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ORIGINAL ARTICLE

Trypanosoma brucei Lipophosphoglycan Activates Host Immune Responses via the TLR-mediated p38 MAP Kinase and NF-κB Pathways

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ABSTRACT

Objective: This study was aimed at investigating the immunoregulatory effects of trypanosomal lipophosphoglycan (LPG) anchored to trypanosome membranes, including the formation of neutrophil extracellular traps (NETs) and neutrophil cytokine release after parasite infection. The interaction of cell surface TLR receptors with LPG, which signals cellular responses during *Trypanosma brucei* infection, was systematically investigated.

Methods: The cytokine expression profile in neutrophils after exposure to *T. brucei* LPG, and the involvement of TLR2, TLR4, p38 MAP kinase, and NF-κB in NET formation were studied with molecular immunological approaches including quantitative PCR, western blotting and immunofluorescence.

Results: *T. brucei*-derived LPG induced phosphorylation of p38 MAP kinase and NF-κB, thereby stimulating neutrophil secretion of IL-1β, IL-8, and TNF-α. The blockade of Toll-like receptor 2/4 and specific inhibitors of MyD88, p38 MAP kinase, and NF-κB decreased cytokine release and the phosphorylation of both kinases. Furthermore, the exposure of neutrophils containing LPG to IL-1β and LPG-induced cell supernatants promoted the release of NETs.

Conclusion: Our findings suggest that *T. brucei* LPG activates neutrophil IL-1β secretion via the TLR-mediated p38 MAP kinase and NF-κB pathways, thereby promoting the formation of LPG-stimulated NETs.

Key words: *Trypanosoma brucei*, IL-1β, lipophosphoglycan, neutrophil, Toll-like receptor, p38 MAP kinase, NF-κB, neutrophil extracellular trap

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INTRODUCTION

African animal trypanosomiasis (also known as Nagana) is caused by infection with *Trypanosoma brucei*, which is transmitted by species of *Tsetse* flies and can infect many mammalian species, including horses, cattle, sheep, and monkeys, thus resulting in widespread distribution of the disease in East and Central Africa, and causing substantial economic losses in these regions [1-3]. Extracellular vesicles originating from the flagellar membrane of *Trypanosoma brucei rhodesiense* convey serum-resistant antigen into *T. brucei*, thus enabling the trypanosomes to resist congenital elimination and increasing

the risk of human infection by trypanosomes of animal origin [4].

Trypanosomes contain various virulence factors on their surfaces that elicit various immune responses inside hosts and facilitate immune evasion. Among these, lipophosphoglycan (LPG) is an important virulence factor on the surfaces of *Trypanosoma* and *Leishmania*. LPG is anchored to the surface of the parasite by a glycosylphosphatidylinositol (GPI) anchor, and it contains a glycan chain with an oligosaccharide cap [5]. As an acidic glycoconjugate, trypanosomatid LPG is characteristic by high levels of glucosamine, sialic acid, and galactosamine; in addition leishmanial LPG contains galactose, phosphatidylinositol, and phosphate [6–8]. *Leishmania* LPG is well known to be involved in the colonization of the parasites in both the sandfly midgut and mammalian macrophages [9,10]. As an immunogen, *Leishmania* LPG triggers non-canonical activation of host caspase-11 and NOD-like receptor protein 3 (NLRP3), increases chemotaxis of CXCR6-dependent macrophages via CXCL16, and induces $CD4^+$ T cell activation in human immunodeficiency viral replication [11–13].

The discovery of pattern recognition receptors and their cognate pathogen-associated molecular patterns have increased understanding of how cells of the innate immune system detect and respond to invading pathogens [14]. The most widely studied pattern recognition receptors are the Toll-like receptors (TLRs), which recognize the structures of lipids, carbohydrates, peptides, and nucleic acids that are specific to mammalian hosts [15]. TLRs consist of an extracellular domain rich in leucine repeats that trigger intracellular signaling cascades after interaction with ligands, either directly or through accessory molecules. In *Trypanosoma* and *Leishmania*, either GPI anchors or glycoinositolphospholipids stimulate immune responses in host innate immune cells. In TLR2 knockout hamster ovary cells transfected with CD14 and TLR2, *T. cruzi*-derived GPIs and glycoinositolphospholipids have been found to increase CD25 expression to levels greater than those in TLR2 knockout cells. More importantly, GPIs purified from *T. cruzi*, containing a longer glycan core and unsaturated fatty acids at the sn-2 position of the alkylglycerolipid, trigger TLR2 activation at nanomolar concentrations [16]. Lipid extracts from *T. cruzi* amastigotes specifically induce liposome formation, macrophage cyclooxygenase 2 expression, TNF-α, and IL-8, as well as nitric oxide release and NF-κB activation via a TLR2/6-dependent pathway, in HEK cells [17,18]. Macrophages infected by *T. cruzi* also release extracellular vesicles that increase the expression of pro-inflammatory cytokines (such as TNF-α, IL-6, and IL-1β), in a process involving TLR2 [19]. Furthermore, peritoneal macrophages from wild-type C57BL/6, TLR2-/-, and TLR-4-/- mice pre-activated with IFN-γ efficiently produce nitric oxide and cytokines (IL-1β, IL-6, IL-12, and TNF-α) after exposure to LPGs of different *Leishmania* species [20]. TLR2 and TLR9 have been suggested to cooperatively control parasite replication in macrophages, and *T. cruzi* infection has been shown to trigger the synthesis of IL-12/IFN-γ via TLR9 and MyD88-dependent pathways [21]. Furthermore, the TLR9 ECD-CpG DNA 3D structural complex, in which the TLR9 extracellular domain is docked with CpG DNA, has been identified in *L. dubliniensis* infection, thus confirming that the LRR11 region of TLR9 is a key region for recognition of CpG DNA of *L. donovani* [22].

As innate immune cells, neutrophils are recruited to focal lesions after pathogen invasion through chemotaxis, and perform phagocytosis or secrete immunologically active factors that confine or destroy pathogens. Neutrophil extracellular traps (NETs), a typical innate immune response mediated by neutrophils and macrophages, have been identified in many infections [23]. They have contrasting roles in not only capturing and destroying pathogens but also increasing inflammation of the affected tissues. Previous studies have indicated that cytokines modulate NET formation. IL-1β in the supernatant of macrophages promotes NET formation by human neutrophils after stimulation with monosodium uric acid crystals, and IL-1β blockade attenuates thrombosis in a NET-dependent breast cancer model [24,25]. Furthermore, we and others previously found that *T. brucei* induces NET formation after interaction with host neutrophils [26–30], and the parasite motility decreases with NET confinement [29]. These findings have indicated that NET formation by neutrophils is an anti-parasite response. We further revealed that *T. brucei* LPG is the main parasite component that, through interaction with neutrophil TLR2/4 receptors, activates NET formation and cytokine secretion [25]; however, the roles of cytokines in this process remain unclear.

In this study, we explored the cytokine expression profiles in neutrophils after exposure to *T. brucei* LPG and the involvement of TLR2, TLR4, p38 MAP kinase, and NF-κB in NET formation. We revealed that *T. brucei* LPG efficiently induces a signaling cascade in neutrophils and cytokine secretion, thus promoting NET formation.

MATERIALS AND METHODS

Animals

China's Liaoning Chang Sheng Biological Technology Company provided us with 6–8 week old female BALB/c mice for experiments. Ethical approval for all animal experiments was obtained from the Shenyang Agricultural University, China (Permit No. SYXK<Liao>2021-00010).

Parasite purification

DE52 cellulose powder was soaked in phosphate solution containing glucose, loaded into a 2.5 cm × 30 cm chromatography column, and allowed to settle freely. The upper layer of liquid was then drained, leaving 1.5 cm of liquid in the column. Two milliliters of blood from

T. brucei Lister 427-infected mice (containing approximately 5×10^7 cell mL^{-1} parasites) with heparin as anticoagulant was added to the chromatography column. Additional phosphate-buffered saline (PBS) was added for the separation of blood cells from the parasites, which appeared first in the fraction. The *T. brucei* parasites were collected and concentrated by centrifugation at 2,500 rpm for 10 min [31].

Lipophosphoglycan purification

The LPG component from isolated parasites (1×10^9) was extracted with previously described methods [28,32]. Briefly, the purified *T. brucei* parasites from infected mouse blood were washed with sodium phosphate buffer and extracted with a 20 × volume of chloroform/methanol/water (4:8:3, *v*/*v*) for 1 h at room temperature with continuous vigorous shaking. The insoluble fraction was collected by centrifugation at 3,500 rpm for 30 min, and the precipitate was extracted twice with water-saturated 1-butanol at 4°C, with continuous stirring, for 18 h each time. The solution was than centrifuged at 10,000 × *g* for 30 min. LPG in the supernatant was lyophilized and washed with chloroform/methanol (2:1, *v*/*v*), then further purified with graded separation by octyl agarose chromatography with a 1-propanol gradient (5–60%) in 0.1 M 2-[tris(hydroxymethyl)methyl]-amino ethanesulfonate buffer.

