p62/SQSTM1 Enhances NOD2-Mediated Signaling and Cytokine Production through Stabilizing NOD2 Oligomerization

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Abstract

NOD2 is a cytosolic pattern-recognition receptor that senses muramyl dipeptide of peptidoglycan that constitutes the bacterial cell wall, and plays an important role in maintaining immunological homeostasis in the intestine. To date, multiple molecules have shown to be involved in regulating NOD2 signaling cascades. p62 (sequestosome-1; SQSTM1) is a multifaceted scaffolding protein involved in trafficking molecules to autophagy, and regulating signal cascades activated by Toll-like receptors, inflammasomes and several cytokine receptors. Here, we show that p62 positively regulates NOD2-induced NF-κB activation and p38 MAPK, and subsequent production of cytokines IL-1β and TNF-α. p62 associated with the nucleotide binding domain of NOD2 through a bi-directional interaction mediated by either TRAF6-binding or ubiquitin-associated domains. NOD2 formed a large complex with p62 in an electron-dense area of the cytoplasm, which increased its signaling cascade likely through preventing its degradation. This study for the first time demonstrates a novel role of p62 in enhancing NOD2 signaling effects.

Introduction

NOD2 belongs to a family of 22 different cytosolic pattern recognition receptors known as NOD-like receptors (NLRs) in humans [1]. NOD2 detects muramyl dipeptide (MDP), a unit of peptidoglycan constituting the bacterial cell wall, and induces the production of cytokines and anti-microbial peptides in myeloid cells and Paneth cells, respectively [2,3]. Mutations in the NOD2 have shown to be associated with chronic inflammatory diseases such as Blau syndrome and Crohn's disease [4,5]. The molecular structure of NOD2 contains three distinct domains: two N-terminal caspase-recruitment domains (CARDs), a central nucleotide-binding domain (NBD) and a leucine-rich repeat (LRR) region at the C-terminus. MDP was recently shown to bind to the NBD of NOD2 [6], which likely causes NOD2 homo-dimerization and interaction with the serine/threonine kinase RIP2 (RICK/CARDIAK/RIPK2). RIP2 physically interacts with NOD2 through CARD-CARD homotypic interactions and undergoes K63-linked poly-ubiquitination. Poly-ubiquitination is an integral part of the NOD2 signaling cascade that eventually leads to the activation of the nuclear transcription factor NF-κB through sequentially activating tumor necrosis factor receptor-associated factor (TRAF6, transforming growth factor-β-activated kinase 1 (TAK1)-binding protein 2, TAK1 and IκB kinase (IKK)) [7,8]. In addition to RIP2, multiple proteins were shown to interact with NOD2 and regulate its downstream signaling events. Some of these proteins include phosphatase 2A [9], ATG16L1 [10], ERBIN (a member of the leucine-rich repeat- and PDZ domain-containing family) [11,12], guanine nucleotide exchange factor H1 [13], caspase-12 [14], CARD8 [15], A20 [16], TRIM27 [17], TRAF4 [18], GRIM-19 (a protein with homology to the NADPH dehydrogenase complex) [19], IPAF/CLAN/NLRC4 [20], and NALP1 [21]. The multimeric complexes of NOD2 are expected to function as a signaling platform referred to as the “NODosome”, homologous to other NOD-like receptor complexes, such as the “inflammasome” [22] and the “apoptosome” [23].

Autophagy was originally described as an energy homeostasis process that degrades and recycles damaged molecules and organelles through the formation of double-membrane vesicles. Recent studies have further revealed its essential roles in innate immune responses including entrapment/killing of intracellular microorganisms, antigen presentation, and cytokine production [10,24,25]. NOD2 has been shown to induce autophagy through a RIP2-dependent manner at least in myeloid and epithelial cells [9,24,26]. A portion of NOD2 was also shown to localize at the plasma membrane and recruit the autophagy processing molecule ATG16L1 at the site of bacterial entry, which was a RIP2-independent process [10]. Autophagy can also function as a negative feedback process of inflammatory responses, since it was shown to suppress signaling events induced by Toll-like receptors and NLRP3 [25]. A study also suggested that human peripheral blood mononuclear cells with defects in autophagy resulting from a mutation in ATG16L1 produced more inflammatory cytokines at
the mRNA level when induced by MDP [27], suggesting that autophagy is also involved in NOD2 signaling.

