Herpes simplex virus infection is sensed by both Toll-like receptors and retinoic acid-inducible gene-like receptors, which synergize to induce type I interferon production


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The innate antiviral response is initiated by pattern recognition receptors, which recognize viral pathogen-associated molecular patterns. Here we show that retinoic acid-inducible gene (RIG)-I-like receptors (RLRs) in cooperation with Toll-like receptor (TLR) 9 is required for expression of type I interferons (IFNs) after infection with herpes simplex virus (HSV). Our work also identified RNase L as a critical component in IFN induction. Moreover, we found that TLR9 and RLRs activate distinct, as well as overlapping, intracellular signalling pathways. Thus, RLRs are important for recognition of HSV infection, and cooperate with the Toll pathway to induce an antiviral response.

The early innate host response to viral infections is characterized by production of interferons (IFNs) as well as proinflammatory chemokines and cytokines (Malmgaard, 2004), which exert direct antiviral activity and regulate cellular processes important for antiviral host defence (Beutler, 2004; Dupuis et al., 2003; Leib et al., 1999; Ank et al., 2006). The antiviral response is initiated by cellular sensor systems that recognize viral molecules and subsequently activate intracellular signal transduction leading to stimulation of antiviral functions (Kawai & Akira, 2006; Mogensen & Paludan, 2005). Among the pattern recognition receptors (PRRs), Toll-like receptors (TLRs) are membrane-bound with ligand-binding domains facing towards extracellular or endosomal surfaces, which recognize viral proteins and nucleic acids, respectively (Boehme et al., 2006; Kurt-Jones et al., 2000; Diebold et al., 2004; Lund et al., 2003). The RNA helicases retinoic acid-inducible gene (RIG)-I and melanoma differentiation-associated gene (MDA) 5 [collectively termed the RIG-I like receptors (RLRs)] are located in the cytoplasm and detect intracellular virus through recognition of viral RNA structures (Yoneyama et al., 2004; Kato et al., 2006; Hornung et al., 2006; Pichlmair et al., 2006) or small self-RNAs generated by RNase L (Malathi et al., 2007).

Upon viral recognition, PRRs activate downstream signal transduction in the cell. Three pathways known to be important for antiviral and inflammatory responses are the nuclear factor (NF)-κB pathway, the mitogen-activated protein kinase (MAPK) pathway and the IFN regulatory factor (IRF) pathway (Mogensen & Paludan, 2005), which together coordinate expression of genes with antiviral activity, including type I IFNs (Kawai & Akira, 2006).

Herpes simplex virus (HSV) type 2 is a DNA virus that can give rise to a number of clinical manifestations, most notably genital and neonatal herpes. Innate host defence against HSV infections is highly dependent on natural killer cells (Biron et al., 1989) and the IFN system (Dupuis et al., 2003; Leib et al., 1999), where rapid recognition of the virus by the innate immune system is essential for activation of these early defence systems. TLR2 and TLR9 have been assigned important functions in this process, and while TLR2 recognizes an unidentified molecular structure on the virion (Aravalli et al., 2005; Kurt-Jones et al., 2004, 2005), TLR9 senses HSV infection through recognition of the viral genomes (Lund et al., 2003; Krug et al., 2004). However, it is known that in addition to TLRs, other mechanisms of recognition are involved in activating the innate antiviral response against HSV (Hochrein et al., 2004; Malmgaard et al., 2004). We recently identified the
downstream RLR adaptor protein mitochondrial antiviral signalling protein (MAVS)/IFN-β promoter stimulator-1 (IPS-1) as a cell-type-specific IFN-α/β inducer during HSV infection (Rasmussen et al., 2007). In addition, we have reported that double-stranded RNA (dsRNA) accumulates in HSV-infected cells (Weber et al., 2006).

In this study we have examined the role of RLRs in recognition of HSV infections and their potential interaction with TLRs. We demonstrate that RLRs are important for recognition of HSV in fibroblasts and macrophages and show that simultaneous stimulation through TLR9 and RLRs during HSV-2 infection is required to activate a full signalling response and induce expression of type I IFN.

