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ALK is required for NLRP3 inflammasome activation in macrophages

Bibo Zhang [a], Wei Wei [b], Jiaming Qiu [b, *]

[a] Department of Critical Care Medicine, The Second People's Hospital of Changshu City, Changshu, Jiangsu, 215500, China
[b] Department of Pathology, The Second People's Hospital of Changshu City, Changshu, Jiangsu, 215500, China

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ABSTRACT

The NLRP3 inflammasome is a key mediator of host immune responses through the induction of pyroptosis and the release of cytokines. Although the pathologic role of inflammasome in infection and sterile inflammation is well known, the mechanism and regulation of NLRP3 inflammasome activation remains obscure. Here, we report that anaplastic lymphoma kinase (ALK) is a novel regulator of NLRP3 inflammasome activation in macrophages. Pharmacologic or genetic inhibition of ALK through targeted drugs (ceritinib and lorlatinib) or RNAi blocked extracellular ATP-induced NLRP3 inflammasome activation in macrophages. Mechanically, ALK-mediated NF-κB activation was required for the priming step of NLRP3 upregulation, whereas ALK-mediated lipid peroxidation contributed to the sensing step of NLRP3-NEK7 complex formation. These studies indicate that inhibition of ALK could be utilized to treat NLRP3-related inflammatory diseases.

1. Introduction

Inflammasomes are large cytosolic protein complexes with multiple components that trigger a rapid immune response against pathogen-associated molecular pattern molecules (PAMPs) during pathogen infections or damage-associated molecular pattern molecules (DAMPs) during tissue damage [1]. Activation of inflammasome leads to pyroptosis, which is a proinflammatory form of regulated cell death initiated by the activation of CASP1/caspase-1 or CASP11/caspase-11 [2]. Although the type and number of inflammasomes are growing, the NLRP3 (NLR family pyrin domain containing 3) inflammasome is the most extensively studied form of inflammasome. The core components of NLRP3 inflammasome is composed of three proteins: NLRP3, PYCARD (PYD and CARD domain containing, also termed ASC), and CASP1. Adenosine triphosphate (ATP), the intracellular energy-carrying molecule and an extracellular DAMP, was the first reported inducer of NLRP3 inflammasome activation [3]. Subsequently, a wide array of stimuli including PAMPs and DAMPs were identified to activate the NLRP3 inflammasome in various cells, especially in macrophages. The pathologic role of the NLRP3 inflammasome in inflammatory and infectious disease has been well documented [4], but the regulation mechanisms and signaling pathways of its activation remain largely unknown [5].

ALK (anaplastic lymphoma kinase) is a tyrosine kinase receptor for pleiotrophin, a growth factor involved in development, inflammation, and immunity [6–9]. Aberrant ALK activity resulting from gene changes (e.g., point mutation, gene amplification, chromosomal translocation, and DNA rearrangement) has been involved in the development of certain human cancers (e.g., non-small-cell lung cancer and anaplastic large cell lymphomas) [10]. Various ALK fusion proteins are constitutively active in cancer cells, contributing to cell proliferation and drug resistance [10]. ALK inhibitors such as ceritinib (the secondary-generation ALK inhibitor) and lorlatinib (the third-generation ALK inhibitor) have been approved for targeted therapy for patients with lung cancer [11]. In addition to playing an oncogenic role in tumorigenesis, ALK exhibits an immunological role in innate immunity and tumor immunity [12–14]. In particular, ALK-mediated TMEM173 (transmembrane protein 173, also termed STING) activation in macrophages and monocytes has been implicated in the pathogenesis of sepsis caused by infection [12].

Abbreviations: PAMP, pathogen-associated molecular pattern molecule; DAMP, damage-associated molecular pattern molecule; NLRP3, NLR family pyrin domain containing; PYCARD, PYD and CARD doma0069n containing; ATP, adenosine triphosphate; ALK, anaplastic lymphoma kinase; TMEM173, transmembrane protein 173; BMDMs, bone marrow-derived macrophages; PBMCs, peripheral blood mononuclear cells; HMDMs, human monocyte-derived macrophages; IMDM, Iscove's Modified Dulbecco's Medium; FCS, fetal calf serum; M-CSF, macrophage colony-stimulating factor; P/S/G, penicillin/streptomycin/glutamine; TBS, Tris-buffered saline; NEK, NIMA-related kinase; LDH, lactate dehydrogenase; ROS, reactive oxygen species; 4-HNE, 4-hydroxynonenal; LPS, lipopolysaccharide.

* Corresponding author. 68 Haiyu South Road, Changshu, Jiangsu, 215500, China.
E-mail address: qiuj76@gmail.com (J. Qiu).

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In this study, we report that ALK is a novel regulator of ATP-induced NLRP3 inflammasome activation in macrophages through regulation of NLRP3 expression at the priming stage and inflammasome assembly at the sensing stage. Our findings uncover an important function of ALK in innate immunity and provide a new strategy for the treatment of NLRP3-related inflammatory diseases.

2. Methods

2.1. Regents

The antibodies to ALK (Cat#sc-398791) and GAPDH (Cat#sc-47724) were purchased from Santa Cruz Biotechnology (Dallas, TX, USA). The antibody to NLRP3 (Cat#AG-20B-0014-C100) was purchased from Adipogen (San Diego, CA, USA). The antibody to NEK7 (Cat#ab133514) was purchased from Abcam (Cambridge, MA, USA). The antibody to IL-1β (Cat#12242) was purchased from Cell Signaling Technology (Danvers, MA, USA). LPS (Cat#tlrl-eblps) and ATP (Cat# tlrl-atp) were purchased from Invivogen (San Diego, CA, USA). Ceritinib (Cat# S7083), lorlatinib (Cat#7536), and parthenolide (Cat#2341) were purchased from Selleck Chemicals (Houston, TX, USA). 4-HNE (Cat#75899-68-2) was purchased from Cayman Chemical (Ann Arbor, Michigan, USA).

