LRRK2 Kinase Plays an Important Role in the Intracellular Survival of Brucella abortus 2308 in Murine Macrophages and in a Mouse Infection Model

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A B S T R A C T

Brucella species are facultative, intracellular bacteria that cause serious diseases in animals and people. Persistent survival inside macrophages is a major cause of chronic infections with Brucella. Leucine-rich repeat kinase 2 (LRRK2), a large multidomain protein kinase, is associated with Parkinson’s disease, chronic inflammation and intracellular infections. Hence, we monitored the activation of the LRRK2 kinase in Brucella-infected cells and evaluated the function of LRRK2 kinase in the intracellular survival of Brucella. Our results show that Brucella abortus 2308 activated the LRRK2 kinase and that the kinase activity was inhibited by a LRRK2-specific inhibitor in a dose-dependent manner. LRRK2 silencing significantly increased the Th1 immune response and reduced the replication of Brucella abortus 2308, both in vitro and in vivo. LRRK2 also enhanced the phosphorylation level of Akt, thereby inhibiting Akt-mediated humoral immune responses during Brucella infection. Collectively, these findings confirm that LRRK2 acts to reduce innate immune responses by activation of PI3K-Akt pathway, thereby contributing to the intracellular survival of Brucella abortus 2308 in murine macrophages and in a mouse infection model. Therefore, LRRK2 kinase play a key role in infections with Brucella abortus 2308, which will provide new insights into the pathogenic mechanisms used by Brucella.

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INTRODUCTION

Brucellosis, a serious zoonotic disease caused by facultative intracellular pathogenic bacteria (Buttigieg et al., 2018) threatens livestock and human health worldwide (Nina et al., 2018), causes economic loses and retards economic development (Huynh Tan et al., 2015). Brucella in fociations cause undulant fever, infertility and spontaneous abortion in livestock (Byndloss et al., 2016). However, no safe and effective Brucella vaccine is currently available for humans or other animals, as the pathogenic mechanisms exploited by Brucella are unclear.

Leucine-rich repeat kinase 2 (LRRK2), a large multidomain protein kinase encoded by the PARK8 gene, plays a key role in chronic diseases, immune response regulation (Kozina et al., 2018), inflammation (Han et al., 2017) and Parkinson's disease (Monfrini et al., 2017). The role played by LRRK2 in immunoregulation occurs in specific cell types under particular circumstances (Moehle et al., 2012). IFN-γ is able to induce LRRK2 production in macrophages, and LRRK2 is essential for bacterial survival in these cells (Wang et al., 2018). Previous studies indicate that LRRK2 has a close relationship with bacterial immune escape in tuberculosis (TB) (Stockman, 2011). Indeed, LRRK2 has been found to negatively regulate phagosome maturation to the benefit of mycobacterial replication while also impairing innate immune responses (Härtlova et al., 2018). That both Brucella and the TB-causing Mycobacterium tuberculosis (Mtb) are intracellular bacteria raises questions as to whether LRRK2 plays an important role in infections with Brucella.

Host invasion by pathogenic microorganisms can activate the PI3K-Akt pathway, thereby inhibiting...
macrophage immune responses (Liu et al., 2017). Previous studies have shown that the PI3K-Akt pathway is differentially activated by Brucella strains 16M and that this pathway can dampen immune responses and inhibit apoptosis during the early stage of a Brucella infection (Zhang et al., 2015). Interestingly, current research indicates that LRRK2 is associated with tumour necrosis factor (TNF-α), and many cell signalling pathways (Moehle et al., 2012), such as the MAPK and PI3K-Akt pathways (Wang et al., 2012).

Currently, the effect of LRRK2 linkage with the PI3K-Akt pathway on the infection of Brucella abortus 2308 and the release of host’s immune factors has not been reported yet. Therefore, this project was planned to explore the relationship between LRRK2 kinase and the intracellular survival of Brucella abortus 2308.

MATERIALS AND METHODS

Bacterial strains, cells and mice: RAW264.7 macrophage cells were obtained from Cell Resource Center, IBMS, CAMS/PUMC (Beijing, China) and were cultured in Dulbecco’s Modified Eagle’s medium (DMEM) supplemented with 10% foetal bovine serum. Brucella abortus 2308 was obtained from the Center of Chinese Disease Control and Prevention, China, was cultured in tryptic soy agar (TSA). Six-week-old female BALB/C mice were purchased from Xinjiang Center for Disease Prevention and Control (Xinjiang, China).

