 and MD2, recognizes LPS from Gram-negative bacteria and TLR2, by forming heterodimers with TLR1 and TLR6, recognizes bacterial peptidoglycans, lipoproteins and lipids from *Trypansome cruzi* [7]. In addition, TLR5 responds to flagellin, TLR3 to double-stranded RNA (dsRNA) and TLR9 to unmethylated CpG motifs. Recent data has demonstrated that single-stranded RNA (ssRNA) signals via TLR7 and that uropathogenic bacteria signals via TLR11 [8,9].

Although it is clear that TLRs sense microbial derived ligands, accumulating evidence surprisingly has shown that TLRs are also able to sense endogenous ligands. Recent studies have added heat shock proteins (HSPs) [10–14], surfactant protein A [15], hyaluronan [16], high mobility group 1 (HMGB1) [17], Chromatin–IgG complexs [18,19], Fibronectin [20], Fibrinogen [20,21], and heparan [21] to the growing list of

* Corresponding author. Tel.: +86 10 62538391; fax: +86 10 62659958.
E-mail address: zhaoy@ioz.ac.cn (Y. Zhao).

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doi:10.1016/j.trim.2006.03.006

Table 1
Endogenous ligands of TLRs

Endogenous stimulus	TLRs involved	Cellular response triggered	References
HSPs			
HSP60	TLR4	NF-κB activation	[10–13]
HSP70	TLR2/4	DC maturation	
GP96	TLR2/4	cytokine synthesis	
Hyaluronan	TLR4	NF-κB activation DC maturation	[15]
Lung surfactant protein-A	TLR4	Cytokine synthesis NF-κB activation	[14]
Necrotic cells	TLR2	NF-κB activation induction of inflammatory and tissue repair genes, DC maturation	[1,19–22]
HMGB1	Not determined	Inflammation	[16]
Chromatin–IgG complexs	TLR9	B-cell activation	[17]
Others			
Fibronectin	TLR4	Inflammatory-gene induction DC maturation	[18–20]
Fibrinogen			
Heparan			

TLR ligands (Table 1). More recently, a murine β-defensin 2 has been found to act directly on immature dendritic cells (DCs) as an endogenous ligand for TLR4, resulting in a type 1 polarized adaptive immune response in vivo [21,22].

TLRs are predominantly expressed on antigen-presenting cells (APCs), such as macrophages and DCs, where ligation leads to DC maturation via up-regulating costimulatory molecules and DC migration via the up-regulation of the CCR7 chemokine receptor [22,23]. The consequent migration to the draining lymph nodes allows DCs to activate naïve T cells resulting in the generation of effectors that can then carry out their adaptive immune functions. This pathway is the proposed basis by which TLRs can "switch on" adaptive immunity [24]. PAMP recognition by TLRs enables the innate immune system to distinguish self from non-self and is important not only for triggering innate immunity against microbial infection but also for priming the adaptive immune responses [25–28]. During the Immunology Congress held in Stockholm in 2000, Matzinger [1] presented the "danger model", according to which TLRs could recognize not only non-self pathogen structures but also altered self structures.

However, this question of endogenous ligands for TLRs does not exclude the possibility of bacterial contaminants, as the ligands used are recombinant proteins expressed in bacteria. Therefore, although it remains an interesting hypothesis, these findings should be interpreted with caution and further validation of these results requires additional studies in systems that can exclude this potential contamination, for example, by using baculovirus expressed proteins [29–32].

3. Signal pathways of TLRs

The activation of TLR signaling pathways originates from the cytoplasmic TIR domains. A crucial role for the TIR domain was first revealed in the C3H/HeJ mouse strain, which had a point mutation that resulted in an amino acid change of the cytoplasmic proline residue at position 712 to histidine [6,7,28]. This praline residue in the TIR domain is conserved among all TLRs, except for TLR3, and its substitution to histidine causes a dominant negative effect on TLR-mediated signaling [22,28]. In the signaling pathway downstream of the TIR domain, a TIR domain-containing adaptor, MyD88, was first characterized as playing a crucial role. In addition, recent accumulating evidence indicates that TLR signaling pathways consist, at least, of a MyD88-dependent pathway that is common to all TLRs, and a MyD88-independent pathway that is peculiar to the TLR3- and TLR4-signaling pathways [28,29,33].

MyD88 possesses the TIR domain in the C-terminal portion, and a death domain in the N-terminal portion. MyD88 associates with the TIR domain of TLRs. Upon stimulation, MyD88 recruits IL-1 receptor-associated kinase (IRAK) to TLRs through interaction of the death domains of both molecules. IRAK is activated by phosphorylation and then associates with TRAF6, leading to the activation of two distinct signaling pathways, and finally to the activation of JNK and NF-κB (Fig. 1) [28,30–32].

