Video Article

Dissecting Innate Immune Signaling in Viral Evasion of Cytokine Production

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Abstract

In response to a viral infection, the host innate immune response is activated to up-regulate gene expression and production of antiviral cytokines. Conversely, viruses have evolved intricate strategies to evade and exploit host immune signaling for survival and propagation. Viral immune evasion, entailing host defense and viral evasion, provides one of the most fascinating and dynamic interfaces to discern the host-virus interaction. These studies advance our understanding in innate immune regulation and pave our way to develop novel antiviral therapies.

Murine γHV68 is a natural pathogen of murine rodents. γHV68 infection of mice provides a tractable small animal model to examine the antiviral response to human KSHV and EBV of which perturbation of *in vivo* virus-host interactions is not applicable. Here we describe a protocol to determine the antiviral cytokine production. This protocol can be adapted to other viruses and signaling pathways.

Recently, we have discovered that γHV68 hijacks MAVS and IKKβ, key innate immune signaling components downstream of the cytosolic RIG-I and MDA5, to abrogate NFKB activation and antiviral cytokine production. Specifically, γHV68 infection activates IKKβ and that activated IKKβ phosphorylates RelA to accelerate RelA degradation. As such, γHV68 efficiently uncouples NFKB activation from its upstream activated IKKβ, negating antiviral cytokine gene expression. This study elucidates an intricate strategy whereby the upstream innate immune activation is intercepted by a viral pathogen to nullify the immediate downstream transcriptional activation and evade antiviral cytokine production.

Video Link

The video component of this article can be found at https://www.jove.com/video/51078/

Introduction

Recent studies have outlined the overall signaling cascades in mounting host innate immune responses. Residing within distinct compartments, pattern recognition receptors (PRRs) detect pathogen-associated molecular patterns (PAMPs) of diverse origin to trigger innate immune signaling[1]. The retinoic acid-induced gene I (RIG-I) and melanoma differentiation antigen 5 (MDA5) proteins are cytosolic sensors that recognize RNA species with specific structural features[2]. Upon activation, RIG-I interacts with its downstream MAVS (also known as IPS-1, VISA, and CARDIF) adaptor that, in turn, activates the IKK (IKKαβγ) and IKK-related kinase (TBK1 and IKKε, also known as IKKi) complexes[3-6]. Activated innate immune kinases phosphorylate key regulators of gene expression, including transcription factors and inhibitors thereof, and enable transcriptional activation of host antiviral genes (*e.g.* IL6, TNFα, CCL5, and IFNβ). These signaling cascades constitute potent intrinsic innate immune responses that establish an anti-microbial state to restrict pathogen propagation during early stages of infection.

Murine γHV68 is closely related to human oncogenic KSHV and EBV. Thus, γHV68 infection of mice provides a tractable small animal model to examine the host immune response to gamma herpes virus infection *in vivo*[7]. Using γHV68, our lab has uncovered an intricate strategy whereby γHV68 hijacks host innate immune signaling to enable viral infection. On one hand, γHV68 activates the MAVS-IKKβ pathway to promote viral transcriptional activation via directing activated IKKβ to phosphorylate replication transactivator (RTA), the viral transcription factor key for γHV68 replication[8]. On the other hand, the IKKβ-mediated phosphorylation primes RelA for degradation and terminates NFKB activation[9]. As such, γHV68 infection effectively avoids antiviral cytokine production. Interestingly, a screening utilizing an expression library of γHV68 identified RTA as an E3 ligase to induce RelA degradation and abrogate NFKB activation[10]. These findings uncover an intricate immune evasion strategy whereby the upstream immune signaling events are harnessed by γHV68 to negate the ultimate antiviral cytokine production.

Here we describe a protocol to measure the antiviral cytokine production in mice infected with γHV68 both *in vivo* and *ex vivo*. In the protocol we further explore the "reconstituted" expression of innate immune components in the knockout embryonic fibroblasts by lentiviral transduction, which pinpoints the function of the specific innate immune molecules in regulating the antiviral cytokine production. This protocol can be easily adapted to other viruses and signaling pathways.

Protocol

Ethics Statement: All animal work was performed under strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Southern California.

