Recent developments in innate immune antiviral sensing and signaling

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Summary

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. There has been remarkable progress in the past few years in understanding the contribution of Toll-like receptors (TLRs), RIG-I like receptors (RLRs), NOD-like receptors (NLRs) and HIN200 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) play a determining role in the host anti-viral response, and 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 Vaccinia virus (VACV) proteins interfere with Toll-like receptor (TLR) signaling was the first indication that TLRs might be important in the sensing of viruses [1]. When either gene was deleted from VACV the resulting virus was attenuated in vivo, pointing to the importance of these proteins in virulence[2, 3]. Subsequently, tremendous progress was made in the uncovering of the role of TLRs and other innate receptors in anti-viral defense[4]. At the cell surface and at endosomal compartments, TLRs recognise both DNA and RNA viruses. Subsequently, additional sensors of viral nucleic acids that occur in the cytosol were found, notably the RIG-I like receptors (RLRs) RIG-I and MDA5, which can both 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, especially NALP3, which activates caspase-1 in the inflammasome leading to the production of the cytokines IL-1β and IL18[4]. More recently, the protein AIM2, which also activates caspase-1, has been shown to sense DNA viruses such as Vaccinia[5]. We therefore have a greatly improved understanding of the innate sensing of viruses. Here we review recent findings in this area, which reveal additional complexity in terms of sensing and signaling of viruses by these receptor systems.

Increased role for TLRs in anti-viral signaling

Work on TLR signaling pathways continues to reveal substantial complexity (see Figure 1). Two recent studies provide us with new insights into the regulation of the activation of IFN regulatory factor 3 (IRF3), one of the critical transcription factors for IFNβ induction, by TLR4 and TLR3. Both of these TLRs can activate IRF3 via the adapter TRIF, which leads to the activation of TBK-1 and subsequent phosphorylation of IRF3. Palsson-McDermott et al have reported on a specific negative regulator of TRAM, the adapter used exclusively by TLR4 to recruit TRIF[6]. 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 complex and limit activation of IRF3. Knockdown of TAG with siRNA boosts the IRF3 pathway and this approach could have use as a vaccine adjuvant to promote Type I IFN production, since the TRAM/TRIF pathway has been shown to be especially important for adjuvancy. When the gene encoding TRAM was inserted into a vaccine DNA vector with a sequence encoding an HIV peptide, the in vivo CD8 response was boosted 3-fold[7]. These kinds of approaches might lead to better vaccine adjuvants.

In another study, the basis for how TLR4 can signal to NFκB (required for both IFNβ and IL-1β induction) on the one hand, and IRF3 on the other has been determined[8] (see Figure 1). The protein TRAF3 is a key regulator of both pathways but it acts in different ways. In the case of NFκB, TRAF3 undergoes degradative ubiquitination during MyD88-dependent signaling, which allows the MyD88 signaling complex to translocate to the cytosol to engage with TAK-1. The K48-linked ubiquitination of TRAF3 is performed by cIAP2, itself an ubiquitin E3 ligase that is activated via K63-linked ubiquitination by TRAF6 after TLR4 stimulation. On the other hand, a separate pool of activated TLR4 complexes translocate to the endosome, where TRAF3 is recruited and becomes K63-linked ubiquitinated. This appears to be required for IRF3 activation by TLR4, and also probably by TLR3[8]. These events are therefore likely to be key for the induction of anti-viral cytokines by these TLRs.

A further role for ubiquitination in TLR3 and TLR4 signalling has been uncovered since it has been shown 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 TBK-1, leading to its activation. Nrdp1 was shown to protect mice from VSV infection because of its ability to promote IFN production. The TRAF3

and Nrdp1 studies reveal an increasingly complex picture of the role of ubiquitin E3 ligases in TLR signaling, which would seem to be at least as important as the role of kinases.

