$G\alpha_{i1}$ and $G\alpha_{i3}$ 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 ($G\alpha_{i1/3}$) 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, $G\alpha_{i1/3}$ 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. $G\alpha_{i1/3}$ deficiency decreased LPS-induced TLR4 endocytosis, which was associated with decreased phosphorylation of IFN regulatory factor 3 (IRF3). $G\alpha_{i1/3}$ knockdown in bone marrow-derived macrophage cells ($G\alpha_{i1/3}$ KD BMDMs) 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, $G\alpha_{i1/3}$ deficiency caused LPS tolerance in mice. In vitro studies revealed that, in LPS-tolerant macrophages, $G\alpha_{i1/3}$ were down-regulated partially by the proteasome pathway. Collectively, the present findings demonstrated that $G\alpha_{i1/3}$ can interact with CD14/Gab1, which modulates macrophage polarization in vitro and in vivo.

Gαi1 | Gαi3 | 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 $F4/80^{hi}CD11b^{hi}$ 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 heterotrimers 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, acquiring its activated state. In the case of $G\alpha_i$ proteins, the activated $G\alpha_i$ subunits, which inhibit adenylyl cyclases, are likely candidates to regulate leukocyte differentiation, thus shaping inflammatory reactions. The $G\alpha_i$ proteins, including $G\alpha_{i1}$, $G\alpha_{i2}$, and $G\alpha_{i3}$, are highly similar, sharing 87–93% of amino acid sequence identity and showing overlapping expression patterns (6). Although $G\alpha_{i1}$ is primarily found in the brain, both $G\alpha_{i1}$ and $G\alpha_{i3}$, here collectively referred to as $G\alpha_{i1/3}$, 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 $G\alpha_i$ proteins in lipopolysaccharide (LPS)-induced inflammatory responses (10–12). However, the molecular mechanisms how $G\alpha_{i1/3}$ regulate septic shock are poorly understood. In addition, the role of $G\alpha_i$ proteins in macrophage polarization and LPS tolerance remains unknown.

Here, we demonstrated that $G\alpha_{i1/3}$ 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 ($G\alpha_{i1/3}$) regulate the downstream signaling pathways of Toll-like receptor 4 (TLR4). We show that $G\alpha_{i1/3}$ 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, $G\alpha_{i1/3}$ act at both the plasma membrane and the endosome levels and are involved in TLR4 endocytosis. Furthermore, $G\alpha_{i1/3}$ participated in the induction of M1 polarization of macrophages, and their decreased expression contributed to LPS tolerance. Thus, $G\alpha_{i1/3}$ are important in controlling inflammation.

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Results

$G\alpha_{i1/3}$ Promote LPS-Induced Inflammatory Response. To explore the influence of $G\alpha_{i1/3}$ in endotoxic shock, we injected a lethal dose of LPS plus D-galactosamine i.p. into WT (129SvEv) and $G\alpha_{i1/3}$ double knockout (DKO) mice. All WT mice died within 8 h after administration while 40% of $G\alpha_{i1/3}$ DKO mice survived (Fig. 1*A*). WT mice showed significantly higher serum levels of TNF-α and IL-6 2 h after LPS administration, compared with $G\alpha_{i1/3}$ DKO mice (Fig. 1 *B* and *C*). These results indicate that $G\alpha_{i1/3}$ 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 $G\alpha_{i1/3}$ in LPS-initiated proinflammatory cytokine production. The results showed that $G\alpha_{i1/3}$ 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 $G\alpha_{i1/3}$ DKO BMDMs after LPS stimulation (Fig. 1 *H*–*K*). Collectively, these data indicate that $G\alpha_{i1/3}$ may play important role in regulating LPS responses.

$G\alpha_{i1/3}$ Deficiency Impairs the TLR4 Triggered NF-κB, MAPKs, and TIR-Domain-Containing Adapter-Inducing Interferon-β Signaling Pathways. To examine roles for $G\alpha_i$ proteins in LPS-mediated signaling, we initially used mouse embryo fibroblasts (MEFs) derived from wild-type (WT) mice or $G\alpha_{i1/3}$ DKO mice. Western blot assay results showed that MEFs lacking $G\alpha_{i1}$ and $G\alpha_{i3}$ (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 $G\alpha_{i1}$ or $G\alpha_{i3}$ in DKO MEFs restored phosphorylation of p-Gab1, Akt-308T, Akt-473S, p38, JNK, and ERK in response to LPS (Fig. 2*A*).

We verified these observations with siRNA strategies in BMDMs. Combined knockdown of $G\alpha_{i1}$ and $G\alpha_{i3}$ inhibited phosphorylation of Akt-308T and Akt-473S in response to LPS (Fig. 2*B*). These data suggested that $G\alpha_{i1/3}$ were required for TLR4-initiated signaling, including activation of NF-κB and MAPKs pathways. Because G proteins partition into lipid rafts which are involved in receptor endocytosis, we wondered whether $G\alpha_{i1}$ and $G\alpha_{i3}$ 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 adaptor molecule (TRAM) and TRIF (13). Thus, we treated WT and $G\alpha_{i1/3}$ DKO BMDMs with LPS for 0–120 min. As shown in Fig. 2*C*, LPS induced the rapid endocytosis of TLR4 in WT BMDMs, but not in $G\alpha_{i1/3}$ DKO cells as analyzed by flow cytometry. Additionally, LPS-induced phosphorylation of IRF3, which occurs only when endocytosis is intact, was abolished in $G\alpha_{i1/3}$ DKO BMDMs (Fig. 2*D*). We also found $G\alpha_{i1/3}$ DKO BMDMs were defective for TRIF-mediated IFNβ production (Fig. 2*E*). Using confocal microscopy, we observed colocalization between the early endosomal marker EEA1 and $G\alpha_{i1}$/$G\alpha_{i3}$ within 3 min of LPS treatment (Fig. 2*F*).

