

Finding NEMO-2: a New Potential Target for Anti-viral Biotherapy

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BACKGROUND

ssRNA viruses, such as RSV, hepatitis C virus, yellow fever virus, and West Nile virus, are a big family of pathogens that can cause various diseases and complications in humans. For example, RSV-induced Lower Respiratory Tract Infection (LRTI) was responsible for 86,000 hospitalizations, 1.7 million office visits, and an estimated annual direct medical cost of \$394 million in the year 2000⁽¹⁾. However, currently there are no effective vaccines or treatments for RSV infection. Studies that can provide a better understanding of pathways involved in ssRNA virus infection are warranted. The purpose of our study was to investigate how RSV activates intracellular signaling and to find potential molecular targets for treating RSV infection.

Retinoic acid inducible gene I / mitochondrial antiviral signaling (RIG-I/MAVS) pathway plays important roles in detecting viral genomes and inducing the activation of two critical transcription factors, nuclear factor kappa B (NF-κB) and interferon regulatory factor 3 (IRF3). The activation of NF-κB requires IκB kinase (IKK) complex, which is composed of IKKα, IKKβ and a regulatory subunit IKKε, also known as NF-κB essential modifier (NEMO). In the activation of IRF3, two IKK-related kinases, TANK-binding kinase 1 (TBK1) and IKKε, play essential roles. TRAF family member-associated NF-κB activator (TANK) links TBK1 and IKKε to the upstream molecules in RIG-I/MAVS pathway. Recently, the interaction between TANK and NEMO has been reported and the "branching point" between NF-κB and IRF3 downstream of RIG-I/MAVS signaling was located on NEMO. In NEMO deficient cells, the activations of both NF-κB and IRF3 were abolished in response to different ssRNA viruses⁽²⁾.

In a previous study, we have identified a 43-kDa protein, NEMO-2, which is an alternative splicing variant of NEMO excluding exon 5⁽³⁾. In this study, we investigated how NEMO and NEMO-2 function differently in response to RSV infection.

MATERIALS AND METHODS

CELL CULTURES. Wild type and NEMO^{-/-} MEFs were cultured in Eagle's minimum essential medium (Gibco). NEMO and NEMO-2 reconstituted stable MEFs were cultured as described previously⁽³⁾.

RT-PCR AND QUANTITATIVE REAL-TIME PCR (QRT-PCR). Experiment was conducted using SYBR green supermix (Bio-Rad) following manufacturer's protocol. The plates were denatured for 90 s at 95°C and then subjected to 40 cycles of 15 s at 94°C, 60 s at 60°C, and 1 min at 72°C in iCycler (BioRAD).

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA). A total of 35 μg whole cell extracts (WCEs) were incubated with 100,000 cpm of 32P-labeled double-stranded oligonucleotide containing NF-κB binding sites⁽⁴⁾ and IRF3 binding site⁽²⁾ in a total volume of 25 μL as describe before. Gels were dried and exposed to BioMax film (Kodak) for autoradiography.

NATIVE PAGE FOR SEPARATION OF IRF-3 DIMERS FROM MONOMERS. 50 μg protein was mixed with native PAGE buffer and run at a constant current (25 mA) for 120 min.

CO-IMMUNOPRECIPITATION AND WESTERN IMMUNOBLOT. Immune complexes were precipitated by adding 50 μL of protein A-Sepharose beads (50% slurry) and incubating for 1 h at 4°C. Immune complexes were fractionated by 10% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane by electroblotting. Membranes were incubated with IRDye 700-conjugated anti-mouse Ab or IRDye 800-conjugated anti-rabbit Ab (Rockland, Inc.), and scanned by an Odyssey infrared scanner.

ELECTROPORATION. Two million freshly isolated MEFs were suspended in 100 μl MEF2 nucleofactor solution (Amaxa), and transfected (program A023) with plasmid DNA as described.

