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Short communication

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.

1. 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-κB 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 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].

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2. Material and methods

2.1. 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 2 mM l-glutamine, 50 IU/mL penicillin, 50 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum in a humidified 5% CO_2 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.

2.2. 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 siRNAs alone had no effect on basal IFN-β gene expression (Fig. 1D).

2.3. 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 2 μl of Turbofect (Thermo Fisher Scientific, Lafayette, CO) and transfected in 500 μl of media in a 24-well plate for 24 h before stimulation with pIC and gene or protein expression analysis.

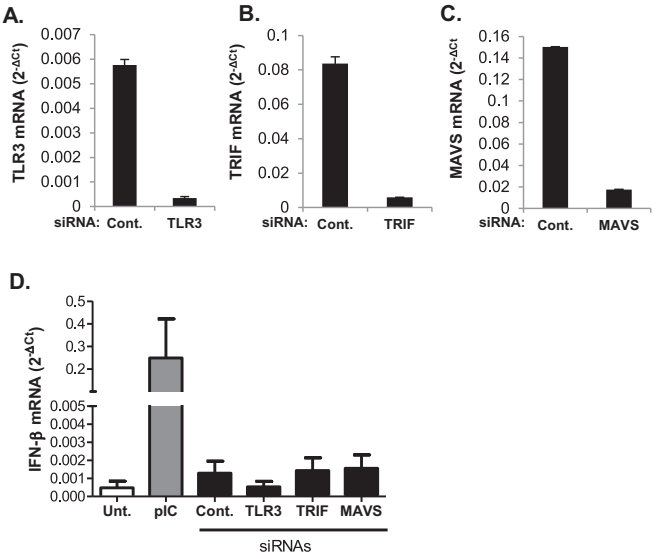

Fig. 1. siRNA transfection efficiency and effect on basal IFN-β levels. Expression of TLR3 (A), TRIF (B) and MAVS (C) following gene silencing using the corresponding siRNA in HCT116. (D) Effect of siRNA treatment alone on basal IFN-β expression in HCT116. Results represent the mean +/− S.E.M of 3 independent experiments. Values are expressed as $2^{-\Delta Ct}$.

2.4. 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 $2^{-\Delta Ct}$. The following oligonucleotides were used: GAPDH-For 5′-GCCTTCCGTGTCCCCACTG, GAPDH-Rev 5′-CGC CTGCTTCACCACCTTC; IFN-β-For 5′-AAACTCATGAGCAGTCTGCA, IFN-β-Rev 5′-AGGAGATCTTCAGTTTCGGAGG; IL-6-For 5′-AGGGCT CTTCGGCAAATGTA, IL-6-Rev 5′-AAGGAATGCCCATTAACAACAAC; TNF-For 5′-CCATCAGAGGGCCTGTACCT, TNF-Rev 5′-GCAGCCTTGG CCCTTGA; TLR3-For 5′-ACCCGATGATCTACCCACAAAC, TLR3-Rev 5′-GTTGGCGGCTGGTAATCTTC.

2.5. 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-IκBα (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).

2.6. 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 $p < 0.05$.

3. Results

3.1. 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,