The E3 Ubiquitin Ligase Neuregulin Receptor Degradation Protein 1 (Nrdp1) Promotes M2 Macrophage Polarization by Ubiquitinating and Activating Transcription Factor CCAAT/Enhancer-binding Protein β (C/EBPβ)*

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Background: Nrdp1 is involved in TLR-triggered classical (M1) macrophage activation.

Results: Nrdp1 up-regulates the characteristic markers of alternatively (M2) activated macrophages and ubiquitinates C/EBPβ to transactivate the Arg1 gene in IL-4-polarized M2 macrophages.

Conclusion: Nrdp1 promotes Arg1 expression and M2 macrophage polarization by ubiquitinating and activating C/EBPβ.

Significance: E3 ubiquitin ligase Nrdp1 is involved in M2 macrophage polarization via ubiquitination and activation of C/EBPβ.

Macrophage polarization, including classical (M1) activation and alternative (M2) activation, plays important roles in host immune response and pathogenesis of diseases. Ubiquitination has been shown to be involved in the differentiation of immune cells and in the regulation of immune responses. However, the role of ubiquitination during M1 versus M2 polarization is poorly explored. Here, we showed that arginase 1 (Arg1), a well recognized marker of M2 macrophages, is highly up-regulated in peritoneal macrophages derived from E3 ubiquitin ligase Nrdp1 transgenic (Nrdp1-TG) mice. Furthermore, other M2 feature markers such as MR, Ym1, and Fizz1, as well as Th2 cytokine IL-10, are also up-regulated in Nrdp1-TG macrophages after IL-4 stimulation. Knockdown of Nrdp1 expression effectively inhibits IL-4-induced expression of M2-related genes in macrophages. Moreover, Nrdp1 inhibits LPS-induced production of inducible NO synthase (iNOS) and pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in macrophages. Immunoprecipitation assays show that Nrdp1 interacts with and ubiquitinates transcriptional factor C/EBPβ via Lys-63-linked ubiquitination. Nrdp1 enhances C/EBPβ-triggered transcriptional activation of the Arg1 reporter gene in the presence of IL-4 stimulation. Thus, we demonstrate that Nrdp1-mediated ubiquitination and activation of C/EBPβ contributes to a ubiquitin-dependent nonproteolytic pathway that up-regulates Arg1 expression and promotes M2 macrophage polarization.

Macrophages are critical innate immune cells in the immune response. In response to different environmental stimuli, macrophages undergo different activation pathways and can be divided into two categories, classically activated macrophage (CAM) and alternatively activated macrophage (AAM) (1). LPS and IFN-γ induce classical (M1) activation of macrophages and result in the CAM, up-regulating inducible nitric-oxide synthase (iNOS) and nitric oxide (NO) production and secreting high levels of IL-12 and pro-inflammatory cytokines, such as TNF-α, IL-1, and IL-6. CAM has highly phagocytic and microbicidal activities, but it also mediates in inflammatory diseases such as rheumatoid arthritis and atherosclerosis (2). Th2-type cytokines (such as IL-4, IL-13, and IL-10) induce alternative (M2) activation of macrophages and result in the AAM, up-regulating arginase 1 (Arg1) production (3). AAM also expresses other unique M2 markers (mannose receptor, MR; a chitinase-like molecule, Ym1; found in inflammatory zone-1, Fizz1) and secretes anti-inflammatory cytokine IL-10. CAM is essential for the immune defense against parasites and associates with collagen synthesis and tissue remodeling (2). However, AAM is also involved in the progress of allergic diseases, inflammatory diseases, and tumors (4, 5), but the underlying molecular pathways for M2 macrophage polarization are far from elucidated (6).

