Gα\textsubscript{i1} and Gα\textsubscript{i3} regulate macrophage polarization by forming a complex containing CD14 and Gab1

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Heterotrimeric G proteins have been implicated in Toll-like receptor 4 (TLR4) signaling in macrophages and endothelial cells. However, whether guanine nucleotide-binding protein G(i) subunit alpha-1 and alpha-3 (G\textsubscript{i1/3}) are required for LPS responses remains unclear, and if so, the underlying mechanisms need to be studied. In this study, we demonstrated that, in response to LPS, G\textsubscript{i1/3} form complexes containing the pattern recognition receptor (PRR) CD14 and growth factor receptor binding 2 (Grb2)-associated binding protein (Gab1), which are required for activation of PI3K-Akt signaling. G\textsubscript{i1/3} deficiency decreased LPS-induced TNF-α production, which was associated with decreased phosphorylation of IFN regulatory factor 3 (IRF3). G\textsubscript{i1/3} knockdown in bone marrow-derived macrophage cells (G\textsubscript{i1/3}KD BMDCs) exhibited an M2-like phenotype with significantly suppressed production of TNF-α, IL-6, IL-12, and NO in response to LPS. The altered polarization coincided with decreased Akt activation. Further, G\textsubscript{i1/3} deficiency caused LPS tolerance in mice. In vitro studies revealed that, in LPS-tolerant macrophages, G\textsubscript{i1/3} were down-regulated partially by the proteasome pathway. Collectively, the present findings demonstrated that G\textsubscript{i1/3} can interact with CD14/Gab1, which modulates macrophage polarization in vitro and in vivo.

G\textsubscript{i1} | G\textsubscript{i3} | macrophage polarization | Toll-like receptor 4 | endosome

The innate immune recognition of bacterial lipopolysaccharide (LPS) is mediated by Toll-like receptor 4 (TLR4) with activation of proinflammatory signaling pathways including activation of nuclear factor (NF)-κB, mitogen-activated protein kinases (MAPKs) and transcription factor IFN regulatory factor 3 (IRF3) (1). Activation of these pathways is essential to protect the host from infection, but this activation must be tightly regulated, because uncontrolled inflammation may have detrimental effects on hosts, resulting in inflammatory diseases including diabetes, hypertension, cardiovascular disorders, and septic shock (2). Macrophages are an essential component of innate immunity and play a central role in inflammation and host defense. Depending on the environmental cues, macrophages can assume a spectrum of activation states ranging from classically activated M1 inflammatory macrophages to various alternatively activated M2 macrophages, the latter being involved in immune regulation and tissue repair (3). The M1 phenotype is characterized by the expression of high levels of proinflammatory cytokines [i.e., tumor necrosis factor α (TNF-α), interleukin (IL)-6, and IL-1], high production of reactive nitrogen and oxygen intermediates, promotion of T helper 1 response, and strong microbicidal and tumoricidal activity. M2 macrophages, however, mainly exert immunoregulatory functions, and are involved in parasite containment, tissue remodeling, and tumor development (4). They are characterized by efficient phagocytic activity, high expression of scavenging molecules, and an F4/80\textsuperscript{hi}CD11b\textsuperscript{hi} phenotype (5). Endotoxin tolerance is a transient state of LPS refractoriness after an initial and nonlethal exposure to LPS. Endotoxin-tolerant macrophages have been found to express a set of molecules that are similar to those expressed by M2-polarized macrophages. G proteins are heterotrimerics composed of α, β, and γ subunits. The α subunit is a GTPase. When a receptor activates a G protein, the α subunit releases GDP and binds GTP and changes conformation, thus shaping inflammatory reactions. The G\textsubscript{i} proteins, including G\textsubscript{i1}, G\textsubscript{i2}, and G\textsubscript{i3}, are highly similar, sharing 87–93% of amino acid sequence identity and showing overlapping expression patterns (6). Although G\textsubscript{i1} is primarily found in the brain, both G\textsubscript{i2} and G\textsubscript{i3}, here collectively referred to as G\textsubscript{i1/3}, are abundantly expressed in the immune system, being involved in many receptors signaling processes, including responses to both GPCRs and non-GPCRs [i.e. FcγRs (refs. 7 and 8) and EGFR (ref. 9)]. Recent studies also have implicated a role of heterotrimeric G\textsubscript{i} proteins in lipopolysaccharide (LPS)-induced inflammatory responses (10–12). However, the molecular mechanisms how G\textsubscript{i1/3} regulate septic shock are poorly understood. In addition, the role of G\textsubscript{i} proteins in macrophage polarization and LPS tolerance remains unknown.