1. Cytokine Gene Expression by Quantitative Real-time PCR and Secretion by ELISA in γHV68-infected Mice

1. Anesthetize gender-matched, 6-8 week-old C57BL/6 (B6) littermates (8-12 mice/group) via intraperitoneally injecting 1.5 mg of ketamine/0.12 mg of xylazine, and inoculate intranasally with 40 PFU of γHV68 in 40 µl (infection detailed in Dong and Feng[11]). At different time points post-infection, euthanize the mice by using CO_2 inhalation followed by cervical dislocation.
2. Open the chest with a left lateral cut along the sternum and cut through the ribs with sharp scissors. Collect the lung tissues.
3. Collect half of the lung tissue (left lobe) into sterile 1.5 ml screw-capped tubes containing 500 µl 1.0 mm Zirconia/Silica beads. Add 1 ml of cold serum-free DMEM into the tube and homogenize the lung tissue by bead-beating for 30 sec. Chill tubes on ice for 2 min and repeat this process once.
4. Centrifuge the tubes (15,000 x g for 10 min) at 4 °C, collect the supernatant and measure the cytokine production as described below.
5. Measure cytokines using commercially available cytokine ELISA kits according to the manufacturer's instruction.
6. Collect the other half of the lung tissue (right lobe) into another bead-containing tube and add 1 ml TRIzol. Homogenize and collect supernatant as described in step 1.3 and extract RNA according to the manufacture's instruction. Determine RNA concentration by taking absorbance readings at 260 and 280 nm.
7. Prepare cDNA with 1 µg of total RNA and reverse transcriptase according to the manufacture's instruction (the total final volume was 20 µl). Dilute cDNA 50 times with DNAse- and RNAse-free water and perform quantitative real-time PCR.
8. Perform the program on a quantitative real-time PCR machine. Each reaction contains 0.5 µl of a pair of gene-specific primers or control primers of GAPDH (the working concentration of the primers were 10 µM, CE 500 nM for each primer), 5 µl of the SYBR master mix and 4 µl of the diluted cDNA. Calculate ΔΔCt to determine the relative quantity of mRNA transcripts of antiviral cytokines.

2. MEF Cell Infection and Cytokine Quantification by ELISA and qRT-PCR

1. Grow *Mavs*$^{+/+}$ and *Mavs*$^{-/-}$ MEF cells to sub-confluent (approximately 80%) density before plating cells.
2. Split MEF cells into 12-well plates at 100,000 cells/well (the cell density will be around 70% on the second day). Carry out γHV68 infection in triplicates, with a multiplicity of infection (MOI) of 5-10 to synchronize and maximize cytokine induction.
3. Prepare γHV68 suspension in complete DMEM medium containing appropriate amount of virus (1 ml for each well).
4. Remove medium and add γHV68-containing suspension to MEF cells.
5. Place the plate in tissue culture incubator, rock every 30 min, and allow a total of 2 hr of incubation.
6. Remove γHV68-containing medium, wash once with PBS, and replace with fresh complete DMEM.
7. At various time points post-infection, harvest medium into 1.5 ml Eppendorf tubes. Wash cells with cold PBS and collect infected cells by trypsin digestion.
8. Measure antiviral cytokines in medium as described in step 1.5.
9. Extract RNA, prepare cDNA and perform qRT-PCR to determine antiviral cytokine gene expression as described in steps 1.5-1.7.

