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TLR3 signaling is downregulated by a MAVS isoform in epithelial cells

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Abstract

Innate immune responses to dsRNA result in signaling through the TLR3 pathway and/or the RIG-I/MDA-5/MAVS pathway which can activate type I IFN, proinflammatory cytokines and apoptosis. It is not clear whether MAVS could play a role in TLR3-dependent responses to extracellular dsRNA. Using a model of epithelial cells that express a functional TLR3 signaling pathway, we found that TLR3-dependent responses to extracellular dsRNA are negatively regulated by MAVS, precisely "miniMAVS", a recently described 50 kDa isoform of MAVS. This regulation of TLR3 by a MAVS isoform constitutes an endogenous regulatory mechanism in epithelial cells that could help prevent a potentially damaging excessive inflammatory response.

INTRODUCTION

Defense against RNA viruses involves detection of viral genetic material by a set of specialized sensors expressed by multiple innate immune cell types, including intestinal epithelial cells [1, 2]. Extracellular viral dsRNA or its synthetic analog poly(I:C) (pIC) can be sensed by TLR3, which triggers TRIF-dependent signaling, whereas RIG-I and MDA-5 detect intracellular dsRNA and signal through the adaptor molecule MAVS (also known as VISA, IPS-1 or CARDIF) to ultimately lead to the activation of the transcription factors NF- κ B and IRF3 and subsequent production of type I IFN and proinflammatory cytokines [3]. TLR3/TRIF can also activate apoptosis through activation of the RIP/FADD/caspase-8 pathway [4, 5]. Studies using intestinal epithelial cells reported that responses to Rotavirus, a dsRNA virus, mostly rely on RIG-I/MDA-5/MAVS signaling to activate a protective type I IFN response [6], although an important role for TLR3 has been suggested [4]. In the airway epithelium, MAVS activation is crucial for induction of interferons in response to Influenza

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Competing Interests

The authors declare no conflict of interest.

A virus [7], but can have some downsides. For instance, respiratory syncytial virus-induced protease expression in the lung, which leads to lung hyperresponsiveness and reduced virus clearance, also depends on MAVS activation [8].

TLR3 and MAVS share many of the same signaling intermediates to activate interferons and previous studies hinted at a possible participation of MAVS in the TLR3 signaling pathway. In one of the first studies describing MAVS [9], an interaction between MAVS and several components of the TLR3 signaling pathway was required for full activation of type I IFN. However, this observation was contradicted by several other studies [10-12]. More recently, a MAVS-TRIF complex was detected in myeloid dendritic cells, the abundance of which was upregulated by pIC [9, 13], but its function remains unknown.

We aimed at evaluating a potential contribution of MAVS to dsRNA-activated TLR3 signaling in intestinal epithelial cells. We used HCT116 cells as a model of epithelial cells as they have been previously reported to respond to extracellular dsRNA stimulation in a TLR3-dependent manner [14]. Using RNA interference, we found that TLR3-dependent responses to extracellular dsRNA are negatively regulated by MAVS. We further observed that the inhibitory effect was mediated by miniMAVS, a recently characterized 50 kDa isoform of MAVS [15].

MATERIAL AND METHODS

Cell culture and reagents

The human colorectal cancer cell line HCT116 and the bronchial epithelial cell line BEAS-2B were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in RPMI 1640 medium (for HCT116) or DMEM/F12 (for BEAS-2B) containing 2mM L-glutamine, 50 IU/mL penicillin, 50 µg/mL streptomycin and 10% heat-inactivated fetal bovine serum in a humidified 5% CO₂ atmosphere at 37°C. Low molecular weight poly(I:C) (pIC) was from Invivogen (San Diego, CA). Stimulation of TLR3 was achieved by adding pIC directly to the cell culture media at a concentration of 10 µg/ml.

Gene silencing

Gene silencing was achieved using Dharmacon siGENOME smart pool siRNA using non-targeting siRNA as control (Thermo Fisher Scientific, Lafayette, CO) at a concentration of 100 nM for HCT116 and 25nM for BEAS-2B cells. SiRNA transfections were performed using Dharmafect 4 transfection reagent according to manufacturer instructions. A minimum 90% decrease in all target genes expression was achieved as verified by real-time PCR (Fig. 1A-C). Transfection of siRNA alone had no effect on basal IFN-β gene expression (Fig. 1D).

Overexpression of miniMAVS

Expression plasmid encoding miniMAVS (50 kDa isoform of MAVS) and the corresponding empty vector control (EV) were generously donated by Dr J. Kagan laboratory (Boston Children's Hospital) [15]. For plasmid transfection, 0.5-0.75 µg of plasmid was incubated with 2µl of Turbofect (Thermo Fisher Scientific, Lafayette, CO) and transfected in 500 µl of

media in a 24-well plate for 24h before stimulation with pIC and gene or protein expression analysis.

