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Toll-like Receptors: Key Players in Antiviral Immunity

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Abstract

TLRs are a family of innate receptors whose specificities are predetermined in the germline. Therefore, TLRs have evolved to recognize conserved features of microbes. Viruses typically lack the conserved features common to other pathogen classes, so the innate immune system has evolved to recognize viral nucleic acid as a hallmark of viral infection. In this review we discuss examples of TLR-mediated viral recognition and the functional consequences of this recognition for antiviral immunity.

Introduction

Overview

Toll-like receptors (TLRs) are an essential arm of the innate immune response to bacteria, viruses and fungi and link recognition of distinct features of these microbes to the induction of pro-inflammatory signaling pathways [1,2]. These receptors are able to respond to broad classes of pathogens because each TLR recognizes specific conserved microbial features. Multiple microbial products can serve as ligands, including LPS (TLR4), lipopeptides (TLR2/1 and TLR2/6), flagellin (TLR5), unmethylated CpG motifs in DNA (TLR9), and RNA (TLRs 3, 7 and 8). It has been proposed that targeting nucleic acids is a necessary strategy for viral recognition by the innate immune system because viruses lack any other shared, conserved features suitable for innate detection [3].

TLRs can be subdivided into two groups based on their localization within the cell and type of ligand they recognize. While TLR1, TLR2, TLR4, TLR5 and TLR6 are localized at the cell surface, the nucleic acid-sensing TLRs, TLR3, TLR7, TLR8 and TLR9 are localized intracellularly within endolysosomes [4]. This intracellular localization facilitates recognition of nucleic acids released from microbes degraded within endolysosomes. It is also likely that localization helps to limit recognition of self nucleic acids, although we will not discuss this aspect of TLR function as the topic has been recently reviewed elsewhere[3].

TLR signaling pathways

TLRs consist of an extracellular ligand-binding domain, a transmembrane domain, and a cytosolic signaling domain called the Toll-interleukin 1 receptor homology domain (TIR)

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[1]. Signaling occurs via homotypic binding of TIR domains on the receptor with those on signaling adaptors. All TLRs, with the exception of TLR3, utilize the common signaling adaptor myeloid differentiation factor 88 (MyD88). MyD88 also contains a death domain that recruits IL-1R-associated kinase (IRAK) family members upon recruitment to the TLR TIR. IRAK proteins then dissociate from the MyD88/TLR complex and interact with tumor necrosis factor receptor-associated factor 6 (TRAF6) to mediate downstream signaling. Both TLR3 and TLR4 are able to recruit a second adaptor, TIR-domain-containing adapter-inducing interferon- β (TRIF). TRIF activation initiates a TRAF3-dependent signaling cascade that results in the dimerization and activation of TANK (TRAF family member-associated NF- κ B activator)-binding kinase 1 (TBK1) and inhibitor of NF- κ B kinase (IKKi). This TBK1/IKKi complex leads to phosphorylation of the transcription factor IRF3, allowing it to translocate into the nucleus.

Type I IFN production by TLRs

One of the most important consequences of TLR activation for antiviral immunity is the induction of type I IFN, and all TLRs implicated in viral recognition can induce expression of this family of cytokines[5]. As mentioned above, TLR3 and TLR4 are able to activate the signaling adaptor TRIF, inducing IRF3 phosphorylation, translocation and the transcription of type I IFN genes. A second pathway for the induction of type I IFN involves the transcription factor IRF7 and takes place downstream of TLR7 and TLR9. Interestingly, this pathway functions primarily in a specialized subset of dendritic cells known as plasmacytoid dendritic cells (pDC) [3]. The ability for these cells to uniquely respond to TLR7 and TLR9 ligands by making IFN has been attributed to their high expression of IRF7. However, by delivering TLR7 and TLR9 ligands to early endosomal compartments via lipid complexes, researchers have been able to activate type I IFNs in macrophages as well [6]. These observations suggest that ligand trafficking may be the unique feature that allows IFN production within pDCs. Whether macrophages are able to make IFN via specialized ligand delivery in vivo is still unknown. However, the importance of this pathway in the recognition and control of viral replication has been repeatedly shown and will be discussed further, below.

