Control of Nipah Virus Infection in Mice by the Host Adaptors Mitochondrial Antiviral Signaling Protein (MAVS) and Myeloid Differentiation Primary Response 88 (MyD88)

Mathieu Lampietro,¹ Noemie Aurine,¹ Kevin P. Dhondt,¹ Claire Dumont,¹ Rodolphe Pelissier,¹ Julia Spanier,² Audrey Vallve,³ Herve Raoul,³ Ulrich Kalinke,^{2,4} and Branka Horvat¹

¹International Center for Infectiology Research (CIRI), Institut National de la Santé et de la Recherche Médicale (INSERM) Unité1111, Centre National de la Recherche Scientifique (CNRS) Unités Mixtes de Recherche (UMR5308), University Lyon 1, Lyon, France, ²Institute for Experimental Infection Research, TWINCORE, Centre for Experimental and Clinical Infection research, a joint venture between the Hanover Medical School and the Helmholtz Centre for Infection Research, Hanover, Germany, ³INSERM, Laboratoire P4 Jean Mérieux, Lyon, France, ⁴Cluster of Excellence—Resolving Infection Susceptibility (RESIST), Hanover, Germany

Interferon (IFN) type I plays a critical role in the protection of mice from lethal Nipah virus (NiV) infection, but mechanisms responsible for IFN-I induction remain unknown. In the current study, we demonstrated the critical role of the mitochondrial antiviral signaling protein signaling pathway in IFN-I production and NiV replication in murine embryonic fibroblasts in vitro, and the redundant but essential roles of both mitochondrial antiviral signaling protein and myeloid differentiation primary response 88 adaptors, but not TRIF (Toll/Interleukin-1 receptor/Resistance [TIR] domain-containing adaptor-inducing IFN- β), in the control of NiV infection in mice. These results reveal potential novel targets for antiviral intervention and help in understanding NiV immunopathogenesis.

Keywords. Nipah virus; innate immunity; mice; MAVS; MyD88; TRIF; TLR; interferon.

Nipah virus (NiV) is a highly pathogenic zoonotic paramyxovirus of *Henipavirus* genus that causes regular human outbreaks in Southeast Asia. In humans, the disease caused by NiV involves severe respiratory and neurological symptoms with a case fatality rate as high as 100% [1]. Despite the recent understandings in the pathogenesis of NiV infection, there is still no approved vaccine or treatment available. High morbidity and mortality rates are observed in numerous mammalian species; however, clinical disease does not develop in mice, though functional entry receptors are expressed on cell surfaces [2].

The innate immune system recognizes viral RNA and protects organisms from infection by RNA viruses through distinct but related systems of recognition, including Toll-like receptors (TLRs) (TLR-3 and TLR-7/8) and Retinoic acid-Inducible Gene (RIG)-I-like receptors (RLRs): RIG-I and Melanoma Differentiation-Associated protein 5 (MDA-5). Driven by different adaptor molecules, these signaling platforms lead to the activation of interferon (IFN) type I [3]. Our group's previous studies demonstrated the critical role of IFN-I signaling in protecting mice from lethal NiV infection [4], but molecular

mechanisms that sense NiV infection and induce IFN-I production in mice remain obscure.

In the current study, we analyzed the importance of different pathogen recognition platforms to sense NiV infection and induce IFN-I production in murine embryonic fibroblasts (MEFs) and to protect from lethal encephalitis, using mice deficient in ≥ 1 innate signaling platform. We demonstrated the critical role of the RLR adaptor mitochondrial antiviral signaling protein (MAVS) (also known as IFN- β Promoter Stimulator-1 [IPS-1], Virus-Induced Signaling Adapter [VISA], or CARD Adapter Inducing Interferon-beta [CARDIF]) in the control of IFN-I production and NiV infection in MEFs in vitro, suggesting the importance of RLR-dependent signaling in sensing NiV infection. Interestingly, in the protection of mice from NiV-induced lethal disease, we showed that another signaling pathway is required in addition to MAVS, a pathway including TLR adaptor myeloid differentiation primary response 88 (MyD88) but not TLR-3 adaptor TRIF (Toll/Interleukin-1 receptor/Resistance [TIR] domain-containing adaptor-inducing IFN- β). Taken together, these results suggest a cell type-specific sensing of NiV in mice in vivo, involving ≥ 2 redundant signaling pathways capable of restraining lethal NiV infection.