Interaction Between $G\alpha_{i1/3}$ Proteins and CD14/Gab1 Is Required for LPS-Dependent Phosphorylation of Gab1 and Its Interaction with p85. We further investigated how $G\alpha_{i1/3}$ mediate TLR4-initiated signal transduction. In agreement with the report of Solomon et al. (14), we also found that $G\alpha_{i1/3}$ coimmunoprecipitate with the LPS coreceptor CD14 (Fig. 3*A*). 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 $G\alpha_i$ proteins in mediating LPS signaling. As a matter of fact, we found LPS induced the association between Gab1 and $G\alpha_i$ proteins in BMDMs (Fig. 3*D*). In addition, LPS induced the association of Gab1 with p85 (Fig. 3*E*), indicating that $G\alpha_i$ 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 $G\alpha_i$-Gab1 association is required for subsequent PI3K-Akt activation.

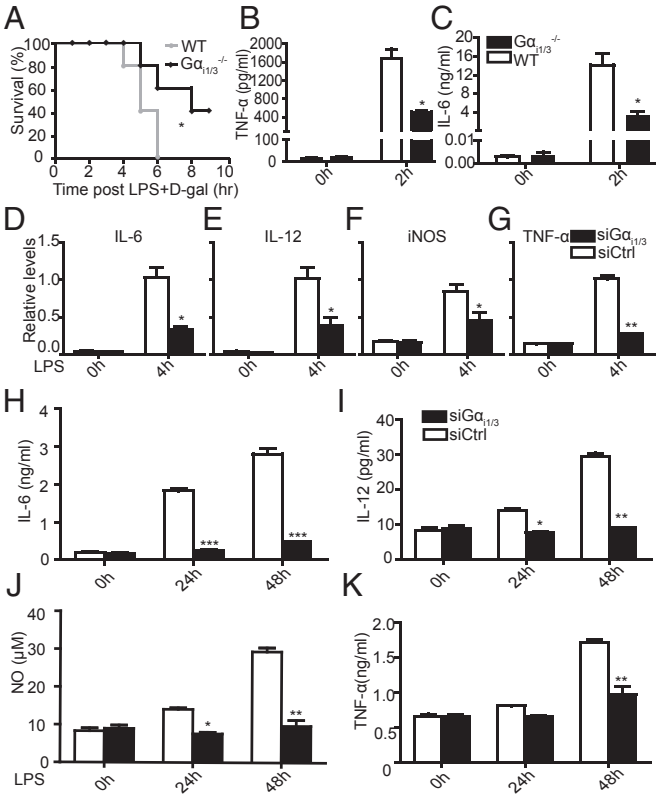

Fig. 1. Survival of WT and $G\alpha_{i1/3}^{-/-}$ mice after LPS and D-galactosamine challenge. (*A*) WT and $G\alpha_{i1/3}$ DKO mice were injected i.p. with 4 mg/kg LPS plus 500 mg/kg D-galactosamine. Survival was monitored over 10 h. Keplan–Meler plots were shown after LPS challenge. Statistical significance was determined by log rank test. Data were representative of at least three independent experiments (five mice per group). $*P < 0.05$. (*B* and *C*) Serum assayed for TNF-α (*B*) and IL-6 (*C*) by ELISA 2 h after 4 mg/kg LPS plus 500 mg/kg D-galactosamine treatment. $*P < 0.05$, $**P < 0.01$. (*D*–*G*) BMDMs from WT mice were transfected with control small interference RNA (siCtrl) or $G\alpha_{i1}$ and $G\alpha_{i3}$-specific siRNA. After 48 h, cells were stimulated with LPS (100 ng/mL) for 4 h. IL-6 (*D*), IL-12 (*E*), iNOs (*F*), and TNF-α (*G*) mRNA expressions were measured by quantitative real-time RT-PCR (QRT-PCR). $*P < 0.05$, $**P < 0.01$. (*H*–*K*) BMDMs from WT mice were transfected with control small interfering RNA (siCtrl) or $G\alpha_{i1}$ and $G\alpha_{i3}$-specific siRNA. After 48 h, cells were stimulated with LPS (100 ng/mL) for 24 and 48 h, and IL-6 (*H*), IL-12 (*I*), NO (*J*), and TNF-α (*K*) were then measured by ELISA in the supernatants. The results shown are representative of three independent experiments. Data are shown as mean ± SD of three independent experiments. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

$G\alpha_{i1/3}$ Regulate Macrophage Polarization. In light of our results that $G\alpha_{i1/3}$ are important for LPS-induced signaling and cytokine release, we speculated that $G\alpha_{i1/3}$ 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^{int}CD11b^{hi} as M1-like and F4/80^{hi}CD11b^{hi} 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, $G\alpha_{i1/3}^{-/-}$ peritoneal macrophages presented reduced M1-like cells compared with

