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Sensing and Signaling in Antiviral Innate Immunity

Minireview

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Viruses are detected by the innate immune system, leading to the initiation of the anti-viral immune response via the production of type I interferons and inflammatory cytokines such as interleukin-1. Remarkable progress has been made over the past few years towards understanding the contribution of Toll-like receptors, RIG-I like receptors, NOD-like receptors and HIN-200 family members to viral detection. Furthermore, new complexities in the signaling pathways activated by these receptors continue to be revealed. Together, these new insights are leading to therapeutically useful information in the fight against viruses.

Introduction

In the first line of defense against viruses, type I interferons (IFNα and IFNβ) and cytokines such as interleukin-1β (IL-1β) initiate the host anti-viral response. Much effort has gone into elucidating the pattern-recognition receptors that sense viral components and the downstream intracellular signaling pathways that lead to IFN and IL-1β induction.

The discovery in 2000 that two proteins from the vaccinia virus interfere with Toll-like receptor (TLR) signaling was the first indication that TLRs might be important in the sensing of viruses [1]: at that point the best characterised TLR was TLR4, which senses lipopolysaccharide, a component of the cell wall of Gram-negative bacteria. When either of the genes encoding these vaccinia proteins was deleted from the viral genome, the infectivity of the resulting virus was attenuated *in vivo*, pointing to the importance of these proteins in virulence [2,3]. Subsequently, tremendous progress was made in revealing the role of TLRs and other innate immune receptors in anti-viral defense [4]. At the cell surface and at endosomal compartments, TLRs recognise both DNA and RNA viruses. In later studies, additional sensors of viral nucleic acids were identified and found to localise to the cytosol, notably the RIG-I-like receptors (RLRs) RIG-I and MDA5, which can sense RNA viruses, and DAI, which can sense DNA viruses [4]. NOD-like receptor (NLR) family members were also shown to engage with both DNA and RNA viruses, in particular the NLR NALP3, which activates caspase-1 in the inflammasome, leading to the production of the cytokines IL-1β and IL-18 [4]. More recently, the HIN-200 family protein AIM2, which also activates caspase-1, has been shown to sense DNA viruses such as vaccinia [5]. We therefore now have a greatly improved understanding of the innate sensing of viruses. Here, we review recent findings in this area that reveal additional complexity in terms of sensing and signaling of viruses by these receptor systems.

Growing Role for TLRs in Anti-Viral Signaling

Work on TLR signaling pathways in viral sensing continues to reveal substantial complexity (Figure 1). Two recent studies provide us with new insights into the TLR3- and TLR4-mediated regulation of the activation of IFN regulatory factor 3 (IRF3), one of the critical transcription factors for IFNβ induction. Both of these TLRs promote activation of IRF3 via the adapter protein TRIF, which leads to the activation of the kinase TBK1 and subsequent phosphorylation of IRF3. Palsson-McDermott *et al.* [6] have reported insights into a specific negative regulator of TRAM, the TRIF-related adapter molecule used exclusively by TLR4 to recruit TRIF: this protein is termed TRAM adapter with GOLD domain (TAG) and is a splice variant of TRAM that acts at the late endosome to displace TRIF from the TLR4–TRAM complex and limit activation of IRF3. Knockdown of TAG with small interfering RNA (siRNA) boosts the IRF3 pathway and this approach could be useful as a vaccine adjuvant to promote type I IFN production, given that the TRAM–TRIF pathway has been shown to be especially important for adjuvancy. Insertion of the gene encoding TRAM into a vaccine DNA vector with a sequence encoding an HIV peptide led to a threefold stimulation of the *in vivo* $CD8^+$ T-cell response to this peptide [7]. These kinds of approach might lead to better vaccine adjuvants.

