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Small molecules that regulate zymosan phagocytosis of macrophage through deactivation of Rho GTPases

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ABSTRACT

Phagocytosis and subsequent degradation of pathogens by macrophages play a pivotal role in host innate immune response to microbial infections. To find small molecule regulators for the investigation of complicated phagocytic process, we screened our in-house chemical library and found chemicals which inhibit phagocytosis of zymosan by macrophages. A representative compound **5a** reduced phagocytosis of zymosan in both peritoneal macrophages and RAW264.7 cells in a dose-dependent manner. Treatment of **5a** led to downregulate the key regulators of phagocytosis, Rac1, Rac2 and Cdc42, and slightly reduced phosphorylation of Akt by zymosan.

1. Introduction

Phagocytosis is a highly conserved and complex mechanism of innate immunity that has been classically defined as internalizing particles greater than 0.5 μm in diameter.[1,2] Phagocytosis is accompanied by intracellular signals that trigger cellular processes as diverse as cytoskeletal rearrangement, alterations in membrane trafficking, activation of microbial killing mechanisms, production of pro- and anti-inflammatory cytokines and chemokines, and production of molecules required for efficient antigen presentation to the adaptive immune system.[3] The phagocytic process is complicated and regulated by a variety of participants including receptors, protein kinases, effector proteins, cytoskeletal rearrangement, and Rho GTPases.[4] The Rho GTPases, Rac1, Rac2 and Cdc42, are widely known as the critical regulators of actin cytoskeletal rearrangements during the phagocytic response to a variety of extracellular stimuli.[5,6] Studies in murine Rac2-deficient macrophages have demonstrated selective defects in phagocytosis of IgG-opsonized particles.[7] Rac1-null neutrophils exhibit decreased actin cytoskeleton assembly and migration, as do Rac2-null neutrophils.[8] It has been speculated that Rac1 and Cdc42 regulate distinct processes during phagocytosis, with Cdc42 regulating pseudopod extension and Rac1 regulating phagosome closure. In addition, the activation patterns of Rac1, Rac2, and Cdc42 correlate with morphological rearrangements of phagocytosis with gain and loss of PI3K activity.[9] Indeed, PI3K signaling has been reported to be involved in the activation of the Rho family GTPases, including Rac1 and Cdc42, leading to initiation of actin polymerization.[10] Activation of macrophages by microbial pathogens is mediated through Toll-like receptors (TLRs). Recent studies also showed that Toll-like receptor (TLR)-dependent signaling induces activation of PI3K, which plays an important role in phagocytosis.[11,15] A heterodimer of TLR2/TLR6 recognizes zymosan, a cell wall preparation of *Saccharomyces cerevisiae*, mediates inflammatory signals in macrophages.[12] However, zymosan phagocytosis is mediated by a variety of phagocytic receptors including the mannose receptor, complement receptors, and Dectin-1 receptors.[13] Recent studies suggested that TLR2 signaling also modulates phagocytosis of zymosan.[14] Actin polymerization inhibitors, cytochalasin D[16] and latrunculins[17] have been reported to regulate phagocytosis of macrophage. In this study, we discovered novel small molecules that modulate phagocytosis of zymosan in macrophages, and investigated the phagocytosis-inhibitory mechanism with an active compound.

2. Results and discussion

2.1. Chemistry

Synthesis of key intermediates **3** and **4** is detailed in Scheme 1. O-alkylation of 4-bromophenol with 1-bromopropane afforded

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1-bromo-4-propoxybenzene (**1**). The Suzuki coupling[18] of compound **1** with 3-cyanophenylboronic acid yielded 4′-propoxybiphenyl-3-carbonitrile (**2**), which was converted to 4′-propoxybiphenyl-3-carboxylic acid (**3**) by hydrolysis. The acid **3** was transformed into the acid chloride **4** by oxalyl chloride. The acid **3** or the acid chloride **4** was coupled with appropriate primary amines[19] to produce biphenyl-3-carboxamide derivatives **5a–5h** (Scheme 2). Synthesis of compound **5i** commenced with 1-bromo-4-methoxybenzene. The preparation of acid **7** was achieved by the Suzuki coupling followed by hydrolysis. Compound **7** was converted to the acid chloride **8** that was coupled with diethylaminopropylamine to provide a methoxy derivative **5i** (Scheme 3).

2.2. Inhibitory activity for zymosan phagocytosis by macrophage

Flow cytometry-based phagocytosis assay was performed to evaluate activity of synthesized compounds in peritoneal macrophages and RAW264.7 (a mouse macrophage cell line), in which Cytochalasin D (CyD), an inhibitor of actin polymerization was used as a positive control (Figure 1). Cytotoxicity analysis was carried out against cells to remove chemicals having cytotoxic effect of more than 20% at 10 μM (data not shown). Approximately a thousand chemicals were screened twice to identify novel modulators for phagocytosis of zymosan by macrophages. Cells were pretreated with 10 μM of chemicals for 30 min and subjected to the phagocytosis analysis. Compound **5a** and its derivatives showed significant inhibitory function to macrophage phagocytosis (Fig. 2). A representative compound **5a** inhibited phagocytosis of zymosan in both peritoneal macrophages and RAW264.7 cells in a dose-dependent manner without cytotoxicity (Fig. 1). As a brief structure-activity relationship analysis, compounds with the *tert*-amine moiety in the side chain showed inhibitory activity compared with **5b**. Reduction of the number of carbons in the side chains (ethyl amine **5e** and methoxy **5h**) led to an almost complete loss of activity. Also, insertion of heteroatom in the *tert*-amine moiety decreased activity. The distance between functional groups and appropriate lipophilicity seemed to be important factor in retaining activity. Further optimization of the alkoxy phenyl moiety is required to establish the structure-activity relationship.