METHODS

Virus, Cells, and Infection

NiV Malaysia (isolate UMMC1; GenBank AY029767) and recombinant NiV (rNiV)-enhanced green fluorescent protein

Correspondence: Branka Horvat, International Center for Infectiology Research, INSERM U1111, 21 Ave Tony Garnier, 69007 Lyon, France (branka.horvat@inserm.fr).

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(eGFP) [5] were prepared by infecting Vero-E6 cells, in the INSERM Jean Mérieux biosafety level 4 (BSL-4) laboratory in Lyon, France. Recombinant vesicular stomatitis virus-expressing green fluorescent protein (VSV-GFP) was obtained and prepared as described elsewhere [6].

Wild-type (WT) MEFs were isolated from murine embryos obtained from pregnant mice 13 days after conception, as described elsewhere [7] and cultured in Dulbecco's modified Eagle medium (DMEM) GlutaMAX (LifeTechnologies) supplemented with 10% heat-inactivated fetal bovine serum (Eurobio), 0.2% 2-mercaptoethanol, 1% HEPES, 1% nonessential amino acids, 1% sodium pyruvate, and 2% penicillin-streptomycin mix (all LifeTechnologies). For infections, MEFs were plated in 12-well plates at 2.10^5 cells per well and cultured with rNiV-eGFP at a multiplicity of infection (MOI) of 0.3 or 3 plaque-forming units (PFUs)/cell for 1 hour at 37°C. Virus-containing medium was then removed, and cells were washed once with $1\times$ phosphate-buffered saline. Finally, fresh DMEM was added to cells that were incubated for 18, 24, or 48 hours at 37°C.

Immunofluorescence was followed using a Zeiss Axiovert 100M microscope, and photographs were taken 24 hours after infection and treated using ImageJ software is version Java 1.8.0_112. L929 (Thymidine kinase-) (LTK) cells (mouse fibroblasts) were obtained from American Type Culture Collection (CRL-10422) and cultured in DMEM GlutaMAX supplemented with 10% heat-inactivated fetal bovine serum and 2% penicillin-streptomycin mix. Infected cell lysates and supernatants were collected using RLT and AVL buffers (Qiagen), according to validated BSL-4 procedures.

Infection of Mice

Different lines of transgenic mice, all on the C57BL/6 genetic background, were used in the study: WT C57BL/6J mice (Charles River) and several knockout (KO) models: mice deleted for IFN-I receptor (IFNAR-KO) [8], RLR adaptor protein MAVS (MAVS-KO) [9], TLR adaptor protein MyD88 (MyD88-KO) [10], and TLR-3 receptor (TLR3-KO) [11], and mice crossed to bear several deletions, including MyD88/TRIF-KO, MyD88/MAVS-KO and MyD88/TRIF/MAVS-KO [12].

Groups of 5–6 mice from each line, 4–6 weeks of age, were anesthetized with isoflurane and infected intraperitoneally with 10^6 PFUs contained in a 200- μ L volume. Animals were monitored for 28 days after infection and manipulated in accordance to good experimental practice and approved by the regional ethics committee CECCAPP (Comité d'Évaluation Commun au Centre Léon Bérard, à l'Animalerie de transit de l'ENS, au PBES et au laboratoire P4) and authorized by the French Ministry of Higher Education and Research (no. 00962.01). Animal experiments were conducted by the animal facility team in the INSERM Jean Mérieux BSL-4 laboratory in Lyon, France.