Ubiquitination is a common post-translational modification of proteins and controls many cell processes, including receptor regulation, cell cycle, and apoptosis. Recently, ubiquitination has been demonstrated in many aspects of the immunological functions, ranging from innate immune responses, antigen presentation, T cell activation, and tolerance to...
immunedefense (7, 8). The ubiquitin system includes ubiquitin and enzymes like E1-activating enzyme, E2-conjugating enzyme, and E3 ubiquitin ligase (9). E3 ubiquitin ligase determinessubstrate specificity and facilitates transfer of the ubiquitin to lysine on substrate proteins, many of which have beenidentified in immune responses (10, 11). It has been known thatpolyubiquitin chains linked through lysine 48 of ubiquitin (Lys-48-linked polyubiquitination) always marks substrates forproteasome-dependent degradation (8). For instance, Lys-48-linkedpolyubiquitination of IkB-α is essential for its degradation andthefollowing activation of NF-κB (12). Besides, Lys-63-linked polyubiquitination mediates someproteasomal independent regulatory functions. For example, oneof our studies showed that CHIP (carboxyl terminus of constitutive heat shock cognate 70-interacting protein) inducesLys-63-linked polyubiquitination of Src and protein kinase Cβ andleads to activation of IL-1 receptor-associated kinase 1 andTANK-binding kinase 1 in Toll-like receptor signaling (13).

Nrdp1 (neuregulin receptor degradation protein-1), an E3ubiquitin ligase, has previously been demonstrated to associate withubiquitination and degradation of ErbB3 (14), which mediates theprogression of breast tumors (15). Nrdp1 also associates with theubiquitination and degradation of BRUCE and initiates cell apoptosis (16). Our results demonstrated thatNrdp1 inhibits LPS-activated secretion of pro-inflammatorycytokines by inducing Lys-48-linked polyubiquitination anddegradation of MyD88 in macrophages (17), indicating thatNrdp1 inhibits LPS-induced M1 activation of macrophages. However, whether E3 ubiquitin ligase Nrdp1 mediates IL-4induced M2 activation of macrophages is still unknown.

Here, we have demonstrated that the expression of Arg1, acharacteristic feature of M2 polarized macrophages, is highlyup-regulated in the macrophages from Nrdp1 transgenic(Nrdp1-TG) mice, and the Arg1 expression is further increasedin macrophages by stimulation with a Th2 cytokine IL-4. Furtherstudies show that other distinct M2 markers like Fizz1, Ym1, and MR are also greatly up-regulated in Nrdp1-TG peritoneal macrophages after IL-4 stimulation. Moreover, Nrdp1 interacts with transcriptional factor C/EBPβ and mediates thepolyubiquitination and activation of C/EBPβ, thus providingthe mechanistic explanation for how Nrdp1 promotes M2 macrophage polarization by increasing Arg1 gene expression.

**EXPERIMENT PROCEDURES**

*Mice, Antibodies, and Reagents—*C57BL/6 mice (7–8 weeks old) were from SIPPR-BK Experimental Animals (Shanghai, China). All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals of China, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai, China. Nrdp1-TG mice were generated as described previously (17). The LPS, Cpg, poly(I:C), and Griess reagent were from Sigma. Recombinant murine IL-4 was from PeproTech. Anti-HA (ab9110) and anti-ubiquitin (Ubi-1, ab7254) were from Abcam. Anti-Arg1 (M-20), anti-C/EBPβ (C-19), anti-STAT6 (AS6 – 10.1.1), and anti-Nrdp1 (D-17) werefrom Santa Cruz Biotechnology; anti-β-actin (AC-15) was fromSigma, and anti-iNOS was from Cell Signaling Technology. Thecorresponding horseradish peroxidase-conjugated secondaryantibodies (TrueBlot) were from eBioscience.

**Cell Culture—**Thioglycollate-elicited peritoneal macrophages from C57BL/6 mice were prepared as described previously and cultured in endotoxin-free RPMI 1640 medium with 10% FCS (17). Murine macrophage RAW264.7 cell line and embryonic fibroblast NIH-3T3 cell line were obtained from American Type Culture Collection and cultured in DMEM with 10% FCS.

