LRRK2 promotes the activation of NLRC4 inflammasome during *Salmonella* Typhimurium infection

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Although genetic polymorphisms in the *LRRK2* gene are associated with a variety of diseases, the physiological function of LRRK2 remains poorly understood. In this study, we report a crucial role for LRRK2 in the activation of the NLRC4 inflammasome during host defense against *Salmonella enteric* serovar Typhimurium infection. *LRRK2* deficiency reduced caspase-1 activation and IL-1β secretion in response to NLRC4 inflammasome activators in macrophages. *Lrk2*−/− mice exhibited impaired clearance of pathogens after acute *S. Typhimurium* infection. Mechanistically, LRRK2 formed a complex with NLRC4 in the macrophages, and the formation of the LRRK2–NLRC4 complex led to the phosphorylation of NLRC4 at Ser533. Importantly, the kinase activity of LRRK2 is required for optimal NLRC4 inflammasome activation. Collectively, our study reveals an important role for LRRK2 in the host defense by promoting NLRC4 inflammasome activation.

INTRODUCTION

The leucine-rich repeat kinase 2 (LRRK2) gene is emerging as a genetic hotspot for disease associations. Pathogenic mutations in LRRK2 are the most prevalent genetic alterations among Parkinson’s disease (PD) patients (Paisán-Ruíz et al., 2004; Zimprich et al., 2004; Cookson, 2010), and single nucleotide polymorphisms in the LRRK2 gene have been linked to a variety of inflammatory diseases including Crohn’s disease, ulcerative colitis, and cancer (Barrett et al., 2008; Franke et al., 2010; Saunders-Pullman et al., 2010; Anderson et al., 2011; Inzelberg et al., 2012). These epidemiological evidences have instigated intense research efforts focusing on the pathogenic mechanisms of LRRK2 variants with the ultimate goal of targeting LRRK2 for treatment.

Despite the growing literature on the roles of the LRRK2 in disease development, much of its physiological function remains elusive (Chia et al., 2014; Cookson, 2015). The expression pattern of LRRK2 points to a critical function in the immune system. LRRK2 can be induced by IFN-γ stimulation in human monocytes, and it is preferentially expressed in mature macrophages and dendritic cells (Gardet et al., 2010). Consistently, accumulating evidence suggests that LRRK2 plays an important role in the host defense against the intracellular pathogens. In humans, an LRRK2 missense single nucleotide polymorphism, which results in an unstable LRRK2 protein, has been shown to confer increased susceptibility to leprosy; a disease caused by *Mycobacterium leprae* infection (Zhang et al., 2009). In the mouse model, LRRK2 was required for the mucosal immunity against the *Listeria monocytogenes* (Zhang et al., 2015b). At the cellular level, LRRK2 was found to colocalize with intracellular *Salmonella enteric* serovar Typhimurium (S. Typhimurium) during bacterial infection in macrophages (Gardet et al., 2010). These evidences collectively indicate that LRRK2 is directly involved in the innate immune response against intracellular bacteria. However, the molecular mechanism by which LRRK2 contributes to the host immunity is unknown.

A major host response against the infection by intracellular bacteria is the activation of NLRC4 inflammasome (Amer et al., 2006; Sutterwala et al., 2007; Suzuki et al., 2007; Case et al., 2009; Miao et al., 2010a). For example, *S. Typhimurium* infection of macrophages induces NLRC4 inflammasome–mediated production of the proinflammatory cytokines IL-1β and IL-18 (Franchi et al., 2006; Miao et al., 2010b). Activation of NLRC4 inflammasome is initiated by the host recognition of cytosolic bacterial components such as flagellin or PrgJ, triggering the oligomerization of NLRC4 proteins (Miao et al., 2010b; Zhao et al., 2011). The NLRC4 oligomers nucleate the filament formation of the adapter protein ASC (apoptotic speck protein containing a caspase recruitment domain) and protease caspase-1 (Hu et
al., 2015; Zhang et al., 2015a). Oligomerization of caspase-1 leads to proximity-induced proteolytic activation and subsequently results in the maturation IL-1β and IL-18 (Vance, 2015). Secreted IL-1β and IL-18 then recruit both the innate and adaptive immune system for the clearance of pathogens (Schroder and Tschopp, 2010).

In this study, we report that LRRK2 is essential for the optimal activation of NLRC4 inflammasome during S. Typhimurium infection. We found that the LRRK2-deficient macrophages showed diminished caspase-1 activation and reduced mature IL-1β secretion in response to NLRC4 inflammasome activators. In addition, Lrk2+/− mice exhibited impaired ability to clear the pathogens during acute S. Typhimurium infection. Mechanistically, LRRK2 formed a complex with NLRC4 in response to S. Typhimurium infection. Structure–function analysis showed that LRRK2 interacted with NLRC4 via the WD40 domain and that the kinase activity of LRRK2 was required for full-scale caspase-1 activation and IL-1β secretion. Moreover, LRRK2 promoted the phosphorylation of NLRC4 at Ser533, a critical modification required for the assembly of NLRC4 inflammasome. In summary, our study discovered a novel role for LRRK2 in host defense against S. Typhimurium via promoting the activation of the NLRC4 inflammasome.

RESULTS

**LRRK2 deficiency impairs NLRC4–dependent inflammasome activation**

To determine the role of LRRK2 in NLRC4 inflammasome activation, we first examined the caspase-1 activation and IL-1β production in response to defined NLRC4 inflammasome activators in LRRK2-deficient and WT macrophages (Fig. 1, a–c and f). Cytosolic delivery of purified flagellin or PrgJ, two NLRC4 inflammasome–specific ligands, induced robust pro–caspase-1 and pro–IL-1β cleavage in the WT but not the LRRK2-deficient macrophages (Fig. 1, a, b, and f). Flagellin or PrgJ are both components of the intracellular pathogen S. Typhimurium. Consistently, we found that LRRK2-deficient macrophages also showed reduced ability to activate pro–caspase-1 and pro–IL-1β as compared with the WT cells in response to S. Typhimurium infection (Fig. 1, c and f).

