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Activation of the NLRP3 inflammasome by intracellular poly I:C

Jayant V. Rajan^{1,2}, Sarah E. Warren^{1,3}, Edward A. Miao^{1,*}, and Alan Aderem^{1,*}

¹Institute for Systems Biology, Seattle, WA 98103

²Department of Medicine, University of Washington Medical Center, Seattle, WA 98195

³Department of Immunology, University of Washington, Seattle, WA 98195

Abstract

Several RNA viruses can be detected by the inflammasome, which promotes IL-1 β and IL-18 secretion, but the underlying mechanisms of detection remain unclear. Cytosolic dsRNA is a replication intermediate of many RNA viruses. We show here that transfection of the dsRNA analogue poly I:C activates the NLRP3 inflammasome via a pathway requiring endosomal acidification. This detection is independent of the other poly I:C sensors: TLR3 and MDA5. These results suggest a mechanism by which cytosolic dsRNA produced during viral infection could activate the NLRP3 inflammasome.

Keywords

Inflammasome; poly I:C; RNA virus; IL-1 β

1. Introduction

Broadly speaking, viruses may be categorized on the basis of their genomic material: DNA vs RNA, and single vs double stranded. Since nucleic acid is a major component of any virus, it is unsurprising that it is detected by multiple pathways. Many nucleic acid sensors induce type I interferon secretion. TLR3, TLR7/8, and TLR9 respond to dsRNA, ssRNA, and dsDNA, respectively, in the vacuolar compartment, while the RLRs MDA5 and RIG-I sense cytosolic viral RNA. Cytosolic DNA also induces type I interferon secretion via an unknown sensor [1].

Cytosolic DNA has also recently been shown to be detected by the AIM2 inflammasome [2–5]. Inflammasomes are platforms for Caspase-1 activation consisting of a regulatory protein (AIM2 or a Nod-like receptor; NLR) and, in most cases, the adaptor protein ASC. Once assembled, the inflammasome promotes Caspase-1 cleavage, resulting in its activation. Activated Caspase-1 cleaves IL-1 β and IL-18 to their mature, secreted forms and induces an inflammatory form of cell death called pyroptosis. Thus IL-1 β and IL-18 secretion are regulated at two steps: NF- κ B dependent transcription, and Caspase-1 dependent processing [6].

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Send correspondence to Edward Miao Telephone: 206-732-1358 Fax: 206-732-1260 emiao@systemsbiology.org.

*These authors contributed equally to this work

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Inflammasome activation can play a role in host defense against viral infection. For instance, mice deficient in NLRP3, ASC or Caspase-1 are more susceptible to influenza A infection [7–9]. Other RNA viruses, including EMCV and VSV, have been shown to activate the inflammasome *in vitro*, but the mechanism of detection is unknown [10].

In addition to being the genomic material for dsRNA viruses, dsRNA is also a replication intermediate of many ssRNA viruses [11]. We therefore hypothesized that cytosolic dsRNA might be detected by the inflammasome. We show here that transfected poly I:C, a synthetic viral dsRNA analogue, rapidly activates the NLRP3 inflammasome in macrophages and dendritic cells.

2. Materials and Methods

Cells and cell culture

Bone marrow cells from 8–12 week old female C57BL/6 mice were differentiated in RPMI + 10% FBS supplemented with 10ng/ml rhM-CSF (macrophages) or 20ng/ml rmGM-CSF (dendritic cells) and refed on day 4. Macrophages were collected on day 6, re-seeded in 96-well plates at 500 cells/ul and stimulated on day 7. Dendritic cells were collected on day 7, re-seeded in 96-well plates at 1000 cells/ul and stimulated. All day 7 cells were cultured in complete RPMI without growth factor. Cells were primed with PAM3 at a concentration of 1 mg/ml for 3 hours. THP-1 cells were seeded in RPMI + 10% FBS at 500cells/ul and differentiated with 10nM PMA. They were stimulated on day 3 and primed with 100ng/ml LPS for 3 hours.

