BIOCHEMICAL PHARMACOLOGY 75 (2008) 589–602

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journal homepage: www.elsevier.com/locate/biochempharm

Commentary

The interplay between viruses and innate immune signaling: Recent insights and therapeutic opportunities

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ARTICLE INFO

Article history:
Received 5 June 2007
Accepted 23 July 2007

Keywords:
Viruses
Innate immunity
Signaling
Pattern-recognition receptors
Viral evasion

ABSTRACT

The immediate response to viral infection relies on pattern-recognition receptors (PRRs), most prominently the Toll-like receptors (TLRs) and the RNA helicases RIG-I and MDA5, as well as double stranded RNA-dependent protein kinase (PKR) and the DNA receptor, DAI. These PRRs recognize pathogen-associated molecular patterns (PAMPs) such as viral proteins and nucleic acids. The engagement of these receptors then initiates intracellular signaling cascades which ultimately cause the activation of transcription factors and the expression of type I interferons and pro-inflammatory cytokines. This innate response establishes an anti-viral state in the infected cell and its neighbours and alerts immune cells to the danger. In order to establish a productive infection, viruses need to overcome this initial anti-viral response. Evasion of innate immune defences is achieved by means of viral proteins that inhibit the signaling cascades emanating from the PRRs. The same innate signal transduction pathways have been implicated in conditions of sterile inflammation, such as rheumatoid arthritis and multiple sclerosis, and in autoimmunity. Because viral proteins target crucial host proteins involved in these pathways, they can point the way to key drug targets. Further, the viral proteins themselves or derivatives of them may be of use therapeutically to curtail inflammation and autoimmunity.

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Abbreviations: AdV, adenovirus; ASFV, African swine fever virus; CARD, caspase recruitment domain; CMV, cytomegalovirus; DC, dentritic cell; ds, double-stranded; EBV, Epstein-Barr virus; eIF, eukaryotic initiation factor; EMCV, encephalomyocarditis virus; FADD, Fas-associated via death domain; HCV, hepatitis C virus; HHV, human herpes virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; IAV, influenza A virus; IFN, interferon; I-κB, inhibitor of nuclear factor κB; IKK, I-κb kinase; IL, interleukin; IPS-1, interferon-β promoter stimulator-1; IRAK, IL-1 receptor associated kinase; IRF, interferon regulatory factor; JEV, Japanese encephalitis virus; JNK, Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MCMV, mouse cytomegalovirus; MDA5, melanoma differentiation-associated gene 5; mDC, myeloid DC; MMTV, mouse mammary tumor virus; MV, measles virus; MyD88, myeloid differentiation primary response gene 88; NAP, NAK-associated protein; NEMO, NF-κB essential modulator; NDV, Newcastle disease virus; NF-κB, nuclear factor κB; PAMP, pathogen-associated molecular pattern; pDC, plasmacytoid DC; PKR, dsRNA-dependent protein kinase; PRR, pattern-recognition receptor; RHIM, RIP homotypic interaction motif; RIG-I, retinoic acid-inducible gene I; RIP, receptor interacting protein; ss, single-stranded; RSV, Rous sarcoma virus; SeV, Sendai virus; TAB, TAK1-binding protein; TAK, TGF-β activated protein kinase; TANK, TRAF family member-associated NF-κB activator; TBK, TANK-binding kinase; TIR, Toll/interleukin-1 receptor; TLR, Toll-like receptor; TNF, tumor necrosis factor; TRAF, tumor necrosis factor receptor-associated factor; TRIF, TIR-domain containing adaptor inducing IFN-β; VACV, vaccinia virus; VSV, vesicular stomatitis virus.

0006-2952/$ – see front matter
doi:10.1016/j.bcp.2007.07.043

1. Introduction

The ability of the innate immune system to detect and respond to pathogens is critical for survival. Although the mammalian response to viruses has been studied for many years, it is only recently that a broad understanding of how pathogen-associated molecular patterns (PAMPs) on viruses are detected by host pattern-recognition receptors (PRRs) has emerged. Viral protein and nucleic acid PAMPs have now been identified as acting on distinct classes of anti-viral PRRs, which when stimulated by virus lead to the initiation of host signaling pathways. For example, the transcription factors interferon regulatory factor 3 (IRF3), IRF7 and nuclear factor κB (NF-κB) are turned on by most anti-viral PRRs and co-operate to induce type I interferons (IFNs), which are critical in initiating and coordinating an anti-viral response. Apart from the identification and characterization of novel PRRs, recent work has revealed how PRRs recognize viral PAMPs at the molecular level and which PRRs are particularly important for detection of distinct viruses. Further, examining the workings of these PRR pathways has revealed much about how transcription factors such as NF-κB and the IRFs are regulated. Viruses have many ways of evading and subverting immune recognition pathways, and studying these mechanisms can provide an insight into the fine detail of such host pathways. Because viruses strategically target crucial host proteins involved in immunity, they can point the way to key drug targets. Further, viral proteins or derivatives of them may be of use as drugs since manipulation of innate immunity is of interest therapeutically. This is because PRR signaling pathways are not only critical to the response to infectious disease, but they also play a role in inflammatory and autoimmune diseases [1].

2. dsRNA-dependent protein kinase (PKR)

The first viral PRR to be proposed was PKR (see Table 1), an intracellular detector of viral dsRNA and poly(I:C). dsRNA has long been recognised to be a candidate viral PAMP, and the synthetic dsRNA poly(I:C) has been extensively used to mimic viral infection. dsRNA is present either as viral genome in dsRNA viruses, or produced as replication intermediate in viruses with a positive strand ssRNA genome or as a by-product of convergent transcription in DNA viruses. PKR is activated upon binding of dsRNA, leading to its dimerization and autophosphorylation. A current model of PKR activation involves the assembly of several PKR monomers on a dsRNA molecule. This is proposed to promote the dimerization of

Table 1 – Detection of viral PAMPs by PRRs, and viral counter-measures

PRR	Viral PAMP	Virus (genome)	Viral evasion protein	References
PKR	dsRNA	HCV (+ssRNA)	NS5A, E2	[59]
	dsRNA	VACV (dsDNA)	K3L, E3L	[59]
	dsRNA	EBV (dsDNA)	EBER-1, -2 RNA	[59]
TLR4	Fusion protein	RSV (−ssRNA)		[16]
	env protein	MMTV (ssRNA)		[91]
	gpG	VSV (−ssRNA)		[17]
TLR3	Genomic dsRNA	Reovirus (dsRNA)		[3]
	?	IAV (−ssRNA)		[92]
	?	CMV (dsDNA)		[93]
	?	VACV (dsDNA)	A46, A52	[32,76,94]
TLR2	HA protein	MV (+ssRNA)		[80]
	?	HSV1 (dsDNA)		[95]
	?	VACV (dsDNA)	A46, A52	[32,76,96]
TLR9	Genomic DNA	HSV1, HSV2 (dsDNA)	ICP0	[9,10,64,97]
	Genomic DNA	MCMV (dsDNA)		[93]
TLR7/8	Genomic ssRNA	HIV (ssRNA)		[98]
	?	SeV (−ssRNA)		[99]
	?	VSV, IAV (−ssRNA)		[14]
RIG-I	ss leader RNA	VSV (−ssRNA)	M	[50,52,100]
	ss leader RNA	MV (−ssRNA)	C	[100,101]
	Genomic RNA	IAV (−ssRNA)	NS1	[50,52]
	?	NDV, SeV (−ssRNA)		[50,99]
	Genomic RNA (3′ and 5′ NTR)	HCV (+ssRNA)	NS3/4A	[41,45]
	?	JEV (+ssRNA)		[50]
	EBER ssRNAs	EBV (dsDNA)	BZLF-1	[51,66]
MDA5	Intracellular dsRNA?	EMCV (+ssRNA)		[49,50]
	?	NDV, SeV (−ssRNA)	V	[38]
DNA receptor	Genomic DNA	CMV (dsDNA)	IE86	[54,102]
	Genomic DNA	HSV1 (dsDNA)	ICP0	[54,64]
	Genomic DNA	AdV (dsDNA)		[55]