2.3. Level of active GTP-bound Rac1, Rac2, and Cdc42 are decreased by treatment with 5a

The transcription levels of phagocytosis associated receptors, Dectin-1, TLR2, TLR6 and TLR1 were not affected by pretreatment with **5a**, indicating that inhibitory activity of **5a** does not result from the down-regulation of TLR recognizing zymosan (Fig. 3a). We next investigated the effect of **5a** on pivotal regulators, the Rho-family GTPases by a pull down assay. As shown in Figure 3c, pretreatment of **5a** decreased significantly levels of active GTP-bound Rac1, Rac2 and Cdc42 under zymosan stimulation, indicating that phagocytosis inhibitory activity of **5a** is caused by deactivation of Rac1, Rac2 and Cdc42. In order to explore the effect of **5a** on activation of PI3K, another key regulator, we analyzed the amount of phosphorylated Akt. Akt is a well-known substrate of PI3K, and phosphorylation of Akt can be used as a marker for PI3K activation.[20] As shown in Figure 3b, stimulation of zymosan enhanced phosphorylation of Akt (Ser473), and zymosan induced phosphorylation of Akt (Ser473) was slightly reduced by treatment with **5a** (Fig. 3b). However, LY294002, a selective PI3K inhibitor did not dramatically reduced the efficiency of phagocytosis in spite of causing complete inhibition of phosphorylation of Akt (Figs. 1b and 3b). These results demonstrate that PI3K plays a partial role in phagocytosis of zymosan by macrophages, and activity of compound **5a** seems to be more implicated in deactivation of Rho GTPases than the PI3K /Akt signaling.

3. Conclusion

In summary, we discovered novel small molecule regulators, which regulate phagocytosis of zymosan in both peritoneal macrophages and RAW264.7 cells. Biphenyl carboxamide derivatives have been synthesized in 4 or 5 steps from 4-bromophenol. Compounds bearing the *tert*-amine group exhibited significant phagocytosis inhibitory activity in macrophages. With **5a** in hand, we investigated its mechanism of action for zymosan phagocytosis. Biochemical data showed that **5a** down-regulated the level of active GTP-bound Rac1/Rac2/Cdc42. **5a** slightly reduced the level of phosphorylation of Akt, a well-known downstream target of PI3K whereas a strong PI3K inhibitor, LY294002, showed less activity than **5a**. Taken together, activity of **5a** resulted from regulation of the Rho family GTPases and PI3K may play a partial role in zymosan phagocytosis in macrophages.

4. Experimental section

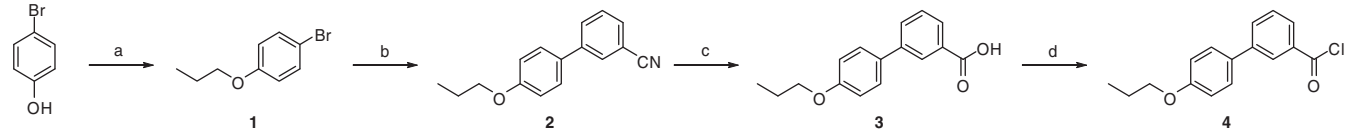
4.1. Chemistry

4.1.1. General information

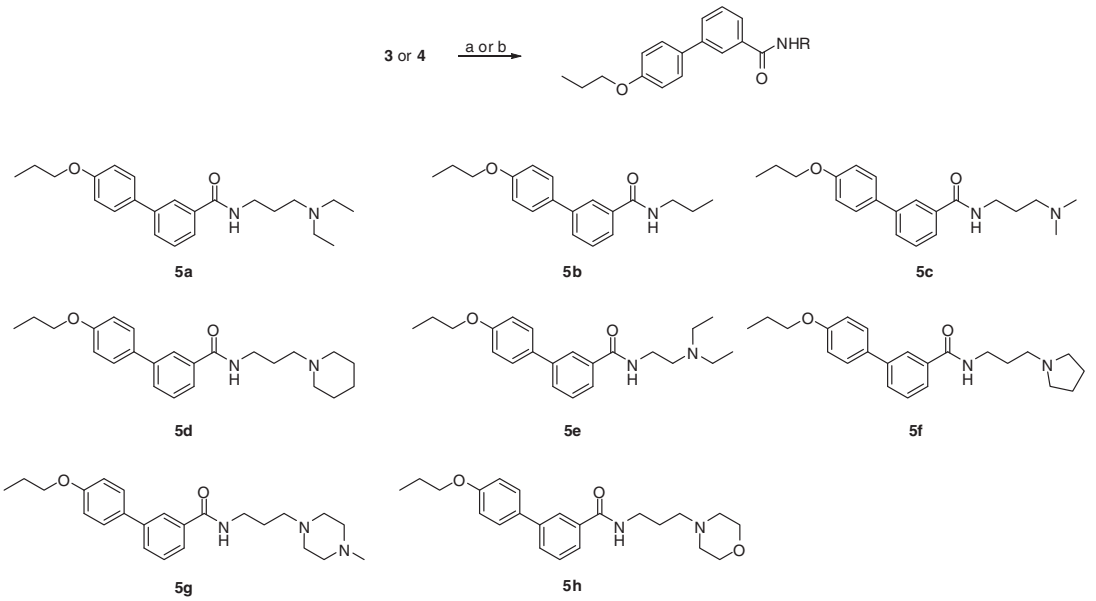
Unless otherwise described, all commercial reagents and solvents were purchased from commercial suppliers and used without further purifications. Flash column chromatography was carried out using silica-gel 60 (230–400 mesh, Merck) and preparative thin layer chromatography was used with glass-backed silica gel plates (1 mm, Merck). Thin layer chromatography was performed to monitor reactions. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA 300 (300 MHz), JEOL JNM-GCX (400 MHz) or BRUKER AMX-500 (500 MHz) spectrometers. Chemical shifts are provided in parts per million (ppm, δ) value relative to an internal standard of tetramethylsilane (internal standard) with coupling constant in hertz (Hz). Multiplicity is indicated by the following abbreviations: singlet (s), doublet (d), doublet of doublet (dd), triplet (t), quartet (q), quintet (quin) multiplet (m) and broad (br). Mass spectra and HRMS were recorded on VG Trio-2 GC-MS instrument and JEOL JMS-AX, respectively.

