MyD88 NEDDylation negatively regulates MyD88-dependent NF- κ B signaling through antagonizing its ubiquitination

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Abstract

Myeloid differentiation factor 88 (MyD88) plays a central role in innate immunity response, however, how its activity is tightly regulated remains largely unknown. In this study, we identify MyD88 as a novel substrate of NEDD8, and demonstrate that MyD88 NEDDylation antagonizes its ubiquitination. Interestingly, in response to the stimulation of IL-1 β , MyD88 NEDDylation is downregulated while its ubiquitination is upregulated. We also show that deNEDDylase NEDP1 serves as a regulator of this process. Furthermore, we demonstrate that NEDD8 negatively regulates the dimerization of MyD88 and suppresses MyD88-dependent NF- κ B signaling. Taken together, this study reveals that NEDDylation of MyD88 regulates NF- κ B activity through antagonizing its ubiquitination, suggesting a novel mechanism of modulating NF- κ B signaling pathway.

Key words:

MyD88 NEDDylation, ubiquitination, NEDP1, IL-1 β , NF- κ B

Introduction

Ubiquitination plays many important roles in eukaryotic cellular processes such as DNA damage repair, cell cycle regulation and immune responses [1,2,3]. Host immune response, a body defense against pathogen infection and tissue injury, needs to be properly initiated, activated and terminated [4]. To ensure that immune response is tightly controlled, ubiquitination functions as an effective regulatory mechanism where ubiquitin molecules are conjugated to diverse target proteins and affect the stability or activity of these proteins [1]. For example, in NF- κ B signaling pathway of innate immunity, TRAF6-mediated K63 poly-ubiquitin chain activates IKK complex for phosphorylation, which subsequently promotes poly-ubiquitination of I κ B α and leads to its degradation, thereby releasing NF- κ B for transcription activation [5,6,7]. As ubiquitin system is largely involved in the immune signaling, its regulatory roles in various signal transduction processes need further exploration.

Besides ubiquitination, other types of post-translational modifications have also been characterized [8]. Among them, NEDDylation has the most similarity with ubiquitination, which also uses a set of enzymatic machinery including E1, E2 and E3 to specifically conjugate the small molecule NEDD8 (neural precursor cell expressed, developmentally down-regulated 8) to the substrates [9,10]. However, unlike ubiquitin, NEDD8 does not target protein for proteasomal degradation. Instead, it mostly affects the localization, activity, or interaction of proteins [11,12]. In contrast to the well-studied substrates of ubiquitin, only a few substrates of NEDD8 have been discovered. Cullin, an ubiquitin E3 ligase subunit, is the largest family of identified

substrates of NEDD8 [13]. NEDDylation of cullin promotes its activity and facilitates protein ubiquitination [14,15]. Recently, non-cullin substrates have also been characterized for their roles in various signaling pathways [13,16,17,18]. For instance, NEDDylation of BCA3 promotes its interaction with p65 subunit and inhibits NF- κ B activation, and the enhanced NEDDylation of IKK γ by TRIM40 also inhibits NF- κ B activity [19,20]. However, whether other pivot proteins in NF- κ B signaling pathway undergo NEDDylation remains unclear. Identifying novel NEDD8 substrates and elucidate their functions will provide us more insights into the physiological role of NEDDylation in innate immunity.

Myeloid differentiation factor 88 (MyD88) is a key adaptor in innate immunity, mediating interleukin-1 receptor (IL-1R) and Toll-like receptor (TLR) signaling pathways [21,22,23]. After the infection of pathogens, activated receptors TLR and IL-1R recruit MyD88 to their intracellular domain as a homodimer, and then transmit the signal through a signaling cascade, which in the end leads to the nuclear translocation of NF- κ B and activates the transcription of several pro-inflammatory factors and cytokines [24,25,26,27]. Owing to the central role of MyD88 in transmitting the signal, it is crucial to investigate how its activity is regulated. It has been reported that lysine 48 (K48)-linked ubiquitination of MyD88 reduces its stability, and inhibition of lysine 63 (K63)-linked ubiquitination of MyD88 negatively regulates MyD88-dependent signaling pathway [28,29,30]. However, the effects of other post-translational modifications on MyD88 activity and NF- κ B pathway remain to be discovered.

In this study, we report that MyD88 is NEDDylated and its NEDDylation antagonizes its ubiquitination to regulate NF- κ B signaling pathway.

2. Materials and Methods

2.1. Plasmids and antibodies

MyD88 was cloned into the pCDNA-3Flag, pEGFP-N3 or pRK-HA vector, NEDP1 and NEDP1 C163S were cloned into pCDNA-3Flag or pCDNA-3Myc vector. Ubiquitin and NEDD8 were cloned into pRK-HA or pEF1-c-6His vector. UBC12 and UBC12 C111S were inserted into pCDNA-3Flag or pCDNA-3Myc vector.