RNA Extraction and Reverse-Transcription Quantitative Polymerase Chain Reaction

Total RNAs were extracted from cells and murine organs using a Nucleospin RNA kit (Macherey-Nagel). Reverse-transcription was performed using an iScript complementary DNA kit (BioRad), and quantitative polymerase chain reaction (qPCR) using a Platinum SYBR Green qPCR SuperMix-UDG kit with ROX (Invitrogen). Data were treated using StepOne software and calculations were performed using the 2-Delta-Delta Cycle Threshold ($2^{\Delta\Delta CT}$) method and normalized with the GlycerAldehyde 3-Phosphate DeHydrogenase (GAPDH) housekeeping gene, as described elsewhere [13]. The primers used for qPCR are described in [Supplementary Table 1](#).

IFN-I Detection Assay

The presence of IFN-I was determined in supernatants of NiV-infected MEFs using a bioassay, as described elsewhere [14]. Briefly, UV-inactivated cell culture supernatants were serially diluted (2-fold) and added to confluent L929 (Thymidine kinase-) (LTK) monolayer cells. After an incubation of 24 hours at 37°C, cells were infected with VSV-GFP at 0.1 PFU/cell. Cytopathic effects were determined after fixation with formalin and crystal violet. Fluorescence was evaluated using a luminometer (TECAN Infinite M200 Pro) 24 hours later. The titration end point represented that dilution at which cells were found to be GFP negative. IFN titers were expressed as international units per milliliter with reference to a standard IFN curve obtained using IFN- α (Sigma).

Statistical Analysis

The calculations were performed using GraphPad Prism 8 software (GraphPad), with tests indicated in the figure legends.

RESULTS

Innate Sensing of NiV Infection in MEFs

To study the implication of TLR and RLR signaling pathways during NiV infection in mice, we initially analyzed in vitro NiV infection of MEFs, which present a convenient model for the first characterization of the cell innate response. MEFs were generated from mice bearing specific deletions in either RLR (MAVS-KO) or TLR (MyD88-KO and TRIF-KO) signaling platforms or their combinations (double MyD88/MAVS-KO and MyD88/TRIF-KO and triple MyD88/TRIF/MAVS-KO mice), and compared with WT MEFs (negative control) and MEFs generated from IFNAR-KO mice (positive controls) ([Figure 1](#)). MEFs were infected with rNiV-eGFP and development of green NiV-infected cells was screened by fluorescent microscopy.

Whereas MEFs deficient for MyD88 and/or TRIF (MyD88-KO and MyD88/TRIF KO) were able to contain NiV replication, similarly to WT MEFs, the MEFs bearing the deletion for RLR-associated sensor MAVS, including MAVS-KO,

Figure 1. Mitochondrial antiviral signaling protein (MAVS) plays a critical role in Nipah virus (NiV) infection in murine embryonic fibroblasts (MEFs). MEFs obtained from transgenic mice deficient in the indicated signaling platforms were infected with recombinant NiV-enhanced green fluorescent protein (eGFP) (multiplicity of infection, 0.3) and cultured for 18 hours. *A*, Cells were analyzed for eGFP expression by means of fluorescence microscopy. *B–D*, Cells were harvested and analyzed by means of reverse-transcription quantitative polymerase chain reaction for NiV nucleoprotein (NiV-N) (*B*), interferon (IFN) α (*C*), and IFN-β (*D*) expression. Results are presented as means with standard errors. The statistical significance of differences between infected wild-type (WT) cells and knockout (KO) cells was analyzed using 1-way analysis of variance, followed by the Tukey multiple comparisons test. *P < .05; †*P* < .01. Abbreviations: IFNAR, Interferon-α/β Receptor; MyD88, myeloid differentiation primary response 88; TRIF, Toll/Interleukin-1 receptor/Resistance (TIR) domain-containing adaptor-inducing IFN-β.

MyD88/MAVS-KO, and MyD88/TRIF/MAVS-KO, elicited rapid spread of NiV-eGFP, similar to IFNAR-KO MEFs (Figure 1A). Moreover, these observations were further confirmed by reverse-transcription qPCR analysis of NiV nucleoprotein (NiV-N) RNA levels in cell lysates and supernatants (Figure 1B and Supplementary Figure 1A). Indeed, though low amounts of NiV-N RNA were detected in WT and TLR-associated KO (MyD88-KO and MyD88/TRIF-KO) in both

MEF cell lysates and supernatants, production of NiV-N RNA was significantly increased in infected MEFs deficient for MAVS adaptor molecule (MAVS-KO, MyD88/MAVS-KO, and MyD88/TRIF/MAVS-KO), similarly to what was observed in IFNAR-KO MEFs.