4.1.2. 1-Bromo-4-propoxybenzene (1)

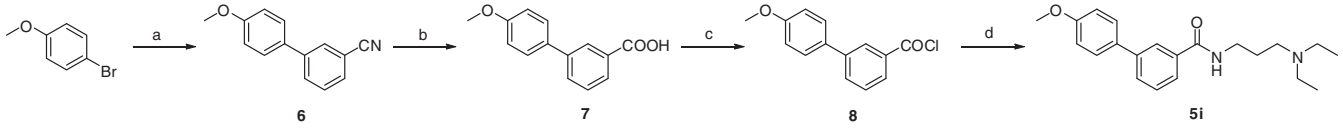
A mixture of 4-bromo-phenol (100 mg, 0.58 mmol), 1-bromopropane (160 μL, 1.73 mmol), potassium carbonate (excess) in DMF (1 mL) was stirred at 90 °C for 3 h. The reaction mixture was diluted with ethyl acetate, filtered, and the filtrate was concentrated in vacuo. The crude product was purified by flash column



Scheme 1. Reagents and conditions: (a) K_2CO_3, DMF, 90 °C, 3 h, 99%; (b) cat. $Pd(PPh_3)_4$, 2 N-$K_2CO_{3(aq)}$, THF, reflux, 1 h, 44%; (c) 2 N-$NaOH_{(aq)}$, MeOH, reflux, 20 h, 99%; (d) oxalyl chloride, cat. DMF, CH_2Cl_2, rt, 30 min.



Scheme 2. Synthesis of derivatives **5a–5h**. Reagents and conditions: (a) amine, EDC, DIPEA, CH_2Cl_2, rt, 1 h; (b) amine, DIPEA or TEA, THF, rt, 30 min.



Scheme 3. Synthesis of derivative **5i**. Reagents and conditions: (a) cat. $Pd(PPh_3)_4$, 2 N-$Na_2CO_{3(aq)}$, THF, reflux, 2 h, 73%; (b) 2 N-$KOH_{(aq)}$, EtOH, microwave, 120 °C, 30 min, 85%; (c) oxalyl chloride, cat. DMF, CH_2Cl_2, rt, 1 h; (d) diethylaminopropylamine, THF, rt, 30 min.

chromatography (EtOAc/hexane = 1:40) to afford **1** (123 mg, 0.58 mmol, 99%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.36 (d, 2H, J = 8.7 Hz), 6.77 (d, 2H, J = 8.7 Hz), 3.87 (t, 3H, J = 5.7 Hz), 1.85–1.73 (m, 2H), 1.02 (t, 3H, J = 7.2 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 158.4, 131.8, 116.0, 112.0, 69.4, 22.2, 9.5; MS(EI) m/z 214[M]$^+$.

4.1.3. 4′-Propoxybiphenyl-3-carbonitrile (2)

To a suspension of 1-bromo-4-propoxybenzene **1** (61.4 mg, 0.29 mmol), 3-cyanophenylboronic acid (50.3 mg, 0.34 mmol) and 2 N-potassium carbonate (0.2 mL, 0.2 mmol) in degased THF (1 mL) was added Tetrakis(triphenylphosphine)palladium(0) (3 mg, 0.03 mmol). The reaction mixture was refluxed for 1 h. The mixture was diluted with ethyl acetate, washed with water and brine, dried over anhydrous $MgSO_4$, filtrated, and concentrated in vacuo. The crude product was purified by flash column chromatography (EtOAc/hexane = 1:10) to afford **2** (30 mg, 0.13 mmol, 44%).

^{1}H NMR ($CDCl_3$, 300 MHz) δ 7.86–7.80 (m, 2H), 7.58–7.50 (m, 4H) 6.95 (d, 2H, J = 8.7 Hz), 3.91 (t, 3H, J = 6.3 Hz), 1.79–1.70 (m, 2H), 1.00 (t, 3H, J = 7.2 Hz); ^{13}C NMR (150 MHz, CD_3OD) δ 159.6, 142.1, 130.8, 130.7, 129.7, 129.5, 127.7, 118.4, 114.7, 112.5, 69.2, 22.2, 9.4; MS(EI) m/z 237[M]$^+$.

4.1.4. 4′-Propoxybiphenyl-3-carboxylic acid (3)

A mixture of 4′-propoxybiphenyl-3-carbonitrile **2** (30 mg, 0.13 mmol) and 2 N-NaOH (1.5 mL, 6 mmol) in methanol (3 mL) was refluxed for 20 h. The mixture was diluted with ethyl acetate, extracted with water, and then water layer was acidified with 1 N-HCl, extracted with EtOAc three times. The organic layer was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. (32 mg, 0.13 mmol, 99%).

^{1}H NMR (acetone-d_6, 300 MHz): δ = 8.26 (s, 1H), 7.99 (d, 1H, J = 8.4), 7.87 (d, 1H, J = 8.4 Hz), 7.65 (d, 2H, J = 8.7 Hz), 7.57 (t, 1H, J = 8.4 Hz), 7.06 (d, 2H, J = 8.7 Hz), 4.02 (t, 2H, J = 5.7 Hz), 1.85–1.78 (m, 2H), 1.05 (t, 3H, J = 7.2 Hz).

4.1.5. 4′-Propoxybiphenyl-3-carbonyl chloride (4)

To a solution of 4′-Propoxybiphenyl-3-carboxylic acid **3** (15 mg, 0.06 mmol) and oxalyl chloride (20 μL, 0.24 mmol) in dichloromethane (2 mL) was added a drop of DMF as a catalyst. The reaction mixture was stirred at room temperature for 1 h. The solvent was evaporated in vacuo. The crude mixture was used directly next step without any other purification.

4.1.6. General procedure for 5a–5g

A mixture of 4′-propoxybiphenyl-3-carbonyl chloride **4**, appropriate primary amine, diisopropylethylamine or diethylamine in THF (anhydrous) was stirred at room temperature for 30 min. The reaction mixture was diluted with ethyl acetate, washed with water and brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash column chromatography (CH_2Cl_2/MeOH = 10–50:1) to afford **5a–5g**.

4.1.7. *N*-(3-(Diethylamino)propyl)-4′-propoxybiphenyl-3-carboxamide (5a)

^{1}H NMR (acetone-d_6, 300 MHz): δ 8.70 (s, 1H), 8.22 (s, 1H), 7.89 (d, 1H, J = 7.5 Hz), 7.75 (d, 1H, J = 7.5 Hz), 7.69 (d, 2H, J = 8.7 Hz), 7.50 (t, 1H, J = 7.5 Hz), 7.03 (d, 2H, J = 8.7 Hz), 4.00 (t, 2H, J = 6.6 Hz), 3.52 (m, 2H), 2.88–2.75 (m, 6H), 1.99–1.77 (m, 4H), 1.17 (t, 6H, J = 7.2 Hz), 1.04 (t, 3H, J = 7.2 Hz); ^{13}C NMR (150 MHz, CD_3OH) δ 169.1, 159.2, 141.2, 134.4, 132.3, 129.3, 128.7, 127.7, 124.9, 114.6, 69.2, 49.7, 46.7, 37.3, 24.8, 22.3, 9.4, 8.8; MS(EI) m/z 368 [M]$^+$.

