Abstract. Previous studies have revealed that activation of the Toll-like receptor 4 (TLR4)-mediated proinflammatory signaling pathway plays an important role in acute inflammation, sepsis and chronic inflammatory disorders. Moreover, TLR4 significantly contributes to lipopolysaccharide (LPS)-induced immune response. Thus, modulation of the TLR4 pathway is an important strategy to specifically target these pathologies. The aim of the present study was to develop a complete human anti-TLR4 IgG2 antibody by screening human TLR4 Fab from a phage-display library and integrating it with constant regions of the heavy chain of human IgG2 via antibody engineering. ELISA, a BLItz system and fluorescence-activated cell sorting were used to assess its affinity. Furthermore, mouse-derived peritoneal macrophages were treated with human anti-TLR4 IgG2 and induced with LPS in vitro. Reverse transcription-quantitative PCR and western blotting were used to determine mRNA expression levels of cytokines and phosphorylation levels of signaling pathways, respectively. It was found that human anti-TLR4 IgG2 bound to TLR4 with a high affinity of 8.713x10^{-10} M, and that preincubation with anti-TLR4 IgG2 inhibited the LPS-induced production of tumor necrosis factor-α, interferon-β and interleukin-6 mRNA expression levels in mouse peritoneal macrophages. It was also demonstrated that human anti-TLR4 IgG2 inhibited LPS-induced TLR4 signaling by reducing the phosphorylation of the NF-κB, mitogen-activated protein kinase and interferon regulatory factor 3 signaling pathways. In addition, human anti-TLR4 IgG2 protected mice from LPS challenge with a survival rate of 40% and also significantly increased the survival time in the cecal ligation and puncture model. Therefore, it was speculated that human anti-TLR4 IgG2 plays a protective role against sepsis-associated injury and is potentially applicable for the treatment of infection-associated immune dysfunction.

Introduction

Toll-like receptors (TLRs), which have the most extensive spectrum of pathogen recognition, detect invading pathogens by recognizing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular pattern molecules (DAMPs) (1,2). TLR4, a TLR family member, plays an important role in innate immunity against allergy (3), obesity-associated metabolic disorders (4), apoptosis (5), infectious diseases (6), and inflammatory bowel diseases (7). Moreover, TLR4 is widely expressed on the surface of immune cells, including macrophages, neutrophils and lymphocytes (8).

Lipopolysaccharide (LPS), an endotoxin on the cell wall of Gram-negative bacteria, is a major PAMP (1). On recognizing LPS, TLR4 interacts with LPS via its cytosolic toll-interleukin (IL)-1 receptor (TIR) domain (9). Furthermore, LPS binds to LPS-binding protein (LBP) and CD14, which then transfers LPS to the TLR4/myeloid differentiation protein-2 (MD2) complex, which dimerizes and translocates to endosomes, triggering myeloid differentiation primary response gene 88 (MyD88)-dependent and MyD88-independent pathways (10). Moreover, the two pathways can induce phosphorylation of
transcription factors, including nuclear factor κB (NF-κB), activator protein 1 and interferon (IFN) regulatory factor 3 (IRF-3), to eventually promote the production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1, IL-6, IL-8, IFN-β and IFN-γ (11-13). The inflammatory response to LPS plays a key role in the defense against bacterial infections; however, excessive host reaction to LPS causes severe inflammatory conditions such as sepsis and fatal septic shock (14,15). Therefore, regulation of TLR4-mediated signaling is critical for maintaining the intensity of the immune response and treating severe sepsis.

Monoclonal antibodies (mAbs) are a widely used pharmacotherapeutic approach in the treatment of various inflammatory diseases (16). Thus, the aims of the present study were to prepare a novel human monoclonal anti-TLR4 immunoglobulin G2 antibody by screening an anti-TLR4 Fab fragment from a human Fab phage-display library, and to examine whether human anti-TLR4 IgG2 decreases LPS-induced immune responses. The present results suggest that the entire human anti-TLR4 IgG2 antibody showed high affinity for TLR4 and functioned well against LPS-induced inflammatory processes in mouse macrophages.