PKR, leading to its autophosphorylation and activation [103]. Once active, PKR dissociates from the RNA and phosphorylates the eukaryotic initiation factor 2α (eIF2α), leading to inhibition of translation. Knock-out mouse studies confirmed a role for PKR in anti-viral innate immunity [2]. However, even though PKR stimulation has been implicated in many signaling pathways including NF-κB activation, the ability of PKR to turn off translation likely represents the unique contribution of this PRR to anti-viral immunity. Since PKR is an IFN-inducible gene, other PRRs likely use PKR as a downstream effector.

3. Toll-like receptors

3.1. TLRs detect viral nucleic acid

The second PRR for viral dsRNA, TLR3 (see Table 1), was discovered based on the observation that mice defective in PKR could still respond to poly(I:C) [3]. Cells from TLR3 −/− mice showed reduced induction of cytokines and type I IFNs in response to poly(I:C) in comparison to wild type cells [3]. Also, reovirus genomic dsRNA failed to induce CD69 expression in splenocytes from TLR3 −/− animals. TLR3 senses viral RNA predominantly in endosomes, but at least in some cells types, also at the cell membrane [4]. The structure of the human TLR3 extracellular domain has now been solved [5,6], which revealed a large horseshoe-shaped solenoid comprising 23 leucine-rich repeats. The extracellular domain is largely covered by carbohydrates, but one face which is glycosylation-free, contained two patches of positively charged residues. Choe et al. [5] and Bell et al. [6] showed direct binding of poly(I:C) to the ectodomain, and Bell et al. went on to locate the poly(I:C) binding site on TLR3 by mutational analysis [7], which confirmed that the glycan-free surface was indeed the site of ligand binding. Although there is no doubt that TLR3 can mediate cellular responses to dsRNA and poly(I:C), its particular role in anti-viral innate immunity is still being defined [8].

TLRs have also been implicated in sensing other types of viral nucleic acid, apart from dsRNA. TLR9 (see Table 1) was first shown to sense unmethylated CpG dinucleotides in bacterial DNA. Herpes simplex virus (HSV), a dsDNA virus with such motifs in its genome, has been shown induce type I IFN in plasmacytoid dendritic cells (pDCs) through TLR9 [9,10]. Like TLR3, TLR9 is located in intracellular endosomal compartments [11], and as such TLR9 can sample endocytosed material and trigger an innate immune response whenever an endocytosed dsDNA virus is present. TLR7 and TLR8, which are more closely related to TLR9 than to other TLRs in terms of sequence similarity and signaling mechanism (see below), also act in endosomes where they have been shown to trigger gene induction in response to viral ssRNA. Guanosine- and uridine-rich ssRNA oligonucleotides derived from HIV-1 were shown to stimulate DCs and macrophages to secrete IFN-α and pro-inflammatory cytokines, via human TLR8 and murine TLR7 [12]. IFN-α induction by murine pDCs in response to influenza virus or vesicular stomatitis virus (VSV) also relied on endosomal TLR7 [13,14]. Diebold et al. [15] determined the molecular basis for the recognition of ssRNA by TLR7 by showing that uridine and ribose are both necessary and sufficient for TLR7 stimulation. Short ssRNAs act as TLR7 agonists in a sequence-independent manner as long as they contain several uridines in close proximity. Both viral and 'self' RNA can trigger TLR7 activation equally efficiently, if delivered to endosomes [15]. This suggests that TLR7 discriminates between viral and 'self' ligands on the basis of endosomal accessibility and uracil content rather than sequence.

3.2. TLRs detect viral proteins

The first viral protein shown to induce cellular responses in a TLR-dependent manner was the fusion (F) protein of respiratory syncytial virus (RSV), which stimulated secretion of IL-6 from wild type cells, but not those isolated from C3H/HeJ mice (which have a point mutation in TLR4) or C57BL10/ScCr mice (in which the gene encoding TLR4 is deleted) [16]. More recently, a VSV protein (glycoprotein G) has been shown to induce IFN-α through TLR4-dependent IRF7 activation in myeloid DCs (mDCs) and macrophages [17]. Likewise, TLR2 has been shown to mediate cellular responses to viral glycoproteins. For cytomegalovirus (CMV), TLR2 was shown to mediate NF-κB activation and cytokine induction in response to UV-inactivated virions, and two CMV envelope glycoproteins, gB and gH, have been shown to mediate these effects, and to coimmunoprecipitate with TLR2 and TLR1 [18].

3.3. Anti-viral signaling pathways activated by TLRs

There is now a detailed molecular description of the signaling pathways activated by TLRs leading to anti-viral effector mechanisms, although some of the detail is inferred from studies examining TIR-dependent pathways stimulated by non-viral agonists (especially interleukin-1 and lipopolysaccharide), and many questions still remain [19]. Fig. 1 shows a schematic diagram of three important anti-viral TLR signaling pathways, namely TLR3, TLR7 and TLR9, which are activated by viral dsRNA, ssRNA and dsDNA, respectively. For simplicity, here we will focus on activation of the key anti-viral transcription factors NF-κB, IRF3 and IRF7. Signaling is initiated intracellularly, in endosomal compartments containing viral nucleic acid, although the demonstration that signaling by TLR3, 7 and 9 is dependent on receptor association with an endoplasmic recticulum (ER)-resident membrane protein, UNC93B, suggests that the ER may be a compartment from which signaling initiates [20]. All TLRs are thought to signal as dimers, and to be preassembled in a low-affinity complex prior to ligand binding [19]. TLRs have a characteristic intracellular signaling domain termed the Toll/IL-1 receptor (TIR) domain, which is critical for mediating downstream signaling through homotypic interactions. Ligand binding likely causes a conformational change in the TLR dimer, which brings the TIR domains into closer proximity. This is thought to create a new signaling surface which then recruits TIR domain-containing adaptor molecules such as myeloid differentiation factor 88 (MyD88) in the case of TLR7 and TLR9, and TIR domain-containing adaptor inducing IFN-β (TRIF) in the case of TLR3 [19].