RESULTS

RSV ONLY ACTIVATED NFκB PATHWAY BUT NOT IRF3 PATHWAY IN NEMO-2^{+/+} CELLS.

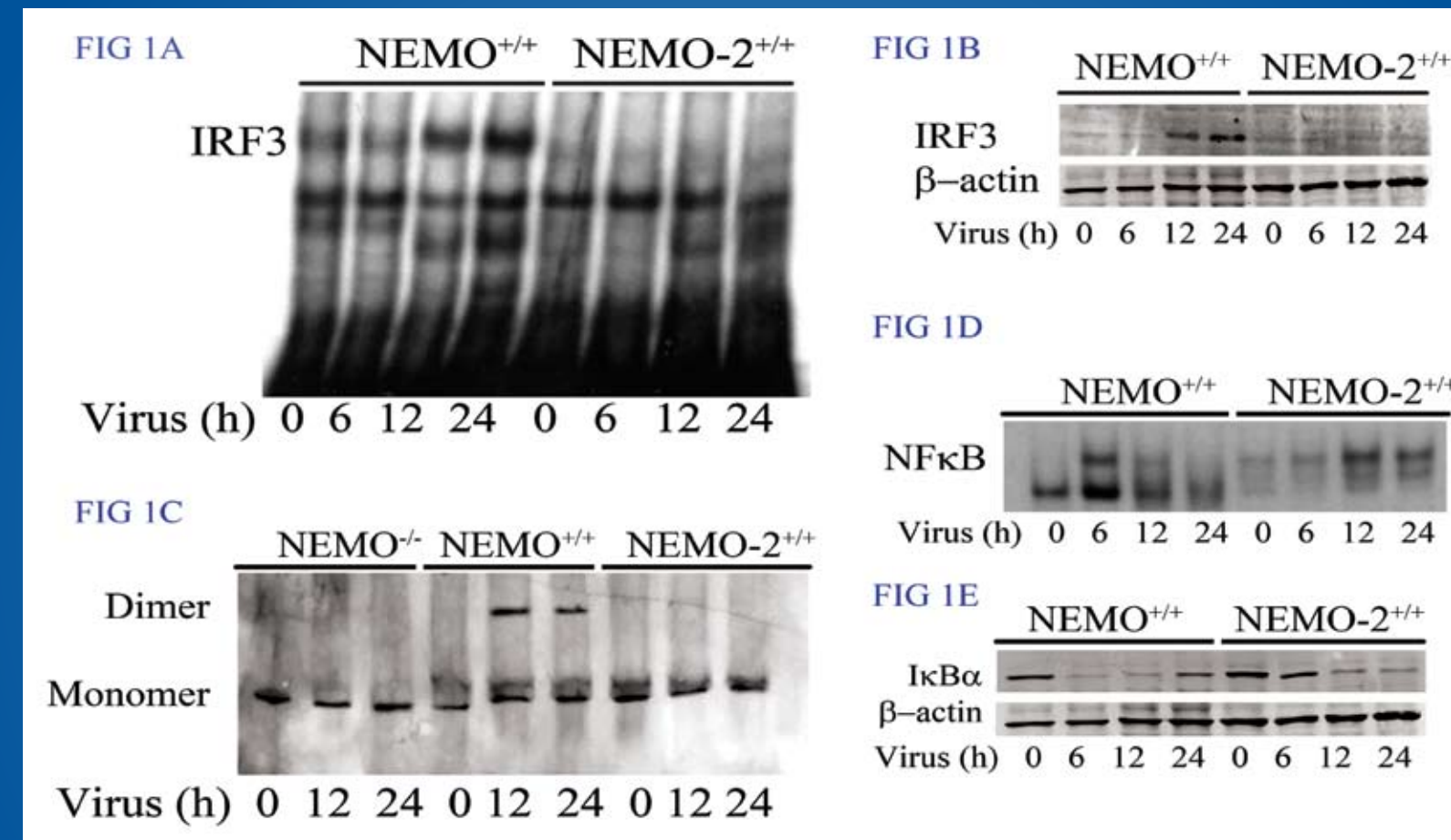


Fig 1: RSV only activated NFκB pathway but not IRF3 pathway in NEMO-2^{+/+} cells. A) EMSA experiment demonstrated that RSV failed to activate IRF3 pathway in NEMO-2^{+/+} cells. B) RSV failed to induce the nuclear translocation of IRF3 in NEMO-2^{+/+} cells. C) RSV-induced dimerization of IRF3 was impaired in NEMO-2^{+/+} cells. D) EMSA experiment showed that RSV activated