Here, we demonstrated that G\textsubscript{i1/3} regulate the downstream signaling pathways of TLR4, namely those leading to activation of NF-κB, MAPKs, and IRF3, and participate in the induction of M1 polarization of macrophages.

Significance

In this study, we demonstrate that guanine nucleotide-binding protein G(i) subunit alpha-1 and alpha-3 (G\textsubscript{i1/3}) regulate the downstream signaling pathways of Toll-like receptor 4 (TLR4). We show that G\textsubscript{i1/3} form complexes containing the pattern recognition receptor (PRR) CD14 and growth factor receptor binding 2 (Grb2)-associated binding protein (Gab1), which are required for activation of PI3K-Akt signaling and NF-κB activation. Besides, G\textsubscript{i1/3} act at both the plasma membrane and the endosome levels and are involved in TLR4 endocytosis. Furthermore, G\textsubscript{i1/3} participated in the induction of M1 polarization of macrophages, and their decreased expression contributed to LPS tolerance. Thus, G\textsubscript{i1/3} are important in controlling inflammation.


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Results

**Gα1/3 Promote LPS-Induced Inflammatory Response.** To explore the influence of Gα1/3 in endotoxic shock, we injected a lethal dose of LPS plus d-galactosamine i.p. into WT (129SvEv) and Gα1/3 double knockout (DKO) mice. All WT mice died within 8 h after administration while 40% of Gα1/3 DKO mice survived (Fig. 1A). WT mice showed significantly higher serum levels of TNF-α and IL-6 2 h after LPS administration, compared with Gα1/3 DKO mice (Fig. 1 B and C). These results indicate that Gα1/3 may play an important role in regulation of LPS-induced TLR4 activation and the inflammatory response. Because macrophages play a major role in inflammation, we used siRNA strategies in bone marrow-derived macrophage cells (BMDMs) as the cell model to study the participation of Gα1/3 in LPS-initiated proinflammatory cytokine production. The results showed that Gα1/3 deficiency significantly impaired LPS-induced IL-6, TNF-α, iNOS, and IL-12 mRNA expression (Fig. 1 D–G). Accordingly, the secretion of IL-6, TNF-α, NO, and IL-12 was also significantly reduced in Gα1/3 DKO BMDMs after LPS stimulation (Fig. 1 H–K). Collectively, these data indicate that Gα1/3 may play important role in regulating LPS responses.

**Gα1/3 Deficiency Impairs the TLR4 Triggered NF-κB, MAPKs, and TIR- Domain-Containing Adapter-Inducing Interferon-β Signaling Pathways.** To examine roles for Gαi proteins in LPS-mediated signaling, we initially used mouse embryo fibroblasts (MEFs) derived from wild-type (WT) mice or Gα1/3, DKO mice. Western blot assay results showed that MEFs lacking Gαi1 and Gαi3 (termed DKO-MEF cells) were severely impaired in LPS-induced phosphorylation of Gab1-627T, Akt-308T, Akt-473S, p38, JNK, and ERK. Moreover, expression of either Gαi1 or Gαi3 in DKO MEFs restored phosphorylation of p-Gab1, Akt-308T, Akt-473S, p38, JNK, and ERK in response to LPS (Fig. 2A).

We verified these observations with siRNA strategies in BMDMs. Combined knockdown of Gαi1 and Gαi3 inhibited phosphorylation of Akt-308T and Akt-473S in response to LPS (Fig. 2B). These data suggested that Gαi1 and Gαi3 had a role in TLR4 endocytosis, a key step for the activation of the transcription factor IFN regulatory factor-3 (IRF3) through the adaptors TIR-domain-containing adapter-inducing interferon-β (TRIF)-related adapter molecule (TRAM) and TRIF (13). Thus, we treated WT and Gαi3 DKO BMDMs with LPS for 0–120 min. As shown in Fig. 2C, LPS induced the rapid endocytosis of TLR4 in WT BMDMs, but not in Gαi3 DKO cells as analyzed by flow cytometry. Additionally, LPS-induced phosphorylation of IRF3, which occurs only when endocytosis is intact, was abolished in Gαi3 DKO BMDMs (Fig. 2D). We also found Gαi3 DKO BMDMs were defective for TRIF-mediated IFNβ production (Fig. 2E). Using confocal microscopy, we observed colocalization between the early endosomal marker EEA1 and Gαi1/Gαi3 within 3 min of LPS treatment (Fig. 2F).

