Virology 514 (2018) 230–239

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/virology

Inhibition of neddylation pathway represses influenza virus replication and pro-inflammatory responses

Haiwei Sun[a,b], Wei Yao[b], Kai Wang[b], Yingjuan Qian[a,1], Hongjun Chen[b,c,**,1], Yong-Sam Jung[a,*,1]

[a] MOE Joint International Research Laboratory of Animal Health and Food Safety, College of Veterinary Medicine, Nanjing Agricultural University, Nanjing, China
[b] Shanghai Veterinary Research Institute, Chinese Academic of Agricultural Sciences, Shanghai 200241, PR China
[c] Animal Influenza Virus Ecology and Pathogenesis Innovation Team, The Agricultural Science and Technology Innovation Program, Shanghai 200241, PR China

ARTICLE INFO

Keywords:
Influenza A virus
Neddylation
MLN4924
Pro-inflammatory cytokine

ABSTRACT

The neddylation pathway belongs post-translational modifications and plays important roles in regulating viral infection and replication. To address the relationship of influenza A virus with the neddylation modification pathway, we demonstrate that IAV infection in A549 cells can activate the neddylation modification pathway to increase virus growth and enhance the expression of pro-inflammatory cytokines to increase pathogenicity. The pre-treatment of Nedd8-activating enzyme subunit 1 (NAE1)-specific inhibitor, MLN4924, interferes with Nedd8 conjugation and NF-κB activity. MLN4924 exhibited pronounced antiviral activity against different subtypes of influenza A virus, including classical H1N1 (PR8), H9N2 subtype, and pandemic H1N1 2009 (pdmH1N1) viruses. Through the inhibition of the CRL/NF-κB pathway, MLN4924 could significantly suppress the expression levels of pro-inflammatory cytokines induced by IAVs. These findings suggest that MLN4924 can be developed as a novel antiviral therapy for influenza infection for anti-viral efficacy and anti-inflammation activity.

1. Introduction

Severe influenza infections cause high levels of morbidity and mortality as influenza viruses lead to an overly aggressive innate immune response with early recruitment of inflammatory leukocytes to the lung microenvironment (Garcia-Sastre, 2010; Garcia-Sastre and Biron, 2006). This phenomenon was addressed as a major contributor to the morbidity of the 1918 influenza pandemic (Kobasa et al., 2007). Recently, the avian high pathogenic H5N1 subtype and 2009 pandemic H1N1 influenza infections have caused excessive pro-inflammatory production and cytokine storm, as documented in the clinical literature, which are significantly associated with high morbidity in humans (de Jong et al., 2006; Takeda et al., 2010; Woo et al., 2010; Yuen and Wong, 2005). However, the detailed mechanisms of the related cellular signaling pathways remain unknown.

Neddylation modification is a post-translational process of adding a neural precursor cell that expresses developmentally down-regulated 8 (NEDD8), a small ubiquitin-like protein modifier, to its target factors (Chadha et al., 2015). It is triggered by the successive reactions of the Nedd8-activating enzyme E1 (NAE1), Nedd8-conjugating enzyme E2 (Ubc12), and Nedd8-E3 ligases (Merlet et al., 2009). The neddylation pathway regulates the localization, stability, and function of targets. To date, the only well-characterized targets of neddylation are Cullins, which function as essential subunits of multi-unit Cullin-RING E3 ligases (CRL) (Luo et al., 2012a). The Cullin family proteins (namely Cullin-1, Cullin-2, Cullin-3, Cullin-4A/Cullin-4B, Cullin-5, Cullin-7, and Cullin-9/Par8) are definitely modified by NEDD8 in eukaryotic cells, which process the degradation of numerous important proteins (Lydeard et al., 2013; Mahon et al., 2014).

MLN4924, a first-in-class inhibitor of NAE1, was initially discovered via high-throughput screening as a Phase I anticancer agent (Gu et al., 2014; Kuo et al., 2015; Lin et al., 2015; Luo et al., 2012b; Nawrocki et al., 2012; Sarantopoulos et al., 2016; Swords et al., 2015; Wei et al., 2014). MLN4924 binds to NAE1 at the active site to form a covalent Nedd8-MLN4924 adduct and thus inactivates the neddylation post-translational modification pathway (Nawrocki et al., 2012). It blocks cullins activation, leading to the accumulation of multiple CRL substrates, such as CDT1 and ORC1 (DNA replication licensing proteins), p21, p27, and Wee1 (cell-cycle inhibitors), and IκB-α, resulting in the inhibition of NF-κB activity (Blank et al., 2013; El-Mesery et al., 2015; Godbersen et al., 2014, 2015; Khalife et al., 2015; Mackintosh et al., 2013; Paiva et al., 2015; Rashidieh et al., 2015; Wan et al., 2015; Wang et al., 2015; Yang et al., 2012). Through these mechanisms, MLN4924 has been shown to suppress lipopolysaccharide (LPS)-induced pro-