The formation of the TLR3/TRIF complex triggers the recruitment of further signaling molecules which interact

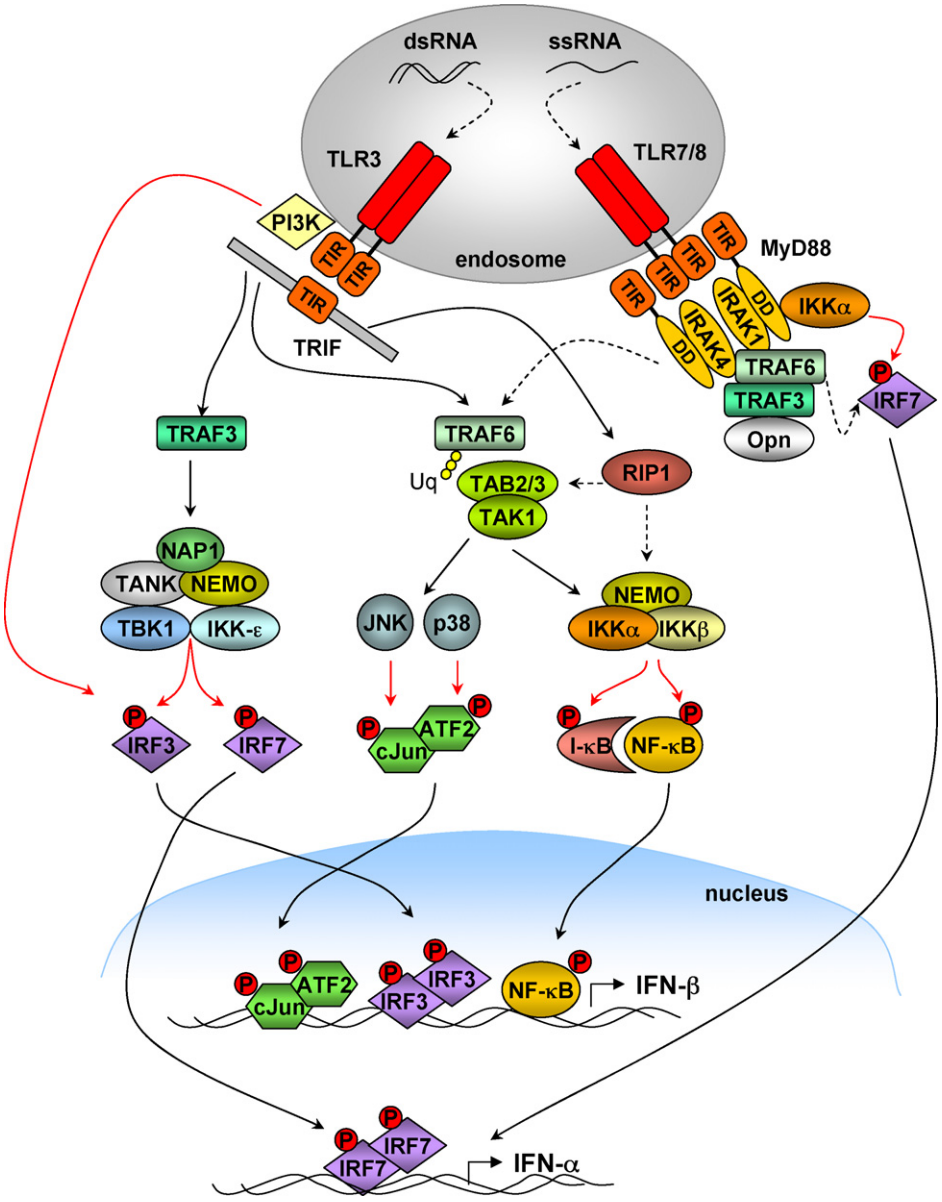

Fig. 1 – Anti-viral TLR signaling pathways involved in interferon induction. Recognition of viral dsRNA (by TLR3) and ssRNA (by TLR7 or TLR8) in the endosomes of plasmacytoid dendritic cells leads to the dimerization of TLRs, and the recruitment of the adaptor proteins MyD88 or TRIF via their TIR domains. This then results in the assmbly of signaling complexes and the initiation of several signaling cascades that ultimately result in the phosphorylation and thus activation of the transcription factors IRF3, IRF7, NF-κB, ATF2 and cJun. Upon activation, the transcription factors translocate to the nucleus and induce the transcription of type I interferons (IFN-α and β). TLR9, which is not shown in the figure for clarity, responds to viral dsDNA in endosomes and is thought to signal similarly to TLR7 and TLR8. Red arrows indicate phosphorylation. Dashed arrows represent less well-defined interactions. Uq, lysine-63-linked ubiquitin chain. See text for further details.

with TRIF. For NF-κB activation, two separate pathways bifurcate from TRIF, and these map to distinct sites at its N- and C-termini. In the N-terminal region of TRIF there are binding motifs for tumor necrosis factor receptor-associated factor 6 (TRAF6), which likely integrate TRAF6 into the TLR3 signaling pathway to NF-κB. TRAF6 is thought to be critical for all TLR pathways to NF-κB, and functions upstream of the inhibitor of NF-κB kinase (IKK) complex which contains IKKα and IKKβ, associated with an essential scaffold protein, NF-κB essential modulator (NEMO). It is now well established that the inhibitor of NF-κB (I-κB) sequesters NF-κB dimers in the cytoplasm until it is phosphorylated by the IKK complex, leading to its lysine-48-linked ubiquitination and degradation. Thereafter, NF-κB is free to translocate to the nucleus to activate its target genes. How TRAF6 participates in IKK activation has become clear in recent years. TRAF6 is a RING-domain ubiquitin E3 ligase which functions with an E2 enzyme complex containing UBC13 and UEV1A to generate lysine-63-linked polyubiquitin chains on target proteins, such as NEMO and TRAF6 itself [21]. Lysine-63-linked polyubiquitin chains

generally influence the signaling properties of a protein, rather than causing its degradation. How exactly TRAF6 E3 ligase activity is turned on by upstream TLR3 signaling or TRIF engagement is still unclear, but it has been shown that lysine-63-linked TRAF6 auto-ubiquitination is essential for downstream IKK activation [22]. The kinase that activates the IKK complex in the TLR pathways is transforming growth factor-β-activated kinase (TAK1), and TAK1 is recruited to TRAF6 and subsequently activated via two adaptor proteins, TAK-binding proteins TAB2 and TAB3, which can associate with ubiquitinated TRAF6 [21]. TAK1 then phosphorylates IKKβ in its activation loop, leading to IKK activation.

There is also a distinct route to NF-κB activation involving the C-terminus of TRIF, which contains a receptor interacting protein (RIP) homotypic interaction motif (RHIM). TRIF recruits RIP1 through this domain, and in mouse embryonic fibroblasts lacking RIP1, poly(I:C)-induced NF-κB activation was completely blocked [23]. Similar to TRAF6, RIP1 can also acquire lysine-63-linked ubiquitin chains, and can engage with TAB2/TAB3/TAK1 in response to tumor necrosis factor signaling. A similar mechanism of activation may apply to the TLR3 pathway. In addition to NF-κB activation, the C-terminal RHIM of TRIF also induces an apoptotic pathway involving RIP1, Fas-associated death domain protein (FADD) and caspase-8 [24].