Importantly, LRRK2-deficient macrophages resembled the WT cells in the ability to produce TNF in response to the tested stimuli (Fig. 1 d), indicating that the defect is specific to inflammasome activation. Additionally, we examined whether LRRK2 is involved in the activation of NLRP3 inflammasome besides NLRC4. We failed to detect noticeable differences between WT and LRRK2-deficient macrophages in their ability to activate NLRP3 inflammasome (Fig. 1, e and f). LPS-primed WT and LRRK2-deficient macrophages showed a similar ability to activate caspase-1 and produce IL-1β in response to ATP or nigericin stimulation (Fig. 1, e and f). Collectively, these data suggest that LRRK2 is specifically required for the optimal activation of the NLRC4 inflammasome.

**LRRK2 is required for host defense against S. Typhimurium infection**

We then sought to determine whether LRRK2 deficiency also reduces NLRC4 inflammasome activation in vivo. A recent study reported that NLRC4–deficient IL-1β production from the neutrophils was crucial for protecting mice from acute *Salmonella* infection delivered through intraperitoneal injection (Chen et al., 2014). After a similar experimental design, we injected Lrk2−/− and WT mice with 10^7 CFUs of S. Typhimurium intraperitoneally to induce peritonitis. IL-1β levels in the peritoneal cavity–flushed fluids (PCFs) and in the sera were significantly reduced in the Lrk2−/− mice compared with that in the littermate control (WT) mice 6 h after infection (Fig. 2 a). The reduction in IL-1β production was associated with reduced infiltration of neutrophils (Fig. 2 b). Furthermore, the neutrophils sorted from infected Lrk2−/− mice produced a smaller amount of IL-1β compared with WT controls (Fig. 2 c), suggesting impaired inflammasome activation in Lrk2−/− neutrophils. Moreover, the reduction in IL-1β production was also associated with increased bacteria colonization in the peritoneal cavity (Fig. 2 d). A prior study showed that IL-1β is essential for controlling the bacteria infection in the S. Typhimurium–induced peritonitis model (Chen et al., 2014). Consistently, the bacterial colonization in the Lrk2−/− mice was effectively attenuated by supplementing the mice with recombinant IL-1β through intraperitoneal injection (Fig. 2 e). Furthermore, although more than half of the WT mice remained alive 6 d after bacteria inoculation, 90% of the Lrk2−/− mice died 4 d after infection (Fig. 2 f). Collectively, these data indicate that LRRK2 is required for the effective clearance of S. Typhimurium, which might be attributed to its role in promoting NLRC4–mediated IL-1β production.

**LRRK2 deficiency reduces ASC speck formation upon NLRC4 inflammasome activation**

A critical question is how LRRK2 regulates NLRC4 inflammasome activation. The activation of NLRC4 leads to the assembly of a macromolecule complex containing ASC and caspase-1. We analyzed the assembly of the NLRC4 inflammasome in LRRK2-deficient and control (WT) macrophages by immunoprecipitation of the endogenous ASC protein (the adapter for inflammasome complex) after S. Typhimurium infection (Fig. 3 a). Western blot analysis showed a substantial reduction of both NLRC4 and caspase-1 in the inflammasome complex, suggesting that LRRK2 is required for the assembly of NLRC4 inflammasome (Fig. 3 a). The assembly of NLRC4 inflammasome concurs with the formation of ASC specks, which is required for NLRC4–mediated caspase-1 activation (Broz et al., 2010). We visualized the endogenous ASC specks in macrophages infected with S. Typhimurium (Fig. 3 b) or treated with purified flagellin using immunofluorescence staining (Fig. 3 c). The frequency of ASC speck–containing cells was decreased by 50% in LRRK2-deficient macrophages compared with...
that in the WT controls (Fig. 3, b and c), suggesting that LRRK2 is required for ASC speck formation upon NLRC4 inflammasome activation.

Biochemical studies have determined that the ASC specks are oligomers of ASC protein that manifest as Triton X-100–insoluble aggregates (Masumoto et al., 1999; Hara et al., 2013). We prepared Triton X-100–soluble and Triton X-100–insoluble fractions from WT and LRRK2-deficient macrophages infected with S. Typhimurium (Fig. 3 d) or transfected with flagellin (Fig. 3 e). The Triton X-100–insoluble fractions were treated with the cross-linking agent disuccinimidyl suberate (DSS) to analyze the oligomerization of ASC. Consistent with decreased ASC speck formation in LRRK2-deficient macrophages (Fig. 3, b and c), the formation of the ASC dimer and oligomer in LRRK2-deficient macrophages was largely reduced compared with that in the WT controls (Fig. 3, d and e). Collectively, these data indicate that LRRK2 deficiency reduces the formation of ASC specks in response to NLRC4 inflammasome activation.