NFκB pathway in NEMO-2^{+/+} cells. E) The degradation of IκBα was observed in NEMO-2^{+/+} cells.

RSV WAS UNABLE TO ACTIVATE TYPE I IFN GENE EXPRESSION IN NEMO-2^{+/+} CELLS.

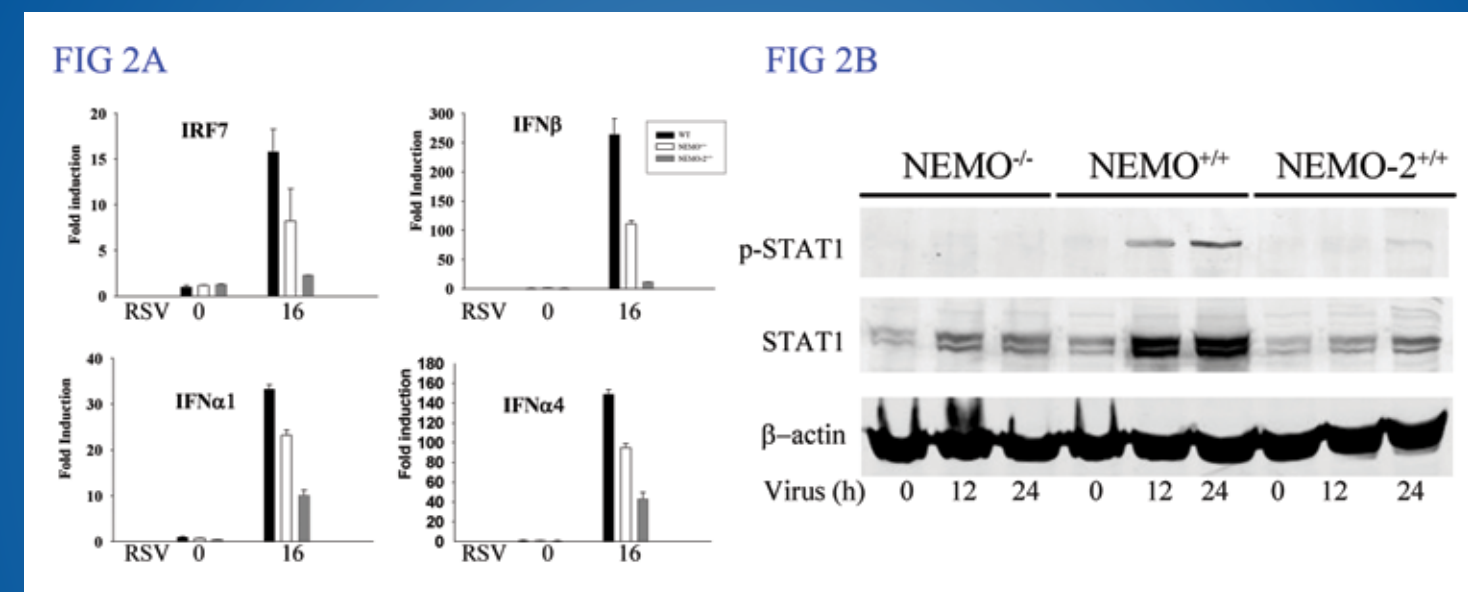


Fig 2: RSV was unable to activate type I IFN gene expression in NEMO-2^{+/+} cells. A) QRT-PCR experiment showed that RSV-induced IRF7, IFN-α1, IFN-α4 and IFN-β expressions were impaired in NEMO-2^{+/+} cells. B) RSV was unable to activate STAT pathway in NEMO-2^{+/+} cells.