Neutrophil extraction

Neutrophils were isolated from healthy mouse blood with a Mouse Peripheral Blood Neutrophil Isolate Kit (Solarbio, Beijing, China) [33-35]. Briefly, 4 mL of Reagent A was added to a centrifuge tube, and 2 mL of Reagent C was carefully added on top of Reagent A. Two milliliters of anticoagulant blood was added on top of the separation solution and centrifuged at room temperature in a horizontal rotor at 1,000 × *g* for 30 min. The layer of neutrophils between Reagents C and A was aspirated, transferred with a pipette to a clean 15-mL centrifuge tube, and washed with 10 mL PBS. The cells were resuspended with 5 mL PBS and incubated at 37°C for 5 min, then washed with PBS after centrifugation at 250 × *g* for 10 min. Purified neutrophils were cultured at 37°C in RPMI 1640 medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal bovine serum in a CO_2 incubator.

Detection of cytokines secreted from neutrophils after treatment with *T. brucei* LPG or LPS

A 50 μL volume of PBS, and 50 μg mL^{-1} *T. brucei* LPG or 100 ng mL^{-1} LPS was mixed with 1×10^6 neutrophils and incubated at 37°C for 20 h. Cytokines in the supernatant were detected with an enzyme-linked immunosorbent assay (ELISA) kit (MEIMIAN, Jiangsu, China). Briefly, 25 μL diluents and 25 μL cell supernatant samples were added to the wells and mixed gently. Subsequently, 100 μL of HRP-conjugate reagent was added to each well (except the blank wells) and incubated at 37°C for 1 h. After the liquid was discarded, 100 μL of wash solution was added to each well and incubated for 60 s; this process was repeated five times. Fifty microliters each of chromogen solutions A and B was added to the wells and incubated for 15 min at 37°C in the dark before termination of the reaction by addition of 50 μL of Stop Solution. The absorbance of each well was measured at A450 nm with a spectrophotometer. The absorbance of the blank wells were set to zero.

Quantitative PCR and western blotting analyses of TLR2 and TLR4 expression in neutrophils after treatment with *T. brucei* LPG

Total RNA was extracted from neutrophils pretreated with PBS, various concentrations of *T. brucei* LPG (0, 5, 25, 50, or 100 μg mL^{-1}), and 100 ng mL^{-1} LPS at 37°C for 20 h. Real-time PCR was performed with SYBR® Premix Ex *Taq*™ (TaKaRa, Otsu, Japan) with a QuantStudio 6 system (ABI, Waltham, MA, USA). The sequences of the specific primers were as follows: *TLR2* (5′-GAC TCT TCA CTT AAG CGA GTC T-3′ and 5′-AAC CTG GCC AAG TTA GTA TCT C-3′); *TLR4* (5′-GCC ATC ATT ATG AGT GCC AAT T-3′ and 5′-AGG GAT AAG AAC GCT GAG AAT T-3′); and β-actin (5′-ACG GTC AGG TCA CTA TCG-3′ and 5′-GGC ATA GAG GTC TTT ACG GAT G-3′). The relative transcription levels of the genes were analyzed with the $2^{-\Delta\Delta Ct}$ method.

The expression of TLR2 and TLR4 was further confirmed by western blotting with protein specific antibodies, with β-actin as a control. The neutrophils mixed with 1 × SDS-PAGE loading buffer were boiled for 10 min, and the denatured proteins were resolved by electrophoresis in a 10% polyacrylamide gel. A Mini Trans-blot system (Bio-Rad, Hercules, CA, USA) was used to transfer the proteins onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated overnight with rabbit polyclonal anti-TLR2 IgG (1: 1,000, Beyotime, Shanghai, China), rabbit polyclonal anti-TLR4 IgG (1: 1,000, Beyotime), and monoclonal anti-β-actin IgG (1: 5,000, Beyotime) after blocking at 37°C for 1 h with 5% (w/v) skimmed milk. After washing, we incubated the polyvinylidene difluoride membranes with horseradish peroxidase-conjugated goat anti-rabbit IgG (1: 1,000, Beyotime) at 37°C for 1 h. Finally, the target proteins were visualized with chemiluminescence (Clinx, Shanghai, China).

Detection of the effect of TLR2/4 blockade with specific antibodies or signaling pathway inhibitors on LPG-induced cytokine responses

Polyclonal anti-TLR2 and/or anti-TLR4 antibodies (20 μg mL^{-1}) was added to 1×10^6 neutrophils in cell culture medium, as described above, and incubated for 1 h at 37°C. Subsequently, 50 μg mL^{-1} *T. brucei* LPG was added and further incubated for 20 h. The levels of IL-1β, IL-8, TNF-α, TGF-β, and MIP-1α in the supernatants were detected with ELISA, as described previously [28].

In another experiment, 1×10^6 neutrophils in cell culture medium were incubated separately with 20 μM T6167923 (MyD88 inhibitor, MCE, New Jersey, USA), 40 μM SB203580 (p38 MAP kinase inhibitor, Selleck, Texas, USA), 20 μM PD98059 (ERK inhibitor, Selleck), 5 μM SC75741 (NF-κB inhibitor, MCE), and the same volume of DMSO at 37°C for 1–2 h; subsequently, 50 μg mL^{-1} *T. brucei* LPG was added and incubated for 20 h [27,36,37]. The supernatant was collected for cytokine detection with ELISA, as described above.

Western blotting analysis of NF-κB p65 and p38 MAP kinase phosphorylation after LPG stimulation

Neutrophils (1×10^6) in cell culture medium were incubated with *T. brucei* LPG (0, 5, 25, 50, or 100 μg mL^{-1}) at 37°C for 3 h. Cells from each well were collected for western blotting analysis. The process was performed as described above, except that the primary antibodies were against the phosphorylated groups on NF-κB p65 (P-p65, 1:1,000, Beyotime) and p38 MAP kinase (P-p38, 1:1,000, Beyotime), and protein-specific antibodies (T-p65 and T-p38, 1:1,000, Beyotime) were used. The secondary antibody was HRP-conjugated goat anti-rabbit IgG (1:1,000, Beyotime) or HRP-conjugated goat anti-mouse IgG (1:1,000, Beyotime). Finally, chromogenic solution A was mixed with B (1:1, v/v), and the recognized proteins were visualized with chemiluminescence, as described above.

In parallel, polyclonal anti-TLR2 and anti-TLR4 antibodies (20 μg mL^{-1}, Beyotime), 20 μM PD98059 (ERK inhibitor), 40 μM SB203580 (p38 MAP kinase inhibitor), 20 μM T6167923 (MyD88 inhibitor), and 5 μM SC75741 (NF-κB inhibitor) were pre-incubated separately with the neutrophils at 37°C for 1–2 h; this was followed by a 3-h stimulation of the cells with 50 μg mL^{-1} *T. brucei* LPG at 37°C. The cells in each well were collected for western blotting, as described above.

Cell counting after treatment with *T. brucei* LPG and LPS

Neutrophils (2.5×10^6 cell mL^{-1}) in cell culture medium were treated with 50 μg mL^{-1} LPG or 100 ng mL^{-1} LPS at 37°C for 20 h, and neutrophils without treatment were used as a control. The cells that did not release NETs were counted according to a previous report [38]. Briefly, approximately 10 μL of trypan blue cell solution was pipetted into each well. Viable cells without release of NET were defined as those that were clear, refractive, and round or elongated. Nonviable cells were defined as those that were similar in shape but blue in color. The total cell count was computed as the sum of viable, NET formation nonviable cell counts.

Indirect immunofluorescence detection of the effects of IL-1β and neutrophil cell supernatants on *T. brucei* LPG-induced NETs

Neutrophils (1×10^7 cell mL^{-1}) in culture medium were added to 24-well plates and incubated with *T. brucei* LPG (50 μg mL^{-1}) for 20 h at 37°C. The supernatants were collected for subsequent experiments. Fresh cells (4×10^5) were deposited onto polylysine-treated coverslips, and reagents were added as described in S1 Table. Anakinra pretreatment was performed at 37°C for 1 h, after which the other reagents were added and cultured for 3 h at 37°C. The treated cells were fixed in 4% paraformaldehyde for 10 min at 37°C and blocked at 37°C for 1 h with 5% (w/v) skim milk. Rabbit monoclonal anti-histone (1:1,000, Thermo Fisher Scientific, Waltham, MA, USA) was added to samples at 37°C for 1 h. After washing, the samples were treated with Alexa Fluor 488 coupled to goat anti-rabbit (1:600, Thermo Fisher Scientific) at 37°C for 1 h in a light-proof environment. The washed coverslips were observed through fluorescence microscopy (Leica Camera AG, Wetzlar, Germany), and the area and proportion of NETs were counted in ImageJ software.

Quantification of NET-DNA released from neutrophils

Neutrophils (8×10^5 cells mL^{-1}) were deposited into 24-well plates and Anakinra, IL-1β, or LPG-induced neutrophil supernatant, and *T. brucei* LPG were added as described in S1 Table. Afterward, the cells were treated with restriction enzymes (*Eco*RI and *Hin*dIII, 20 U/mL; Takara, Otsu, Japan) at 37°C for 2 h. The amount of NET-DNA in the supernatant was quantified with PicoGreen's double stranded DNA kit (Invitrogen, California, USA), in accordance with the manufacturer's instructions, and detected with a fluorescent enzyme marker (PerkinElmer, VICTOR Nivo, MA, USA) [39].