Flag antibody (F3165) and HA antibody (H9658) were purchased from Sigma Aldrich (USA). Myc antibody (M047-3), His antibody (D291-3), GFP antibody (598) and Actin antibody (PM053) were purchased from MBL (Japan). NEDP1 antibody (F1512) was from Santa cruz (USA).

2.2. Cell culture and transfection

HEK293T wide-type and NEDP1^{-/-} cells were cultured in the Dulbecco's Modified Eagle medium (Gibco, USA) with 10% fetal bovine serum (Gibco), and transfected using PEI transfection agent (Polyscience, USA).

2.3. Ni²⁺ pull-down assay

Detection of NEDDylation or ubiquitination by Ni²⁺ pull-down assay was achieved following the protocol described previously [31].

2.4. Co-Immunoprecipitation

HEK293T cells were harvested in cold PBS at 36 h after transfection with indicated plasmids, and then lysed in NP-40 lysis buffer for 1 h at 4°C with protein

inhibitors. The lysates were then centrifugated at 13000 rpm for 15 min, and the supernatants were incubated with the primary antibody for 3 h and precipitated with protein G for 3 h at 4°C. The beads were washed with NP-40 lysis buffer for three times, and then boiled with SDS loading buffer.

2.5. Luciferase reporter assay

293T cells were transfected with NF- κ B firefly reporter, Renilla reporter and other indicated plasmids, and then luciferase reporter assay was performed following the instructions of reporter assay system (Promega, USA). All experiments were repeated three times with triplicates and data was shown as mean $\pm$ SD using one representative experiment.

3. Results

3.1 MyD88 is a novel substrate of NEDD8

To explore whether MyD88 undergoes NEDDylation, we performed Ni^{2+} pull-down assay. As shown in Fig. 1A, besides ubiquitin, multiple NEDD8 molecules were conjugated to MyD88. We further investigated whether MyD88 NEDDylation depends on specific NEDDylation machinery. After treatment with MLN4924, a specific inhibitor of NEDDylation E1 enzyme, the level of MyD88 NEDDylation decreased significantly (Fig. 1B, lane 3 vs lane 2). We also found that the enzymatic mutant UBC12 C111S abolished the NEDDylation of MyD88 in comparison to UBC12 wide-type, indicating that UBC12 is the E2 of MyD88 NEDDylation (Fig. 1B, lane 5 vs lane 4, lane 5 vs lane 2). Next, we showed that NEDP1, a deNEDDylase, but not its enzymatic mutant NEDP1 C163S largely reduced MyD88 NEDDylation (Fig. 1C, Fig 1D, lane 1-3). Furthermore, when NEDP1 was deleted, NEDDylation of MyD88 was enhanced significantly (Fig. 1D, lane 4 vs lane 1), and only NEDP1 but not NEDP1 C163S abolished the increase (Fig. 1D, lane 5 vs lane 4). These results indicate that MyD88 is NEDDylated and NEDP1 acts as its deNEDDylase.

3.2 MyD88 NEDDylation antagonizes its ubiquitination.

Next, we sought to determine the relationship between MyD88 NEDDylation and its ubiquitination. We found that NEDD8 markedly suppressed MyD88 ubiquitination (Fig. 2A). Likewise, ubiquitin inhibited MyD88 NEDDylation as well (Fig. 2A). Given that NEDP1 deNEDDylated MyD88, we investigated the effect of NEDP1 on MyD88

ubiquitination. As shown in Fig. 2B, NEDP1 promoted MyD88 ubiquitination (lane 4 vs lane 3), in comparison to the negative effect of NEDP1 on MyD88 NEDDylation (lane 2 vs lane 1). Moreover, MyD88 ubiquitination decreased in NEDP1^{-/-} cells compared to the increased NEDDylation (Fig. 2C, lane 4 vs line 3, lane 2 vs lane 1), indicating that NEDDylation of MyD88 antagonized its ubiquitination.

As Nrdp1 acts as an ubiquitin E3 ligase of MyD88 [28], we then examined whether MyD88 NEDDylation was affected by Nrdp1. As shown in Fig. 2D, MyD88 ubiquitination was enhanced by Nrdp1 (lane 6 vs lane 5), however, its NEDDylation was inhibited significantly (lane 3 vs lane 2). Collectively, these results reveal that MyD88 NEDDylation antagonizes its ubiquitination, and vice versa.