2.2. Cell cultures

Mouse bone marrow-derived macrophages (BMDMs), human peripheral blood mononuclear cells (PBMCs), and human monocyte-derived macrophages (HMDMs) were produced as previously described [15]. Briefly, bone marrow cells from femurs and tibia (6–8 week old C57BL/6 background mice) were used to generate BMDMs using Iscove's Modified Dulbecco's Medium (IMDM) containing 20% fetal calf serum (FCS), 20 ng/ml mouse macrophage colony-stimulating factor (M-CSF), and 1% penicillin/streptomycin/glutamine (P/S/G). After seven days, non-adherent cells were removed and adherent cells were used as indicated. Human PBMCs were isolated from freshly drawn peripheral venous blood with LSM-Lymphocyte Separation Medium. PBMCs were cultured in RPMI 1640 medium containing 10% FCS and 1% P/S/G. HMDMs were generated from $CD14^+$ monocytes by differentiation for seven days in IMDM supplemented with 20% FCS, 20 ng/ml human M-CSF, and 1% P/S/G.

2.3. Priming and stimulation of NALP3 inflammasome

Cells were seeded in 12-well culture dishes (1×10^6/well) and cultured overnight. Culture medium was replaced with 1 mL pre-warmed Opti-MEM medium. Cells were primed for 4 hfour hours with 200 ng/ml LPS and then stimulated by 5 mM ATP for 1 hone hour as previously described [15,16].

2.4. Cell transfection

Pooled siRNAs against mouse or human *Alk* and *Nek7* were purchased from Thermo Fisher Scientific (Pittsburgh, PA, USA). The Neon® Electroporation System from Invitrogen was used to deliver siRNAs into BMDMs according to the manufacturer's instructions. Transfected cells were recovered in complete Dulbecco's modified Eagle's medium. The medium was replaced at 3 h post electroporation. The cells were cultured for 48 h before further examination.

2.5. Gene expression analysis

Total RNA was isolated from cells using TRIzol™ Reagent (Cat#15596026, Thermo Fisher Scientific). Total RNA (2 μg) was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcription Kit (Cat# 4368813, Thermo Fisher Scientific). cDNA from cell samples was amplified with specific primers (human *Nlrp3*: 5′- GGACTGAAGCACCTGTTGTGCA -3′and 5′-TCCTGAGTCTCCCAAGGCATTC -3'; mouse *Nlrp3*: 5′-TCACAACTCGCCCAAGGAGGAA-3′and 5′- AAGAGACCACGGCAGAAGCTAG-3'; human *Gapdh*: 5′- GTCTCCTCTGACTTCAACAGCG-3′and 5′-ACCACCCTGTTGCTGTAGCCAA-3'; mouse *Gapdh*: 5′-CATCACTGCCACCCAGAAGACTG-3′and 5′- ATGCCAGTGAGCTTCCCGTTCAG-3′). Quantitative real time PCR was performed using ssoFast EvaGreen Supermix (Cat# 1725200, Bio-Rad Laboratories, Hercules, CA, USA) on the C1000 Touch Thermocycler CFX96 Real Time System (Bio-Rad Laboratories) according to the manufacturer's protocol. The expression levels of Nlrp3 mRNA were calculated by using the comparative Ct method ($2^{-\Delta Ct}$ formula) after normalization to *Gapdh*.

2.6. NF-κB transcription activity assay

NF-κB transcription activity assay was evaluated using NF-κB p65 Transcription Factor Assay Kit (Cat#ab210613) from Abcam (Cambridge, MA, USA) according to the manufacturer's instructions. A specific double-stranded DNA sequence containing the NF-κB p65 consensus binding site (5′-GGGACTTTCC-3′) was immobilized onto a 96-well plate. Active NF-κB p65 present in the nuclear extract specifically bonds to the oligonucleotide. NF-κB p65 was detected by a primary antibody that recognizes an epitope of NF-κB p65 accessible only when the protein was activated and bound to its target DNA. An HRP-conjugated secondary antibody provided sensitive colorimetric readout that at OD 450 nm.

2.7. Western blot

Indicated cells were washed with cold phosphate-buffered saline and harvested with Cell Lysis Buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na3VO4, 1 μg/ml leupeptin) and the Protease/Phosphatase Inhibitor Cocktail (Cat#5872, Cell Signaling Technology). Whole-cell lyses were separated using SDS-PAGE and were blotted onto a polyvinylidene fluoride membrane. After blocking with 3% bovine serum albumin in a Tris-buffered saline (TBS) buffer with 0.1% Tween-20, the membrane was probed with the primary antibody. After rinsing twice with TBS buffer, the membrane was incubated with a horseradish peroxidase-conjugated secondary antibody and washed, followed by visualization using Pierce™ ECL Western Blotting Substrate (Cat #32106, Thermo Fisher Scientific).

2.8. Immunoprecipitation assay

Pierce™ IP Lysis Buffer (Cat#87787, Thermo Fisher Scientific) including 25 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40 and 5% glycerol was used to lysate cells. The lysates of cells were then immunoprecipitated overnights at 4 °C with 2 μg/ml antibody and protein G-agarose beads. After extensive washing with lysis buffer, the immunocomplexes and any non-covalently bound proteins were dissociated by boiling in 5 × loading buffer (250 mM Tris HCl, pH 6.8, 10% SDS, 30% glycerol, 10 mM DTT, 0.05% bromophenol blue) and subjected to SDS-polyacrylamide gel electrophoresis, followed by western blot.

2.9. Cytotoxicity assay

Lactate dehydrogenase (LDH) release was evaluated using

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