Materials and methods

Reagents and mice. LPS used to stimulate inflammation responses was obtained from Sigma-Aldrich (Merck KGaA). RPMI-1640 medium, DMEM/F12 and FBS used for cell culture were purchased from Gibco (Thermo Fisher Scientific, Inc.). Diagnostic ELISA kits for the measurement of mouse TNF-α (cat. no. MTA00B), IL-6 (cat. no. M6000B) and IFN-β (cat. no. MIFNB0) were obtained from R&D Systems, Inc. C57BL/6J female mice (age, 6‑8 weeks; weight, 20‑25 g) were purchased from SLAC Laboratory Animal Company. The following specific antibodies were obtained from Cell Signaling Technology, Inc.: Anti-phosphorylated (p)-p38 (cat. no. 9215), anti-p38 (cat. no. 8690), anti-p-p65 (cat. no. 3033), anti-p-p65 (cat. no. 8242), anti-p-JNK (cat. no. 4668), anti-JNK (cat. no. 9258), anti-p-ERK (cat. no. 4376), anti-ERK (cat. no. 1695), anti-p-inhibitor of κB (IκB) (cat. no. 2859), anti-IκB (cat. no. 4812), anti-p-IRF-3 (cat. no. 29047), anti-IRF-3 (cat. no. 11904), anti-p-IκB kinase (IKK) (cat. no. 2697), anti-IKK (cat. no. 8943) and anti-β-actin (cat. no. 8457). All the animal experiments were performed according to protocols that were approved by the Ethics Committee of Huadong Medical Institute of Biotechniques.

Cells and cell culture. Mouse peritoneal macrophages (MPM) were isolated by peritoneal lavage 3‑4 days after intraperitoneal injection of mice with 2 ml sterile 5% thioglycolate broth, as previously described (17). MPM were cultured in RPMI-1640 medium supplemented with 10% FBS and 1% antibiotic-antimycotic (Gibco; Thermo Fisher Scientific, Inc.) at 37°C with 5% CO₂.

Preparation of human anti-TLR4 IgG2. A phage-displayed library with >10¹³ phage clones was used to screen the human Fab against TLR4, as previously described (18). A total of seven rounds of screening with precoted recombinant TLR4 protein were performed to ensure the specificity of the binding phage. VBASE2 database (vbase2.org/vbhelp.php) was used for analyzing the sequence of Fab. Cloned anti-TLR4 Fab was selected to develop complete human IgG2 via gene synthesis. The heavy (H) and light (L) chains were cloned separately into the pMHC vector (AmProtein-China, Inc.). Recombinant IgG2 expression vectors (pMHC-anti-TLR4-IgG2-H and pMHC-anti-TLR4-IgG2-L) were expressed in Chinese hamster ovary (CHO) cells (American Type Culture Collection) which were cultured in DMEM/F12 supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% antibody-antimycotic. Then, the cell culture media was centrifuged at 200 x g for 5 min at 4°C, and the supernatant was harvested after a transient transfection (Lipofectamine 2000; Thermo Fisher Scientific, Inc.) for 6 days and purified with a Protein A column (Cytiva). The purified protein was separated via 12% SDS-PAGE and visualized by staining with 0.1% Coomassie brilliant blue R250 at room temperature for 1 h.

ELISA. ELISA was used to assay the affinity of human anti-TLR4 IgG2. Briefly, 96-well plates were precoated with 50 ng TLR4 antigen (R&D Systems, Inc.) per well in coating buffer (50 mM sodium carbonate buffer, pH 9.6) overnight at 4°C. After blocking with 5% non-fat milk at room temperature for 1 h, 100 µl human anti-TLR4 IgG2 at different concentrations was added to the wells (1:2 serial dilution; 3 wells per dilution) for incubation at 37°C for 1 h. The plates were washed three times with 250 µl PBS containing 0.1% Tween 20. Horseradish peroxidase (HRP)-conjugated goat anti-human IgG (1:3,000; cat. no. AP113P; Sigma-Aldrich; Merck KGaA) was then added. The absorbance values of the wells were determined at 450 nm and analyzed by GraphPad Prism software version 5.0 (GraphPad Software Inc.). The negative control comprised PBS (10mM). The experiment was performed in triplicate.

