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TLR-Mediated Activation of Type I IFN During Antiviral Immune Responses: Fighting the Battle to Win the War

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Abstract Toll-like receptors (TLRs) are crucially important in the sensing of viral infections and viral nucleic acids. TLR triggering leads to the induction of specific intracellular signaling cascades that result in the activation of two major families of transcription factors; the IFN-regulatory factors (IRFs) and nuclear factor-kappa B (NF-κB). IRFs and NF-κB work together to trigger the production of type I interferons (IFNα/β) or inflammatory cytokines leading to the maturation of dendritic cells and the establishment of antiviral immunity. This review will focus on the most recent findings relating to the regulation of IRF activity by TLRs, highlighting the increasing complexity of TLR-mediated signaling pathways.

1 Introduction

The mission of a virus inside the host is to multiply. This task is counteracted by strong and precise host immune responses. The first warriors to combat virus infections were discovered 50 years ago by Isaacs and Lindenmann as soluble proteins released by almost all cell types capable of interfering with virus replication, and referred to as the interferons (IFNs) (Isaacs and Lindenmann 1957). Type I IFNs belong to a multiprotein family that consists of about 30 members sharing a variable degree of structural homology (Hardy et al. 2004; Pestka et al. 2004; van Pesch et al. 2004). Type I IFNs include multiple *Ifn-αs*, *Ifnb*, *Ifnω*, *Ifnκ*, and *Ifne* genes; during viral and bacterial infections, the main type I IFNs that are synthesized are IFN-αs and IFN-β (Bogdan et al. 2004; Coccia et al. 2004). In the past few years, the regulation and function of these IFNs have been extensively characterized.

The discovery of the Toll-like receptors (TLRs) represents a key milestone in understanding how virus-infected cells recognize and react to invading pathogens (Janeway and Medzhitov 2002). At present, 13 TLRs have been identified: TLR1–9 are common to mouse and human, while TLR10 is unique to humans and TLR11–13 are unique to the mouse (Tabeta et al. 2004; Takeda et al. 2003; Zhang et al. 2004). TLRs play a key role in detecting microbial products derived from a broad range of pathogens, often referred to as pathogen-associated molecular patterns (PAMPs). Several lines of evidence indicate that the TLRs involved in the recognition of molecular structures unique to bacteria and fungi (TLR1, TLR2, TLR4, TLR5, TLR6) are localized to the plasma membrane and can be recruited to the phagosome, whereas the TLRs that detect viral and bacterial nucleic acids (TLR3, 7, 8, and 9) are localized in the endosomal compartment. Bacterial and viral double-stranded (ds) DNA is detected by TLR9. TLR7 and TLR8 are closely related and are involved in recognizing virus-derived single-stranded (ss) RNAs. Furthermore, dsRNA, which is generated in infected cells as an intermediate of virus replication, triggers TLR3.

TLRs are transmembrane proteins: their extracellular domains contain a repetitive structure rich in leucine residues, the leucine-rich repeats (LRRs), that are involved in ligand recognition. The intracellular region includes a common structure to all TLRs and IL-1 receptor family members, and is referred to as the Toll/IL-1 resistance (TIR) domain, which is essential for signal transduction. Every TLR triggers a specific cellular activation program via the recruitment of different combinations of specific adaptor molecules to its TIR domain. These adapters include myeloid differentiation factor 88 (MyD88) (Muzio et al. 1997), MyD88 adapter-like (Mal) (Fitzgerald et al. 2001) (also

called TIRAP; Horng et al. 2001), TIR-domain-containing adapter inducing interferon-β (TRIF) (Yamamoto et al. 2002; Hoebe et al. 2003) (also called TICAM1; Oshiumi et al. 2003a) and TRIF-related adapter molecule (TRAM) (Fitzgerald et al. 2003b) (also called TICAM2; Oshiumi et al. 2003b). Only recently, another TIR-domain-containing adapter has been described, SARM (SAM- and ARM-containing protein), which contains sterile alpha (SAM) and HEAT/Armadillo (ARM) motifs, as well as a TIR domain (Liberati et al. 2004). SARM has recently been shown to act as a negative regulator of TLR signaling (Carty et al. 2006). The recruitment of these TIR-domain-containing adapters to the TIR domain of activated TLRs leads to the activation of several transcription factors, including NF-κB and the IFN-regulatory factors (IRFs), with the subsequent induction of type I IFNs and IFN-dependent responses.

In this review, we have focused on the role of TLRs and associated signaling molecules in innate immunity to viruses in order to give a complete overview of how TLRs are involved in sensing and initiating immune responses to viruses.

2
ER-Localized TLRs: The Specialists in Virus Recognition

2.1
TLR3

The innate immune system is the first line of defense against virus infection and involves the release of proinflammatory cytokines, type I IFNs, and activation of adaptive immune responses. A number of viral products are sensed by cells of the innate immune system; among them, dsRNA is a common signature of viral replication and is generated in infected cells by most (if not all) viruses. In 2001, it was described for the first time that TLR3 mediates responses to poly (I:C), a synthetic analog of dsRNA. Indeed TLR3 knockout mice were resistant to poly (I:C)-induced shock compared to wild-type mice (Alexopoulou et al. 2001). Since the inhibition of endosomal acidification abrogates poly (I:C) signaling, it has been assumed that TLR3 is localized to the endosomal compartment. In fact, TLR3 has been shown to reside in multivesicular bodies, a subcellular compartment situated in the endocytic trafficking pathway in dendritic cells (DC) and could not be detected on the cell surface (Matsumoto et al. 2003). This intracellular localization of TLR3 is thought to be important for encountering dsRNA.