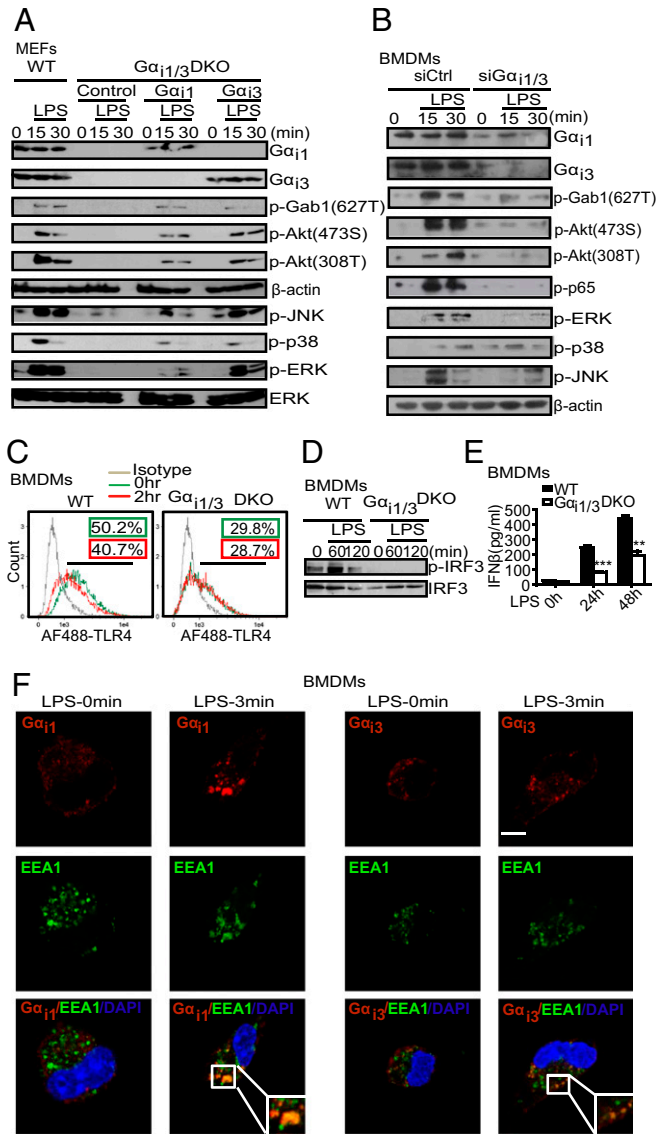

Fig. 2. $G\alpha_{i1/3}$ deficiency impairs the TLR4 triggered NF-κB, MAPKs, and TRIF signaling pathways. (*A*) Exogenous $G\alpha_{i1}$ or $G\alpha_{i3}$ rescue the activation of Akt and MAPKs signaling in DKO MEFs in response to LPS. DKO MEFs were transfected with vectors encoding WT $G\alpha_{i1}$ and $G\alpha_{i3}$ (4 μg per well). After 48 h, cells were treated with LPS (1 μg/mL) for the indicated times. $G\alpha_{i1}$, $G\alpha_{i3}$, p-Gab1 (T627), p-Akt (473S), p-Akt (308T), β-actin, p-JNK, p-p38, p-ERK, and total ERK were detected by Western blot. (*B*) Knockdown of the expression of $G\alpha_{i1/3}$ decreases the activation of Akt and MAPKs by LPS in BMDMs. BMDMs were transfected with control small interfering RNA (siCtrl) or $G\alpha_{i1}$ and $G\alpha_{i3}$-specific siRNA. After 48 h, cells were stimulated with LPS (100 ng/mL) for the indicated times. $G\alpha_{i1}$, $G\alpha_{i3}$, p-Gab1 (627T), p-Akt (473S), p-Akt (308T), p-ERK, p-p38, p-JNK, and β-actin were detected by Western blot. (*C*) BMDMs from WT and $G\alpha_{i1/3}$ DKO mice were treated or not with LPS (100 ng/mL) for 2 h. Flow cytometry was then used to examine TLR4 endocytosis by determining the surface levels of TLR4. (*D*) BMDMs from WT and $G\alpha_{i1/3}$ DKO mice were treated or not with LPS (100 ng/mL) for the times indicated. The presence of phosphorylated IRF3 in cell extracts was determined. (*E*) BMDMs were treated with LPS (100 ng/mL) for the indicated times and the amounts of secreted IFN-β were determined by ELISA. (*F*) BMDMs were stimulated with LPS (100 ng/mL) for the times indicated and processed for confocal microscopy to detect the presence of $G\alpha_{i1}$, $G\alpha_{i3}$, or EEA1. (Scale bar: 5 μm.) All images are representative of at least three independent experiments where over 500 cells were examined per condition and >95% of the cells displayed similar staining.

WT macrophages (8.85% versus 16.9%) (Fig. 4*A*), suggesting that $G\alpha_{i1/3}$ may participate in these shifting endotoxin responses.

To understand the cellular mechanisms by which $G\alpha_{i1/3}$ regulate macrophage polarization, we measured $G\alpha_{i1}$ and $G\alpha_{i3}$ expression in M1 and M2 macrophages induced by LPS/IFN-γ or IL-4, respectively. The results showed a significantly different expression of $G\alpha_{i1}$ and $G\alpha_{i3}$ in M1 and M2 macrophages (Fig. 4*B*). We next transfected BMDMs with siRNA specific for $G\alpha_{i1/3}$ and stimulated with 5 ng/mL LPS plus 10 U/mL IFN-γ 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 $G\alpha_{i1/3}$-shRNA–transfected macrophage than in sc-shRNA transfected macrophages (Fig. 4*C*). Accordingly, LPS/IFN-γ–stimulated secretion of TNF-α and IL-12 was significantly reduced in $G\alpha_{i1/3}$-shRNA expressing BMDMs (Fig. 4 *D* and *E*). Whereas TGF-β secretion was increased in $G\alpha_{i1/3}$-shRNA–expressing BMDMs compared with sc-shRNA–expressing BMDMs (Fig. 4*F*). Similar results were also seen in human monocytic THP-1 cells (Fig. 4 *G* and *H*). These findings suggested that $G\alpha_{i1/3}$-shRNA could suppress LPS/IFN-γ–induced M1-like macrophages, which presented a plausible mechanism for the resistance of $G\alpha_{i1/3}$DKO mice to LPS-induced endotoxin shock.