3. Lentivirus Production, Infection, and Generation of Stable Cell Lines

1. Split 293T cells one day before transfection and allow the cells to reach ~40% confluency at the time of transfection.
2. Transfect 10 cm dish of 293T cells with the packaging plasmids (1.2 µg of pVSV-G and 6 µg of pDR8.9) and 7.2 µg of pCDH-FLAG-MAVS or pCDH control by calcium phosphate precipitation.
3. Replace medium at 6 to 8 hr post-transfection with fresh complete DMEM.
4. At 72 hr post-transfection, collect medium containing lentivirus, centrifuge at 4,000 x g for 15 min, and filter with 0.22 µm membrane. To increase the lentivirus titer, we centrifuge the virus-containing medium at 110,000 x g for 2.5 hr at 4 °C. Carefully resuspend the viral pellet in small volume of medium of interest.
5. Seed *Mavs*$^{-/-}$ MEF cells or other knockout MEF cells one day before infection in 6-well plates at ~30-40% confluency.
6. Infect MEF cells with 1 ml lentivirus and 2 ml fresh complete DMEM, supplemented with polybrene to a final concentration of 10 µg/ml.
7. Centrifuge the plate at 500 x g at 30 °C for 30 min and transfer the plate to a tissue culture incubator.
8. Replace medium at 6 hr post-infection with fresh complete DMEM.
9. Split MEF cells at 24 hr post-infection and add puromycin to a final concentration of 1 µg/ml at 48 hr post-infection to select cells stably expressing MAVS.
10. Maintain MEF cells in puromycin-containing medium and verify protein expression by immunoblotting with corresponding antibodies.
11. Cells can be used for viral infection, cytokine gene expression and secretion experiments as described in Protocol 2.

Representative Results

Three representative figures are shown here, including cytokine production in the lung of γHV68-infected *Mavs*$^{+/+}$ and *Mavs*-$^{/-}$ mouse, cytokine secretion and gene expression level of γHV68-infected *Mavs*$^{+/+}$ and *Mavs*$^{-/-}$ MEFs, and cytokine mRNA levels of γHV68-infected *Mavs*$^{-/-}$ MEFs "reconstituted" with MAVS. These representative experiments utilize gene knockout mice to investigate antiviral cytokine production *in vivo* and explore knockout MEFs to dissect the mechanism of regulated cytokine production *ex vivo*. Specifically, γHV68 infection of *Mavs*$^{-/-}$ mice led to

significantly higher production of CCL5 *in vivo*. *Mavs*$^{-/-}$ MEFs produced more antiviral cytokine than *Mavs*$^{+/+}$ MEFs during γHV68 infection, which recapitulates the *in vivo* phenotype. "Reconstituted" expression of MAVS in *Mavs*$^{-/-}$ MEFs decreased CCL5 production. Collectively, these results demonstrate that MAVS is necessary for γHV68 to suppress antiviral cytokine production both *in vivo* and *ex vivo*.