RNA purification and real-time PCR

For quantitative real-time polymerase chain reaction (qPCR) analysis, total RNA was purified using the RNeasy kit (Qiagen, Germantown, MD). Then, cDNAs were synthesized out of total RNA using the Iscript reverse transcription kit (Biorad, Hercules, CA) according to manufacturer's directions. qPCR reactions were performed using SYBR Green PCR master mix on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). A standard amplification protocol was used (95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min) and each reaction was run in triplicate. Relative mRNA expression was normalized to GAPDH expression, calculated using the delta Ct and expressed as fold change or 2^{-Ct} . The following oligonucleotides were used : GAPDH-For 5'-GCCTTCCGTGTCCCCACTG, GAPDH-Rev 5'-CGCCTGCTTCACCACCTTC ; IFN- β -For 5'-AAACTCATGAGCAGTCTGCA, IFN- β -Rev 5'-AGGAGATCTTCAGTTTCGGAGG ; IL-6-For 5'-AGGGCTCTTCGGCAAATGTA, IL-6-Rev 5'-AAGGAATGCCATTAACAACAAC ; TNF-For 5'-CCATCAGAGGGCCTGTACCT, TNF-Rev 5'-GCAGCCTTGGCCCTTGA ; TLR3-For 5'-ACCCGATGATCTACCCACAAAC, TLR3-Rev 5'-GTTGGCGGCTGGTAATCTTC

Protein quantification

For immunoblot analysis, proteins were extracted using RIPA buffer containing sodium orthovanadate, protease inhibitors and PMSF (Santa Cruz Biotechnology, Dallas, TX). Antibody against actin (1/5000) was from Sigma (St. Louis, MO), anti-TRIF (1/500), anti-p-IRF3 (1/500), anti-p-I κ B α (1/500) were from Cell Signaling Technology (Danvers, MA). Antibody against MAVS (1/1000) was obtained from Bethyl Laboratories (Montgomery, TX). Nitrocellulose membranes were scanned and analyzed in a Li-Cor Odyssey Imager (LI-COR Biotechnology, Lincoln, NE). For measurement of IFN- β in supernatants, we used the Human IFN- β ELISA Kit from PBL (Piscataway, NJ).

Statistics

Data was analyzed using GraphPad Prism 5 software and presented as mean and the standard error of the mean (s.e.m.). Two-tailed unpaired Student's t-tests were used to analyze the results. Differences were considered significant when $p < 0.05$.

RESULTS

Extracellular dsRNA signaling in HCT116 relies on TLR3/TRIF and is negatively regulated by MAVS

Extracellular sensing of dsRNA in HCT116 relied on TLR3 and TRIF, as silencing of either one led to a significant reduction in IFN- β expression following pIC stimulation (Fig. 2A-B). In striking contrast, MAVS silenced cells activated with pIC manifested a significant increase in IFN- β expression and secretion (Fig. 2A-B). Similar to IFN- β , the pro-inflammatory cytokines IL-6 and TNF were also upregulated in response to pIC in the absence of MAVS (Fig. 2C-D). This suggested that MAVS was acting as an inhibitor of

TLR3 signaling. The upregulation caused by MAVS silencing was not observed if TLR3 or TRIF were silenced together with MAVS (Fig. 2E), which suggested that a functional and activated TLR3/TRIF pathway was required for the regulatory activity of MAVS to take place.

We next wanted to test whether a similar observation could be made in other epithelial cells known to respond to extracellular pIC in a TLR3/TRIF-dependent manner. A robust response to extracellular pIC was produced by the bronchial epithelial cell line BEAS-2B, and similar to HCT116, IFN- β expression was TRIF-dependent and was upregulated in MAVS silenced cells (Fig. 2F). This result demonstrated that the phenotype observed is not specific to HCT116 and may well be taking place in epithelial cells from other mucosal sites.

MAVS silencing upregulates NF- κ B and IRF3 signaling without affecting TLR3/TRIF levels

Negative regulation of TLR3 signaling has been linked to the degradation of TRIF [16-18]. In HCT116, pIC-induced degradation of TRIF was not affected in MAVS silenced cells (Fig. 3A) and TLR3 expression was not different between control and MAVS silenced cells (Fig. 3B), thus indicating that the MAVS effect did not involve a regulation of TLR3 and TRIF protein levels.