TLR3 has been implicated in the immune response to several viruses. TLR3 controls inflammatory cytokine and chemokine production in respiratory syncytial virus (RSV)-infected cells (Rudd et al. 2005). RSV-induced CXCL10 and

CCL5 production, but not CXCL8 production or viral replication, were shown to be impaired in the absence of TLR3. Hoebe et al. reported that mice homozygous for the *Lps2* mutation, a distal frameshift error in TRIF, are hypersusceptible to mouse cytomegalovirus (MCMV) (Hoebe et al. 2003), and a role for TLR3 in the response to MCMV was confirmed using TLR3 knockout mice (Tabeta et al. 2004). A major function for TLR3 in antiviral responses involves its role in promoting the cross-priming of cytotoxic T lymphocytes (CTLs). This occurs in cells that are themselves not directly infected. Murine CD8α^+ DCs can be activated in this manner by dsRNA present in virally infected cells taken up by phagocytosis (Schulz et al. 2005). These observations may explain the subcellular localization pattern of TLR3 in the endosomal compartment.

In some circumstances, the TLR3-mediated response can be detrimental to the host. During infection with West Nile Virus (WNV), a mosquito-borne ssRNA flavivirus, TLR3-deficient mice were found to be more resistant to lethal WNV infection. TLR3-deficient mice had increased viral load in the periphery (Wang et al. 2004). TLR3-dependent inflammatory response modulates the ability of WNV to invade the central nervous system after replicating in the periphery by inducing a reversible breakdown of the blood–brain barrier. TLR3 knockout mice also have an unexpected advantage upon influenza A virus challenge: a reduction in TLR3-mediated inflammatory response reduces the clinical manifestation of the influenza A-induced pneumonia (Le Goffic et al. 2006). In both of these cases, the virus appears to benefit from its interaction with TLR3.

In addition to viral RNA, heterologous RNA released from or associated with necrotic cells, likely through secondary structure, also stimulates TLR3 and induces immune activation (Kariko et al. 2004). Thus, RNA escaping from damaged tissues or contained within endocytosed cells could serve endogenous danger signals and be sensed by TLR3.

2.2 TLR7 and TLR8

TLR7 and TLR8 have been shown to recognize viral nucleic acids. Firstly, TLR7 and 8 were shown to trigger IFN production in response to the imidazoquinolines, imiquimod, and resiquimod (or R-848). These are low–molecular–weight immune response modifiers with potent antiviral and antitumor properties that are used clinically in the treatment of external genital warts caused by human papilloma virus infection (Hemmi et al. 2002). Using MyD88 and TLR7 knockout mice, Hemmi et al. showed that the imidazoquinolines activate murine immune

cells in a TLR7- and MyD88-dependent manner. Moreover, R-848 can be recognized either by human and murine TLR7 or human TLR8 but not murine TLR8, suggesting that TLR8 is not functional in mice, in accordance with the observation that TLR7-deficient mice do not respond to R-848, even though TLR8 is present (Jurk et al. 2002). Since this initial discovery, the immunostimulatory action of several additional guanine nucleoside analogs has been shown to be controlled exclusively via TLR7 (Lee et al. 2003) and this activity in human cells appeared to require endosomal acidification.

The first evidence of TLR7 and 8 triggering by physiological ligands was reported by Heil et al. (2004). Indeed they described the ability of guanosine- and uridine-rich ssRNA oligonucleotides derived form immunodeficiency virus-1 (HIV-1) to stimulate DCs and macrophages to secrete IFN-α and proinflammatory cytokines via murine TLR7 and human TLR8. In the same issue of *Science*, another group also reported the capacity of TLR7 to sense synthetic ssRNA (polyU) or ssRNA derived from wild-type Influenza virus (Diebold et al. 2004). Viral genomic ssRNA could substitute for intact Influenza in triggering IFN-α and cytokine production by murine plasmacytoid DCs (pDCs) and only background levels of IFN-α were measured in pDCs derived from TLR7$^{-/-}$ and MyD88$^{-/-}$ mice, further supporting the hypothesis that ssRNA is a TLR7 ligand. The recognition of another ssRNA virus, vesicular stomatitis virus (VSV), was also shown to be TLR7/MyD88-dependent (Lund et al. 2004).

Influenza virus, like VSV, is internalized into an endocytic compartment where viral fusion and release into the cytosol occurs; this suggests that the recognition by TLR7 might occur in the endosomal compartment. In fact, both Diebold and Heil's reports showed that virus-induced IFN-α production in pDCs required intact endocytic pathways (Diebold et al. 2004; Heil et al. 2004). This is consistent with the idea that viral nucleic acids would be sensed from an intracellular compartment.

Because GU-rich sequences are found in viral as well as endogenous RNA, TLR7 and 8, as has been described for TLR3, may also detect self-RNA acting in this way as sensors of endogenous danger signals (Heil et al. 2004). Accordingly, small nuclear ribonucleoproteins (snRNPs), which are a major component of the immune complexes associated with the pathogenesis of the autoimmune disease systemic lupus erythematosus (SLE) activate human pDCs to produce IFN-α, proinflammatory cytokines and to upregulate costimulatory molecules when the U1snRNA is intact (Savarese et al. 2006). The recognition of U1snRNA is dependent on TLR7. Therefore in certain circumstances, detection of self-RNA by these TLRs can contribute to autoimmune disease.

