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Original Article

A humanized anti-Toll like receptor 4 antibody Fab fragment inhibits pro-inflammatory responses induced by lipopolysaccharide through TLR4 in vitro and in vivo

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Abstract

Introduction: Toll like receptor 4 (TLR4) and its co-receptor MD-2 recognize bacterial lipopolysaccharide (LPS), initiating responses to infections caused by Gram-negative bacteria. TLR4 also plays a role in various pathological processes, including viral infections and sterile inflammation. However, effective methods to inhibit LPS/TLR4-mediated inflammation remain elusive. This study aimed to evaluate the inhibitory effects of a constructed hTLR4-Fab on LPS-induced inflammation in both in vitro and in vivo settings.

Methodology: In vitro, mouse dendritic cells (DCs), human macrophages, and human DCs were incubated with hTLR4-Fab and then stimulated with LPS. In vivo, mice were pre-treated with a humanized anti-TLR4 antibody Fab prior to LPS injection. We examined the activation of various signaling pathways to elucidate the molecular mechanism underlying the inhibition of LPS-induced inflammation by hTLR4-Fab.

Results: We observed that the binding affinity of hTLR4-Fab to TLR4 on mouse bone marrow-derived dendritic cells (DCs) was approximately 81.8%, while the binding affinity to human blood monocyte-derived macrophages and DCs exceeded 90%. Pretreatment with hTLR4-Fab significantly reduced both mRNA and protein levels of LPS-induced proinflammatory cytokines. In vivo, a significant suppression of serum cytokine expression was driven by hTLR4-Fab treatment.

Conclusions: The results demonstrated that the antibody Fab could impede the phosphorylation of downstream components, including the nuclear factor κB (NF-κB) signaling pathway, the mitogen-activated protein kinase (MAPK) signaling pathway, and IFN regulatory factor 3 (IRF-3), all of which are activated by TLR4. Consequently, our study demonstrates that our hTLR4-Fab is effective in mitigating LPS-induced inflammation, both in vitro and in vivo.

Key words: TLR4 signaling; sepsis; anti-inflammatory therapy lipopolysaccharide; toll like receptor 4 signaling; inflammation.

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Introduction

Immune cells, such as monocytes, macrophages, and dendritic cells (DCs), which express Toll-like receptors (TLRs) play a crucial role in the immune response against invading pathogens [1,2]. Serving as the primary detectors of pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs), TLRs are essential in microbe-host interactions and innate immunity [3]. TLR4, a member of the TLR family, functions as the pattern recognition receptor (PRR) for lipopolysaccharide (LPS), a major endotoxin and component of gram-negative bacterial cell walls [2,4]. Independent studies have shown that LPS, along with LPS-binding protein, binds to CD14 and subsequently interacts with the TLR4/MD-2 complex. This interaction stimulates TLR4 expression on immune cell surfaces and activates the TLR4 signaling pathway [5].

The TLR4 signaling pathway involves two crucial routes: the MyD88-dependent pathway and the TIR-domain-containing adapter-inducing interferon-β (TIRF) mediated pathway. Once activated, the MyD88 pathway triggers the release of pro-inflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12, and tumor necrosis factor (TNF) – α [6,7]. Meanwhile, the TIRF pathway induces the IFN-β expression and initiates innate immune and inflammatory responses [8]. Evidence suggests that activation of the LPS/TLR4 signaling pathway may lead to chronic inflammation, a key factor in various chronic degenerative pathologies including cancer, cardiovascular diseases, and renal damage [8,9]. This suggests that targeting these pathways, such as through anti-LPS strategies, anti-cytokine therapies, and agents that neutralize LPS or inhibit TLR4/MyD88 signaling, could be effective in treating TLR4-mediated diseases [10,11]. However, to date, these approaches have seen limited success. TLR4-knockout mice exhibit resistance to traditional murine models of LPS-induced sepsis, and TLR4-deficient macrophages do not induce the production of genes typically induced by LPS [12]. Therefore, we propose that blocking the TLR4 receptor might be an effective approach to inhibit LPS-induced inflammation. Through our previous research utilizing phage antibody library technology, we have successfully developed a humanized anti-TLR4 Fab antibody and assessed its effects on mouse macrophages. Our findings indicate that this antibody effectively suppresses the expression of pro-inflammatory cytokines induced by LPS in mouse macrophages, as well as inhibits the activation of NF-κB, MAPK, and IRF-3 signaling pathway.