In another study, the basis for how TLR4 can signal to NFκB (which is required for both IFNβ and IL-1β induction) on the one hand, and IRF3 on the other has been determined [8] (Figure 1). Tumor necrosis factor (TNF) receptor associated factor 3 (TRAF3) is a key regulator of both pathways but it acts in different ways. In the case of NFκB activation, TRAF3 undergoes ubiquitin-mediated degradation during signaling mediated by the MyD88 adaptor protein, and this degradation of TRAF3 allows the MyD88 signaling complex to translocate to the cytosol to engage with the kinase TAK-1. The degradation-associated K48-linked ubiquitination of TRAF3 is performed by cIAP2, itself a ubiquitin E3 ligase that is activated via non-proteolytic, regulatory K63-linked ubiquitination by TRAF6 after TLR4 stimulation. In the case of IRF3 activation, a separate pool of activated TLR4 complexes comprising TLR4 and TRAM translocates to the endosome, where TRAF3 is recruited and undergoes K63-linked ubiquitination. This modification of TRAF3 appears to be required for IRF3 activation by TLR4 and probably also by TLR3 [8]. These events are therefore likely to be important for the induction of anti-viral cytokines by these TLRs.

A further role for ubiquitination in TLR3 and TLR4 signalling has been uncovered by the finding that the E3 ubiquitin ligase Nrdp1 can preferentially promote the induction of type I IFNs by these TLRs, at the expense of pro-inflammatory gene expression [9]. Nrdp1 mediates K48-linked ubiquitination of MyD88 leading to MyD88 degradation, while also stimulating K63-linked ubiquitination of TBK1, leading to its activation and IFN induction. Nrdp1 was shown to protect mice from vesicular stomatitis virus infection because of its ability to promote IFN production. The TRAF3 and Nrdp1 studies reveal an increasingly complex picture of ubiquitin E3 ligases in TLR signaling, which would seem to have a role at least as important as that of kinases.

As well as TLR3 and TLR4, TLR2 has recently been a surprising addition to the list of TLRs capable of mediating

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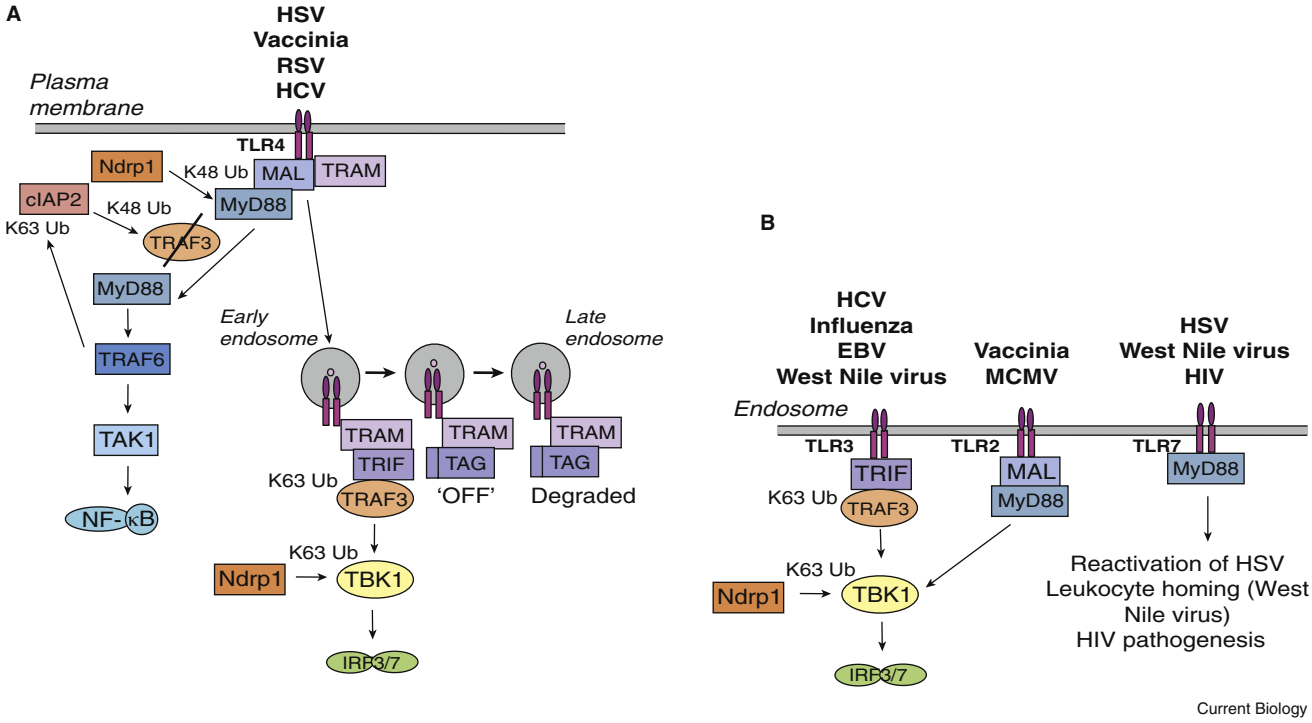

Figure 1. Recent developments in TLR-mediated sensing of viruses.