Statistical analyses

We analyzed all data in GraphPad Prism 5.0 (GraphPad Software, CA, USA) and IBM Statistics 18 (IBM Corporation, NY, USA). A t-test was used to compare two groups of data, and the mean and standard deviation (SD) were calculated with at least three biological replicates. Results were considered significant at $p < 0.05$ and highly significant at $p < 0.01$ and $p < 0.001$.

RESULTS

T. brucei LPG induces TLR2 and TLR4 expression on neutrophils

To investigate whether *T. brucei* LPG might upregulate TLR2/4 expression in neutrophils, we added 50 μg/mL LPG to neutrophils. *T. brucei* LPG significantly increased the transcription of TLR2 and TLR4 genes in neutrophils, a finding similar to the increase induced by the LPS positive control (Fig 1A). To explore whether LPG had a concentration-dependent effect on TLR2/4 expression, we treated neutrophils with four concentrations of LPG. The transcription of *TLR2* (~5–9-fold increases) and *TLR4* (~5–8-fold increase) was significantly greater in the treated group than the untreated group (Fig 1B and 1C).

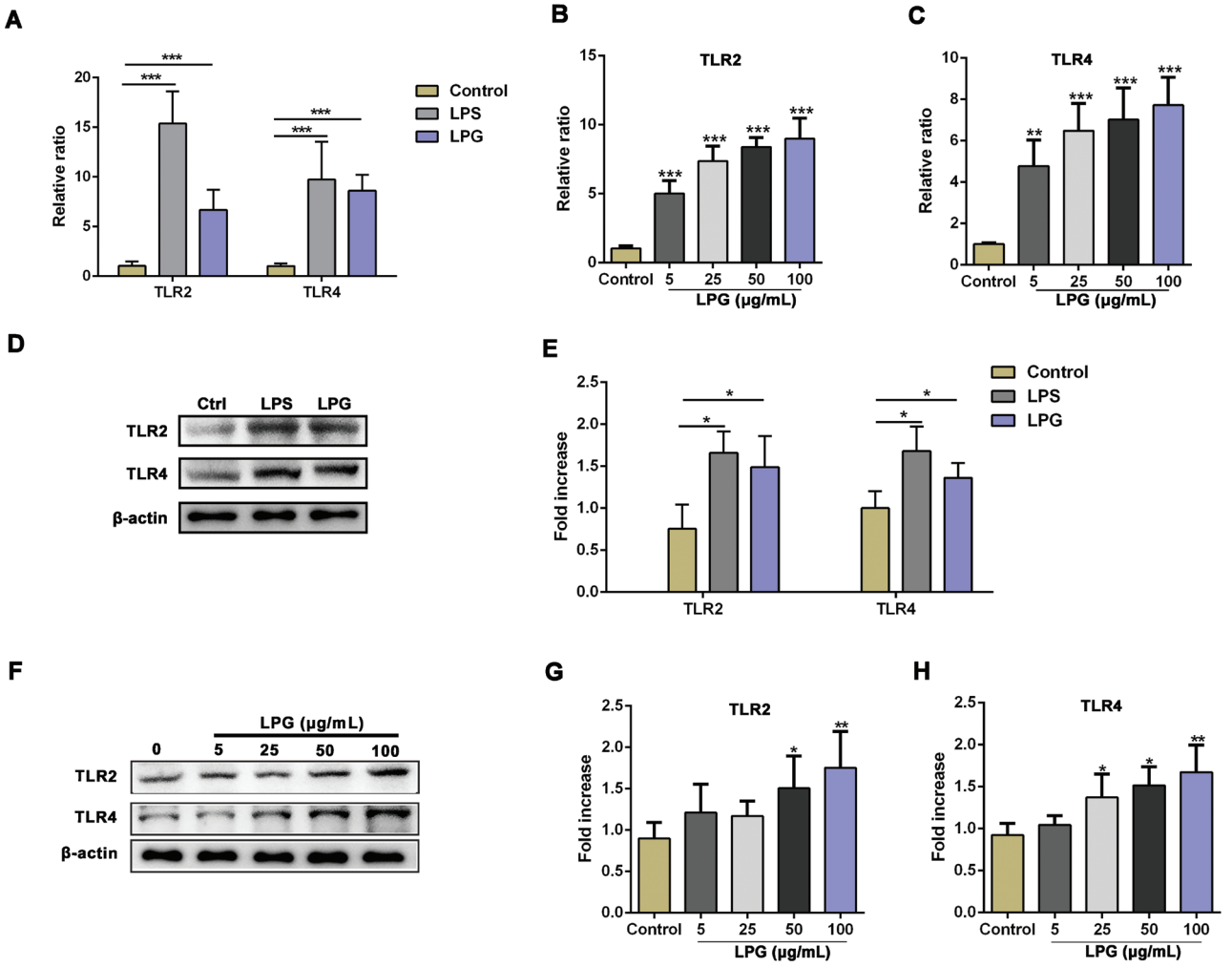

FIGURE 1 | *T. brucei* LPG induces TLR2 and TLR4 expression on neutrophils.
(A) The transcription of TLR2 and TLR4 is significantly greater than that of β-actin in neutrophils treated with *T. brucei* LPG, cells treated with LPS (positive control), and untreated cells (negative control). (B, C) Dose-dependent responses of TLR 2/4 transcription to LPG. Data are shown as the mean ± standard deviation of three independent experiments. (D) Western blotting assays detecting the expression of TLR2, TLR4, and β-actin in neutrophils after stimulation with *T. brucei* LPG; cells treated with LPS are a positive control. (E) Histogram corresponding to Fig. D. (F–H) Western blotting and histograms of the expression of TLR 2/4 in neutrophils stimulated with different concentrations of *T. brucei* LPG and bacterial LPS. The error bars represent the mean ± standard deviation of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Moreover, the expression of both TLR2 and TLR4 significantly increased, to ~1.5-fold that of the negative control (Fig 1D and 1E). However, TLR2 expression showed a significant increase only when the concentration of LPG reached 50 μg mL^{-1} (Fig 1F and 1G), whereas significant differences in TLR4 expression were observed with 25 μg mL^{-1} LPG (Fig 1F and 1H).

Neutrophils secrete cytokines after stimulation by *T. brucei* LPG

The cytokine responses, primarily those involving IL-1β, TNF-α, IL-8 TGF-β, and MIP-1α, of neutrophils to *T. brucei* LPG were investigated. Compared with non-stimulated controls, LPG at 50 μg mL^{-1} concentration significantly stimulated the expression of IL-1β (~12-fold increases), IL-8 (~10-fold increases), and TNF-α (~2-fold increases) in neutrophils. In a positive control, LPS (100 ng mL^{-1}) increased the expression of IL-1β (~13-fold increase), IL-8 (~10-fold increase), TNF-α (~3-fold increase), TGF-β (~1.5-fold increase), and MIP-1α (~2-fold increase) relative to non-stimulated neutrophils (Fig 2).

TLR2 and TLR4 blockade inhibits neutrophil responses to LPG and cytokine secretion

After treatment with anti-TLR2 antibodies, the expression of IL-1β, TNF-α, and IL-8 in responses to LPG in neutrophils decreased by 42%, 28%, and 23%, respectively (Fig 3A-C). Similarly, anti-TLR4 antibodies down-regulated IL-1β, TNF-α, and IL-8 expression in neutrophils by 59%, 55%, and 37%, respectively (Fig 3A-C). In contrast, a 58%, 59%, and 48% decrease in IL-1β, IL-8,

