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Deubiquitinase USP12 promotes LPS induced macrophage responses through inhibition of IκBα

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ABSTRACT

Post translational modifications, ubiquitination and its reversal by deubiquitination play an important role in regulating innate immune system. USP12 is a poorly studied deubiquitinase reported to regulate T-cell receptor signalling however the functional role of USP12 in macrophages, the principal architects of inflammation, is unknown. Thus, in this study we probed the involvement of USP12 in macrophage mediated inflammatory responses using bacterial endotoxin, LPS, as the model system. Here, we observed that the expression of USP12 was altered in time dependent manner in LPS stimulated RAW 264.7 macrophages at both mRNA and protein levels as revealed by qPCR and western blot analysis, respectively. Further analysis showed that LPS reduced the levels of Sp1 which enhanced the transcriptional levels of USP12. We observed that siRNA mediated ablation of USP12 expression in mouse macrophages suppressed the induction of LPS-induced iNOS and IL-6 expression but failed to alter IFN-β synthesis, oxidative stress and phagocytic ability of macrophages. Mechanistic analysis suggest that USP12 may be required for the activation of NFκB pathway as knockdown of USP12 reduced the inhibitory phosphorylation of IκBα, a well characterized inhibitor of NFκB nuclear translocation. Further, USP12 was observed to be required for LPS elicited phosphorylation of ERK1/2 and p38. Collectively, our data suggest that USP12 may be a key mediator of LPS stimulated macrophage responses.

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1. Introduction

Post translational modifications (PTMs) expand the repertoire of mechanisms that regulate diverse cellular processes including immune responses. Phosphorylation events controlled by kinases and phosphatases are the most well studied PTMs in the regulation of immune responses. Of late, there is a surge in interest to study the role of another PTM, ubiquitination, in the control of inflammatory responses. Ubiquitination levels are maintained by the opposing actions of ubiquitinases and deubiquitinases. Several deubiquitinases which act to reverse the ubiquitination process by cleaving ubiquitin chains are implicated in macrophage inflammatory responses. Prominent among them are A20 and CYLD which were reported to suppress NFκB activation through the inactivation of E3 ubiquitin ligase, TRAF6 [1]. Deubiquitinase, DUBA, was demonstrated to cleave lysine-63 linked polyubiquitin chain of another E3 ligase TRAF3 resulting in its dissociation from downstream signalling leading to dampened IFN-β production [2].

USP12 (Ubiquitin Specific Peptidase 12) belongs to the ubiquitin specific peptidase sub-family of deubiquitinases. The enzymatic activity of USP12 is enhanced by its interaction with adaptor proteins WDR48 [3] and WDR20 [4]. It was shown that USP12 deubiquitinates histones H2A and H2B both *in vitro* and *in vivo* [3]. USP12 is poorly studied with very few known functions. Over-expression of USP12 in Xenopus embryos caused gastrulation defects [3]. Recently, USP12 was reported to negatively control notch signalling by influencing the lysosomal mediated degradation of notch receptor [5]. Further, USP12 was shown to stabilize T-cell receptor complex at the cell surface and thereby sustain T-cell receptor mediated signalling [6].

In this study we specifically examined the role of USP12 in LPS mediated activation of macrophages and identified that USP12 is required for select LPS elicited responses of macrophages. Mechanistic analysis showed that USP12 is required for LPS induced

Abbreviations: CYLD, Cylindromatosis; DUBA, deubiquitinating enzyme A; iNOS, inducible nitric oxide synthase; PTMs, Post translational modifications; TRAF3, TNF receptor associated factor 3; TRAF6, TNF receptor associated factor 6; USP12, Ubiquitin Specific Peptidase 12; WDR20, WD repeat containing protein 20; WDR48, WD repeat containing protein 48.

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phosphorylation of IκBα and MAPKs ERK1/2 and p38. Collectively, this study suggested a positive regulatory role for USP12 in promoting activation of the critical components of NFκB pathway and further studies may delineate its additional effects on LPS-induced macrophage inflammatory responses.

2. Materials and methods

2.1. Cells, antibodies and reagents

RAW 264.7 were maintained in Dulbecco's Modified Eagle's medium (Gibco, Life Technologies, Grand Island, NY, USA) and were grown in 10% heat-inactivated Fetal Bovine Serum (Gibco, Life Technologies, Grand Island, NY, USA). *E. coli* LPS (0127:B8) was obtained from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Recombinant mouse IFNγ was procured from Peprotech (Rocky Hill, NJ, USA) and USP-12 siRNA and negative control siRNA were obtained from Ambion (Ambion, Austin, TX, USA). The lyophilized pellets were dissolved in nuclease free water as per manufacturer's instructions. Antibodies specific for iNOS (BD Biosciences, San Jose, CA, USA), β-actin (Calbiochem, San Diego, CA, USA) were used and phospho-specific antibodies were obtained from Cell Signalling Technology (Beverly, MA, USA). USP12 antibody was from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO, USA). Anti-mouse HRP and anti-rabbit HRP antibodies were obtained from Genscript (Genscript, Piscataway, NJ, USA). DCFDA dye was purchased from Life technologies (Life Technologies, Grand Island, NY, USA) and were resuspended in DMSO as per manufacturer's guidelines.