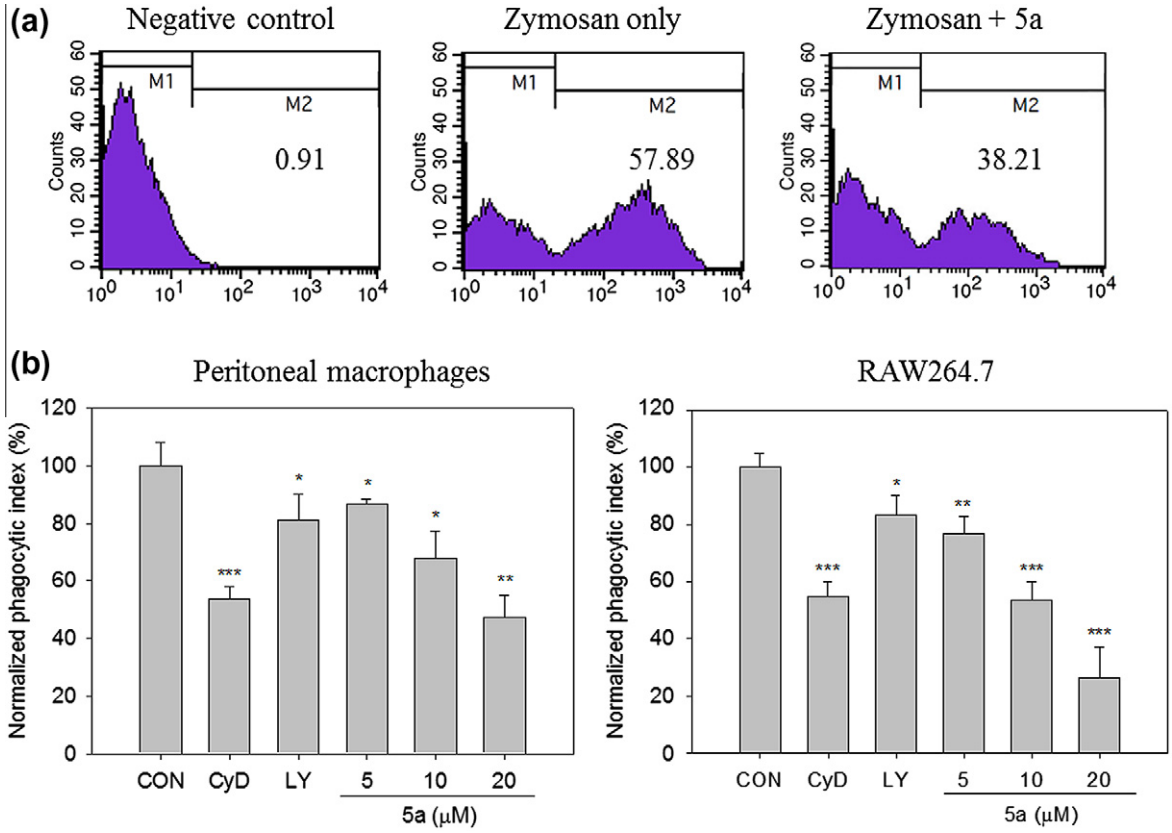


Figure 1. Phagocytosis assay using flow cytometry and the inhibitory effect of **5a** on phagocytosis of zymosan by macrophages. (a) Phagocytosis of zymosan was analyzed by flow cytometry. **5a** exhibited inhibitory activity for phagocytosis at 10 μM. (b) **5a** (5, 10 and 20 μM) inhibited phagocytosis of zymosan both in peritoneal macrophages and RAW264.7 cells in a dose-dependent manner. CyD (Cytochalasin D) and LY (LY294002) were tested at 5 and 20 μM, respectively. Error bars are in ± SD. (*P <0.05; **P <0.01, ***P <0.005, Student's t-test).

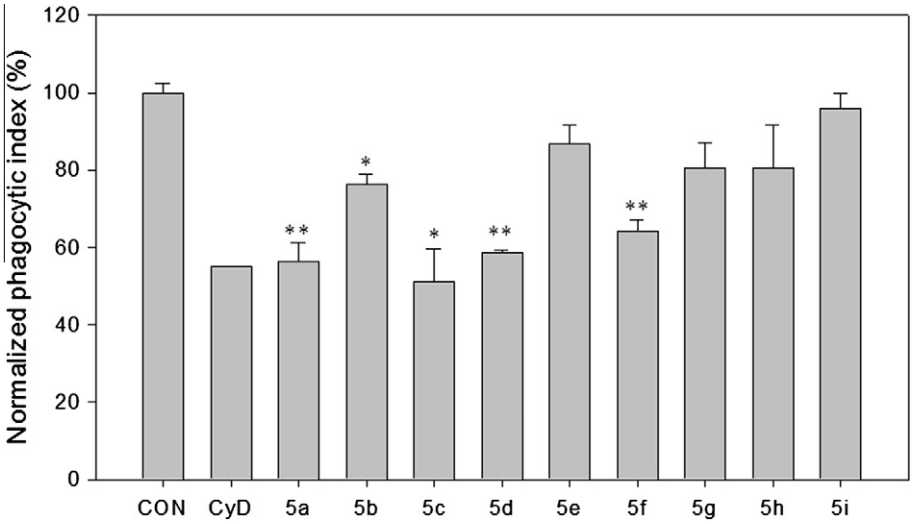


Figure 2. Activities of compounds **5a**–**5i** for phagocytosis at 10 μM. Relative phagocytic indexes (the phagocytic efficiency of cells treated with each compound relative to control cells) of RAW264.7 cells were measured for zymosan. A positive control, Cytochalasin D (CyD) was tested at 5 μM. Error bars are in ± SD. (*P <0.05; **P <0.01, Student's t-test).