THE REPLICATION OF RSV WAS INCREASED IN NEMO-2^{+/+} CELLS.

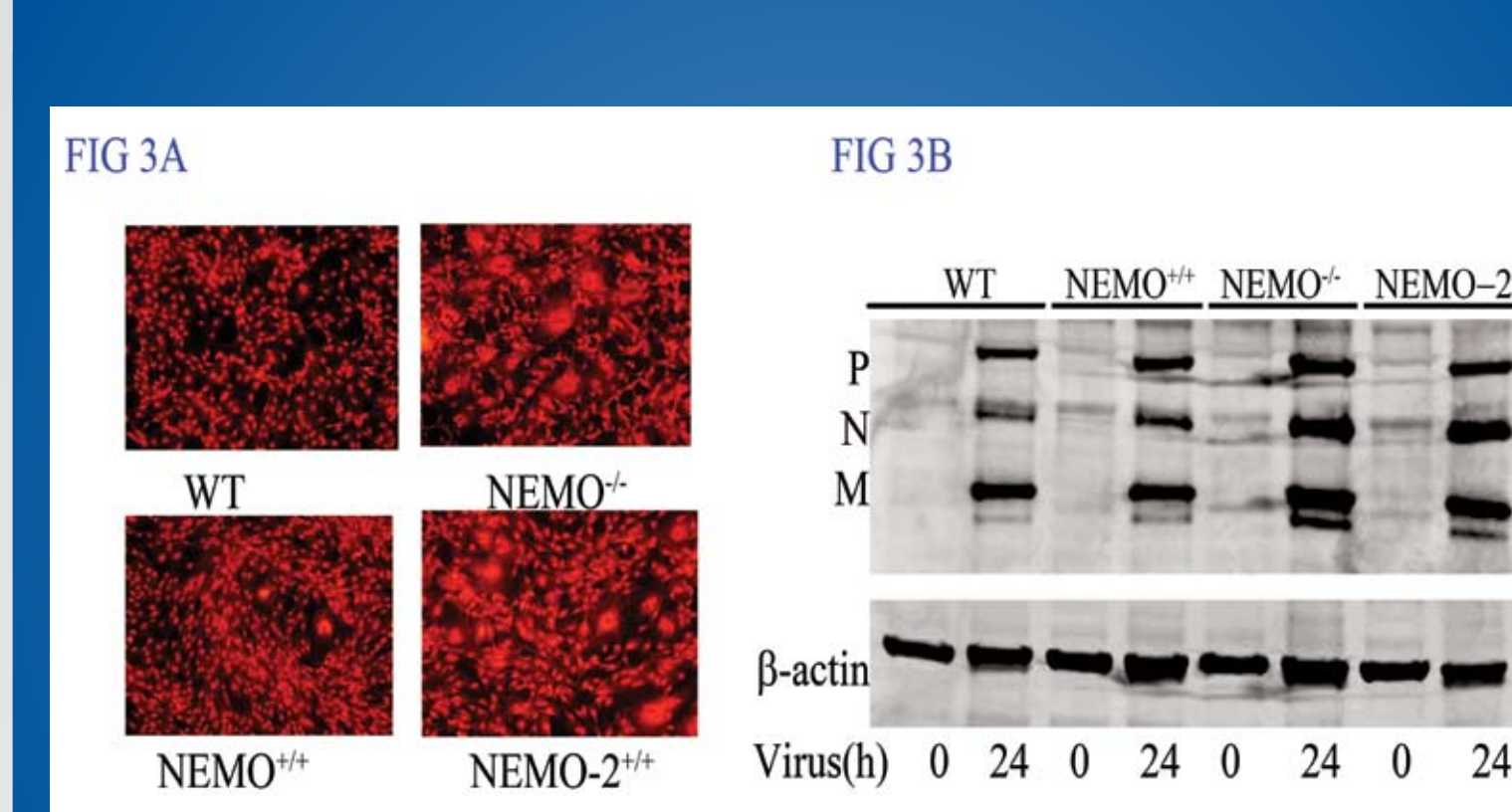


Fig 3: The replication of RSV was increased in NEMO-2^{+/+} cells. A) Western immunoblot demonstrated the increase of viral protein P, N and M in NEMO-2^{+/+} cells in response to RSV infection. B) Immuno-staining showed the increased numbers of multinucleated cells in NEMO-2^{+/+} cells.

NEMO, BUT NOT NEMO-2, WAS ASSOCIATED WITH TANK.