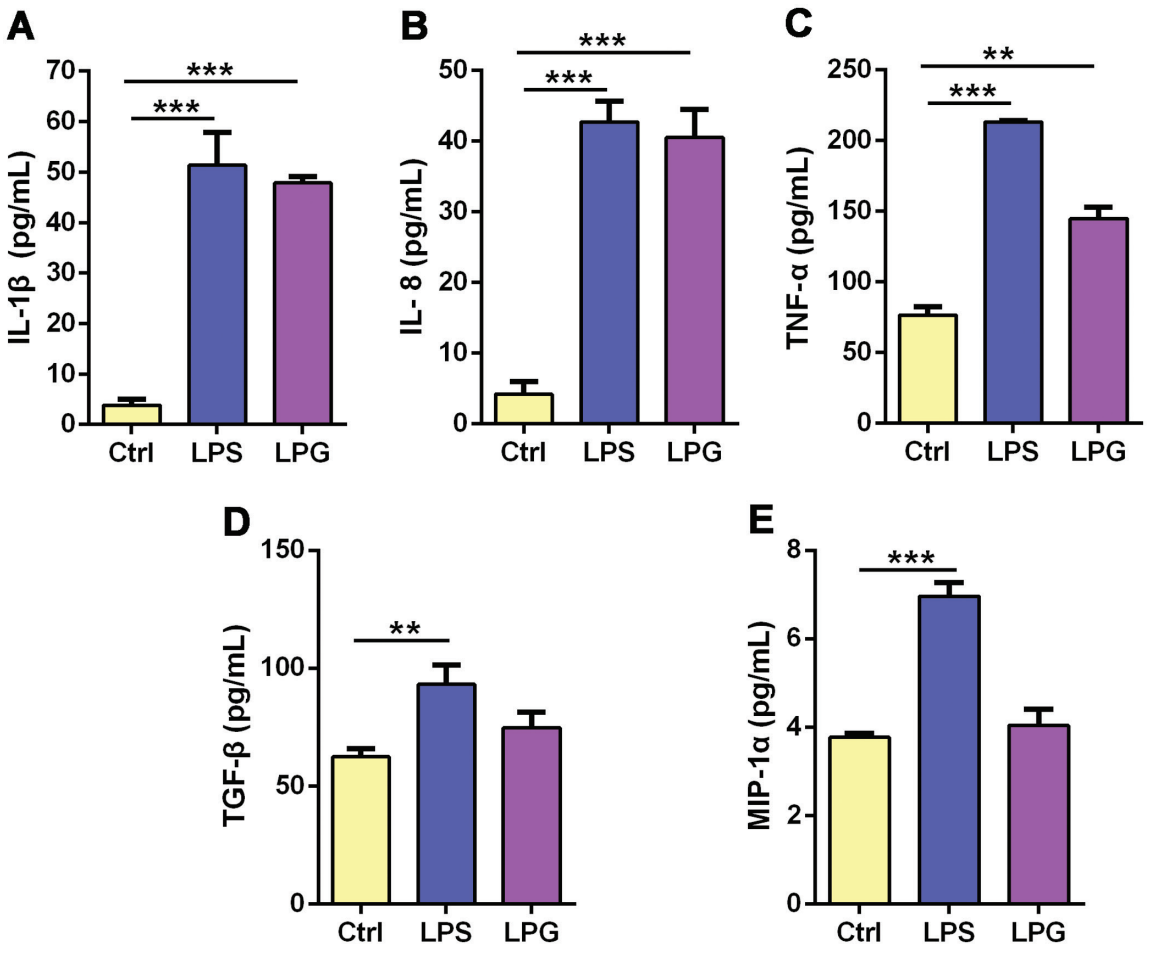

FIGURE 2 | Neutrophils secrete cytokines in response to stimulation by *T. brucei* LPG.
Neutrophils (1×10^6 cell mL^{-1}) were cultured in RPMI 1640 medium in the presence of LPG (50 μg mL^{-1}); LPS (100 ng mL^{-1}) served as a positive control, and RPMI 1640 medium (unstimulated neutrophils) served as a negative control. *T. brucei* LPG promoted the expression of (A) IL-1β, (B) IL-8, (C) TNF-α, (D) TGF-β, and (E) MIP-1α, with effects similar to those of bacterial LPS. In all cases, the error bars indicate the mean ± standard deviation from three independent experiments. ** $p < 0.01$, *** $p < 0.001$.

and TNF-α expression, respectively, was observed after blockade of TLR4 and TLR2 with specific antibodies (Fig 3A-C). However, the blockade of TLR4 and TLR2 did not affect TGF-β and MIP-1α expression in neutrophils (Fig 3D and 3E).

Inhibition of p38 MAP kinase and NF-κB decreases the effect of *T. brucei* LPG on cytokine expression in neutrophils

The p38 MAP kinase and NF-κB pathways play central roles in the regulation of cytokine responses. Therefore, we examined the IL-1β, TNF-α, IL-8, TGF-β, and MIP-1α responses in neutrophils to *T. brucei* LPG after inhibition of p38 MAP kinase and/or NF-κB. The expression of IL-1β, TNF-α, and IL-8 significantly increased when *T. brucei* LPG was added to neutrophils (Fig 4), whereas the responses were significantly inhibited by the addition of a p38 MAP kinase inhibitor (SB203580) and NF-κB inhibitor (SC75741) (Fig 4A-C). However, the expression of TGF-β and MIP-1α did not change significantly (Fig 4D and 4E).

T. brucei LPG induces the phosphorylation of NF-κB p65 and p38 MAP kinase via the TLR2 and TLR4 pathways

We investigated the phosphorylation of NF-κB p65 and p38 MAP kinase downstream of stimulation of TLR2 and TLR4 by *T. brucei* LPG. The phosphorylation of NF-κB p65 increased significantly after the LPG concentration reached 25 μg mL^{-1}, and peaked or saturated phosphorylation was observed when the LPG concentration exceeded 50 μg mL^{-1} (Fig 5A and 5B). Simultaneously, the phosphorylation of p38 MAP kinase significantly increased as the concentration of LPG increased (Fig 5C).

Compared with the group with LPG alone, the group pretreated with SB203580 (p38 MAP kinase inhibitor) and T6167923 (MyD88 inhibitor) showed lower phosphorylation of p38 MAP kinase and NF-κB p65 (Fig 5D and 5E). However, treatment with SC75741 (NF-κB inhibitor) did not decrease the LPG-induced phosphorylation of NF-κB p65 (Fig 5D).

To explore the roles of TLR2 and TLR4 in LPG-induced phosphorylation of p38 MAP kinase and NF-κB

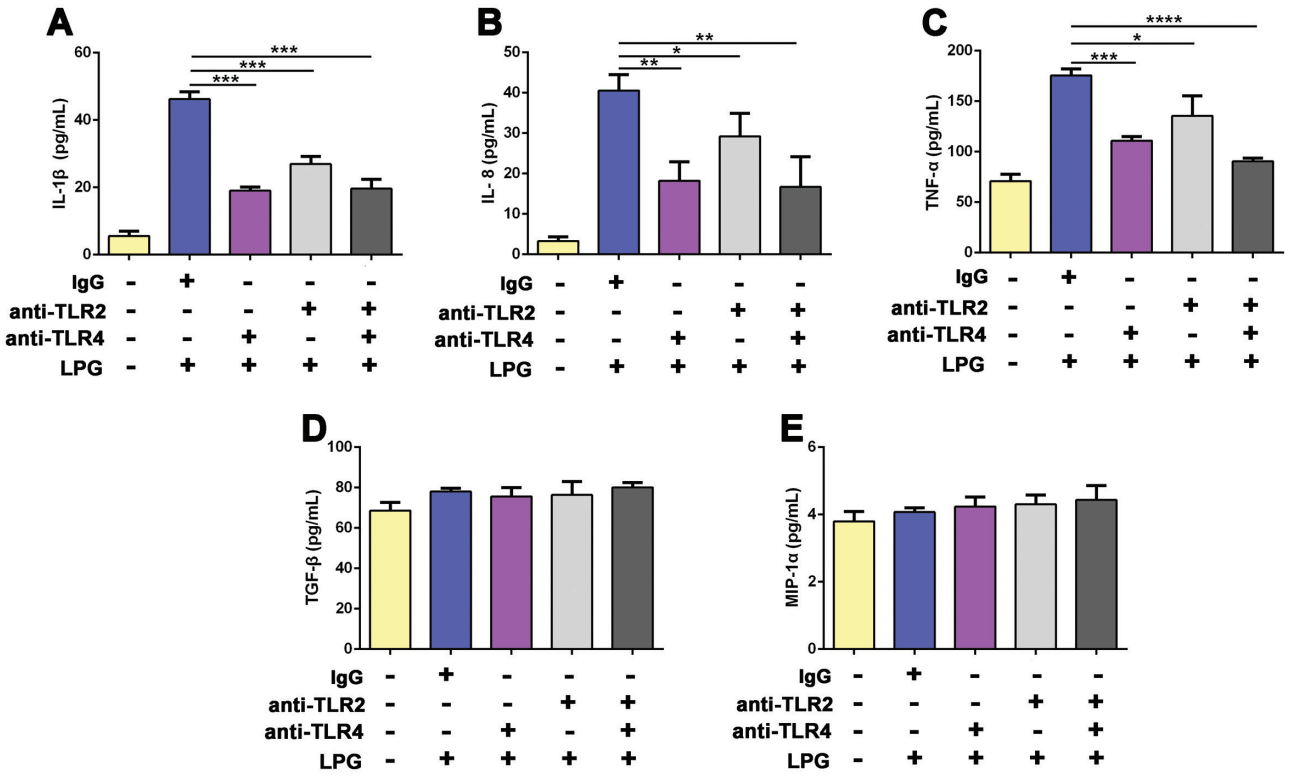

FIGURE 3 | TLR2 and TLR4 blockade with specific antibodies inhibits LPG-induced cytokine expression.
TLR2 and TLR 4 blockade inhibits the expression of (A) IL-1β, (B) IL-8, and (C) TNF-α, but not that of (D) TGF-β or (E) MIP-1α, in neutrophils in response to *T. brucei* LPG stimulation. In all cases, error bars indicate the mean ± standard deviation from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