New insights have emerged regarding the regulation of TLR4 and TLR3 signaling, as well as new roles for TLR2 and TLR7 in the response to certain viruses. (A) TRAF3 is an important inhibitor of plasma membrane signaling by TLR4, a TLR implicated in the response to herpes simplex virus (HSV), vaccinia, Rous sarcoma virus (RSV) and hepatitis C virus (HCV). TRAF3 is degraded following cIAP2-mediated, K48-linked ubiquitination (K48 Ub), which requires TRAF6-mediated K63-linked ubiquitination (K63 Ub) of cIAP2. This releases the MyD88 signaling complex, which engages via TRAF6 with TAK1 leading to NF-κB activation. The TLR4–TRAM complex translocates to the early endosome, where, via TRIF, it activates TBK1 leading to IRF3 activation. This also requires TRAF3, but in this case, TRAF3 is modified by K63-linked (not K48-linked) ubiquitination. The TRAM splice variant TAG then displaces TRIF to turn off the signal ('OFF'), with TLR4 ultimately being degraded in the late endosome. TBK1 also undergoes K63-linked ubiquitination, mediated by Nrdp1. (B) At the endosome, TLR3, which is involved in the sensing of West Nile virus, influenza virus, HCV and Epstein-Barr virus (EBV; via the sensing of EBER), engages with TRIF and TRAF3 (which is modified by K63-linked ubiquitination) to activate TBK1. TLR2 has also been shown to engage at the endosome with TBK1 via the MAL–MyD88 complex, in response to vaccinia and murine cytomegalovirus (MCMV). Recent findings on endosomal TLR7 indicate an important role in leukocyte trafficking in response to West Nile virus, reactivation of HSV in response to vesicular stomatitis virus and finally in the pathogenesis of AIDS by HIV.

type I IFN induction. Barbalat *et al.* [10] showed that vaccinia and murine cytomegalovirus can induce type I IFNs via TLR2. A particular cell type in the bone marrow and spleen — Ly6C^{hi} inflammatory monocytes — was shown to be the key cell type involved in this response, which may explain why this phenomenon was missed in previous studies. Known bacterial ligands for TLR2 were unable to drive this IFN response, which was shown to require TLR2 internalisation. TLR2 can therefore recognize a viral-associated molecular pattern and drive type I IFN production.

A further insight into the role of TLRs in viral diseases came from Town *et al.* [11], who showed that deficiency in either TLR7 or MyD88 dramatically impaired leukocyte homing during infection with West Nile virus, resulting in increased mortality and higher viral burdens. Interestingly, IL-23, which is required for acquired immunity and leukocyte trafficking, was shown to be a particularly important effector cytokine in this model of infection. TLR7 activation by West Nile virus has also been shown to promote migration of Langerhans cells from the skin to lymph nodes, which could be an important mechanism for triggering adaptive immunity to the virus [12]. The current treatment for West Nile viral infection is very limited, so TLR7 agonists might be of use here.

A different study, however, showed that ligation of TLR7 might actually affect viral latency [13]. Agonists specific for TLR7/8 have been shown to reactivate latent Kaposi's sarcoma-associated herpesvirus, inducing viral lytic gene transcription and replication. Vesicular stomatitis virus, which is sensed by TLR7/8, also reactivated this herpes virus from latency, suggesting that infections sensed by TLR7 might be important triggers for episodic reactivation of latent herpes viruses.