$G\alpha_{i1/3}$ Degradation Is Involved in LPS Tolerance. To determine whether $G\alpha_{i1/3}$ expression is changed in endotoxin tolerance, we preinjected mice with saline or a low dose of LPS, then challenged them with a lethal dose of LPS plus D-galactosamine 16 h later. As expected, preexposure to a low dose of LPS resulted in much better survival with lethal dose of LPS (Fig. 5*A*). We also found a decreased $G\alpha_{i1/3}$ 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 $G\alpha_{i1/3}$ inhibits the production of proinflammatory cytokines and dampens inflammatory responses to improve survival during acute sepsis. Further, we examined the arginase 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

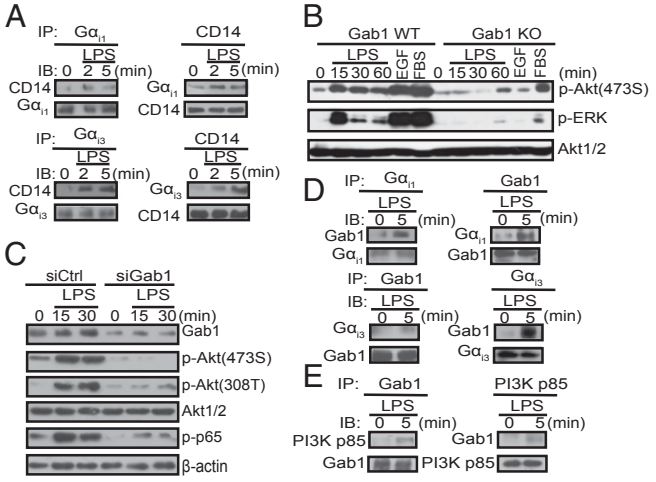

Fig. 3. Binding of $G\alpha_{i1/3}$ proteins to CD14/Gab1 is required for LPS-dependent phosphorylation of Gab1 and its interaction with p85. (*A*) LPS induces the association of CD14 with $G\alpha_{i1/3}$. BMDMs were treated with LPS (100 ng/mL) for the indicated times and were then lysed. CD14, $G\alpha_{i1}$, or $G\alpha_{i3}$ was immunoprecipitated as described in *SI Materials and Methods*. (*B*) Gab1 WT and Gab1 KO MEFs were treated with LPS (1 μg/mL). p-Akt (473S), p-ERK1/2, and Akt1/2 were detected by Western blot. (*C*) BMDMs from WT mice were transfected with control small interfereing RNA (siCtrl) or Gab1-specific siRNA. After 48 h, cells were stimulated with LPS (100 ng/mL) for the indicated times. Gab1, p-Akt (473S), p-Akt (308T), total Akt, p-p65, and β-actin were detected by Western blot. (*D* and *E*) LPS induces the association of $G\alpha_{i1/3}$ with Gab1, followed by the association of Gab1 with PI3K p85. Gab1, $G\alpha_{i1}$, $G\alpha_{i3}$, or Gab1 was immunoprecipitated as described in *SI Materials and Methods*.