Interconnection Between Gα1/3 Proteins and CD14/Gab1 Is Required for LPS-Dependent Phagocytosis of Gab1 and Its Interaction with p85.

We further investigated how Gα1/3 mediate TLR4-initiated signal transduction. In agreement with the report of Solomon et al. (14), we also found that Gα1/3 coimmunoprecipitate with the LPS coreceptor CD14 (Fig. 3A). Grb2-associated binder 1 (Gab1), the scaffolding/adaptor protein, mediates signal transduction of many receptors. Phosphorylated Gab1 recruits downstream effectors such as p85 to activate downstream signaling effectors. In concert with the previous study (15), loss of Gab1 severely impaired Akt-308T and Akt-473S phosphorylation in response to LPS in both MEFs and BMDMs (Fig. 3 B and C), indicating that Gab1 lies downstream of Gαi proteins in mediating LPS signaling. As a matter of fact, we found LPS induced the association between Gab1 and Gαi proteins in BMDMs (Fig. 3D). In addition, LPS induced the association of Gab1 with p85 (Fig. 3E), indicating that Gαi proteins are required for the interaction between Gab1 and p85, and the subsequent activation of PI3K-Akt signaling. Overall, our findings suggest that in response to LPS, a Gαi1Gab1 association is required for subsequent PI3K-Akt activation.

**Gα1/3 Regulate Macrophage Polarization.** In light of our results that Gα1/3 are important for LPS-induced signaling and cytokine release, we speculated that Gα1/3 DKO macrophages might have an M2-like bias or antiinflammatory traits. To test this hypothesis, WT peritoneal macrophages were profiled by flow cytometry to assess macrophage subtypes at steady state (16), designating F4/80+CD11b+ as M1-like and F4/80-CD11b+ as M2-like. WT mice exhibited a clear population shift away from the M2-like toward the M1-like phenotype in response to LPS. However, Gα1/3−/− peritoneal macrophages presented reduced M1-like cells compared with
To understand the cellular mechanisms by which \( \text{G}_{\alpha \gamma i1/3} \) regulate macrophage polarization, we measured \( \text{G}_{\alpha i1} \) and \( \text{G}_{\gamma i1} \) expression in M1 and M2 macrophages induced by LPS/IFN-\( \gamma \) or IL-4, respectively. The results showed a significantly different expression of \( \text{G}_{\alpha i1} \) and \( \text{G}_{\gamma i1} \) in M1 and M2 macrophages (Fig. 4B). We next transfected BMDMs with siRNA specific for \( \text{G}_{\alpha i1/3} \) and stimulated with 5 ng/mL LPS plus 10 U/mL IFN-\( \gamma \) to induce the M1-like phenotype. The results indicated that the LPS-induced activation of the MAPKs and Akt signaling pathways was less in intensity and signaling in \( \text{G}_{\alpha i1/3} \)-shRNA–transfected macrophage than in sc-shRNA transfected macrophages (Fig. 4C). Accordingly, LPS/IFN-\( \gamma \)-stimulated secretion of TNF-\( \alpha \) and IL-12 was significantly reduced in \( \text{G}_{\alpha i1/3} \)-shRNA expressing BMDMs (Fig. 4D and E). Whereas TGF-\( \beta \) secretion was increased in \( \text{G}_{\alpha i1/3} \)-shRNA-expressing BMDMs compared with sc-shRNA-expressing BMDMs (Fig. 4F). Similar results were also seen in human monocytic THP-1 cells (Fig. 4 G and H). These findings suggested that \( \text{G}_{\alpha i1/3} \)-shRNA could suppress LPS/IFN-\( \gamma \)-induced M1-like macrophages, which presented a plausible mechanism for the resistance of \( \text{G}_{\alpha i1/3} \) mice to LPS-induced endotoxin shock.