Of the more than 30 different proteins involved in autophagy, p62 (also known as sequestosome-1), is an adaptor protein which sequesters poly-ubiquitinated proteins [28] and Salmonella-containing vacuoles [29] to autophagy through interacting with microtubule-associated protein 1 light chain 3 (LC3). In addition to these catabolic roles, p62 has also been shown to regulate various signaling events. For example, it up-regulates signaling events initiated by receptors activated by tumor necrosis factor (TNF-α, IL-1, nerve growth factor, and RANK-L (receptor activator of NF-κB-ligand) through scaffolding for TRAF6 and atypical protein kinase C with these receptors [30]. In contrast, p62 suppresses Toll-like receptor signaling cascades by inducing MyD88-aggregation and down-regulation of MyD88-TRAF6 complex formation [31]. In light of the multifaceted roles of p62 in autophagy and signal transduction, we examined the role of p62 in NOD2 signaling. This study found that p62 interacted with NOD2 and enhanced its signaling response toward NF-κB activation, and TNF-α and IL-1β production, through stabilizing NOD2 signaling complexes.

Methods

Cells and Cell culture

The human monocytic cell line THP-1 (ATCC® TIB-202) was maintained in complete RPMI 1640 medium containing 8% heat-inactivated fetal bovine serum (FBS, Sigma Aldrich), 1 mM MEM non-essential amino acids solution, 1 mM sodium pyruvate, and antibiotics (mixture of 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate). HEK293T and RAW 264.7 macrophages (kindly provided by Dr. J. Han, The Scripps Research Inst., La Jolla, CA) were cultured in complete DMEM medium containing 8% FBS, and the same reagents as in complete RPMI 1640. Cells were grown at 37°C in a humidified atmosphere containing 5% CO₂.

Plasmids and transfections

pcDNA3-Myc-RIP2 was provided by Dr. Inohara (University of Michigan, Ann Arbor, MI), and plasmids expressing HA-p62 full-length and its mutants (ΔPB1, ΔTRAFF6, ΔAUB) were obtained from Dr. Moscat (Sanford/Burnham Institute, La Jolla, CA). Additional deletion mutants (PB1, TRAF6, UBA) and full-length p62 (NM_003900) were subcloned into pEGFP-C1 vector at EcoRI and BamHI sites for co-immunoprecipitation. The full-length human pcDNA3.1-NOD2 cDNA was obtained from Dr. Nunez (University of Michigan, Ann Arbor, MI). NOD2 full-length and mutants (ΔLRK, CARD, NBD) were further subcloned into pCMV-Myc vector (Clontech) for co-immunoprecipitation, pDsRed-Monomer-C1 or pEGFP (Clontech) for confocal imaging, and the mammalian retroviral expression vector pLNCX (Clontech) for stable transfections into HEK293T cells. TRAF6 cDNA sequences (NM_004620) were cloned and inserted into pCMV-Myc vector for co-immunoprecipitation experiments.

Retrovirus production and cell infection were performed as previously described [32]. Briefly, pLNCX-NOD2 recombinant retroviruses were generated in Phoenix Amphotropic producer cells using the calcium phosphate method of transfection. Viruses were produced at 32°C, and virus-containing medium was collected 24 h post-transfection and filtered through a 0.45 μm filter. HEK293T cells were plated in six-well plates at a density of 5 x 10⁵ cells/well. One round of retroviral infection was performed by replacing medium with 2 mL of pLNCX-NOD2 virus (containing 4 μg of Polybrene per mL), followed by centrifugation of the six-well plates at 2,000 RPM for 40 min at 32°C. On the third day, culture media were replaced with selection media containing 10 μg/mL of Puromycin (Calbiochem).

Co-immunoprecipitation for poly-ubiquitination and protein-protein interactions

Twenty-four hours after transfection, cells were washed twice in PBS, overlaid with RIPA lysis buffer (50 mM TrisCl, 150 mM NaCl, 1% Igepal CA-630 (NP-40), 0.5% Sodium Deoxycholate, and 0.1% SDS) and harvested by scraping. Cells were homogenized by pipetting on ice, centrifuged at 12,000 g for 15 min at 4°C, and supernatants were transferred to new 1.5 mL tubes. Ubiquitin-conjugated lysates or total lysates were combined with either anti-Myc or anti-GFP antibodies (Clontech) for 1, 2, 4 h or overnight at 4°C. Twenty microliters of Protein G Sepharose™ Fast Flow Beads (Sigma-Aldrich) were added to lysates and incubated for an additional 1 h. Immunobeads were recovered by centrifugation at 10,000 g for 30 sec and washed five times by resuspension and centrifugation in the same lysis buffer. The immunocomplex was eluted with 4X SDS loading buffer (125 mM Tris-HCl [pH 6.8], 4% SDS, 50% Glycerol, 0.08% Bromophenol Blue, and 5% β-mercaptoethanol).