IRF3 activation by TLR3 also proceeds via TRIF. The main IRF3 kinase activated by TLR3 is TANK-binding kinase TBK1, although IKKε is also likely involved in some contexts. A TBK1 complex, which also contains the adaptor TRAF-associated NF-κB activator (TANK) and possibly IKKε, is recruited to the TRIF N-terminal region and subsequently activated in a process involving both TRAF3 and NAK-associated protein (NAP) 1, although the fine detail of this process is still unclear [19]. This TBK1-containing complex can also phosphorylate and activate IRF7. Once phosphorylated, IRF3 or IRF7 homodimerize and translocate to the nucleus. In addition to this TRIF-dependent pathway, phosphoinositol-3 (PI3) kinase recruitment to TLR3 is also essential for full IRF3 activation [25]. PI3 kinase is recruited to phosphorylated tyrosine residues in the cytoplasmic domain of TLR3 and, via the downstream kinase Akt, causes additional phosphorylation of IRF3, which is necessary for the interaction with the transcriptional co-activator CBP [25].

TLR7/8 and 9 signal though the TIR-containing adaptor MyD88. Formation of the TLR7- or TLR9-MyD88 complex leads to the recruitment of the IL-1 receptor associated kinase 1 (IRAK-1) and IRAK-4. IRAK-4 had been proposed to have a role in TLR7 and TLR9 signaling both as an early activated kinase (which phosphorylates IRAK-1) and as a scaffold protein (i.e. in a function independent of its kinase activity). Mice lacking IRAK-4 display attenuated TLR9-induced cytokine responses, and studies using 'knock-in' mice where the wild type IRAK-4 gene was replaced by a mutant gene encoding kinase-deficient IRAK-4 have clarified the contribution of IRAK-4's kinase activity to TLR7 and TLR9 signaling. This showed that IRAK-4 kinase activity was required for TLR7-induced NF-κB activation and for TLR7- and TLR9-dependent production of cytokines [26–28]. Further, IFN-α production in response to TLR7 and TLR9 ligation was also severely curtailed, and consistent with this, TLR9-mediated IRF7 activation was blocked [27]. Thus the kinase activity of IRAK-4 is necessary for both NF-κB and IRF7 activation by TLR7 and TLR9. In contrast to IRAK-4, the role of IRAK-1, a key substrate for IRAK-4 kinase activity, is more restricted in TLR7 and 9 signaling. Although IRAK-1 was originally thought to have a central role in the TLR-NF-κB pathway, IRAK-1 was dispensable for TLR9-induced NF-κB activation and pro-inflammatory cytokine production in dendritic cells [29]. Moreover, IRF7 has been identified as a target of IRAK-1 kinase activity [29]. Thus, for TLR7 and TLR9, the pathways to NF-κB and IRF7 activation bifurcate upstream of IRAK-1, at the level of IRAK-4. The activation of NF-κB by TLR7 and TLR9 probably also involves stimulation of TRAF6 auto-ubiquitination, and subsequent TAK1 recruitment, as in the case of TLR3. In contrast to TLR7 and TLR9 signaling, there does not seem to be a role for IRAK-1 [30] or IRAK-4 [31] in TLR3 signaling, although there is some evidence that IRAK-2 is important [32].

In terms of IRF7 activation, the formation of an IRF7/IRAK-1 complex followed by IRAK-1-mediated phosphorylation of IRF7 appears to be a prerequisite for IFN-α induction by TLR7 [33]. MyD88 has also been shown to interact with IRF7, as has TRAF6, and both proteins are essential for TLR9-induced IFN-α production [33]. As in the case of NF-κB activation, the ubiquitin E3 ligase activity of TRAF6 is necessary for IRF7 activation. Like TRAF6, IKKα is a signaling molecule which was thought to be primarily involved in NF-κB activation, but has more recently been shown to be essential for TLR7/9-induced IRF7 phosphorylation [34]. Finally, TRAF3 and osteopontin have also been shown to be required for this pathway, although their mechanism of involvement is as yet undefined [33]. After TLR9 engagement, colocalization of osteopontin and MyD88 was found to be associated with IRF7-dependent IFN-α induction, whereas IRF7 nuclear translocation was deficient in plasmacytoid DCs lacking osteopontin [35].

It is now clear that the endosomal TLR7 and TLR9 pathways to IRF7 activation account for the production of IFN-α in plasmacytoid DCs, known to play a crucial role during viral infection. TLR9 is also expressed in myeloid DCs and macrophages where it can induce type I IFNs through IRF1, rather than IRF7. However, TLR engagement does not account for virally induced type I IFN production in many other cell types, such as epithelial cells [8]. Further, *in vivo*, TLR3 may have alternative roles in anti-viral immunity not directly linked to type I IFN induction [8]. These and other observations suggested that further anti-viral PRRs must exist.

4. The helicases RIG-I and MDA5

4.1. *Discovery*

While PKR and TLR3 can bind dsRNA and contribute to the innate immune response, gene targeting in mice revealed that neither of the two proteins are essential for the initial type I interferon response in virally infected cells or in response to poly(I:C) (reviewed in [4]), suggesting that an additional dsRNA sensor may exist. In 2004, the two RNA helicases RIG-I and MDA5 (see Table 1) were identified as cytoplasmic sensors of viral RNA [36–38].

RIG-I was identified by screening a cDNA library for clones that enhanced the induction of IRF activation following

treatment with poly(I:C) [36], while MDA5 was found to be a binding target for the V protein of paramyxoviruses, which inhibits IFN-β production by dsRNA [38]. The two proteins are closely related members of the DExD/H box-containing RNA helicase family, with a conserved domain structure. They contain two N-terminal caspase recruitment domain (CARD) motifs, and a C-terminal DExD/H box helicase domain. The CARD motifs of RIG-I and MDA5 activate downstream signaling cascades that result in the activation of IRF3, IRF7 and NF-κB.

A third member of the RIG-I/MDA5 helicase family, LGP2, possesses a helicase domain which is closely related to that of RIG-I and MDA5, but lacks an N-terminal CARD domain [37]. Thus, LGP2 is unlikely to activate downstream signaling pathways on its own, and probably plays a regulatory role.

4.2. *How RIG-I signals*

The expression of RIG-I, MDA5 and LGP2 is induced at the transcriptional level by retinoic acid, type I IFNs, and dsRNA. Once present in the cell, their signaling capacity is tightly regulated. In the case of RIG-I, it has been suggested that the helicase domain can act as a molecular switch, which controls whether the CARD domain is available for the activation of downstream signaling. This has been proposed based on the observation that only those RNA molecules that are efficiently unwound by RIG-I are potent activators of the type I interferon response [39]. Thus, it can be envisaged that the remodelling of RNA structures by the helicase activity of RIG-I may lead to a conformational change which exposes the N-terminal CARD domains, allowing them to activate downstream signaling pathways. A repressor domain at C-terminus of RIG-I co-operates with the helicase domain during RNA binding, and inhibits the multimerization of RIG-I and its binding to its downstream adaptor [40,41]. LGP2, which contains a similar repressor domain, may use this to inhibit RIG-I in trans, by interacting directly with RIG-I and masking its CARD domain [40,41]. MDA5 does not possess a repressor domain, and neither autorepression nor regulation by LGP2 have been observed [40,41].

