The NLRP3 Inflammasome Detects Encephalomyocarditis Virus and Vesicular Stomatitis Virus Infection

Jayant V. Rajan,1,2 David Rodriguez,1 Edward A. Miao,1†* and Alan Aderem1†

Institute for Systems Biology, Seattle, Washington 98104,1 and Department of Medicine, University of Washington Medical Center, Seattle, Washington 981952

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Inflammasomes are cytosolic protein complexes that regulate caspase-1 activation and the secretion of interleukin-1β (IL-1β) and IL-18. Several different inflammasome complexes have been identified, but the NLRP3 inflammasome is particularly notable because of its central role in diseases of inflammation. Recent work has demonstrated an essential role for the NLRP3 inflammasome in host defense against influenza virus. We show here that two other RNA viruses, encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV), activate the NLRP3 inflammasome in dendritic cells and macrophages through a mechanism requiring viral replication. Inflammasome activation in response to both viruses does not require MDA5 or RIG-I signaling. Despite the ability of the NLRP3 inflammasome to detect EMCV and VSV, wild-type and caspase-1-deficient mice were equally susceptible to infection with both viruses. These findings indicate that the NLRP3 inflammasome may be a common pathway for RNA virus detection, but its precise role in the host response may be variable.

Interleukin-1β (IL-1β) and IL-18 are two pleiotropic cytokines that play crucial roles in inflammatory responses in addition to instructing adaptive immune responses. Secretion of both is controlled at two steps: transcription and posttranslational processing. Therefore, any pathogen that elicits IL-1β/IL-18 secretion must be able to provide both signals. Expression of both pro-IL-1β and pro-IL-18 is induced by NF-κB (for example via Toll-like receptor [TLR] activation) although pro-IL-18 is expressed at a basal level. Posttranslational processing and secretion of IL-1β and IL-18 are controlled by caspase-1, whose activity is regulated by a cytosolic protein complex known as the inflammasome (4).

Most inflammasomes consist of a member of the Nod-like receptor (NLR) family of cytosolic receptors that either directly interacts with caspase-1 or is indirectly coupled to it by the adaptor protein ASC (apoptosis-associated speck-like protein containing a CARD). When an agonist is detected by an NLR, the NLR oligomerizes and recruits caspase-1, which is proteolytically processed to its active form. Activated caspase-1 in turn processes IL-1β and IL-18, resulting in the secretion of the mature cytokines (14).

Several inflammasomes have now been described. The NLRP3 inflammasome is unique in one respect: unlike other inflammasomes, which detect discrete agonists, the NLRP3 inflammasome is activated by a broad array of agonists, including extracellular ATP, intracellular nucleic acids, crystals, bacteria, and fungi. How these agonists induce NLRP3 activation is not clear although several hypotheses have been proposed (13, 22, 26).

Recent work suggests that the inflammasome may play a role in antiviral host defense. Both vaccinia virus and murine cytomegalovirus are detected by the AIM2 inflammasome, which responds to cytosolic double-stranded DNA (18), while influenza A virus is detected by the NLRP3 inflammasome (1, 9, 21). In this work, we show that the RNA viruses encephalomyocarditis virus (EMCV) and vesicular stomatitis virus (VSV) are also detected by the NLRP3 inflammasome.

MATERIALS AND METHODS

Cells and cell culture. Bone marrow was harvested from 8- to 12-week-old mice. All mice were on a C57BL/6 background, with Casp1−/−, Asc−/−, Nlrp3−/−, and Mda5−/− mice all backcrossed at least five generations. Bones from RIG-I−/− and control mice were obtained from the M. Gale lab, University of Washington, and were on a Swiss ICR background. For bone marrow-derived dendritic cells (BMDC), bone marrow cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 20 ng/ml recombinant murine granulocyte-macrophage colony-stimulating factor (rmGM-CSF) on day 0. Cells were reseeded on day 4 and collected by centrifugation at 1,500 rpm on day 7, at which point they were seeded at 1,000 cells/µl in RPMI medium supplemented with 10% FBS. For bone marrow-derived macrophages (BMDM), bone marrow cells were cultured in RPMI medium supplemented with 10% FBS and 10 ng/ml recombinant human macrophage colony-stimulating factor (rhMCSF) on day 0. Cells were reseeded on day 4, collected in phosphate-buffered saline (PBS) with 1 mM EDTA on day 6, centrifuged at 1,500 rpm, seeded at 500 cells/µl in RPMI medium with 10% FBS and rhMCSF, and stimulated on day 7.