A cell model of Brucella infection is established: In order to establish a cell model of Brucella infection, Cells were treated with or without inhibitor (GSK2578215A: LRRK2 inhibitor, LY294002: PI3K-Akt inhibitor) for 1 h and then infected with Brucella abortus 2308 at an MOI of 100. After 1 h post-infection, gentamicin (30 g/ml, sigma-Aldrich) was added into the cell medium for 30 min to kill all of the extracellular Brucella. Cells received different treatments at 0, 4, 8, 12, 24 and 48 h.

Assay of Brucella survival inside macrophages and Cytokine production: According to the Brucella infection models. At different time post-infection, the cells lysed with 0.1% TritonX-100. The number of bacterial colony forming units (CFUs) were counted after plating them onto TSA. At 12 h post-infection, ELISAs (Thermo Fisher Scientific, USA) were used to measure TNF-α and IFN-γ expression in the supernatants.

Western blotting: According to the Brucella infection models, at different time post-infection, the cells were lysed for 20 min in cold RIPA lysis buffer. Supernatant proteins were boiled for 10 min at 100°C. After electrophoresis, separated proteins were transferred onto nitrocellulose membranes (Millpore, USA) for 1 h. The membranes were blocked with 5% skim milk for 1.5 h at 37°C and incubated with a 1:200 dilution of antibodies specific to Akt or LRRK2 (Santa Cruz Biotechnology, USA) for 1 h at 37°C. The membranes were then incubated with goat anti-rabbit IgG for 1 h. The antigens were visualised using DAB substrate (Thermo Fisher Scientific, USA).

Real-time PCR (RT-PCR): According to the Brucella infection models, at different time post-infection, 1 ml of TRIzol was added to each well and the lysate was mixed by repeated pipetting. After total RNA was extracted and purified from the lysate, avian myeloblastosis virus reverse transcriptase (Takara, Japan) was used for cDNA preparation, following the manufacturer’s instructions. Using the cDNA as a template, we performed RT-PCR using Light-Cycler 480 (Roche, Switzerland) instrumentation. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal reference. RT-PCR amplification primers as shown in the Table 1.

| Genes   | Primers           | Primer sequences (5'-3') |
|---------|-------------------|-------------------------|
| Akt     | Akt-F             | GCCATGAGGAGCATATCGATTCC |
| Akt-R   |                   | AGCCACCTGAAAGCAGAGCA    |
| LRRK2   | LRRK2-F           | CTGCCATTGCTTCCTTGTC     |
|         | LRRK2-R           | CATTGCTGTCGAAATGGTGC    |
| Interleukin | IL-1β-F | GTGCCATTGCTTCCTTGTC     |
|         | IL-1β-R           | CACAGCGACCACATCAACAG    |
| IL-6    | IL-6-F            | CTCGTTGGAAAATCTGGAATT  |
|         | IL-6-R            | CACATTGGTGCAGGATCCATC   |
| Interferon | IFN-γ-F | GCTTCCATTGGCTGGATTC    |
|         | IFN-γ-R           | GAGATCATCCATTGATTTGG    |
| GAPDH   | GAPDH-F           | CTGCCCAAGACATATCCCTC    |
|         | GAPDH-R           | GA CACATTGGGTTAGGACAC  |

Brucella infections in mice: Mice (n=20) were injected with 10 mg/kg/day GSK2578215A dissolved in PBS buffer. After 6 days of pretreatment, Brucella abortus 2308 was intraperitoneally injected into the mice, and the control mice (n=20) also were injected with PBS buffer. In the mouse survival test, mice (n=20/40) were divided into 4 groups with 5 mice in each group. We counted the numbers of dead mice once per day until the end of the experiment. For the mouse infection model, the mice were euthanised on days 3, 6 and 9 post-infection. The spleen and liver tissues were embedded in paraffin and sectioned conventionally, before staining with haematoxylin and eosin (HE) solution. The spleens were removed aseptically and weighed, and the number of bacterial CFUs in each spleen was measured. We measured TNF-α and IFN-γ levels in the blood samples collected from the mice using ELISAs kit (Thermo Fisher Scientific, USA).