TLR7 has also proved to be relevant to HIV pathogenesis as it may be important for explaining why HIV-1-infected women tend to have lower viral loads early in HIV-1 infection but progress more quickly to AIDS for a given viral load compared with men [14]. Meier *et al.* [14] demonstrated significant sex differences in the response of plasmacytoid dendritic cells to HIV. Plasmacytoid dendritic cells from women produced markedly more IFNα in response to HIV-1-encoded TLR7 ligands when compared with those from men. Considerably higher levels of CD8$^+$ T-cell activation were also found in treatment-naïve women chronically infected with HIV-1. It is likely that there is higher immune activation in women in response to HIV, which might lead to faster HIV disease progression. The mechanism here is not clear but could involve the presence of increased cell

numbers (both dendritic cells and $CD4^+$ T cells) for infection by the virus and possibly enhanced infection due to induction of receptors such as the C-type lectin DC-SIGN on dendritic cells. The data suggest that inhibition of the TLR7 pathway in plasmacytoid dendritic cells might represent a new approach for treating HIV-1 infection.

Apart from TLR7, another TLR recently implicated in the pathogenesis of human viral disease is TLR3. Most Epstein-Barr virus (EBV) infections are asymptomatic, but in certain cases the virus can trigger infectious mononucleosis and EBV-associated hemophagocytic lymphohistiocytosis, thought to be a result of sudden release of inflammatory cytokines. Iwakiri *et al.* [15] showed that this cytokine production is probably induced by the release of small viral RNAs called EBERs from infected cells, which activate immune cells (probably human blood dendritic cells) via TLR3 signalling to produce IFNγ and TNF. EBERs are non-coding RNAs that form stem-loop structures by intermolecular base-pairing, giving rise to double-stranded RNA (dsRNA)-like molecules. Thus, TLR3 significantly contributes to the pathogenesis of EBV infection.

Cytosolic Detection of Viruses by NLRs and RLRs

Viral infection is known to promote IL-1β production, which is important for the inflammatory response to viruses and also for the induction of fever, an important clinical feature of viral diseases. IL-1β is produced in a pro-IL-1β form that must be cleaved by caspase-1 to become active. Caspase-1 is found within multiprotein complexes termed inflammasomes, the best-characterized of which contains the NLR NALP3, which has been shown to be required for IL-1β processing in response to both adenovirus [16] and RNA derived from influenza (Figure 2) [17]. NALP3 is unlikely to bind directly to viral nucleic acids, however, and is more likely to sense an as yet ill-defined event, possibly membrane perturbation. In addition to NALP3, an IFN-inducible HIN-200 family protein termed AIM2 has been shown to activate caspase-1 in response to cytosolic DNA [5,18–20] and vaccinia [5] (Figure 2). Apart from its DNA-binding HIN domain, AIM2 (like NALP3) has a pyrin domain, the domain required for recruitment of caspase-1 via the inflammasome component ASC. Both NALP3 and AIM2 are likely to be important for the induction of fever during viral infections and also for the production of IL-18, which is an important cytokine for promoting responses from type 1 T helper cells during viral infections.

Similar to NALP3 and AIM2, the RLR RIG-I has also now been shown to mediate IL-1β production in response to certain viruses. Poeck *et al.* [21] showed that RIG-I-dependent viruses and a synthetic RIG-I ligand were capable of activating the caspase-1-dependent inflammasome in a NALP3-independent manner by a novel pathway involving a complex containing RIG-I and ASC, and not requiring the adapter protein MAVS [21] (Figure 2) — the first example of MAVS-independent RIG-I signaling. In contrast, viruses sensed by the RLR MDA5 did require NALP3 for inflammasome activation [21].

NOD2 is another NLR that has recently been implicated in the anti-viral response. NOD2 was previously thought only to sense the bacterial peptidoglycan breakdown product muramyl dipeptide, but it has now been shown to bind viral single-stranded RNA (ssRNA) and activate IRF3, leading to IFNβ production [22]. NOD2 was also shown to sense Rous sarcoma virus and mediate viral killing in 293 cells and was required *in vivo* for host defense against this virus. NOD2 signaled via MAVS, which again is something of a surprise, as MAVS was thought to be involved only in RLR signaling (Figure 2).