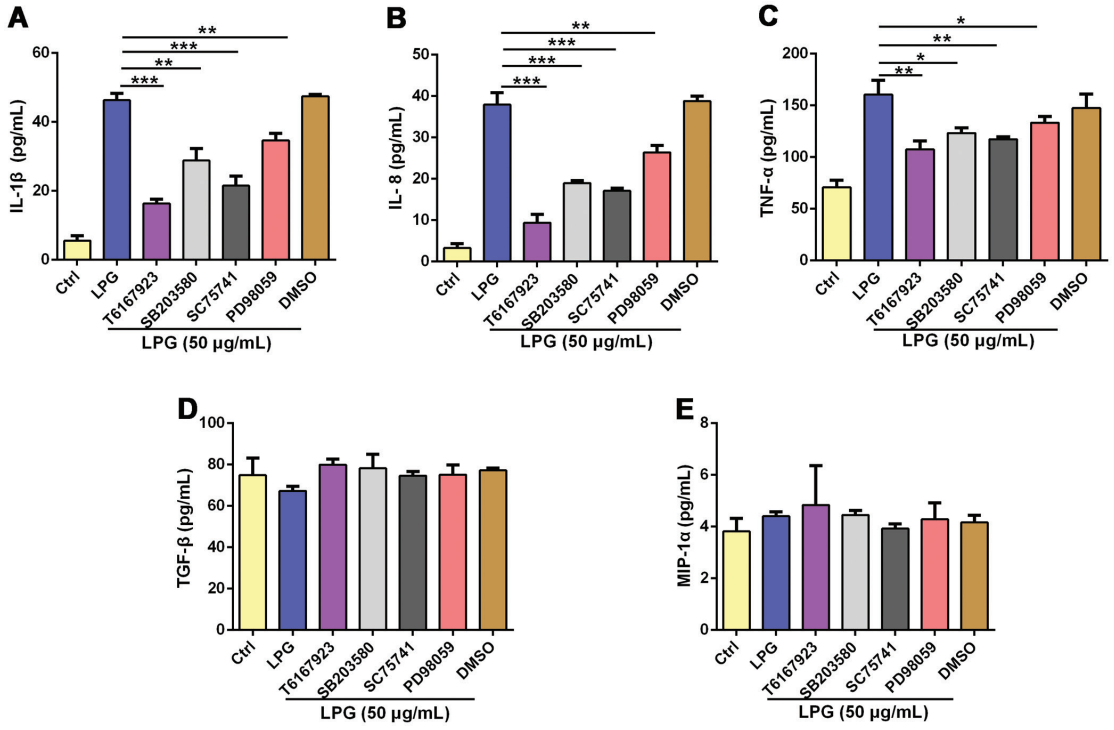

FIGURE 4 | Inhibition of p38 MAP kinase and NF-κB decreases cytokine expression induced by *T. brucei* LPG.
Neutrophils (1×10^6) were cultured in the presence of 20 μM T6167923 (MyD88 inhibitor), 40 μM SB203580 (p38 MAP kinase inhibitor), 20 μM PD98059 (ERK inhibitor), 5 μM SC75741 (NF-κB inhibitor), or the same volume of DMSO for 1–2 h at 37°C, then treated with *T. brucei* LPG (50 μg mL^{-1}) for 20 h. The expression of (A) IL-1β, (B) IL-8, and (C) TNF-α was significantly decreased by the inhibitors; however, no decrease in (D) TGF-β or (E) MIP-1α was observed. In all cases, error bars indicate the mean ± standard deviation from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

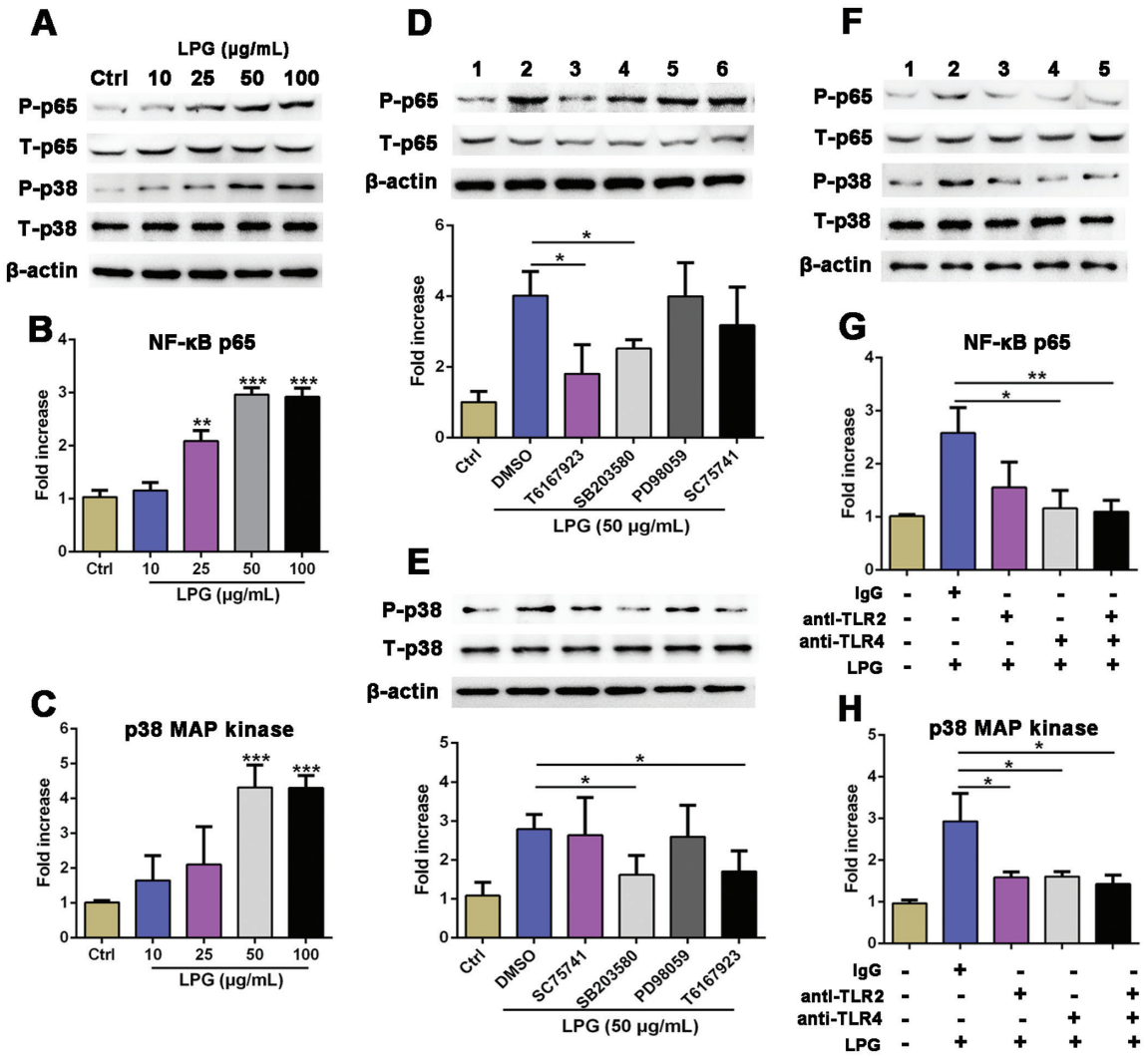

FIGURE 5 | *T. brucei* LPG induces the phosphorylation of NF-κB p65 and p38 MAP kinase via the TLR2 and TLR4 pathways. (A–C) Different concentrations (5, 25, 50, and 100 μg mL^{-1}) of LPG were added to neutrophils (1 × 10^6) for 3 h. The phosphorylation of NF-κB p65 and p38 MAP kinase was assayed with western blotting with primary antibodies for phosphorylated groups on p65 (P-p65) and p38 (P-p38), and protein-specific antibodies (T-p65 and T-p38) with an anti-β-actin antibody served as a loading control. (B and C) Histograms corresponding to the western blotting. (D and E) The neutrophils were grown in RPMI 1640 medium containing *T. brucei* LPG (50 μg mL^{-1}), with or without 20 μM T6167923 (MyD88 inhibitor), 40 μM SB203580 (p38 MAP kinase inhibitor), 20 μM PD98059 (ERK inhibitor), 5 μM SC75741 (NF-κB inhibitor), or the same volume of DMSO solvent pretreatment. Untreated neutrophils served as a control. The phosphorylation of NF-κB p65 and p38 MAP kinase was assayed with western blotting with specific antibodies, as described in A–E. (F–H) Culturing of neutrophils in RPMI 1640 medium with *T. brucei* LPG (50 μg mL^{-1}) was performed in the presence or absence of anti-TLR 2/4 (20 μg mL^{-1}) antibody or irrelevant IgG pretreatment. Anti-TLR2 and anti-TLR4 antibodies significantly inhibited the expression of NF-κB p65 and p38 MAP kinases. Error bars for all values represent the mean ± SD of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

p65, we added anti-TLR2 and anti-TLR4 antibodies. Both antibodies exhibited an inhibitory effect on the phosphorylation of both NF-κB p65 and p38 MAP kinase (Fig 5F-H).