2.3 TLR9

Unmethylated CpG motifs are a feature of bacterial but not vertebrate genomic DNA and TLR9 was originally shown to be activated by these molecules (Hemmi et al. 2000). Oligodeoxynucleotides (ODNs) containing CpG motifs activate host defense mechanisms leading to innate and acquired immune responses. The concept of immunostimulatory DNA was borne as a result of studies on attenuated mycobacteria bacillus Calmette Guerin (BCG)-mediated tumor resistance. The component of BCG for activating natural killer (NK) cells and inducing tumor regression in mice was subsequently found to be the DNA (Tokunaga et al. 1984). Purified BCG DNA induced NK cell activity and the production of type I and II IFNs in vitro (Yamamoto et al. 1988). Cloning and synthesizing mycobacterial genes helped to elucidate that certain self-complementary palindromes in these ODNs were responsible for the immune stimulatory effects (Yamamoto et al. 1992). The active palindromes contained at least one CpG dinucleotide. CpG dinucleotides are more common in the bacterial genome (Kuramoto et al. 1992) and are not methylated in bacterial DNA but are routinely methylated at the 5′ position of the cytosines in vertebrate DNA (for extensive reviews see Krieg 2002 and Tokunaga et al. 1999). Several groups reported that the immunostimulatory CpG-ODNs directly activate macrophages (Sparwasser et al. 1997; Stacey et al. 1996) and murine DCs (Sparwasser et al. 1998) to upregulate co-stimulatory molecules and produce proinflammatory cytokines. Interestingly, expression patterns for TLRs differ between different subpopulations of dendritic cells. Plasmacytoid DCs (pDCs) predominantly express TLR7 and TLR9, whereas myeloid DCs express TLR1–6 and TLR8, but not TLR7 and TLR9 (Hornung et al. 2002; Jarrossay et al. 2001; Kadowaki et al. 2001). Accordingly, only human pDCs (as well as human B cells) respond to CpG-DNA.

The CpG motifs are also found in abundance in some viral genomes, such as the dsDNA virus, Herpes simplex virus (HSV). The pDCs respond to HSV-1 by secreting high levels of type I IFNs, releasing IL-12 and upregulating co-stimulatory molecules (Dalod et al. 2002) and the pDC responsiveness to HSV-1 in vitro is indeed mediated by the TLR9/MyD88 pathway (Krug et al. 2004). Similar results have been reported in HSV-2-infected pDCs (Lund et al. 2003); in this case, however, they also demonstrated that purified HSV-2 DNA was able to trigger IFN-α production in pDCs. In $TLR9^{-/-}$ mice infected with HSV-2, no IFN-α was detected (Lund et al. 2003). Moreover, the recognition of HSV-2 by pDCs is dependent on an intact endocytic pathway, since inhibitors of endosomal acidification such as chloroquine or bafilomycin inhibit these responses. This is consistent with the fact that TLR9 is located and signals from an intracellular

endosomal compartment (Ahmad-Nejad et al. 2002; Latz et al. 2004). Ahmad-Nejad et al. and Latz et al. reported that CpG-ODNs move into early endosomes and are then transported to a tubular lysosomal compartment. In accordance with this, TLR9 redistributes from the ER to these structures where the CpG-ODNs are located and where MyD88 can also accumulate.

It is highly likely that other large DNA viruses whose genomes are rich in CpG motifs are also recognized by TLR9. Only very recently, Basner-Tschakarjan et al. reported that the dsDNA virus, adenovirus efficiently activates pDCs in a TLR9-dependent manner, resulting in maturation and IFN-α production (Basner-Tschakarjan et al. 2006).

Another intriguing aspect of TLR9 function is that its activation can also be triggered by self-DNA. DNA-containing immune complexes (ICs) isolated from sera of SLE patients have been shown to trigger TLR9 (Boule et al. 2004; Leadbetter et al. 2002; Means et al. 2005), and this stimulation is inhibited either by agents that block TLR9 signaling or by directly inhibiting TLR9 itself (Leadbetter et al. 2002). Thus, a mechanism must exist to ensure that TLRs involved in nucleic acid recognition (TLR3, 7, 8, 9) can discriminate between foreign and self nucleic acids. Recently, Barton et al. very elegantly described that a chimeric TLR9 receptor, which localizes to the cell surface, responded normally to synthetic CpG-DNA but not to nucleic acids contained in viral particles. However the relocated chimeric TLR9 gained the ability to recognize self-DNA, which does not stimulate wild-type TLR9 (Barton et al. 2006). So, it appears that the intracellular localization of TLR9 is not required for ligand recognition as was initially proposed but instead controls access of the receptor to different sources of DNA. Viral DNA can be methylated as is the case for self-DNA; therefore the immune system has adopted a strategy for viral recognition: the recognition of viral nucleic acids within endosomal compartments. This can be a critical mechanism to properly discriminate between self or foreign nucleic acids and to maintain homeostasis within the immune system.

In addition to the recognition of viral nucleic acids, it has also been reported that several viral proteins are detected by TLRs located on the surface of host cells. The hemagglutinin (HA) protein of measles virus activates human cells in a TLR2-dependent manner (Bieback et al. 2002). Human cytomegalovirus (HCMV) has also been shown to trigger TLR2 signaling (Compton et al. 2003). A role for TLR4 in virus recognition was first described in the case of the fusion (F) protein of RSV (Kurt-Jones et al. 2000). More recently, the envelope proteins (env) from both mouse mammary tumor virus (MMTV) and Moloney murine leukemia virus (MMLV) (Burzyn et al. 2004) activate murine monocytes and bone-marrow-derived macrophages, respectively, in a TLR4-dependent manner.