2.2. Nucleofection

RAW 264.7 cells were cultured in 10% fetal bovine serum (FBS) containing DMEM medium at 37 °C in 5% CO_2 environment. All transfections were carried out in nucleofection apparatus using Solution V (Lonza, Allendale, NJ, USA) as per the manufacturer's instructions with some modifications. Briefly, 12×10^6 cells were nucleofected with 300 nM of control or USP12 siRNA after gently resuspending them in 100 μl of nucleofector solution V. Post-nucleofection, 500 μl of pre-warmed medium was added to the transfected cells and were then transferred to the plate containing 10 ml of FBS containing DMEM. Plates were then incubated for a minimum of 16 h at 37 °C and transfected cells were then seeded at equal density and were stimulated as mentioned in figure legends.

2.3. Cloning of USP12 promoter and promoter assays

A 2.8 kb fragment encompassing the promoter region upstream of human USP12 gene was cloned into a gateway compatible luciferase reporter plasmid using Gate way cloning technology following manufacturer's instructions (Invitrogen, Carlsbad, CA). Transient transfections in HEK 293T cells were performed using PEI reagent. HEK293T cells were transfected with 1 μg of empty vector, p65, STAT1, PKAC, Sp1 constructs separately along with 200 ng of 2.87 Kb upstream region of USP12 gene and 50 ng of renilla luciferase constructs. Twenty four hours post transfection, cells were lysed and luciferase assay was performed as described previously [7]. Briefly, cells were lysed in passive lysis buffer and the lysates were mixed with required volumes of luciferase assay buffer and the firefly luciferase activity was measured using Sirius Luminometer (Berthold Detection Systems GmbH, Germany). Renilla luciferase activity was measured using renilla luciferase assay buffer. The data was plotted by normalizing firefly luciferase values with the corresponding renilla luciferase values.

2.4. Cell stimulation, cell lysis and immunoblotting

RAW 264.7 cells are treated with 1 μg/mL of LPS or 100 ng/mL of IFNγ for indicated time points. Post stimulation cells were washed in phosphate buffered saline (PBS) and were subsequently lysed in TN1 lysis buffer containing protease inhibitors (50 mM Tris pH 8.0, 10 mM EDTA, 10 mM $Na_2P_2O_7H_2$, 10 mM NaF, 1% Triton-X 100, 125 mM NaCl, 10 mM Na_3VO_4, 10 μg/ml each of aprotinin and leupeptin and 1 mM PMSF). The total protein was estimated by Bradford assay and protein matched lysates were denatured and resolved on 10% SDS PAGE. The proteins separated by electrophoresis were next transferred on to PVDF membrane and the membrane was incubated with desired primary antibody for overnight at 4 °C. This was followed by washes with Tris buffered saline with 0.1% Tween20 (TBST) for a minimum of three times and was finally probed with the horse radish peroxidase (HRP) conjugated secondary antibody at room temperature for 1 h. The protein of interest was then detected using enhanced chemiluminescence (ECL) (GE Life Sciences, Piscataway, NJ, USA).

2.5. ELISA for cytokine analysis

RAW 264.7 cells were stimulated with LPS for 10 h and the culture supernatants collected were centrifuged at 14,000 rpm for 3 min at 4 °C to remove any cell debris. The amount of IL-6 (R&D systems, Minneapolis, MN, USA) in the supernatants was analyzed using ELISA kits as per manufacturer's instructions. IFN-β in the supernatants was measured as previously described [7]. All the cytokines were analyzed at 450 nm using Wallac 1420 Victor Multimode Plate Reader (Perkin-Elmer, Waltham, Massachusetts, USA).

2.6. Phagocytosis measurements

RAW 264.7 cells phagocytic efficiency was measured using fluorescent *E. coli* bacteria K-12 strain (Molecular Probes- Invitrogen, CA, USA). The lyophilized bacteria were resuspended in PBS as per manufacturer's instructions and RAW 264.7 cells transfected with control or USP12 siRNA were left un-infected or infected with a multiplicity of infection (MOI) of 10. The infected cells were then centrifuged at 650 g for 2 min for establishing optimal contact between bacteria and macrophages. The infection was performed for 3 h at 37 °C, bacterial supernatant was removed and fluorescence due to non-phagocytosed bacteria was quenched by 500 μg/mL of trypan blue (Sigma Aldrich, St. Louis, MO, USA) of pH 4.4 (set with citrate buffer). The cells were then carefully washed with ice-cold PBS, lysed in 0.1% Triton X-100 and the uptake of fluorescent bacteria by RAW 264.7 cells was determined in the cell lysates using multiplate reader (Perkin-Elmer, Waltham, Massachusetts, USA) with excitation at 485 nm and emission at 535 nm.

2.7. Oxidative stress measurement

Post-transfection, RAW 264.7 cells stimulated with 1 μg/ml LPS for 24 h or left un-stimulated (Resting, R) were incubated with 5 μM DCFDA (Life Technologies, Grand Island, NY, USA) dye for 30 min in dark. Subsequently cells were carefully washed using ice-cold PBS, were lysed in 0.1% Triton X-100. Amount of fluorescence in the cell lysates was measured using a multiplate reader (Perkin-Elmer, Waltham, Massachusetts, USA) with excitation at 485 nm and emission at 535 nm.

2.8. RNA isolation and quantitative real time PCR analysis

Post-stimulation, cells were carefully washed with PBS and total

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