IL-1β promotes the formation of LPG-induced NETs

Not all mature neutrophil cells release NETs after stimulation. This study indicated that, similarly to bacterial LPS stimulation, approximately 30% of neutrophils responded to *T. brucei* LPG stimulation and released NETs (Fig 6A). Furthermore, IL-1β alone did not stimulate NET release from neutrophils. When IL-1β, neutrophil supernatant pretreated with LPG, and LPG were added to fresh neutrophils, NETs were released, as indicated by histone staining (Fig 6B). This result was confirmed by quantification of the area and proportion of histones (Fig 6C and 6D). However, pretreatment with Anakinra, an interleukin inhibitor, significantly decreased the effects of IL-1β and neutrophil supernatant (Fig 6B-D). Furthermore, NET-DNA assays indicated that IL-1β and cell supernatant significantly promoted the release of NET-DNA, and that IL-1β receptor antagonists inhibited their effects (Fig 6D). Therefore, *T. brucei* LPG stimulated IL-1β secretion from neutrophils via the TLR-mediated p38 MAP

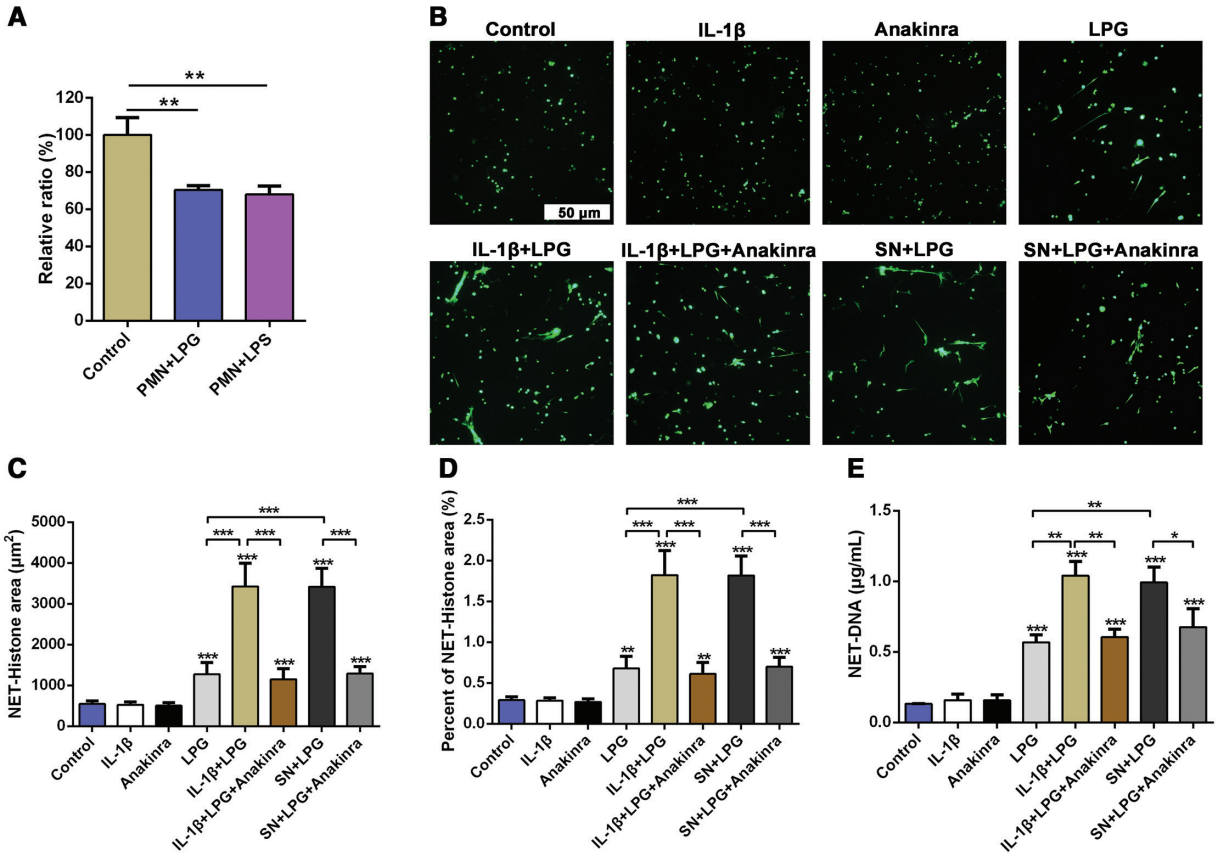

FIGURE 6 | IL-1β promotes the formation of *T. brucei* LPG-induced NETs.
(A) In RPMI 1640 medium, polymorphonuclear neutrophils (PMN, 2.5 × 10^6 cell mL^{-1}) were cultured with LPG (50 μg mL^{-1}); LPS (100 ng mL^{-1}) served as a positive control, and untreated cells served as a negative control. The relative ratio of LPG-treated cells was significantly lower than that in the untreated group. (B) The neutrophils were cultured with IL-1β (100 ng mL^{-1}), Anakinra (149 μg mL^{-1}), 500 μL supernatant (SN) of neutrophils treated with LPG, and/or LPG (50 μg mL^{-1}). Histones were detected through indirect immunofluorescence with rabbit monoclonal anti-histone and Alexa Fluor 488 coupled to goat anti-rabbit as primary and secondary antibodies, respectively. Histones were observed by fluorescence microscopy, on the basis of green staining; scale bar: 50 μM. (C and D) Histogram of NET-histone area and percentage corresponding to Fig B. (E) NET-DNA in treated neutrophil supernatants was analyzed with PicoGreen's double stranded DNA kit and detected by enzymatic labeling; untreated neutrophil supernatants served as a negative control. The error bars represent the mean ± standard deviation of three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

kinase and NF-κB pathways, thereby promoting the formation of LPG-stimulated NETs (Fig 7).

DISCUSSION

Neutrophils are the most abundant innate immune cells and are considered critical in responses to pathogenic invasions, including phagocytosis, degranulation, the release of active factors, and production of NETs [40–42]. Previously, mature neutrophils were commonly believed to lack transcriptional activity and to rarely be involved in protein synthesis. However, recent studies have shown that neutrophils are also capable of *de novo* synthesis of cytokines and exerting immunomodulatory functions [43]. Human neutrophils have been shown to express and produce many pro- and anti-inflammatory cytokines, chemokines, colony-stimulating factors, angiogenic factors, TNF family members, and growth factors *in vitro* and *in vivo* [44]. These findings indicate that neutrophils participate in a wide variety of physiological and pathological processes.

LPG is a polymer that was first identified in *Leishmania*, and was later identified in *Trypanosoma* [8,45]. TLR2 on dendritic cells recognizes *L. mexicana* LPG and promotes the release of IFN-γ from the dendritic cells, thereby activating the expression of IL-12p70 in NKT cells [26]. *L. infantum* LPG activates macrophage release of MCP-1, IL-6, TNF-α, and IL-12p70, whereas *L. mexicana* LPG induces the expression of TNF-α, IL-10, IL-12p40, IL-12p70, and IL-1β by macrophages [27,46]. *T. cruzi* surface glycoproteins or glycosylphosphatidylinositol triggers the expression of inflammatory cytokines, such as TNF-α, IFN-γ, IL-1β, and IL-6, as well as chemokines, such as IP-10, MCP-1, and RANTES/CCL5, in macrophages [47-49]. These inflammatory cytokines/chemokines induce macrophages to produce superoxide and nitric oxide, which directly kill *T. cruzi* [50]. Furthermore, the lipopolysaccharide moiety has been identified as a virulence factor in bacteria, and is commonly used to stimulate neutrophils to produce a variety of cytokines, such as IL-1β, MIP-1α, IL-8, TGF-β, and TNF-α [51-54].

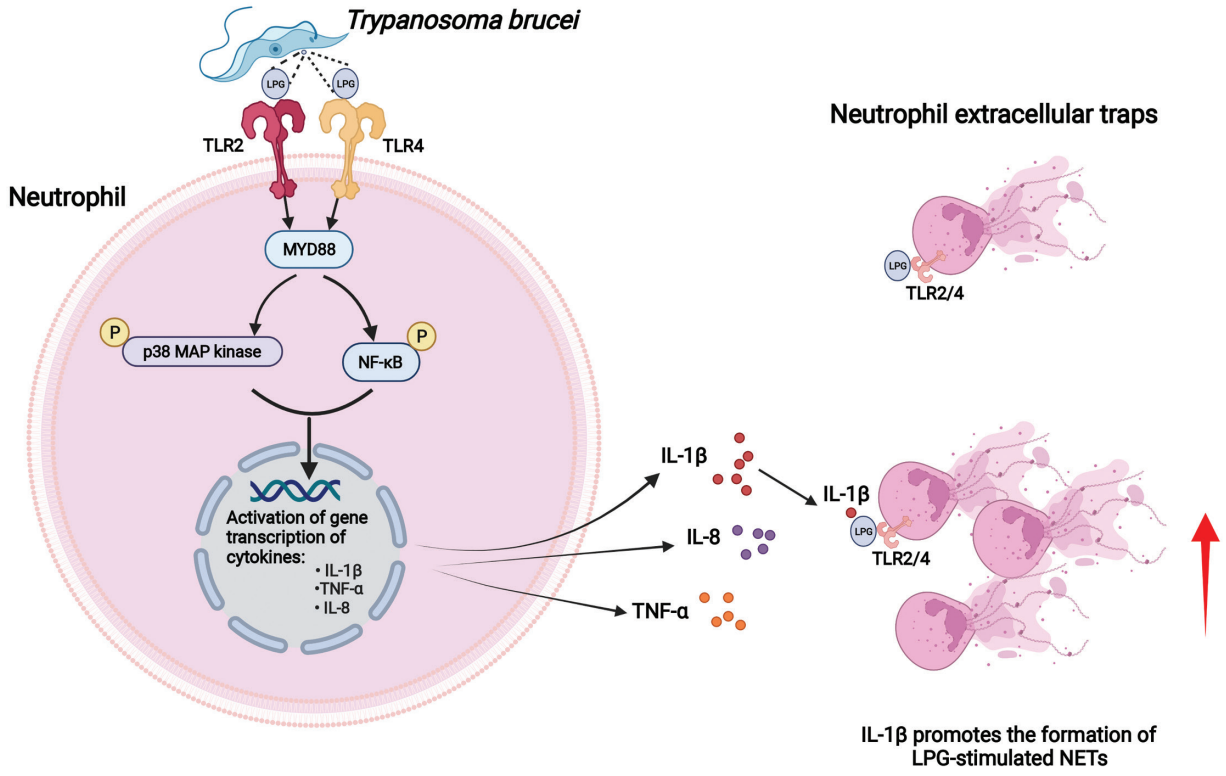

FIGURE 7 | Schematic illustration of *T. brucei* LPG-mediated stimulation in neutrophils, followed by a cascade reaction of IL-1β secretion and promotion of NET formation.