Although RLRs now have an established role in the cytosolic detection of multiple RNA viruses [23], it is only very recently that the exact viral RNA moiety they recognize has become apparent. For RIG-I, previous reports had shown that a 5′ triphosphate group in the RNA ligand was required for stimulating this RLR, which had superseded the idea that RIG-I recognizes dsRNA, since some ssRNAs with 5′ triphosphate groups are also recognized [24,25]. However, two groups recently showed that pure ssRNA with a 5′ triphosphate group is unable to activate RIG-I [26,27]. Rather, when using chemically synthesized oligoribonucleotides, the optimal RIG-I agonist was shown to be blunt-ended 5′ triphosphate dsRNA at least 20 base pairs in length [27]. The reason for the previous confusion is that *in vitro* transcribed ssRNA often contains base-paired structures as a result of 'copy back' from the 3′ end. These studies explain how RIG-I detects negative-stranded RNA viruses, such as rabies, which lack stretches of dsRNA but contain blunt, short 5′ triphosphate dsRNA in the panhandle region of their single-stranded genome [26,27]. For MDA5, it had been assumed that the ligand for this RLR was long dsRNA, and indeed Pichlmair *et al.* [28] showed that RNA extracted from cells infected with encephalomyocarditis virus or vaccinia could induce MDA5-dependent type I IFN production. However, the stimulatory activity of the RNA resided in higher-order structures containing both ssRNA and dsRNA, leading the authors to conclude that MDA5 recognises branched RNA in web-like structures rather than long linear molecules of dsRNA.

As is the case for TLRs, new roles for ubiquitin and E3 ligases have recently been revealed in the RLR signaling pathways. It was already known that the E3 ligase TRIM25 can positively regulate RIG-I, while, similar to TLR3/4-mediated IRF3 activation, TRAF3 also has a role in RLR signaling and IRF3 activation downstream of MAVS [29]. The importance of ubiquitination in the RLR pathway was further underscored by Zeng *et al.* [30], who showed that the ubiquitin-conjugating enzyme (E2) Ubc5 has an essential role in IRF3 activation downstream of MAVS, probably by catalyzing the addition of K63-linked chains to components of the MAVS signaling complex, to recruit TBK1 via the ubiquitin-binding domains of NEMO.

In another study, a novel pathway that downregulates RLR signaling via degradation of MAVS was uncovered [31]. This pathway involves the protein PCBP2, which was previously shown to have a role in regulating mRNA stability and translation, but was shown here to be upregulated in response to viral infection and to interact with MAVS. PCBP2 mediated MAVS degradation by recruiting the E3 ligase AIP4 to polyubiquitinate MAVS. In cells lacking AIP4, the RLR-mediated antiviral response was exaggerated and prolonged [31]. Thus, although PCBP2 has a defined role in regulation of RNA stability and translation, it can also 'moonlight' as a more direct regulator of innate immune signaling. This is reminiscent of another recently identified component of RLR signaling, DEAD-box protein 3 (DDX3), which has a well-defined role in multiple aspects of RNA metabolism but has now also been shown to directly regulate the TBK1–IKKε complex required for IRF3 activation downstream of RIG-I [32,33].