**Two-dimensional DIGE—**Total protein samples of mouse peritoneal macrophages were prepared with lysis buffer containing 7 M urea, 2 M thiourea, 50 mM Tris, and 2% CHAPS, purified by the two-dimensional clean-up kit (GE Healthcare). Protein concentration was determined using the Bradford assay (Bio-Rad). For labeling proteins, 50 μg of each protein sample was incubated with 400 pmol of CyDye (Cy3 and Cy5), according to the manufacturer’s guidelines. A pool containing equal amounts of all samples was also prepared and labeled with Cy2 to be used as an internal standard. First dimension isoelectric focusing was performed in an IPGphor™ III instrument (GE Healthcare). After equilibration, the strips were transferred onto 12% homogeneous polyacrylamide gels (2.6% C) using anEttan-DALT™ six system (GE Healthcare). Two-dimensional DIGE gel images were analyzed using DeCyder™ (GE Healthcare) software to determine protein spots significantly different in abundance. Samples were re-electrophoresed on two-dimensionalgels loaded with 500 μg of unlabeled protein, and theindicated protein spots were excised and send to ShanghaiApplied Protein Technology (Shanghai, China) for matrix-assistedlaser desorption ionization/time of flight mass spectrometry (MALDI/TOF MS) analysis.

**Quantitative PCR—**Total cellular RNA was extracted using TRIzol reagent (Invitrogen). An amount of 1 μg of RNA was used in a reverse transcription reaction using the First Strand cDNA synthesis kit (Toyobo), and then the cDNA was diluted into 80 μl as the template of quantitative PCR (Q-PCR) with the following primers: Nrdp1 F, 5’-CCT GCC ATG TTA TGT TAC-3’; and Nrdp1 R, 5’-CAT GGC ATA TGA CTT CTC-3’; iNOS F, 5’-ACA TCG ACC CGT CCA CAG TAT-3’; iNOS R, 5’-CAG AGG GGT AGG CTT GTC TC-3; Arg1 F, 5’-CTC AAA GCC AAA GTC CTT AGAG-3; Arg1 R, 5’-ACA TCG ACC CGT CCA CAG TAT-3’; Ym1 F, 5’-CAG GTC TGG CAA TTC TTC TGAA-3; and Ym1 R, 5’-CTC CAA GCC AAA GTC CTT AGAG-3’. Q-PCR was performed on a Light Cycler (Roche Applied Science), according to the manufacturer’s protocol.

**Immunoblot Analysis—**A total of 1 × 10⁶ cells was seeded in 6-well plates and cultured overnight. After stimulation, the cells were washed twice with cold PBS and lysed with M-PER protein extraction reagent (Pierce) supplemented with protease inhibitor “mixture” (Calbiochem). Equal amounts of protein were subjected to SDS-PAGE and transferred onto nitrocellulose
transiently transfected into NIH-3T3 cells as indicated using INTERFERin (PolyPlus) (R&D Systems) according to the manufacturer’s instruction.

**RNA Interference Assay**—For transient transfection, small interfering RNA (siRNA) was synthesized by Shanghai GenePharma (Shanghai, China) as follows: siRNA-Nrdp1, 5′-UGC GGA ACA UGU UGU CAA-3′; siRNA-C/EBPβ, 5′-CCC UGC GGA ACU UCA A-3′; and a scrambled oligonucleotide served as control (siRNA-Ctrl), 5′-AAT CAG TCA CGT TAA TGG TCG-3′. siRNA-Nrdp1 duplex was transfected into peritoneal macrophages, and siRNA-C/EBPβ was transfected into NIH-3T3 cells as indicated using INTERFERin (PolyPlus) according to the manufacturer’s protocol.

**Plasmid Construction and Transfection**—Recombinant pcDNA3.1 vectors encoding murine Nrdp1 (BC049078), murine Cebpb (NM_009883), and murine Stat6 (NM_009284) were constructed by PCR-based amplification of cDNA from RAW264.7 cells with the following primers, respectively: Nrdp1 F, 5′-CGG GAT CCT GTC TTG-3′; C/EBPβ F, 5′-GCC CCA CGG CAT ATC CGC TCT ACT-3′, and C/EBPβ R, 5′-CTC TTG ATC GTC ACG CAC GAT TT-3′; STAT6 F, 5′-CGG AAT TCA CAC TGC TCT AG-3′, and STAT6 R, 5′-CGG GAT CCT TCA GCT GCT CAC AG-3′. pcDNA3.1-HA-ubiquitin (BC0100341) plasmid was constructed as described (18). Lys-63-mutant ubiquitin vector (pcDNA3.1-HA-K63-ubiquitin) (Lys-63 to Arg-63) was obtained by PCR-based mutation and amplification of pcDNA3.1-HA-ubiquitin vector. Arg1 luciferase reporter plasmid was constructed as described (19). The pRL-TK-luciferase plasmid was from Promega. Plas-
mids were transiently transfected into NIH-3T3 cells with jet-PEI (Polyplus) reagents according to the manufacturer’s instructions.