4.1.8. 4′-Propoxy-*N*-propylbiphenyl-3-carboxamide (5b)

^{1}H NMR (acetone-d_6, 300 MHz): δ 8.11 (s, 1H), 7.90 (s, 1H), 7.83 (dd, 1H, J = 1.8, 7.8 Hz), 7.74 (dd, 1H, J = 1.8, 7.5 Hz), 7.63 (d, 2H, J = 9.0 Hz), 7.50 (t, 1H, J = 7.8 Hz), 7.04 (d, 2H, J = 9.0 Hz), 4.00 (t, 2H, J = 6.3 Hz), 3.41–3.34 (m, 2H), 1.87–1.75 (m, 2H), 1.69–1.57 (m, 2H), 1.04 (t, 3H, J = 7.5 Hz), 0.95 (t, 3H, J = 7.5 Hz); ^{13}C NMR (150 MHz, CD_3OH) δ 168.9, 159.1, 141.09, 134.9, 132.4, 129.1, 128.6, 127.7, 124.9, 114.5, 41.4, 22.3, 10.4, 9.4. MS (EI) m/z 297 $[M]^+$.

4.1.9. *N*-(3-(Dimethylamino)propyl)-4′-propoxybiphenyl-3-carboxamide (5c)

^{1}H NMR (acetone-d_6, 300 MHz): δ 8.68 (s, 1H), 8.21 (s, 1H), 7.88 (dd, 1H, J = 1.8, 7.8 Hz), 7.76 (dd, 2H, J = 1.8, 7.8 Hz), 7.69 (d, 2H,

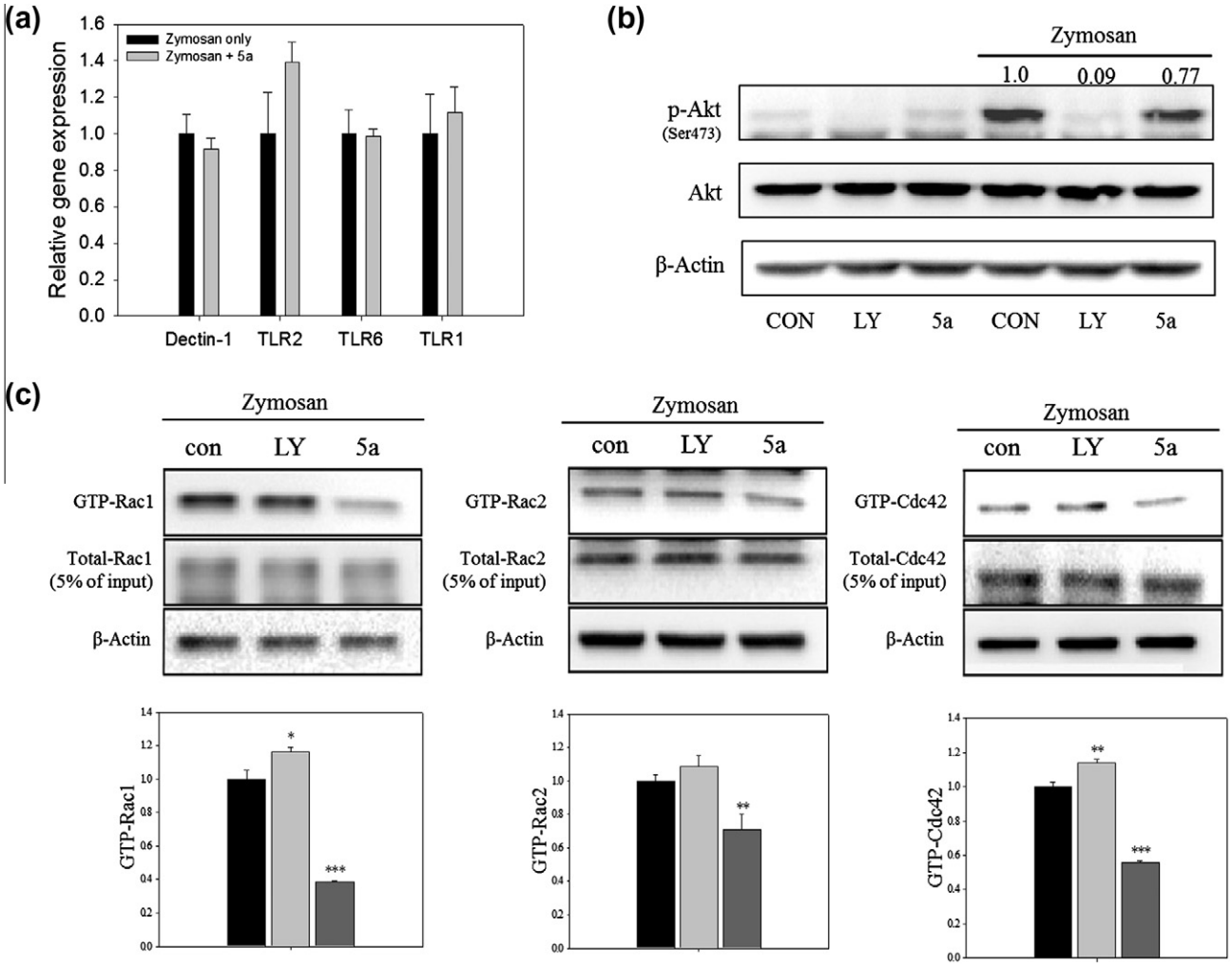


Figure 3. (a) Expression levels of phagocytic receptors, Dectin-1, TLR2, 6, and 1 were not altered by **5a** in macrophages. (b) Macrophages were pretreated with LY294002 (LY, 20 μM) or **5a** (20 μM) for 15 min, then incubated with zymosan (200 μg/ml) for 30 min. The expression levels of each molecule were determined by western blot analysis. Levels of p-Akt were quantitated by densitometric analysis and expressed as fold change above blots. (c) RAW264.7 cells were pretreated with LY294002 (20 μM) or **5a** (20 μM) for 20 min (Rac1 and Rac2), 5 min (Cdc42) then incubated with zymosan (200 μg/ml) for 15 min. GTP-bound proteins were determined by western blot analysis, and quantitated by densitometry and normalized to β-actin levels. The experiment was repeated three times and representative blots from a single experiment are shown. Error bars are in ± SD, ($^{*}P$ <0.05; $^{**}P$ <0.01; $^{***}P$ <0.005, Student's *t*-test).