We previously found that *T. brucei* LPG stimulates NET formation through interaction with TLR2 and TLR4 [28]. To better understand the interaction between *T. brucei* and the host immune system, primarily neutrophil responses, we added LPG extracted from *T. brucei* to neutrophils and further investigated the cytokine responses. *T. brucei* LPG upregulated the expression of TNF-α, IL-8, and IL-1β in neutrophils (Figs 1 and 2). In *Leishmania*, LPG activates NK cells via TLR2, thus increasing the secretion of IFN-γ and TNF-α, and promoting the secretion of TNF-α, IL-10, IL-12p40, IL-12p70, and IL-1β by human macrophages via the TLR2 and TLR4 pathways [27,55]. These findings imply an important role of TLRs in response to LPG stimulation. Furthermore, blockade of TLR2 and TLR4 with specific antibodies curtailed the ability of *T. brucei* LPG to induce TNF-α, IL-8, and IL-1β expression in neutrophils (Fig 3). However, the amount of LPG used in the *in vitro* study was not comparable to that of parasites in actual infection, owing to the insolubility of LPG extract.

The pathways of cytokine production in neutrophils and macrophages triggered by activators are diverse. The LPG of *Giardia duodenalis* and *Leishmania* activates cytokine expression in macrophages through the p38 MAP kinase and ERK pathways, whereas bacterial LPS stimulates host immune responses through the NF-κB and MAP kinase pathways [27,56,57]. To investigate the pathway of *T. brucei* LPG-induced cytokine expression in neutrophils, we incubated MyD88, p38 MAP kinase, ERK, and NF-κB inhibitors with LPG with neutrophils; all these inhibitors inhibited cytokine expression, thus suggesting that LPG might stimulate neutrophil-derived cytokine responses through multiple signaling pathways (Fig 4). The blockade of TLR2, TLR4, MyD88, and p38 MAP kinase inhibitors decreased the phosphorylation of p38 MAP kinase and NF-κB p65 (Fig 5), and consequently decreased the expression of cytokines (Fig 4). NF-κB inhibitor did not affect NF-κB p65 phosphorylation (Fig 5D), possibly because SC75741 only inhibits the binding of activated NF-κB p65 to DNA in the nucleus, thereby affecting the expression of downstream cytokine genes [37].

Furthermore, the inflammatory cytokines IL-1β, TNF-α, and IL-8 released from neutrophils induced by *T. brucei* LPG may further activate cellular immune responses. Macrophage-derived IL-1β has been found to enhance the formation of sodium urate crystal-triggered NETs [24], and recombinant IL-1β has been found to promote the formation of NETs in patients with septic arthritis, gangrenous sepsis, and acne [58]. In addition, the blockade of IL-1β has been reported to attenuate thrombosis in a NET-dependent breast cancer model [25]. In this study, we also investigated the potential role of IL-1β in NET formation. Recombinant IL-1β and the supernatant from LPG stimulated neutrophils indeed promoted NET formation (Fig 6). Thus, *T. brucei*-derived LPG stimulates IL-1β secretion from neutrophils, and IL-1β further promotes NET formation.

In summary, *T. brucei* LPG was found to be recognized by TLR2 and TLR4 on the surfaces of neutrophils; to activate intracellular p38 MAP kinase and NF-κB phosphorylation; and to upregulate the expression of IL-1β in neutrophils, thereby promoting the formation of NETs (Fig 7). Our study deepens understanding of the innate immune response of neutrophils to trypanosomal infection.

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CONFLICTS OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

REFERENCES

1. Guerrini L, Bouyer J. Mapping African animal trypanosomosis risk: the landscape approach. Vet Ital. 2007;43:643-654.
2. Odeniran PO, Onifade AA, MacLeod ET, Ademola IO, Alderton S, Welburn SC. Mathematical modelling and control of African animal trypanosomosis with interacting populations in West Africa—Could biting flies be important in maintaining the disease endemicity? PLoS One. 2020;15:e0242435.
3. Ebhodaghe F, Ohiolei JA, Isaac C. A systematic review and meta-analysis of small ruminant and porcine trypanosomiasis prevalence in sub-Saharan Africa (1986 to 2018). Acta Tropica. 2018;188:118-131.
4. Szempruch AJ, Sykes SE, Kieft R, Dennison L, Becker AC, Gartrell A, et al. Extracellular vesicles from *Trypanosoma brucei* mediate virulence factor transfer and cause host anemia. Cell. 2016;164:246-257.
5. Forestier CL, Gao Q, Boons GJ. *Leishmania* lipophosphoglycan: how to establish structure-activity relationships for this highly complex and multifunctional glycoconjugate? Front Cell Infect Microbiol. 2014;4:193.
6. Singh BN, Lucas JJ, Beach DH, Costello CE. Expression of a novel cell surface lipophosphoglycan-like glycoconjugate in *Trypanosoma cruzi* epimastigotes. J Biol Chem. 1994;269:21972-21982.
7. Turco SJ, Wilkerson MA, Clawson DR. Expression of an unusual acidic glycoconjugate in *Leishmania donovani*. J Biol Chem. 1984;259:3883-3889.
8. Hublart M, Tetaert D, Mendonca-Previato L, Degand P. Presence of a lipophosphoglycan in two variants of *Trypanosoma brucei brucei*. Biochem Biophys Res Commun. 1988;153:1257-1266.
9. Sacks DL, Saraiva EM, Rowton E, Turco SJ, Pimenta PF. The role of the lipophosphoglycan of *Leishmania* in vector competence. Parasitology. 1994;108(Suppl):S55-62.
10. Spath GF, Epstein L, Leader B, Singer SM, Avila HA, Turco SJ, et al. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. Proc Natl Acad Sci U S A. 2000;97:9258-9263.
11. de Carvalho RVH, Andrade WA, Lima-Junior DS, Dilucca M, de Oliveira CV, Wang K, et al. *Leishmania* lipophosphoglycan triggers caspase-11 and the non-canonical activation of the NLRP3 inflammasome. Cell Rep. 2019;26:429-437 e425.
12. Chaparro V, Leroux LP, Zimmermann A, Jardim A, Johnston B, Descoteaux A, et al. *Leishmania donovani* lipophosphoglycan increases macrophage-dependent chemotaxis of CXCR6-expressing cells via CXCL16 induction. Infect Immun. 2019;87:e00064-19.
13. Wolday D, Akuffo H, Demissie A, Britton S. Role of *Leishmania donovani* and its lipophosphoglycan in CD4+ T-cell activation-induced human immunodeficiency virus replication. Infect Immun. 1999;67:5258-5264.
14. Tarleton RL. Immune system recognition of *Trypanosoma cruzi*. Curr Opin Immunol. 2007;19:430-434.
15. Trinchieri G, Sher A. Cooperation of Toll-like receptor signals in innate immune defence. Nat Rev Immunol. 2007;7:179-190.
16. Campos MA, Almeida IC, Takeuchi O, Akira S, Valente EP, Procopio DO, et al. Activation of Toll-like receptor-2 by glycosylphosphatidylinositol anchors from a protozoan parasite. J Immunol. 2001;167:416-423.
17. Kavoosi G, Ardestani SK, Kariminia A. The involvement of TLR2 in cytokine and reactive oxygen species (ROS) production by PBMCs in response to *Leishmania major* phosphoglycans (PGs). Parasitology. 2009;136:1193-1199.
18. Bott E, Carneiro AB, Gimenez G, Lopez MG, Lammel EM, Atella GC, et al. Lipids From *Trypanosoma cruzi* amastigotes of RA and K98 strains generate a pro-inflammatory response via TLR2/6. Front Cell Infect Microbiol. 2018;8:151.
19. Cronemberger-Andrade A, Xander P, Soares RP, Pessoa NL, Campos MA, Ellis CC, et al. *Trypanosoma cruzi*-infected human macrophages shed proinflammatory extracellular vesicles that enhance host-cell invasion via toll-like receptor 2. Front Cell Infect Microbiol. 2020;10:99.
20. Vieira TDS, Rugani JN, Nogueira PM, Torrecilhas AC, Gontijo CMF, Descoteaux A, et al. Intraspecies polymorphisms in the lipophosphoglycan of L. braziliensis differentially modulate macrophage activation via TLR4. Front Cell Infect Microbiol. 2019;9:240.
21. Bafica A, Santiago HC, Goldszmid R, Ropert C, Gazzinelli RT, Sher A. Cutting edge: TLR9 and TLR2 signaling together account for MyD88-dependent control of parasitemia in *Trypanosoma cruzi* infection. J Immunol. 2006;177:3515-3519.
22. Gupta CL, Akhtar S, Waye A, Pandey NR, Pathak N, Bajpai P. Cross talk between *Leishmania donovani* CpG DNA and Toll-like receptor 9: an immunoinformatics approach. Biochem Biophys Res Commun. 2015;459:424-429.
23. Brinkmann V, Reichard U, Goosmann C, Fauler B, Uhlemann Y, Weiss DS, et al. Neutrophil extracellular traps kill bacteria. Science (New York, N.Y.). 2004;303:1532-1535.
24. Sil P, Wicklum H, Surell C, Rada B. Macrophage-derived IL-1β enhances monosodium urate crystal-triggered NET formation. Inflamm Res. 2017;66:227-237.
25. Gomes T, Varady CBS, Lourenco AL, Mizurini DM, Rondon AMR, Leal AC, et al. IL-1β blockade attenuates thrombosis in a neutrophil extracellular trap-dependent breast cancer model. Front Immunol. 2019;10:2088.
26. Zamora-Chimal J, Fernandez-Figueroa EA, Ruiz-Remigio A, Wilkins-Rodriguez AA, Delgado-Dominguez J, Salaiza-Suazo N, et al. NKT cell activation by *Leishmania mexicana* LPG: description of a novel pathway. Immunobiology. 2017;222:454-462.
27. Rojas-Bernabe A, Garcia-Hernandez O, Maldonado-Bernal C, Delegado-Dominguez J, Ortega E, Gutierrez-Kobeh L, et al. *Leishmania mexicana* lipophosphoglycan activates ERK and p38 MAP kinase and induces production of proinflammatory cytokines in human macrophages through TLR2 and TLR4. Parasitology. 2014;141:788-800.
28. Zhang K, Jiang N, Sang X, Feng Y, Chen R, Chen Q. *Trypanosoma brucei* lipophosphoglycan induces the formation of neutrophil extracellular traps and reactive oxygen species burst via toll-like receptor 2, toll-like receptor 4, and c-Jun N-Terminal Kinase Activation. Front Microbiol. 2021;12:713531.