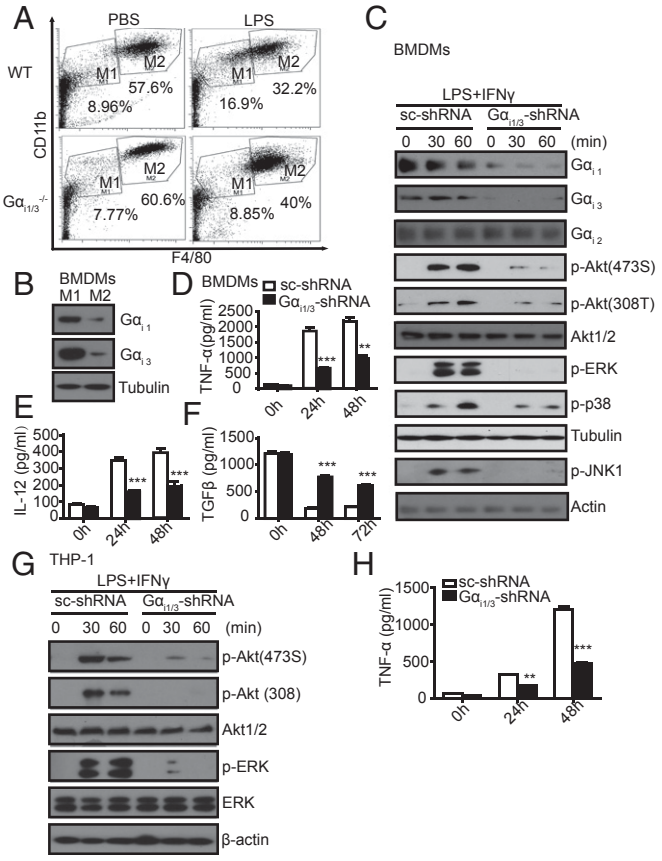

Fig. 4. $G\alpha_{i1/3}$ regulate macrophage polarization. (*A*) Peritoneal cells extracted from WT and $G\alpha_{i1/3}$ DKO mice were analyzed by flow cytometry for CD11b and F4/80 to discriminate macrophage subtypes. WT or $G\alpha_{i1/3}$ DKO mice were treated i.p. for 6 h with 20 mg/kg LPS or PBS. Peritoneal macrophages were extracted and subpopulations of macrophages were analyzed by flow cytometry with F4/80 and CD11b. Population M1 ($F4/80^{int}CD11b^{int}$) and population M2 ($F4/80^{hi}CD11b^{hi}$) were gated. (*B*) $G\alpha_{i1/3}$ protein expression in M1 and M2 macrophages. BMDMs were treated with LPS (5 ng/mL) plus IFN-γ (10 U/mL) or IL-4 (10 U/mL), respectively, and tested for the M1 and M2 macrophage formation. After 24 h, cells were lysed. $G\alpha_{i1}$ and $G\alpha_{i3}$ were detected by Western blot. (*C*) Knockdown of $G\alpha_{i1/3}$ decreased the activation of Akt and MAPKs in M1 macrophages. BMDMs were transfected with control small RNA (Ctrl) or $G\alpha_{i1}$ and $G\alpha_{i3}$-specific siRNA. After 48 h, BMDMs were treated with LPS (5 ng/mL) plus IFN-γ (10 U/mL) for the indicated times and were then lysed. $G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, p-Akt (473S), p-Akt (308T), total Akt, p-ERK1/2, p-p38, Tubulin, p-JNK, and β-actin were analyzed by Western blot. (*D*–*F*) Knockdown of $G\alpha_{i1/3}$ decreases the expression of M1 cytokines: TNF-α (*D*), IL-12 (*E*), but increase the expression of the M2 cytokine TGF-β (*F*). BMDMs were transfected with control small RNA (Ctrl) or $G\alpha_{i1}$ and $G\alpha_{i3}$-specific siRNA. After 48 h, BMDMs were treated with LPS (5 ng/mL) plus IFN-γ (10 U/mL) for the indicated times, and TNF-α, IL-12, and TGF-β in the supernatants were measured by ELISA. $^{**}P < 0.01$, $^{***}P < 0.001$. (*G*) Knockdown of $G\alpha_{i1/3}$ decreases the activation of Akt and ERK in the THP-1 cell line. THP-1 cells were treated with LPS (5 ng/mL) plus IFN-γ (10 U/mL) for the indicated times and were then lysed. P-Akt (473S), p-Akt (308T), total Akt, p-ERK, total ERK, and β-actin were analyzed by Western blot. (*H*) Knockdown of $G\alpha_{i1/3}$ decreases the expression of the M1 cytokine TNF-α in THP-1 cells. THP-1 cells were transfected with control small interfering RNA (Ctrl) or $G\alpha_{i1}$ and $G\alpha_{i3}$-specific siRNA. The level of TNF-α in the supernatants was measured by ELISA. $^{**}P < 0.01$, $^{***}P < 0.001$.

the same dose of LPS (100 ng/mL). We found increased arginase activity in BMDMs rechallenged with LPS, which resembles IL-4–stimulated M2 polarization (Fig. 5*E*). We also examined the expression of $G\alpha_{i1/3}$ 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 $G\alpha_{i1/3}$ was increased in naive or medium-pretreated macrophages, whereas it was decreased in LPS-pretreated or IL-4 treated macrophages (M2-like) (Fig. 5*F*). Next, we examined whether there was a change in $G\alpha_{i3}$ mRNA level. In concert with $G\alpha_{i3}$ protein, there was a decrease of $G\alpha_{i3}$ mRNA in LPS rechallenged BMDMs (Fig. 5*G*). Because elimination of G proteins by ubiquitination has been found in model organisms (17), we explored a role for the proteasome in degradation of $G\alpha_{i1/3}$ in tolerance. Significantly, proteasome inhibition by MG-132 prevented $G\alpha_{i1/3}$ loss in endotoxin-tolerized macrophages (Fig. 5*H*). A previous study reported that Gα-interacting protein (GAIP) N terminus interacting protein (GIPN) was a putative E3 ubiquitin ligase, which could promote $G\alpha_{i3}$ 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. 5*I*). Therefore, GIPN up-regulation might be responsible for $G\alpha_{i3}$ degradation in LPS-pretreated cells.

Discussion

Our current study demonstrated that $G\alpha_{i1/3}$ is involved in three major downstream signaling pathways of TLR4: (*i*) TLR4-induced production of proinflammatory cytokines, possibly by CD14–$G\alpha_{i1/3}$–Gab1–PI3K–Akt–NF-κB complex formation; (*ii*) $G\alpha_{i1/3}$-MAPK signaling; and (*iii*) the $G\alpha_{i1/3}$-IRF3 signaling pathway (Fig. 6). A previous study had also shown that PI3K 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, $G\alpha_{i1/3}$ recruited Gab1, which further interacted with PI3K regulatory subunit p85 to promote Akt activation in macrophages. Thus, $G\alpha_{i1/3}$ may be one of the most important adaptors that trigger PI3K/Akt signaling in the LPS response. We also found that $G\alpha_{i1/3}$ 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 hck can be stimulated by a Gα protein (21). In this study, we found that LPS induces the formation of a complex among CD14, $G\alpha_{i1}$, and $G\alpha_{i3}$. Recruitment of $G\alpha_{i1}$ and $G\alpha_{i3}$ 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 PI3K. The phosphotyrosine-containing motifs form complexes with proteins having an SH2 domain, for example, the p85 regulatory subunit of PI3K, 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 $G\alpha_i$ proteins in response to LPS. $G\alpha_{i1/3}$ interacted with Gab1 and promoted its phosphorylation by an unknown kinase. Activated Gab1 interacts with the 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 PIP3 (phosphatidylinositol 3,4,5-trisphosphate), which in turn interacts with the pleckstrin homology domains of Akt and PDK1, and PDK1 phosphorylates Akt on Thr^{308}. 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-$G\alpha_i$ signaling. As a consequence, here we did not use PTX as inhibitor to study the action of $G\alpha_{i1}$ and $G\alpha_{i3}$ on LPS stimulation. Fan et al. demonstrated that constitutively active $G\alpha_{i2}$ and $G\alpha_{i3}$, which incorporate a Q204L mutation that impairs their GTPase activities, potentiated TLR4-induced

ERK1/2 phosphorylation (10), indicating that TLR4 may transactivate a GPCR to promote signaling (25). Moreover, Dauphinee et al. demonstrated that $G\alpha_i$ proteins modulate endothelial TLR signaling independent of TRAF6 (26). Thus, we propose