J = 9.0 Hz), 7.50 (t, 1H, *J* = 7.8 Hz), 7.04 (d, 2H, *J* = 9.0 Hz), 4.00 (t, 2H, *J* = 6.3 Hz), 3.51 (q, 2H, *J* = 6.0 Hz), 2.82 (t, 2H, *J* = 6.9 Hz), 2.53 (s, 6H), 2.00–1.92 (m, 2H), 1.86–1.75 (m, 2H), 1.04 (t, 3H, *J* = 7.5 Hz); ^{13}C NMR (150 MHz, CD_3OH) δ 169.0, 159.2, 141.2, 134.5, 132.3, 129.3, 128.6, 127.7, 124.9, 114.6, 69.2, 56.3, 43.3, 37.3, 26.0, 22.3, 9.4. MS (EI) *m/z* 340 $[M]^+$.

4.1.10. *N*-(3-(Piperidin-1-yl)propyl)-4′-propoxybiphenyl-3-carboxamide (5d)

^{1}H NMR (acetone-d_6, 300 MHz): δ 8.69 (s, 1H), 8.21 (s, 1H), 7.89 (dd, 1H, *J* = 2.4, 7.5 Hz), 7.76 (dd, 1H, *J* = 1.8, 7.8 Hz) 7.69 (d, 2H, *J* = 8.7 Hz), 7.51 (t, 1H, *J* = 7.8 Hz), 7.04 (d, 2H, *J* = 8.7 Hz), 4.01 (t, 2H, *J* = 6.3 Hz), 3.51 (q, 2H, *J* = 6.3 Hz), 2.73 (s, 6H), 1.98–1.92 (m, 2H), 1.87–1.70 (m, 4H), 1.52–1.46 (m, 2H), 1.04 (t, 3H, *J* = 7.5 Hz); ^{13}C NMR (150 MHz, CD_3OH) δ 169.3, 159.2, 141.2, 134.3, 132.3, 129.4, 128.7, 127.7, 125.0, 124.9, 114.6, 69.2, 55.2, 53.3, 37.0, 24.7, 23.7, 22.3, 22.2, 9.4. MS (EI) *m/z* 380 $[M]^+$.

4.1.11. *N*-(2-(Diethylamino)ethyl)-4′-propoxybiphenyl-3-carboxamide (5e)

^{1}H NMR (acetone-d_6, 300 MHz): δ 8.17 (s, 1H), 7.85 (dd, 1H, *J* = 1.5, 7.5 Hz), 7.76 (dd, 1H, *J* = 1.8, 7.5 Hz), 7.67 (d, 2H, *J* = 9.0 Hz), 7.51 (t, 1H, *J* = 7.8 Hz), 7.03 (d, 2H, *J* = 9.0 Hz), 4.00 (t, 2H, *J* = 6.6 Hz), 3.55 (q, 2H, *J* = 6.3 Hz), 2.84–2.68 (m, 6H), 1.87–1.75 (m, 2H), 1.10 (t, 3H, *J* = 7.2 Hz), 1.04 (t, 3H, *J* = 7.2 Hz); ^{13}C NMR (150 MHz, CD_3OH) δ 169.1, 159.2, 141.19, 134.4, 132.3, 129.3, 128.7, 127.7, 124.9, 114.6, 69.2, 51.3, 47.0, 36.5, 22.3, 9.6, 9.4. MS (EI) *m/z* 352 $[M]^+$.

4.1.12. 4′-Propoxy-*N*-(3-(pyrrolidin-1-yl)propyl)biphenyl-3-carboxamide (5f)

^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.48 (s, 1H), 8.17 (s, 1H), 7.87 (d, 1H, *J* = 7.5 Hz), 7.68 (d, 1H, *J* = 7.5 Hz), 7.61 (d, 2H, *J* = 8.7 Hz), 7.47 (t, 1H, *J* = 7.2 Hz), 6.74 (d, 2H, *J* = 8.7 Hz), 3.96 (t, 2H, *J* = 6.6 Hz), 3.66 (q, 2H, *J* = 5.7 Hz), 3.13–3.05 (m, 6H), 2.14–2.06 (m, 8H), 1.89–1.77 (m, 2H), 1.05 (t, 3H, *J* = 7.5 Hz); ^{13}C NMR (150 MHz, CD_3OH) δ 169.4, 159.2, 141.25, 139.3, 134.2, 132.2, 129.5, 128.7, 127.7, 124.9, 114.6, 69.2, 53.8, 52.8, 36.6, 26.5, 22.6, 22.3, 9.4. MS (EI) *m/z* 366 $[M]^+$.

4.1.13. *N*-(3-(4-Methylpiperazin-1-yl)propyl)-4′-propoxybiphenyl-3-carboxamide (5g)

^{1}H NMR (acetone-d_6, 300 MHz): δ 8.26 (s, 1H), 8.09 (s, 1H), 7.82 (d, 2H, *J* = 7.8 Hz), 7.75 (dd, 1H, *J* = 2.1, 7.8 Hz), 7.64 (d, 2H,

J = 9.0 Hz), 7.51 (t, 1H, J = 7.8 Hz), 7.04 (d, 2H, J = 9.0 Hz), 3.48 (q, 2H, J = 6.6 Hz), 2.48 (t, 2H, J = 6.6 Hz), 2.35 (s, 8H), 2.11 (s, 3H), 1.87–1.75 (m, 2H), 1.04 (t, 3H, J = 7.2 Hz); ^{13}C NMR (150 MHz, CD_3OH) δ 168.8, 159.2, 141.2, 134.8, 132.4, 129.2, 128.6, 127.7, 124.9, 114.6, 69.2, 55.9, 54.1, 52.1, 44.3, 38.3, 25.7, 22.3, 9.4. MS (EI) m/z 395 $[M]^+$.