Statistical analysis: SPSS Statistics 17.0 software was used for the analyses. Student’s t tests were used to compare the groups.

RESULTS

GSK2578215A (LRRK2 inhibitor) and LY294002 (PI3K-Akt inhibitor) each had an effect on cell viability: We used MTT assays to test the viability of the cells following 24 h pre-treatment with various concentrations of the LRRK2 inhibitor, GSK2578215A, or the PI3K-Akt inhibitor, LY294002. Our results indicate that both of these inhibitors reduced the cell viability at concentrations above 20 μM (Fig. 1). In contrast, at concentrations below 20 μM, neither inhibitor affected the cell viability. DMSO was used as the control.
The viability of RAW264.7 cells treated with 40 or 60 μM of each inhibitor differed significantly compared with the DMSO-treated control cells. The results are the mean values from three experiments ± SD. Statistical significance is indicated as *P<0.05 or **P<0.01.

**Table 2: Data of IL-1β, IL-6, and IFN-γ mRNA expression in the inhibitor-treated Brucella-infected cells**

| Genes    | Mock | Control | GSK2578215A group (15 μM) | LY294002 group (15 μM) |
|----------|------|---------|--------------------------|-------------------------|
| IL-1β    | 2.3±0.55  | 3.1±0.32 | 3.9±0.15***               |                         |
| IL-6     | 3.0±0.23   | 4.10±0.33** | 4.72±0.27**               |                         |
| IFN-γ    | 2.3±0.21   | 3.58±0.14** | 3.82±0.17**               |                         |

The results are the mean values from three experiments ± SD, and the statistical significance is indicated as *P<0.05 or **P<0.01.

**Infection with Brucella abortus 2308 induced LRRK2 mRNA transcription and protein expression:** We investigated whether infection with *Brucella abortus* 2308 induced LRRK2 mRNA transcription and protein expression in RAW264.7 cells. At 0, 4, 8, 12, 24, and 48 h post *Brucella* infection, western blots showed that infection with *Brucella abortus* 2308 induced varying degrees of LRRK2 protein expression between 0 and 12 h post-infection, with expression peaking at the 12 h time point, after which the protein level fell steadily. At the same time, we used RT-PCR to analyse LRRK2 mRNA transcription levels, the result showed that LRRK2 mRNA expression in the *Brucella*-infected cells was significantly higher than in the uninfected control group (P<0.05 or P<0.01), peaking at 12 h post-infection (Fig. 2A). Thus, *Brucella abortus* 2308 infection was able to induce mRNA transcription and protein expression, time-dependently.

We next investigated whether GSK2578215A affected LRRK2 expression in *Brucella*-infected cells. Western blots showed that GSK2578215A inhibited the activation of LRRK2 in a dose-dependent manner after infection with *Brucella abortus* 2308 in RAW264.7 cells. Notably, when the cells were incubated with 10 μM GSK2578215A, LRRK2 protein expression was almost completely abrogated. Additionally, after treatment with GSK2578215A at different concentrations, the LRRK2 transcript levels in the *Brucella*-infected cells were significantly higher than those in the *Brucella*-infected cells pre-treated with 10 μM of this inhibitor (Fig. 2B; P<0.05). Thus, inhibitor GSK2578215A affected LRRK2 protein and mRNA transcript levels during the *Brucella* infection in a dose-dependent manner.

**LRRK2 kinase activity affected the immune cytokine response to Brucella infection in RAW264.7 cells:** To determine the role played by LRRK2 in the humoral immune response, cells were incubated with inhibitor for 1 h. and then infected with *Brucella abortus* 2308 for 12 h. We used ELISAs to detect TNF-α (Fig. 3A) and IFN-γ (Fig. 3B) expression in the supernatants from the *Brucella*-infected RAW264.7 cells. *Brucella abortus* 2308 induced TNF-α and IFN-γ production during the early infection stages. However, the *Brucella abortus* 2308-infected inhibitor-treated cells produced significantly higher levels of TNF-α and IFN-γ at concentrations of 5, 10 and 15 μM than those of the *Brucella abortus* 2308-infected cells (P<0.05 or P<0.01). These results indicate that GSK2578215A treatment heightened the Th1 immune response in the *Brucella abortus* 2308-infected macrophages and confirm that the presence of LRRK2 protein affected cytokine production in these cells after *Brucella abortus* 2308 invasion.