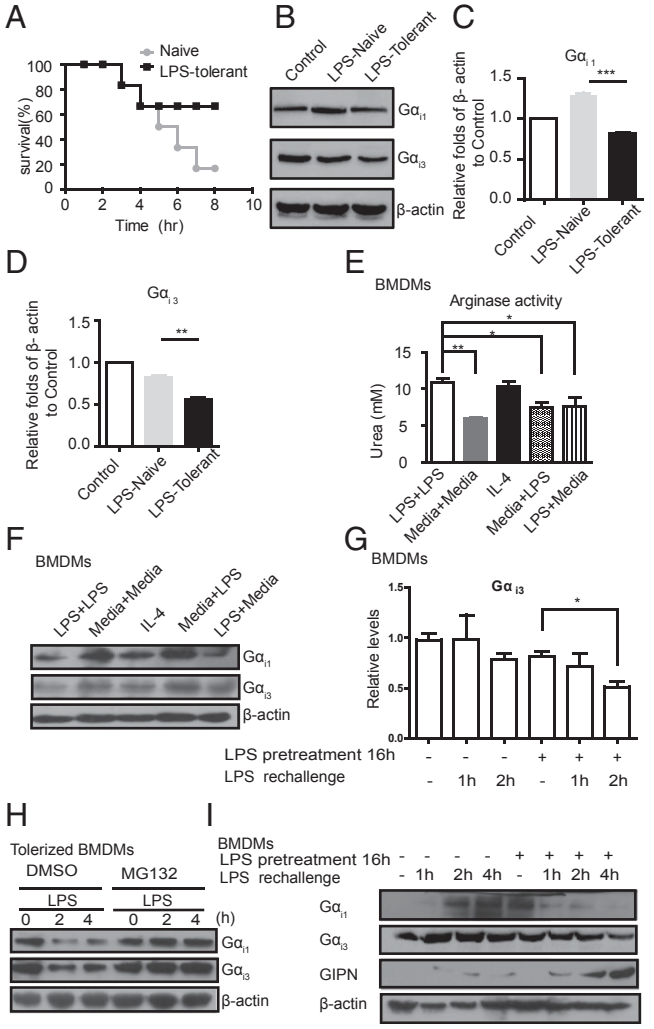

Fig. 5. $G\alpha_{i1/3}$ degradation is involved in LPS tolerance. (*A*) Survival of mice ($n = 6$ per group) given saline (Naive) or preexposure to a low dose of LPS (LPS-tolerant) were challenged with a lethal dose of LPS (4 mg/kg. i.p.) plus D-galactosamine (500 mg/kg). Survival was monitored over 10 h. (*B*) $G\alpha_{i1/3}$ expression in peritoneal macrophages (primary Mφ) isolated from mice treated as in *A* for 2 h. $G\alpha_{i1}$, $G\alpha_{i3}$ was detected by Western blot. (*C* and *D*) Expression of $G\alpha_{i1/3}$ level was quantified from Western blot assays. The data are expressed as the mean ± SE of the ratios of indicated protein to β-actin. $^{**}P < 0.01$, $^{***}P < 0.001$. (*E*) BMDMs (2×10^5 cells per well) were tolerized 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 $G\alpha_i$ protein in BMDMs tolerized or not with 100 ng/mL LPS for 16 h, washed, and rechallenged with 100 ng/mL LPS. After 8 h of incubation, cells were lysed. $G\alpha_{i1}$, $G\alpha_{i3}$, and β-actin were assessed by Western blot. (*G*) BMDMs were vehicle-treated or tolerized with 100 ng/mL LPS for 16 h, washed, and rechallenged with 100 ng/mL LPS for the indicated times. $G\alpha_{i3}$ mRNA expression was measured by QRT-PCR. $^{*}P < 0.05$, $^{**}P < 0.01$. (*H*) BMDMs tolerized overnight with LPS (100 ng/mL) were pretreated for 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. $G\alpha_{i1}$, $G\alpha_{i3}$, and β-actin were assessed by Western blot. (*I*) BMDMs were vehicle-treated or tolerized with 100 ng/mL LPS for 16 h, washed, and rechallenged with 100 ng/mL LPS for indicated times. $G\alpha_{i1}$, $G\alpha_{i3}$, GIPN, and β-actin were assessed by Western blot.