We next analyzed IFN-I transcription and production in infected MEFs. Although expression of both IFN-α and IFN-β in MEFs deficient for MyD88 and/or TRIF (MyD88-KO and

MyD88/TRIF-KO) was similar to that in WT MEFs, the deficiencies for MAVS (MAVS-KO, MyD88/MAVS-KO, and MyD88/TRIF/MAVS-KO mice) were associated with a significant decrease in RNA levels for both IFN- α and IFN- β (Figure 1C and 1D). Similar results were obtained when a higher MOI was used (MOI, 3; data not shown). Those results were further confirmed at the protein level using a bioassay to quantify IFN-I production after NiV infection. Indeed, IFN-I production in MEFs deficient for MAVS adaptor protein (MAVS-KO and MyD88/MAVS-KO) was highly reduced below the limit of detection (Supplementary Figure 1B). Altogether, these results highlight the major role of MAVS adaptor protein in the induction of the IFN-I expression, inhibiting thus NiV infection in MEFs in vitro.

Involvement of TLR and RLR Signaling–Associated Adaptor Molecules in NiV Infection in Mice

We next compared the susceptibility to NiV infection in vivo of mice bearing deletions in either RLR (MAVS-KO) or TLR (MyD88-KO and TLR3-KO) signaling platforms or their combinations (double MyD88/TRIF-KO and MyD88/MAVS-KO mice) (Figure 2). After intraperitoneal NiV infection, mice were monitored daily for 28 days for clinical signs and death. Although MAVS-KO, MyD88-KO, TLR3-KO, and MyD88/TRIF-KO mice did not show any clinical signs during the study, double-deficient MyD88/MAVS-KO mice presented symptoms of severe neurological syndrome (lordosis, prostration, and paralysis), similar to what was observed in IFNAR-KO mice (Figure 2A). IFNAR KO mice succumbed to NiV infection at

Figure 2. Mitochondrial antiviral signaling protein (MAVS) and myeloid differentiation primary response 88 (MyD88) signaling affects the capacity of Nipah virus (NiV) to infect mice. Wild-type (WT) mice and mice deficient in several pathogen recognition signaling pathways were infected intraperitoneally with 10^6 plaque-forming units of NiV (5 or 6 animals per group). **A**, Survival of mice infected by NiV and followed up for 4 weeks. $P < .01$ (Mantel-Cox χ^2 test). **B**, NiV nucleoprotein (NiV-N) replication in murine organs, harvested on the day of death or at the end of protocol for different genotypes, was determined by means of reverse-transcription quantitative polymerase chain reaction (RT-qPCR). Results represent mean and standard errors for each group. $*P < .05$; $†P < .01$ (Kruskal-Wallis test). **C**, Analysis of interferon (IFN) α and IFN- β expression by means of RT-qPCR in organs harvested 6–14 days after infection. All samples were analyzed using 2-way analysis of variance, followed by the Tukey multiple comparisons test (differences were not significant). **D**, Schematic representation of the innate immunity system sensing NiV infection, addressed in this study. After NiV infection, pattern recognition receptors recognize viral single-stranded RNA (ssRNA) or double-stranded RNA (dsRNA). Those receptors implicate Retinoic acid-Inducible Gene (RIG)-I-like receptors (RLRs) RIG-I and Melanoma Differentiation-Associated protein 5 (MDA-5), capable of recognizing dsRNA in the cytosol and stimulating the MAVS signaling pathway, and Toll-like receptors (TLRs) TLR-7, recognizing ssRNA, and TLR-3, recognizing dsRNA in endosomes, and leading to the activation of MyD88 and TRIF (Toll/Interleukin-1 receptor/Resistance (TIR) domain-containing adaptor-inducing IFN- β) signaling pathways, respectively. Induction of those signaling cascades results in IFN-I production and activation of the antiviral stage. Our results suggest that although MAVS is sufficient in the control of NiV infection in murine embryonic fibroblasts (MEFs) in vitro, both MyD88 and MAVS (red boxes) display a redundant but essential role in the control of NiV infection in vivo. Abbreviations: IKK, I Kappa B Kinase; IRAK, interleukin 1 receptor-associated kinase; IRF, Interferon-Regulatory Factor; TBK, Tank-Binding Kinase; TRAF, tumor necrosis factor α receptor-associated factor.

day 6, and MyD88/MAVS-KO mice died between days 9 and 17; the difference was not statistically significant.

