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Summary: Innate sensors of viral infection detect viral products and initiate the signal cascades that lead to the antiviral response. Several proteins have been identified to play a role in this process, mostly members of the Toll-like receptor and retinoic acid-inducible gene I-like receptor families. These receptors have been demonstrated to function in part by recognizing a diverse yet unique repertoire of nucleic acid substrates. Upon recognition of their ligands, these sensors activate distinct signaling pathways that lead to the secretion of type I interferon and inflammatory cytokines. It remains to be seen, however, if these sensors are redundant or whether each serves a unique function. In this work, we review the current knowledge of viral sensors, speculate on how they may function *in vivo*, and explore the potential reasons for their diversity.

Keywords: innate immunity, Toll-like receptor, RIG-I-like receptor, pattern recognition receptors, viral immunity

Introduction

Viral pathogens have been discovered in all species from single-cell bacteria to the most complex of mammals. To protect themselves from pathogenic effects of these invaders, organisms have been required to develop mechanisms to detect and prevent viral infection. In higher mammals, this requirement has evolved the adaptive immune system, which is able to generate highly specific antibodies and T cells that recognize specific viral proteins and peptides that either block infection or target infected cells for destruction. Initiation of the adaptive immune response, however, is a slow process that requires days to weeks for maximum effect. To provide protection during the initial hours and days of infection, we have maintained a system of pattern recognition receptors (PRRs) first seen in lower organisms that recognize broad motifs common to viral pathogens and thus serve as the initial sensors of viral infection. These sensors serve to initiate and maintain an antiviral response while the more specific adaptive response develops.

There are a wide variety of viral pathogens that are known to infect humans. Viruses can have genomes composed of double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), or DNA and can replicate at different locations in the

cell through different mechanisms that proceed through unique intermediates. Designing a system that can broadly detect all of these possibilities is challenging to say the least. To accomplish this, organisms use a variety of sensors from two main classes: Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs). These sensors protect different cellular compartments and signal through different adaptors to activate an antiviral response.

RLRs

RLRs are cytoplasmic proteins that recognize viral products that have gained access to the cytosol. There are currently three known members of this family: RIG-I, melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology-2 (LGP2) (1). Both RIG-I and MDA5 contain a DExD/H box helicase domain that binds dsRNA and two N-terminal caspase recruitment (CARD) domains

involved in signaling (2–5). LGP2 contains the helicase domain but lacks the CARD domains and is thought to be a negative regulator (6, 7). Both RIG-I and LGP2 also contain a C-terminal repressor domain that blocks signaling in the absence of ligand binding (5). RIG-I binds preferentially to ssRNAs that are phosphorylated at the 5′-end (8, 9) and contain homopolyuridine or homopolyriboadenine motifs as well as short dsRNA (10–12). MDA5 recognizes long dsRNAs and does not require 5′-phosphorylation (11–14). The differences in ligand preferences of the two proteins result in specificity for the recognition of individual viruses, which is discussed later.

The pathways by which RLHs signal are shown in Fig. 1. Both MDA5 and RIG-I signal through CARD–CARD interactions with interferon-β (IFN-β) promoter stimulator 1 (IPS-1) [also known as mitochondrial antiviral signaling (MAVS), virus-induced signaling adapter (VISA), or CARD adapter-