Small interfering RNA (siRNA) and small hairpin RNA (shRNA)-mediated silencing

Pre-designed siRNAs directed against human p62 (NM_003900) were purchased from Ambion Life Technologies (ID# s16960; Sense, GGAGACAGGGAGGAAAAGAtt; Anti-sense, UCUUUUUCGCCGUCUGCCAc). On-TARGETplus SMARTpool mouse p62 (NM_011018) siRNAs were obtained from Thermo Scientific (target sequence; ACAGAUGCCAGAUCGGGA, CUGUCACAGGAGGAGAU, GAACAGAUGUCGGGAA, CCAUGGGUUUCUCGGGAUGA). The mouse map1lc3a (mLC3, NM_025735) specific siRNAs were obtained from Ambion, Life technologies. The shRNA-mediated silencing of p62 was performed using calcium phosphate transfection of human p62 specific shRNA constructs (F1336477,-8, -9 and F1336480) and –scramble shRNA (TR30015) (HuSH™, Origene) in THP-1 monocytic cells as described in “Methods” for stable transfection. Transfected THP-1 cells were re-plated with selection media containing 10 μg/mL of puromycin (Calbiochem) to obtain cell colonies stably transfected with p62 shRNA plasmids. For transient gene knock down, HEK293T and RAW 264.7 cells were transfected with scramble siRNA or p62-specific siRNA using LipoFasttrans™ RNAiMAX (Invitrogen). Knock down of specific gene products was confirmed by Western blot analysis.

Confocal microscopy

HEK293T cells were seeded at 0.5 x 10⁶ cells overnight on coverslips and transfection was performed with GFP-p62, in combination with DsRed-NOD2, DsRed-NBD, DsRed-LRR, or GFP-LC3 and DsRed-NOD2 for 24 hours in complete DMEM media at 37°C in a 5% CO₂ incubator.

The cells were fixed with 4.0% paraformaldehyde in PBS (pH 7.4) at RT for 5 min and rinsed twice with PBS at RT for 5 min. Confocal images were obtained using a Zeiss LSM510 META confocal microscope and analyzed with ZEN software.

Immunoblot analysis

Total cell lysates were resolved by SDS-PAGE, transferred to PVDF (PALL Life Sciences) or nyloncellulose (BioRad) membranes, and blocked in 5% skim milk in 1 X TBST (0.05% Tween
NOD2, p62 and p38.

Column and each fraction was analyzed by Western blots for kDa). Fractions (5 ml) were collected after injecting on to the reporter detector (Sirius luminometer, Berthold).

NF-κB luciferase assay

HEK293T cells were transfected with pCMV-Myc vector alone, NOD2 (50 ng of Myc-NOD2), Igk luciferase reporter plasmid (100 ng; kindly provided by Dr. Girardin, University of Toronto, Canada), and β-gal (100 ng). At the same time, gMDP (5 μg/mL, Invitrogen) was added with Lipofectamine™ 2000 (Invitrogen) in the presence or absence of p62-specific siRNA targeting human p62, and then measured 24 h after co-incubation. NF-κB activity was measured with a luciferase reporter detector (Sirius luminometer, Berthold).

ELISA and TNF-α bioassay

TNF-α levels in cell culture supernatants were measured by ELISA or bioassay. ELISA was followed by manufacturer’s instruction (eBioscience). Bioassay for TNF-α concentrations in cell culture supernatants were measured as previously described [34]. Briefly, murine L929 fibroblasts were seeded to each well of a 96-well tissue culture (7 × 10^4 cells/well) for 4 h. Cell culture media were then replaced with media containing cyclohexamide (2,000 kDa), calibrated with standard proteins (Sigma) containing blue Dextran (2,000 kDa) and crystals were solubilized in 50% acetic acid (Caledon) for 2 h. Following fixing, cultures were washed 5 times in PBS-BSA for 5 min each, and grids were blocked in 1% BSA (rabbit; Abcam) and anti-GFP (mouse; Clontech) antibodies at 1:200 and 1:10 dilution, respectively, for 2 h at room temperature. Grids were viewed using a Phillips CM10 transmission electron microscope at 60 kV.