We have previously shown that HSV is recognized through both TLR-dependent and -independent mechanisms (Malmgaard et al., 2004; Rasmussen et al., 2007). In order to examine whether RLRs were involved in this process, we used low passage mouse embryonic fibroblasts (MEFs) with targeted deletions in RIG-I, MDA5 or MAVS/IPS-1. The cells were seeded and treated with HSV-2. RNA was harvested 4 h post-infection (p.i.) and levels of IFN-β were measured by qPCR and normalized to β-actin. The ability of HSV to induce expression of IFN-β was not impaired in either RIG-I−/− or MDA5−/− cells (Fig. 1a, b), but was, however, strongly reduced in MAVS/IPS-1−/− cells (Fig. 1c). The MAVS/IPS-1-dependent IFN response was not mediated by cytosolic DNA recognition, since transfection with DNA induced comparable induction of IFN-β mRNA in wild-type and MAVS/IPS-1−/− MEFs (data not shown). Interestingly, HSV-2-induced IFN-β expression was also impaired in RNase L−/− fibroblasts (Fig. 1d), previously reported to generate small self-RNAs stimulating the IFN response through both RIG-I and MDA5 (Malathi et al., 2007). Thus, the results suggest that HSV-2 infection is sensed by RLRs and that RNase L is essential for this process.

To examine the role of RLRs in HSV recognition in macrophages, which seem to detect this virus through mechanisms very similar to fibroblasts (Rasmussen et al., 2007), we generated RAW264.7 cells stably transfected with a dominant-negative RIG-I mutant (helicase and C-terminal domains; dnRIG-I) or empty vector (pcDNA3) (Melchjorsen et al., 2005). The cells were seeded and stimulated with polyinosinic : polycytidylic acid (polyIC), oligodeoxynucleotides (ODN; ODN1826) (both InvivoGen), vesicular stomatitis virus (VSV) or encephalomyocarditis virus (EMCV) to check specificity. Extracellular polyIC, ODN1826, VSV and EMCV activate TLR3, TLR9, RIG-I and MDA5, respectively (Alexopoulou et al., 2001; Malmgaard et al., 2004; Kato et al., 2006). Expression of dnRIG-I did not affect stimulation through TLR3 or TLR9 (Fig. 2a and data not shown) but strongly inhibited cellular activation by VSV (Fig. 2b). In addition, EMCV-induced cytokine expression was reduced in cells expressing dnRIG-I (Fig. 2c). By using MEFs from RIG-I+/− versus RIG-I−/− mice, as well as from C57BL/6 versus MDA5−/− mice, we demonstrated that the preparation of VSV used in this study induced IFN-β in a manner dependent on RIG-I and independent of MDA5, whereas the preparation of EMCV triggered expression of the cytokine in an MDA5-dependent and RIG-I-independent fashion (data not shown). Thus, the cells expressing dnRIG-I were fully capable of signalling through TLRs, but displayed impaired ability to transduce signals downstream of both RIG-I and MDA5.

We next examined the ability of HSV-2 to propagate in RAW-pcDNA3 and RAW-dnRIG-I cells, and found that infection with a dose similar to the one used for cytokine induction experiments (m.o.i. of 3, 3 × 10⁶ p.f.u.) led to recovery of about 100 times less virus at 20 h p.i. than was used for infection; modestly higher levels of virus were produced by the RAW-dnRIG-I than the RAW-pcDNA3 cell line (data not shown). To examine for cytokine expression, the RAW-pcDNA3 and RAW-dnRIG-I cells were infected with HSV for different time periods, after which supernatants were harvested and cytokines were measured. RANTES was measured by ELISA (using matched antibody pairs from R&D Systems) and IFN-α/β levels were measured by bioassay (Melchjorsen et al., 2005). HSV-2-induced expression of RANTES and IFN-α/β was strongly reduced in the RAW-dnRIG-I cell line (Fig. 2d, e). Similar results were obtained when virus-induced IFN-β expression was assessed by real-time RT-PCR (data not shown).
To look further into the mechanisms underlying the observation that RIG-I and TLR9 cooperatively induce expression of cytokines, we examined how these PRRs affected the ability of HSV-2 infection to activate signal transduction. RAW-pcDNA3 and RAW-dnRIG-I cells as well as RAW264.7 cells incubated in the presence or absence of ODN2088 were infected with HSV-2 for up to 12 h before harvest of cell lysates (BioRad). Phosphorylation of IκBα and MAPK isoform p38 was determined by using Luminex Technology (kits from BioRad). Activation of the NF-κB pathway was reduced in cells expressing dnRIG-I, whereas p38 was activated to even higher levels in RAW-dnRIG-I cells compared with RAW-pcDNA3 cells (Fig. 3a, b).