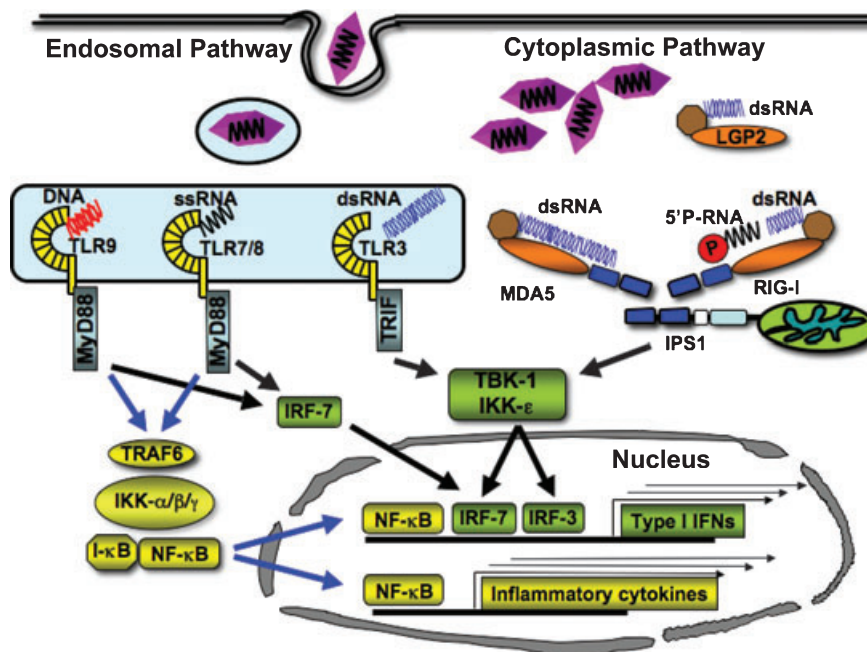


Fig. 1. Cytoplasmic and endosomal sensors of viral nucleic acids. This figure illustrates the detection of viral products by retinoic acid-inducible gene I (RIG-I)-like receptor (RLR) and Toll-like receptor (TLR) family members. TLR3, TLR7/ 8, and TLR9 are located on endosomal compartments in which they sense their double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), and CpG DNA ligands, respectively. TLR3 signals through the adapter protein Toll/interleukin-1 (IL-1) receptor (TIR) domain-containing adapter-inducing interferon-β (TRIF), which activates tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) (not shown) and the TANK-binding kinase 1 (TBK-1) complex leading to interferon (IFN) regulatory factor 7 (IRF-7) activation and IFN signaling. TRIF also signals through TRAF6, which leads to nuclear factor κB (NF-κB) activation and inflammatory cytokine production. TLR7, TLR8, and TLR9 signal through the MyD88 adapter. MyD88 signals through a protein complex consisting of TRAF6 and IL-1 receptor-associated kinase 1/4 (IRAK1/4) (not shown), leading to the activation of type I IFN and NF-κB signaling. RLR family members, melanoma differentiation-associated gene 5 (MDA5), retinoic acid-inducible gene I (RIG-I), and laboratory of genetics and physiology-2 (LGP2), are cytoplasmic proteins that detect viral products within the cytosol. MDA5 and RIG-I signal through IFN-β promoter stimulator 1 (IPS-1), which is located on the mitochondrial membrane. IPS-1 signals through TRAF3 and the TBK-1/inhibitor of NF-κB kinase ε complex to activate IRF-3 and IRF-7 and then type I IFN. IPS-1 also signals through FAS-associated death domain-containing protein (FADD) leading to the activation of caspase-8 and caspase-10 (not shown), which causes NF-κB activation and inflammatory cytokine production. LGP2 does not signal through IPS-1 and is considered to be a negative regulator of RIG-I.

inducing IFN- β (Cardif)], which is located on the outer mitochondrial membrane (15–18). Downstream of IPS-1 (19), tumor necrosis factor (TNF) receptor-associated factor 3 (TRAF3) activates TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor κ B (NF- κ B) kinase ϵ (IKK ϵ), which phosphorylate IFN regulatory factor 3 (IRF-3) and IRF-7 (20, 21). Activated IRF-3 and IRF-7 translocate into the nucleus and bind IFN-stimulated response elements (ISREs), inducing the expression of type I IFNs (22). IPS-1 also interacts with FAS-associated death domain (FADD)-containing protein (23). FADD activates caspase-8 and caspase-10, and the activation of the caspase death effector domains activates NF- κ B, leading to the production of inflammatory cytokines (24). Thus, MDA5 and RIG-I appear to activate both the IFN and inflammatory responses.

TLRs

TLRs are transmembrane proteins that contain luminal leucine-rich repeats (LRRs) that sense pathogen-associated molecular patterns and cytoplasmic Toll/interleukin-1 (IL-1) receptor homology (TIR) domains that signal through downstream adapters (1). There are 10 members of the TLR family in humans and 13 in mice. TLRs involved in the detection of viral nucleic acids are located on the cell surface (TLR3) or in endosomal compartments (TLR3, TLR7, TLR8, and TLR9) (25). TLR3 recognizes dsRNA, which constitutes the genome of dsRNA viruses and is also an intermediate produced during replication of ssRNA viruses (26). TLR7 and TLR8 recognize ssRNA as well as imidazolequinilone compounds, which are known to have antiviral properties (25, 27–30). TLR9 recognizes unmethylated CpG-containing DNA, which is commonly found in the genomes of DNA viruses (31, 32).