**Immunoprecipitation**—Cells were lysed with radiolabeled immunoprecipitation assay buffer (Cell Signaling Technology) supplemented with protease inhibitor mixture. Protein concentrations of the extracts were measured by BCA assay (Pierce). The lysates of NIH-3T3 cells or macrophages were immunoprecipitated for 3 h with constant mixing at 4 °C with 2 µg/ml anti-Nrdp1 or anti-C/EBPβ antibody and protein A-agarose beads (Sigma) as indicated. After extensive washing with lysis buffer, the immunocomplexes were boiled in 2× loading buffer (Sango, Shanghai) and subjected to SDS-PAGE, followed by immunoblotting (20).

**Assay of Luciferase Reporter Gene Expression**—NIH-3T3 cells were co-transfected with the mixture of Arg1 luciferase reporter plasmid, pRL-TK-luciferase plasmid, and the other indicated plasmids. Total amounts of plasmid DNA were equalized with empty control vector (Mock). After 24 h, the cells were treated with IL-4 (10 ng/ml). Dual-Luciferase Reporter assay system (Promega) was used for measuring luciferase activity. By dividing Firefly luciferase activity with that of Renilla luciferase, the data were normalized for transfection efficiency.

**Statistical Analysis**—All experiments were independently performed three times. The statistical significance was determined with Student’s t test. p values of less than 0.05 were considered to be statistically significant.

**RESULTS**

**Nrdp1 Up-regulates Arg1 Expression in Macrophages**—Our previous study shows that Nrdp1 mediates Toll-like receptor-triggered activation of macrophages (17). So we used two-dimensional DIGE plus MS analysis to study the possible proteins regulated by Nrdp1 in macrophages of Nrdp1-TG mice, and we found that Arg1 was significantly up-regulated in Nrdp1-TG peritoneal macrophages (Fig. 1A). Then we used Q-PCR and Western blot to validate the results we received from two-dimensional DIGE, and we showed that Arg1 mRNA and protein levels were indeed remarkably increased in peritoneal macrophages from Nrdp1-TG mice compared with those from wild-type (WT) mice (Fig. 1, B and C). Therefore, Nrdp1 up-regulates Arg1 expression in macrophages.

**Nrdp1 Increases Arg1, Fizz1, Ym1, MR Expression, and IL-10 Secretion in IL-4-stimulated Macrophages**—Arg1 is a well recognized marker of M2 macrophage polarization (21). A variety of signals can regulate Arg1 expression in macrophages. Among them, IL-4 is by far the most potent cytokine that polarizes macrophages to AAM (22). As the Arg1 level was significantly higher in Nrdp1-TG macrophages than that in WT
macrophages, we further explored whether Nrdp1 influences the M2 macrophage polarization triggered by IL-4. Our results showed that, compared with those in WT macrophages, Arg1 mRNA and protein levels in Nrdp1-TG macrophages were markedly up-regulated after IL-4 stimulation (Fig. 2A).

Nrdp1 enhances IL-4-stimulated Arg1 production in macrophages, indicating Nrdp1 favors a M2 macrophage polarization. It is well known that some other proteins, such as Fizz1, Ym1, MR, as well as cytokine IL-10, are also distinct markers of M2-polarized macrophages (3). Thus, we explored the effect of Nrdp1 on these characteristic M2 marker expressions in macrophages after IL-4 stimulation. As shown in Fig. 2B, compared with WT macrophages, Nrdp1-TG macrophages expressed significantly higher levels of Ym1, Fizz1, and MR mRNA after IL-4 stimulation. Consistently, Nrdp1-TG macrophages also produced much more IL-10 than WT macrophages (Fig. 2C). Altogether, Nrdp1 promotes IL-4-stimulated M2 macrophage polarization by increasing Arg1, Fizz1, Ym1, MR expression, as well as Th2 cytokine IL-10 production.