4.1.14. *N*-(3-Morpholinopropyl)-4′-propoxybiphenyl-3-carboxamide (5h)

To a mixture of 4′-propoxybiphenyl-3-carboxylic acid **3** (16 mg, 0.06 mmol), 3-morpholinopropylamine (18.2 μL, 0.13 mmol), diisopropylethylamine (60 mg, 0.31 mmol) in dichloromethane (2 mL), was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide at room temperature. The reaction mixture was stirred at room temperature for 1 h. The reaction mixture was diluted with dichloromethane, washed with water and brine, dried over anhydrous magnesium sulfate, and concentrated in vacuo. The crude product was purified by flash column chromatography (dichloromethane/methanol = 20:1) to afford **5h** (8.5 mg, 0.02 mmol, 35.6%).

^{1}H NMR (acetone-d_6, 300 MHz): δ 8.01 (m, 2H), 7.82 (d, 1H, J = 7.8 Hz), 7.75 (d, 1H, J = 7.8 Hz), 7.64 (d, 2H, J = 8.7 Hz), 7.52 (t, 1H, J = 7.8 Hz), 7.04 (d, 2H, J = 8.7 Hz), 4.01 (t, 2H, J = 6.3 Hz), 3.60 (t, 2H, J = 4.5 Hz), 3.58–3.45 (m, 2H), 2.50–2.40 (m, 6H), 1.85–1.75 (m, 4H), 1.05 (t, 3H, J = 7.2 Hz); ^{13}C NMR (150 MHz, CD_3OH) δ 168.8, 159.2, 141.2, 134.8, 132.3, 129.2, 128.6, 127.7, 124.9, 114.6, 69.2, 66.2, 56.4, 53.3, 38.1, 25.5, 22.3, 9.4. MS (EI) m/z 382$[M]^+$.

4.1.15. 4′-Methoxybiphenyl-3-carbonitrile (6)

Compound **6** was prepared from 1-bromo-4-methoxybenzene in a manner similar to that described for compound **2**. The crude product was purified by flash column chromatography (EtOAc/hexane = 1:10) to afford **6** (411.4 mg, 1.97 mmol, 73%).

^{1}H NMR (CD_3OD, 300 MHz): δ 7.92(s, 1H), 7.88 (d, 1H, J = 7.5 Hz), 7.65–7.55 (m, 4H), 7.03 (d, 2H, J = 8.7 Hz), 3.84 (s, 3H).

4.1.16. 4′-Methoxybiphenyl-3-carboxylic acid (7)

A mixture of 4′-methoxybiphenyl-3-carbonitrile **6** (411 mg, 1.97 mmol), 3 N-potassium hydroxide (2 mL, 6 mmol) in ethanol (5 mL) was stirred at 120 °C for 2 h in microwave reactor. After removing ethanol in vacuo, the reaction mixture was diluted with ethyl acetate, basified with 1 N-KOH, and extracted with water. Water layer was acidified with 1 N-HCl, and extracted with EtOAc. The extract was washed with brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash column chromatography (EtOAc/hexane = 1:2–5) to afford **7** (379 mg, 1.66 mmol, 84.5%)

^{1}H NMR (CD_3OD, 300 MHz): δ 8.21 (s, 1H), 7.94 (d, 1H, J = 7.5 Hz), 7.80 (d, 1H, J = 7.5 Hz), 7.58 (d, 2H, J = 9.0 Hz), 7.51 (t, 1H, J = 7.5 Hz), 7.02 (d, 2H, J = 9.0 Hz), 3.84 (s, 3H); ^{13}C NMR (150 MHz, CD_3OD) δ 168.5, 159.7, 141.0, 132.4, 131.0, 130.6, 128.5, 127.6, 127.4,, 127.2, 114.0, 54.3; MS(EI) m/z 228 $[M]^+$.

4.1.17. 4′-Methoxybiphenyl-3-carbonyl chloride (8)

4′-Methoxybiphenyl-3-carboxylic acid **7** (100 mg, 0.44 mmol), oxalyl chloride (187 μL, 2.19 mmol) were dissolved in dichloromethane (5 mL). And dimethylformamide was added to mixture as a catalyst. The mixture was stirred at room temperature for 1 h. The solvent and oxalyl chloride were evaporated in vacuo. The crude mixture was used directly next step without any other purification.

4.1.18. *N*-(3-(Diethylamino)propyl)-4′-methoxybiphenyl-3-carboxamide (5i)

To a solution of 4′-methoxybiphenyl-3-carbonyl chloride **8** (108 mg, 0.44 mmol) in anhydrous THF (10 mL), was added dropwise N^1,N^1-diethylpropane-1,3-diamine (103.2 μL, 0.66 mmol) at room temperature. The reaction mixture was stirred at room temperature for 30 min. The mixture was diluted with ethyl acetate, washed with 1 N-KOH, water and brine, dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash column chromatography (CH_2Cl_2/MeOH/Et_3N = 30:2:0.1) to afford **5i** (54 mg, 0.16 mmol, 36.4%).

^{1}H NMR ($CDCl_3$, 300 MHz): δ 8.75 (s, 1H), 8.08 (s, 1H), 7.79 (d, 1H, J = 6.6 Hz), 7.66 (d, 1H, J = 7.8 Hz), 7.59 (d, 2H, J = 9.0 Hz), 7.46 (t, 1H, J = 7.5 Hz), 6.98 (d, 2H, J = 9.0 Hz), 3.86 (s, 3H), 3.62 (q, 2H, J = 5.7 Hz), 2.77 (s, 6H), 1.14 (s, 6H); ^{13}C NMR (150 MHz, CD_3OH) δ 168.9, 159.7, 141.1, 134.6, 132.4, 129.3, 128.7, 127.7, 125.0, 124.9, 114.0, 54.4, 49.8, 46.5, 37.7, 25.0, 9.2. MS (EI) m/z 340 $[M]^+$.

4.2. Mice

Male C57BL/6 mice, 8–10 weeks old, were obtained from the Orient Bio Inc. Animals were housed in environmentally controlled, pathogen-free animal facility for the duration of the study. They were given free access to tap water and chow daily, and maintained under the following laboratory conditions of constant temperature (21 ± 3 °C), relative humidity (50 ± 10%), and illumination (12 h light/dark cycles).