TLR Recognition of Viruses

As discussed above, nucleic-acid sensing plays an important role in recognizing viral genomes. This requirement for sensing nucleic acid is due, in part, to limited viral 'foreignness.' Viruses utilize host machinery and nucleotides to assemble into infectious particles, and the innate immune system relies on ligand delivery of viral nucleic acid to intracellular compartments to distinguish viral ligands from self. Although there are examples of recognition of viral envelope proteins by TLRs, we will first discuss nucleic acid sensing and touch upon these other forms of recognition later.

Recognition of DNA viruses by TLR9

Unmethylated CpG motifs within bacterial nucleic acid were the first ligands identified to activate TLR9 [7]. Since then, many DNA viruses have also been shown to activate this TLR, including those from the herpesvirus[8-17], adenovirus[18], poxvirus[19] and torquetenovirus (TTV) families[20]. Both α - and β -herpesviruses have genomes that are rich in CpG motifs. Herpes simplex viruses (HSV) 1 and 2 have been shown to activate TLR9 in spleen and bone marrow cultures to produce pro-inflammatory cytokines and type I IFN [16,17], and similar results have also been observed with mouse cytomegalovirus (MCMV) [10,21,22]. Although the contribution of TLR9 recognition of these viruses has clearly been reported in vitro, the phenotype of TLR9-deficient mice in vivo does not completely phenocopy that of mice deficient in the signaling adaptor MyD88. These results suggested

that other members of the TLR/IL-1R family might play a redundant role in recognition of these viruses, and it was later shown that the remaining MyD88-dependent recognition may be mediated by detection of viral envelope proteins by TLR2 and possibly RNA intermediates produced during viral replication by TLR3 and TLR7 (discussed later) [9,10,23].

It is noteworthy that very few examples exist of viruses suppressing CpG motifs within their genomes as a strategy to evade nucleic acid detection. This lack of evidence can perhaps be explained by the constraint on mutability of compact viral genomes with few non-coding sequences. One example of CpG suppression has recently been reported for a member of the γ -herpesvirus family, murine herpesvirus 68 (MHV68) [24]. In this study it was reported that CpG suppression, most likely mediated by cytosine to thymine conversion, within the genome of MHV68, allows the virus to evade TLR9 detection and largely avoid detection in vivo. Further, during latent stages of infection, CpG motifs within the viral genome were found to be methylated, and therefore unable to stimulate TLR9. These alterations in genome composition lead to reduced type I IFN and pro-inflammatory cytokine production. Nevertheless, it is difficult to prove that TLR9 recognition was the selective pressure leading to suppression of these motifs.

Recognition of ssRNA by TLR7/8

The first ligands identified to stimulate TLRs 7 and 8 were members of the imidazoquinolones family which share structural similarity with ribonucleosides [25]. The imidazoquinolones were long known to stimulate a potent antiviral response, and it wasn't until the discovery of these TLRs that the receptors mediating this response were appreciated. The precise structural motifs or ssRNA sequences (analogous to CpG motifs for TLR9 stimulation) that are capable of stimulating TLR7 are not precisely known. G-U rich and even PolyU RNA are capable of stimulating TLR7 [26]. Single stranded RNA is extremely labile, and host-derived RNA rarely comes into contact with intracellular TLRs because it is rapidly broken down by extracellular RNases. In contrast, viral particles protect viral RNA from degradation; destruction of viral particles within phagosomes releases viral RNA for recognition by TLRs 7 and 8.

Similarly to TLR9, TLRs 7 and 8 are capable of inducing type I IFNs in pDCs via the signaling adaptor MyD88. HIV, influenza and vesicular stomatitis virus (VSV) have all been reported to stimulate TLR7[26-28]. The genomic RNA of influenza virus is capable of inducing IFN α from mouse dendritic cells in a TLR7 dependent manner, and similar results were obtained when comparing stimulations of wildtype and TLR7-deficient cells with the U5 region of HIV-1 RNA.