Silencing of Nrdp1 Inhibits IL-4-induced Expression of M2-related Markers—To further characterize the function of Nrdp1 during IL-4-triggered M2 polarization, we synthesized siRNA specifically against Nrdp1 (siRNA-Nrdp1) and examined the effect of silencing of Nrdp1 expression on M2 macrophage polarization. In murine peritoneal macrophages, siRNA-Nrdp1 transfection effectively decreased the mRNA and protein levels of Nrdp1 (Fig. 3A). We further showed that siRNA-Nrdp1 transfection effectively blocked the up-regulation of Arg1 triggered by IL-4 in peritoneal macrophages, compared with siRNA-control (si-Ctrl) transfection (Fig. 3B). In addition, siRNA-Nrdp1 also decreased the mRNA level of M2-related markers mentioned above after IL-4 stimulation, including Arg1, Ym1, Fizz1, and MR (Fig. 3C). All these results show that silencing of Nrdp1 can hamper IL-4-stimulated M2 macrophage polarization. Nrdp1 Inhibits LPS-stimulated iNOS Expression and Pro-inflammatory Cytokine Secretion in Macrophages—The above results show that Nrdp1 enhances IL-4-stimulated Arg1 expression in macrophages and favors an M2 macrophage polarization. We then explored whether Nrdp1 regulates M1 polarization induced by LPS, and we found that LPS treatment significantly induced iNOS expression in WT peritoneal macrophages. However, LPS-induced up-regulation of iNOS...
was markedly reduced in Nrdp1-TG macrophages (Fig. 4A). In M1 polarized macrophages, iNOS catabolizes arginine to produce nitric oxide (NO), which is important in host defense to pathogens (2). We then examined the production of NO in Nrdp1-TG macrophages after LPS stimulation and found that LPS-stimulated NO secretion was also markedly inhibited in Nrdp1-TG macrophages (Fig. 4B).

Differential cytokine production is a key feature of macrophage polarization. The M1 polarized macrophages secrete a high level of pro-inflammatory cytokines, including IL-6, TNF-α, and IL-1β, whereas the production of anti-inflammatory cytokine IL-10 is remarkably suppressed (1). We further measured the cytokine levels in Nrdp1-TG macrophages stimulated by Toll-like receptor agonists (Fig. 4C). The results showed that LPS increased IL-12, IL-6, TNF-α, and IL-1β production in WT macrophages. However, cytokines induced by LPS were markedly abrogated in Nrdp1-TG macrophages, with TNF-α decreased more than 70% and IL-12 decreased about 60%. In contrast to the inhibitory effects on pro-inflammatory cytokine production, the production of anti-inflammatory cytokine, IL-10, was greatly increased over 50% in Nrdp1-TG macrophages after LPS stimulation (Fig. 4D). Altogether, Nrdp1 inhibits iNOS expression, NO production, down-regulates pro-inflammatory cytokine secretion, but up-regulates anti-inflammatory cytokine production in LPS-stimulated macrophages, thus hampering M1 macrophage polarization.

**Nrdp1 Interacts with Transcription Factor C/EBPβ**—We went further to investigate the underlying molecular mechanism for how Nrdp1 up-regulates Arg1 mRNA expression and polarizes M2 macrophage generation. Previous studies show that signal transducer and activator of transcription 6 (STAT6) and C/EBPβ are essential transcription factors for IL-4-stimulated production of Arg1 (19). We then investigated whether
Nrdp1 enhanced Arg1 gene transcription by regulating the activity of STAT6 and C/EBPβ.

NIH-3T3 cells were co-transfected with pcDNA3.1-STAT6 or pcDNA3.1-C/EBPβ along with pcDNA3.1-Nrdp1, and the interactions between STAT6, C/EBPβ, and Nrdp1 were examined by immunoprecipitation assay. In this overexpression system, we detected C/EBPβ in the products pulled down by anti-Nrdp1 antibody from NIH-3T3 cell lysates, but we did not detect STAT6 in the immunoprecipitant (Fig. 5A). This finding indicates the association of Nrdp1 with C/EBPβ. Moreover, this interaction was further enhanced by IL-4 stimulation. We further showed, in reversed immunoprecipitation assay, Nrdp1 was also detected in the products pulled down by anti-C/EBPβ antibody from NIH-3T3 cell lysates, confirming that Nrdp1 interacts with C/EBPβ in NIH-3T3 cells (Fig. 5B).