3.3 MyD88 NEDDylation decreases in response to IL-1 β

To gain insights into the function of MyD88 NEDDylation in the cellular process of immune responses, we then tested the dynamics of MyD88 NEDDylation in response to IL-1 β stimulation. The results showed that MyD88 NEDDylation gradually decreased with the time extended (Fig. 3A), and ectopic NEDP1 further reduced the level of MyD88 NEDDylation in cells treated with IL-1 β (Fig. 3B). However, in NEDP1^{-/-} cells, there was no evident change of MyD88 NEDDylation under IL-1 β stimulation (Fig. 3C), indicating a crucial role of NEDP1 in downregulating MyD88 NEDDylation triggered by IL-1 β . We suspected that the gradual decrease of MyD88 NEDDylation is induced by the recruitment of NEDP1 to MyD88. To testify this hypothesis, we detected the interaction between MyD88 and NEDP1 in cells after treatment with

IL-1 β . As expected, a much stronger interaction between NEDP1 and MyD88 was observed after IL-1 β stimulation compared to the untreated cells (Fig. 3D). Taken together, these results suggest that NEDP1 is recruited to and deNEDDylates MyD88 when the signaling pathway is activated.

3.4 NEDD8 regulates MyD88 ubiquitination in response to IL-1 β and reduces NF- κ B activity

Based on the previous observation that MyD88 NEDDylation antagonizes its ubiquitination, we speculated that IL-1 β stimulation would also lead to a change of MyD88 ubiquitination. As expected, MyD88 ubiquitination gradually increased under the stimulation of IL-1 β (Fig. 4A). And we also found that NEDD8 blocked the increase of MyD88 ubiquitination in response to IL-1 β (Fig. 4B). Interestingly, when NEDP1 was co-expressed, the peak level of MyD88 ubiquitination appeared earlier after stimulation (Fig. 4C), which indicated that NEDP1 promoted the increase of ubiquitination. This result is consistent with the observation that NEDP1 deneddylates MyD88 in response to IL-1 β . Furthermore, when NEDP1 was deleted in HEK293T cells, the increase of MyD88 ubiquitination was inhibited (Fig. 4D). These results demonstrate that MyD88 ubiquitination increases when MyD88-dependent NF- κ B pathway is activated by IL-1 β , and this process is regulated by NEDDylation.

As activation of MyD88-dependent signaling pathway will affect modifications of MyD88, to further address the regulatory role of MyD88 ubiquitination and NEDDylation, we next investigated whether these modifications of MyD88 influenced

downstream signal transduction. First, Co-IP assay was used to test the interaction between Flag-tagged MyD88 and GFP-tagged MyD88. And we confirmed that MyD88 dimerization increased under the stimulation of IL-1 β (Fig. 4E), which is in agreement with the previous studies that MyD88 forms a dimer to transmit the signal [25,27]. Then we examined whether NEDDylation regulated the level of MyD88 dimerization under different conditions. Expression of NEDD8 suppressed interaction between two MyD88 molecules (Fig. 4F, lane 3 vs lane 2), while ubiquitin strongly enhanced their interaction (Fig. 4F, lane 4 vs lane 2). Furthermore, when NEDDylation was inhibited by NEDP1, the dimerization of MyD88 also significantly increased compared to the vector control (Fig. 4F, lane 5 vs lane 2). In addition, a luciferase reporter assay was conducted to analyze the effect of MyD88 modifications on NF- κ B transcriptional activity. As shown in Fig. 4G, MyD88 NEDDylation suppressed transcriptional activity of NF- κ B (lane 3 vs lane 2) while MyD88 ubiquitination activated NF- κ B activity (lane 4 vs lane 2). Together, these results indicate that MyD88 NEDDylation inhibits IL-1 β -induced signal transduction while its ubiquitination promotes downstream signal activation.

4. Discussion

Recent studies have demonstrated that MyD88 is a substrate of ubiquitin, and k48-linked poly-ubiquitination leads to degradation of MyD88 [28,29]. Here we identified that MyD88 is conjugated to NEDD8, and elucidated an underlying mechanism in regulation of IL-1 β -induced signaling transduction through MyD88 NEDDylation.

MyD88 is a vital adaptor in the TLR signaling pathway, and MyD88 binds to almost all TLRs but not TLR3 [29]. Thus, modulation of MyD88 has an important role in regulating downstream signals. As MyD88 undergoes both NEDDylation and ubiquitination, we wondered whether these two modifications keep a balance to modulate and control the signal transduction. Interestingly, in response to IL-1 β treatment, NEDP1 is recruited to MyD88 and deNEDDylates MyD88, resulting in a significantly decrease of MyD88 NEDDylation and an increase of ubiquitination. These results indicate a proper model that MyD88 NEDDylation is vital in the quiescent cells to avoid unnecessary signaling; when cells are faced with signals, IL-1 β as an example, NEDDylation is downregulated, leading to the upregulation of ubiquitinated MyD88, thus promoting the dimerization of MyD88 and the downstream signals.