Apart from TLR3 and TLR4, TLR2 has also recently been added to the list of TLRs capable of mediating Type I IFN induction, which was a surprise. Barbalat et al showed that VACV and murine cytomegalovirus can induce Type I IFNs via TLR2[10]. A specialist cell type in the bone marrow and spleen termed Ly6C^{hi} inflammatory monocytes were shown to be the key cell type involved in this response, which is probably 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 receptor internalisation. TLR2 can therefore recognize a viral PAMP and drive Type I IFNs.

A further new insight into the role of TLRs in viral diseases came from Town et al who showed that deficiency in either TLR7 or MyD88 dramatically affected leukocyte homing during infection with West Nile virus, which results in increased mortality and higher viral burdens[11]. Interestingly, IL23, 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, 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, being mainly supportive, so TLR7 agonists might be of use here.

However a different study 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 (KSHV), inducing viral lytic gene transcription and replication. VSV,

which is sensed by TLR7/8, also reactivated KSHV from latency. This suggests 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 in that it may be key to explaining why HIV-1-infected women tend to have lower viral loads early in HIV-1 infection but progress faster to AIDS for a given viral load compared to men[14]. Meier et al demonstrated significant sex differences in the response of plasmacytoid dendritic cells (pDCs) to HIV. pDCs from women produced markedly more interferon-alpha in response to HIV-1-encoded TLR7 ligands when compared to pDCs from men. There were also considerably higher levels of CD8+ T cell activation 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 higher HIV disease progression. The mechanism here is not clear, but could involve increased cell numbers to infect (both DCs and CD4+ T cells) and possibly enhanced infection due to induction of receptors such as DC-SIGN on DCs. The data suggest that inhibition of the TLR7 pathway in pDCs might represent a new approach to treating HIV-1 infections.

Apart from TLR7, another TLR recently implicated in the pathogenesis of human viral disease is TLR3. Most Epstein-Barr virus (EBV) infections are asymptomatic, however in certain cases the virus can trigger infectious mononucleosis and EBV-associated hemophagocytic lymphohistiocytosis, thought to be due to sudden release of inflammatory cytokines. Iwakiri et al showed that this is likely caused by the release of small viral RNAs called EBERs from infected cells, which activate immune cells (likely human blood DCs) via TLR3 signalling to produce IFNg and TNF[15]. EBERs are noncoding 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. Figure 1 summarises these recent advances in understanding how TLRs sense viruses.

Cytosolic detection of viruses by NLRs and RLRs

Viruses are known to be activators of 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 form that must be cleaved by caspase 1 to be active. Caspase 1 occurs in multiprotein complexes termed inflammasomes. The best-characterized inflammasome contains the NLR NALP3, and this protein has been shown to be required for IL-1β processing in response to adenovirus[16] and RNA derived from influenza (see Figure 2) [17]. NALP3 is unlikely to be directly binding to viral nucleic acids however, and it is more likely that it is sensing 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 VACV[5] (Figure 2). Apart from its DNA binding HIN domain, AIM2 (like NALP3) has a pyrin domain, the key domain required for recruitment of caspase-1 via the protein ASC. Both NALP3 and AIM2 are likely to be important for the induction of fever during viral infections, and also the production of IL18, which is an important cytokine for promoting Th1 responses during viral infections.

Similar to NALP3 and AIM2, the RLR RIG-I has also now been shown to mediate ILβ production in response to certain viruses[21]. Poeck et al 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 MAVS[21] (Figure 2). This is the first example

of MAVS-independent RIG-I signaling. In contrast, viruses acting via the RLR MDA5 did require NALP3 for inflammasome activation[21].

NOD2 is another NLR recently implicated in the anti-viral response. NOD2 was previously thought to only sense the bacterial peptidoglycan breakdown product muramyl dipeptide, but it has now been shown to bind viral ssRNA and activate IRF3, leading to IFNβ production[22]. NOD2 was also shown to sense RSV and mediate viral killing in 293 cells, and in vivo was required for host defense against RSV. NOD2 signalled via the adapter MAVS, which again is something of a surprise since MAVS was thought to be only involved 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 the RNA ligand needed to contain a 5' triphosphate group in order to stimulate the receptor, which had superseded the idea that RIG-I recognizes dsRNA, since some ssRNAs with 5' triphosphate are also recognized[24, 25]. However two groups recently showed that in fact pure ssRNA with a 5' triphosphate 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 5¹ triphosphate dsRNA with at least 20 base pairings [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 that lack stretches of dsRNA but contain blunt short double stranded 5' triphosphate RNA in the panhandle region of their single stranded genome[26, 27]. For MDA5, it had been assumed that the ligand was long dsRNA, and indeed Pichlmair et al showed that RNA