Furthermore, we examined whether this interaction occurred endogenously in macrophages and found that in the immunoprecipitant pulled down by anti-Nrdp1 antibody from Nrdp1-TG macrophages, the C/EBPβ was detected by immunoblot analysis, but STAT6 was still not detected. Also, the association between Nrdp1 and C/EBPβ could be enhanced by IL-4 stimulation (Fig. 5C). Altogether, these results suggest that Nrdp1 interacts with C/EBPβ but not STAT6 in macrophages.

**Nrdp1 Enhances IL-4-induced Ubiquitination of C/EBPβ—**

Data above showed that Nrdp1 interacts with transcription factor C/EBPβ, suggesting Nrdp1 may increase Arg1 expression by regulating the activity of C/EBPβ. Nrdp1 is an E3 ligase involved in ubiquitination and degradation of ErbB3 and BRUCE (15, 16) and also promotes ubiquitination and activation of TBK1 in macrophages (17). We wondered whether Nrdp1 regulated the ubiquitination of C/EBPβ. We found that IL-4 stimulation induced the ubiquitination of C/EBPβ, and this ubiquitination was remarkably enhanced in Nrdp1-TG macrophages (Fig. 6A). However, Nrdp1 did not affect the total protein level of C/EBPβ (Fig. 6B). Thus, E3 ubiquitin ligase Nrdp1 enhances the ubiquitination of C/EBPβ but does not induce its degradation.

Polyubiquitin chains are linked through one of the seven lysine residues of ubiquitin, including the most common two, Lys-48 and Lys-63. It is believed that Lys-48-linked polyubiquitination mediates proteasome-dependent degradation of substrates, whereas Lys-63-linked polyubiquitination mediates protein-protein interaction or downstream kinase activation. Thus, we wondered whether Nrdp1 ubiquitinated C/EBPβ via Lys-63–polyubiquitin pathway. pcDNA3.1-C/EBPβ and pcDNA3.1-Nrdp1, together with pcDNA3.1-HA-ubiquitin or pcDNA3.1-HA–ΔK63-ubiquitin, were co-transfected into NIH-3T3 cells. We found that Nrdp1 and ubiquitin overexpression enhanced ubiquitination of C/EBPβ (Fig. 6C); however, pcDNA3.1-HA–ΔK63-ubiquitin transfection inhibited IL-4-stimulated ubiquitination of C/EBPβ. In all, these results indicate that Nrdp1 may function as an E3 ubiquitin ligase for Lys-63-linked polyubiquitination of transcription factor C/EBPβ.
Nrdp1 overexpression increased Arg1 reporter gene activation in the presence of IL-4 stimulation. Moreover, pcDNA3.1-Nrdp1 transfection notably enhanced C/EBPβ-induced Arg1 gene transcription in a dose-dependent manner.

We further used siRNA-C/EBPβ to knock down C/EBPβ expression in NIH-3T3 cells (Fig. 7B), and we examined its effect on Nrdp1-induced activation of Arg1 gene. As shown in Fig. 7C, silencing of C/EBPβ significantly impaired Nrdp1-enhanced Arg1 gene activation after IL-4 stimulation. Thus, Nrdp1 enhances IL-4-mediated activation of Arg1 gene by activating transcriptional activity of C/EBPβ.