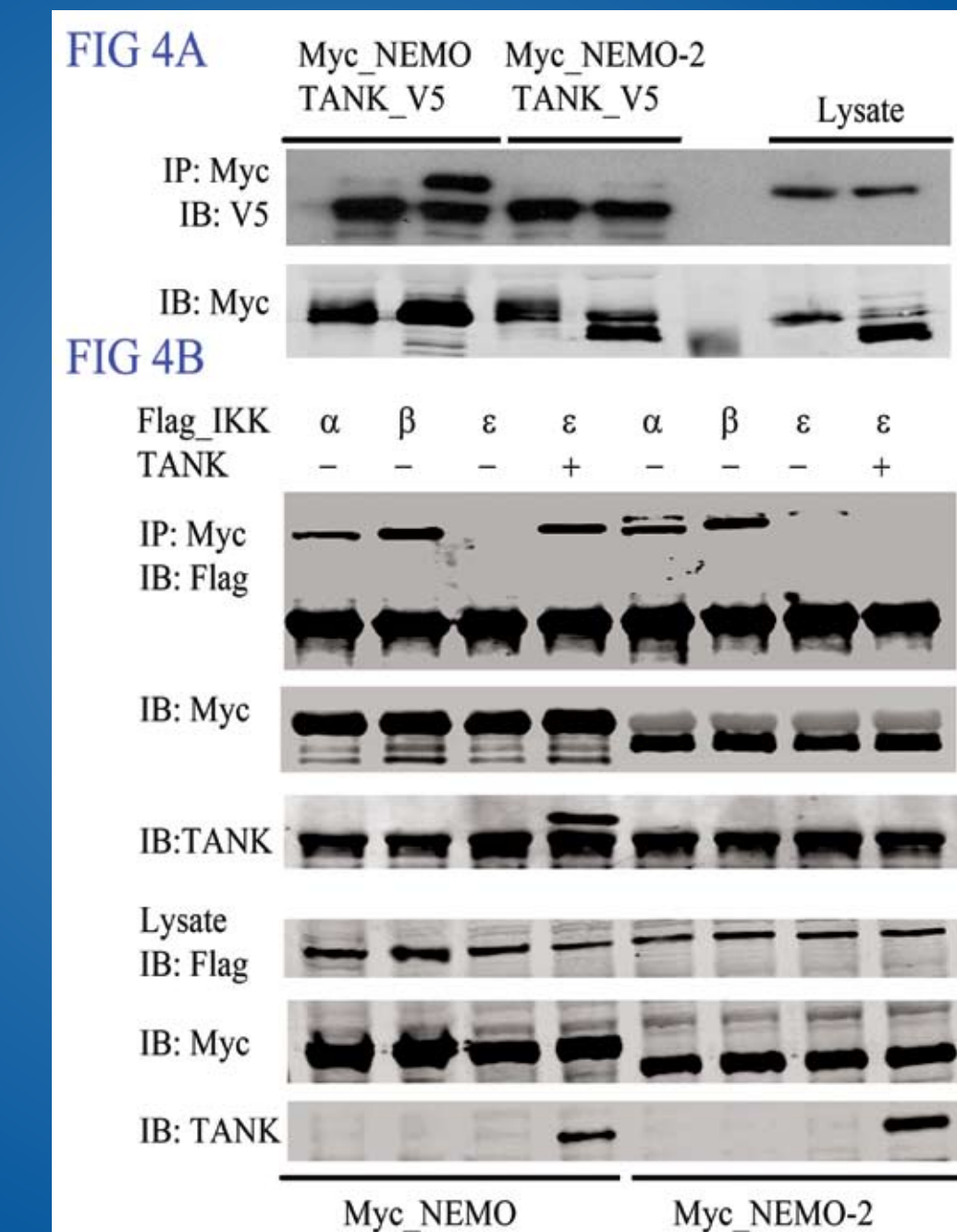


Fig 4: NEMO was associated with TANK, but not NEMO-2. A) Co-immunoprecipitation experiment demonstrated NEMO-2 failed to recruit TANK. B) Co-immunoprecipitation experiment showed that NEMO-2 was unable to bind IKKε.

CONCLUSION

A major dilemma for effective treatment of infections caused by single stranded RNA (ssRNA) viruses, such as respiratory syncytial virus (RSV), is how to reduce host inflammatory reactions without affecting viral clearance. Our data suggests that the exon 5 of NEMO is an essential domain for the activation of IRF3, and that NEMO-2, a splicing variant with this domain eliminated, is only involved in the activation of NF-κB, but not IRF3. Therefore, therapeutic strategies targeting NEMO-2 may significantly inhibit virus-induced inflammatory reactions by down-regulating NF-κB, without slowing down the clearance of virus since the IFN signaling is not affected. Thus, NEMO-2 may serve as a promising molecular target for therapies against ssRNA infection.

DISCUSSION

The NEMO gene is located on chromosome Xq28 and contains 10 exons⁽¹²⁾. Mutations in the NEMO gene, including different truncations of the NEMO protein, have been linked to syndromes of incontinentia pigmenti and anhidrotic ectodermal dysplasia associated with immunodeficiency in human⁽¹³⁾. In a previous study, our group first discovered a NEMO transcript with exon 5 deletion and named it as NEMO-2. Using a specific reverse transcription-PCR assay, we demonstrated that NEMO-2 is widely expressed in cultured human cells and normal human tissues⁽⁵⁾.