TLR3 signals through the adapter protein TIR domain-containing adapter-inducing IFN- β (TRIF) (33, 34) (Fig. 1). TRIF interacts with TRAF3 and TRAF6 through TRAF-binding motifs and with receptor-interacting protein 1 (RIP1) and RIP3 through RIP homotypic interaction motifs (RHIM) (35–37). TRAF3 leads to the secretion of type I IFNs, while TRAF6 and RIP1 lead to NF- κ B activation and production of inflammatory cytokines (38). TLR7, TLR8, and TLR9 signal through the adapter protein myeloid differentiation primary response gene 88 (MyD88). MyD88 contains a TIR domain as well as a death domain that allows it to serve as an adapter for TLR signaling. MyD88 associates with a signaling complex consisting of TRAF6, Bruton's tyrosine kinase (BTK), IL-1 receptor-associated kinase 4 (IRAK4), and IRAK1 (39). Signaling through this complex activates IRF7, NF- κ B, and

mitogen-activated protein kinase pathways (40–42). Thus, although RLRs and TLRs signal through different pathways, both appear to be able to activate the production of type I IFNs (i.e. IFN- α and IFN- β) and inflammatory cytokines.

Two additional TLR family members that signal through MyD88 have been implicated in the recognition of additional viral components. TLR2 is known to detect a variety of lipoproteins as well as yeast-associated zymosan; however, it has also been demonstrated to have a role in the recognition of viral envelope proteins (43). Similarly, while TLR4 has traditionally been known as the sensor of LPS, it can also respond to virus-derived envelope glycoproteins (44). In this review, we focus primarily on TLRs that recognize nucleic acids.

Additional sensors

The TLRs and RLRs have been shown to play a role primarily in RNA virus infection. Recently, the array of innate immune sensors of viral infection has been shown to include two additional cytosolic proteins that are involved in the recognition of DNA viruses. A DNA-binding protein, named DNA-dependent activator of IFN-regulatory factors (DAI), Z-DNA-binding protein 1 (ZBP1), or DLM-1, binds cytosolic DNA, inducing type I IFN and other genes involved in innate immunity (45, 46). Accordingly, RNA interference of mRNA for DAI in cells inhibits DNA-mediated antiviral responses. Furthermore, Nacht domain-, LRR-, and PYD-containing protein 3 (NALP3), a component of the cytosolic molecular complex termed the inflammasome, has been shown to recognize adenoviral DNA, inducing activation of caspase-1 and maturation of pro-IL-1 β in macrophages (47). Correspondingly, mice lacking NALP3 or its signaling adapter, apoptosis-associated speck-like protein containing a C-terminal caspase (ASC), display reduced innate inflammatory responses to adenovirus particles. The discovery of these sensors has provided further insight into the innate response against DNA viruses.

Besides the RLR and TLR classes of sensors, other proteins are known to detect viral products and contribute to the immune response, especially RNase L and protein kinase R (PKR). RNase L has recently been reported to be involved in the RLR response to viral nucleic acids (48). It is proposed that 2',5'-linked oligoadenylate generated by viral infection activates RNase L to cleave self-RNA into small RNA products, which are responsible for RLR signaling. However, it is not yet known how these small self-RNAs are recognized by MDA5 and RIG-I. PKR has been shown to dimerize upon binding of dsRNA. The activated PKR dimer phosphorylates

eukaryotic initiation factor 2-a (eIF2-a), which results in the inhibition of translation, preventing viral replication (49). Like RLRs, RNase L and PKR are upregulated in response to type I IFN, demonstrating their important role in the preprogrammed antiviral response.