DISCUSSION

As one of key players in the immune responses, macrophages undergo M1 or M2 polarization in response to different environmental stimuli. M1 polarized macrophages up-regulate iNOS expression and secrete high levels of pro-inflammatory cytokines, whereas M2 polarized macrophages up-regulate the expression of Arg1, MR, Ym1, and Fizz1 and increase anti-inflammatory cytokine IL-10 production. M2-polarized macrophages are essential for anti-parasite immunity, collagen synthesis, tissue remodeling, as well as allergic diseases and tumors, but the underlying molecular pathways regulating M1/M2 polarization need further investigation (1, 6). In this study, using two-dimensional DIGE and MS analysis, we found that Arg1 expression was up-regulated in Nrdp1-TG peritoneal macrophages, which was verified by Q-PCR and Western blot. We further found that Nrdp1-TG macrophages expressed much higher levels of other M2 feature markers, Fizz1, Ym1, MR, and IL-10, especially after IL-4 stimulation. Moreover, siRNA-Nrdp1 transfection effectively reversed the up-regulation of Arg1, Ym1, Fizz1, and MR in Nrdp1-TG peritoneal macrophages stimulated with IL-4. All the results demonstrate the critical role of Nrdp1 in promoting IL-4-induced M2 macrophage polarization.

However, Nrdp1 significantly inhibited LPS-induced pro-inflammatory cytokine secretion, which is consistent with our previous data (17). Moreover, we showed in this study that Nrdp1 remarkably suppressed the iNOS expression and NO production in LPS-stimulated macrophages, indicating Nrdp1 impaired the M1 macrophage polarization induced by LPS. In addition, macrophages from mutant-Nrdp1 (lacking E3 ubiquitin ligase) transgenic mice also showed similar impaired expression of Arg1 after LPS stimulation (data not shown), suggesting that Nrdp1 inhibits iNOS expression in an E3 ubiquitin ligase-independent manner.

iNOS protein level in cells can be up-regulated by increased transcription of iNOS gene or the decreased ubiquitination- and proteasome-mediated degradation of iNOS protein (22, 23). A variety of transcription factors, such as nuclear factor-κB (NF-κB), activating protein-1 (AP-1), C/EBP, C/EBPβ, octamer-binding transcription factor-1 (Oct-1), have been proved to bind with the iNOS promoter and enhance the transcription of the iNOS gene. Transcription factors activating the iNOS gene vary in different cells or in response to different stimulators, of which NF-κB is an essential component for iNOS gene transcription in macrophages in response to LPS (24). As Nrdp1 can effectively inhibit NF-κB and AP-1 activation by promoting
ubiquitination and degradation of MyD88 in LPS-stimulated macrophages (17), in this study, the suppressed activation of NF-κB and AP-1 may account for the impaired iNOS expression in Nrdp1-TG peritoneal macrophage after LPS stimulation. In addition, we cannot detect the interaction between Nrdp1 and iNOS (data not shown); therefore, the decreased protein level of iNOS in Nrdp1-TG peritoneal macrophages after LPS treatment could not be attributed to Nrdp1-mediated ubiquitination and degradation of iNOS.

Arg1 expression and l-arginine metabolism play important roles in M2 macrophage polarization (25). In the CAM, iNOS catalyzes l-arginine to produce NO and citrulline, whereas in the AAM, Arg1 competes with iNOS for l-arginine to produce urea and ornithine (26), thereby suppressing the production of NO in macrophages. Our results showed that Nrdp1 promoted IL-4-induced Arg1 expression and M2 macrophage polarization; we then further explored the underlying molecular mechanisms. It is well-known that transcription factor-mediated transcription is critical for Arg1 gene expression. Arg1 expression in macrophages is regulated cooperatively by transcription factor STAT6 and C/EBPβ in response to IL-4 (27, 28). In this study, we investigated whether Nrdp1 enhances Arg1 gene transcription by regulating the activity of STAT6 and C/EBPβ. We showed that exogenous overexpression of C/EBPβ and Nrdp1 resulted in the interaction between Nrdp1 and C/EBPβ in NIH-3T3 cells; however, in the same overexpression system, we cannot detect the interaction between Nrdp1 and STAT6, either before or after IL-4 stimulation. In addition, the endogenous interaction between Nrdp1 and C/EBPβ was further confirmed in Nrdp1-TG peritoneal macrophages by immunoprecipitation assay. Altogether, Nrdp1 interacts with transcription factor C/EBPβ but not STAT6 in macrophages. In addition, because C/EBPβ is required for basal expression of Arg1 in macrophages (28), the steady interaction between Nrdp1 and C/EBPβ may account for the constitutive up-regulation of Arg1 in Nrdp1-TG macrophages. Recently, El Kasm et al. (29) demonstrated that Mycobacterium tuberculosis-induced expression of Arg1 in macrophages depends on C/EBPβ signaling but is independent of the STAT6 signaling, and Mycobacterium-induced IL-6, IL-10, and G-CSF production accounts for the increased Arg1 expression in an autocrine-paracrine manner by activating the STAT3-signaling pathway (30), which is consistent with our results and establishes the crucial role of C/EBPβ in the expression of Arg1 gene.