In this study, we conducted in vitro and in vivo experiments to further investigate the mechanism of action of our humanized anti-TLR4 antibody Fab. The results demonstrated that hTLR4-Fab can bind to TLR4 receptors on immune cells from different species, and effectively inhibit LPS-induced inflammation in these cells. Additionally, the anti-TLR4 antibody significantly suppressed the level of pro-inflammatory cytokines in serum following LPS injection.

Methodology

Reagents and Animals

The hTLR4-Fab has been previously described. LPS from E. coli 055: B5 was purchased from Sigma (St. Louis, MO, USA). RPMI-1640 and fetal bovine serum (FBS) for cell culture were obtained from Gibco (Carlsbad, CA, USA). ELISA kits for TNF-α, IL-1, and IL-6 were purchased from R&D systems (Minneapolis, MN, USA). IFN-β ELISA kits were obtained from PBL (Piscataway, NJ, USA). Anti-phospho-p38, -phospho-JNK1/2, -phospho-ERK1/2, -phospho-p65, -phospho-IKK, phosphor-IκB and -phospho-IRF3 were obtained from Cell Signaling Technology (Danvers, MA, USA). Mouse IL-4, GM-CSF, and M-CSF as well as human IL-4, GM-CSF, and M-CSF were purchased from R&D systems. C57BL/6J mice weighing 15-20 g were used in this study. Mice were obtained from SLAC Laboratory Animal Company (Shanghai, China) and housed in microisolator cages under specific pathogen-free conditions. They were fed with autoclaved food and used for experiments at 4-8 weeks of age. All animal experiments adhered to the National Institutes of Health Guide for Care and Use of Laboratory Animals and received approval from the Research Institute for Medicine of Nanjing Command (2019020).

Cells

Murine bone marrow-derived dendritic cells (DCs) were obtained from the femurs of 4-week-old C57BL/6J mice, as previously described. Briefly, the mice were euthanized by rapid cervical dislocation, and their bone marrow was flushed with RPMI-1640 medium. The collected cell suspensions were centrifuged at room temperature for 5 minutes at 800 × g. The resulting cell pellets were resuspended in DC complete medium containing RPMI-1640 supplemented with 10% FBS, IL-4 (2ng/mL), and GM-CSF (10 ng/mL), subsequently added to each well in six-well plates (3×10^6 cells/well) with 3 ml of DC complete medium, and cultured at 37°C and 5% CO_2. Every two days, 1mL of fresh DC complete medium was added to each well. After seven days, the adherent cells were assessed for cell-surface markers CD11C and MHC II, indicating an approximately 70% pure DC population. These cells were then utilized for subsequent experiments.

Human blood monocytes were isolated from healthy individuals to obtain macrophages and dendritic cells. This isolation process followed established methods, involving the separation of peripheral blood mononuclear cells (PBMCs) through Ficoll-paque centrifugation (GE Healthcare UK Ltd., Buckinghamshire, England). Subsequently, peripheral blood monocytes were isolated from PBMCs using $CD14^+$ magnetic beads (Miltenyi Biotec GMBH, Gladbach, Germany). The cells were then cultured in either 4 ml of DC complete medium (RPMI-1640 supplemented with 10% FBS, IL-4 10 ng/mL, GM-CSF 10ng/mL) or macrophage complete medium (RPMI-1640 supplemented with 10% FBS, M-CSF 20ng/mL),

at a density of 3 × 10^6 cells/well in six-well plates. Every two days, the medium was replenished through the addition of 1 ml of complete medium to each well specific to either DCs or macrophages. After 7 days, the adherent cells were purified based on their expression of cell-surface markers: CD11C and MHC II for DCs (70% purity), and CD11b and CD14 for macrophages (68.9% purity). These cells were then utilized in subsequent experiments.