In the past few years it has become apparent that a MyD88-independent TLR signaling pathway also exists. Initially, an alternative adaptor protein named TIRAP or Mal was thought to be a candidate signal adaptor in the MyD88-independent pathway [33–35]; however, later studies using TIRAP-deficient animals revealed that TIRAP is an essential cofactor for TLR2 and TLR4 signaling upstream of MyD88 [36,37]. Subsequently, TLR3 and TLR4 were found to initiate the upregulation of interferons (IFNs), which are important for an antiviral immune response, because they activate the transcription factor IRF3 via a MyD88-independent pathway [38]. Later studies determined the identity of the TLR adaptor protein used in this pathway as TICAM-1 or TRIF. Together, these studies show that TLR4 signaling has dual characteristics and is dependent on both MyD88 and TICAM-1, with possible crosstalk between the two pathways [38,39]. Recently, another adaptor protein named TRAM has been found to transduce TLR4-dependent MyD88-independent signaling pathways [40,41]. Thus, it is apparent that TLR signaling occurs via different pathways, leading to a tailored immune response (Fig. 1).

In addition, different endogenous activators of TLRs probably trigger multiple pathways and induce distinct subsets of effector genes, as seen with different microbial agents. However, comparative analysis of either target genes induced by different endogenous agents or endogenous versus microbial agents has not been performed. Such studies might go a long way towards defining crucially important differences between host responses to endogenous versus microbial agents [21–26,32,33].

4. TLRs and adaptive immune response

Several lines of investigation have provided evidence that signaling via TLRs is critical for the development of Th_1-dependent immune responses (driven by IL-12, IL-23 and IL-

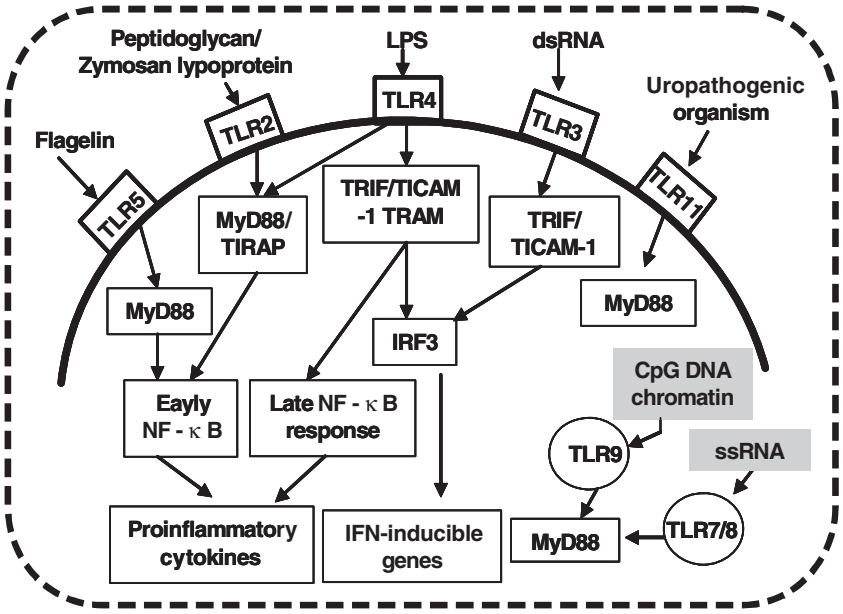

Fig. 1. TLRs and intracellular signal transduction pathways. Various distinct pathogenic motifs that can signal via TLRs (e.g. TLR7–TLR9) are thought to reside in intracellular compartments (not shown). All TLRs, with the possible exception TLR3, can signal via MyD88, leading to the translocation of NF-κB and the production of pro-inflammatory cytokines. Signaling via TLR3 and TLR4 can result in an upregulation of IFN-inducible genes via the adaptor protein TICAM-1 or TRIF. In addition, TLR4 can signal via another adaptor protein, TRAM, and can induce a late NF-κB response.

