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NEMO differentially regulates TCR and TNF-α induced NF-κB pathways and has an inhibitory role in TCR-induced NF-κB activation

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ABSTRACT

NF-κB essential modulator (NEMO), the regulatory subunit of the IκB kinase (IKK) complex, is an essential adaptor both for inflammation stimuli and TCR-induced NF-κB activation. However, the exact mechanism of its function has not been fully understood. Here, we report that knockdown of NEMO by RNA interference in Jurkat E6.1 cells enhanced TCR-induced NF-κB report gene activity and IL-2 production by promotion of IκBα degradation and p65 nuclear translocation, whereas inhibited TNF-α and LPS-induced IκBα degradation without influencing the phosphorylation of MAPKs. In human primary T and Jurkat E6.1 cells, both CD3/CD28 and PMA/Ionomycin induced NF-κB activation showed a para-curve correlation with the dosage of small interfering RNA targeting NEMO (siNEMO): the NF-κB report gene activity was increased along with ascending doses of transfected siNEMO and reached the highest activity when knockdown about 70% of NEMO, then turned to decline and gradually be blocked once almost thoroughly knockdown of NEMO. Meanwhile, TNF-α induced NF-κB was always inhibited no matter how much NEMO was knockdown. Subcellular fractionation results suggested that upon CD3/CD28 costimulation, NEMO and IKKβ may not cotranslocate to cytoskeleton fraction as a conventional NEMO/IKK complex with a static stoichiometric ratio, instead the ratio of NEMO: IKKβ continuously shift from high to low. Depletion of NEMO accelerated TCR-induced cytoskeleton translocation of IKKβ. Altogether, this study suggests that NEMO may function as a rheostat exerting a negative action on TCR-induced NF-κB activation and differentially regulates TNF-α and TCR-induced NF-κB pathways.

1. Introduction

NF-κB is a family of essential transcription factors involved in a wide array of biological processes through regulating a large number of genes. The NF-κB family consists of NF-κB1 (p50 and its precursor p105), NF-κB2 (p52 and its precursor p100), RelA (also called p65), c-Rel, and RelB. NF-κB family members are normally sequestered in the cytoplasm by inhibitors of NF-κB (IκBs) or the unprocessed forms of NF-κB1 and NF-κB2. The canonical NF-κB signaling pathway employed by most stimuli is centered on activation of IKK (IκB kinase) complex-the catalytic subunits IKKα and IKKβ and the regulatory/scaffold subunit IKKγ (also called NEMO for NF-κB essential modulator), which leads to phosphorylation and degradation of IκBs and then nuclear translocation of p65:p50 and c-Rel:p50 heterodimers [1]. The non-canonical NF-κB signaling pathway employed by several stimuli, is mediated by NF-κB-induced kinase (NIK) to activate and promote IKKα binding to p100, leading to the proteolytic processing of p100/p52 to yield translocation-competent p52-containing NF-κB complexes [2].

The canonical NF-κB signaling pathway is widely adopted by a variety of signals originating from antigen receptors, pattern-recognition receptors and receptors for the members of TNF and IL-1 cytokine families. In these canonical NF-κB pathways, IKKβ and especially NEMO are obligatory components [1]. It is believed that NEMO promotes the assembly of IKK complex and bridges this complex to upstream signaling molecules. Meanwhile, a number of NEMO-binding proteins including positive and negative regulators of NF-κB have been reported. By far, the exact functions of NEMO in NF-κB pathway are not clear [3]. In T cells, upon aggregation of T cell receptor (TCR) and CD28 coreceptors, protein kinase C-θ (PKCθ) is recruited to the immunological synapse (IS) and activated, and in turn phosphorylates CARMA1 and facilitates its association with Bcl10-MALT complex, leading to the assembly of CARMA1-Bcl10-MALT1 (CBM) complex [4–8]. The CBM complex then promotes the ubiquitination of NEMO and stimulates IKK/NF-κB pathway [9]. TCR signaling can also stimulate non-canonical NF-κB pathway indirectly [10].