Recognition of dsRNA by TLR3

TLR3 recognizes dsRNA as well as the synthetic analog, polyI:C [29-32]. This receptor signals through TRIF and leads to production of IFN in all cell types in which it is expressed. Despite the discovery that TLR3 is a potent inducer of IFN in response to dsRNA, in vivo evidence that TLR3 deficiency results in exacerbated disease in response to viral infection was lacking for many years —leading some to believe that TLR3 may not actually be a receptor of viral dsRNA directly [33].

It has since been appreciated, however, that TLR3 may play a role in recognition of dsRNA intermediates within engulfed apoptotic cells that have been infected with ssRNA viruses [34]. The viruses used in this study were encephalomyocarditis virus and Semliki Forest virus, both of which are ssRNA viruses, lending to the possibility that dsRNA, produced during the replication of these viruses, is detected by TLR3 within the apoptotic cells after

phagocytosis. Interestingly, several reports have also shown that in models of West Nile virus and Influenza A virus (both ssRNA viruses), pro-inflammatory cytokines produced in a TLR3-dependent manner can actually lead to increased viral pathology [35-39]. These reports further confirm the notion that dsRNA intermediates are likely generated and detected in vivo. Adding further complexity to how TLR3 may participate in viral recognition, TLR3-deficient mice are more susceptible to the DNA virus MCMV [40]. Also, human patients with deficiencies in TLR3 or signaling molecules downstream of this receptor show predisposition to HSV-induced encephalitis [41-45]. This suggests that dsRNA may be produced by the secondary structures of viral transcripts derived from the viral genome of DNA viruses.

Non-nucleic acid recognition of Viral ligands by TLRs

As mentioned earlier, the recognition of viral nucleic acid by TLRs may be the most reliable method of detecting viruses because of ligand delivery, the constraints of mutating viral genomic contents, and the fact that the viral particle is assembled using host machinery. Despite these conceptual considerations, there are a handful of examples of viral proteins serving as TLR ligands. In principle, the sequence or structure of these detected proteins must be constrained for this strategy to work. Mutating the regions that are detected by TLRs should make the viral particle non-functional; otherwise, the virus would easily be able to mutate these proteins to avoid detection. The clear exception to this idea is when TLR-mediated inflammation is important for viral pathology, as is supported in some infection models discussed below.

HSV-1, HSV-2, HCMV, MCMV, VV, respiratory syncytial virus (RSV) and the hemagglutinin protein of measles virus have all been shown to activate TLR2 [23,46-51]. In response to VV infection, TLR2 on inflammatory monocytes is capable of inducing type I IFN, and depletion of these cells leads to elevated levels of Vaccinia virus in ovaries of mice [51]. TLR2 signaling has also been shown to be important in CD8 T cells where it is critical for clonal expansion and memory formation following VV infection [52]. These examples suggest that TLR2-mediated detection of VV is important for the host to be able to generate a protective response against the virus.

In a very different example, TLR2 activation by certain isolates of HSV-1 seems to induce excessive inflammation. In this model, TLR2-deficient animals are somewhat protected from HSV-induced encephalitis [50]. The responding cells in this HSV example may be generating a pro-inflammatory response without the production of protective IFN, aberrantly leading to increased encephalitis pathology.

Mouse mammary tumor virus (MMTV) and RSV have both been implicated to activate TLR4 [53-57]. In the case of MMTV, TLR4 leads to the production of IL-10, an anti-inflammatory cytokine, and allows for the virus to enter a stage of persistence by dampening the immune response against it [57]. On the other hand, the role of TLR4 detection of RSV seems to be protective. TLR4-deficient mice have reduced IL-6 production in response to RSV and infants with polymorphisms in TLR4 are at higher risk for severe RSV infection [55,56].