extracted from encephalomyocarditis virus- or VACV-infected cells could induce MDA5-dependent Type I IFN[28]. However the stimulatory activity of the RNA resided in higher order structures containing both ss and dsRNA, leading the authors to conclude that MDA5 recognises an RNA web 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 downstream of MAVS[29]. The importance of ubiquitination in the RLR pathway was further underscored by Zeng et al who showed that the ubiquitin-conjugating enzyme (E2) Ubc5 has an essential role in IRF3 activation downstream of MAVS, probably by catalyzing the formation of K63 chains in the MAVS signaling complex, to recruit TBK1 via NEMO ubiquitin binding domains[30].

In another study, a novel pathway which downregulates RLR signaling via degradation of MAVS was uncovered[31]. This involved 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 antiviral response was exaggerated and prolonged[31]. Thus although PCBP2 has a defined role in regulation of RNA stability and translation, it can 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 also now been shown to directly regulate the TBK1/IKKe complex required for IRF3 activation downstream of RIG-I[32] [33].

Cytosolic DNA sensors: the knowns and the known unknowns

One recent area of 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 linked to DAI recently is human CMV, since in transformed human fibroblasts DAI had a role in stimulation of innate immune signalling in response to the virus, and was also required to restrict viral replication[37]. Interestingly, DDX3 was also implicated in sensing CMV, 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-null mice, responses to poly(dA-dT), a synthetic dsDNA that seems to induce IFNβ in all cell types, were normal[38], while DAI but not TBK1 was 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 STING (or MITA). Previously using siRNA STING/MITA was shown to be a novel downstream component of both RLR and DNA sensor pathways inducing IFN[39] [40]. Recently the STING -/- mouse was generated and this confirmed that STING has an essential role in mediating exogenous DNA, and VSV- and

HSV-mediated IFN induction, and also in cytotoxic T cell responses to plasmid DNA vaccination[41].

A novel DNA sensor pathway, which accounts for the response of many cells to poly(dA-dT), was unexpectedly found to involve RIG-I and MAVS[42, 43] [44]. In HEK293 cells, poly(dA-dT)-induced IFNβ was found to be impaired by siRNA knockdown of RIG-I and MAVS, but not of MDA5[42] [44]. Interestingly, HEK293 cells only seem to induce IFNβ in response to poly(dA-dT) and not other DNAs, 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, 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 DAI in terms of which cell types it operates in, 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 disruption of Pol III (using a chemical inhibitor) inhibited the ability of EBV EBERs to induce IFN[44], or the induction if IFN by adenovirus[43] [42] and HSV[42] in cell lines. The role of Pol III-RIG-I in responding to DNA viruses in primary cells remains to be clarified, as does the particular viruses this pathway responds to in vivo. Further, we can assume that other DNA sensors that account for the innate immune response to DNA viruses remain to be discovered (Figure 2)

Conclusion

How innate immune receptors sense viruses has been an area of immunology research that has seen remarkable progress in the past 5 years. TLRs, RLRs, NLRs and the HIN200 family of proteins have all been implicated, and the complexities of the signaling pathways

activated continue to be revealed. However, there is still a need to decisively prove which receptors, or more likely, 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 are the precise effector mechanisms 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, since 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.