The adaptor which connects RIG-I and MDA5 to the downstream effects was identified by four independent groups as a CARD-containing protein, named IFN-β promoter stimulator-1 (IPS-1) [42], mitochondrial anti-viral signaling (MAVS) [43], virus-induced signaling adaptor (VISA) [44] and CARD adaptor inducing INF-β (Cardif) [45]. Here we will refer to the adaptor as IPS-1. IPS-1 harbors a CARD domain near its N-terminus, which is highly homologous to the CARD domains of the helicases RIG-I and MDA5. As Fig. 2 shows, IPS-1 transduces signals from the two helicases by means of CARD–CARD interactions, and is essential for the dsRNA-induced activation of NF-κB, IRF3 and IRF7 [42–46]. IPS-1 has been suggested to make use of IKKα, IKKβ, IKKε and TBK-1 to activate the IRFs and NF-κB. The interaction between IPS-1 and the IRF-activating kinases TBK1 and IKKε is bridged by TRAF3, a signaling factor also involved in TLR-activated pathways [47]. Recently a novel role for NEMO in the RIG-I-IRF pathway has been shown, whereby NEMO associated with TANK facilitates the recruitment of TBK1 and IKKε to the IPS-1-TRAF complex, leading to TBK1 and IKKε activation [48]. IPS-1 has also been shown to interact with TRAF2, TRAF6, FADD and RIP1, all of which are involved in the NF-κB activation pathway [42,44]. However, the precise role of these proteins in the signaling cascades emanating from IPS-1 is still unclear. The involvement of TRAF6, in particular, is controversial, as IPS-1 can induce the IFN-β promoter in mouse embryonic fibroblasts lacking TRAF6, and mutation of the TRAF6 interaction motif does not abrogate its signaling capacity [43]. While FADD and RIP1 are also dispensable for IFN-β promoter activation, they appear to be required for the activation of NF-κB by IPS-1 [42,43]. It was suggested that IPS-1 interacts with TRIF and participates in TLR3-dependent signaling [44]. However, more recent experiments using IPS-1 knock-out mice show that IPS-1 is dispensable for TLR signaling, but is specifically required for the innate immune response against intracellular dsRNA and infection by RNA viruses [46].

Another intriguing feature of IPS-1 is its localization to the outer mitochondrial membrane, mediated by a short transmembrane domain near the C-terminus [43]. While the functional significance of this observation has yet to be defined, it is clear that the mitochondrial localization of IPS-1 is essential for its ability to activate signaling pathways, as an IPS-1 construct lacking the transmembrane domain loses its ability to signal [43]. Furthermore, the NS3/4 protease of hepatitis C virus (HCV) interferes with this anti-viral signaling pathway by cleaving off the transmembrane domain, rendering IPS-1 inactive [45].

4.3. *RNA recognition by RIG-I and MDA5*

Originally, both RIG-I and MDA5 were thought to recognise cytoplasmic double-stranded RNA during viral infection [37]. However, the generation of knock-out mice lacking either RIG-I or MDA5 revealed that the two helicases are not redundant in their ability to recognize viral RNA. While MDA5 is essential for the recognition of picornaviruses such as encephalomyocarditis virus (EMCV) [49,50], RIG-I is required for the recognition of many other negative strand RNA viruses, such as Sendai virus, vesicular stomatitis virus and influenza A virus, as well as the positive strand Japanese encephalitis virus [50]. RIG-I is also involved in sensing the small Epstein-Barr virus (EBV) non-translated RNAs EBER-1 and -2 [51]. Furthermore, RIG-I but not MDA5 appears to sense the presence of RNA species transcribed *in vitro* by viral polymerases, while MDA5 is the principal intracellular receptor for synthetic poly(I:C) [49,50]. Thus, it is clear that the two helicases, despite their apparent similarities, are not functionally equivalent.

In line with these differences, recent observations have challenged the notion that the PAMP sensed by both MDA5 and RIG-I is dsRNA. Picornaviruses, which are recognised by MDA5 do produce large quantities of dsRNA during their replication cycle, supporting the hypothesis that MDA5 might recognise a dsRNA structure. However, viruses that produce undetectable amounts of dsRNA, such as influenza A virus, are nevertheless potent activators of RIG-I [52]. Indeed, more detailed experiments revealed that both single- or double-stranded RNA with a triphosphate moiety at the 5′ end can activate type I interferon production via RIG-I [52,53]. It cannot be excluded, however, that RIG-I activation requires double-stranded regions within an RNA molecule, as RNA commonly folds into hairpins and loops even in regions of low complimentarity.