Figure 1. LRRK2 is critical for NLRC4 inflammasome activation. (a and b) LPS-primed WT and Lrrk2−/− peritoneal macrophages were treated with 1 µg/ml LFn-PrgJ and anthrax-protective antigen (PA) or 1 µg/ml LFn-flagellin + anthrax-protective antigen for 1 h (b). Cell lysates and culture supernatants (Sup) were collected and immunoblotted with the indicated antibodies. (c) Peritoneal macrophages from littermate control (WT) and Lrrk2−/− mice were infected with S. Typhimurium at an MOI of 100 for 2 h. Cell lysates and culture supernatants were collected and immunoblotted with the indicated antibodies. (d) EUSA of TNF-α in cell-free supernatants from WT and Lrrk2−/− peritoneal macrophages that were either infected with S. Typhimurium at an MOI of 100 for 2 h or pretreated with LPS (500 ng/ml) for 4 h followed by stimulation with LFn-flagellin + anthrax-protective antigen (1 µg/ml), LFn-PrgJ anthrax-protective antigen (1 µg/ml), ATP (5 mM), or nigericin (20 µM) for 1 h. (e) WT and Lrrk2−/− peritoneal macrophages were primed with 500 ng/ml LPS for 4 h and then treated with ATP (5 mM) or nigericin (20 µM) for 1 h. Cell lysates and culture supernatants were collected and immunoblotted with the indicated antibodies. "Ctrl" indicates the control group. (f) EUSA of IL-1β in cell-free supernatants from WT and Lrrk2−/− peritoneal macrophages that were treated as stated in d. Data are shown as means ± SEM; *, P < 0.05; **, P < 0.01; ***, P < 0.001; determined by Student's t test; all conditions were determined in triplicate. In each panel, data are representative of at least three independent experiments.

Figure 2. WD40 of LRRK2 and LRR of NLRC4 are required for LRRK2–NLRC4 interaction

The reduced ASC speck formation in LRRK2-deficient macrophages prompted us to further examine the interaction between the LRRK2 and NLRC4 inflammasome pathways. Upon S. Typhimurium infection, both NLRC4 and NLRP3 inflammasomes are activated (Man et al., 2014; Qu et al., 2016). However, coimmunoprecipitation experiments indicated that LRRK2 only formed a complex with NLRC4 but not NLRP3 in macrophages after S. Typhimurium infection (Fig. 4 a). Consistently, LRRK2 specifically pulled down NLRC4 but not NLRP3 or ASC in HEK293T cells (Fig. 4 b), suggesting that LRRK2 may specifically interact with NLRC4. Indeed, recombinant LRRK2 protein was able to form a complex with purified NLRC4 in vitro (Figs. 4 c and S1). LRRK2 is a large cytosolic protein consisting of a leucine-rich repeat (LRR) domain, a Ras of complex (ROC) GTPase domain, a C terminus of ROC (COR) domain, a kinase domain, and a WD40 repeat domain. We performed structure–function analysis to determine the relative contribu-
tions from the different domain or domains to the interaction with NLRC4. Using overexpressed full-length and different domains of LRRK2, we found that LRRK2 interacted with NLRC4 primarily via the WD40 domain (Fig. 4d). In addition, we generated deletion mutants of NLRC4 lacking the caspase activation and recruitment domain (CARD; ΔCARD), the LRR domain (ΔLRR), or both the CARD and LRR domains (ΔCARD + LRR) and tested their ability to interact with LRRK2. Interestingly, deleting the LRR domain of NLRC4 was sufficient to abolish its interaction with LRRK2 (Fig. 4e). Further analysis showed that the WD40 domain of LRRK2 formed a complex with the LRR domain of NLRC4 when coexpressed in 293T cells, implicating them as the interacting surfaces for the LRRK2 and NLRC4 (Fig. 4f). The crystal structure of NLRC4 suggests that the NLRC4 LRR domain sequesters the protein as a monomeric state in an auto-inhibitory conformation (Hu et al., 2013). The LRR deletion leads to a constitutively active NLRC4 in processing of IL-1β. Therefore, our data imply that LRRK2 may be required for releasing the self-inhibition of NLRC4 to activate inflammasome assembly.

Kinase activity of LRRK2 is required for NLRC4 inflammasome activation

Interestingly, the LRRK2 kinase inhibitor GSK2578215A (Reith et al., 2012; Saez-Atienzar et al., 2014) greatly reduced the formation of the endogenous LRRK2–NLRC4 complex in S. Typhimurium–infected macrophages (Fig. 4g). Pretreatment with the LRRK2 inhibitors (LRRK2–IN-1 [Deng et al., 2011] or GSK2578215A) noticeably attenuated ASC polymerization, caspase-1 activation, and IL-1β cleavage in S. Typhimurium–infected peritoneal macrophages (Fig. 5, a and b). Of note, LRRK2 inhibitors prevented the phosphorylation of LRRK2 at Ser935 in macrophages, an event previously shown to correlate with suppression of LRRK2 kinase activity (Fig. 5b). Furthermore, these LRRK2 inhibitors were able to suppress the caspase-1 activation and pro–IL-1β cleavage induced by the NLRC4 inflammasome activator flagellin (Fig. 5c), implicating the kinase activity of LRRK2 in NLRC4 inflammasome activation.