Transfections/stimulations

All transfections were performed using Lipofectamine 2000 or FuGene. Each agent was incubated for 5 minutes at room temperature prior to mixing with nucleic acid. Complexes were formed by mixing nucleic acid with preincubated transfection agent for 30 minutes at room temperature and subsequently added to cells. Stimulations with Alum (Pierce 77161) were performed by adding it to media to a final concentration of 500ug/ml. poly I:C was obtained from Amersham and Sigma, while poly dA:dT, poly I, poly C, and poly U were all purchased from Sigma. All ligands were transfected at a final concentration of 1ug/ml unless otherwise indicated.

Electroporation

Electroporation was performed using 'intracellular buffer' [12]. Cells were primed using 100ng/ml LPS and 2×10^6 cells were electroporated with 10ug of nucleic acid in 200ul.

ELISA

IL-1 β and IL-6 ELISAs were performed on cell-free supernatants using ELISA duosets obtained from R&D systems (DY201, DY401, DY406). IFN- β sandwich ELISA was performed using antibodies purchased from US Biological (I7662-10A) and PBL InterferonSource (PBL 32400-1). A plate reader was used to measure OD450 to calculate cytokine concentration.

3. Results

Transfected poly I:C rapidly activates the NLRP3 inflammasome

To determine if cytosolic dsRNA induces Caspase-1 activation, we transfected poly I:C into bone marrow derived macrophages (BMDM). Transfected poly I:C induced IL-1 β secretion within 4 hours (Fig. 1a), as did transfected poly dA:dT as a control (Fig. 1c). No signal was

observed in the absence of the transfection reagent, suggesting that cytosolic delivery is required. In both cases, priming with a TLR agonist to induce pro-IL-1 β transcription was required (Fig. 1b,1d). poly I:C induced IL-1 β secretion required Caspase-1, ASC and NLRP3. In contrast, IL-1 β secretion in response to poly dA:dT did not require NLRP3, consistent with its detection by AIM2. Extracellular poly I:C alone induced IFN- β (Fig. 1e) but not IL-1 β (Fig. 1f) secretion at 20 hours. Finally, although THP-1 cells secreted IL-1 β in response to transfected poly dA:dT, transfected poly I:C had no effect (Fig. 1f). These results show that transfected poly I:C activates the NLRP3 inflammasome in BMDM.

MDA5, IPS-1, TLR3 and TRIF are not required for activation of the inflammasome by transfected poly I:C

Cytosolic poly I:C is detected by MDA5 which signals via the adaptor protein IPS-1 to induce IFN- β secretion. We found that MDA5^{-/-} and IPS-1^{-/-} BMDMs retained the ability to secrete IL-1 β after poly I:C transfection (Fig. 2a,2b). Vacuolar poly I:C is detected by TLR3 which signals through the adaptor protein TRIF. However, IL-1 β secretion in response to transfected poly I:C was still observed in TLR3^{-/-} or TRIF^{-/-} BMDMs (Figure 2a). There was a reproducible reduction in IL-1 β secretion in both IPS-1^{-/-} and TRIF^{-/-} cells that was specific, as IL-6 secretion was not reduced (data not shown). While the mechanism of this reduction is unclear, these cells retain the ability to respond to transfected poly I:C through Caspase-1 (Fig. 2a, 2b). IFN- β was measured as a control and was decreased in all knockout lines in response to both extracellular (Fig. 2c) and transfected (Fig. 2d,2e) poly I:C. Thus NLRP3 activation in response to transfected poly I:C does not require established dsRNA detectors.

Dose and ligand dependence of poly I:C activation of the inflammasome

We next examined the sensitivity and structural requirements for poly I:C detection by the inflammasome. A dose titration determined the threshold of poly I:C detection to be between 0.1 μ g/ml and 1 μ g/ml (Fig. 3a). This likely represents the efficiency of poly I:C packaging by lipofectamine; inefficient packaging likely occurs at both high and low ratios of nucleic acid to lipofectamine reagent.