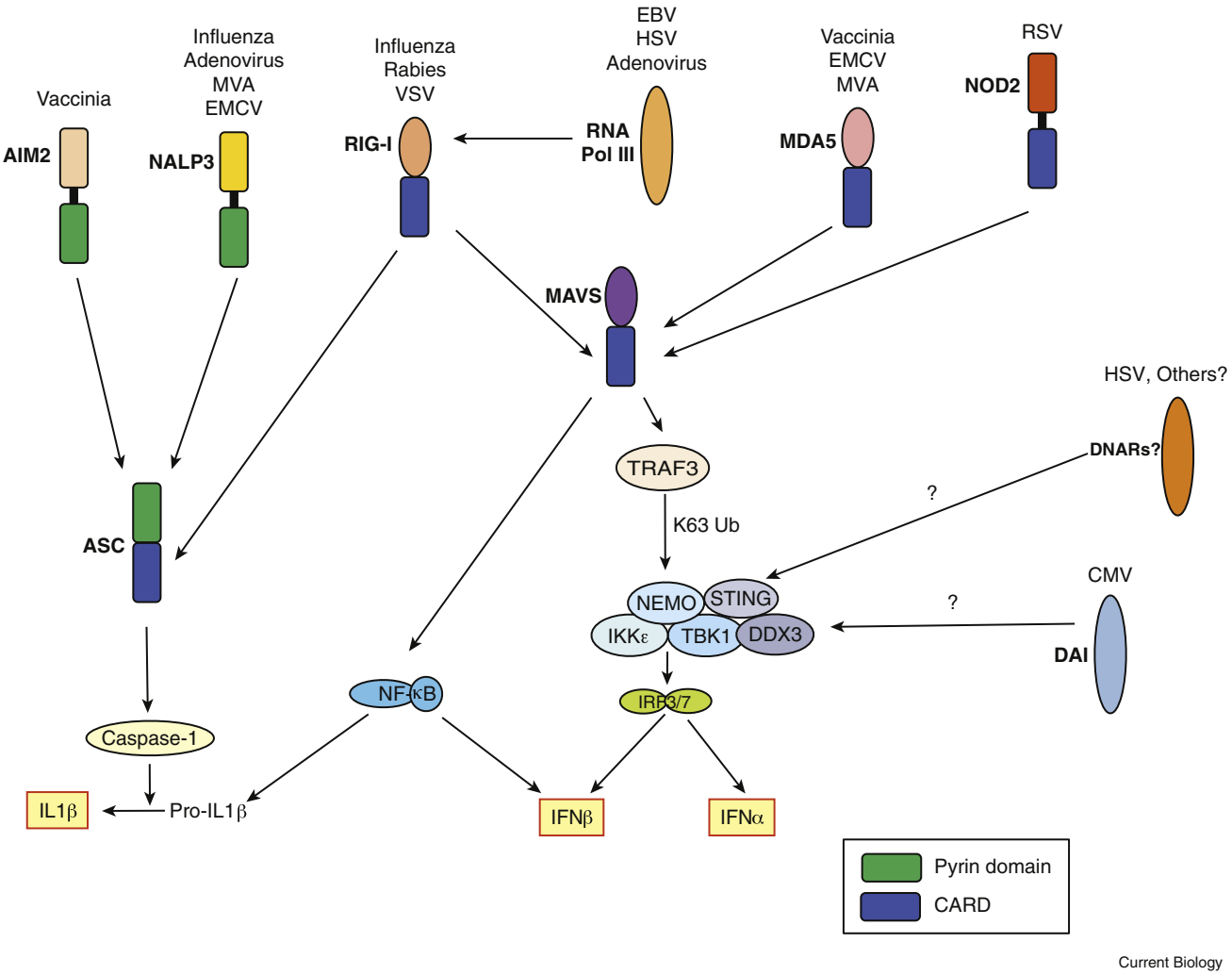

Figure 2. Recent developments in viral nucleic acid cytosolic detection pathways.

A number of novel receptors for viral nucleic acid have recently been identified, mediating both IL-1β and IFNβ production. Production of IL-1β in response to viruses requires activation of the inflammasome, which involves ASC and caspase-1. For vaccinia, inflammasome activation requires the upstream DNA sensor AIM2, for vesicular stomatitis virus (VSV), RIG-I is required, while for influenza, adenovirus, modified vaccinia Ankara virus (MVA) and encephalomyocarditis virus (EMCV), NALP3 is essential. MAVS is a central player in the activation of the TBK1 complex via a process requiring TRAF3-dependent formation of K63-linked ubiquitin chains, and involving the signaling components DDX3 and STING. The TBK1 complex phosphorylates IRF3 and IRF7, leading to induction of IFNα and IFNβ. Multiple upstream nucleic-acid-sensing receptors detect viruses and access this MAVS pathway: RIG-I for many RNA viruses, including rabies and VSV; MDA5 for vaccinia, EMCV and MVA; and NOD2 for RSV. Further, RNA polymerase III transcribes DNA from EBV, HSV and adenovirus into RNA ligands for RIG-I that also stimulate the MAVS pathway. CMV has been shown to activate the TBK1 complex in a DAI- and DDX3-dependent manner, via a poorly characterized signaling pathway, while HSV and other viruses also act through STING to activate TBK1, which may involve as yet undiscovered DNA sensors.