Cytokine response to viruses

IFNs

The initiation of IFN production is an essential step in the antiviral response. Type I IFNs fight viruses both directly by inhibiting viral replication in cells and indirectly by stimulating the innate and adaptive immune responses. IFN- α and IFN- β bind to the IFN- α receptor (IFNAR) in an autocrine or paracrine manner. Activation of this receptor leads to Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signal transduction pathways (50, 51). These genes increase the cellular resistance to viral infection and sensitize virally infected cells to apoptosis (52). Interestingly, several viral sensors are among those genes induced by IFN. They in turn enable the production of IFN, creating a positive feedback loop that creates a local cellular response. In addition, type I IFNs directly activate dendritic cells (DC) and natural killer (NK) cells and promote the survival and effector functions of T and B cells, providing a link between the innate response to infection and the adaptive immune response (53–56).

TLRs signaling pathways also induce the recently identified type III IFNs. These include three proteins, named IFN- λ 1, IFN- λ 2, and IFN- λ 3, or IL-29 (λ 1) and IL-28A/B (λ 2/3). Although genetically distinct from type I IFNs, type III IFNs have similar biological antiviral functions (57–59). Whether RIG-I and MDA5 transmit signals leading to the expression of type III IFN is yet unknown.

Inflammatory cytokines

In addition to IFN signaling, viral sensors are also known to initiate signaling for inflammatory cytokine and chemokine secretion. Both DCs and macrophages produce TNF α , IL-6, monocyte chemotactic protein 1 (MCP-1), and IL-12 in response to viral infection. In addition, these same inflammatory cytokines are often detected in the serum of virally infected animals. Inflammatory cytokines activate the vascular endothelium as well as stimulate the recruitment of immune cells such as monocytes and neutrophils. While the resulting inflammatory response is important in the clearance of viral infection, a prolonged inflammatory state can also lead to adverse reactions including necrosis of local tissue and autoimmune diseases.

Understanding the diversity between viral sensors

It is not entirely clear whether viral sensors serve redundant or non-redundant functions. One way in which viral sensors can be seen to have differential effects is by the recognition of different viruses. However, the sensors may also recognize different components of the same virus. Additionally, diversity could insure that different sensors activate the production of distinct cytokines. Finally, the differential expression of viral sensors in tissues and cell types is likely to contribute to their distinct roles in viral infection. In the following sections, we explore whether there is true redundancy or if there is specialization between the RLR and TLR families of sensors. This is also illustrated in Fig. 2.

Diversity by recognition of different viruses

RLRs

Among the RLRs, ligand preferences appear to determine which virus is recognized by which sensor. The current paradigm is that RIG-I recognizes RNA-containing 5'-triphosphates, while MDA5 recognizes dsRNA. Therefore it is not surprising that RIG-I has been shown to detect influenza A and B viruses, vesicular stomatitis virus (VSV), and some flaviviruses (Japanese encephalitis virus and hepatitis C virus) (13, 60, 61). Likewise, MDA5 detects picornaviruses such as encephalomyocarditis virus (EMCV), Mengo virus, and Theilers virus (13, 14) as well as caliciviruses (62). These

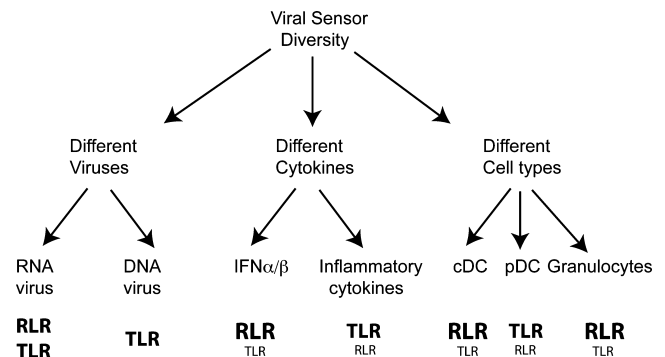


Fig. 2. Hypothetical model of functional diversity among viral sensors. Three potential mechanisms by which viral sensors perform unique functions: (i) viral sensors detect different ligands resulting in the recognition of a variety of viral families and/or the recognition of different components of the same virus. (ii) Toll-like receptor (TLR) and retinoic acid-inducible gene I-like receptor (RLR) signaling results in the production of different cytokine responses (i.e. type I interferon versus pro-inflammatory cytokines). (iii) The expression of TLRs and RLRs in different cell types results in unique responses to viruses in different cells and tissues. Predominant sensors are indicated in bold, while secondary sensors are indicated below in smaller font.