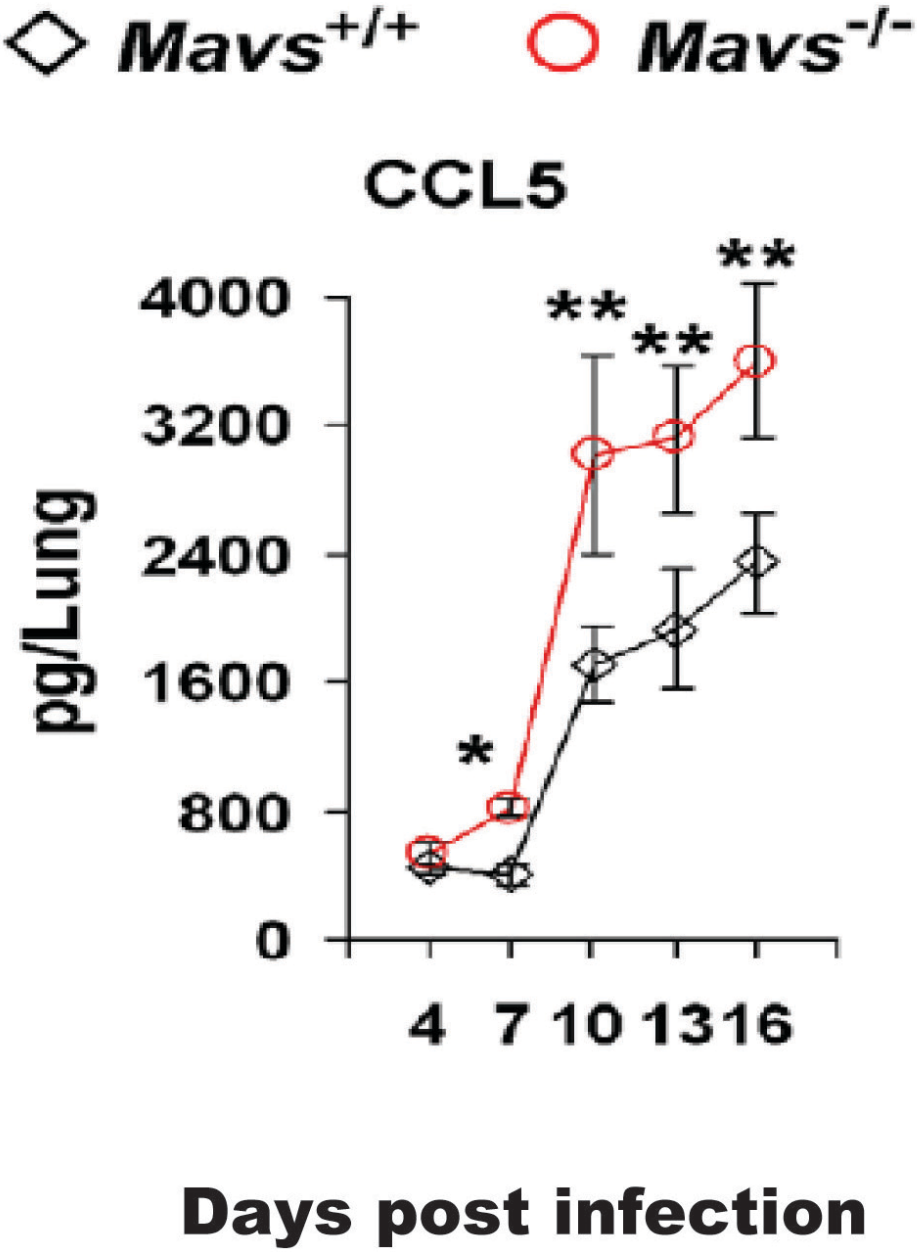
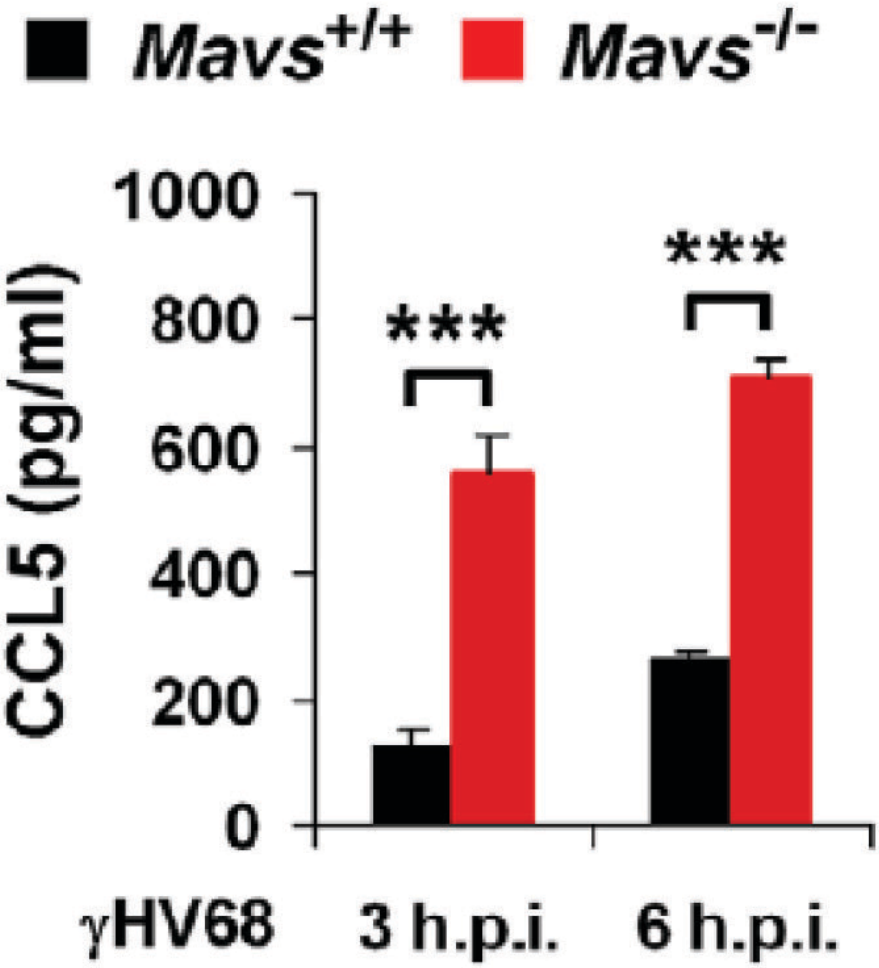
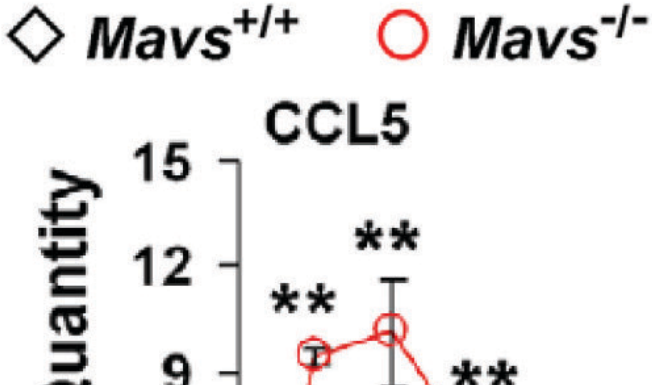