**LRRK2 protein expression increased Brucella survivability in RAW264.7 cells:** We evaluated whether pretreatment with 10 μM of the GSK2578215A affected *Brucella abortus* 2308 survival and intracellular growth. From 4 to 12 h post-infection we found that the number of bacterial CFUs in untreated RAW264.7 cells infected with *Brucella abortus* 2308 gradually increased. However, compared to *Brucella*-infected untreated cell groups, the number of *Brucella* in cells decreased significantly after treatment with the GSK2578215A inhibitor (10 μM) from 8 to 24 h post-infection (Fig. 4, P<0.05 or P<0.01). Therefore, inhibition of LRRK2 expression decreased the survivability of *Brucella abortus* 2308 in RAW264.7 cells.

**Inhibition of LRRK2 expression protected mice from infection with Brucella abortus 2308:** We next evaluated whether the LRRK2 protein plays a role in protecting mice from *Brucella abortus* 2308. Mice were pretreated with GSK2578215A (10 μM) for 6 days before infection with *Brucella abortus* 2308. Administering GSK2578215A to the mice significantly increased their survival during infection with *Brucella abortus* 2308 compared with the untreated mice infected with this bacterium. However, the inhibitor treatment did not affect mouse survival per se (Fig. 5A). Furthermore, the spleens from the inhibitor-treated mice infected with *Brucella abortus* 2308 (1x10⁶ CFUs) contained significantly fewer bacterial CFUs and the weights were significantly lighter than those from the mice infected with *Brucella abortus* 2308 at 6, and 9 days post-infection (P<0.05; Fig. 5B and C). IFN-γ and IL-6 expression in the sera from mice treated with GSK2578215A was also evaluated at 3, 6, and 9 days post *Brucella abortus* infection. Mice infected with *Brucella abortus* 2308 and treated with GSK2578215A secreted higher levels of IFN-γ and IL-6 compared with the untreated mice infected mice (P<0.05; Fig. 5D). Thus, *in vivo* LRRK2 protein expression reduced Th1 humoral immunity to the benefit of *Brucella abortus* 2308, while administration of the LRRK2 GSK2578215A restrained *Brucella* growth in the mice.
Fig. 2: LRRK2 kinase activity is activated by Brucella abortus 2308 to varying degrees, and the GSK2578215A inhibitor affects LRRK2 protein and mRNA transcript levels during a Brucella infection. Western blot and RT-PCR analysis of LRRK2 kinase mRNA transcription and protein expression in Brucella-infected RAW264.7 cells at different time points (A). Cells were treated with GSK2578215A inhibitor (5, 10, and 15 μM) and then infected with Brucella abortus 2308 for 12 h. The LRRK2 kinase LRRK2 protein and mRNA transcript levels were detected by western blot and RT-PCR analysis. β-actin was used as the reference protein, and untreated cells were used as the control group (B). The results are the mean values from three experiments ± SD, and the statistical significance is indicated as *P<0.05 or **P<0.01.

Fig. 3: TNF-α (A) and IFN-γ (B) expression levels in GSK2578215A-treated RAW264.7 cells and in control cells infected with Brucella 2308. The results are the mean values from three experiments ± SD, and the statistical significance is indicated as *P<0.05 or **P<0.01.

Fig. 4: Decreased Brucella 2308 survival inside RAW264.7 cells after GSK2578215A treatment. The results are the mean values from three experiments ± SD, and the statistical significance is indicated as *P<0.05 or **P<0.01.

On day 9 post-infection with Brucella, the livers and spleens collected from the mice were used to produce pathological sections. HE staining showed massive hyperaemia and inflammatory cell infiltration in the spleens from the mice infected with Brucella 2308. We also observed severe cell degeneration, necrosis and congestion in the livers from the Brucella 2308-infected mice (Fig 6C, 6F). Moreover, after GSK2578215A administration, we observed a small amount of congestion, inflammatory cell infiltration and multinuclear cell proliferation in the spleens from the mice infected with Brucella abortus 2308. Slight cell necrosis and atrophy of the hepatic cords in the livers from...
Fig. 5: GSK2578215A protects mice from infection with Brucella. (A) Mice were pre-treated with GSK2578215A for 6 days before injecting 5×10⁸ CFU of virulent or avirulent strains of Brucella 2308, and the bacterial numbers were counted for 10 days. (B)(C) The number of Brucella 2308 bacteria in mouse spleens and spleen weight assessments. (D) IL-6 and IFN-γ serum secretion levels were determined in the live-infected mice at 3, 6, and 9 days post-infection. The results are the mean values from three experiments ± SD, and the statistical significance is indicated as *P<0.05 or **P<0.01.