In conclusion, there is ample evidence that TLRs participate in viral recognition. TLR2 and TLR4 recognize viral glycoproteins on virions while the intracellular TLR3, 7, 8 and 9 detect naked viral nucleic acids.

3
IFN Gene Induction During Viral Infections: Pathways Activated by TLRs

3.1
MyD88 or TRIF? This Is the Question!

Among the five different adapter molecules containing the TIR domain, MyD88 was the first identified and shown to be critical for TLR and IL1R family signaling (Kawai et al. 1999). MyD88 can associate with all TLRs (Medzhitov et al. 1998) with the exception of TLR3 (Oshiumi et al. 2003a; Yamamoto et al. 2003). MyD88 has an amino terminal death domain (DD) and a carboxy-terminal TIR domain. The TIR domain is involved in the interaction with TLRs and other adapters (see below) while the death domain associates with members of the IL-1R-associated kinase (IRAK) family (Martin and Wesche 2002). IRAK-1 is recruited to MyD88 via DD–DD interactions within a complex with another protein termed Toll-interacting protein (Tollip) (Burns et al. 2000). This IRAK1-MyD88 association triggers hyperphosphorylation of IRAK1 by itself as well as phosphorylation by the related kinase, IRAK-4 (Cao et al. 1996; Li et al. 2002). These events lead to the dissociation of IRAK1 from MyD88 and Tollip and its interaction with the downstream adaptor tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF-6) (Burns et al. 2000). TRAF-6, a RING domain ubiquitin ligase activates the TAK1 kinase through K63-linked polyubiquitination (reviewed in Chen 2005). TAK1 in turn activates the IKK complex, which phosphorylates IκBs and targets these NF-κB inhibitors for ubiquitination and degradation by the proteosome. NF-κB is then released and translocates to the nucleus where it can induce several hundred target genes (Medzhitov et al. 1997; O'Neill 2002).

The diversity of TLR signaling pathways was revealed following the analysis of the response of MyD88-deficient macrophages to Gram-negative bacteria-derived lipopolysaccharide (LPS) (Kawai et al. 1999). LPS, which signals via TLR4 and MD2, can still trigger the activation of NF-κB and MAPK in cells from MyD88 knockout mice, albeit with delayed kinetics compared with wild type cells, whereas most other TLR ligands are completely ineffective at triggering these events in the absence of MyD88. Although MyD88-deficient mice lose their ability to induce proinflammatory cytokines in response to LPS, they are still able to upregulate co-stimulatory molecules and induce type I

IFNs and IFN-inducible genes (ISGs) (Kaisho et al. 2001; Kawai et al. 2001). Subsequent studies from several groups identified another adapter TRIF that regulates these MyD88-independent pathways (Fitzgerald et al. 2003b; Hoebe et al. 2003; Yamamoto et al. 2003). TRIF knockout mice are compromised in the induction of type I IFNs and the expression of ISGs in response to LPS and the dsRNA mimetic poly(I:C), a TLR3 ligand. Both TLR4 (Navarro and David 1999) and TLR3 (Fitzgerald et al. 2003b; Oshiumi et al. 2003a; Yamamoto et al. 2002) signaling cascades activate the nuclear translocation and DNA binding of the transcriptional regulator, IRF3, a key regulator of IFN-β and ISGs, a process mediated solely by TRIF in the case of TLR3 signaling (Fitzgerald et al. 2003b; Hoebe et al. 2003; Yamamoto et al. 2003). In the case of TLR4 signaling, an additional adapter, TRAM is also required to recruit TRIF to TLR4 (Bin et al. 2003; Fitzgerald et al. 2003b; Oshiumi et al. 2003b). TRAM is modified by N-terminal myristoylation, which is important in tethering TRAM to the plasma membrane, where it co-localizes with TLR4 (Rowe et al. 2006). This function of TRAM appears to be important in recruiting TRIF to membrane-localized TLR4. A fourth adapter molecule Mal (also called TIRAP) also participates in TLR4 signaling. In contrast to TRIF and TRAM, however, Mal appears to be important in the recruitment of MyD88 to TLR4 to regulate inflammatory cytokine genes (Fitzgerald et al. 2001; Horng et al. 2001; Kagan and Medzhitov 2006).

TLR3-mediated NF-κB activation is also triggered by a TRIF-dependent mechanism. The C-terminus of TRIF associates with the serine threonine kinase receptor interacting protein-1 (RIP1) through a RIP homotypic interaction motif (Meylan et al. 2004). RIP-1-deficient cells fail to activate NF-κB in response to poly (I:C) (Meylan et al. 2004), whereas IRF3 activation remains intact (Cusson-Hermance et al. 2005). The TRIF N-terminal region has also been shown to associate with TRAF6 in overexpression systems (Sato et al. 2003). Studies using macrophages from TRAF6-deficient mice, however, suggest that the exact requirement for TRAF6 in the TLR3 response to NF-κB is still a little unclear, probably due to functional redundancy with other TRAF proteins in certain cell types (Gohda et al. 2004). TAK-1 is also involved in TLR3-mediated NF-κB and MAPK activation (Sato et al. 2005). Recent studies have also shown that TRIF and MyD88 can bind to a second TRAF family member TRAF3, which activates IRFs to induce type IFNs. TRAF3 does not appear to be required for the induction of proinflammatory cytokines, however (Hacker et al. 2006; Oganesyan et al. 2006).