Affinity and kinetic assay of human anti-hTLR4 IgG2. Affinity and kinetic assays of human anti-hTLR4 IgG2 were performed using a BLItz system (Fortebaio, Inc.). TLR4 was diluted to 50 ng/µl using PBS and then loaded to the biosensor (cat. no. 18-5012; Fortebaio, Inc.), while the anti-TLR4 IgG2 was diluted in different concentrations (100-1,600 nM). The association time was 120 sec and the dissociation time was 120 sec. Then, BLItz Pro 1.0.1 software (Fortebaio, Inc.) was used to analyze sensograms.

Fluorescence-activated cell sorting. Each group of MPM (1x10⁶ cells) was pretreated with 5 ng/µl human anti-TLR4 IgG2 at 37°C for 1 h, washed three times with PBS and probed with FITC-conjugated anti-human IgG (1:1,000; cat. no. F9512; Sigma-Aldrich; Merck KGaA) at 37°C for 1 h. A LSRII flow cytometer (BD Biosciences) was used to detect the fluorescence intensity of cells after washing three times with PBS.

Reverse transcription-quantitative PCR (RT-qPCR) analysis. To optimize the pretreatment concentration, MPM were cultured in 24-well plates (5x10⁵ cells/well), pretreated with human anti-TLR4 IgG2 at different concentrations (1, 5 and 10 ng/µl) at 37°C for 2 h and then stimulated with 1 ng/µl LPS at 37°C for 4 h. The optimum concentration of human
anti-TLR4 IgG2 (5 ng/µl) was used for subsequent analyses. After stimulation, total RNA was extracted using the RNaFast200 kit (cat. no. 220010; ShanghaiFastagen Biotech Co., Ltd.) and RT RNA to cDNA (37˚C for 15 min) using the PrimeScript RT Master Mix kit (cat. no. RR036A; Takara Bio, Inc.). RT-qPCR was performed using SYBR-Green I kit (cat. no. DRR041A; Takara Bio, Inc.) under the following conditions: Initial denaturation at 95˚C for 30 sec, followed by 40 cycles of 95˚C for 5 sec, 58˚C for 10 sec and 72˚C for 30 sec and an end-up synthesis at 72˚C for 30 sec. The relative expressions of cytokines were normalized to those of β-actin, using the 2^−ΔΔCq method (19). The primers, as previously described, were shown in Table I (20).

**Western blot analysis.** In order to analyze the inhibitory effect of human anti-TLR4 IgG2 on TLR4 signal transduction, western blotting analysis of phosphorylation levels of the NF-κB, mitogen-activated protein kinase (MAPK) and IRF-3 pathways was performed as previously described (20). The mouse macrophages, cultured in 6-well plates (10⁶ cells per well), were pre-incubated with the human anti-TLR4 IgG2 (5 ng/µl) at 37˚C for 2 h and induced with LPS (1 ng/µl) at 37˚C for 0, 30 or 60 min. Cells were lysed in a RIPA lysis buffer supplemented with protein inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Lysates were mixed and centrifuged at 12,000 x g for 15 min at 4˚C. A total of 30 µg protein/lane was then loaded onto an SDS-PAGE gel (12% resolving gel) and electrotransferred onto a nitrocellulose membrane after determining the protein concentration by BCA protein assay kit (cat. no. 23225; Thermo Fisher Scientific Inc.). After blocking with 5% non-fat milk in TBST (0.1% Tween 20) at 37˚C for 1 h, the membranes were incubated with primary antibodies (anti-p-p38, anti-p38, anti-p-p65, anti-p65, anti-p-JNK, anti-JNK, anti-p-ERK, anti-ERK, anti-p-IkB, anti-IkB, anti-p-IRF3, anti-IRF3, anti-p-IKK, anti-IKK and anti-β-actin antibody) diluted in 5% non-fat milk in TBST overnight at 4˚C. The membranes were then washed three times with TBST and probed with HRP-conjugated secondary antibodies (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) in 5% non-fat milk in TBST at room temperature for 1 h. Then, the bands were visualized by an enhanced chemiluminescence kit (cat. no. 1805001; Tanon Science and Technology Co., Ltd.). The relative protein expression levels were analyzed using Gel-Pro-analyzer software 4.0 (Media Cybernetics, Inc.).