To validate this observation, we reconstituted NLRC4 inflammasome components in HEK293T cells, which allowed us to test LRRK2 mutants with defective and enhanced ki-
nase activity. In the reconstituted system, although overexpression of WT LRRK2 significantly increased caspase-1 activation (Fig. 5 d) and IL-1β secretion (Fig. 5 e), the kinase-dead mutant LRRK2 D2017A (Johnson et al., 1996; Nolen et al., 2004; Matikainen-Ankney et al., 2016) failed to promote IL-1β production (Fig. 5, d and e). In contrast, the PD-associated G2019S LRRK2 mutant, which possesses enhanced kinase activity (Zimprich et al., 2004; Inzelberg et al., 2012), exhibited increased ability to induce IL-1β production compared with the WT protein (Fig. 5, d and e). These data suggest that LRRK2 promotes NLRC4 inflammasome activation through its kinase activity. We then sought to confirm this finding by testing macrophages expressing WT and LRRK2 mutants. Because primary macrophages are resistant to viral transduction, we used CRISPR–Cas9 to generate LRRK2-deficient immortalized BMDMs (iBMDMs). The LRRK2-deficient iBMDMs were restored with WT LRRK2, LRRK2 G2019S (hyperactive), LRRK2 D2017A (kinase...
Figure 4. **LRRK2 directly interacts with NLRC4 to promote inflammasome assembly.** (a) Peritoneal macrophages from WT and *Lrk2*<sup>−/−</sup> mice were infected with S. Typhimurium at an MOI of 50 for 45 min. Uninfected cells were included as controls. Cell lysates were then immunoprecipitated (IP) using anti-LRRK2 antibody and were immunoblotted with the indicated antibodies. Whole-cell lysates are shown as the input. (b) LRRK2 interacts with NLRC4 but not NLRP3 and ASC. The HEK293T cells were transfected with Myc-tagged LRRK2 and cotransfected with/without Flag-tagged NLRC4, NLRP3, or ASC as indicated. Cell lysates were immunoprecipitated with anti-Flag and analyzed by immunoblot. (c) In vitro pull-down assay using recombinant LRRK2 and NLRC4 proteins: 50 ng purified 3×Flag-NLRC4 protein was incubated with 200 ng recombinant LRRK2 protein followed by immunoprecipitation with anti-LRRK2 antibody and Western blot analysis. (d) Flag-tagged WT full-length LRRK2 or different LRRK2 domains (LRR, ROC, C terminus of ROC [COR], kinase, and WD40) were coexpressed with Myc-tagged NLRC4 in 293T cells, immunoprecipitated with anti-Flag, and analyzed by immunoblot. (e) Flag-tagged full-length NLRC4 and the NLRC4 mutants ΔLRR (NLRC4 lacking LRR domain), ΔCARD (NLRC4 lacking CARD), or only nucleotide-binding domain (NBD) do-
dead), or empty vectors. Consistent with our finding in the reconstituted system, the LRRK2 D2017A (kinase dead) failed to restore ASC oligomerization (Fig. 5 f), caspase-1 activation, and IL-1 β cleavage (Fig. 5 g) in response to S. Typhimurium infection, whereas the hyperactive LRRK2 G2019S mutant showed an enhanced ability to promote inflammasome activation (Fig. 5, f and g) in the iBMDMs. Collectively, our results demonstrate that the kinase activity of LRRK2 is required for NLRC4 inflammasome activation.

LRRK2 phosphorylates NLRC4 at Ser533 upon inflammasome activation

The reduced ASC speck formation in LRRK2-deficient macrophages (Fig. 3) indicated that ASC oligomerization was impaired in the absence of LRRK2 during NLRC4 inflammasome activation. Biochemical evidence suggests that formation of ASC oligomers is triggered by and subsequently to the activation of NLRC4 protein (Broz et al., 2010). A critical step in the activation of NLRC4 is the phosphorylation at the Ser533 on NLRC4 (Qu et al., 2012; Matusiak et al., 2015). Because LRRK2 forms a complex with NLRC4 and the kinase activity of LRRK2 is required for optimal NLRC4 inflammasome activation, we hypothesize that LRRK2 may promote NLRC4 phosphorylation.

To determine LRRK2-mediated NLRC4 modification, we performed tandem mass spectrometry analysis for NLRC4 protein immunoprecipitated from LRRK2 and NLRC4-coexpressing HEK293 cells. With 66% of the NLRC4-unique peptides captured from the sample, pSer533 was the only phosphorylated residue detected (Fig. S2, a and b). In support of this, NLRC4 Ser533 phosphorylation was significantly attenuated in the LRRK2-deficient macrophages in response to NLRC4 inflammasome activators (Fig. 6, a–c). Moreover, overexpression of LRRK2 enhanced phosphorylation of WT NLRC4 but not NLRC4 S533A in HEK293T cells (Fig. 6 d). Consistently, immunoprecipitated LRRK2 from HEK293T cells induced the phosphorylation of purified NLRC4 (Fig. 6 e). Correlated with exaggerated NLRC4 inflammasome activation (Fig. 5, d and e), LRRK2 G2019S protein showed an enhanced ability to promote NLRC4 Ser533 phosphorylation compared with the WT protein (Fig. 6 e). However, the LRRK2 kinase-dead mutant D2017A failed to induce NLRC4 phosphorylation (Fig. 6 e). These data suggest that NLRC4 may be a direct substrate for LRRK2. PKCδ was the first kinase identified to phosphorylate NLRC4 Ser533 during S. Typhimurium infection (Qu et al., 2012). Using PKCδ as a positive control, recombinant LRRK2 was indeed able to phosphorylate NLRC4 at Ser533 (Figs. 6 f and S1). Consistently, LRRK2 inhibitors suppressed LRRK2- but not PKCδ-induced NLRC4 Ser533 phosphorylation in the in vitro kinase assay (Fig. 6 g). We noted that LRRK2 deficiency did not completely abolish the phosphorylation of NLRC4, suggesting that an additional kinase or kinases may contribute to NLRC4 phosphorylation (Fig. 6, a–c). Knockdown of PKCδ in LRRK2-deficient BMDMs further attenuated NLRC4 Ser533 phosphorylation and completely abolished caspase-1 and pro–IL-1β cleavage (Fig. 6 h), suggesting that LRRK2 and PKCδ may play complementary roles in phosphorylating NLRC4 at Ser533 and promoting NLRC4 inflammasome activation.

LRRK2 kinase activity is required for host defense against S. Typhimurium infection

Given the critical role of LRRK2-mediated NLRC4 Ser533 phosphorylation during NLRC4 inflammasome activation, we tested whether LRRK2 kinase activity is required for host defense against S. Typhimurium infection. To this end, we used the intraperitoneal injection of the S Typhimurium model in which LRRK2 is required for NLRC4-dependent IL-1β production and pathogen clearance (Fig. 2). Intraperitoneal injection of the LRRK2 inhibitor GSK2578215A 1 h before infection completely abolished LRRK2 Ser935 phosphorylation in peritoneal cells from S. Typhimurium–infected mice (Fig. 7 a). IL-1β levels in the PCF and in the sera were significantly reduced in the LRRK2 inhibitor–treated mice compared with that in the control mice (Fig. 7 b). The reduction in IL-1β production was associated with reduced cell infiltration in the peritoneal cavity (Fig. 7 c) and increased colonization and dissemination of bacteria (Fig. 7 d).