Fig. 6: Pathological changes induced by Brucella abortus 2308 in the mouse model. Liver pathological sections (A to C): A: uninfected control mouse liver; B: the effect of 2308 on the liver following GSK2578215A treatment; C: the effect of 2308 on the liver. Spleen in the pathological sections (D to F): D: uninfected mouse spleen control, E: the effect of 2308 on the spleen following GSK2578215A treatment; F: the effect of 2308 on the spleen. Magnification: 400 ×

Fig. 7: PI3K/Akt signalling is involved in the LRRK2-mediated inflammatory response. (A) Western blots were performed to determine the protein levels of Akt at 4, 8, 12, 24 h post-infection with Brucella abortus 2308. (B)(C) Cells were pre-incubated with different inhibitors (15 μM of GSK2578215A or LY294002) for 1 h and then infected with Brucella abortus 2308 for 12 h, after which they were western blotted and RT-PCR was used to analyse Akt transcript and protein levels. (D) IFN-γ, IL-1β and IL-6 mRNA expression was examined by RT-PCR in the inhibitor-treated Brucella-infected cells. The results are the mean values from three experiments ± SD, and the statistical significance is indicated as *P<0.05 or **P<0.01.
the mice infected with *Brucella abortus* 2308 were also observed (Fig 6B, 6E). Thus, LRRK2 is beneficial to *Brucella abortus* 2308 persistence in mice and inhibiting its expression can augment host defence mechanisms against infection with this pathogen.

**Brucella activated the PI3K-Akt pathway, and LRRK2 linkage with the PI3K/Akt pathway is involved in the host immune response:** To further explore the regulatory mechanism exerted by LRRK2 on the *Brucella*-induced immune response, we examined the effect of LRRK2 on PI3K-Akt pathway activation. The results indicate that infection with *Brucella abortus* 2308 activates the PI3K-Akt pathway at the early stage of infection Fig 7A, 7B. Down-regulation of LRRK2 was found to significantly decrease the phosphorylation level of Akt at the mRNA and protein level (Fig 7C). In addition, compared to control group, the PI3K-Akt inhibitor (LY294002) significantly promoted the transcription of inflammatory cytokines (IL-1β, IL-6, IFN-γ) in *Brucella*-infected cells, an effect similar to LRRK 2 silencing (Fig 7D; Table 2). Thus, LRRK2 silencing may inhibit activation of the PI3K-Akt pathway, leading to activation of humoral immune defences against *Brucella* infection.

**DISCUSSION**

Macrophages, the first-line host defense against pathogenic microorganisms, are activated by cytokines (e.g., TNF-α, IFN-γ, and IL-4) and bacterial virulence factors (Mitchell et al., 2016; Salim et al., 2016). Activated macrophages trigger the activation of immunity-related signalling pathways, leading to the elimination of pathogenic bacteria (Kim et al., 2017). Previous studies have shown the clearance of almost 90% of invading *Brucella* by macrophages, but the remaining 10% can establish a safe niche for growth and reproduction in the endoplasmic reticulum (Von et al., 2012). During the infection process, *Brucella* virulence factors can change the expression of immune cell-related proteins, thereby inducing a microenvironment beneficial to its survival (Smith et al., 2013; Sankarasubramanian et al., 2015).