27, and characterized by IFN-γ production) after immunization with ovalbumin (OVA) peptide in Freund's adjuvant containing heat-killed *Mycobacterium tuberculosis* which can activate TLR2 and 4 [42]. However, Th_2 immune responses (IgG1 and IgE productions) remained intact. Another study demonstrated that MyD88-deficient $CD4^+$ T cells defaulted to a Th_2 phenotype (driven by IL-4, IL-5 and IL-13 cytokines, and characterized by IgE and IgG1 productions) after immunization with *Toxoplasma gondii* extracts [27,28,43]. Hence, these studies imply that MyD88 signaling may be important in determining whether an immune response develops a Th_1 or Th_2 phenotype. When MyD88 knockout mice were immunized with OVA, and then challenged with the same antigen, the antigen-specific Th_1 cell responses including cell proliferation as well as the productions of IFN-γ and Th_1-dependent IgG2a were impaired [42–44]. In contrast, Th_2 immune responses including the production of total IgE, OVA-specific IgE and Th_2-dependent IgG1 were not affected [43]. So, MyD88 is necessary for Th_1-specific immunity. All studies suggest that TLRs signals are critical to develop a Th_1 or Th_2 phenotype of adaptive immune response. However, whether MyD88 directly or indirectly acts on T-cells needs to be identified [35,43].

5. TLR and graft rejection

Despite the sentinel role of innate immunity in driving and shaping adaptive immunity, its direct contribution to graft rejection has generally been ignored. It is traditionally recognized that innate immunity involves recognition of structures displayed by pathogens but not by eukaryotic, multicellular hosts [1,2,44–46]. Therefore, it would seem unlikely that innate immune system elements should recognize mammalian tissues. However, increasing evidence has displayed that innate recognition of allogeneic and particularly xenogeneic cells does exist [47], and that innate immunity promotes direct rejection of transplanted mammalian tissues, particularly those from other species (xenografts) [44–46]. Neutrophil and monocyte/macrophage responses to allogeneic and xenogeneic cells were also observed in severe combined immunodeficiency disease (SCID) mice and were therefore T-cell-independent. Furthermore, injection of syngeneic cells induces little or no neutrophil and monocyte/macrophage response [41,43]. Xenogeneic cells elicited far greater numbers of neutrophils and monocytes/macrophages than allogeneic cells. The reason for this is unclear, although there are far more molecular differences between than within species, which encompass a range of molecules (e.g. oligosaccharides) in addition to polymorphic molecules such as major histocompatibility complex (MHC) antigens [39,44].

The role of TLR4 signaling in acute rejection is controversial. Methe et al. investigated the role of innate immunity especially TLRs in the development of allograft rejection in mice by comparing the TLR4 mRNA levels in acute and chronic rejection. Rejecting mice exhibited elevated mRNA levels for mTLR4, suggesting that activation of innate immunity in heart-transplant recipients through TLR4 contributes to the development of chronic rejection after cardiac transplantation [48]. Goldstein et al. showed that $TLR2^{-/-}$ mice had a small but significant, prolongation of survival of their HY-mismatched skin allografts, but $TLR4^{-/-}$ mice had no such difference when compared to wild type control mice [49]. Samstein et al. used a skin graft transplantation model to examine the role of TLR4 in graft rejection. Using two TLR4-deficient strains of mice demonstrated that dysfunction of TLR4

did not in fact delay the rejection of either major or minor MHC-mismatched skin allografts [50]. Goldstein et al. [49,51] recently observed that skin allograft rejection did not occur in mice with targeted disruption of MyD88, but did occur in those with disruption of TLR2 or TLR4.

Studies in humans, however, suggested that TLR4 may play an important role in the development of acute lung allograft rejection [52,53]. Palmer's group conducted studies in which they screened DNA from 147 lung transplantation recipients and their respective donors for heterozygosity of the two TLR4 polymorphisms. Other researchers' studies have shown that recipients heterozygous for a mutation in the TLR4 gene had reduced acute allograft rejection after lung transplantation [54–57]. TLR4 is expressed on macrophages, DCs, B cells, and airway epithelial cells [28]. Yet, only recipient, not donor, polymorphisms in TLR4 influenced rejection. This suggests that recipient leukocytes with TLR4 polymorphisms, rather than donor cells, are pivotal in reducing acute allograft rejection. Furthermore, increasing evidence suggests that recipient epithelial cells traffic to the donor lung and become integrated with donor epithelial cells [51,53]. These resulted in the donor lung having recipient epithelial cells that expressed TLR4 polymorphisms as well as demonstration of reduced responsiveness to airway bacterial colonization and/or infection.