viruses contain a 5'-VPg cap instead of 5'-triphosphate and make large amounts of dsRNA during replication. However, other results do not neatly fit this paradigm. RIG-I and MDA5 play redundant roles in the recognition of West Nile virus (63), Dengue virus, (61) paramyxovirus, and reovirus (61), most of which contain 5'-triphosphates. In addition, although Sendai virus has been shown to activate RIG-I, it encodes for a protein, the V protein, that is a specific inhibitor of MDA5 (64). Furthermore, murine hepatitis virus, a member of the coronavirus family that does not contain VPg, has recently been shown to be recognized by MDA5 (65). One explanation is that, although RIG-I preferentially recognizes 5'-triphosphates and polyuridine-rich regions, it can also recognize short dsRNA, while MDA5 recognizes long dsRNA (11). The ability of MDA5 and RIG-I to specifically detect certain viruses while also detecting common pathogens illustrates the need for multiple sensors to recognize and control the wide variety of viral pathogens.

TLRs

Compared with that of the RLRs, the role of TLRs in antiviral responses is more intricate (66). TLR3 was originally shown to detect dsRNA (26). Accordingly, TLR3 has been implicated in the detection of several RNA viruses such as EMCV (67), respiratory syncytial virus (RSV) (68, 69), West Nile virus (70), and Punta Toro virus (PTV) (71). However, in one study TLR3 did not contribute to viral pathogenesis in VSV, lymphocytic choriomeningitis virus (LCMV), and reovirus infections (72). To make matters more confusing, TLR3 has been implicated in recognition of DNA viruses. TLR3-deficient mice are more susceptible to murine cytomegalovirus (MCMV) infection than wildtype mice (73). Moreover, a recent human study has demonstrated that a dominant negative form of TLR3 causes susceptibility to neonatal herpes simplex-1 encephalitis (HSE) (74). However, it is unclear why TLR3 plays such a major role in HSE, whereas it has no obvious role in other HSV-1 infections, such as skin, eye, or mouth infections, or sepsis or in other DNA virus infections. Thus, TLR3 may recognize not only RNA viruses but also DNA viruses, most probably through RNA intermediates that are generated during viral replication.

TLR7 has been shown to contribute to the detection of RSV, Sendai virus, influenza, human immunodeficiency virus (HIV), VSV, and coxsackie virus B3 (CVB3) (75), while TLR8 has been implicated in the detection of influenza and paramyxovirus as well as HIV (28, 44, 76). TLR9 plays a role in the recognition of herpes simplex virus and cytomegalovirus infection (73, 77–79). TLR2 and TLR4 have been shown to

play a role in the recognition of enveloped viruses. Both herpes viruses, which contain a DNA genome, and RSV, which has a ssRNA genome, have been reported to be recognized by these sensors (80–82). In summary, TLR7 and TLR8 recognize ssRNA viruses, while TLR9 recognize DNA viruses. TLR2 and TLR4 recognize enveloped viruses, while TLR3 plays a role in the recognition of both RNA and DNA viruses. Overall, TLR viral specificities exhibit a significant overlap with those of RLRs.

Diversity by distinct cytokine responses

Another potential explanation for the presence of different classes of sensors could be the induction of different cytokine responses. Although both RLRs and TLRs appear to be able to signal through both IFN and inflammatory cytokine pathways, there is evidence that the different classes have distinct functions in signal propagation leading to different immune responses.

Indeed, initial characterization of TLR3-deficient mice in response to dsRNA analog polyinosinic–polycytidylic acid (polyI:C) revealed a defect in IL-12 not type I IFN secretion in serum (26). Subsequently, it was shown that TLR3 induces a T-helper 2 (Th2)-type inflammatory response in airway epithelia cells infected with RSV infection (68, 69). Another study showed that TLR3 triggers an inflammatory response against West Nile virus infection that breaks down the blood–brain barrier, facilitating viral penetration and spreading in the brain and subsequent neuronal damage (70). Similarly, TLR3 plays an important role in liver pathology caused by PTV infection through the overproduction of inflammatory mediators (71). Altogether, these studies have suggested that TLR3 promotes a strong inflammatory response to RNA viruses, whereas it seems to have limited impact in type I IFN responses that control viral replication.