C/EBPβ is a member of the basic leucine zipper family transcription factors and is involved in expression of many immune-related genes, such as Arg1, IL-6, and iNOS. C/EBPβ transcription efficacy can be regulated by lots of post-translational modifications, including phosphorylation (31, 32), SUMOylation (33), acetylation (34), and methylation (35). In addition, it has been shown that phosphorylation of C/EBPβ can abrogate its methylation by protein-arginine methyltransferase 4 PRMT4/CARM1, thus demonstrating the cross-talk between phosphorylation and epigenetic methylation of C/EBPβ (36). Ubiquitination is a common post-translational modification of proteins and controls many aspects of immune responses. Several E3 ubiquitin ligases have been identified in the immune system, and classical Lys-48-linked polyubiquitination mediates degradation of target substrates. However, Lys-63-linked polyubiquitination displays proteasome-independent regulatory functions. Our laboratory has previously shown that E3 ubiquitin ligase Nrdp1 stimulates Lys-48-linked ubiquitination and proteasomal degradation of MyD88 to suppress pro-inflammatory cytokine production; at the same time, it also triggers Lys-63-linked ubiquitination and activation of TBK1 in LPS-treated macrophages (17). Here, we showed that IL-4 stimulation induced the ubiquitination of C/EBPβ, and this ubiquitination was remarkably enhanced in Nrdp1-TG macrophages. Moreover, although Nrdp1 ubiquitinated C/EBPβ, it did not decrease protein levels of C/EBPβ; therefore, Nrdp1 did not cause degradation of C/EBPβ. We later demonstrated that pcDNA3.1-HA-ubiquitin transfection enhanced IL-4-stimulated ubiquitylation of C/EBPβ. However, pcDNA3.1-HA-ΔK63-ubiquitin transfection impaired the ubiquitination, so Nrdp1 may function as an E3 ubiquitin ligase to induce Lys-63-linked polyubiquitination and activation of C/EBPβ. In addition, Nrdp1 overexpression notably enhanced the transactivation efficacy of C/EBPβ to activate the Arg1 reporter gene, and siRNA-C/EBPβ significantly decreased Nrdp1-enhanced Arg1 reporter gene activation in the presence of IL-4 stimulation. Altogether, Nrdp1 ubiquitinates and enhances transcriptional activity of C/EBPβ during the IL-4-mediated activation of the Arg1 gene and established an additional ubiquitination-mediated regulatory mechanism of C/EBPβ to trigger Arg1 expression. However, the possible cross-talk between phosphorylation, acetylation, methylation, and ubiquitination of C/EBPβ needs further investigation. Moreover, like other modification events, ubiquitination is transient and can be reversed by the deubiquitination system (37), so whether there is any deubiquitination involved in regulatory Arg1 expression in M2 macrophages is still unknown.

In summary, the expression of Arg1 is highly up-regulated in Nrdp1-TG macrophages, and Nrdp1 promotes IL-4-induced M2 macrophage polarization. Nrdp1 interacts with and ubiquitinates transcriptional factor C/EBPβ, thus enhancing transcriptional efficacy of C/EBPβ to activate the Arg1 gene in M2-polarized macrophages. Nrdp1-mediated ubiquitination of C/EBPβ is a new regulatory mechanism of Arg1 expression and M2 macrophage polarization. Given the critical role of M2 macrophages in the regulation of immune responses and the pathogenesis of diseases (38), a better understanding of the role and molecular mechanisms by which Nrdp1 regulates M2 macrophages will be helpful to develop a novel therapeutic strategy for diseases such as protozoan infection, cancer, and allergic and metabolic diseases (4, 5, 39).

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