We then reasoned that silencing MAVS may affect signaling events mediated through TRIF. Downstream of TRIF, signaling is transduced by TRAF3 and TRAF6 leading to the activation of IRF3 and NF- κ B signaling pathways [1, 5]. We evaluated IRF3 and NF- κ B activation by determining the levels of IRF3 and I κ B- α phosphorylation (Fig. 3C-D). IRF3 and I κ B- α phosphorylation was detected 3h after stimulation of HCT116 cells with pIC. As expected, no phosphorylation was detected in TLR3 and TRIF silenced cells but MAVS silencing resulted in increased IRF3 and I κ B- α phosphorylation. Previous reports showed that TRIF may interact with MAVS in some cell types [9, 13] but no evidence of such interaction in HCT116 was found using co-immunoprecipitation (data not shown).

MiniMAVS overexpression downregulates IFN- β expression following TLR3 stimulation

Some studies showed that MAVS can exist in multiple isoforms [15, 19]. A 50 kDa isoform named miniMAVS was recently described as an inactive variant that could compete with canonical MAVS for TRAFs protein binding, leading to downregulation of MAVS dependent gene expression [15].

In HCT116, we detected 2 major forms of MAVS, the canonical, full length 75 kDa form and a 50 kDa form corresponding to miniMAVS. MAVS silencing led to reduction in both forms (Fig. 4A). We hypothesized that miniMAVS also impairs TLR3 signaling by competing with TRIF for TRAFs binding. In HCT116 cells overexpressing miniMAVS, we found that IFN- β expression following pIC-activated TLR3 signaling in HCT116 cells was reduced by 30-40% compared to stimulated cells transfected with the empty vector control (Fig. 4B,D). Downregulation of IFN- β by miniMAVS was dose-dependent (Fig. 4C). Of note, HCT116 cells transfected with pIC to activate RIG-I/MDA-5/MAVS-dependent signaling also had decreased IFN- β expression in cells overexpressing miniMAVS compared to cells expressing the control vector (Fig. 4E), indicating that miniMAVS could also inhibit

canonical MAVS signaling, as previously reported [15]. In addition, overexpression of miniMAVS led to downregulation of IL-6 and TNF gene expression (Fig. 4F,G) as well as downregulation of p-IRF3 and p-I κ B- α protein levels (Fig. 4H) in pIC activated cells. Taken together, our data indicated that the 50 kDa isoform of MAVS (miniMAVS) can dampen TLR3 dependent responses.

DISCUSSION

In the present study we report new findings regarding the regulation of the TLR3 signaling *in vitro* in epithelial cells. Using HCT116 cells as a model, we found that TLR3 signaling activated by extracellular dsRNA is negatively regulated by MAVS, precisely one of its isoform.

The idea that MAVS could be a part of the TLR3 signaling pathway was previously proposed. Xu and collaborators found that MAVS interacted with TRIF and TRAF6, and silencing MAVS led to a reduced IFN promoter activation in pIC-activated 293 cells [9]. While the described effect of MAVS is the opposite of our finding, this study was the first to suggest an interaction between TLR3 signaling and MAVS. More recently, Zhang et al. described the existence of a TRIF-MAVS complex in myeloid dendritic cells but did not comment on the functional consequences of such interaction [13]. These studies conflict with other reports that did not find a requirement for MAVS in the TLR3 pathway [10-12]. It appears that the existence of a MAVS interaction with TLR3 signaling components, such as TRIF and TRAFs, and its outcomes are likely cell-type dependent and may rely on the involvement of MAVS isoforms, which was not accounted for in these studies. Therefore, additional studies are needed to fully understand the role and requirement of MAVS and its isoforms in regulating cell signaling.

Multiple MAVS isoforms can be expressed [19] and a 50 kDa MAVS variant named miniMAVS has recently been characterized by Brubaker and colleagues [15]. MiniMAVS is the product of alternative translation that lack of the N-terminal CARD domain required for signal transduction. MiniMAVS has been shown to interfere with the canonical MAVS induced gene expression due to its ability to bind TRAFs, which are key signaling molecules normally recruited by the activated canonical MAVS. We found that overexpression of miniMAVS led to reduced IFN- β induction following TLR3 activation. Since TRAF proteins are also recruited by TRIF for signaling, a similar interference of miniMAVS, i.e through binding of TRAFs, is likely taking place when TLR3/TRIF signaling is activated.

Due to the multifactorial role of TLR3 in viral defense [20] and the fact that miniMAVS is expressed only in primates and higher mammals [15], further studies are required to address the physiological importance of TLR3 signaling downregulation by miniMAVS. Nevertheless, the regulatory mechanism we describe may be involved in controlling type I IFN levels in epithelial cells, which could help set, in a specific context, an adequate immune response to prevent excessive, tissue damaging responses.