29. Grob D, Conejeros I, Velásquez ZD, Preußer C, Gärtner U, Alarcon P, et al. *Trypanosoma brucei brucei* induces polymorphonuclear neutrophil activation and neutrophil extracellular traps release. Front Immunol. 2020;11:559561.
30. Zhang K, Jiang N, Chen H, Zhang N, Sang X, Feng Y, et al. TatD DNases of African trypanosomes confer resistance to host neutrophil extracellular traps. Sci China Life Sci. 2021;64:621-632.
31. Lanham SM, Godfrey DG. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp Parasitol. 1970;28:521-534.
32. McConville MJ, Bacic A, Mitchell GF, Handman E. Lipophosphoglycan of *Leishmania major* that vaccinates against cutaneous leishmaniasis contains an alkylglycerophosphoinositol lipid anchor. Proc Natl Acad Sci U S A. 1987;84:8941-8945.
33. Boyum A. Separation of blood leucocytes, granulocytes and lymphocytes. Tissue Antigens. 1974;4:269-274.
34. Swamydas M, Lionakis MS. Isolation, purification and labeling of mouse bone marrow neutrophils for functional studies and adoptive transfer experiments. J Vis Exp. 2013;77:e50586.
35. Zhang L, Cheng Q, Li C, Zeng X, Zhang XZ. Near infrared light-triggered metal ion and photodynamic therapy based on AgNPs/porphyrinic MOFs for tumors and pathogens elimination. Biomaterials. 2020;248:120029.
36. Wu Y, Ma L, Cai S, Zhuang Z, Zhao Z, Jin S, et al. RNA-induced liquid phase separation of SARS-CoV-2 nucleocapsid protein facilitates NF-κB hyper-activation and inflammation. Signal Transduct Target Ther. 2021;6:167.
37. Ehrhardt C, Ruckle A, Hrincius ER, Haasbach E, Anhlan D, Ahmann K, et al. The NF-κB inhibitor SC75741 efficiently blocks influenza virus propagation and confers a high barrier for development of viral resistance. Cell Microbiol. 2013;15:1198-1211.
38. Sarkar S, Lund SP, Vyzasatya R, Vanguri P, Elliott JT, Plant AL, et al. Evaluating the quality of a cell counting measurement process via a dilution series experimental design. Cytotherapy. 2017;19:1509-1521.
39. Guimaraes-Costa AB, Nascimento MT, Froment GS, Soares RP, Morgado FN, Conceicao-Silva F, et al. Leishmania amazonensis promastigotes induce and are killed by neutrophil extracellular traps. Proc Natl Acad Sci U S A. 2009;106:6748-6753.
40. Hirsch JG, Cohn ZA. Degranulation of polymorphonuclear leucocytes following phagocytosis of microorganisms. J Exp Med. 1960;112:1005-1014.
41. Winterbourn CC, Kettle AJ. Redox reactions and microbial killing in the neutrophil phagosome. Antioxid Redox Signal. 2013;18:642-660.
42. Papayannopoulos V. Neutrophil extracellular traps in immunity and disease. Nat Rev Immunol. 2018;18:134-147.
43. Cassatella MA. The production of cytokines by polymorphonuclear neutrophils. Immunol Today. 1995;16:21-26.
44. Tamassia N, Bianchetto-Aguilera F, Arruda-Silva F, Gardiman E, Gasperini S, Calzetti F, et al. Cytokine production by human neutrophils: revisiting the "dark side of the moon". Eur J Clin Invest. 2018;48(Suppl 2):e12952.
45. Orlandi PA Jr, Turco SJ. Structure of the lipid moiety of the *Leishmania donovani* lipophosphoglycan. J Biol Chem. 1987;262:10384-10391.
46. Lima JB, Araujo-Santos T, Lazaro-Souza M, Carneiro AB, Ibraim IC, Jesus-Santos FH, et al. *Leishmania* infantum lipophosphoglycan induced-Prostaglandin E2 production in association with PPAR-γ expression via activation of Toll like receptors-1 and 2. Sci Rep. 2017;7:14321.
47. Almeida IC, Camargo MM, Procopio DO, Silva LS, Mehlert A, Travassos LR, et al. Highly purified glycosylphosphatidylinositols from *Trypanosoma cruzi* are potent proinflammatory agents. EMBO J. 2000;19:1476-1485.
48. da Fonseca LM, da Costa KM, Chaves VS, Freire-de-Lima CG, Morrot A, Mendonca-Previato L, et al. Theft and reception of host cell's sialic acid: dynamics of *Trypanosoma Cruzi* trans-sialidases and mucin-like molecules on chagas' disease immunomodulation. Front Immunol. 2019;10:164.
49. Aliberti JC, Souto JT, Marino AP, Lannes-Vieira J, Teixeira MM, Farber J, et al. Modulation of chemokine production and inflammatory responses in interferon-γ- and tumor necrosis factor-R1-deficient mice during *Trypanosoma cruzi* infection. Am J Pathol. 2001;158:1433-1440.
50. Martins GA, Cardoso MA, Aliberti JC, Silva JS. Nitric oxide-induced apoptotic cell death in the acute phase of *Trypanosoma cruzi* infection in mice. Immunol Lett. 1998;63:113-120.
51. Fujishima S, Hoffman AR, Vu T, Kim KJ, Zheng H, Daniel D, et al. Regulation of neutrophil interleukin 8 gene expression and protein secretion by LPS, TNF-alpha, and IL-1 beta. J Cell Physiol. 1993;154:478-485.
52. Jenkins JK, Malyak M, Arend WP. The effects of interleukin-10 on interleukin-1 receptor antagonist and interleukin-1 beta production in human monocytes and neutrophils. Lymphokine Cytokine Res. 1994;13:47-54.
53. Kita H, Abu-Ghazaleh R, Sanderson CJ, Gleich GJ. Effect of steroids on immunoglobulin-induced eosinophil degranulation. J Allergy Clin Immunol. 1991;87:70-77.
54. Sohn EJ, Paape MJ, Connor EE, Bannerman DD, Fetterer RH, Peters RR. Bacterial lipopolysaccharide stimulates bovine neutrophil production of TNF-alpha, IL-1beta, IL-12 and IFN-gamma. Vet Res. 2007;38:809-818.
55. Becker I, Salaiza N, Aguirre M, Delgado J, Carrillo-Carrasco N, Kobeh LG, et al. *Leishmania* lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. Mole Biochem Parasitol. 2003;130:65-74.
56. Lai JL, Liu YH, Liu C, Qi MP, Liu RN, Zhu XF, et al. Indirubin inhibits LPS-induced inflammation via TLR4 abrogation mediated by the NF-κB and MAPK signaling pathways. Inflammation. 2017;40:1-12.
57. Pu X, Li X, Cao L, Yue K, Zhao P, Wang X, et al. *Giardia duodenalis* induces proinflammatory cytokine production in mouse macrophages via TLR9-mediated p38 and ERK signaling pathways. Front Cell Dev Biol. 2021;9:694675.
58. Mistry P, Carmona-Rivera C, Ombrello AK, Hoffmann P, Seto NL, Jones A, et al. Dysregulated neutrophil responses and neutrophil extracellular trap formation and degradation in PAPA syndrome. Ann Rheum Dis. 2018;77:1825-1833.