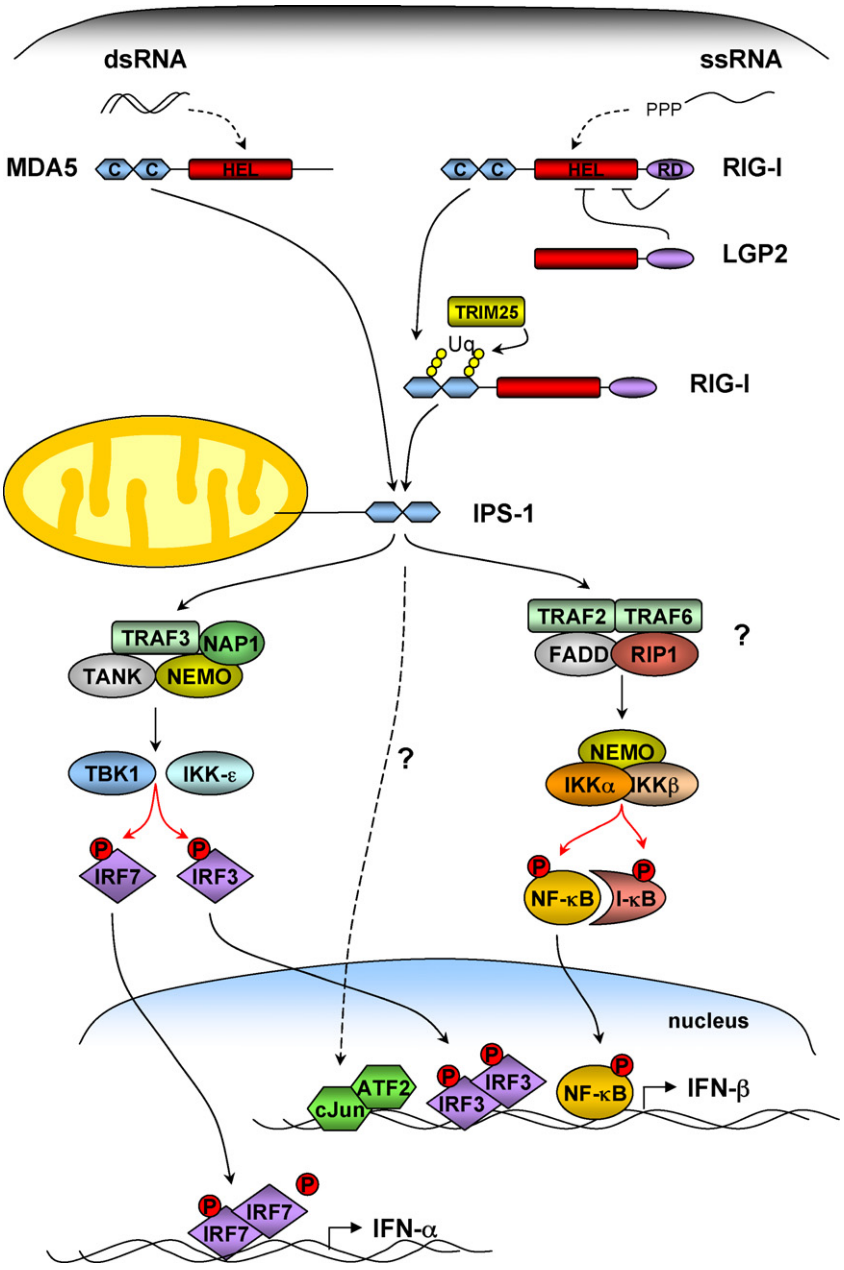

Fig. 2 – Anti-viral signaling by RIG-I and Mda5. ssRNA with a 5′ triphosphate group and dsRNA are detected by the cytoplamsic RNA helicases RIG-I and MDA5, respectively. Activation of RIG-I involves lysine-63-linked ubiquitination (Uq) by the ubiquitin ligase TRIM25. This activation is inhibited by the repressor domains (RD) and the helicase domains (HEL) of LGP2 and RIG-I itself. Both MDA5 and RIG-I activate the adaptor protein IPS-1 via CARD domain (C) interactions. IPS-1 then induces signaling pathways that result in the activation of the transcription factors IRF3, IRF7 and NF-κB. The exact role of TRAF2, TRAF6, FADD and RIP1 in this pathway is yet to be defined. IPS-1 signaling likely also involves a MAP kinase cascade for the activation of cJun and ATF2 (dashed arrow). Red arrows indicate phosphorylation. See text for further details.

More recent studies on the molecular basis of RNA recognition by RIG-I provide some insight into how the innate immune system distinguishes viral RNAs from the abundance of cellular RNA species. Ribosomal RNA (rRNA) and transfer RNA (tRNA), which make up the bulk of the RNA present in a cell, possess a 5′ monophosphate, and thus would not be detected as triphosphate-containing viral RNA by RIG-I. Analogously, pre-microRNA transcripts generated by the ribonuclease III enzyme Drosha and mature microRNAs processed by DICER possess a 5′ monophosphate, and thus may not efficiently target RIG-I *in vivo*. In addition, their 2 nucleotide 3′ overhang appears to be important in allowing them to escape detection by RIG-I or other dsRNA sensors in the cell [39]. The 5′ end of mature messenger RNAs (mRNAs)

and small nuclear RNAs (snRNAs) is modified with a methylguanosine cap. In addition, many abundant cellular RNAs are posttranscriptionally modified, and possess modified bases or a methylated sugar backbone. Hornung et al. [53] showed that some common eukaryotic posttranscriptional modifications, such as pseudouridine, 2-thiouridine or 2′-O-methylation suppress the immunostimulatory activity of RNA, even if the RNA contains a 5′ triphosphate. Thus, it seems that several features of endogenous RNAs co-operate to mark 'self' molecules. Some abundant cellular non-coding RNAs possess a 5′ triphosphate group, but do not activate the innate immune system. This is the case for example for the abundant 7SL RNA which is part of the signal recognition particle that accompanies nascent proteins to the endoplasmic reticulum. It is likely that additional, as yet undefined, distinguishing features are recognized by the innate immune system. Also, it has to be kept in mind that RNA generally does not exists as 'naked' molecule in a cellular environment, but forms complexes with a multitude of RNA-binding proteins. Thus, it is conceivable that protein components of the complex may also play a role in marking certain RNA species as 'self'.

5. Recognition of cytoplasmic DNA

DNA can be detected by TLR9 in endosomes, and this pathway is of particular importance in plasmacytoid dendritic cells. However, an additional, non-TLR-dependent pathway appears to exist in other cell types including macrophages and non-immune cells, where dsDNA is recognized in the cytoplasm [54]. In the experimental systems where cytoplasmic DNA recognition was first described, dsDNA originating from a variety of sources was able to elicit a type I interferon response. This was true for DNA isolated from bacteria, viruses and mammals, and for the synthetic DNA molecule poly(dA-dT) [54]. In these cases, the DNA was introduced into the cells by transfection. A similar DNA-dependent response was observed during infection with the bacterium *Listeria monocytogenes*, which invades the cytoplasm of infected cells, and activates TLRs and nucleotide-binding and oligomerization domain proteins 1 and 2 (NOD1/2), as well as an independent DNA-mediated signaling pathway [54]. Furthermore, when bacterial DNA or DNA from apoptotic cells was able to enter the cytoplasm through a bacterial type IV secretion system or the protein Listeriolysin O, this also caused the induction of IFN-β in macrophages [54]. Infection with adenovirus, which possesses a DNA genome, also activated the interferon response in macrophages and primary lung fibroblasts in a DNA-dependent and TLR-independent manner [55]. Finally, a further study uncovered this pathway in deoxynuclease II-deficient mice that accumulate DNA of engulfed apoptotic cells in macrophages and mount a strong TLR-independent type I interferon response [56]. Thus, it appears that not only microbial, but also 'self' DNA can stimulate the innate immune system, as long as the DNA accumulates in the cytoplasm.

In macrophages and non-immune cells the recognition of cytoplasmic DNA leads to the activation of IRF3, and possibly also NF-κB, and consequently the induction of IFN-β [54,56]. One cellular receptor for dsDNA has now been identified as the Z-DNA binding protein 1 (ZBP-1), also called DNA-dependent activator of IRFs (DAI) [57]. The expression of DAI is strongly induced in mouse embryonic fibroblasts following treatment with DNA or IFN-β. DAI binds dsDNA, and also associates with IRF3 and TBK1 upon stimulation with DNA. Mouse fibroblasts expressing small interfering RNAs for the depletion of DAI produced less IFN-β in response to synthetic, viral and bacterial DNA, and following infection with the DNA virus HSV1 [57]. Mice lacking the gene encoding DAI will need to be generated, in order to establish whether DAI is the only cytoplasmic DNA receptor, and to what extent it contributes to innate immunity *in vivo*.

The signaling pathway that is activated by dsDNA is only starting to be characterized. While the kinases TBK-1 and IKKε do play a role in this elusive pathway [54], none of the known adaptor proteins that activate these kinases in other instances appear to be involved. Thus, induction of IFN-β in response to cytoplasmic DNA is unimpaired in mouse fibroblasts lacking the TLR adaptors MyD88 and TRIF, the helicases RIG-I or MDA5, the NOD-interacting protein RIP1 or the DNA repair factors DNA-PK, p53 or ATM [54,56]. While IPS-1 was originally implicated in the DNA recognition pathway using small interfering RNAs in human embryonic kidney cells [54], experiments in IPS-1-null mouse embryonic fibroblasts clearly indicate that this adaptor is dispensable in this system [46,58]. Following on from the identification of the DNA receptor DAI, additional adaptors and regulators of this pathway will certainly be identified in the near future.