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Extracellular poly(I:C) activates TLR3-dependent IFN- β expression in HCT116

MAVS silencing upregulates IFN- β production via upregulation of NF- κ B and IRF3 signaling.

TLR3 signaling is regulated by miniMAVS, a 50kDa isoform of MAVS.

Figure 1. siRNA transfection efficiency and effect on basal IFN- β levels

Expression of TLR3 (A), TRIF (B) and MAVS (C) following gene silencing using the corresponding siRNA in HCT116. (D) Effect of siRNA treatment alone on basal IFN- β expression in HCT116. Results represent the mean $\pm$ S.E.M of 3 independent experiments. Values are expressed as $2^{-\Delta Ct}$.

Figure 2. Extracellular dsRNA signaling in HCT116 relies on TLR3/TRIF and is negatively regulated by MAVS

Expression of IFN- β (A), IL-6 (C) and TNF (D) in HCT116 cells treated with non-targeting control (Cont.), TLR3, TRIF and MAVS siRNA, followed by stimulation with pIC (10 μ g/ml) for 3h. (B) IFN- β in supernatants was detected after 6h of pIC stimulation (10 μ g/ml). (E) Expression of IFN- β in pIC stimulated HCT116 cells (10 μ g/ml – 3h) treated with MAVS siRNA alone and in combination with TRIF and TLR3 siRNAs. (F) Expression of IFN- β in BEAS-2B bronchial epithelial cells treated with TRIF and MAVS siRNA, followed by 3h stimulation with pIC (10 μ g/ml). Values are expressed as fold change compared to untreated (Unt.). N.S: not significant; * = $p < 0.05$ compared to control. Results represent the mean $\pm$ S.E.M of 3 independent experiments.

Figure 3. MAVS silencing upregulates NF- κ B and IRF3 signaling without affecting TLR3/TRIF levels

(A) TRIF protein levels in untreated and pIC (10 μ g/ml) treated HCT116 cells following gene silencing with Control, TLR3, TRIF and MAVS siRNAs. Protein extracts were collected 16 hours post-stimulation. Results from 1 representative experiment out of 2 performed.

(B) TLR3 expression in HCT116 cells following silencing of MAVS. Values are expressed as 2^{-Ct} . Results of 1 representative experiment out of 2 performed.

(C) Blot of phosphorylated IRF3 and I κ B- α expressed in HCT116 silenced for the expression of TLR3, TRIF and MAVS and stimulated with pIC (10 μ g/ml) for 3h. The histogram (D) represents the quantification of gel band intensities expressed as a ratio of the fluorescent signal measured for p-IRF3 and I κ B- α to the signal of actin. $\emptyset$ = no siRNA.

N.S: not significant; $\ast$ = $p < 0.05$ compared to control. Results of 1 representative experiment out of 3 performed.

Figure 4. MiniMAVS overexpression downregulates IFN-β expression following TLR3 stimulation

(A) Immunoblot of MAVS protein expression in HCT116 cell lysates. 2 major bands are observed at 75 kDa and 50 kDa, corresponding to full length MAVS and miniMAVS respectively. Cells treated with MAVS siRNA show reduction in the expression levels of both forms of MAVS. (B) Immunoblot of miniMAVS protein expression following transfection of HCT116 with an empty vector control (EV) or vector encoding miniMAVS (constitutive expression). (C) Expression of IFN-β in HCT116 cells stimulated with extracellular pIC (10 μg/ml – 3 h), following transfection with increasing concentration of miniMAVS plasmid (0.2–1.5 μg). Results of 1 representative experiment out of 2 performed. (D) Expression of IFN-β in HCT116 cells stimulated with extracellular pIC, following transfection with an empty vector or miniMAVS expression plasmid. IFN-β gene expression was measured after 3 h stimulation with pIC (10 μg/ml). Result represents the mean $\pm$ S.E.M of 3 independent experiments. (E) Expression of IFN-β in HCT116 stimulated with intracellular pIC, following transfection with an empty vector or miniMAVS expression plasmid. IFN-β gene expression was measured after 6 h of transfection with pIC (1 μg). (F–G) Expression of IL-6 (F) and TNF (G) in HCT116 cells stimulated with extracellular pIC following transfection with an empty vector or miniMAVS expression plasmid. Gene expression was measured after 3 h stimulation with pIC (10 μg/ml). Result represents the mean $\pm$ S.E.M of 3 independent experiments. * = $p < 0.05$. Values are expressed as fold change compared to control. (H) Immunoblot of p-IRF3 (top) and p-IκB-α (bottom) protein expression following 3 h stimulation with pIC (10 μg/ml) in cells transfected with the empty vector control (EV) or vector encoding miniMAVS. ∅ = untreated.