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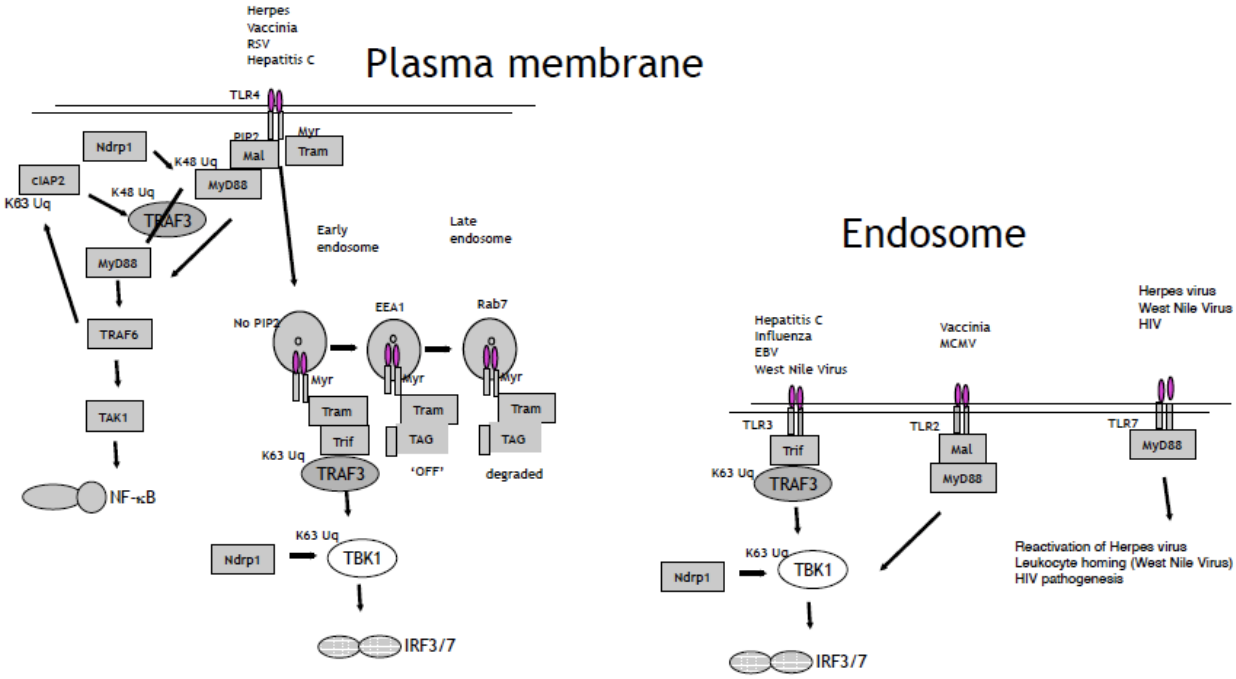

Figure 1: Recent developments in TLR sensing of viruses.

New insights have emerged in the regulation of TLR4 and TLR3 signaling, as well as new roles for TLR2 and TLR7 in the response to certain viruses. TRAF3 is an important inhibitor of plasma membrane signaling by TLR4, a TLR implicated in the response to Herpes virus, VACV, RSV and HCV. It is degraded in response to K48-linked ubiquitination by cIAP2, which itself requires K63-linked ubiquitination by TRAF6. This releases the MyD88 signaling complex, which engages with TAK1 leading to NF-kappaB activation. The TLR4/TRAM complex translocates to the early endosome, where via TRIF, it activates TBK-1 leading to IRF3 activation. This also requires TRAF3, but in this case, TRAF3 is modified by K63-linked ubiquitination. The TRAM splice variant TAG then displaces TRIF to limit the signal, with TLR4 ultimately being degraded in the late endosome. TBK-1 also undergoes K63-linked ubiquitination, mediated by Nrdp1. Similarly, TLR3, which is involved in the sensing of West Nile Virus, Influenza virus, HSV and EBV (via the sensing of EBER), engages with TRIF and TRAF3 (modified by K63-linked ubiquitination) to activated TBK-1. TLR2 has also been shown to engage with TBK1 via MyD88, in response to VACV and MCMV. Recent findings

on TLR7 indicate and important role in leukocyte trafficking in response to West Nile Virus, reactivation of HSV in response to VSV and finally in the pathogenesis of AIDS by HIV.