Next, we took advantage of a transgenic mouse strain that carries a hyperactive LRRK2 mutant gene, the LRRK2 G2019S, to further validate the physiological function of LRRK2 kinase activity during host defense. Genotyping of LRRK2 G2019S transgenic mice by RT-PCR and sequencing of cDNA from macrophages and brain tissues suggested human LRRK2 G2019S expression (Fig. S3). Restoration of LRRK2-deficient iBMDMs with LRRK2 G2019S led to enhanced NLRC4 inflammasome activation after S. Typhimurium infection (Fig. 5 g). Consistently, macrophages from transgenic mice expressing LRRK2 G2019S showed exaggerated caspase-1 activation, IL-1β production, and ASC polymerization in response to S. Typhimurium infection compared with the littermate controls (Fig. 7, e and f). The LRRK2 G2019S transgenic mice also exhibited enhanced IL-1β production (Fig. 7 g), increased cell infiltration (Fig. 7 h), and improved pathogen clearance (Fig. 7 i) in response to acute...
LRRK2 kinase activity is required for NLRC4 inflammasome activation. (a) LPS-primed peritoneal macrophages were treated with LRRK2 inhibitors 2 µM LRRK2–IN-1 (IN-1) or 2 µM GSK2578215A for 1 h and then infected with S. Typhimurium at an MOI of 50 for 1 h. Cells were lysed with Triton X-100–containing buffer followed by cross-linkage of insoluble fractions with DSS to capture ASC oligomers. Insoluble fractions (Insoluble + DSS) and soluble fractions were detected with antibody to ASC by immunoblot. (b and c) LPS-primed macrophages were treated with LRRK2 inhibitors either by 2 µM LRRK2–IN-1 or 2 µM GSK2578215A for 1 h and then infected with S. Typhimurium at an MOI of 100 for 2 h (b) or stimulated with 1 µg/ml LFn-flagellin and anthrax-protective antigen (PA) for 1 h (c). Cell lysates and culture supernatants (Sup) were collected and blotted with the indicated antibodies. (d and e) Reconstitution of the NLRC4 inflammasome activation in HEK293T cells: HEK293T cells were plated in six-well microplates. The cells were transfected with plasmids expressing Flag-tagged human pro–IL-1β (500 ng/well), pro–caspase-1 (300 ng/well), and NLRC4 (500 ng/well) with or without plasmid encoding Myc-tagged LRRK2 WT, G2019S, or D2017A mutants for 24 h. IL-1β cleavage was assessed by immunoblot (d) and ELISA analysis of IL-1β level (e) in the supernatants. Data are shown as means ± SEM; ***, P < 0.001; determined by one-way ANOVA followed by Bonferroni’s post hoc test. (f) LRRK2-deficient iBMDMs were generated by CRISPR-Cas9–mediated deletion. For rescue experiments, Myc-tagged human LRRK2 WT, G2019S, or D2017A mutants were stably expressed in the LRRK2-deficient iBMDMs. These cells were primed with LPS and then infected with S. Typhimurium at an MOI of...
S. Typhimurium infection in the peritoneal cavity. Collectively, these data show that LRRK2 kinase activity is required for host defense against S. Typhimurium infection.

DISCUSSION

In this study, we report an important physiological role for LRRK2 in the host defense against S. Typhimurium infection. We found that the activation of NLRC4 but not NLRP3 inflammasome was impaired in LRRK2-deficient macrophages. Lrrk2−/− mice exhibited decreased IL-1β production after S. Typhimurium infection, resulting in increased bacterial colonization and a higher mortality rate. Mechanistically, LRRK2 was shown to be in a complex with NLRC4 in macrophages after S. Typhimurium infection, implicating the LRRK2–NLRC4 interaction during NLRC4 inflammasome activation. In support of this, LRRK2 deficiency blunted NLRC4 Ser533 phosphorylation induced by NLRC4 inflammasome activators, and the kinase activity of LRRK2 is required for the optimal activation of NLRC4 inflammasomes. Moreover, the in vitro kinase assay showed that purified LRRK2 was able to promote the phosphorylation of NLRC4 at Ser533. In summary, our study identified a novel function of LRRK2 as a critical kinase for NLRC4 activation during the host defense response.

The activation of NLRC4 is an important biochemical event during NLRC4 inflammasome activation. A previous study has shown that the LRR domain helps to sequester NLRC4 in an autoinhibitory conformation (Hu et al., 2013). We found that the LRR domain of NLRC4 is required for its interaction with LRRK2 (Fig. 4 e). It is possible that the LRR domain of NLRC4 mediates the interaction with LRRK2. The binding of LRRK2 to the LRR domain of NLRC4 might result in conformational change that activates NLRC4, permitting subsequent phosphorylation and oligomerization of NLRC4.

Our data also indicate that the kinase activity of LRRK2 is required for the optimal activation of the NLRC4 inflammasome (Fig. 5). The kinase activity of LRRK2 has been of particular interest because most of the pathogenic LRRK2 mutations appear to have increased kinase activity (Rudenko et al., 2012). Intriguingly, we found that expression of LRRK2 G2019S, the most common pathogenic mutant associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f). This observation is consistent with the emerging concept of PD as an inflammatory disease. A recent study showed that increased serum levels of IL-1β are associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f).