**\( \text{G}_{\alpha i1/3} \) Degradation Is Involved in LPS Tolerance.** To determine whether \( \text{G}_{\alpha i1/3} \) expression is changed in endotoxin tolerance, we preincubated mice with saline or a low dose of LPS, then challenged them with a lethal dose of LPS plus \( \beta \)-galactosamine 16 h later. As expected, preexposure to a low dose of LPS resulted in much better survival with lethal dose of LPS (Fig. 5A). We also found a decreased \( \text{G}_{\alpha i1/3} \) expression in peritoneal macrophages of LPS-tolerant mice compared with that in saline-treated mice (Fig. 5 B−D). We suggest that the decreased expression of \( \text{G}_{\alpha i1/3} \) inhibits the production of proinflammatory cytokines and dampens inflammatory responses to improve survival during acute sepsis. Further, we examined the arginine activity in macrophages that were first incubated for 16 h with medium alone, or with 100 ng/mL LPS, and then stimulated for 8 h with
In BMDMs that were first incubated for 16 h with medium alone, or with LPS (100 ng/mL), and then stimulated for an 8-h time course with the same dose of LPS. We found that the expression of Goi1/3 increased in naive or medium-pretreated macrophages, whereas it decreased in LPS-pretreated or IL-4 treated macrophages (M2-like) (Fig. 5F). Next, we examined whether there was a change in Goi1/3 mRNA level. In concert with Goi3 protein, there was a decrease of Goi3 mRNA in LPS rechallenged BMDMs (Fig. 5G). Because elimination of G proteins by ubiquitination has been found in model organisms (17), we explored a role for the proteasome in degradation of Goi1/3 in tolerance. Significantly, proteasome inhibition by MG-132 prevented Goi1/3 loss in endotoxin-tolerized macrophages (Fig. 5H). A previous study reported that Goi-interacting protein (GAPI) N terminus interacting protein (GIPN) was a putative E3 ubiquitin ligase, which could promote Goi3 down-regulation (18). We further investigated the expression of GIPN in vehicle-pretreated (naive) or LPS-pretreated (tolerized) BMDMs. The results revealed that GIPN increased significantly in LPS-pretreated BMDMs (Fig. 5J). Therefore, GIPN up-regulation might be responsible for Goi3 degradation in LPS-pretreated cells.

**Discussion**

Our current study demonstrated that Goi1/3 is involved in three major downstream signaling pathways of TLR4: (i) TLR4-induced production of proinflammatory cytokines, possibly by CD14-Goi1/3-Gαι1/3-PLCγ1–PI3K–Akt–NF-κB complex formation; (ii) Goi1/3-MAPK signaling; and (iii) the Goi1/3-IRF3 signaling pathway (Fig. 5E). A previous study had also shown that P38 and Akt are activated by TLR4 in macrophage (15); however, the underlying mechanisms were not well characterized. Here, we demonstrated that, in response to LPS, Goi1/3 recruited Gab1, which further interacts with P38 regulatory subunit p85 to promote Akt activation in macrophages. Thus, Goi1/3 may be one of the most important adaptors that trigger P38/Akt signaling in the LPS response. We also found that Goi1/3 act at the endosome level and are involved in endocytosis of TLR4 in macrophages.

Heterotrimeric G proteins serve as signal transducers for GPCRs at the plasma membrane (6). However, it is also known that G proteins reside intracellularly (19, 20), indicating that they may have cytoplasmic functions. Indeed, Ma et al. have reported that c-src and lck can be stimulated by a Ga protein (21). In this study, we found that LPS induces the formation of a complex among CD14, Goi3, and Ga3. Recruitment of Goi3 and Ga3 to the receptor may occur directly or through a GPCR.