Viruses. All viruses were grown in BHK-21 cells, and supernatants were harvested at maximal cytopathic effect. Supernatants were clarified by centrifugation at 2,500 rpm for 30 min at 4°C, aliquoted, and stored at −80°C. Viral titers were determined by standard plaque assay on Vero cells. Viruses were UV inactivated by irradiation for 5 min in a Stratagene 2400 UV cross-linker.

In vitro infections/stimulations. Day 7 BMDC or BMDM were seeded as described above in complete RPMI medium with or without 1 mg/ml Pam3 (tripalmitoyl). Three hours after seeding, BMDC were infected at a multiplicity of infection of (MOI) of 5. Six hours after infection, cell-free supernatants were collected for subsequent assay by enzyme-linked immunosorbent assay (ELISA).

Cell lysates were collected for Western blotting by lysis in 1% Triton X-100 (final concentration).

Mice and infections. Wild-type (WT) C57BL/6 mice were obtained from Jackson Laboratories. Casp1−/− mice were backcrossed into a BL6 background for at least five generations. All mice were aged 10 to 12 weeks at the time of infection. For EMCV infections, 2× the 50% lethal dose (LD50) of viral stock...
was prepared in PBS, and 250 μl was injected into mice intraperitoneally on day 0. For VSV infections, mice were anesthetized by injecting 1% ketamine intraperitoneally. VSV stock virus was prepared in PBS, and 2 × 10⁵ PFU was administered in the left nare in a 15-μl volume. Mice were monitored daily, with the frequency increased to twice daily when they began to show signs of clinical illness. They were observed for survival although when mice were clinically morbid or showed frank hindlimb paralysis, they were sacrificed.

**ELISA and Western blotting.** IL-1β and IL-6 ELISAs were performed using sandwich ELISA DuoSets purchased from R&D Systems (catalog items DY401 and DY406, respectively). IFN-γ ELISA was performed by sandwich ELISA using antibodies purchased from US Biological (item I7662-10A) and PBL InterferonSource (PBL 32400-1). For Western blotting, protein samples were run on 4 to 12% bis-acrylamide gels (Invitrogen) and blotted for caspase-1 in 5% milk and Tris-buffered saline–Tween 20 (TBST) using a commercially available antibody (sc-514; Santa Cruz Biotechnology).

**RESULTS**

**EMCV infection activates the NLRP3 inflammasome.** In order to determine if EMCV is detected by the inflammasome, we infected murine bone marrow-derived dendritic cells (BMDC). The TLR2 agonist Pam3 was used to induce pro-IL-1β expression in the cells prior to infection. We found that EMCV infection induced IL-1β secretion (Fig. 1a) and caspase-1 cleavage (Fig. 1b) within 6 h.

IL-1β secretion in response to EMCV infection was not observed in Casp1−/− BMDC (Fig. 1a), indicating that our observations were not due to caspase-1-independent viral cellular lysis. NLRP3 and its adaptor protein, ASC, were also required (Fig. 1a). These results show that EMCV infection of dendritic cells activates the NLRP3 inflammasome.

**EMCV does not prime pro-IL-1β production in BMDC.** IL-1β secretion requires two signals: transcription of pro-IL-1β and processing by caspase-1. To examine whether EMCV infection provides the transcriptional signal, we infected unprimed BMDC. No IL-1β secretion was observed in the absence of priming (Fig. 1c). Western blot analysis also showed no pro-IL-1β in response to viral infection at either 6 or 18 h postinfection (Fig. 1d).