The recombinant expression vector was transfected into CHO cells, which were cultured for 6 days. Cells were centrifuged and the supernatant was purified with a Protein A affinity column and examined via SDS-PAGE, followed by Coomassie Brilliant Blue staining (Fig. 1D). It was identified that the purification efficiency was ~95% with 2.5 mg/ml human anti-TLR4 IgG2.

**Verification of specific binding of human anti-TLR4 IgG2 to TLR4.** The present study determined whether human anti-TLR4 IgG2 can specifically and selectively bind to human TLR4 using ELISA. Gradient dilutions of human anti-TLR4 IgG2 were prepared for ELISA and it was found that this antibody can specifically bind to TLR4 in a dose-dependent manner (Fig. 2A). The antigen-antibody affinity constant was assessed to analyze the binding affinity of human anti-TLR4 IgG2 to TLR4. The mouse macrophages, cultured in 6-well plates (10⁶ cells per well), were pre-incubated with the human anti-TLR4 IgG2 (5 ng/µl) at 37˚C for 2 h and induced with LPS (1 ng/µl) at 37˚C for 0, 30 or 60 min. Cells were lysed in a RIPA lysis buffer supplemented with protein inhibitor cocktail (Sigma-Aldrich; Merck KGaA). Lysates were mixed and centrifuged at 12,000 x g for 15 min at 4˚C. A total of 30 µg protein/lane was then loaded onto an SDS-PAGE gel (12% resolving gel) and electrotransferred onto a nitrocellulose membrane after determining the protein concentration by BCA protein assay kit (cat. no. 23225; Thermo Fisher Scientific Inc.). After blocking with 5% non-fat milk in TBST (0.1% Tween 20) at 37˚C for 1 h, the membranes were incubated with primary antibodies (anti-p-p38, anti-p38, anti-p-ERK, anti-ERK, anti-p-IkB, anti-IkB, anti-p-IRF3, anti-IRF3, anti-p-IKK, anti-IKK and anti-β-actin antibody) diluted in 5% non-fat milk in TBST overnight at 4˚C. The membranes were then washed three times with TBST and probed with HRP-conjugated secondary antibodies (1:2,000; cat. no. 7074; Cell Signaling Technology, Inc.) in 5% non-fat milk in TBST at room temperature for 1 h. Then, the bands were visualized by an enhanced chemiluminescence kit (cat. no. 1805001; Tanon Science and Technology Co., Ltd.). The relative protein expression levels were analyzed using Gel-Pro-analyzer software 4.0 (Media Cybernetics, Inc.).
IgG2 to TLR4. Data obtained from BLItz system analysis had an equilibrium dissociation constant (KD) of 8.713x10^{-10} M (Fig. 2B), indicating that human anti-TLR4 IgG2 selectively and effectively bound to TLR4.

The binding ability of human anti-TLR4 IgG2 was further assessed via flow cytometric analysis of TLR4-positive MPM. Compared with the untreated control group, specific binding of the human anti-TLR4 IgG2 to TLR4 reached ~66% (Fig. 2C), suggesting that human anti-TLR4 IgG2 effectively binds to TLR4 on the mouse cell surface.

**Human anti-TLR4 IgG2 inhibits LPS-induced production of inflammatory cytokines in vitro.** To determine the optimal human anti-TLR4 IgG2 concentration that can inhibit LPS-stimulated MPM, the optimal concentrations of human anti-TLR4 IgG2 and LPS were examined. LPS was used at concentrations ranging from 0.01-1 ng/µl, and it was demonstrated that the mRNA expression levels of TNF-α and IFN-β were increased in a concentration-dependent manner compared with the L + A group (Fig. S1A and B). In addition, it was found that 1 ng/µl LPS induced significant inflammation, on which the human anti-TLR4 IgG2 showed a higher inhibition efficiency. Furthermore, compared with 1 ng/µl human anti-TLR4 IgG2, a concentration of 5 ng/µl showed improved inhibition efficiency on IFN-β and TNF-α mRNA expression levels (Fig. 3). However, 10 ng/µl human anti-TLR4 IgG2 did not have a higher performance compared with the concentration of 5 ng/µl. Moreover, treatment with 5 ng/µl human anti-TLR4 IgG2 reduced TNF-α, IFN-β and IL-6 expression levels by approximately 50, 90 and 40%, respectively, compared with levels after LPS treatment (Fig. 3). Therefore, human anti-TLR4 IgG2 and LPS concentrations of 5 and 1 ng/µl, respectively, were used in the subsequent experiments.