Results and Discussion

p62 enhances NOD2 signaling in HEK293T cells

We first examined if p62 is involved in NOD2 signaling using an NF-κB-driven luciferase reporter system in HEK293T cells. Cells were treated with p62 or scrambled small interference RNAs (si-p62; Fig. 1A, upper panel), and transiently transfected with Myc-tagged NOD2 and Igk luciferase reporter plasmids. As shown previously [35,36], over-expression of NOD2 alone caused about a 3-fold increase in NF-κB reporter activity, which was further increased by the NOD2 ligand N-glycolyl muramyl dipeptide (gMDP; Fig. 1A, lower panel). However, cells knocked down in p62 failed to respond to NOD2 over-expression alone or gMDP treatments. To further examine the role of p62 in NOD2 signaling, we examined its downstream signaling events: poly-ubiquitination of RIP2 [8], TRAF6 [37] and p38 mitogen-activated protein kinase phosphorylation [38]. For RIP2 and TRAF6, cells stably transfected with NOD2 were transiently transfected with HA-tagged ubiquitin and Myc-tagged Ryp2 (Myc-RIP2) or TRAF6, together with scrambled siRNAs or si-p62. Cells transfected with scrambled siRNAs showed apparent ubiquitinations of RIP2 (Fig. 1B) and TRAF6 (1C) or ubiquitinated proteins co-immunoprecipitated with RIP2 and TRAF6 in response to gMDP; however, cells treated with si-p62 showed no or much less such ubiquitated proteins. Similarly, treatments of gMDP in cells stably transfected with NOD2 also gradually induced p38 MAPK tyrosine phosphorylation, which was inhibited in cells knocked down in p62 (Fig. 1D). Collectively, these data suggest that p62 enhances NOD2 signaling cascades.

p62 has been shown to recruit signaling molecules for post-translational modifications. For example, p62 recruits the E3 ligase cullin 3 and induces poly-ubiquitination of caspase-8, resulting in protein aggregation and full activation of caspase-8 [39]. Similarly, p62 recruits another E3 ligase, TRAF6, and sustains the activation of NF-κB induced by RANK-ligand [40] and nerve growth factor [41]. NOD2 activation induces K63-linked poly-ubiquitination of RIP2 at K209, resulting in oligomerization of RIP2 and NF-κB.
activation [8]. To date, the E3 ligase responsible for RIP2 poly-ubiquitination is unknown. Since RIP2 was shown to interact with TRAF1, TRAF5 and TRAF6, but not TRAF2, TRAF3 and TRAF4 [42], it is possible that p62 scaffolds NOD2, RIP2, and TRAF1/5/6 interactions and enhances poly-ubiquitination of RIP2. Therefore, we examined if knocking down p62 affected NOD2 and TRAF6 interaction through performing co-immunoprecipitation experiments. As expected, immunoprecipitation of Myc-TRAF6 also co-precipitated HA-NOD2; however, knocking down p62 had no effects on the level of HA-NOD2 co-precipitation (Supplemental Fig. S1). These results suggest that p62 positively regulates NOD2 signaling, involving events before RIP2 activation.

NOD2 interacts with p62 through the NBD domain of NOD2 and UBA or TRAF6 domain of p62

To examine if p62 interacts with NOD2, co-immunoprecipitation analyses were performed in HEK293T cells transfected with Myc-NOD2 and GFP-conjugated p62 (GFP-p62) plasmids. As shown in Fig. 2A-B, immunoprecipitations using anti-GFP or anti-Myc monoclonal antibodies were able to co-immunoprecipitate with Myc-NOD2 or GFP-p62, respectively. Also, cells treated with gMDP (5 µg/mL) further enhanced the Myc-NOD2 and GFP-p62 interaction (Fig. 2C), suggesting that p62 interacted with NOD2 better when NOD2 was activated.