Figure 1. γHV68 acute infection induced higher cytokine production in the lungs of *Mavs*$^{-/-}$ mice than that in *Mavs*$^{+/+}$ mice. Age- and gender-matched *Mavs*$^{+/+}$ and *Mavs*$^{-/-}$ mice were intranasally infected with 40 PFU of γHV68 and CCL5 in the infected lungs at indicated days post-infection was measured by ELISA. γHV68 infection of *Mavs*$^{-/-}$ mice led to significantly higher production of antiviral cytokine CCL5, which suggested that MAVS is necessary for γHV68 to suppress antiviral cytokine production. Data are presented as the mean ± the standard error of the mean (SEM). The statistical significance: *,p<0.05; **,p<0.02. Click here to view larger image.

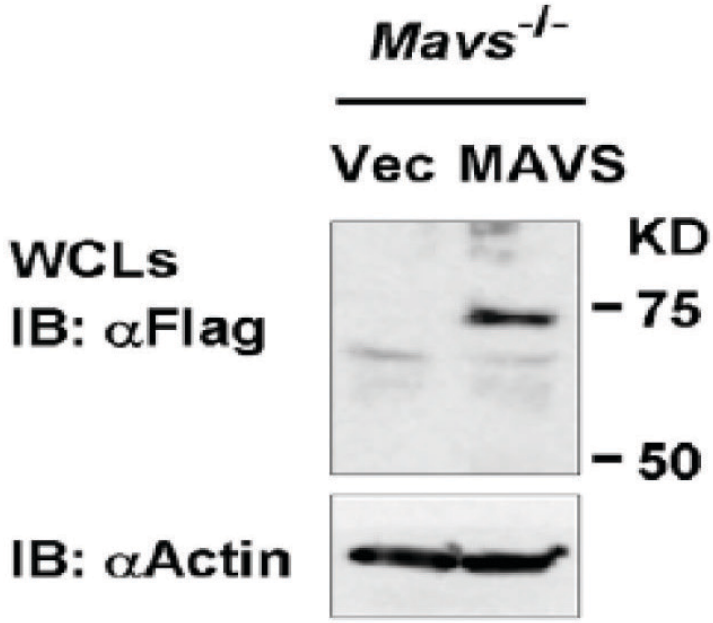
A)


Mavs+/+
Mavs-/-
CCL5 (pg/ml)
1000
800
600
400
200
0
***
***
γHV68
3 h.p.i.
6 h.p.i.