* Corresponding author.
** Corresponding author at: Shanghai Veterinary Research Institute, Chinese Academic of Agricultural Sciences, Shanghai 200241, PR China.
E-mail addresses: yqian@njau.edu.cn (Y. Qian), vetchj@shvri.ac.cn (H. Chen), ysjung@njau.edu.cn (Y.-S. Jung).
[1] Y.Q, Y.-S.J, and H.C are co-senior authors of the paper.

https://doi.org/10.1016/j.virol.2017.11.004
Received 4 August 2017; Received in revised form 1 November 2017; Accepted 6 November 2017

inflammatory cytokine production in the macrophages (Chang et al., 2012; Li et al., 2013) and inhibit TLR3/4- and retinoic acid-inducible gene-I-induced IFN-β expression in different cells (Song et al., 2016). Neddylation also affects the function of dendritic cells and T cells during inflammatory responses (Godbersen et al., 2015; Mathewson et al., 2013; Wang et al., 2015). These findings demonstrate the critical role of the neddylation pathway in immune regulation during hypercytokinemia and suggest that neddylation inhibitors are potential anti-inflammation agents (de Jong et al., 2006; Song et al., 2016; Takeda et al., 2010).

MLN4924 influenced a decrease H1N1 subtype influenza virus infection in the A549 cells (Le-Trilling et al., 2016). However, the potential role of neddylation in the regulation of the pro-inflammatory responses and the mechanisms of the Nedd8 conjunctive CRL complex activating NF-κB pathway (Nedd8-CRL/NF-κB) induced by influenza viruses have not been well defined. It is very important to determine the expression patterns of the Nedd8-CRL/NF-κB pathway in influenza A virus (IAV) infections. This would be helpful for understanding the mechanisms of immune responses in influenza A virus infections.

2. Materials and methods

2.1. Ethics statement

Balb/c mice studies were performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of Shanghai Veterinary Research Institute, CAAS (ID: SHVRI-PO-2015-0098), and the animal research was approved by the Animal Association of Science and Technology Commission of Shanghai Municipality, China (Permit Number: 2013-11). Nine-day-old specific pathogen-free (SPF) chicken embryos were obtained from Merial Vital Co. (Beijing, China).

2.2. Cell culture and reagents

The human alveolar type II epithelial cell line A549 (ATCC CCL-185) was grown in Dulbecco's Modified Eagle's medium (DMEM) (Thermo Fisher Scientific Inc., Waltham, MA, USA) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin-streptomycin (Kobasa et al.) solution (Thermo) at 37 °C with 5% CO_2. The Madin–Darby canine kidney epithelial (MDCK) cell line (ATCC CCL-34) was maintained in Modified Eagle's medium (Ebrahimi et al. 2010) (Gibco) containing 5% FBS and 1% PS. The human embryonic kidney 293T cell line (ATCC CCL-3216) was grown in Opti-MEM® I Reduced-Serum Medium (Gibco, Grand Island, NY, USA) containing 10% FBS and 1% PS. NAE1-specific inhibitor MLN4924 (Active Biochem, Maplewood, NJ, USA) was synthesized and dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich Corp., St. Louis, MO, USA) and stored at −20 °C for *in vitro* studies. In mice experiments, the drug was dissolved in hydroxypropyl-beta-cyclodextrin (HP-β-CD) (Sangon Biotech Inc., Shanghai, China) when ready to use.