Transcriptional regulation of the IFN-β gene requires the activation of IRF3, ATF-2/c-Jun, and NF-κB. These transcription factors form a multiprotein complex, the enhanceosome on the IFN-β enhancer (Maniatis 1986). In the resting state, IRF3 is localized to the cytoplasm. In response to a viral challenge, IRF3 is phosphorylated on multiple serine/threonine residues, which control

its dimerization. In this active form, IRF3 then translocates to the nucleus and associates with the coactivators CREB-binding protein (CBP)/p300 on the IFN-β enhancer. The IκB-related kinases, inhibitory protein κB kinase (IKK)ε (also called IKK*i* ;Shimada et al. 1999) and TANK-binding kinase (TBK1) (also called NAK [Tojima et al. 2000] or T2K [Bonnard et al. 2000]), phosphorylate IRF3 (Fitzgerald et al. 2003a; Sharma et al. 2003). IKKε and TBK1 are structurally related to IKKα and IKKβ. but, unlike IKKα or IKKβ, do not appear to be involved in NF-κB activation (McWhirter et al. 2004; Sharma et al. 2003). Sharma et al. and Fitzgerald et. al. showed that blocking IKKε and TBK1 activity using RNA interference prevented Sendai virus-induced IRF3 phosphorylation and subsequent activation of the IFN promoter (Fitzgerald et al. 2003a; Sharma et al. 2003). Fitzgerald et al. also described a requirement for IKKε and TBK1 in poly (I:C)-induced IRF3 activation via TLR3 and TLR4 (Fitzgerald et al. 2003a; McWhirter et al. 2004). TBK1$^{-/-}$ embryonic fibroblasts fail to activate IRF3 and induce IFN-β, IFN-α, or ISGs in response to virus, LPS or poly (I:C) (McWhirter et al. 2004). TBK1 is ubiquitously expressed, while IKKε expression is restricted to lymphoid cells, even if it can be inducible in several other cell types. Moreover, IKKε may be functionally redundant with TBK1 in cells where both are expressed (Hemmi et al. 2004; Perry et al. 2004). Perry et al. showed that the Sendai virus-induced IFN response in TBK1$^{-/-}$ embryonic fibroblasts could be partially restored by reconstitution with wild-type IKKε but not with a mutant lacking the kinase activity (Perry et al. 2004).

A schematic representation of the signaling pathways downstream of TLR3 and TLR4 and the role of the adapters TRIF and TRAM in regulating these events are shown in Fig. 1.

3.2 MyD88-Dependent Pathways in pDCs

The first report that described cells with plasma cell morphology in the T cell areas of human reactive lymph nodes was published in 1958 (Lennert and Remmele 1958). These cells were named T-associated plasma cells. Only in 1999, after much debate and several controversial manuscripts, Siegal et al. (1999) reported that the plasmacytoid DCs indeed represented the previously characterized IFN-producing cells (Fitzgerald-Bocarsly 1993; Svensson et al. 1996). In the intervening years, the morphology and functions of pDCs have been fully characterized, together with their intracellular signaling cascades (Barchet et al. 2005; Liu 2005). Following viral infections, human and mouse pDCs are capable of producing up to 10 pg/cell of type I IFNs, making them 10- to 100-fold more efficient than other cell types, including mDCs (Fitzgerald-Bocarsly et al. 1988; Siegal et al. 2001). Moreover, unlike mDCs, pDCs do not express

TLR2, TLR3, TLR4, or TLR5, and therefore they do not respond to the ligands of these TLRs. Remarkably, the TLRs expressed by pDCs are restricted to those that enable recognition of DNA and RNA viruses. In fact, human and murine