The absence of a major role for TRIF signaling was observed in both MyD88/TRIF-KO and TLR3-KO mice, because TLR-3 uses TRIF as a signaling adaptor protein (Figure 2D). NiV replication in mice, as evaluated by the production of NiV-N RNA in different organs, was significantly higher in mice bearing both MAVS and MyD88 deletions than in WT mice or mice bearing MyD88 and TRIF deletions (Figure 2B). The absence of viral clearance in MyD88/MAVS-KO mice was associated with lower production of both IFN- α and IFN- β (Figure 2C). Therefore, in contrast to in vitro data, where MAVS was sufficient for IFN-I induction in NiV-infected MEFs, during in vivo experiments both MAVS and MyD88 were required for the control of NiV replication in different organs and the protection of mice against NiV infection. These results suggest a redundant role of MAVS and MyD88 pathways during NiV infection (Figure 2D).

DISCUSSION

Despite expression of NiV entry receptors ephrin B2 and B3, and in contrast to findings in many other mammals, clinical signs of NiV infection do not develop in mice [15, 16]. The type of immune response capable of efficiently restraining lethal NiV infection is still largely unknown. We addressed this question using a large panel of mice deficient in different components of the innate immune system. Because our group's previous work demonstrated the crucial role of IFN-I signaling in the control of NiV infection [4], in the current study we analyzed the nature of intracellular sensing during viral infection, critical for the induction of a protective IFN-I response. Our results demonstrate the essential role of MAVS in the control of NiV infection in vitro. Because the adaptor MAVS is the critical component of signaling pathway of RLR receptors RIG-I and Melanoma Differentiation-Associated protein 5 (MDA-5), known as important sensors for viral RNA in both human and murine cells (Figure 2D), we hypothesized that NiV-mediated counteraction of RLR signaling in human cells, described elsewhere [17], is not functional in mice, preventing NiV infection in murine MAVS-expressing cells.

However, regulation of NiV infection in vivo seems to be more complex: although individual signaling pathways through either RLRs or TLRs, analyzed using MAVS- or MyD88-deficient mice, respectively, are independently sufficient for protection, MyD88/MAVS double-deficient mice succumbed to NiV infection at a rate similar to that in mice deficient for IFN-I receptors. These results suggest the existence of a redundant role of MAVS and MyD88 pathways in the control of NiV infection in different cell types, indicating that concomitant TLR and RLR signaling pathways are involved in the efficient protection against lethal NiV infection. In addition, because TLR7/MyD88 signaling is specific to immune cells [18], these data indicate an important function of cells of the immune system in the

control of NiV infection in mice. The role of several signaling pathways was observed for some other RNA viruses, including VSV [12] or rotavirus [19]. In certain viral infections, however, including Ebola virus or rhinovirus infection, either MAVS or MyD88 signaling alone, respectively, was shown to be sufficient to induce efficient antiviral protection [20, 21]. Interestingly, although TLR3/TRIF signaling was shown to be important in certain viral infections [11], it does not seem to be critical in NiV infection in mice.

These results present the first evidence for a dual MAVS- and MyD88-mediated containment of NiV infection in vivo. However, some of the MyD88/MAVS-KO animals still managed to control and survive the infection with this pathogen, suggesting the existence of another yet undetermined signaling pathway (or pathways), functional in protecting against death from NiV in mice. Together, these data reveal novel aspects of innate immunoregulation of NiV infection, which should be further evaluated in human cells and considered in the development of new strategies to control this highly lethal infectious disease.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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