Increased bacterial colonization and a higher mortality rate are associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f). This observation is consistent with the emerging concept of PD as an inflammatory disease. A recent study showed that increased serum levels of IL-1β are associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f). This observation is consistent with the emerging concept of PD as an inflammatory disease. A recent study showed that increased serum levels of IL-1β are associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f). This observation is consistent with the emerging concept of PD as an inflammatory disease. A recent study showed that increased serum levels of IL-1β are associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f). This observation is consistent with the emerging concept of PD as an inflammatory disease. A recent study showed that increased serum levels of IL-1β are associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f). This observation is consistent with the emerging concept of PD as an inflammatory disease. A recent study showed that increased serum levels of IL-1β are associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f). This observation is consistent with the emerging concept of PD as an inflammatory disease. A recent study showed that increased serum levels of IL-1β are associated with PD, enhanced caspase-1 activation and IL-1β production in response to NLRC4 inflammasome activation (Fig. 7, e and f).
LRRK2 phosphorylates NLRC4 Ser533 for NLRC4 inflammasome activation. (a–c) BMDMs from littermate control (WT) and Lrrk2−/− mice were infected with S. Typhimurium at an MOI of 50 for 1 h (a) and then stimulated with 1 µg/ml LFn-flagellin and anthrax-protective antigen (PA) for 30 min (b) or 1 µg/ml LFn-PrgJ and PA (c) for 20 min. Cell lysates were collected and blotted with the indicated antibodies. Relative quantitation of NLRC4 phosphorylation was performed by ImageJ. Each relative pNLRC4 value was normalized to total NLRC4 protein and to the WT control. Each condition was performed at least in triplicate. Data are shown as means ± SEM; *, P < 0.05; determined by Student’s t test. (d) Flag-tagged WT or NLRC4 mutant S533A were coexpressed with Myc-tagged LRRK2 in HEK293T cells. Cell lysates were collected and blotted with the indicated antibodies. (e) In vitro kinase assay. Immunoprecipitated Myc-tagged LRRK2 WT, G2019S, or D2017A were mixed with purified Flag-tagged WT or NLRC4 mutant S533A (50 ng as substrates), respectively. pNLRC4 and protein inputs were analyzed by immunoblot. (f) In vitro kinase assay. Purified human LRRK2 (200 ng) or PKCδ protein (50 ng) were mixed with immunoprecipitated Flag-tagged WT or NLRC4 mutant S533A (50 ng as substrates), respectively. NLRC4 phosphorylation was analyzed by immunoblot. (g) In vitro kinase assay with inhibitors. Purified recombinant LRRK2 (200 ng) or PKCδ protein (50 ng) were treated with LRRK2 inhibitor GSK2578215A (GSK) and mixed with purified Flag-tagged WT NLRC4 (substrates). NLRC4 phosphorylation and protein inputs were analyzed by immunoblot.
and all chemical reagents were bought from Sigma-Aldrich unless otherwise specified.

Cell culture
Unless otherwise indicated, all cells were cultured at 37°C in 95% air and 5% CO₂. Peritoneal macrophages were elicited by intraperitoneal injection of 1 ml BBL thioglycollate medium, brewer modified (4%; BD), and then they were recovered 4 d later by peritoneal lavage with 5 ml PBS. The peritoneal macrophages were cultured in DMEM cell culture medium (Gibco) containing 10% FBS, 1% penicillin, and streptomycin. BMDMs were obtained from bone marrow of the tibia and femur and cultured in DMEM with 20% FBS, 20 ng/ml mouse M-CSF (PeproTech), and penicillin/streptomycin for differentiation and proliferation. Cells were differentiated for 6 d and then replated and used for experiments the next day. HEK293T cells were cultured in DMEM cell culture medium containing 10% FBS, 1% penicillin, and streptomycin.

Salmonella infection in vitro
The S. Typhimurium (14028; ATCC) strain was grown in Luria-Bertani medium at 37°C for overnight culture. Overnight-cultured bacteria were diluted at 1:100 and cultured for another 3 h to induce SPI-1 expression. Bacteria were added to macrophages at a multiplicity of infection (MOI) of 50–100, and gentamycin (50 µg/ml) was added after 30 min to kill the remaining extracellular bacteria.

Inflammasome activation
To induce inflammasome activation, 1 × 10⁶ cells were plated in a 12-well plate overnight, and the medium was changed to Opti-MEM the next morning before stimulation. For NLRP3 inflammasome activation, cells were primed with LPS (500 ng/ml) for 4 h. After that, the cells were stimulated with ATP (5 mM) or nigericin (10 µM) for 1 h. For NLRP3 inflammasome activation, the cells were either infected with ATP (5 mM) or nigericin (10 µM) for 1 h. For NLRP3 inflammasome activation, cells were either infected with S. Typhimurium (MOI 50–100) or purified S. Typhimurium flagellin (2 µg/ml; Sigma-Aldrich) was delivered in the cytosol using DOTAP following the manufacturer’s protocol (Roche). Alternatively, cells were treated with anthrax-protective antigen (1 µg/ml) and 1 µg/ml LFn-flagellin or LFn-PrgJ. For all experiments, cells were lysed with 1% NP-40 supplemented with complete protease inhibitor cocktail (Roche), and cell-free supernatant was either collected for ELISA analysis or concentrated with methanol and chloroform for immunoblot.

Plasmids
The plasmid pCMV-2×Myc-LRRK2 expressing either Myc-tagged WT or mutant G2019S was purchased from Addgene (plasmids 25361 and 25362), cDNAs encoding human pro–IL-1β, ASC, and pro–caspase-1 were inserted into the mammalian expression vector pCMV-3×Flag. LRRK2 WT and various domains mutants or NLRP4 WT and domain deletion mutants were also cloned into pCMV-3×Flag vector. NLRP4 mutant S33A construct was generated by site-directed point mutagenesis. All constructs were confirmed by sequencing.