6. Viral counter-measures against innate signaling pathways

In parallel to the discovery and definition of the anti-viral signaling pathways described above, viral anti-immunity mechanisms which target these systems have been discovered. These discoveries have not only thrown further light on the anti-viral response, but also will likely lead to novel therapeutic strategies to manipulate innate immune signaling (as discussed in Section 7). Viruses need to evade or counteract the immediate cellular defenses, such as the IFN system, in order to establish a productive infection. Thus, even small viruses that possess only very few open reading frames, invest a considerable portion of their genomes into modulating the innate immune system.

6.1. *Inhibition of PKR*

PKR's role as a critical anti-viral effector protein leading to inhibition of translation and hence shut-down of infected cells, make it a target for viral evasion strategies. The vaccinia virus (VACV) protein E3L, influenza virus NS1 and the reovirus σ3 protein all sequester dsRNA and thus prevent it from activating PKR or other dsRNA receptors [59]. In addition, several viruses express double-stranded or structured RNA molecules that act as competitive inhibitors of dsRNA binding by PKR. These include the adenovirus VAI RNA, the EBV RNAs EBER-1 and EBER-2 and the region of the HCV genome containing a structured internal ribosomal entry site (IRES)

[59]. Furthermore, PKR is also inhibited by direct interaction with several viral proteins, such as the HCV proteins NS5A and E2, VACV E3L, and the Ebola virus protein VP35 [59,60]. In a further strategy to prevent a block in translation, several viruses encode eIF2α homologues, such as the VACV K3 protein, which acts as a pseudosubstrate for PKR [61].

6.2. *Inhibition of IRF3 and IRF7*

Viruses that possess only few genes need to focus their disruptive efforts to a limited number of host targets that play a central role in the anti-viral response. The many different signaling pathways that are activated by PAMP recognition converge at the level of transcription factor activation, which in turn results in the expression of a multitude of anti-viral genes. Thus, transcription factors are particularly good targets for viruses that need to prevent the activation of the innate immune system with a limited toolbox of viral protein products.

IRF3 and IRF7 are prime candidates for viral inhibition, as they are crucial for the initial induction of type I interferons during viral infection. The phosphoproteins (P) of many non-segmented negative strand RNA viruses, such as Rabies virus, Borna disease virus and Ebola virus (where the analogous protein is called VP35), prevent the activation of IRF3 by inhibiting the kinase TBK1 [53]. In cells expressing the P or VP35 protein, IRF3 is not phosphorylated, and does not dimerize or translocate to the nucleus. Thus, even after viral engagement of PRRs, the expression of IFN-β cannot be induced.

Human herpes virus 8 (HHV-8) blocks IRF signaling in a different way: it possesses several IRF analogues which have dominant negative activity, and thus interfere with the function of cellular IRF proteins [62]. Yet another strategy that is adopted by some viruses is to eliminate IRF3 altogether. For example, the ICP0 protein from bovine herpes virus has been shown to interact with IRF3 and to mediate its proteasome-dependent degradation [63]. Notably, the function of ICP0 appears to be only partially conserved in other herpes viruses. HSV ICP0 also interacts with IRF3, but instead of causing its degradation, it sequesters IRF3 and its co-activator CBP/p300 in specialized nuclear bodies and thus prevents it from binding to DNA [64].

It is evident that different viruses employ different strategies to prevent IRF3 from fulfilling its role as a transcriptional activator. The observation that so many different viruses target IRF3 reflects the notion that IRF3 plays a central role in the activation of the initial anti-viral response. While IRF3 and its viral antagonists have been studied extensively for a number of years, the role of IRF7 as a key regulator of the anti-viral response has only recently been recognized [65]. The EBV immediate early protein BZLF-1 binds IRF7 directly, and prevents it from activating type I IFN promoters [66].

In many cell types IRF7 protein stability is dynamically regulated. IRF7 is generally a short-lived protein that is continually turned over in a proteasome-dependent manner. In fibroblasts, IRF7 is destabilized further by infection with Newcastle disease virus (NDV) [67]. Proteasome-dependent degradation of IRF7 is also promoted by the rotavirus NSP1 protein, which in addition causes the degradation of IRF3 and IRF5 [68]. Intriguingly, splenocytes and thymocytes appear to be resistant to NDV-induced IRF7 turnover, and indeed exhibit greater IRF7 stability after viral infection or interferon treatment [67].

Further, it is likely that many of the viral countermeasures that target IRF3 also act on IRF7, in particular those that impair the function of the upstream kinases TBK1 and IKKε.

6.3. *Modulation of NF-κB activity*

Although NF-κB is required for IFN-β induction, not all viruses aim to inhibit NF-κB, since this transcription factor has other functions that would favour certain viruses such as in preventing apoptosis and promoting cell proliferation. Indeed, apart from activation of NF-κB due to virally triggered PRR activation, some viruses have evolved mechanisms to actively stimulate NF-κB activation [69]. In fact the genomes of HIV-1, CMV, HPV-16, EBV and hepatitis B virus all possess gene promoters that contain NF-κB binding sites, and thus rely on elevated NF-κB activity for their life cycle (reviewed in [69]).

However, given the role of NF-κB in IFN-β and pro-inflammatory cytokine induction, many viruses do seek to curtail NF-κB activation, particularly at early stages of infection when the virus is most vulnerable to elimination by the innate immune system. For example, African swine fever virus (ASFV) regulates NF-κB activity in a biphasic manner, where virus-induced NF-κB activation is initially inhibited by the early viral protein A238L which acts as a degradation-resistant I-κB homologue [70]. However, once ASFV infection has progressed further, NF-κB activity is stimulated by the late viral protein A224L, which has homology to cellular inhibitors of apoptosis (IAPs) and acts on both NF-κB and caspases in order to prevent apoptosis and prolong the infection [71].

Inhibition of NF-κB activity often targets the step of I-κB degradation which controls the release of the activated NF-κB dimer (prototypically p50/p65) into the nucleus. The 3C protease from coxsackievirus cleaves I-κBα, creating a fragment that accompanies NF-κB subunits into the nucleus and prevents their activation [72]. The relationship between viruses and host gene expression is undoubtedly complex, and given the multiple cellular mechanisms for regulating transcription factor activation, it is likely that many new examples of viral manipulation of NF-κB will continue to become apparent upon more detailed investigation.

6.4. *Inhibition and subversion of PRR signaling pathways upstream of transcription factor activation*

In addition to targeting the transcription factors which turn on anti-viral genes, some viruses also interfere with more upstream components of the signaling pathways, in order to obtain a more complete, or perhaps more specific, inhibition of the innate immune system. Due to genomic space constraints, viral proteins are often multifunctional, and a single viral protein may target several independent components of a signaling cascade. Viruses with larger genomes have the luxury of availing of a particularly large toolbox of many multifunctional immunomodulatory factors, and appear to

interfere with virtually every step of innate immune activation [73,74].