Abbreviations: NEMO, NF-κB essential modulator; siRNA, small interfering RNA; TCR, T cell receptor; CBM, CARMA1-Bcl10-MALT1 complex; IS, Immunological synapse; shRNA, short hairpin RNA; SM, Soluble membrane; DI, Detergent Insoluble cytoskeleton fraction; AICD, activation-induced cell death; PBMC, prepared human peripheral blood monocytes; NBD, NEMO-binding domain; EAE, experimental allergic encephalomyelitis.

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More complicated than other biological receptors, the TCR is composed of a clonotypic αβ TCR heterodimer that recognizes a peptide–MHC complex in combination with a signal-transduction complex of six subunits (CD3γε, CD3δε and CD3ζζ pairs). The striking diverse TCR-peptide-MHC complexes cooperate with various co-receptors and intracellular regulators such as protein kinases and ubiquitination E3 ligases to integrate a broad range of signal strengths, manipulating transduction of TCR signaling accurately and meticulously and ensuring TCR to identify subtle stimulatory variations and to produce proper immune response [11].

In order to understand the sophisticated TCR signaling, we studied the effect of NEMO on TCR-induced NF-κB pathway and the mechanism underlying. We found that partially knockdown of NEMO by RNA interference (shNEMO or siNEMO) in Jurkat or human primary T cells blocked inflammation signals such as TNF-α and LPS-induced NF-κB activation as expected, but increased TCR-induced NF-κB activation unexpectedly, whereas more thoroughly knockdown of NEMO blocked TCR-induced NF-κB. Upon TCR/CD28 costimulation, cytoskeleton translocation of IKKβ was much quicker and BCL-10 was slower in shNEMO cells than they were in control cells, and the stoichiometric ratio of NEMO:IKKβ in cytoskeleton gradually changed from high to low. Taken together, our results suggest that NEMO differentially regulates TNF-α/LPS and TCR-induced NF-κB activation, and plays a negative regulatory role in TCR-induced NF-κB activation, which may be an important mechanism for TCR signaling.

2. Materials and methods

2.1. Plasmids and antibodies

NEMO (accession: NM_003639) was amplified by PCR from the cDNA of Jurkat E6.1 cells and cloned into pFLAG-CMV2. Lck-NEMO was constructed by fusing Lck (aa 1–12) to the N-terminal of NEMO and then cloned into pFLAG-CMV2. Antibodies against TRAF6, Bcl10, NEMO, p65, IKKβ, IκBα, pJNK, pERK, pp38, JNK, ERK, p38, lamin-B, β-actin, and Lck were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against PKCθ, Calnexin were purchased from Abcam (Cambridge, MA, USA). The mouse FLAG antibody (M2), PMA, TNF-α, LPS (*Escherichia coli* 0111:B4) and Ionomycin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The HRP-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-human CD3 mAb (UCHT1) and the anti-human CD28 mAb (CD28.2) were from BD Pharmingen (San Diego, CA, USA).

2.2. Stable cell line construction

Scrambled control nucleotides (5′-CGCTAATTCGACTCGGATA-3′), RNA interference sequences against NEMO including nucleotides 529–547 of NEMO mRNA (5′-GGAGTTCCTCATGTGCAAG-3′, targeting ORF) and nucleotides 2021–2039 of NEMO mRNA (5′-CCAGTCACTATTGATGGAC-3′, targeting 3′-UTR) were constructed into RNA interference expression vector pSUPER.Retro.Neo-GFP (OligoEngine, Seattle, USA), respectively. Jurkat E6.1 cells were transfected with pSUPER.Retro.Neo-GFP based shRNA using the Amaxa 4D-Nucleofector according to user manual and selected for 15 days with 500 μg/ml G418 after 24 h transfection. Mono clones were isolated, amplified and cultured for about 1 month. Then SDS-PAGE was employed to analysis the protein level of NEMO in these stable cell lines.