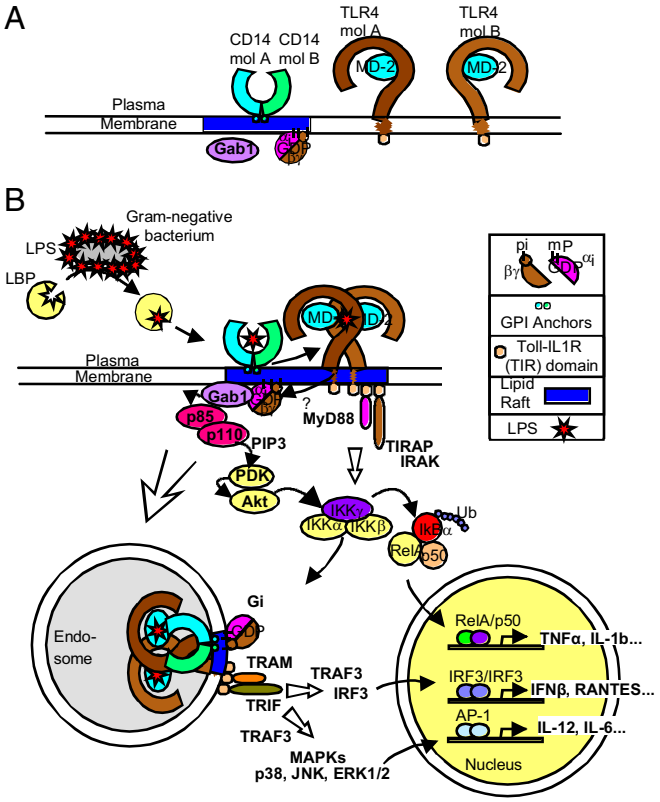

Fig. 6. Model of $G\alpha_{i1/3}$-mediated activation of TLR4 in response to LPS. (*A*) Distribution of CD14, TLR4, $G\alpha_{i1}$, $G\alpha_{i3}$, and Gab1 in rested macrophage. (*B*) LPS induces formation of a complex between CD14, $G\alpha_{i1}$ and $G\alpha_{i3}$. However, whether and how $G\alpha_{i1}$ and $G\alpha_{i3}$ combine to TLR4 is not clear. Subsequently, $G\alpha_{i1}$ and $G\alpha_{i3}$ 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 PIP3 (phosphatidylinositol 3,4,5-trisphosphate), which in turn interacts with the pleckstrin homology domain of Akt and PDK1, and PDK1 phosphorylates Akt on Thr^{308}. When Akt is activated, it may phosphorylate IKKs, which further promotes NF-κB activation. Besides, $G\alpha_{i1}$ and $G\alpha_{i3}$ also involve in TLR4 endocytosis. We also propose that Gαi proteins may couple to the MAPK pathway. However, the exact mechanism merits further investigation.

a signaling model of $G\alpha_{i1}$ and $G\alpha_{i3}$-mediated activation of the Gab1-PI3K-Akt-NF-κB pathway in response to LPS. $G\alpha_{i1}$ and $G\alpha_{i3}$ also contribute to the endocytosis 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 $G\alpha_{i1/3}$ and TIRAP-MyD88 adaptors to activate inflammatory cytokine expression. CD14 and $G\alpha_{i1/3}$ 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 tumorigenesis, wound healing, and resolution of inflammation, and its deregulation underlies both tumor progression and chronic inflammatory diseases (27, 28). We have shown that $G\alpha_{i1/3}$ 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 $G\alpha_{i2}$-deficient mice with elevated $G\alpha_{i1/3}$ expression develop colitis due to unresolved inflammations (29) and Fan et al. also found that splenocytes from $G\alpha_{i2}^{-/-}$ mice exhibit augmented IFN-γ and IL-12

production by LPS, compared with WT mice (30). Therefore, $G\alpha_{i1/3}$ expression seems to be a common participant favoring M1 macrophages, and a critical contributor to their effector functions. Moreover, as $G\alpha_{i1}$ is primarily found in the brain, and $G\alpha_{i3}$ is also expressed in brain, it implicates that $G\alpha_{i1/3}$ could be involved in chronic inflammation diseases such as obesity, type 2 diabetes, and aging, especially because NF-κB in the brain has been appreciated 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 $G\alpha_{i1/3}$ expression in LPS-tolerant macrophage, which was consistent with the study by Makhlouf et al. (34, 35). Because there is no report of $G\alpha_{i1}$-specific E3 ubiquitin ligase, we only demonstrated the mechanism of $G\alpha_{i3}$ 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 $G\alpha_{i1/3}$ in controlling inflammation, we propose that controlling $G\alpha_i$ expression or targeting associated ubiquitin E3 ligases may represent approaches to control inflammation.

Materials and Methods

Mice. $G\alpha_{i1/3}^{-/-}$(*Gnai1/3*$^{-/-}$) mice on 129SvEv background were generated by breeding homozygous DKO mice (36). Studies used 6- to 8-wk-old $G\alpha_{i1/3}^{-/-}$ and age-matched 129SvEv WT mice for all of the experiments. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of Center for New Drug Safety Evaluation and Research, China Pharmaceutical University.

Cell Lines and Cell Culture. WT and $G\alpha_{i1/3}$ DKO MEFs were derived from WT, and $G\alpha_{i1/3}$ doubly deficient mouse embryonic day 14.5 embryos. The MEFs (5×10^5 to 10×10^5) 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_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_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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Supporting Information

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SI Materials and Methods

Reagents. LPS (0111:B4), MG132 were purchased from Sigma-Aldrich. Antibodies to total Akt and Akt phosphorylated at Ser473 or Thr308, and antibodies to phosphorylated ERK1/2, p38, JNK, Gab1, as well as p-Gab1, were from Cell Signaling Technology. Antibodies against $G\alpha_{i1}$ (sc-391), $G\alpha_{i3}$ (sc-262), Gab1 (sc-9049), and Akt1/2(sc-8312), as well as goat antibody against rabbit IgG (IgG) conjugated to horseradish peroxidase (HRP) (sc-2030) were purchased from Santa Cruz Biotechnology. Anti-GIPN was purchased from Abgent. Goat antibody against mouse IgG-HRP (s0368G) was purchased from Beijing Biosynthesis Biotechnology (Bioss). Recombinant mouse IL-4, IFNγ and recombinant human IFN-γ were from Peprotech. D-galactosamine was purchased from TCI.

Western Blot Analysis. Aliquots of 30 μg of protein from each sample (treated as indicated in the legends) were subjected to 12% SDS–polyacrylamide gel electrophoresis (SDS/PAGE) and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 10% instant nonfat dry milk for 30 min, membranes were incubated with specific antibodies overnight at 4 °C followed by incubation with secondary antibodies (HRP-conjugated anti-rabbit or anti-mouse IgG at the appropriate dilutions) for 45 min to 1 h at room temperature. Antibody binding was detected with the enhanced chemiluminescence (ECL) detection system (Pierce).