Vaccinia virus (VACV) is a large DNA virus with over 200 open reading frames, many of which are involved in host–pathogen interactions. Two VACV proteins, A46 and A52 have been found to be specific inhibitors of TLR-dependent signaling [75]. A46 binds the TLR adaptors MyD88, Mal, TRIF and TRAM leading to inhibition of downstream signaling, while A52 targets TRAF6 and IRAK-2 which disrupts formation of signaling complexes [32,76]. Viruses lacking either of the two proteins are attenuated in mouse infection models [32,76], despite the fact that VACV also possesses additional proteins that interfere with the activation of NF-κB and IRF3 further downstream [77–79]. Thus, it appears that blocking several steps in a given pathway might be necessary for optimal inhibition of the innate immune system, as the cell may itself have developed complex and partially redundant signaling networks to avoid viral countermeasures. Alternatively, in an *in vivo* infection, different VACV signaling inhibitors may come into play in different cell types or at different stages of infection. Another viral protein that acts on the TLR signaling pathway is the NS3/4A protease from HCV, an RNA virus with great expertise in immune evasion. During chronic infection with HCV, the NS3/4A protease cleaves the TLR3 adapter TRIF, thus disrupting the anti-viral response triggered by dsRNA in endosomes [45].

As well as inhibition of TLR signaling, viruses have also been shown to hijack TLR pathways for their own purposes. For example, in the case of measles virus, activation of TLR2-dependent signaling by hamagglutinin likely contributes to virus spread by upregulating CD150, the measles virus cellular receptor [80]. Mouse mammary tumor virus infection is dependent on TLR4 signaling, which triggers production of the immunoregulatory cytokine IL-10 [81]. Further, in mice lacking functional TLR4, the virus was eliminated by the cytotoxic immune response [81]. Interestingly, VACV A52 can also manipulate IL-10 via the TLR pathway, since as well as inhibiting NF-κB activation and chemokine production, A52 expression also enhances MAP kinase activation via TRAF6, leading to induction of the IL-10 promoter and enhancement of TLR-induced IL-10 production [82]. Hence in VACV-infected cells where TLRs have been activated, A52 may direct the signaling pathway towards IL-10 induction and away from chemokine production. Consistent with this, many other viruses have mechanisms to enhance host IL-10 production, or in fact encode their own IL-10 orthologs, and it has been shown that elevated IL-10 production is important for the persistence of the RNA virus lymphocytic choriomeningitis virus *in vivo* [83].

In the short time since the discovery of RIG-I and MDA5, many viral proteins have been shown to antagonize these pathways. The influenza virus NS1 protein interacts with IPS-1 and RIG-I and inhibits their function [84], while the V protein of many other paramyxoviruses interacts with MDA5 [38]. As well as acting on TRIF, HCV NS3/4A also cleaves IPS-1 [45]. Cleavage of IPS-1 releases it from the mitochondrial membrane, and this abolishes its signaling activity. Hepatitis A virus, which belongs to a different RNA virus family, also causes the degradation of IPS-1 using the 3ABC protein [85]. A transmembrane domain targets 3ABC to mitochondrial membranes where IPS-1 resides, and mitochondrial targeting, as well as protease activity are essential for 3ABC function. The identification of such specific inhibition of the RNA helicase pathway caused by a variety of viruses emphasizes the importance of these PRRs. As in other cases, the host factors that are inhibited by a certain virus often provide some information towards which signaling pathways are involved in the detection of the virus, thus validating the role of newly identified signaling components.

7. Therapeutic opportunities

The growing understanding of the mechanisms of innate immune signaling pathways has led to an appreciation of their role not only in infectious disease, but also in the initiation and progression of inflammatory disease and of autoimmunity [1]. This is because as well as responding to PAMPs, PRRs can also in certain contexts be stimulated by self molecules. At present the number of disease states in which a role for TLRs is proposed is increasing rapidly, although the other innate immune pathways more recently described are also likely to play a role. Inflammation is a complex process underlying a large number of acute and chronic diseases such as sepsis, rheumatoid arthritis, multiple sclerosis and colitis. Most therapeutic approaches to blocking inflammation target individual effector cytokines such as TNF, often with good effect. Given the evidence that TLRs have a crucial role in the production of such effector cytokines, targeting the initial intracellular TLR signaling cascades directly is ultimately likely to be an even more effective approach. As TLRs are involved in both pathogen-induced and sterile inflammation, there is therapeutic interest not only in PRR agonists (to enhance an anti-pathogen response, for example as an adjuvant during vaccination) but also in antagonists (to suppress inflammation and autoimmunity). It is likely that particular PRRs, and certain downstream signaling pathways, make different relative contributions to distinct disease states. For example, TLR2 has been implicated in rheumatoid arthritis and atherosclerosis, while TLR7, TLR8 and TLR9 have been strongly linked to autoimmunity [86]. In particular, TLRs are thought to contribute to the autoimmune disease systemic lupus erythematosus due to the inappropriate recognition of 'self' nucleic acids and related immune complexes by TLR7 and TLR9 leading to the release of autoantibodies from B cells and IFN-α from plasmacytoid DCs [87].

There are a number of ways that interactions between viruses and PRR signaling pathways can be exploited therapeutically. Firstly, understanding the molecular basis of the viral PAMP–PRR interaction will help inform the design of synthetic PRR agonists for use as adjuvants. Secondly, since viruses strategically target crucial host signaling proteins, they can point the way to key drug targets, which may not otherwise become apparent.

Finally, viral proteins themselves, or derivatives of them (such as peptides or peptidomimetics), may be useful as drugs. Viral immunosuppressive proteins have been finely tuned and honed by evolution to target the host immune system with maximal effectiveness. This is analogous to a 'naturally occurring drug development programme', whereby the protein

has already undergone cycles of modification due to natural selection, leading to enhanced inhibitory function. This might be expected to give rise to optimized viral protein interaction sites which potently bind to host proteins and disrupt their function, and therefore peptides based on these sites may be useful.

Peptides based on a particular region of host TIR adaptor proteins such as MyD88 called the 'BB loop' have recently been shown to block TLR signaling [88]. These BB loop peptides display some differential sensitivity to different TLR pathways and act presumably by disrupting protein–protein interactions between two TIR proteins (such as TLR9 and MyD88), and thus suppressing the formation of signaling complexes. Viral peptides would be expected to act in a similar manner, but with greater potency. Of note, short cell-permeable peptides derived from the VACV TLR inhibitor A52 have been shown to inhibit TLR-induced production of key inflammatory mediators *in vitro* [89], while also being effective at reducing LPS-induced inflammation, liver damage and mortality in mice [89,90]. Since A52 inhibits NF-κB and not IRF activation by TLRs [32,76], use of A52-derived peptides might preserve anti-viral immunity while inhibiting an over-active inflammatory response that causes pathogenesis. Certainly, exploring the therapeutic use of peptides and peptidomimetics derived from viral PRR signaling inhibitors warrants further focus.

Acknowledgements

Work in the author's lab is funded by Science Foundation Ireland, Enterprise Ireland and the Irish Health Research Board. LU is supported by a Marie Curie Intra-European Fellowship.

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