4.3. Cell culture

RAW264.7 macrophage cell lines were obtained from the American Type Culture Collection (Manassas, USA) and maintained in DMEM medium (Cellgro, Manassas) supplemented with 10% heat-inactivated FBS (Fetal Bovine Serum, Cellgro), 2 mM L-glutamine, 100 U/ml penicillin and streptomycin (Cellgro) at 37 °C in a 5% CO_2 humidified incubator. The thioglycollate-elicited peritoneal cells were harvested by peritoneal lavage from C57BL/6 male mice 96 h after i.p. injection with 1 mL of sterile 4% brewer thioglycollate (Sigma Aldrich, B2551). Red blood cells were eliminated by ACK buffer and the cells were washed and resuspended in RPMI 1640 supplemented with 10% inactivated FBS, 10 mM HEPES, 2 mM glutamine, and 100 U/ml penicillin-100 mg/ml streptomycin. MACS buffer was supplemented with 0.5% bovine serum albumin (BSA), 2 mM EDTA and 100 U/ml penicillin–100 mg/ml streptomycin in PBS. Peritoneal macrophages were obtained from twenty wild-type mice to harvest enough cells for western blot analysis.

4.4. Reagents and antibodies

CD11b MicroBeads (130-049-601), MiniMACS column (130-042-201), MACS multi stand were purchased from Miltenyi Biotec. Fluorescein isothiocyanate (FITC)-conjugated zymosan (Z-2841) and unlabeled zymosan (Z-2850) were obtained from molecular probes. LY294002 (L9908) and Cytochalasin D (CTC, C2618) were purchased from Sigma Aldrich. Capture and detection antibodies (Abs) used in ELISA for TNF-α and IL-6, and FITC-mouse-anti-CD11b antibody were purchased from BD Biosciences. Rabbit Akt monoclonal Ab (mAb), mouse phosphor-Akt (Ser473) mAb, mouse IκB-α mAb, mouse phospho-IκB-α (Ser32/36) mAb, rabbit anti-Cdc42 mAb, and rabbit anti-β-actin mAb were obtained from cell signaling. Goat anti-Rac2 polyclonal Ab were purchased from Abcam. Phycoerythrin (PE) conjugated mouse anti-TLR2 mAb was purchased from eBioscience. Active Rac1 pull-down and detection kit were obtained from Thermo Scientific. The chemical compounds were provided by Professor Min KH (College of Pharmacy, Chung-Ang University).

4.5. Phagocytosis assay

Mouse peritoneal macrophages ($CD11b^+$) and RAW264.7 cells were plated 4×10^6, 2×10^6 cells/well in 24-well plates and

adhered for 2 h, respectively. Then, the cells were pretreated with CTC or other inhibitors or chemical compounds for 30 min. After pretreatment, the cells were scraped, washed, and then incubated in medium containing FITC-conjugated zymosan (5 particles/cell) for 1 h at 37 °C. After thorough washing to remove unbound zymosan, the cells were resuspended in PBS buffer and analysis was performed using FACSCalibur and Cellquest Pro software (BD biosciences, San Jose).

4.6. Pull-down assay and Western blot analyses

Utilizing respective antibodies, the Active Pull-Down and Detection Kit (Thermo Scientific, Rockford, IL, USA) was used to extract Rac1, Rac2 and Cdc42. Heat-denatured (5 min, 95 °C) cell lysates and precipitation products were separated by SDS–PAGE on 10% or 15% gels and transferred to nitrocellulose membranes. Membranes were blocked (1 h, room temperature) and incubated at 4 °C overnight with specific Abs against mouse anti-Rac1 mAb (Thermo Scientific, 1:1000), goat anti-Rac2 polyclonal Ab (abcam, 1:1000) and rabbit anti-Cdc42 mAb (cell signaling, 1:1000), rabbit anti-Akt mAb, mouse anti-phosphor-Akt (Ser473) mAb (cell signaling, 1:1000, respectively), mouse anti-IκB-α mAb, mouse anti-phospho-IκB-α (Ser32/36) mAb (cell signaling, 1:2000, respectively), rabbit anti-β-actin mAb (cell signaling, 1:5000) according to the antibody data sheets. After incubation, membranes were washed three times with TBS-T for 5 min, incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (1 h, room temperature, anti-mouse or rabbit or goat-HRP IgG, cell signaling, 1:5000), and washed again three times with TBS-T. Signals were detected using the ChemiGlow West (Alpha Innotech) by measuring chemiluminescence (BioRad Chemidoc system) and analyzed using the software Quantity One (Bio-Rad).

4.7. Total RNA isolation and Real-time PCR analysis

Cells were incubated for 30 min at 37 °C with chemical 10 (20 μM), before addition of zymosan (200 μg/ml) for 6 h. Total RNA was isolated from each sample using Trizol reagent (INVITROGEN, Carlsbad). RNA was transcribed at 42 °C for 1 h in a volume of 25 μl containing 5 × RT buffer, 10 mM dNTPs 200 units Maloney myrine leukemia virus reverse transcriptase and 100 pmole of oligo-dT primer. cDNA were amplified by real-time PCR using SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara, Shiga, Japan) on an MyiQ™2 Two-Color Real-Time PCR detection system (Bio-rad). To confirm the PCR specificity, PCR products were subjected to a melting-curve analysis. The comparative threshold method was used to calculate the relative amount of mRNA of treated sample in comparison with control samples. The primers used for the study included:

GAPDH, 5′-AATGGTGAAGGTCGGTGTGAAC-3′ (forward) and 5′-GAAGATGGTGATGGGCTTCC-3′ (reverse); Dectin-1, 5′-AGGCCCAGGGGATCAGAGAAAGG-3′ (forward) and 5′-ACCCAGCACTGCAGCAACCA-3′ (reverse); TLR2, 5′-CGGGACTTCGTTCCGGGCAA-3′ (forward) and 5′-GGCCGCGTCGTTGTTCTCGT-3′ (reverse); TLR6, 5′-AAGAAAATGGTACCGTCAGTGCTGG-3′ (forward) and 5′-AAGGCCAGGGCGCAAACAAAGT-3′ (reverse); TLR1, 5′-AAGGCCAGGGCGCAAACAAA-3′ (forward) and 5′-TGCAAGGGTAGGTCCTTGGGCA-3′ (reverse).

The number of amplification cycles was optimized in preliminary experiments to ensure that the PCR has not reached its plateau.

4.8. Statistical analysis

Data are expressed as mean ± SD, and statistical analysis were performed by using a Student's *t* test. *P* values of <0.05 were considered statistically significant.

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