To assess whether human anti-TLR4 IgG2 reduces the production of LPS-induced inflammatory cytokines, the mRNA expression levels of TNF-α, IFN-β and IL-6 were examined at different time points. MPM were pretreated with 5 ng/µl human anti-TLR4 IgG2 for 2 h and induced with 1 ng/µl LPS for 2, 4 and 8 h. It was demonstrated that TNF-α, IFN-β and IL-6 were significantly upregulated in LPS-induced groups compared with those in the untreated controls; however, these were significantly downregulated after pretreatment with...
Human anti-TLR4 IgG2 specifically recognizes TLR4 with high affinity. (A) ELISA results. TLR4 was used to coat ELISA plates. The wells were incubated with serial dilutions of human anti-TLR4 IgG2. The specific binding of human anti-TLR4 IgG2 and TLR4 was concentration-dependent. (B) A BLItz System was used to determine the binding affinity of TLR4. TLR4 affinity and kinetics assays yielded five curves with the concentrations of human anti-TLR4 IgG2 ranging from 100-1,600 nM. Equilibrium dissociation constant = 8.71x10^{-10} M with TLR4. (C) Affinity of human anti-TLR4 IgG2 for TLR4 was quantified via flow cytometry analysis. (a) Blank; (b) group treated without human anti-TLR4 IgG2; (c) group treated with human anti-TLR4 IgG2. OD450, optical density at 450 nm; TLR4, Toll-like receptor 4.

Human anti-TLR4 IgG2 inhibits phosphorylation levels of TLR4 signaling after LPS stimulation. To investigate the inhibition of human anti-TLR4 IgG2 on LPS-induced TLR4 signaling, western blotting was used to analyze the phosphorylation of the NF-κB, MAPK and IRF-3 signaling pathways, which are downstream effectors of the TLR4 pathway. After treatment with LPS, the phosphorylation of p65, p38, JNK, ERK, IκB, IKK and IRF-3 increased, but decreased after pretreatment with 5 ng/µl human anti-TLR4 IgG2 (Figs. 5-7). Thus, these results indicated that human anti-TLR4 IgG2 inhibited LPS-induced inflammatory responses in MPM by blocking TLR4.

Human anti-TLR4 IgG2 protects mice from LPS-induced sepsis in vivo. An in vivo protection assay was carried out in the CLP model. After LPS administration, the mice treated with human anti-TLR4 IgG2 had a lower mortality rate compared to the control group.
with PBS in the control group died within 35 h. Moreover, in the Group L + A receiving the antibody, human anti-TLR4 IgG2 protected mice from LPS challenge with a survival rate of 40% and significantly increased the survival time, compared with the Group L (Fig. 8). Furthermore, the present study examined the serum levels of inflammatory factors by ELISA. It was identified that treatment with human anti-TLR4 IgG2 reduced LPS-initiated inflammatory responses, as it reduced TNF-α, IFN-β and IL-6 levels by ~80, 75 and 60% at 4 h after LPS injection, respectively (Fig. S2). Collectively, these results were consistent with those from the in vitro inhibition assay.

Discussion

Excessive host responses to LPS can lead to systemic inflammatory conditions, including sepsis and fatal septic shock (22-24). The mortality rate of severe sepsis can reach 30-50% worldwide, possibly due to the lack of efficient therapies (22-24). Previous findings have confirmed the role of inflammatory pathways stimulated by the interaction between TLR4 and LPS (13); therefore, blocking of LPS-TLR4 signaling is important.