Recently, Ducloux et al. assessed the occurrence of the two TLR4 allele variants in a cohort of renal transplant recipients (RTR), and analyzed the relations among these polymorphisms, the risk of severe infections, the incidence of acute rejection, and the development of atherosclerotic complications. Results displayed that RTR with TLR4 polymorphism present a lower risk of post-transplant atherosclerotic events and acute allograft rejection, but severe infectious episodes were experienced more frequently [58]. This subset of RTR may benefit from a less potent immunosuppression regimen, along with increased preventive measures against infectious agents. These data suggest that TLR4 plays a pivotal role in atherogenesis after renal transplantation. It would be of interest to investigate whether transplant recipients with the mutated TLR4 gene (Asp299Gly or Thr399Ile) had defects in adaptive immune function after transplantation in addition to reduced rejection rates. Do lymphocytes from these patients demonstrate reduced IFN-γ production in response to donor antigen? Is there a reduction in the number of donor-specific antibodies in these recipients? These are important questions since work in experimental models has demonstrated that mice that are deficient in an important TLR signal adaptor protein, MyD88, have impaired priming and production of helper T-cell type 1 immune response in infectious and transplant models [53,58].

In hematopoietic stem cell transplantation recipients, the presence of TLR4 mutations in recipient or donor has been reported to possibly be associated with reduced risk of acute graft-versus-host disease and an increased risk of Gram-negative bacteria without, however, reaching statistical significance [59].

Researchers believed that there are several likely important explanations for the differences between their animal model and clinical studies. First, environmental exposure directly into the allograft makes clinical lung transplant unique. Inhalational exposure to air pollution (including endotoxin), infectious agents (such as Gram-negative bacteria), and other noxious toxins occurs on a regular basis after lung transplantation. As a result, TLR4 may be of particular importance in the initiation of innate and adaptive immune responses after lung transplantation. Genetic differences in TLR4 signaling, therefore, might exert a greater influence on posttransplant rejection in lung transplants as compared with other organs. Moreover, in contrast to the murine model used by Goldstein, in which mice were identical at the MHC loci, almost all human lung allograft recipients have multiple MHC mismatches with the donor [60,61]. MHC matching is not performed because of short cold ischemic times tolerated by lung allografts. The absence of a significant effect with the TLR4 disruption in the murine model does not address the impact of impaired TLR4 signaling in the setting of multiple MHC differences.

The mechanisms for the role of TLRs in graft rejection have not been well defined so far. The self/non-self model of discrimination proposes that TLRs are used to distinguish self from foreign antigens [2]. However, this previously proposed model has problems dealing with allografts that may not possess obvious PAMPs disparate from those of the host [12,13,28]. However, it has been shown that TLRs may be activated by a variety of endogenous ligands, including HSPs, hyaluronan, and fibronectin, all of them implicated in the pathogenesis of acute allograft rejection, and which induce the synthesis and release of inflammatory cytokines and chemokines, and costimulatory molecules [10–20]. Importantly, some of these endogenous ligands (e.g. HSPs) are known to be up-regulated during transplant rejection [21–34]. An alternative explanation is that contaminating microorganisms of the allograft stimulates TLRs using PAMPs. Indeed, there are several reports that infectious agents, mainly viruses, can modify acute rejection in experimental and clinical transplantation [22,26]. Interestingly, TLR4 does not appear to have a direct effect in reducing allograft rejection in pathogen-free animals [62]. Further studies should determine the precise mechanisms linking TLR4 polymorphisms and alloreactive responses.

It is likely that more than one TLR is involved in allograft rejection. A recent article has demonstrated that LPS expression is increased in reperfusion injury in a rat model of hepatic transplantation [63]. If LPS recognition by TLR4 were a major mechanism of allograft rejection, one would have expected $TLR4^{-/-}$ recipients to have manifested a delay in allograft rejection, which was not the case. So, we cannot exclude the possibility that other receptors are important, too.

Most TLRs signal through a common adaptor protein, MyD88, which was named MyD88-dependent control mechanisms [18,19,28]. TLR signal adaptor protein MyD88 has an important role in solid-organ transplantation. Goldstein and Tesar [53] employed a skin allograft model using mice with targeted deletion of the universal TLR signal adaptor protein, MyD88. Results displayed that minor antigen-mismatched (HY-mismatched) allograft rejection cannot occur in the absence of MyD88 signaling, demonstrating that MyD88 signaling is critical for allograft rejection [64,65]. This is an important

finding, since in vivo investigation of TLRs has predominately involved infectious models. This study also provided key evidence that adaptive alloimmunity could be controlled by the innate immune system and that TLRs, through MyD88, may play an important role in rejection of minor antigen-mismatched allografts. MyD88$^{-/-}$ animals are unable to reject a minor antigen mismatched allograft. However, acute graft rejection in mice with a deficiency in MyD88 can be restored by an adoptive transfer of primed spleen cells from normal donors or the presence of normal APCs from either the donor or the recipient, indicating that this mechanism is acting at the initiation phase of the immune response. Although MyD88-deficient is sufficient to prevent minor MHC-mismatch related graft rejection, the exact types of TLRs involved in this process remain to be elucidated.