2.3. Cell culture, transfection and stimulation

293 T cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) plus with 100 U/ml streptomycin, and 100 U/ml penicillin (Invitrogen) at 37 °C, 5% CO2. Plasmids transfection was carried out with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Jurkat E6.1 cells and ZAP-70-/- T cells were cultured in RPMI supplemented with 10% FBS and transfected by using Amaxa 4D-Nucleofector according to user manual. shNEMO stable cell lines were cultured in RPMI supplemented with 10% FBS and 500 ng/ml G418. In the research, cells were untreated or stimulated for the indicated times with PMA (100 ng/ml), LPS (1 μg/ml), Ionomycin (1 μM), TNF-α (20 ng/ml), anti-CD3 (10 μg/ml) and anti-CD28 mAbs (2 μg/ml), which were crosslinked with goat-anti-mouse IgG (10 μg/ml).

2.4. Western blot and co-immunoprecipitation

For western blot, the cells were extracted in a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, pH 8.0, 5 mM NaPPi, 1 mM sodium orthovanadate (Na3VO4), 1 mM PMSF, 1% NP-40, and 10 μg/ml each aprotinin and leupeptin). Cell lysates were centrifuged at 10,000 *g* for 10 min at 4 °C and supernatants were subjected to SDS-PAGE, transferred to PVDF membranes and followed by immunobloting. Immunoblots were developed with the ECL reagent (GE Healthcare) according to the manufacturer's instructions. For immunoprecipitation, whole cell lysate was prepared and incubated with 0.5 μg antibodies for 2 h and then precipitated with 25 μL protein G-Sepharose (GE Healthcare) for 4 h at 4 °C, washed five times with lysis buffer, denatured by boiling in 2× sample buffer for SDS–PAGE, and then subjected to immunoblot analysis with the indicated antibodies.

2.5. Subcellular fractionation

Subcellular fractionation was a modified version of methods as previously described [1]. Briefly, Jurkat T cells were resuspended in hypotonic buffer (42 mM KCl, 10 mM Hepes pH 7.4, 5 mM MgCl2, 10 μg/ml each aprotinin and leupeptin). Nuclei homogenates were removed by centrifugation. Cytosol, SM (Soluble membrane) and DI (Detergent Insoluble cytoskeleton fraction) were separated by two steps of centrifugation at 25,000 *g* for 90 min at 4 °C.

2.6. Reporter gene assay

Dual luciferase reporter system was employed (Promega) to examine the activity of NF-κB. Cells were transfected with constructs of NF-κB luciferase reporter gene plasmid by using BioRad Gene Pulser X cell electroporation. Renilla luciferase control reporter gene vector was used for normalization. 24 h after transfection, cells were treated with the indicated stimuli, then washed with PBS and lysed. Luciferase activities were measured by BioLuminometer (Berthold).

2.7. ELISA

ShNEMO and NC cells were cultured in 24-well plate with coated CD3 (5 μg/ml) and soluble CD28 (2 μg/ml) for 48 h. The concentration of supernatant IL-2 was measured by ELISA kits (eBioscience) according to the manufacturer's protocol.

2.8. Preparation of PBMC, culture and siRNA nucleofection

Peripheral blood mononuclear cells (PBMC) obtained from healthy donors were isolated by centrifugation over Ficoll-Hypaque (Hao Yang Co., Tianjin, China) according to the manufacturer's instructions. Isolated PBMCs were stimulated with 5 μg/ml PHA (Sigma) and 10 ng/mL rhIL-2 (R&D Systems) for 2 days in order to enrich the cells prior to Nucleofection, then cultured in RPMI 1640 supplemented with 10% FBS (Hyclone, Logan, UT, USA) and 10 ng/mL rhIL-2 at 37 °C, 5% CO2. The scrambled control siRNA, small interference RNA against NEMO (accession: NM_003639) including si001 (5′-CCAGUCACUAUUGAUGGACdTdT-3′,