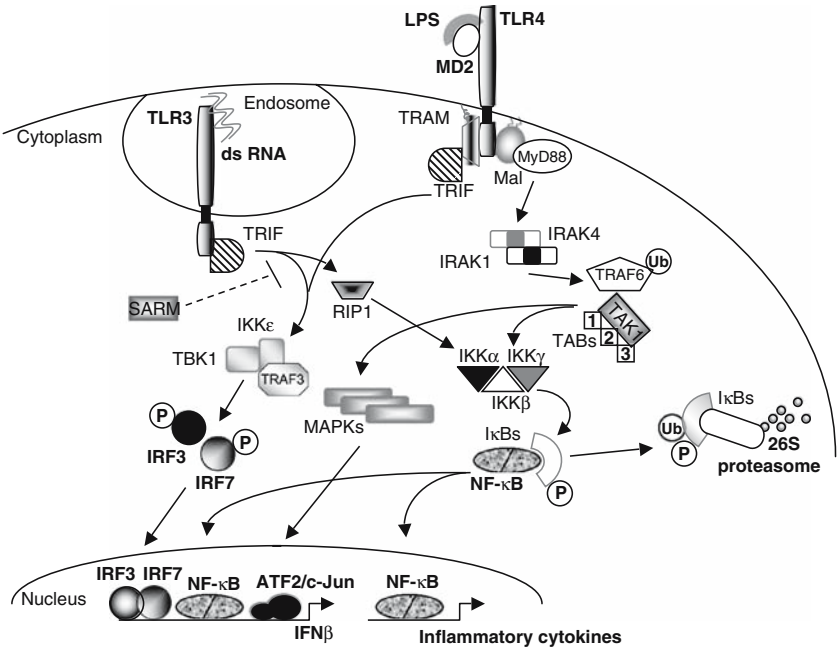

Fig. 1 TRIF-dependent pathways regulating TLR3- and TLR4-mediated activation of IRF3/7 and NF-κB. The adapter molecule Mal/TIRAP contains a phosphatidylinositol 4,5-bisphosphate (PIP2) binding domain, which is important in mediating the recruitment of MyD88 to TLR4. MyD88 associates with the downstream serine/threonine kinases IRAK-1 and -4. A dimeric E2 (or ubiquitin conjugating enzyme) consisting of Ubc13 and Uev1A polyubiquitinates target proteins, including TRAF6. K63-polyubiquintated TRAF6 mediates activation of TAK1-associated proteins TAB2 and TAB3, which interact with K63-ubiquitin chains. The IKK complex is then activated, leading to NF-κB activation. TLR3 signaling to this pathway bypasses MyD88 and IRAKs and possibly TRAF6. Instead TLR3 uses RIP1, which may also be ubiquitinated by TRAF6. Both TLR3 and TLR4-mediated activation of IRF3/7 and the induction of IFN-β take place in a MyD88-independent manner and require TRIF and the IKK-related kinases, IKKε and TBK1. The adapter TRAM (TRIF-related adaptor molecule) is tethered to the plasma membrane via N-terminal myristoylation, which is required to recruit TRIF to the TLR4 cytoplasmic domain. IRF7 is also activated by the IKKε/TBK1 pathway, although it is unclear if transcriptional regulation via IFN-β is required or if this is direct. The TRIF-dependent pathways are negatively regulated by SARM

pDCs express only TLR7 and TLR9 (Bauer et al. 2001; Boonstra et al. 2003; Iwasaki and Medzhitov 2004; Jarrossay et al. 2001; Kadowaki et al. 2001; Krug et al. 2001) and can promptly produce large amounts of type I IFNs in response to either imidazoquinoline compounds (Ito et al. 2002), ssRNA-ODNs, ssRNA viruses (Heil et al. 2004), or CpG-ODNs and DNA viruses (Kadowaki et al. 2001; Krug et al. 2001).

TLR7 is closely related to TLR9 phylogenetically and as such these two receptors have several features in common (Wagner 2004). The signaling pathways activated by these TLRs are completely dependent on MyD88, and there is no evidence that other TIR-domain-containing adapters are involved (Hemmi et al. 2003). In contrast to what was observed in TLR3- and TLR4-activated signaling to IFN genes, TRIF is completely dispensable for type I IFN gene induction in the TLR7 and TLR9 pathways (Hemmi et al. 2000, 2002, 2000). Because the induction of type I IFNs is crucially dependent on the activation of IRFs, this raised the intriguing question of how these TLRs could activate IRFs without the help of TRIF. Compared to mDCs, pDCs express constitutively very high levels of IRF7 (Coccia et al. 2004; Izaguirre et al. 2003). Most cell types, including mDC, require upregulation of IRF7 in response to type I IFN feedback signaling, in order to secrete IFN-α subtypes. In contrast, pDCs are capable of rapidly secreting IFN-α even in the absence of the IFN autocrine loop due to this high basal expression of IRF7 (Barchet et al. 2002). Come clarity to this issue was provided by the observation that the engagement of TLR7 and TLR9 did not lead to the activation of IRF3, but instead activated the related factors IRF7 (Honda et al. 2004; Kawai et al. 2004) and IRF5 (Schoenemeyer et al. 2005). In a key paper from Honda et al., IRF7 has been named the master regulator of type I IFN-dependent immune response (Honda et al. 2005). Using splenic pDCs purified from IRF7 knockout mice, the authors demonstrated that the induction of IFN-α and IFN-β upon HSV-1 and VSV infection, which activate TLR9 (Krug et al. 2004) and TLR7 (Lund et al. 2004), respectively, is completely dependent on IRF7, whereas no difference was observed in IRF3-deficient pDCs. Type I IFN induction was also completely IRF7-dependent when the cells were stimulated with the TLR9 ligand, CpG-ODNs (Honda et al. 2005). Thus in the pDCs, IRF7 and not IRF3 is the key mediator of IFN-α and IFN-β gene expression.

Major advances in understanding how type I IFN production is triggered in the TLR7 and TLR9-activated pathways have been made with the discovery that IRF7 interacts directly with MyD88 to form a complex in the cytoplasm (Honda et al. 2004; Kawai et al. 2004). Moreover, this complex involves the IRAK1/4 kinases and TRAF6 (Honda et al. 2004; Kawai et al. 2004). Data from Kawai et al. has suggested that in addition to being phosphorylated, IRF7 is also ubiquitinated and that the ubiquitin ligase activity of TRAF6 is important

for this event (Kawai et al. 2004). Although IRF7 activation can occur via phosphorylation through the action of the IKKε and/or TBK1 kinases as part of the secondary feedback loop (Caillaud et al. 2005; Sharma et al. 2003), it is unclear at present if either of these kinases participate in TLR7/9 signaling to IRF7 in pDCs. What is clear is that the IRAK kinases participate in the phosphorylation of IRF7 in pDCs (Uematsu et al. 2005). IRAK1 interacts with and phosphorylates IRF7 in vitro and the kinase activity of IRAK1 is necessary for the activation of IRF7. TLR7 and TLR9 ligands are severely impaired in their ability to activate IRF7 and induce IFN-α in IRAK1- and IRAK4-deficient pDCs. A very recent study has also identified a role for IKKα in IRF7 activation in TLR7/9 signaling (Hoshino et al. 2006). Hoshino et al. demonstrated that TLR7/9-induced IFN-α production was severely impaired in IKKα-deficient pDCs and a kinase-deficient IKKα blocked the ability of MyD88 to activate the IFN-α promoter in synergy with IRF7 in overexpression experiments. All of these findings highlight the importance of IRF7 in TLR7 and TLR9 signaling and are summarized in Fig. 2.