We next examined whether the lack of pro-IL-1β expression was specific to this cytokine. Cytosolic EMCV RNA can activate both NF-κB and interferon (IFN) regulatory factor-3 and -7 (IRF3/7)-dependent transcriptional responses via MDA5/IFN promoter-stimulating factor 1 (IPS-1) signaling (24). We thus measured IL-6 and IFN-β secretion as markers for NF-κB and IRF3/7 activation, respectively. Both IL-6 and IFN-β were secreted in the absence of priming (Fig. 1e and f), albeit at low levels for IL-6 relative to TLR agonists.

These results suggest that while infection of dendritic cells with EMCV activates the NLRP3 inflammasome, it does not provide an IL-1β priming signal. The observation that IL-6 is expressed suggests that NF-κB is activated by EMCV infection yet fails to induce sufficient pro-IL-1β production for detection by Western blotting.

**VSV infection activates the NLRP3 inflammasome.** We next examined a second RNA virus with different structural characteristics. While EMCV is a nonenveloped positive-sense single-stranded RNA (ssRNA) virus, VSV is an enveloped negative-sense ssRNA virus. Primed BMDC secreted IL-1β and induced caspase-1 processing in response to VSV within 6 h of infection (Fig. 2a and b). Similar to EMCV, IL-1β secretion required caspase-1, ASC, and NLRP3 (Fig. 2a). Therefore, both viruses trigger NLRP3 activation.

**VSV M protein inhibits pro-IL-1β production.** VSV is known to inhibit host cell transcription and nuclear export of RNAs via its M protein (19). In accordance with this, VSV infection of unprimed BMDC also did not induce IL-1β secre-
tion (Fig. 2c), and this could be attributed to a lack of pro-IL-1β expression (Fig. 1d). Unlike EMCV, VSV infection did not induce IL-6 or IFN-β secretion in unprimed BMDC (Fig. 2d and e).

To confirm that our observations could be explained by the activity of the M protein, we examined a strain of the virus with an inactivating mutation (M51R) (12). Interestingly, M51R induced the expression of IL-6 and IFN-β and also induced IL-1β secretion from unprimed BMDC (Fig. 2d to f). As with WT VSV, M51R induction of IL-1β secretion required NLRP3 (Fig. 2g). These results show that the VSV M protein inhibits production of pro-IL-1β in infected cells.

**RLR signaling is not required for inflammasome activation by EMCV and VSV.** RIG-I-like receptor (RLR) signaling plays a central role in mediating type I interferon responses to RNA viral infection (10, 24, 25). We therefore investigated whether RLR signaling also played a role in inflammasome activation. Neither MDA5 nor RIG-I was required for induction of IL-1β secretion in response to EMCV (Fig. 3a and c) or VSV (Fig. 3b and d). As controls, secretion of type I IFN in response to

![Graphs and images](http://jvi.asm.org/)

**FIG. 2.** VSV activates the NLRP3 inflammasome. Unprimed and Pam3-primed WT, Casp1−/−, Asc−/−, and Nlrp3−/− BMDC were infected with VSV at an MOI of 5. Supernatants and cell lysates were harvested at 6 h postinfection and assayed for IL-1β (a and c). Caspase-1 cleavage was assessed by Western blotting in WT BMDC (b). Unprimed WT BMDC were infected with VSV and with the VSV M51R mutant. IL-6 (d), IFN-β (e), and IL-1β (f) were measured by ELISA. Pam3-primed WT and Nlrp3−/− BMDC were infected with VSV M51R, and supernatants were assayed for IL-1β by ELISA at 6 h postinfection (g). *nonspecific band.