The present study extracted an anti-TLR4 Fab from a human phage library and transformed it to IgG2 with an affinity of 8.713x10^-10 M, which has stronger affinity to TLR4 compared with the Fab region alone. The eukaryotic expression vector pMH3-hTLR4-IgG2 was successfully constructed and using a BLItz system and ELISA, the specific binding ability of the human anti-TLR4 IgG2 to TLR4 was assessed. The present results indicated that the H and L chains of human anti-Tlr4 IgG2 were efficiently assembled into the active form, and the approaches to prepare mAb did not change the specificity of the human anti-TLR4 IgG2.

LPS increases the secretion of numerous inflammatory cytokines by activating the phosphorylation of the TLR4-mediated NF-κB, MAPK and IRF3 pathways, and also elevates the production of T proinflammatory cytokines (11-13,25). Thus, the present study evaluated human anti-TLR4 IgG2 by measuring TNF-α, IFN-β and IL-6 levels, which are involved in the MyD88 pathway, after LPS stimulation. The results showed that human anti-TLR4 IgG2 at 5 ng/µl was sufficient for blocking TLR4 on the surface of MPM, and did not show a higher inhibitory effect at an increased concentration (10 ng/µl). Moreover, RT-qPCR results identified a significant increase in LPS-induced production of TNF-α, IFN-β and IL-6 mRNA expression levels, but this production decreased after pretreatment with human anti-TLR4 IgG2. Western blotting results demonstrated that LPS-stimulated phosphorylation of p65, p38, JNK, ERK, IκBα, IKKaβ and IRF3. However, these results were reversed by preincubation with human anti-TLR4 IgG2, which was consistent with the decreased expression levels of proinflammatory cytokines. In addition, given the high homology between mice and humans, an in vivo neutralization assay as performed using the mouse CLP model in which LPS was injected peritoneally.
Figure 4. Human anti-TLR4 IgG2 inhibits LPS-induced production of inflammatory cytokines in mouse macrophages. (A) TNF-α, (B) IFN-β and (C) IL-6 expression levels were quantified via reverse transcription-quantitative PCR and normalized to the internal control, β-actin. All experiments were performed independently ≥3 times. Data are presented as the mean ± SD. N=3. *P<0.05, **P<0.01, vs. LPS control. LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; L, LPS; A, human anti-TLR4 IgG2; IL, interleukin; IFN-β, interferon-β; TNF-α, tumor necrosis factor-α.

Figure 5. Human anti-TLR4 IgG2 activates the MAPK signal pathway after LPS stimulation. Cells were pretreated with human anti-TLR4 IgG2 (5 ng/µl) for 2 h and further incubated in the presence or absence of LPS (1 ng/µl) for 0, 30 and 60 min. After immunoblotting, phospho-specific antibodies were used to probe the regions containing p-ERK1/2, p-JNK1/2 and p-p38. β-actin was used as the internal loading control. *P<0.05, **P<0.01, ***P<0.001 vs. LPS group. LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; L, LPS; A, human anti-TLR4 IgG2; p-, phosphorylated.
the half-life of antibody is short, mice were immunized via intravenous injection to increase the absorption rate. It was found that human anti-TLR4 IgG2 efficiently protected mice from LPS challenge with a survival rate of 40% and inhibited LPS-induced sepsis in mice by decreasing serum levels of proinflammatory cytokines. Thus, it was speculated that human anti-TLR4 IgG2 could rescue mice from severe sepsis. However, while this mouse model is used for several purposes, such as for investigating pathogenic mechanisms and evaluating new therapeutic approaches (26-28), individual gene activation in humans may not necessarily be predicted by the ortholog in the corresponding mouse model (29), which is a limitation to the neutralization assay. In addition, the experiments performed in mouse macrophages do not completely mimic human inflammatory responses (29-32). Thus, further studies are required to assess the inhibitory effects of human anti-TLR4 IgG2 in human macrophages.