3.3 IRF5: The Outsider

Many members of the IRF family are important in innate and/or acquired immunity. Although they share a similar DNA-binding domain at their N-terminus, the different IRFs possess unique characteristics that result in unique protein–protein and protein–DNA interactions leading to unique functions. In most viral infections, dsRNA and LPS signaling can activate IRF3 and IRF7 (Doyle et al. 2002; Fitzgerald et al. 2003b; Kawai et al. 2001). In contrast, the activation of IRF5 is much more restricted. It occurs upon infection with Newcastle disease virus (NDV), VSV, and HSV (Barnes et al. 2002, 2003), while no effect has been detected following Sendai virus infection or dsRNA treatment (Schoenemeyer et al. 2005). Recently, an important role for IRF5 in TLR signaling has been emphasized (Schoenemeyer et al. 2005; Takaoka et al. 2005). IRF5 seems to be highly involved in the induction of proinflammatory cytokines, such as TNF-α, IL-12, and IL-6; in fact, their expression is severely impaired upon TLR4, 5, 7, and 9 triggering in various cells from IRF5 knockout mice (Takaoka et al. 2005). Putative IFN-stimulated response elements in the promoters of these inflammatory cytokines are suggested to bind IRF5. TLR7 and 8 triggering by the imidazoquinoline R-848 induced nuclear translocation of IRF5 in murine macrophages (Schoenemeyer et al. 2005), whereas IRF5 could not be activated by either the TLR3/TRIF pathway or upon SV infection. Data from several groups have shown that SV is detected by the recently identified RNA helicase RIG-I (Rothenfusser et al. 2005; Yoneyama et al. 2004).

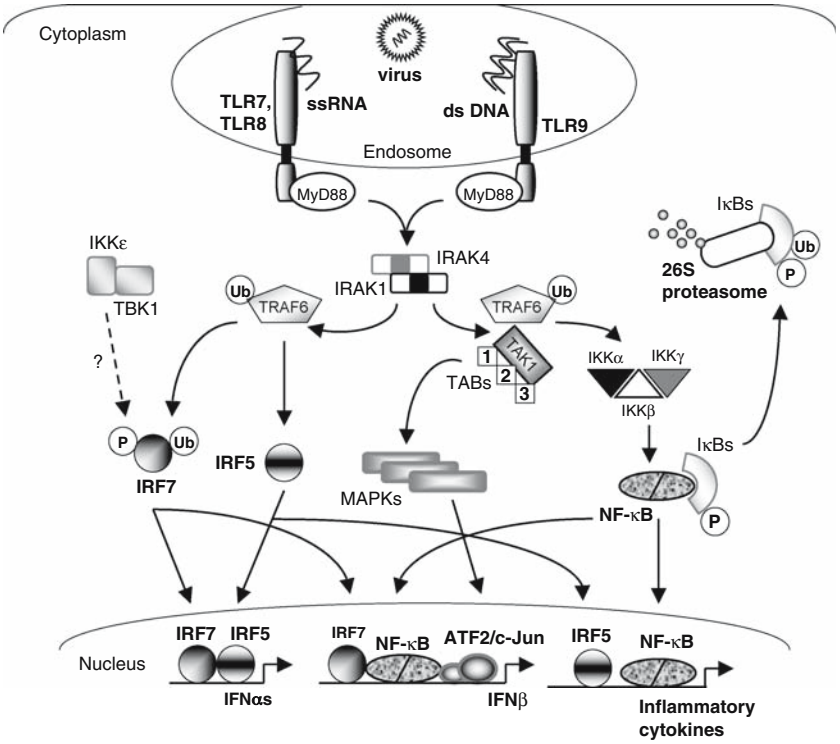

Fig. 2 MyD88-dependent pathways in pDCs. Recognition of viral ssRNA and dsDNA via TLR7/8 and TLR9, respectively, triggers the recruitment of MyD88, which in turn interacts with IRAKs and TRAF6. TRAF6-mediated ubiquitination leads to the activation of TAK1 and ultimately to NF-κB and MAPK activation. IRF5 and IRF7 are also activated, via MyD88. IRAK1 is required to phosphorylate IRF7. IRF7 is also ubiquitinated via K63-polyubiquintation. The activated form of IRF7 can translocate to the nucleus and activate the transcription of IFN-β and IFN-α genes. TRAF6 and IRAK1 are also involved in the activation of IRF5, which is essential for inflammatory cytokine gene induction. IRF5 is activated by all TLRs which signal via MyD88

Several earlier studies had shown that IRF5 and IRF7 could regulate the expression of overlapping as well as distinct IFN-α subtypes (Barnes et al. 2002). In human cells, Schoenemeyer et al. demonstrated that ectopic expression of IRF5 enabled type I IFN production following TLR7 triggering and that knockdown of IRF5 by siRNA in human monocytes reduced this response. In contrast, Takaoka et al. showed that the induction of IFN-α in response to the TLR9

ligand, CpG-ODNs was normal in pDCs derived from IRF5-deficient mice. Observations from Mancl et al. identified nine distinct alternatively spliced IRF5 mRNAs (V1-V9) that have cell type-specific expression, localization, inducibility, and function in virus-mediated type I IFN gene induction (Mancl et al. 2005). Further investigations are needed to better understand the exact role of IRF5 in IFN induction in different pathways and in different cell types.