Gab1 is characterized by an N-terminal PH domain, a central proline-rich domain interacting with an SH3 domain, and multiple conserved tyrosine residues favored by various SH2 domain-containing proteins (22). The PH domain binds to PIP3, the main product of PIP2. The phosphatidylinositol-containing motifs form complexes with proteins having an SH2 domain, for example, the p85 regulatory subunit of P38, PLCγ, and SHP-2, thus providing a platform for interaction of additional signaling protein(s). Here, we found that Gab1 is an intracellular target of Goi proteins in response to LPS. Goi1/3 interacted with Gab1 and promoted its phosphorylation by an unknown kinase. Activated Gab1 interacts with the regulatory subunit of P38 (p85), which leads to the activation of the catalytic subunit of P38 (p110). Active P38 phosphorylates PIP2 (phosphatidylinositol4,5-bisphosphate) to generate PIP3 (phosphatidylinositol 3,4,5-trisphosphate), which in turn interacts with the pleckstrin homology domains of Akt and PDK1, and PDK1 phosphorylates Akt on Thr308. When Akt is activated, it may phosphorylate IKKα, which further promotes NF-κB activation (23).

Nishida et al. have demonstrated that TLR4 is one of two receptors of pertussis toxin (PTX) (24), thus PTX is not a specific inhibitor of receptor-Goi signaling. As a consequence, here we did not use PTX as inhibitor to study the action of Goi3 and Ga3 on LPS stimulation. Fan et al. demonstrated that constitutively active Goi3 and Ga3, which incorporate a Q204L mutation that impairs their GTPase activities, potentiated TLR4-induced
ERK1/2 phosphorylation (10), indicating that TLR4 may trans-activate a GPCR to promote signaling (25). Moreover, Daphinene et al. demonstrated that Goi proteins modulate endothelial TLR signaling independent of TRAF6 (26). Thus, we propose a signaling model of Goi1 and Goi3-mediated activation of the Gab1-Pi3K-Akt-NF-κB pathway in response to LPS. Goi1 and Goi3 also contribute to the endotoxins of TLR4. A model depicting interaction cascades that are mediated by CD14 in order to promote TLR4 signaling is shown in Fig. 6. CD14 chaperones LPS molecules to the plasma membrane localized complex of TLR4 and MD2, which signals through the Goi1,2,3 and TIRAP-MD88 adaptors to activate inflammatory cytokine expression. CD14 and Goi1,2,3 then transport TLR4 to endosomes, where TRAM-TRIF signaling activates IRF3, which further initiates the expression of IFNs. The shift in macrophage polarization is now recognized as a relevant event in tumorogenesis, wound healing, and resolution of inflammation, and its deregulation underlies both tumor progression and chronic inflammatory diseases (27, 28). We have shown that Goi1,3 play an important role in maintaining homeostasis of macrophage responses and are required for normal polarization. This polarization was associated with enhanced NF-κB responses and constitutive expression of polarized markers. In concert with our results, Rudolph et al. reported that Goi4,2-deficient mice with elevated Goi3 expression develop colitis due to unresolved inflammations (29) and Fan et al. also found that splenocytes from Goi2−/− mice exhibit augmented IFN-γ and IL-12

Fig. 6. Model of Goi1/3-mediated activation of TLR4 in response to LPS. (A) Distribution of CD14, TLR4, Goi1, Goi3 and Gab1 in rested macrophage. (B) LPS induces formation of a complex between CD14, Goi1, and Goi3. However, whether and how Goi1,2,3 and Gab1 combine to TLR4 is not clear. Subsequently, Goi1 and Goi3 interact with Gab1, which promotes its phosphorylation by an unknown kinase. Activated Gab1 interacts with regulatory subunit of PI3K (p85), which leads to the activation of the catalytic subunit of PI3K (p110). Active PI3K phosphorylates PIp2 (phosphatidylinositol 4,5-bisphosphate) to generate Pi3p (phosphatidylinositol 3,4,5-trisphosphate), which in turn interacts with the pleckstrin homology domain of Akt and PDK1, and PDK1 phosphorylates Akt on Thr308. When Akt is activated, it may phosphorylate IKKs, which further promotes NF-κB activation. Besides, Goi1 and Goi3 also involve in TLR4 endocytosis. We also propose that Goi proteins may couple to the MAPK pathway. However, the exact mechanism merits further investigation.