As an adaptor protein, NEMO is known to be associated with many proteins involved in the IRF3 and NF-κB activation, including upstream kinases such as IKKα, IKKβ, TBK1 and IKKε. In addition, NEMO is also a target for direct IKK activators such as RIG I and MAVS. In mapping studies, NEMO was shown to bind IKKα and β subunits through its NH2-terminal 119 aa, a region unaffected in the NEMO-2 splice variant^(15, 16, 17). Consistent with this finding, our co-immunoprecipitation experiments demonstrated that NEMO-2 binds both IKKα and IKKβ, which may explain why NEMO-2 is still able to activate NF-κB pathway. Another adaptor protein, TANK, links NEMO and TBK1•IKKε, and passes the upstream signal from RIG I/MAVS to IRF3 pathway⁽¹⁰⁾. Our study demonstrated that NEMO-2, which lacks 174-224aa, was unable to bind TANK (illustrated in Fig 5). Consistent with these findings, our result showed that NEMO-2 can not activate IRF3 pathway and consequently fails to induce type I interferon production.

In summary, our study demonstrated that different from NEMO which links both NF-κB and IRF3, NEMO-2 is a "pure" NF-κB adaptor (illustrated in Fig 6). Bio-therapy targeting NEMO-2, by either inhibiting its function or decreasing the process of NEMO-2 from NEMO, provides a possibility to partially down-regulate NF-κB (lower inflammatory response), and at the same time, allow greater signaling passed to IRF3 pathway through NEMO (faster viral clearance).

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