Generation of LRRK2 knockout iBMDMs by CRISPR-Cas9-mediated genome editing
The immortalized macrophage line iBMDMs derived from C57BL/6 mice (Shi et al., 2015) were provided by F. Shao. Lentiviral CRISPR–Cas9 targeting guide RNA–expressing vector (lentiCRISPRv2) was obtained from Addgene (52961). The LRRK2-knockout target sequence used was 5′-TTAGTGAGAACCACACGTG-3′. To generate LRRK2-knockout iBMDMs, lentiviruses containing the LRRK2 target sequence were used to transduce iBMDMs. Puromycin-positive iBMDMs were used by a limited dilution assay for single clones. Candidate knockout clones were screened by the T7 endonuclease I-cutting assay and identified by immunoblotting with anti-LRRK2 antibody.

Reconstitution of LRRK2-deficient iBMDMs
LRRK2-deficient iBMDMs were transfected with Myc-tagged LRRK2 WT or mutants using Lipofectamine 2000 (Thermo Fisher Scientific). After 3 d, transfected cells were selected with Geneticin (Gibco) for 1–2 wk. Expression of reconstituted LRRK2 proteins was determined by immunoblot.

siRNA-mediated interference
Primary WT and Lrrk2−/− BMDM in 12-well cell culture plates were transfected with 25 µM siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific). The siRNA specific for mouse PKCδ was obtained from Addgene and the scrambled siRNA were bought from GE Healthcare. The knockdown efficiency was determined by immunoblot.

Cytokine measurements
 Supernatants from cell cultures, serum, or PCF were analyzed for mouse IL-1β, IL-18, IL-6, or TNF with ELISA kits from eBioscience. All procedures followed the manufacturer’s instructions.

(h) Primary BMDMs from littermate control (WT) and Lrrk2−/− mice were transfected with scramble siRNA (siCon) or siRNA-targeting PKCδ (siPKCδ) and stimulated with LPS plus S. Typhimurium infection. Cell lysates and culture supernatants (Sup) were collected and blotted with the indicated antibodies. All data are representative of three independent experiments.
ASC oligomerization assay
For the generation of Triton X-100–soluble and –insoluble fractions, differentially stimulated macrophages were lysed with 50 mM Tris–HCl, pH 7.6, containing 0.5% Triton X-100, EDTA-free protease inhibitor cocktail, and phosphatase inhibitor cocktail (Roche). The lysates were centrifuged at 6,000 g for 15 min at 4°C, and the pellets and supernatants were used as the Triton X-100–insoluble and –soluble fractions, respectively. For the detection of ASC oligomerization, the Triton X-100–insoluble pellets were washed twice with TBS buffer and then were resuspended in 300 μl TBS buffer. The resuspended pellets underwent cross-linkage for 30 min at room temperature with 2 mM DSS (Thermo Fisher Scientific) and then were centrifuged for 15 min at 6,000 g. The pellets were dissolved in SDS sample buffer and subjected to immunoblotting.

NLRC4 inflammasome reconstitution in HEK293T cells
HEK293T cells were plated in six-well microplates and incubated overnight. The cells were transfected with plasmids...
expressing Flag-tagged human pro–IL-1β (500 ng/well), pro-
caspase-1 (300 ng/well), and NLRC4 (500 ng/well) with or
without plasmid-encoding Myc-tagged LRRK2 (1,500 ng/
well) or its mutants LRRK2 G2019S and D2017A using Li-

netic beads at 4°C overnight.

incubated with anti-ASC antibody and protein A/G mag-

NP-40 lysis buffer with complete protease inhibitor and then
propionimidate-2HCl (DTBP) was added to cultures 30 min
′

A cleavable imidoester cross-linker dimethyl 3,3-
dithiobis-
infected with S. Typhimurium at an MOI of 50 for 1–2 h.

The cell lysates were incubated with anti-Flag antibody and protein A/G magnetic
beads (Biotool) and subjected to immunoblot analysis. For the
endogenous interaction assay, macrophages were lysed
with NP-40 lysis buffer with complete protease inhibitor.
The cell lysates were incubated with anti-LRRK2 anti-
body and protein A/G magnetic beads at 4°C. For immu-

oblot analysis, all protein samples were dissolved in SDS

membrane was then blocked with 5% nonfat milk. After in-

munoprecipitated LRRK2 and NLRC4 in the kinase assay
samples were subjected to immunoblot analysis. 10% of the
mixture of LRRK2 and NLRC4 proteins was shown as input.

Immunofluorescence staining
Cells were treated as indicated. Cells were washed and fixed
in 4% paraformaldehyde, permeabilized with 0.1% Triton
X-100, and blocked with 1% BSA in PBS for 30 min. The
cells were incubated with anti-ASC (Santa Cruz Biotechnol-
om), horseradish per-
iody, Inc.) and then with Alexa Fluor 488–labeled antibody
to rabbit IgG (Invitrogen). Nuclei were stained with DAPI.

Cross-linking
Peritoneal macrophages from Lrrk2−/− and WT mice were
infected with S. Typhimurium at an MOI of 50 for 1–2 h.
A cleavable imidoester cross-linker dimethyl 3,3′-dithiobis-
propiomimidate–2HCl (DTBP) was added to cultures 30 min
before cell lysis. Cell extracts were obtained by addition of
NP-40 lysis buffer with complete protease inhibitor and then
incubated with anti-ASC antibody and protein A/G mag-
netic beads at 4°C overnight.

In vitro kinase assays
In vitro kinase assays were performed either by mixing im-
munoprecipitated LRRK2 and NLRC4 in the kinase assay
buffer for 60 min at 30°C or by incubating purified re-
combinant LRRK2 and NLRC4 protein in kinase buffer
under the same conditions.

NLRC4 WT and S533A mutant purification: HEK293T
cells transfected with plasmids expressing 3×Flag NLRC4 WT
or S533A mutant were lysed in NP-40 lysis buffer. The cell
lysates were incubated with anti-Flag antibody and protein
A/G magnetic beads at 4°C. Beads were washed extensively
with NP-40 lysis buffer with excessive salt concentration and
eluted with 0.5 mg/ml 3×Flag peptide (Biotool) in PBS.