Commercially available poly I:C preparations are heterogeneous, leading us to compare two different preparations by agarose gel electrophoresis (Fig. 3b). Despite significant differences in their relative sizes, transfection of both reliably induced IL-1 β secretion (Fig. 3c). Finally, to investigate the structural requirements for inflammasome activation, we transfected BMDM with single stranded poly I, poly C or poly U. None of these ligands induced IL-1 β secretion (Fig. 3d), showing that double stranded structure is required for IL-1 β secretion in response to transfected RNA.

Electroporation of poly I:C elicits IL-1 β secretion

To rule out the possibility that our results were an artifact of lipid transfection, we introduced poly I:C into BMDM by electroporation. As with our transfection experiments, we found that electroporation of poly I:C resulted in Caspase-1 dependent IL-1 β secretion and that this required priming with a TLR ligand (Fig. 3e,3f). Activation of the inflammasome by cytosolic nucleic acid was thus not a function of the mode of delivery.

Inflammasome activation by transfected poly I:C requires endosomal acidification

Several inhibitors are known to have differential effects on NLRP3 activity. Cytochalasin D prevents phagocytosis and blocks NLRP3 activation by crystals. We found that inhibition of phagocytosis did not affect IL-1 β secretion in response to transfected poly I:C, but

completely inhibited alum induced IL-1 β secretion (Fig. 4a, 4c). Like poly I:C, poly dA:dT was unaffected by cytochalasin treatment (Fig. 4b).

Bafilomycin inhibits the vacuolar ATPase, blocking acidification, and partially blocks IL-1 β secretion in response to crystals. We found that bafilomycin treatment significantly diminished IL-1 β secretion in response to transfected poly I:C (Fig. 4a). While bafilomycin also inhibited inflammasome activation by both alum and poly dA:dT, the effect was not as strong as it was for poly I:C (Fig. 4b,4c). Interestingly, bafilomycin treatment did not inhibit induction of IL-1 β secretion by electroporation of both poly I:C and poly dA:dT (Fig. 4d, 4e).

Dendritic cells secrete IL-1 β in response to transfected poly I:C

Finally, we examined whether bone marrow derived dendritic cells (BMDC) also responded to transfected poly I:C. BMDCs secreted IL-1 β in response to transfected poly I:C with a dose response that was similar to that observed for BMDM (Fig. 4f) and this response was Caspase-1 dependent (Fig. 4g). These results show that the detection pathway for transfected poly I:C is present in both DCs and macrophages.

4. Discussion

In this study, we have shown that transfected dsRNA induces activation of the NLRP3 inflammasome, eliciting IL-1 β secretion in macrophages and dendritic cells. This is the first demonstration that transfected dsRNA activates the NLRP3 inflammasome.

Other investigators have examined inflammasome activation by extracellular RNA and have shown that poly I:C and bacterial RNA induce IL-1 β secretion only after prolonged stimulation (24h) [13,14]. The delayed activation by extracellular poly I:C in these reports contrasts with rapid activation by an NLRP3 agonist such as ATP. We find that transfected poly I:C induces IL-1 β secretion relatively quickly (4 hours), but were unable to observe IL-1 β secretion in response to overnight stimulation with extracellular poly I:C. The difference between our results and what has previously been reported may be due to variation in experimental conditions and/or cell types used.

This poly I:C detection pathway is likely conserved as Rintahaka, *et al.* showed that transfected poly I:C induces IL-1 β secretion in human monocyte derived macrophages, although the NLR responsible was not identified [15]. However, Muruve *et al.* showed that THP-1 cells do not secrete IL-1 β in response to transfected poly I:C and we verified their results [16]. The differences between primary monocytes derived macrophages and THP-1 cells remain to be elucidated.

NLRP3's central role in host defense and disease pathogenesis has been widely documented [17]. It has been reported to sense extracellular ATP, crystals, and pore forming toxins, as well as bacterial, viral, and fungal infections [18]. It is unlikely that NLRP3 itself directly senses each of its many agonists. Several mechanisms have been proposed and have recently been reviewed [19–21]. One of these proposed mechanisms involves lysosomal disruption.