2.3. Construction of expression plasmids and transient transfection

The full-length DNA fragment encoding the wild-type Cullin-1 protein was amplified by PCR from the A549 cDNA with forward primer Cullin-1-F (5′-CGGGATCCATCCTTTCTGAGCTGCTGTGA-3′) and reverse primer Cullin-1-R (5′- ATGCGGCCGCCAACATGAACTATTTGC TGC-3′) and then cloned into a pcDNA3.1-HA expression vectors *Bam*HI via *Not*I and sites. All constructs were verified by DNA sequencing. Transfection was performed using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4. Viruses

A/swine/Jiangsu/C1/08 (H9N2) (H9C1) was isolated from a Jiangsu swine farm in 2008 with mild respiratory disease. The genomic accessions in GenBank are from No. KX867822 to KX867829 (Qi Huang et al., 2017). Mouse-adapted A/California/04/09 (H1N1) (maCa04) was a mutated virus of Ca04 blindly passaged into DBA mice, a gift from Dr. Daniel R. Perez (Ye et al., 2010). A/Puerto Rico/8/34 (H1N1) (PR8) was amplified in nine-day-old SPF embryo eggs at 37 °C for 48 h. The allantoic fluid was collected in vials and stored at −80 °C until use. The viral titers were determined and calculated according to the Reed–Muench method for a 50% tissue culture infectious dose ($TCID_{50}$) on MDCK cells with MEM with 2% bovine serum albumin (BSA) and 1 μg/mL trypsin treated with L-(tosylamido-2-phenyl) ethyl chloromethyl ketone (TPCK).

2.5. ATPlite cell viability assay

Cells were seeded in 12-well plates with 1×10^5 cells per well, cultured for 24 h, and treated with MLN4924 at a concentration of 0.1, 0.33, 1, 2, or 3 μM for 36 h. Cell viability was determined at indicated times using the ATPlite™ luminescence assay system according to the manufacturer's instructions (PerkinElmer, Waltham, MA, USA).

2.6. Virus infections and quantitation of viral loads

The A549 cells (1×10^6/well) were passaged into a 100-mm dish with 90–95% confluence overnight. The cells were pre-treated with MLN4924 at different doses. After 12 h, the cells were infected with influenza A viruses at a multiplicity of infection (m.o.i) of 1 per cell and incubated at 37 °C for 2 h. After incubation, the viral supernatants were removed and replaced with fresh culture media. The cells and media were collected at 2, 4, 6, 8, 10, 12, and 24 h post-infection (Hjerpe et al. 2012) to determine the viral loads by virus titration and TaqMan probe real-time reverse transcription-polymerase chain reaction (rRT-PCR) using the Uni12M universal primer of influenza A viruses (AGCRAAAGCAGG, R = A/G) for cDNA reverse transcription. Besides MLN4924 pre-treatment, A549 cells were infected with influenza A virus (1 m.o.i) for 2 h, and subsequently treated with MLN4924 as indicated concentrations and times. The copies quantitation of the M segment was determined with the primers Flu-RT-F GACCRATCCT GTCACCTCTGAC and Flu-RT-R (AGGGCATTYTGGAC AAAKCGTCTA). In addition, the probe was a Flu-RT-probe (5′-FAM-TGCAGTCCTCGC TCA CTGGGCACG-BGQ2-3′).

2.7. Pro-inflammatory cytokine levels by rRT-PCR

The infected cells were thawed and the total RNA of 200-μl supernatants was treated and extracted with an RNeasy mini kit (Qiagen) from the tissues above according to the manufacturer's instructions. The mRNA was primed using Oligo $(dT)_{18}$ (Promega Co., Madison, WS, USA), and the cDNA synthesis was driven by AMV transcriptase (Promega) in 20-μl volumes at 30 °C for 10 min, 42 °C for 2 h, and then 70 °C for 15 min. The primers of RT-PCR were designed using PrimerQuest Tool. The sequences of the primers were as follows: *β-actin-107F*: GGACCTGACTGACTACCTCAT; *β-actin-107R*: CGTAGCACA GCTTCTCCTTAAT. *IL-1β-102F*: CATGGGATAACG AGGCTTATGT; *IL-1β-102R*: CATATGGACCAGACATCACCAA. *IL-6-107F*: CA CTCACCTCTTCAGAACGAAT; *IL-6-107R*: GCTGCTTTCACACATGTTA CTC. *TNF-α-106F*: CCAGGGACC TCTCTCTAATCA; *TNF-α-106R*: TCAG CTTGAGGG TTTGCTAC. The reactions were conducted by rRT-PCR analysis with AceQ® qPCR SYBR® Green Master Mix (Vazyme Inc., Nanjing, China) according to the following cycle protocol: 95 °C for 5 min, 40 cycles at 95 °C for 10 s, and 60 °C for 30 s or followed by the melt curve stage (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s). The reaction results were represented by threshold cycle (Ct) values. As in