Purified recombinant LRRK2 (A15197) and PKCδ
protein (P2293) were purchased from Invitrogen. Purified
human LRRK2 (200 ng) or PKCδ protein (50 ng) were incu-
bated with/without purified 3×Flag-NLRC4 WT or S533A
mutant (50 ng) for kinase assays. The reaction was stopped by
the addition of 2×SDS sample buffer and heating for 5 min
at 95°C. Proteins were subjected to SDS–PAGE followed by
immunoblotting for NLRC4 phosphorylation analysis.

In vitro pull-down assay
50 ng purified 3×Flag-NLRC4 protein was mixed with/with-
out 200 ng recombinant LRRK2 protein and incubated with
rabbit IgG or anti-LRRK2 antibody and protein A/G mag-
netic beads overnight at 4°C in cold NP-40 lysis buffer (50 mM
Tris, pH 7.6, 150 mM NaCl, 1% [vol/vol] NP-40, and complete
protease inhibitors). The beads were washed four times
with NP-40 lysis buffer. The bound proteins were eluted from
the beads by boiling in SDS–PAGE sample buffer. The eluted
samples were subjected to immunoblot analysis. 10% of the
mixture of LRRK2 and NLRC4 proteins was shown as input.

Immunoprecipitation and immunoblot
24 h after transient transfection as described in Fig. 4 (b
and d–f), Fig. 5 (d and e), Fig. 6 d, and Fig. S2 a, HEK293T
cells were lysed in cold NP-40 lysis buffer (50 mM Tris,
pH 7.6, 150 mM NaCl, 1% [vol/vol] NP-40, and complete
protease inhibitors). Cell lysates were incubated with an-
ti-Flag or anti–Myc antibody at 4°C. The proteins bound
by antibody were pulled down by protein A/G magnetic
beads (Biotool) and subjected to immunoblot analysis. For the
endogenous interaction assay, macrophages were lysed
with NP-40 lysis buffer with complete protease inhibitor.
The cell lysates were incubated with anti-LRRK2 anti-
body and protein A/G magnetic beads at 4°C. For immu-

oblot analysis, all protein samples were dissolved in SDS
sample buffer and resolved by 7.5–15% SDS–PAGE. After
electrophoresis, separated proteins were transferred onto
polyvinylidenedifluoride membrane (EMD Millipore). The
membrane was then blocked with 5% nonfat milk. After in-
cubation with primary antibody, horseradish peroxi-
dase–conjugated secondary antibody was applied. The
positive immune reactive signal was detected by enhanced
chemiluminescence (EMD Millipore).

In vitro kinase assays
HEK293T cells were transfected with plasmids expressing
Flag-tagged human NLRC4 with or without plasmid-en-
coding human LRRK2 using Lipofectamine 2000. Cell ly-
sates were immunoprecipitated with anti-Flag and protein
A/G magnetic beads. Proteins eluted with 3×Flag peptide
were resolved by SDS–PAGE, and bands corresponding with
NLRC4 were excised, reduced, alkylated, and digested with
in situ trypsin. Tryptic peptides were analyzed with a QExac-
tive hybrid quadrupole–Orbitrap mass spectrometer (Thermo
Fisher Scientific). Tandem mass spectra were searched against
target protein database using the MASCOT search en-
gine (Matrix Science Ltd).

RT-PCR and sequencing
LRRK2 G2019S transgenic mice and littermate control WT
mice were sacrificed, and peritoneal macrophages and brains
were subsequently separated and snap frozen on liquid ni-
trogen. RNA was isolated using TRIzol (Invitrogen) follow-
ing the manufacturer’s instructions. cDNA was synthesized
with a PrimeScript RT Master Mix kit (Takara Bio Inc.).
PCR was performed with Q5 High-Fidelity DNA Poly-

In vivo infection
For survival experiments, Lrrk2<sup>−/−</sup> and littermate control WT mice were infected intraperitoneally with 1 × 10<sup>2</sup> CFUs of S. Typhimurium, and mice survival was monitored daily. For the acute S. Typhimurium model, WT and Lrrk2<sup>−/−</sup> mice were infected with 1 × 10<sup>6</sup> CFUs S. Typhimurium for 6 h. Then, WT and Lrrk2<sup>−/−</sup> mice were sacrificed, and serum and PCF were collected for detection of IL-1β, IL-6, and TNF analysis by ELISA. Meanwhile, peritoneal cells were obtained, counted, and analyzed by flow cytometry. CD11b and Ly6G double-positive cells were identified as neutrophils. For bacterial burden analysis, 24 h after S. Typhimurium infection, WT and Lrrk2<sup>−/−</sup> mice were sacrificed, and then blood, PCF, and spleens were harvested, weighted, and homogenized. Systemic bacterial burdens were determined by plating dilutions on Luria-Bertani agar plates, then colonies were counted, and CFU/g of tissue was determined.

Statistical analysis
The p-values were determined by unpaired Student’s t tests unless otherwise specified as in Figs. 2 e and 5 e, where a one-way ANOVA was used followed by Bonferroni’s post hoc test. Survival rates were analyzed by the Kaplan–Meier method. P-values <0.05 were considered to be significant. All values are presented as means ± SEM unless otherwise specified.

Online supplemental material
Fig. S1 shows Coomassie blue staining of purified recombinant LRRK2 and NLRC4 proteins (NLRC4 WT and S533A mutant) and is related to Fig. 6. Fig. S2 indicates identification of phosphorylation at Ser533 of NLRC4 by mass spectrometry and is related to Fig. 6. Fig. S3 shows genotyping of LRRK2 G2019S transgenic mice by RT-PCR and cDNA sequencing and is related to Fig. 7.

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