**FIG. 3.** RLR signaling is not required for EMCV and VSV to activate the Nlrp3 inflammasome. Pam3-primed WT and Mda5−/− BMDC were infected with EMCV (a) and VSV (b) at an MOI of 5. Supernatants were assayed for IL-1β by ELISA at 6 h postinfection. Pam3-primed WT and RIG-I−/− BMDC were infected with EMCV (c) and with VSV and the VSV M51R mutant (d) at an MOI of 5. Supernatants were assayed for IL-1β by ELISA at 6 h postinfection. IFN-β (e and g) and IL-6 (f and h) were also measured by ELISA as controls.
EMCV required MDA5 (Fig. 3e) while secretion of type I IFN in response to M51R was dependent on RIG-I (Fig. 3g). Although IL-6 secretion did not require either MDA5 or RIG-I (Fig. 3f and h), we did note a decrease in IL-6 secretion in response to EMCV in MDA5−/− BMDC and in response to M51R in RIG-I−/− BMDC, consistent with IPS-1-dependent NF-κB activation. These results show that RLR signaling does not play a role in induction of inflammasome activation in response to EMCV or VSV, in contrast with a prior report (17).

Viral replication is required for activation of the inflammasome by EMCV and VSV. We next examined whether viral replication was required for EMCV and VSV detection through NLRP3. EMCV and VSV were both inactivated by UV irradiation as confirmed by plaque assay (Fig. 4a and b). Unlike live virus, UV-irradiated EMCV was unable to induce IL-1β secretion from primed BMDC (Fig. 4c). IL-6 and IFN-β secretion was used as a control for virus inactivation. As expected, Pam3-stimulated BMDC secreted IL-6, and this was potentiated by EMCV but not by UV-inactivated EMCV (Fig. 4d). UV-inactivated EMCV also failed to induce IFN-β secretion from unprimed cells (Fig. 4e).

VSV-induced IL-1β secretion from primed cells was also blocked by UV inactivation (Fig. 4f). Again, IL-6 and IFN-β were used as controls. In contrast to EMCV, live VSV inhibited Pam3-induced IL-6 production, while UV-inactivated virus did not (Fig. 4g), consistent with the function of the viral M protein. As expected, IFN-β secretion was inhibited by M protein expression in live virus and was not induced by UV-inactivated virus (Fig. 4h).

These results show that detection of both EMCV and VSV by the inflammasome requires viral replication.

Inflammasome activation by RNA viruses occurs in macrophages. To determine whether the induction of NLRP3 inflammasome activity was specific to dendritic cells, we infected bone marrow-derived macrophages (BMDM) with EMCV and VSV (Fig. 5). Infection of BMDM with both viruses induced IL-1β secretion (Fig. 5a and d) within 6 h of infection, demonstrating the same kinetics we observed in BMDC. However, the amount of IL-1β secreted by BMDM was severalfold lower, as was IFN-β secretion in response to EMCV (Fig. 5c). IL-6 secretion by primed BMDM, however, was robust (Fig. 5b and e). These data show that the ability of EMCV and VSV to activate the inflammasome is not limited to BMDC but that these cells may be more responsive to RNA viral infection than BMDM.

Caspase-1 activity is not required for host defense against EMCV and VSV. Inflammasome activity is required for host defense against influenza A virus, another RNA virus that activates the NLRP3 inflammasome. To determine whether this requirement might be broadly true for this class of pathogens, we infected WT and Casp1−/− mice with EMCV or VSV. In both cases, we observed no difference in survival rates between WT and Casp1−/− mice (Fig. 5f and g). These results indicate that NLRP3 inflammasome activation does not play a general role in host defense against RNA viruses, in contrast with the essential role of type I IFNs.

**DISCUSSION**

In this report, we show that the RNA viruses EMCV and VSV both activate the NLRP3 inflammasome, that replication is required for activation, that it occurs in both BMDM and BMDC, and that it does not play an obvious role (detrimental or beneficial) in host defense against either virus. Although the ability of NLRP3 to detect EMCV and VSV parallels recent reports for influenza A virus (1, 9, 21), the lack of a role for the inflammasome in host defense against EMCV and VSV is an important difference.