As many NEDDylated substrates share the same E3 ligase with its ubiquitination [13], and MyD88 is reported to be poly-ubiquitinated by Nrdp1 [28], we explored whether Nrdp1 acts as an E3 ligase of MyD88 NEDDylation. Unexpectedly, Nrdp1 promotes ubiquitination of MyD88 but inhibits its NEDDylation, which further confirms

that ubiquitination of MyD88 antagonizes its NEDDylation. On the other hand, MLN4924 and UBC12 C111S specifically blocks NEDDylation of MyD88, indicating a conserved enzymatic system of NEDDylation is used to NEDDylate MyD88, thus which E3 ligase is utilized to mediate MyD88 NEDDylation is worth to be investigated in the future.

It has been reported that MyD88 undergoes both K48-linked ubiquitination and K63-linked ubiquitination [28,30], K63-linked ubiquitination is mainly responsible for the signaling transduction, and K48-linked ubiquitination is helpful for targeting MyD88 to proteasomal degradation. Here we showed that in response to IL-1 β treatment, MyD88 poly-ubiquitination is increased and further regulates the dimerization of MyD88 and NF- κ B signaling, thus it is likely that NEDDylation of MyD88 modulates NF- κ B signaling through antagonizing K63-linked ubiquitination. Further studies are necessary to address if NEDDylation of MyD88 regulates its stability.

Taken together, our work reveals a vital mechanism of MyD88-dependent regulation of TLR signaling pathway through NEDDylation, in which NEDD8 antagonizes the ubiquitination of MyD88 and inhibits its dimerization. These data provide a new insight into the regulation of NF- κ B activity by affecting MyD88 NEDDylation.

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Figure legends

Figure 1. MyD88 is a novel substrate of NEDD8. (A) MyD88 is NEDDylated in HEK293T cells. (B) MLN4924 and UBC12 C111S inhibit MyD88 NEDDylation. (C) MyD88 is deNEDDylated by NEDP1. (D) MyD88 NEDDylation is increased in NEDP1^{-/-} cells and NEDP1 C163S loses the activity to deNEDDylate MyD88. In A-D, HEK293T wide-type cells or NEDP1^{-/-} cells were transfected with indicated plasmids, and then NEDDylation and ubiquitination of MyD88 were analyzed by Ni²⁺ pull-down assay and western blotting.

Figure 2. MyD88 NEDDylation antagonizes its ubiquitination. (A) NEDD8 antagonizes ubiquitination of MyD88, and vice versa. (B) NEDP1 promotes MyD88 ubiquitination. (C) MyD88 ubiquitination decreases in NEDP1^{-/-} cells. In A-C, NEDDylation or ubiquitination of MyD88 was analyzed by Ni²⁺ pull-down assay as described above. (D) Nrdp1 promotes MyD88 ubiquitination but inhibits Myd88 NEDDylation. HEK293T cells were transfected with Flag-MyD88, His-NEDD8 or His-ubiquitin, and empty vector or myc-Nrdp1, and then immunoprecipitation was performed with Flag antibody to analyze the effect of Nrdp1 on MyD88 NEDDylation and its ubiquitination.

Figure 3. MyD88 NEDDylation decreases in response to IL-1 β . (A) NEDDylated MyD88 decreases in response to IL-1 β treatment. (B) NEDP1 promotes the decrease of MyD88 NEDDylation. (C) NEDP1 is essential for the decrease of MyD88 NEDDylation. In A-C, HEK293T cells were transfected with indicated plasmids, 36 h

later, cells were treated with IL-1 β for indicated time, and then NEDDylation of MyD88 was analyzed by Ni²⁺ pull-down assay as described above. (D) NEDP1 binds to MyD88 after IL-1 β treatment. HEK293T cells transfected with Flag-MyD88, Myc-NEDP1 were treated with IL-1 β , and subjected to co-IP assay to detect the interaction between MyD88 and NEDP1.

Figure 4. NEDDylation regulates MyD88 ubiquitination in response to IL-1 β and reduces NF- κ B activity. (A) MyD88 ubiquitination increases in response to IL-1 β treatment. (B) NEDD8 blocks the increase of MyD88 ubiquitination. (C) NEDP1 promotes the increase of MyD88 ubiquitination. (D) The increase of MyD88 ubiquitination is inhibited in NEDP1^{-/-} cells. In A-D, 293T cells were treated with IL-1 β after transfection for 36 h, and then ubiquitination or NEDDylation of MyD88 was analyzed as described above. (E) MyD88 forms a dimer in response to IL-1 β treatment. (F) Ubiquitin and NEDP1 promotes MyD88 dimerization. In E & F, immunoprecipitation was performed with Flag antibody to analyze the dimerization of MyD88. (G) Ubiquitin promotes but NEDD8 inhibits MyD88-dependent NF- κ B activation. HEK293T cells were transfected with indicated plasmids, and Luciferase reporter assay was carried out using Promega reporter assay system, data was shown as mean $\pm$ SD from one representative experiment.