To further detail the interaction, different deletion mutants of NOD2 and p62 were examined for their interactions. As aforementioned, NOD2 comprises three distinct motifs: CARD, NBD and LRR (Fig. 3A, left panel). Myc-tagged LRR-deleted NOD2 mutant (MycΔLRR) was strongly co-immunoprecipitated...
Figure 2. NOD2 physically interacts with p62. **A–C.** HEK293T cells were transiently transfected with expression vectors encoding GFP-tagged p62 (GFP-p62) and/or Myc-tagged NOD2 (Myc-NOD2). After 24 h, total cell lysates were subjected to immunoprecipitation using anti-GFP (A) or anti-Myc (B) antibodies and the immune complexes were resolved by SDS-PAGE followed by immunoblotting against GFP and HA. **C.** Similar experiments as A–B were performed but with or without N-glycorylated muramylidipeptide (gMDP: 5 μg/mL) treatments for 4 h. Data shown are representative images of 3 independent experiments.
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Figure 3. The NBD of NOD2 interacts with both TRAF6 and UBA domains of p62. **A.** NOD2 (left top panel) and p62 (right top panel) structures, and their mutant constructs are schematically presented. **B.** HEK293T cells were transfected with GFP-p62 and Myc-NBD (left middle panel), GFP-p62 and Myc-CARD (center middle panel) or GFP-p62 and Myc-ΔLRR (LRR region-deleted NOD2) (right middle panel). **C.** Similarly, HEK293T cells were transiently transfected with GFP-TRAF6 domain of p62 and Myc-ΔLRR (left bottom panel), GFP-UBA domain of p62 and Myc-ΔLRR (middle bottom panel), and GFP-PB1 domain of p62 and Myc-ΔLRR (right bottom panel); co-immunoprecipitation assays were performed as described in legend to Fig. 2. Data shown are representative images of 3 independent experiments.
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with GFP-tagged p62. However, Myc-tagged proteins containing only CARD (Myc-CARD) or LRR (Myc-LRR) failed to co-immunoprecipitate with GFP-p62 (Fig. 3B). These results suggest that NOD2 interacts with p62 through its NBD domain.

p62 contains at least four distinct motifs (Fig. 3A, right panel): Phox and Bem 1p (PB1), zinc finger (ZZ), TRAF6-binding (TRAF6) and ubiquitin-associated (UBA) domains [43]. The N-terminal PB1 domain is known to accommodate p62 homodimerization as well as hetero-dimerization with various signaling molecules including PKCζ/ι/λ, MEKK3, MEK5 and ERK1. ZZ and TRAF6 domains were shown to be involved in the interaction with RIP1 and TRAF6, respectively. The C-terminal UBA domain preferentially binds to K63-linked poly-ubiquitin chains [44] and the LC3-interacting region (LIR), located between UBA and TRAF6 domains, interacts with LC3. Therefore, p62 is expected to function as an autophagy cargo molecule that targets aggregated proteins, cellular organelles and microbes for degradation [45]. We examined how p62 interacted with NOD2 using a similar co-immunoprecipitation approach with different p62 mutants and LRR-deleted NOD2 (ΔLRR) to maximize the interaction. Interestingly, both GFP-TRAF6 and GFP-UBA, but not GFP-PB1, domains were co-immunoprecipitated with Myc-ΔLRR (Fig. 3C). Consistent with these results, ΔLRR was also co-immunoprecipitated with TRAF6 binding domain-deleted (ΔTRAF6) or UBA domain-deleted mutants of p62 (Supplemental Fig. S2). Collectively, these results suggest that the NBD domain of NOD2 interacted with either the TRAF6-binding or UBA domain of p62. We found that NOD2 undergoes both K48- and K63-mediated polyubiquitinations (data not shown), which likely contributes interaction between UBA domain of p62 and NOD2. Further detailed experiments are required to elucidate whether p62 binding to NOD2 through UBA domain requires ubiquitination of NOD2, and how p62 interacting through TRAF6-binding domain and UBA domain affect NOD2 signaling.

NOD2 is co-localized with p62 in the cytoplasm as a granulated form

Previous studies demonstrated that NOD2 could be localized in both the plasma membrane and cytosol as speckles [10,13,46]. Indeed, DsRed-NOD2 was localized in both intracellular compartments in punctate form and the plasma membrane in HEK293T cells (Fig. 4A, left panel). Furthermore, cytosolic DsRed-NOD2 positive speckles, but not the plasma membrane associated, were co-localized with p62 (right panel). In line with co-immunoprecipitation results (Fig. 3B), DsRed-conjugated with the NBD of NOD2 also prominently co-localized with GFP-p62 (Fig. 4B, upper lane); whereas, no such co-localization was detected in NOD2 only containing LRR motif (lower lane). To further examine co-localization of these molecules, cells over-expressing both GFP-p62 and HA-tagged full length NOD2 were viewed through EM after immunogold labeling against GFP and HA. As shown in Fig. 4C, aggregated patterns of both GFP-NOD2 (10 nm gold particles) and HA-p62 (18 nm gold particles) were detected in electron-dense areas. However, it could not be determined whether the electron-dense NOD2 and p62-positive