We observed that bafilomycin inhibits IL-1 β secretion in response to transfected poly I:C. There are two possible mechanisms to explain this observation. First, transfection of poly I:C may result in acidification dependent lysosome disruption, which has been proposed to activate NLRP3 [22]. Alternatively, endosomal acidification may promote fusion of liposomes with the endosomal membrane, increasing the efficiency of nucleic acid delivery to the cytosol. The latter possibility is supported by the observations that bafilomycin

partially inhibits poly dA:dT detection by, but does not inhibit IL-1 β secretion in response to electroporated poly I:C.

Recently, it was reported that the RNA viruses EMCV, VSV, and influenza activate the inflammasome [7–10]. A tantalizing hypothesis would be that recognition of dsRNA produced during viral infections provides a unifying mechanism of RNA virus detection by the inflammasome via NLRP3. However, based on the available data, this seems unlikely. We show that inflammasome activation by transfected poly I:C was NLRP3 dependent and MDA5 independent. In contrast, activation of the inflammasome by EMCV required MDA5 and NLRP3, while activation by VSV required RIG-I. Influenza A viral infection is detected via the NLRP3 inflammasome, though a role for RLRs has not been investigated. Instead, a mechanism involving endosomal ion fluxes due to the influenza M protein (an ion channel) has been proposed [23].

In conclusion, two main points can be drawn from our work and that of others: 1) NLRP3 plays a central role in the detection of some RNA viral infections, and 2) there are likely multiple pathways by which RNA viruses can activate the inflammasome. Future work will clarify the role of cytosolic dsRNA detection by the inflammasome in response to RNA viral infection.

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Figure 1. Activation of the NLRP3 inflammasome by transfected poly I:C

Primed (A,C) and unprimed (B,D) wild-type, Caspase-1^{-/-}, ASC^{-/-} and NLRP3^{-/-} BMDM were transfected with poly I:C and IL-1β measured by ELISA 4H later. (E) WT BMDM were stimulated with poly I:C for 20H and IL-1β measured by ELISA. (F) THP1 cells were transfected with poly I:C, and assayed for IL-1β by ELISA 4H later.

Figure 2. MDA5, TLR3 and TRIF are not required for poly I:C induced IL-1 β secretion (A,B) WT, MDA5^{-/-}, TLR3^{-/-}, TRIF^{-/-} and IPS-1^{-/-} BMDM were transfected with poly I:C using FuGene and assayed for IL-1 β by ELISA 4H later. Unprimed BMDM were stimulated with (C) extracellular (100ug/ml) and (D,E) transfected (FuGene) poly I:C and IFN- β measured by ELISA 20H later.

Figure 3. Dose and ligand dependence of poly I:C activation of the inflammasome
 (A) WT BMDM were transfected with poly I:C while maintaining a fixed amount of Lipofectamine and IL-1 β measured by ELISA 4H later. (B) 30 μ g of poly I:C was run on a 0.8% agarose gel and visualized with Gel Green stain (C) poly I:C was transfected into WT BMDM and IL-1 β measured 4H later. (D) poly I:C, poly I, poly C and poly U were transfected into WT BMDM and IL-1 β secretion measured by ELISA 4H later. (E,F) poly I:C and poly dA:dT were electroporated into WT and Caspase-1 BMDM and IL-1 β measured by ELISA 4H later.

Figure 4. Inhibition of poly I:C induced IL-1 β secretion by bafilomycin and activation in dendritic cells

(A–C) WT BMM were primed for 3 hours and treated with 1 μ M Cytochalasin D or 50nM Bafilomycin for the final hour before (A) poly I:C transfection, (B) poly dA:dT transfection, (C) alum (500ug/ml), (D) poly I:C electroporation, or (E) DNA/poly dA:dT electroporation. IL-1 β was measured by ELISA 4H later. (F) poly I:C dose curve in BMDC as in Fig 1e. (H) primed WT and Caspase-1^{-/-} BMDC were transfected with poly I:C and IL-1 β measured by ELISA 4H later.