Interestingly, when signalling through TLR9 was blocked, we observed the opposite effect, i.e. unaltered activation of NF-κB but reduced activation of p38 (Fig. 3c, d). Finally, we examined the involvement of TLR9 and RIG-I in activation of IRF-3 by HSV-2, which peaked at 3 h p.i. (Fig. 3e). Inhibition of shown). Thus, HSV infection is recognized by an RLR-dependent mechanism in RAW264.7 macrophage-like cells.

TLR9 has been reported to recognize HSV infection (Lund et al., 2003; Krug et al., 2004). To evaluate the role of TLR9 in HSV-induced cytokine expression in RAW264.7 cells, we used the TLR9 antagonist ODN2088, the specificity of which is shown in Fig. 2(f). RAW264.7 cells were incubated in the presence or absence of ODN2088 and infected with HSV-2 for the times indicated in Fig. 2; type I IFN and interleukin (IL)-6 were measured in the supernatants. Expression of both cytokines was totally blocked in the presence of ODN2088, regardless of the time of sampling (Fig. 2g and data not shown). Thus, TLR9 plays an important role in HSV-induced cytokine expression in RAW264.7 cells.

The role of TLR2, previously shown to recognize HSV (Kurt-Jones et al., 2004, 2005), was examined using RAW264.7 cells stably expressing a dominant-negative mutant of the TLR2 adaptor Mal. However, no role for Mal in HSV-2-induced IFN expression was observed (not shown).

The above data suggest that both RLRs and TLRs are required for HSV-induced IFN expression in RAW264.7 macrophages. To examine if this was also the case in fibroblasts, we investigated the effects of myeloid differentiation primary response gene (88) (MyD88) and TIR domain-containing adapter-inducing IFN-β (TRIF) by examining the ability of MyD88−/−, TRIF−/− MEFs to induce expression of IFN-β in response to HSV infection. As shown in Fig. 2(h), the ability of HSV infection to trigger IFN-β expression was totally inhibited in MyD88−/−, TRIF−/− MEFs, which did respond to DNA transfection with an unaltered IFN response (data not shown). These findings, together with the data shown in Fig. 1(c), support the conclusion that signalling through both TLRs and RLRs is required to stimulate IFN expression in fibroblasts in response to HSV infection.
either RIG-I or TLR9 led to reduced activation of IRF-3 after HSV infection, and combined inhibition of both PRRs led to nearly total abrogation of HSV-2-activated phosphorylation of IRF-3 (Fig. 3f). Thus, RIG-I and TLR9 control activation of overlapping, yet distinct, sets of signalling pathways in response to HSV-2 infection in RAW264.7 cells.

In this work, we have shown that type I IFN production during HSV-2 infection is dependent on RLRs and RNase L. We have also shown that RLRs and TLR9 cooperatively induce expression of cytokines during HSV-2 infection. These findings thus identify RLRs as PRRs that recognizes HSV-2 infection, and also show that cytosolic receptors act in concert with TLR9 to induce cytokine expression. Our results also identify RNase L as an important part of this induction.

Until recently, it was believed that RIG-I only recognizes 5′-triphosphate ends of RNA (Hornung et al., 2006; Pichlmair et al., 2006), a structure assumed not to be present during HSV replication. It is now known that RIG-I also activates antiviral immune responses upon binding to dsRNA (Takahasi et al., 2008), and we have previously shown that dsRNA does indeed accumulate during HSV infection (Weber et al., 2006). In addition, RNase L augments IFN-β expression through both RIG-I and MDA5, by generating small self-RNAs (Malathi et al., 2007). The results presented here show that HSV-induced IFN expression is dependent on RNase L. Thus, it is possible that both viral and self-RNAs stimulate IFN induction in an RLR-dependent manner during HSV infection.

Another important finding is that RLRs cooperate with TLR9 in induction of cytokine expression, and that the two PRRs differentially stimulate signalling pathways. Two previous studies have shown that RIG-I and TLR3 coordinately activate the host response against influenza A virus and respiratory syncytial virus infections (Le Goffic et al., 2007; Liu et al., 2007), and our work further supports the idea that a full innate antiviral response is induced through activation of several PRRs that act in concert to mediate host defence.

Collectively, our work has identified RLRs as PRRs recognizing HSV infection, and shown that simultaneous stimulation through two or more PRRs impacts on the nature of the antiviral response.

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