Figure 4. p62 co-localizes with NOD2 through the NBD domain of NOD2. A. HEK293T cells were transfected for 24 h with scramble siRNA (left top panel), si-p62 (right top panel), and GFP-NOD2. GFP-NOD2 was visualized using confocal microscopy as described in “Methods”. B. Similarly, DsRed-NBD domain, LRR region or full-length NOD2 and GFP-p62 expression vectors were transfected in HEK293T cells and co-localization of these proteins was examined using confocal microscopy. C. Immunogold staining of co-localized pCMV-HA-p62 (18 nm colloidal gold) and GFP-NOD2 (10 nm colloidal gold) in HEK293T cells. Cells on grids were viewed using a transmission electron microscope. Scale bars: 500 nm (left bottom), 100 nm (middle, right bottom). D. HEK293T cells were transfected with DsRed-NOD2 and GFP-LC3 plasmids. Cells were observed by confocal microscopy and images were acquired using ZEN software.
areas were autophagosomes, because our immunogold EM staining could not clearly resolve membrane structures. Since p62 associates with autophagosomes through interacting phosphatidyethanolamine conjugated microtubule-associated protein 1 light-chain 3 (LC3) during autophagy [47,48,49], we examined whether LC3 co-localized with the cytosolic NOD2-positive granules. LC3-GFP was detected throughout the cytoplasm and as granular forms close to the nucleus. However, DsRed-NOD2-positive granules were localized in distinct locations from those of LC3-GFP (Fig. 4D). These results suggest that cytosolic NOD2-p62 aggregates were not autophagosomes.

Previously, p62 was shown to aggregate with several signaling molecules to enhance their signaling effects. For example, p62 aggregates with poly-ubiquitinated caspase-8 that leads to full activation and processing of the enzyme [39]. p62 is also involved in the formation of large structures known as aggresome-like induced structures (ALIS) [50]. Unlike aggresomes, which are rapidly degraded through a proteasomal route, ALIS are devoid of proteasomes and transient in nature, and recruit ubiquitination enzymes including the ubiquitin-activating enzyme E1, the ubiquitin-conjugating enzyme E2 and the ubiquitin ligase E3 [51,52]. ALIS were shown to be induced by Toll-like receptors or various stresses [53] and ubiquitinated proteins associated with ALIS were shown to have a much longer half-life than those present in the cytosol [52]. Several features such as the granular aggregation of NOD2 with p62 in non-autophagic vacuoles may point that the electron-dense organelles are ALIS or ALIS-related structures. However, further studies are required to determine if NOD2 is indeed localized in bona fide ALIS.

**p62 stabilizes NOD2 oligomerization**

Considering the multiple roles of p62 in protein modifications (through recruiting E1/E2/E3 proteins or other signaling molecules) and stabilization of proteins through forming ALIS, p62 could have enhanced NOD2 signaling through recruiting TRAF6 to nodosomes or stabilizing NOD2 oligomers. However, knocking down p62 had little effects on recruiting TRAF6 to NOD2 (Supplemental Fig. S1). Thus, we examined whether p62 was involved in stabilization of NOD2 at protein levels. To this end,
HEK293T cells stably expressing NOD2 were knocked down in p62 by si-p62 and NOD2 protein levels were examined in the presence of the broad translation inhibitor cyclohexamide (CHX; 100 μg/mL) and gMDP (5 μg/mL). In cells treated with si-Scrambled, both NOD2 and p62, but not p38, gradually degraded over 12 hours (Fig. 5A). However, in cells knocked down in p62, NOD2 degradation was significantly faster. These results are in line with a recent study shown that NOD2 undergoes the 26S proteasome-mediated degradation, which negatively regulates its signaling [17]. Thus, it is possible that p62 leads p62-NOD2 complexes to avoid their degradation by proteasomes. Indeed, the 26S proteasome inhibitor MG132 prevented fast degradation of NOD2 in gMDP-treated p62 knock-down cells (Supplemental Fig. S3). Next, we examined whether p62 also enhanced oligomerization of NOD2. Cells were transiently transfected with both HA- and Myc-conjugated NOD2 expression vectors together with si-Scramble or si-p62, and oligomerization of NOD2 was examined through co-immunoprecipitation. As shown in Fig. 5B, HA-NOD2 was co-immunoprecipitated by anti-Myc antibody, which was diminished by si-p62, suggesting that dimerization or multimerization of NOD2 is formed through a p62-dependent manner. In addition, size-exclusion gel filtration chromatography was used to examine the degree of NOD2 complex formation. Myc-NOD2 was eluted between fraction number 5 and 7 (Fig. 5C, left panels), suggesting that Myc-NOD2 complex was less than 2000 kDa but higher than 200 kDa in size (Supplemental Fig. S4-A). In gMDP-treated cells, Myc-NOD2 was eluted in earlier fractions (number 3-7), suggesting a higher degree of complex formation with about 2000 kDa in size. However, the gMDP-induced Myc-NOD2 complex was not detected in cells treated with si-p62 (Fig. 5C, right panels). Elution of p38 was used as a control and showed no differences in elution patterns between g-MDP or si-p62 treated or non-treated cells. p62 was detected in fractions between 6 and 8, which partially overlapped with those of Myc-NOD2 (Supplemental Fig. S4-B). In the presence of gMDP, p62 was also formed a higher degree of complex which was eluted in fractions 4-9,
which fully overlapped with those of Myc-NOD2. Collectively, these results suggest that, in the presence of gMDP, p62 forms a higher degree of complex with NOD2 that may prevent the 26S proteasomal degradation of NOD2.