Detecting the contribution of non-nucleic acid-sensing TLRs to the overall innate immune response may require that mice deficient in TLR2 or TLR4 are crossed to those lacking nucleic acid-sensing TLRs that can mask the contribution of viral protein detection. Infection of these mice will allow us to gain insight into the potentially wider contribution of TLR detection of viral proteins as a strategy for host protection or a viral strategy for exploiting these signals.

Functional Consequences of TLR activation

Viral evasion strategies

As discussed above, there are many examples of TLRs detecting viral nucleic acid or viral envelope proteins. Concomitant with the evolution of these innate detection strategies, viruses have acquired genes whose products inhibit TLR signaling pathways, thus evading detection by TLRs or specific downstream consequences of TLR activation. While not the main focus of this review, we have outlined examples of viral evasion of TLR signaling pathways in Table 1. Notably, there are relatively few examples of viral gene products that specifically inhibit TLR signaling, especially when compared to the many examples of viral inhibition of cytosolic innate immune sensors. Furthermore, several of the downstream TLR signaling components targeted by viruses are common to both TLR and RLR signaling. Even TRIF has recently been implicated as a downstream adaptor for the cytosolic dsRNA-sensing helicases DDX1, DDX21, and DHX36 [58].

An important consideration when evaluating the selective pressure responsible for these evasion strategies is that TLRs detect viral particles that fail to infect cells. In fact, the ability of innate immune cells to recognize viruses without being infected is likely an important mechanism for the host to avoid viral immune evasion strategies. Even viruses within infected cells that undergo apoptosis can be detected by TLRs expressed by phagocytic cells. Once viruses have infected a cell, their genomes, replication intermediates, and products of gene expression can be sensed by innate cytosolic sensors. This potential recognition coupled with the ability to express evasion genes in infected cells may explain why so many viruses have evolved strategies to block or circumvent cytosolic innate sensing.

Activation of TLRs on antigen-presenting cells, such as macrophages and dendritic cells (DCs), leads to upregulation of costimulatory molecules, the production of inflammatory cytokines, and the generation of a functional adaptive immune response. We focus on these functional consequences of TLR signaling in the next section as a means of understanding precisely how TLR-detection of viruses contributes to induction of antiviral immunity.

Phagosome-autonomous responses

A specific feature of TLR responses that does not apply to cytosolic sensors is the detection of defective viral particles that do not cause active infection but activate TLRs upon degradation in the phagosome. This also applies to the engulfment of apoptotic virally infected cells. The detection of these viral products highlights the phagosome-autonomous functions of TLR signaling, outlined in Figure 1. As mentioned, TLR3, TLR7 and TLR9 reside within the phagosome and TLR4 has been shown to be internalized with ligand upon activation [79]. Once activated, these TLRs induce more rapid acidification of the phagosome, presumably through the recruitment of the V₁ sector subunits A and E (of the Vacuolar-ATPase) to the phagosomal membrane [80-83]. The release of reactive oxygen and nitrogen species by recruitment of NADPH oxidase or mitochondria to the phagosome is another hallmark of phagosome-autonomous TLR-mediated maturation [84-86]. Along with proteases that are active at the low pH of the mature phagosome, viral proteins are degraded into peptides, loaded into MHC class II and shuttled to the surface of the cell. Further, antigens present within this mature phagosome can be loaded into MHC class I for activation of cytotoxic CD8 T cells via a process known as cross presentation [87-89]. The precise details of the cross presentation pathway are still elusive, however, it is widely accepted that engulfed, virally-infected dead or dying cells activate TLRs and that their viral antigens are capable of being presented in class I MHC after engulfment by CD8 α^+ dendritic cells [34].

Upregulation of Cell Surface Molecules

TLR signaling also leads to the upregulation of costimulatory molecules on the surface of the signaling cell. These include CD80, CD86 and CD40 (Figure 1). These molecules are required along with antigen presentation for the activation of T lymphocytes. Class II MHC molecules presenting viral antigens are also upregulated on the cells surface. These molecules, upregulated via TLR signaling, serve to activate T cells for protective immunity.