The LRRK2 kinase is an emerging genetic hotspot for disease associations, but its physiological function is poorly understood. Previous studies have reported that LRRK2 can modulate the immune response (Moehle et al., 2012; Wandu et al., 2015) and is especially involved in the IFN-γ response and other host responses to pathogens. Currently, studies are finding that LRRK2 is not only associated with dominantly inherited and sporadic Parkinson’s disease, but is also absolutely required for intracellular bacterial survival in the host (Härtlova et al., 2018). There is evidence supporting the notion that abrogating LRRK2 activity in macrophages may limit Mtb replication and survival (Stockman, 2011). In LRRK2 knock-out mice, the IFN-γ expression levels were significantly higher than those of the control group upon Mtb infection. As an intracellular pathogen, *Brucella* shares similar pathogenic and immune escape mechanisms with Mtb. However, the relationship between LRRK2 kinase activity and *Brucella* survival awaits exploration. Thus, we hypothesise that LRRK2 may be an important molecule that camps in the cells at the early stage of a *Brucella* infection. Coincidentally, in this study, we found that *Brucella abortus* 2308 activated the LRRK2 kinase to varying degrees. GSK2578215A is an effective and highly selective LRRK2 kinase inhibitor (Qin et al., 2017), which can inhibit LRRK2 kinase activity after infection with *Brucella abortus* 2308 in a concentration-dependent manner. A previous study showed that LRRK2 inhibits the production of inflammatory factors in macrophages by negatively regulating activated T cell nuclear factor 1 (Liu et al., 2011). Thus, we investigated whether the loss of LRRK2 would affect *Brucella*-mediated immune responses and bacteria growth. Our results indicate that LRRK2 expression reduced the expression of immune related factors and early innate immune responses against *Brucella abortus* 2308, both in vivo and in vitro. IFN-γ, a key Th1-type immune cytokine, is required for the bactericidal activity of macrophages, and as (Liu et al., 2011; Kursunel et al., 2016). TNF-α is one of the most important pro-inflammatory and pro-immune cytokines involved in type 1 cell-mediated immune responses to intracellular pathogens. TNF reportedly plays a crucial role in the control of *Brucella* infection and invasion (Lobet et al., 2018). IL-6 is important for this immune response also (Poffenberger et al., 2012). The immune capabilities of the host can directly affect the replication and internalization of intracellular bacteria, and strong immunity can inhibit the development of a persistent *Brucella* infection (Ahmed et al., 2016). IFN-γ and TNF-α, both of which are Th1-type cytokines, when produced can affect the intracellular survival of *Brucella* (Paul et al., 2015). Here, we confirmed that LRRK2 was a negative regulator of immune-related factors, such as IFN-γ and TNF-α, which could be beneficial to *Brucella abortus* 2308 survival in cells. That we observed the same results in the animal experiments as in the cell experiments underlines this point. Importantly, GSK2578215A directly decreased the growth of *Brucella abortus* 2308 in mice, weakened the liver and induced spleen lesions. GSK2578215A administration to mice also significantly increased their immune cytokine levels. Therefore, taken together, LRRK2 inhibition can be said to have successfully enhanced the Th1 immune response and reduced *Brucella abortus* 2308 replication in murine macrophages as well as in our mouse infection model.

One study reported that LRRK2 is not only associated with immune responses (Kozina et al., 2018), but may also be involved in signalling cascades such as MAPK and PI3K-Akt (Vermilyea et al., 2018). Another study reported that LRRK2 mediated PI3K complex recruitment and regulated phagosome maturation by modulating PI3P levels on phagosomes (Härtlova A et al., 2018). Akt often regulates the effects of PI3K in promoting cell survival (Damrauer et al., 2018), and activation of the PI3K-Akt pathway reportedly increased *Brucella* 16M survival and decrease Th1 immune factor release (Zhang et al., 2015). In this respect, the Th1 immune response appears to be a key driver for *Brucella* infection clearance, a conclusion we have also reached in the present study. Thus, LRRK 2 may be associated with the PI3K-Akt signalling pathway during *Brucella* infections. Our results indicate that GSK2578215A and
LY2942002 inhibitors each inhibit the phosphorylation of Akt. Interestingly, we found that both of these inhibitors acted to increase the expression of immune cytokines during Brucella abortus 2308 infections. Therefore, Akt/LRRK2 regulates macrophage function in innate immunity.

Conclusions: In summary, we identified the LRRK2 kinase as a key player in the intracellular survival of Brucella abortus 2308, both in vivo and in vitro. LRRK2 negatively regulates the immune response by recruiting the PI3K-Akt complex, thereby altering the intracellular survival and reproduction of Brucella. Collectively, these results indicate the LRRK2 linkage with the PI3K signaling pathway. Plos One 12:e0170666.

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Authors contribution: Experimental work and data collection: JY, XD and ZS; experimental facilities: YW, T

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