p62 is required for cytokine production mediated by NOD2 in macrophages

To confirm the role of p62 in physiologically relevant cell types, we used two macrophage cell lines of murine and human origin: RAW 264.7 (mouse) or THP-1 (human). RAW 264.7 cells express low levels of NOD2 which is rapidly induced by LPS [54]. Consistently, gMDP alone did not induce expression of pro-IL-1β in si-Scramble RNA-transfected RAW 264.7 cells (Fig. 6A). LPS induced pro-IL-1β expression at low levels. In cells pretreated with LPS for 4 h, gMDP significantly enhanced pro-IL-1β expression, as similarly demonstrated before [36,53]. However, in RAW 264.7 cells transfected with si-p62, no such enhancing effect was detected. Knocking down p62 had no effects on LPS-induced pro-IL-1β expression. In addition, production of TNF-α in response to gMDP was measured in LPS-primed RAW 264.7 cells with or without si-p62. LPS alone induced TNF-α production which was further increased by gMDP (Fig. 6B). However, si-p62 significantly prevented gMDP-induced TNF-α in LPS-primed cells. To further examine the role of p62 in human macrophages, THP-1 cells were knocked down in p62, no such enhancing effect was detected. Knocking down p62 had no effects on LPS-induced pro-IL-1β expression. In addition, production of TNF-α in response to gMDP was measured in LPS-primed RAW 264.7 cells with or without si-p62. LPS alone induced TNF-α production which was further increased by gMDP (Fig. 6B). However, si-p62 significantly prevented gMDP-induced TNF-α in LPS-primed cells. To further examine the role of p62 in human macrophages, THP-1 cells were knocked down in p62 using small hairpin RNAs (shRNA-p62). Three THP-1 cell clones stably knocked down in p62, pooled clones stably transfected with scrambled sh-RNAs (sh-Scramble), and non-infected wild-type cells were treated with gMDP (Fig. 6B). THP-1 cells responded to gMDP without priming with LPS and induced high levels of TNF-α in wild-type and sh-Scramble transfected clones. However, all three clones knocked down in p62 failed to respond to gMDP. Collectively, these results suggest that p62 was indeed required for optimal IL-1β and TNF production in response to NOD2 in mouse and human macrophages, respectively.

p62 traffics ubiquitinated molecules to autophagosomes through interacting with LC3 [48]. Therefore, we examined the involvement of LC3 in p62-mediated regulation of NOD2. RAW 264.7 cells were knocked down in LC3 using si-RNAs (si-LC3) and pro-IL-1β expression in response to gMDP, LPS and LPS+gMDP were examined. However, knocking down LC3 had no effects on IL-1β production induced by LPS or LPS+gMDP (Supplemental Fig. S5). These results, together with data shown in Fig. 4D suggest that the enhancing effects of p62 in NOD2 stabilization and signaling are not mediated through autophagy.