Cytokine production

As discussed previously, the intracellular nucleic acid sensing TLRs are capable of inducing type I IFN in pDCs and TLR2 and TLR4 both recognize viral products and lead to the induction of IFN in certain cell types. The overall importance of this cytokine is exemplified by the extreme susceptibility of type I IFN receptor mice to viral infections [5]. Signaling via the type I IFN receptor leads to the upregulation of many genes that serve the function of shutting off host translation and inducing apoptosis, thus rendering the cell inhospitable for viral replication. This cytokine is also critically important for other immune responses. For example, IFN can lead to the differentiation and activation of natural killer (NK) cells. These cells serve the function of killing virally infected cells that try to evade antigen presentation via MHC down-regulation [22,90,91]. Type I IFN has also been shown to induce cross presentation by dendritic cells and leads to the upregulation of MHC Class I, further increasing the activation of CD8 T cells.

Other pro-inflammatory cytokines that are produced downstream of TLRs include IL-6, IL-12 and TNF- α . These cytokines serve numerous functions including promoting the survival and proliferation of B and T cells, activating NK cells, and inducing local inflammation to recruit other cells to sites of infection. In one circumstance, as discussed above, the production of IL-10 downstream of TLR4 seems to be exploited by MMTV because IL-10 is a potent suppressant of immune cell function.

Conclusion

Our conceptual understanding of how TLRs recognize viruses has expanded significantly over the past few years. Recent discoveries demonstrate a role for TLR3 in recognizing virally infected apoptotic cells and for the detection of HSV in the central nervous system. Further, the role of TRIF as an adaptor for cytosolic RNA sensors and the surprising finding that TLR2 is capable of making IFN in certain cell types have been reported. A common recurring theme is that viral recognition by TLRs always seems to lead to the production of type I IFN. The role of this cytokine in the generation of an effective antiviral response has been repeatedly shown, and inhibiting these signaling pathways seems to be a common viral evasion mechanism. Detection of non-nucleic acid viral ligands by TLRs has also been more recently appreciated. The implications of these detection strategies may be that viruses have begun to suppress nucleic acid motifs that can be detected by TLRs, although there remain very few examples of this evasion mechanism. Further understanding of the interactions between virally infected cells and TLR signaling pathways may provide important advances in the study of protective adaptive immunity and vaccine development.

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Highlights

- TLRs detect viral nucleic acid and envelope proteins
- Defective viral particles that do not infect cells can also activate TLRs
- TLRs can detect viral nucleic acid by engulfing infected apoptotic cells
- TLR signaling upregulates costimulatory molecules for T cell activation
- Selection of antigens for presentation is mediated by phagosomal TLR activation

Figure 1. Functional Consequences of TLR Signaling

Engulfed viruses are degraded in endosomes and/or phagosomes. Released viral nucleic acid stimulates intracellular nucleic acid-sensing TLRs (red) leading to phagosome autonomous responses including recruitment of V-ATPase (green) and NADPH oxidase (purple). Viral antigens generated upon degradation of the virus are loaded into MHC class II and shuttled to the surface (pink). In certain cell types, antigens are loaded into MHC class I via cross presentation (dashed line, green). TLR activation also leads to the upregulation of costimulatory molecules, CD40 (brown) and CD86 (lime), as well as a signaling cascade that leads to the transcription of cytokine genes.

Target	Viruses	References
MyD88, IRAK1/2/4	Herpes simplex virus, Poxviruses, Hepatitis C virus	[59-61]
RIP1, TRAF3/6	West Nile virus, Respiratory syncytial virus	[62-64]
TRIF	Influenza, Reovirus, Coxsackievirus, Kaposi's sarcoma-associated herpes virus, African swine fever virus, Hepatitis A/C viruses	[58,65-69]
TBK1, IKKi, IRF3/7	Ebola virus, Hepatitis C virus, Vaccinia virus, Varicella-Zoster virus, Rabies virus, Foot-and-mouth disease virus, Epstein-Barr virus, Measles virus	[70-78]