Figure 6. Human anti-TLR4 IgG2 activates the NF-κB signaling pathway after LPS stimulation. Cells were pretreated with human anti-TLR4 IgG2 (5 ng/µl) for 2 h and further incubated in the presence or absence of LPS (1 ng/µl) for 0, 30 and 60 min. After immunoblotting, phospho-specific antibodies were used to probe the regions containing p-IKK, p-IκB and p-p65. β-actin was used as the internal loading control. "P<0.01, "**P<0.001 vs. LPS group. LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; L, LPS; A, human anti-TLR4 IgG2; p-, phosphorylated.

Figure 7. Human anti-TLR4 IgG2 activates IRF-3 after LPS stimulation. Cells were pretreated with human anti-TLR4 IgG2 (5 ng/µl) for 2 h and further incubated in the presence or absence of LPS (1 ng/µl) for 0, 30 and 60 min. The phospho-specific antibody was used to detect phosphorylation level of IRF-3. β-actin was used as the internal loading control. "P<0.05, "***P<0.001 vs. LPS group. LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; L, LPS; A, human anti-TLR4 IgG2; p-, phosphorylated.
anti-TLR4 IgG2 for the treatment of infection-associated immune dysfunction in humans.

Previous studies have been aimed at evaluating TLR4 inhibition by targeted small-molecule compounds or antibodies for the therapy of multiple inflammatory responses (33-37). In addition to the human anti-TLR4 IgG2 designed in the present study, several mAbs against TLR4 have been reported, which can be divided into two categories. Firstly, agonistic mAbs, such as UT12 and Sa15-21, which induce NF-κB activation and protect mice from subsequent lethal LPS challenges (36,37); this phenomenon is called LPS tolerance. However, UT12 is significantly distinguished from Sa15-21 as the latter enhances LPS-induced TNF-α production, while Sa15-21 alone induces minimal TNF-α production (36). The other category is antagonistic mAbs, such as MTS510, which inhibit LPS-induced NF-κB activation in TLR4-expressing cells (34). The human anti-TLR4 IgG2 designed in the present study belongs to the second category. Moreover, there are two advantages in the application of the human anti-TLR4 IgG2. Firstly, previous reported antibodies are mouse mAbs or humanized murine mAbs (20,34,36-38), and these antibodies contain some non-human components that could cause antigen-reactive responses. Furthermore, the human anti-TLR4 IgG2 used in the present study was a complete human antibody, without any potential to elicit mAb production in humans. Moreover, human anti-TLR4 IgG2 was produced using a eukaryotic expression system that had post-translational modification capabilities, while also eliminating the effect of Escherichia coli endotoxin, which is prevalent in anti-TLR4-Fab produced in prokaryotic expression systems (20,38).

Previous findings have characterized the three-dimensional structures of LPS receptors (39-42). It has also been reported that the LBP binds firstly to LPS and presents it to CD14, and then CD14 transfers LPS to the TLR4/MD2 complex to form the M-shaped TLR4/MD2/LPS complex dimer (40). The TIR domains of TLR4 are located in close spatial contiguity upon dimer formation, activating downstream signaling molecules and promoting the secretion of inflammatory cytokines (40,41). Therefore, it was speculated that the human anti-TLR4 IgG2 may prevent the interaction of MD2 with TLR4, thus blocking LPS-induced TLR4 signal pathway transduction. However, the protective mechanism of the human anti-TLR4 IgG2 requires further investigation.

In conclusion, the present study established a full human anti-TLR4 IgG2 that bound specifically to TLR4 with high affinity, inhibited the TLR4/MAPKs/NF-κB signaling pathway and reduced the production of downstream inflammatory mediators, such as TNF-α, IL-6 and IFN-β. Therefore, the specific blockade of TLR4 activation by the human anti-TLR4 IgG2 may be promising in the treatment of infection-associated diseases in the future.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions
YW performed most of the experiments, analyzed the data and wrote the manuscript. DG helped with the partial plasmid construction. FZ and TZ helped with the preparation of experimental samples. CY and QC helped with experimental operations. MW helped with experiment design. JZ and XZ designed the research and revised the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
All animal experiments in this study were performed according to the protocols approved by the Ethics Committee of Huadong Medical Institute of Biotechniques.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References