p62 was first found to be required for NF-κB activation induced by IL-1 [56] or NGF [41,57] and its sustained activation in RANK (Receptor Activator of Nuclear Factor κ B)-activated osteoclasts [40]. Consistently, p62-deficient mice have defects in sustaining activation of NF-κB in T cells [58]. It was shown that p62 interacts with TRAF6, protein kinase C, MAP kinase kinases and PDK1, which enhances NF-κB and Akt activation [59,60]. However, p62 was also found to be involved in both positive and negative regulation of NF-κB by interacting with a deubiquitinating enzyme, CYLD [61,62]. In macrophages, p62 is involved in both TLR- and NLRP3-mediated signaling events. It plays a suppressive role in interferon γ and CpG DNA (TLR9 ligandre) induced cytokine production [63]; whereas, only a partial effect has been detected on TLR4-induced signaling events, namely, activation of the p38 and c-Jun N-terminal kinase but not NF-κB, and production of IL-6 but not TNF-α [31]. p62 also plays a critical role in NLRP3 inflammasome activation induced by *Mycobacterium abscessus* [64] but at the same time limits NLRP3 inflammasome activation through targeting inflammasomes to autophagy-mediated destruction as a feedback mechanism [65]. Therefore, p62 is involved in multiple signaling cascades with different roles. Here, we demonstrated that p62 plays a positive role in NOD2-mediated signaling cascades probably through forming aggregation of NOD2/p62 oligomers that prevents their degradation. Further studies are required to delineate the mechanism of p62 in preventing degradation of NOD2 and its physiological significance in NOD2 innate immune function.

Supporting Information

**Figure S1** p62 has no effects on TRAF6 and NOD2 interaction. HEK293T cells were transiently transfected with pCMV-HA-NOD2 and pCMV-Myc-TRAF6 with or without si-p62 using a PolyJetTM (SignaGen Laboratories). Myc-TRAF6 was immunoprecipitated with anti-Myc antibody (the second lane). HA-NOD2 was co-precipitated with Myc-TRAF6 regardless of the presence of small interference RNAs against p62 (si-p62). Endogenous p62 was knocked down by si-p62 (bottom lane). (TIF)

**Figure S2** NOD2 interacts with TRAF6 or UBA domain deletion mutants. A. The schematic structure of p62 and mutant constructs are shown. B. HEK293T cells were transiently transfected with expression vectors encoding Myc-tagged LRR region deleted NOD2 (Myc-DLRR) and/or different mutants of deletion mutant of p62. Twenty four h post-transfection, total cell lysates were subjected to immunoprecipitation using anti-GFP antibodies and the immune complexes were resolved by SDS-PAGE, followed by immunoblotting against anti-Myc antibodies. Both TRAF6-interacting domain or UBA domain deletion mutants co-immunoprecipitated with NOD2. Data shown are representative images of 3 independent experiments. (TIF)

**Figure S3** Degradation of NOD2 in p62 knocked down cells was prevented by the 26S proteasome inhibitor MG132. HEK293T cells stably transfected with pLNCX-NOD2 were treated with si-p62 using a PolyJetTM (SignaGen Laboratories) for 24 h. Cells were then treated with the translation inhibition cyclohexamide (CHX, 100 μg/ml) and gMDP (5 μg/ml) with or without the 26S proteasome inhibitor MG132 (25 μM) for the time indicated. Stability of NOD2 was analyzed by Western blots using anti-NOD2 (4A11). Western blots for p38 were used as loading controls. (TIF)

**Figure S4** Size exclusion gel filtration analysis and formation of a higher form p62 complex formation by gMDP. A. A mixture of 2000 kDa (Blue dextran; Peak I), 200 kDa (β-amylose; Peak II) and 66 kDa (Bovine serum albumin; Peak III) proteins were eluated through Superdex™ 200 gel filtration column. Elution of standard proteins were detected by UV light. B. HEK293T cells were transiently transfected with pCMV-Myc-NOD2 using a PolyJetTM (SignaGen Laboratories) for 24 h. Cells were then treated with gMDP (5 μg/ml) for 4 h and cell extracts were loaded onto the gel filtration column. Elution of p62 was analyzed using Western blots against p62 on each fraction (left panel) and intensities of immuno-reacted bands were plotted (right panel, n = 2). In non-treated cells, p62 complexes were eluted between 2000 kDa-200 kDa fractions; whereas, in gMDP-treated cells, p62 was eluted in ≥ 2000 kDa fractions. These results indicate that gMDP caused a higher degree of p62 complex formation. (TIF)
Figure S5  Knocking down LC3 has no effects on p62-mediated NOD2 signaling regulation. RAW264.7 cells were treated with scrambled- or map1lc3a (LC3)-specific siRNA for 24 hr. Cells were treated with LPS (50 ng/mL) for 4 hr and were rinsed twice with fresh media, followed by a subsequent treatment with gMDP (5 μg/mL) for an additional 4 hr. Total cell lysates were resolved by 14% SDS-PAGE, transferred onto PVDF and blotted with anti-LC3 and anti-IL-1β antibodies. (TIFF)
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