However, when Goldstein and coworkers [49,51,53] studied the rejection of MHC-mismatched skin and cardiac grafts in mice, there was a slight delay in cardiac rejection when MyD88 was absent from the recipient alone or from both recipient and donor, and no detectable difference in skin allograft rejection in MyD88-deficient mice. Absence of MyD88 did not impair the ability of DCs to express costimulatory molecules during acute allograft rejection or the ability of allogeneic APCs and APCs derived from rejecting transplant recipients to stimulate alloprimed T cells [64]. The lack of protection of fully MHC-mismatched grafts in MyD88 deficient mice indicates that the alloantigen-dependent immune response is still the major factor that determines the recognition and tolerance of the grafts [53,64]. In addition, Schmidt et al. [66] observed the acute cellular xenograft rejection in a strain of mice with a targeted gene disruption of the TLR signal adaptor protein MyD88, and concluded that porcine islet xenograft rejection persists in mice lacking the TLR adaptor protein MyD88. Whether there is a difference in the rejection of allografts and xenografts in MyD88-deficient mice needs to be further addressed.

6. TLRs and immune tolerance

It has been reported that viruses provide TLR signals that are required for bypassing regulatory T cell (Treg)-mediated tolerance and emphasize the importance of persistent TLR signals for immunotherapy in the setting of established tolerance [60]. Treg cells play a great role in modulating immune response and immune tolerance. TLRs can modulate the adaptive immune response through either stimulation or inhibition of Treg cell functions [63–72]. But whether a TLR signal is a kind of important mechanism to control immune tolerance remains to be elucidated. Although production of the pro-inflammatory cytokines TNF, IL-1 is normal, IL-10 release is severely impaired in the TLR2$^{-/-}$ mice [70]. This is accompanied by a 50% decrease in the CD4^{+}CD25^{+} Treg cell population in TLR2$^{-/-}$ mice [69,71]. In vitro studies confirmed that enhanced survival of Treg cells was induced by TLR2 agonists [73–75]. Takekazu et al. [69] examined that the requirements for murine Treg-suppressive activity and proliferation in the context of the maturation of myeloid DCs. They found that the suppressive function of Treg cells is critically dependent on immature DCs and is readily reversed by the maturation of DCs induced by GM–CSF, but does not require TLR-induced activation of either DCs or Treg cells. In contrast, reversal of Treg cell anergy is dependent on TLR-induced activation of DCs, and involves the potentiation of Treg cell responsiveness to IL-2 by cooperative effects of IL-6 and IL-1, both of which are produced by TLR-activated, mature DCs. This implies that proinflammatory cytokines produced by TLRs-activated, mature DCs are required for reversal of Treg cell anergy, but are not required to overcome the suppression of Treg cells. The above mentioned studies provide some clues that the function and number of Treg cells may be significantly related to TLRs-mediated activation—namely immune tolerance also may be related to TLRs signal pathways. Nevertheless, accumulating data indicate that TLRs are directly related to immune tolerance and immune rejection.

7. Future considerations and closing remarks

Studies in both our clinical samples and animal models suggest an important and previously unrecognized role for innate immunity in the development of allograft rejection [69,74–77]. The question is whether targeting TLR and/or MyD88 would be beneficial in reducing allograft rejection. One concern would be the potential risk of having an already immunosuppressed transplant recipient who is more susceptible to infections because of reduced pathogen recognition. Previous studies have demonstrated that patients with TLR4 polymorphisms experience reductions in acute phase reactants, inflammatory cytokines, and soluble adhesion molecules and are at increased risk of Gram-negative infections [70,71]. Nevertheless, further investigation on the potential mechanisms for the involvement of TLRs and/or MyD88-mediated pathways in graft rejection may greatly enhance our ability to prevent clinical rejection.

Acknowledgements

The authors wish to thank Drs. David E. and Hong Shen for their critical review of the manuscript. This work was supported by grants from National Natural Science Foundation for Distinguished Young Scholars (C03020504, Y.Z.), the National Basic Research Program (973 Program, 2003CB515501, Y.Z.), 100 Quality Vocational Colleges of Chinese Academy of Sciences (2003-85, Y.Z.), the Scientific Research Foundation for the Returned Overseas Chinese Scholars of State Education Ministry (2005-546, Y.Z.), the Knowledge Innovation Program of Chinese Academy of Sciences (KSCX2-SW-333, Y.Z.), Chinese Postdoctoral Research Funds (30020040300, G.L.) and Chinese Academy of Sciences Wangkuancheng Postdoctoral Research Funds (2004, G.L.).

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