**Fig. 5.** Goi1/3 degradation is involved in LPS tolerance. (A) Survival of mice (n = 6 per group) given saline (Naive) or preexposure to a low dose of LPS (LPS-tolerant) were challenged with a lethal dose of LPS (4 mg/kg, i.p.) plus β-galactosamine (500 mg/kg). Survival was monitored over 10 h. (B) Goi1/3 expression in peritoneal macrophages (primary M0) isolated from mice treated as in A for 2 h. Goi1,2,3 detected by Western blot. (C and D) Expression of Goi1/3 level was quantified from Western blot assays. The data are expressed as the mean ± SE of the ratios of indicated protein to β-actin. *P < 0.05, **P < 0.01, ***P < 0.001. (E) BMDMs (2 × 10⁵ cells per well) were treated or not with 100 ng/mL LPS for 16 h, washed, and rechallenged with 100 ng/mL LPS. After 8 h of incubation, arginase activity was assessed by an assay of urea production from arginine substrate. *P < 0.05, **P < 0.01. (F) Expression of Goi protein in BMDMs treated or not with 100 ng/mL LPS for 16 h, washed, and rechallenged with 100 ng/mL LPS for the indicated times. Goi1 mRNA expression was measured by QRT-PCR. *P < 0.05, **P < 0.01. (H) BMDMs treated overnight with LPS (100 ng/mL) were treated with 30 min with either vehicle only (DMSO) or MG132 (25 μM). Cells were subsequently stimulated with LPS (100 ng/mL) for the indicated times and lysed. Goi1, Goi3, GIPN, and β-actin were assessed by Western blot.
production by LPS, compared with WT mice (30). Therefore, G\textsubscript{i1/3} expression seems to be a common participant favoring M1 macrophages, and a critical contributor to their effector functions. Moreover, as G\textsubscript{i2} is primarily found in the brain, and G\textsubscript{i3} is also expressed in brain, it implicates that G\textsubscript{i1/3} could be involved in chronic inflammation diseases such as obesity, type 2 diabetes, and aging, especially because NF-kB in the brain has been reported to be a central cause for neural inflammation and diseases as reported by Zhang et al. and Yan et al. (31, 32). Inhibition of inflammation could revert aging related-degenerative symptoms (33). We also confirmed decreased G\textsubscript{i1/3} expression in LPS-tolerant macrophage, which was consistent with the study by Makhlouf et al. (34, 35). Because there is no report of G\textsubscript{i1}-specific E3 ubiquitin ligase, we only demonstrated the mechanism of G\textsubscript{i1/3} degradation, involving altered gene transcription and proteasomal degradation. However, the contribution of altered gene transcription and protein degradation to LPS tolerance merits further investigation.

Collectively, our study suggested that LPS unresponsiveness occurs by programming macrophages to the M2 phenotype. Given the importance of G\textsubscript{i1/3} in controlling inflammation, we propose that controlling G\textsubscript{i1} expression or targeting associated ubiquitin E3 ligases may represent approaches to control inflammation.

Materials and Methods

Mice. G\textsubscript{i2}–/–/G\textsubscript{i3}–/– mice on 129SvEv background were generated by breeding homozygous DKO mice (36). Studies used 6- to 8-week-old G\textsubscript{i1/3}–/– and age-matched 129SvEv WT mice for all of the experiments. All animal experiments were performed in accordance with the National Institutes of Health guidelines for the Care and Use of Laboratory Animals, with the approval of the Center for New Drug Safety Evaluation and Research, China Pharmaceutical University.

Cell Lines and Cell Culture. WT and G\textsubscript{i1/3}–/– DKO MEFs were derived from WT, and G\textsubscript{i1/3} doubly deficient mouse embryonic day 14.5 embryos. The MEFs (5 × 10\textsuperscript{4} to 10 × 10\textsuperscript{5}) were then immortalized by transfection with the total SV40 genome (plasmid pSV40WT) and subcultured several times with DMEM supplemented with 10% (vol/vol) FCS (37). WT and Gab1-deficient MEFs were used as previously described (38). BMDMs were obtained as described (39) and were maintained in DMEM supplemented with 10% (vol/vol) FBS and 10% (vol/vol) supernatants of L929 mouse fibroblasts as conditioned medium, providing macrophage colony-stimulating factor at 37 °C in humidified air with 5% CO\textsubscript{2} for 6 d. THP-1 cells were maintained in medium 1640 containing 10% (vol/vol) heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C under 5% CO\textsubscript{2} in air.

Statistical Analysis. Statistical significance was determined with a Student t test, and P < 0.05 is considered to be statistically significant. Standard methods were used for bone marrow macrophage isolation and culture, transfections, and Western blot analyses. For further details including primer sequences, see SI Methods and Materials.

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