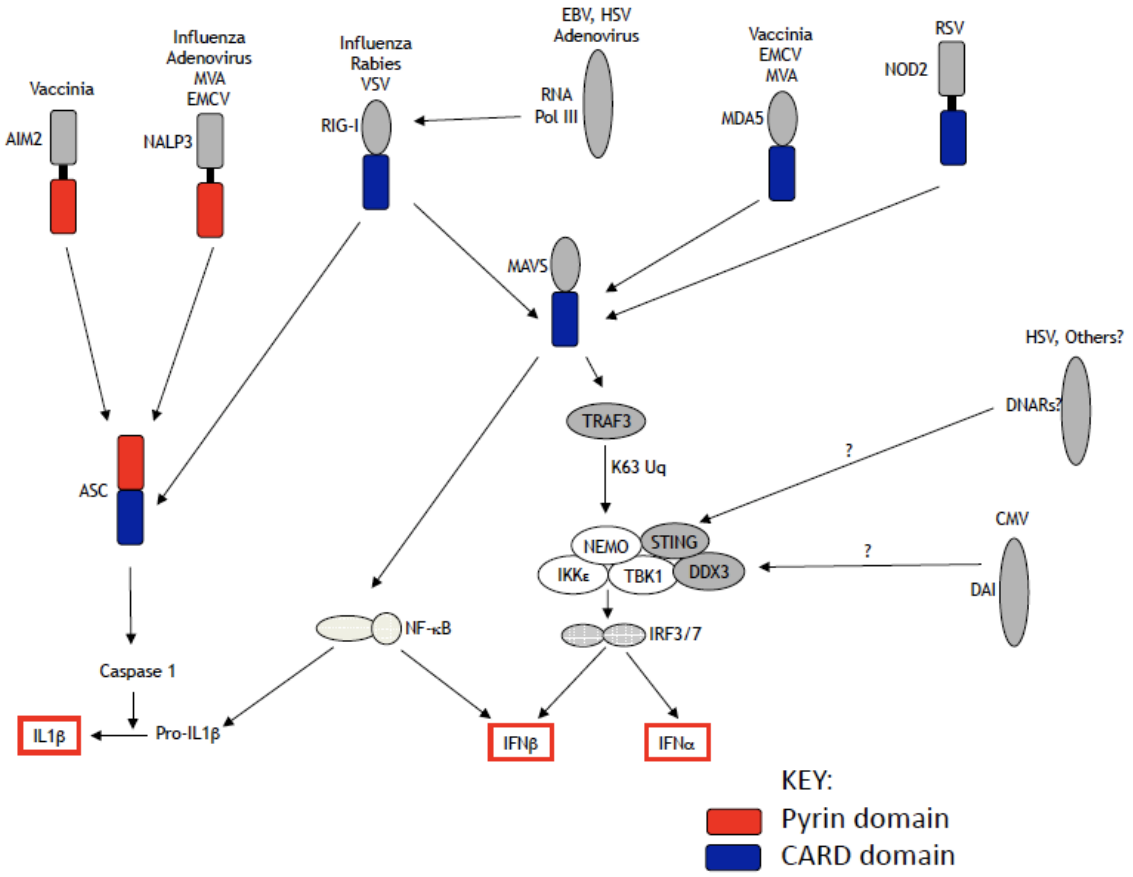

Figure 2: Recent developments in viral nucleic acid cytosolic detection pathways.

A number of novel receptors for viral nucleic acid have recently been identified, mediating both IL1β and IFNβ production. Production of IL1β in response to viruses requires activation of the inflammasome, which involves ASC and Caspase 1. For vaccinia, this requires the upstream DNA sensor AIM2, VSV requires RIG-I, while for Influenza, Adenovirus, MVA and EMCV, NALP3 is essential. MAVS is a central player in activation of the TBK1 complex in a process requiring TRAF3-dependent formation of K63-linked ubiquitin chains, and involving the novel signalling components DDX3 and STING. The TBK1 complex phosphorylates IRF3 and IRF7, leading to induction of 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 signalling pathway, while HSV and other viruses also act through STING to activate TBK1, which may involve yet-to-be discovered DNA sensors.

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