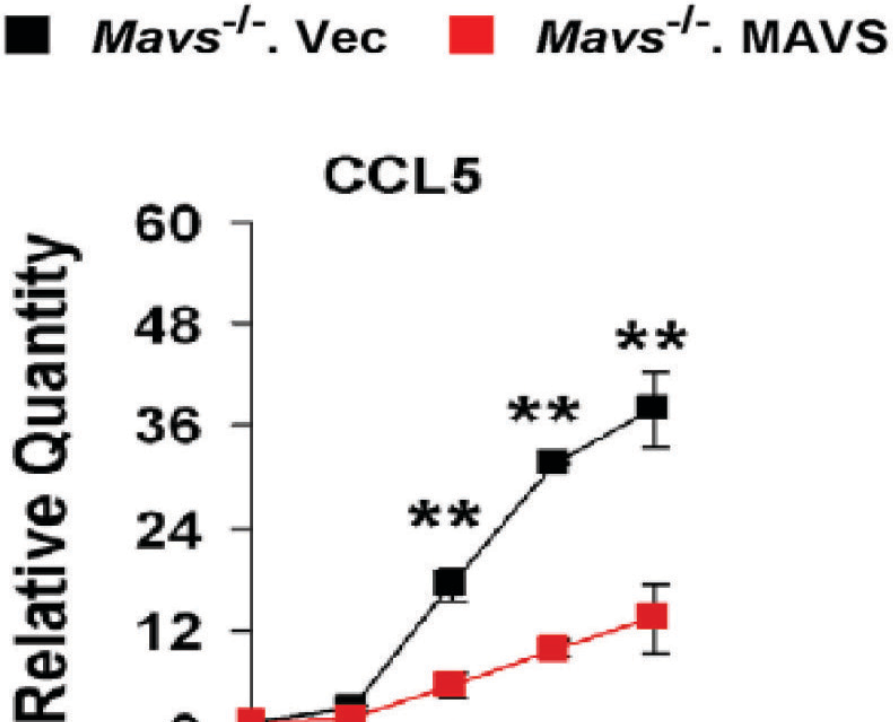
B)


Mavs+/+
Mavs-/-
CCL5
15
12
**
**
**

A)


Mavs-/-
Vec MAVS
KD
WCLs
IB: αFlag
75
50
IB: αActin

B)


Mavs-/-. Vec
Mavs-/-. MAVS
CCL5
Relative Quantity
60
48
36
24
12
**
**
**

Discussion

Viral immune evasion is one of the most dynamic and fascinating interactions interfacing viral offense and host defense[9]. The host innate immune components are structured such that signal transduction is effectively initiated and faithfully transmitted. Delineating the hierarchy and regulation of signaling cascades is a preeminent topic of innate immunity. Here, we introduce a protocol to identify the regulatory roles of an innate immune component, MAVS, in viral evasion of cytokine production. The protocol comprises a method determining cytokine production in virus-infected mouse and assessing cytokine gene expression and production in MEFs. The gene knockout MEFs and mouse provide tools that enable the genetic and biochemical dissection of protein function *in vivo* and *ex vivo*. Built on the genetically modified mouse strains, many of which are commercially available and number of which are increasing at an accelerating pace, this protocol further explores the "reconstituted" expression of wild-type or mutated protein of interest by lentivirus. One potential limitation of the "reconstituted" expression of innate immune components is the undesired immune signaling triggered by exogenous expression, which may impact viral infection and signal transduction thereof. Optimization to achieve low level of expression comparable to endogenous levels may reduce the side effect. In the event a mutant protein carrying targeted "lesions" is used, it will allow us to pinpoint the function of a particular domain, post-translational modification, or protein-protein interaction in immune regulation during viral infection. If "reconstituted" cells are transplanted into knockout mouse, *in vivo* function of selected immune components or targeted mutant can be examined.

Compared to the "knock-in" or transgenic expression of mutant proteins, "reconstituted" expression of wild-type or mutated protein of interest by lentivirus is more cost- and time-effective, permitting the investigation with diverse gain- or loss-of-function mutants. The critical step of the whole protocol is that the reconstituted protein should be comparable to endogenous protein level, especially when the protein is an enzyme or transcription factor. Over-expression of some proteins may lead to aberrant activation of the signaling pathway and ambiguous results.

Although our protocol focuses primarily on mouse fibroblasts, which is largely due to the intrinsic regulation of innate immune component on γHV68 replication, similar applications can be extended to other immune constituents, including macrophage and dendritic cells. Additionally, these approaches can be adapted to mechanistic studies entailing diverse pathogens. The application is emerging[12] and will likely take more center-staged roles in the future.

Disclosures

The authors declare no conflicts of interest.

Acknowledgements

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