Cytosolic DNA Sensors

One area of recent intense research has been the search for cytosolic DNA-sensing pathways that would account for the ability of exogenously added DNA (such as that introduced by invading DNA viruses) to induce IFNβ, and also for the adjuvant effect of non-TLR9-activating DNA in vaccines. DAI/ZBP1, a Z-DNA binding protein, was the first such receptor identified in recent years, and it was initially shown that DAI expression in murine L929 fibroblasts led to enhanced IFNβ induction by multiple types of exogenously added DNA, including viral DNA [34]. However, the role of DAI is cell-type specific, and it may not be relevant to the majority of viruses, since knocking down DAI expression in other cell types by siRNA had very little effect on cellular responses to exogenous DNA [35,36]. One virus that has been recently linked to DAI is human cytomegalovirus; in transformed human fibroblasts DAI had a role in stimulation of innate immune signaling in response to the virus, and was also required to restrict viral replication [37]. Interestingly, DDX3 was also implicated in sensing cytomegalovirus, while MAVS was dispensable, demonstrating a role for DDX3 not only in RLR signaling but also in DNA-sensing pathways [37] (Figure 2). Importantly, though, in cells from DAI-deficient mice responses to poly(dA-dT), a synthetic dsDNA that seems to induce IFNβ in all cell types, were normal [38], and DAI, but not TBK1, was also dispensable for DNA-vaccine-induced immunogenicity [38]. Clearly other DNA sensor pathways must exist that initiate signaling leading to TBK1 activation and subsequent systemic responses to DNA.

Apart from TBK1, another signaling molecule recently implicated in DNA-mediated innate immune and vaccine adjuvant responses is the membrane protein STING (also known as MITA). Previously, siRNA-mediated knockdown of STING/MITA revealed that it is a novel downstream component of both RLR-mediated and DNA-sensing pathways that induce IFN production [39,40]. Recent studies in STING-deficient mice confirmed that STING has an essential role in mediating IFN induction in response to exogenous DNA, and vesicular stomatitis virus and herpes simplex virus, and also in promoting cytotoxic T-cell responses to plasmid DNA vaccination [41].

A novel DNA sensor pathway that accounts for the response of many cells to poly(dA-dT) was unexpectedly found to involve RIG-I and MAVS [42–44]. In HEK293 cells, poly(dA-dT)-induced IFNβ was impaired by siRNA-mediated knockdown of RIG-I and MAVS, but not MDA5 [42,44]. Interestingly, HEK293 cells only seem to induce IFNβ in response to poly(dA-dT) and not other types of DNA (which probably explains their usefulness in DNA plasmid transfection studies). Surprisingly, the poly(dA-dT)–RIG-I–MAVS–IFNβ response was shown to require the transcription of poly(dA-dT) into a RIG-I RNA agonist by RNA polymerase III (Pol III), thus implicating yet another protein with a classical role in RNA biology in innate immune sensing. The role of Pol III–RIG-I in sensing DNA seems less restricted than that of DAI in terms of cell type, since it can at least partly account for poly(dA-dT) responses in human cell lines [42], and in primary human and murine dendritic cells [44]. DNA sensing via RIG-I–Pol III has also been linked to DNA viruses, since inhibition of Pol III blocked the ability of EBV EBERs to induce IFN [44], and prevented the induction of IFN by adenovirus [42,43] and herpes simplex virus [42] in cell lines. The role of Pol III–RIG-I in responding to DNA viruses in primary cells and the particular viruses to which this pathway responds *in vivo* remain to be clarified. Further, we can assume that other DNA sensors that account for the innate immune response to DNA viruses are yet to be discovered (Figure 2).

Conclusions

How innate immune receptors sense viruses has been an area of immunological research that has seen remarkable progress in the past five years. TLRs, RLRs, NLRs and the HIN200 family of proteins have all been implicated, and the complexities of the signaling pathways that are activated by these receptors continue to be revealed. However, there is still a need to prove decisively which receptors or, more likely, which combination of receptors are detecting live viruses in primary cells, and there remain a number of important questions. Firstly, how are the various receptor systems integrated and what precise effector mechanisms are engaged? This is an important challenge for the development of new vaccines since an optimal adjuvant will likely engage with multiple receptor systems. Secondly, what receptors remain to be discovered, given that, for example in the case of DNA sensing, our understanding remains incomplete? Thirdly, and perhaps most challengingly, how do these systems relate to human viral disease pathogenesis? Ongoing studies are focusing on these questions, and the answers will provide important information that will hopefully lead to new treatments, both preventive and therapeutic, for the many viral diseases that remain a burden on humanity.

Acknowledgements

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