Consistent with a role for IRF5 in the regulation of inflammatory cytokine production, Schoenemeyer et al. showed that IRF5 is part of a complex with MyD88 and TRAF6 (Schoenemeyer et al. 2005), similarly to IRF7 (Kawai et al. 2004). This resemblance between MyD88-mediated activation of IRF7 and IRF5 is further enforced by the observation that IRAK-1 kinase is important in IRF5 activation (Schoenemeyer et al. 2005). IRF5 can also be phosphorylated and activated upon ectopic expression of TBK1 and IKKε (Cheng et al. 2006). The physiological relevance of these observations remains to be clarified, however, since inflammatory cytokine production (which is controlled by IRF5) is induced normally in TBK1 or IKKε knockout cells (Hemmi et al. 2004; N. Goutagny and K.A. Fitzgerald, unpublished data). MyD88 also interacts with IRF4, which appears to negatively regulate the IRF5 signaling pathway (Negishi et al. 2005). IRF4 deficiency does not affect the ability of TLR7/9-stimulated pDCs to secrete IFN-α but caused overproduction of inflammatory cytokines. This was accompanied by enhanced activation of NF-κB and MAPKs. This hyperreactivity is observed not only in TLR7/9 but also in TLR2/4 signaling. IRF4, but not IRF7, can compete with IRF5 for association with MyD88, which can account for this phenotype of the IRF4 knockout mice. Our current understanding of the role of IRF5 in the antiviral immune responses is shown in Fig. 2.

3.4 Negative Regulators of MyD88 and TRIF Signaling

Several endogenous negative regulators of TLR signaling have been described for the MyD88-dependent pathway. MyD88s is the short form of MyD88 and its overexpression inhibits IL-1- and LPS- but not TNF-induced NF-κB activation (Janssens et al. 2003). Another inhibitor of the MyD88-mediated pathway is IRAK-M, a member of the IRAK kinase family (Wesche et al. 1999), which has been shown to block the formation of IRAK1–TRAF6 complexes (Kobayashi et al. 2002). A different level of regulation occurs through SOCS1, one of eight members of the SOCS family important in suppressing cytokine signaling (Alexander 2002). SOCS1 represses LPS-induced NF-κB activation in a TLR4- and MD2-dependent manner (Kinjyo et al. 2002), and Mansell et al. demonstrated recently that SOCS1 is required for the ubiquitin-proteasome-mediated degradation of Mal (Mansell et al. 2006). The inhibitory effect of

SOCS1 on TLR signaling can also be indirect by blocking type I IFN signaling itself (Baetz et al. 2004; Gingras et al. 2004). Several additional negative regulators of the MyD88 pathway have been described, including PI3K (Fukao et al. 2002), Tollip (Zhang and Ghosh 2002), A20 (Boone et al. 2004), ST2 (Brint et al. 2002), SIGIRR (Wald et al. 2003), and RIP105 (Divanovic et al. 2005), all acting at different levels of the intracellular cascade.

Much less is known about negative regulation of the TRIF-IRF3 response. Carty et al. recently demonstrated that the fifth TIR-domain containing adapter SARM acts as a negative regulator of TRIF signaling (Carty et al. 2006). SARM interacts directly with TRIF leading to a block in gene induction downstream of TRIF. SARM does not target the MyD88 pathway. Knockdown of SARM by siRNA leads to enhanced TRIF-dependent cytokine and chemokine induction.

As discussed above, the IKK-related kinases TBK1 and IKKε are involved in IRF3 activation downstream of TRIF. SIKE (for suppressor of IKKε) is a protein that interacts with both TBK1 and IKKε and dissociates from them upon viral infection or TLR3 stimulation (Huang et al. 2005). Overexpression of SIKE blocks the interaction of TBK1 and IKKε with TRIF and IRF3, but does not influence the interaction of TRIF with TRAF6 or Rip1, essential for NF-κB activation. siRNA targeting of SIKE potentiated virus- and TLR3-induced IRF3 responses. Very recently, Saitoh and colleagues demonstrated that the peptidyl prolyl isomerase Pin1 also negatively regulates the IRF3 pathway. Pin1 associates with activated IRF3 and promotes the ubiquitin-mediated degradation by the proteosome. Phosphorylation of IRF3 on Ser339/Pro440 upon stimulation with poly (I:C), LPS or Newcastle virus is associated with this destabilization of IRF3 (Saitoh et al. 2006). IRF3 and Pin1 interact only when IRF3 is phosphorylated on Ser339. Ectopic expression of Pin1 blocks IRF3 activation and IFN-β production downstream of TLR3 and 4 and the RIG-I pathway. As expected, Pin1-deficient mice produce much more IFN-β in response to dsRNA compared to wild type mice in vivo.

4 Concluding Remarks and Some Speculations

In the last few years we have witnessed an enormous improvement in our understanding of the delineation of TLR signaling, particularly in relation to the pathways that regulate IRF activation. The TLR pathway is particularly important in the pDCs for the detection of viral RNA and DNA associated with endocytosed viral particles. However, it is now becoming increasingly clearer that, in most other cell types, TLR-independent sensors are more critical for antiviral defenses. These TLR-independent sensors include the recently

discovered cytoplasmic RNA helicases, RIG-I (Yoneyama et al. 2004) and Mda-5 (Kang et al. 2004) and a putative cytosolic DNA sensor, which remains to be defined. Signaling through these cytoplasmic receptors converges on many of the same signaling intermediates as those employed by the TLRs. Elucidation of the cross-talk between these different sensors and pathways in the response to a given virus remains a key challenge in our quest to understand innate immunity to viruses.

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