More recent studies have revealed differential signaling by RLRs and TLRs in individual cell types. One study found that influenza infection in bronchial epithelial cells led to TLR3-dependent inflammatory cytokine induction and RIG-I-dependent IFN response (83). Another recent study has demonstrated that human keratinocytes contain functional TLR, RLR, and PKR signaling pathways and, with the use of small interfering RNA (siRNA) and small molecule inhibitors, has shown that TLR3 provides the main stimulus for NF- κ B signaling, while RLRs are the primary initiators of IRF3 and IFN signaling in this cell type (84).

The stimulation of different signaling pathways by TLRs and RLRs could have important implications. There are several

viruses that are known to stimulate both TLR and RLR pathways, depending on whether infection is detected in endosomal or cytoplasmic compartments. It has been assumed that these sensors activate the same general cytokine responses; however, if the TLR and RLR pathways do indeed produce different cytokine products, then it is likely that they also produce different effects on the subsequent immune response. For example, it is known that the inflammatory cytokines can function not only to limit viral infection but also to cause excessive immunopathology. If we are able to distinguish which receptor is preferentially responsible for the production of each cytokine, then we may be able to target small molecules to the endosomal or cytoplasmic compartments to affect cytokine response. This could be a tremendous advantage to the fields of vaccine development as well as infectious diseases and tumor immunology, as antiviral sensors and their ability to induce cytokines are potential targets for therapy.

Diversity by differential distribution of sensors

The distribution of viral sensors in different cell and tissue types may be another mechanism to differentiate their actions. This is easily seen in comparison between conventional DCs (cDCs) and plasmacytoid DCs (pDC). cDCs are specialized for pathogen detection and antigen presentation. pDCs specialize in the secretion of type I IFNs in response to viruses (25, 85). In humans, cDCs express TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, and TLR8, while pDCs preferentially express TLR7 and TLR9. cDCs are capable of expressing high levels of RIG-I and MDA5, while pDCs also express these cytoplasmic sensors, but, paradoxically, the sensors do not appear to function (86). Nevertheless, both cell types are able to respond to viruses. In human cDCs, this occurs in an MDA5- and TLR3-dependent manner, leading to the production of IFN- β , autocrine activation via IFNAR, and the production of IFN- α . In pDCs, however, TLR3 is not present, MDA5 and RIG-I may

not be functional, and autocrine secretion and activation via IFN- β does not occur, but high levels of IFN- α are produced (87). This occurs because pDCs utilize TLR7 and TLR9 and express endogenously high levels of IRF7 (22), which primes them for IFN- α production. In this situation, we can see that by varying the expression of TLRs and signaling components, different cell types have unique ways to detect pathogens. Extending this observation, a recent study demonstrated that human neutrophils respond to polyI:C through the RLR pathway rather than the TLR3 pathway (88).

A similar situation may occur in tissues. RIG-I and MDA5 are IFN-inducible genes that may be expressed in all cell types, while TLRs have a more restricted tropism. It has been demonstrated that MDA5 and RIG-I are the predominant sensors for polyI:C and RNA virus infection in bone marrow-derived DCs, macrophages, and fibroblasts. However, TLR3 has also been shown to play a role in different cell types. This may occur as a result of differential expression of the various sensors or their downstream adapter proteins. One specific example may occur in the brain. Several groups have shown that TLR3 is expressed in the brain, while MDA5 does not appear to be expressed. Most probably, this differential expression of the various viral sensors contributes to their importance in viral infection.

Conclusions

Both the TLR and RLR families of receptors contain multiple sensors that are important in viral infection. In this review, we examine the diversity of these sensors and the potential explanations for this diversity. The role of each sensor potentially may be distinguished by recognition of distinct viral pathogens, by stimulation of distinct cytokine signaling pathways, or by distribution of individual sensors in different cell and tissue types. Further studies are necessary to determine which of these possibilities most contributes, as well as the role of the individual sensors in viral infection *in vivo*.

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