Prior work by Poeck et al. also examined inflammasome activation by EMCV and VSV (17). Our two reports agree that EMCV is detected via NLRP3, but in other respects our results are divergent. Poeck et al. have proposed a role for RLRs in the detection of both EMCV and VSV. According to their work, MDA5 plays an undefined but essential role in activation of the NLRP3 inflammasome by EMCV. They further propose that VSV is detected by a novel RIG-I/ASC/caspase-1 inflammasome that does not require NLRP3. Our results do not support the model proposed by Poeck et al. We have consistently observed a requirement for NLRP3 in response to both...
EMCV and VSV have found no evidence that RLR detection is required for inflammasome activation in response to either virus. The reasons for our discrepant results are not clear. While further work will be required to better delineate how RNA viruses activate the inflammasome, our work in combination with the work of other investigators on influenza A virus suggests that NLRP3 may be a common pathway. A second difference between our results and those of Poeck et al. regards the role of priming. We found that EMCV and VSV do not induce detectable pro-IL-1β expression in the absence of exogenous priming. For VSV, this lack of priming is at least in part due to viral inhibition of host gene transcription via its M protein. In contrast to our finding that EMCV does not prime pro-IL-1β expression, Poeck et al. found that exogenous priming was not required for IL-1β secretion in response to either EMCV or VSV. The most likely explanation for our divergent findings is technical. For example, variation between the viral stock preparations or infection methodologies could result in differential exposure of viral pathogen-associated molecular patterns (PAMPs) to vacuolar TLRs. Both our experiments and those of Poeck et al. examine viral infection of dendritic cells in culture, enabling dissection of cell-intrinsic events. In vivo, however, pro-IL-1β expression could be induced in response to other cytokines and inflammatory mediators secreted by neighboring cells (3, 5).

The broader question of how NLRP3 is activated by EMCV, VSV, and influenza A virus infection remains unanswered. These viruses could trigger NLRP3 activation via different mechanisms. For example, Ichinohe et al. have proposed that the influenza A virus matrix protein (an ion channel) triggers NLRP3 activation (8). Alternately, there may be a common pathway leading to NLRP3 activation. In this context, our results showing that viral replication is a requirement for detection of both EMCV and VSV may provide some insights. Double-stranded RNA (dsRNA) is produced during replication of both EMCV and VSV in the cytosol (11, 23), suggesting that cytosolic dsRNA could trigger NLRP3 activation. Under this hypothesis, a novel dsRNA sensor could act upstream of NLRP3 since our data indicate that neither MDA5 nor RIG-I is involved. Many other cellular perturbations occur during viral infection, and another possibility is that one of these may be sensed through NLRP3. For instance, some viruses (including both influenza A virus and VSV) enter cells by endocytosis and require endosomal acidification for delivery of their nucleic acid cargo to the cytosol (6, 16). A pathway involving lysosomal destabilization has been proposed for crystal induced activation of the NLRP3 inflammasome, and it is possible that some viruses could trigger this pathway (7). In fact, recent work suggests that adenovirus, a DNA virus, activates the NLRP3 inflammasome via lysosomal destabilization (2). Future work will further define the mechanisms of detection.

Our results with EMCV and VSV suggest that the role of the NLRP3 inflammasome in host defense against RNA viral infection is complex. Why inflammasome activity is required for host defense against influenza virus but not for EMCV or VSV is not immediately clear. One obvious difference is the target organ tropism of the viruses; influenza is a pulmonary infection while EMCV and VSV both affect the central nervous system (CNS). The influenza A virus inflammasome phenotype is known to be primarily due to IL-1 signaling (9, 20), suggesting that it is more protective in the lung than in the CNS. Indeed, IL-1β has been shown to play a detrimental role in the host response to a CNS-tropic strain of Sindbis virus (an RNA virus that is a member of the Alphavirus family). One intriguing possibility is that EMCV and VSV have evolved mechanisms to prevent inflammasome activation in vivo. This is analogous to the observation that Salmonella enterica serovar Typhimurium can be detected by NLRC4 in vitro but that this response is largely evaded in vivo (15). The lack of detectable pro-IL-1β priming observed in vitro would support such a mechanism. Further work will clarify the mechanisms by which NLRP3...
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REFERENCES