Immunoprecipitation. Cells treated with the appropriate stimuli were lysed with lysis buffer [200 mM NaCl (pH 7.4), 1% Triton X-100, 10% glycerol, 0.3 mM EDTA, 0.2 mM Na_3VO_4, and protease inhibitor mixture (Roche Diagnostics)]. Aliquots of 500-μg proteins from each sample were precleared by incubation with 35 μL of protein A/G Sepharose beads (Santa Cruz Biotechnology) for 2 h at 4 °C. Precleared samples were incubated with specific antibodies in lysis buffer overnight at 4 °C. To this mixture was added 20 μL of protein A/G beads, and the samples were incubated for 2 h at 4 °C. The beads were washed five times with PBS and once with lysis buffer, boiled, subjected to 12% SDS/PAGE, and transferred onto a PVDF membrane followed by Western blot analysis as described above.

Flow Cytometric Analysis. For in vivo studies, sterile PBS was injected into the peritoneal cavity of treated mice and cells lavaged. Cells collected were washed with PBS and then stained with FITC-F4/80, PE-CD11b (E-Biosciences). Staining was assessed with a Amnis Flowsight (Millipore). Samples were compensated and analyzed by using Amnis IDEAS software.

RNA Quantification. Total RNA was prepared by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA by reverse transcriptase (Takara). Quantitative real-time RT-PCR (QRT-PCR) analysis was performed with LightCycler (Roche Diagnostics) and the SYBR RT-PCR kit (Takara) as described (1). Primers used for quantitative-PCR amplification of mouse GAPDH mRNA were 5′-AGTGGCAAAGTGGAGATT-3′(sense) and 5′-GTGGAGTCATACTGGAACA-3′ (antisense), of mouse IL-6 mRNA were 5′-ACCTGTCTATACCACTTC-3′(sense) and 5′-GCATCATCGTTGTTCATA-3′ (antisense), of mouse TNFα mRNA were 5′-TTCTGTCTACTGAACTTC-3′(sense) and 5′-CCATAGAACTGATGAGAG-3′ (antisense), of mouse IL-12 mRNA 5′-ACATCTGCTGCTCCACAAG-3′(sense) and 5-′GGTGCTTCACACTTCAGGAA-3′(antisense), mouse iNOS mRNA 5′- TTGACGCTCGGAACTGTA-3′(sense) and 5′-ACCTGATGTTGCCATTGTT-3′ (antisense), mouse $G\alpha_{i3}$ mRNA 5′-GTGATTACGACCTTGTTCTG -3′ (sense) and 5′-AACCTGTGTATTCTGGATAAC-3′(antisense). Data were normalized by the level of GAPDH expression in each sample.

Interfering RNAs. $G\alpha_{i1}$- and $G\alpha_{i3}$-specific RNAi duplexes were purchased from Santa Cruz Biotechnology. Twenty microliters of $G\alpha_{i1}$-specific (sc-41751) and $G\alpha_{i3}$-specific (sc-29325) RNAi duplexes (10 μM, diluted in siRNA Dilution Buffer from Santa Cruz Biotechnology) were mixed with liposome 2000 (Invitrogen). The complex were added to the well containing 2 mL of medium with a final siRNA concentration of 100 nM. BMDMs (1×10^6) were seeded into each well of 6-well plates and incubated overnight, and then transfected with siRNA duplexes. After 24 h, the supernatant was removed and fresh medium was added. The cells were cultured for another 24 h before further experiments. We also use shRNA for $G\alpha_{i1}$ (sc-41751-SH) and $G\alpha_{i3}$ (sc-29325-SH) in macrophage phenotype research.

Gab1-specific siRNA: 5′-AGGAGACAAACAAGUCGAATT-3′ (sense)
/5′-UUCGACUUGUUUGUCUCCUTT -3′ (antisense);
Gab1 nonsense control sequence: 5′-UUCUCCGAACGUGUCACGUTT-3′ (sense)
/5′- ACGUGACACGUUCGGAGAATT -3′ (antisense).

Cytokine Assays. Production of the cytokines IL-6, TNF-α, IFNβ, and IL-12 were measured in the serum or supernatants by ELISA by using kits from R&D Systems.

Nitric Oxide Detection. Cells plated at 1.5×10^5cells per well in 24-well culture dishes were incubated overnight before stimulation. After the cells were treated with 100 ng/mL LPS for 24 h, the culture medium was collected for analysis by using the Griess Reagent kit (Bytime). Nitrite concentrations were determined by the measurement of the optical density at 570 nm.

Determination of Arginase Activity. Arginase activity was measured in cell lysates as described (1). Briefly, cells were lysed with 100 μL of 0.1% Triton X-100. After 30 min on a shaker, 100 μL of 25 mM Tris·HCl was added. To 100 μL of this lysate, 10 μL of 10 mM $MnCl_2$ was added, and the enzyme was activated by heating for 10 min at 56 °C. Arginine hydrolysis was conducted by incubating the lysate with 100 μL of 0.5 M L-arginine (pH 9.7) at 37 °C for 15–120 min. The reaction was stopped with 900 μL of H_2SO_4 (96%)/H_3PO_4 (85%)/H_2O (1/3/7, vol/vol/vol). The urea concentration was measured at 540 nm after addition of 40 μL of α-isonitrosopropiophenone (dissolved in 100% ethanol) followed by heating at 95 °C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of urea per min.

Microscopy. BMDMs were cultured as 5×10^5 cells per well in 6-well plates on sterile coverslips overnight and stimulated with LPS (100 ng/mL) for indicated times. Then cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized with 0.1% Triton X-100, and incubated with $G\alpha_{i1}$, $G\alpha_{i3}$ antibody and EEA1 overnight. Binding was detected by using Alexa Fluor 488-labeled goat anti-rabbit IgG antibody or Alexa Fluor 647-labeled goat anti-mouse IgG antibody (Molecular Probes). Fixed cells imaging were performed by using a Leica TCS SPE.

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