Flow cytometry

Cells (mouse DCs, human DCs, human macrophages) were incubated with hTLR4-Fab on ice for 60 minutes, followed by three washes with phosphate-buffered saline (PBS). The cells were subsequently incubated in the dark at 4°C for 30 minutes with FITC-conjugated goat anti-human IgG (Fab specific) and analyzed using a FACS Calibur instrument from Becton Dickinson in Oxford, UK.

Cytokine mRNA levels analysis

The mRNA levels of various cytokines were quantified using real-time quantitative PCR (qPCR). Cells (murine bone marrow-derived DCs, and human blood monocyte-derived macrophages and DCs) were cultured at a density of 2.5-3.5 × 10^5 cells/well in 24-well plates and pre-incubated with hTLR4-Fab (1μg/mL) for 2 hours. The cells were then washed three times with PBS before being treated with LPS (1000 ng/mL). After stimulation by LPS for 4, 8, or 12 hours, the cells were collected and RNA was extracted using a total RNA kit (Omega, Norcross, GA, USA), following the manufacturer's protocol. Subsequently, 1 microgram of total RNA was utilized as a template for cDNA synthesis using the PrimeScript RT Master Mix kit (Takara Bio, Shiga, Japan) following the manufacturer's protocol. Quantitative real-time PCR was performed using a standard SYBR Green PCR kit (Takara Bio, Shiga, Japan) to detect the relative levels of expressed cytokines (TNF-α, IFN-β, IL-1, IL-6) utilizing a Real-time PCR machine (ABI7500HT, Applied Biosystems, Foster City, CA).

The primers utilized in this investigation are listed in Table 1 (in the 5' to 3' direction). The 2-△△Ct method was utilized for relative quantification, with cytokine genes normalized to corresponding β-actin/GAPDH results.

Cytokine protein levels analysis

The expression levels of related cytokine proteins were measured using ELISA (Enzyme-linked immunosorbent assay). Cell culture supernatants were collected following 8 hours of LPS stimulation, with or without pre-treatment with hTLR4-Fab. The concentrations of cytokines (TNF-α, IFN-β, IL-1, IL-6) in the cell culture supernatants then were measured using human and mouse TNF-α, IL-1, IL-6 (R&D Systems, Minneapolis, MN, USA), and IFN-β ELISA kits (PBL, Piscataway, NJ, USA) according to the manufacturer's protocols.

Western bolt analysis

Western blotting was utilized to investigate the TLR4 signaling pathways in mouse DCs, human DCs, and human macrophages. Specifically, respective cells (1 × 10^6) were incubated with hTLR4-Fab for 2 hours before being stimulated by LPS for varying time periods (15 minutes, 30 minutes, 60 minutes). Following stimulation, the cells were lysed at 4°C using RIPA buffer (50 mM Tris-HCl pH 8, 1% Triton X-100,

Table 1. Mouse and human TNF-α, IFN-β, IL-1, IL-6, and β-actin gene-specific primer sequences for quantitative PCR.

Primer name		Primer sequence
mouse TNF-α	forward primer	GACGTGGAACTGGCAGAAGAG
	reverse primer	TTGGTGGTTTGTGAGTGTGAG
mouse IFN-β	forward primer	CAGCTCCAAGAAAGGACGAAC
	reverse primer	GGCAGTGTAACTCTTCTGCAT
mouse IL-1	forward primer	GCAACTGTTCCTGAACTCAACT
	reverse primer	ATCTTTTGGGGTCCGTCAACT
mouse IL-6	forward primer	TAGTCCTTCCTACCCCAATTTCC
	reverse primer	TTGGTCCTTAGCCACTCCTTC
mouse β-actin	forward primer	AGTGTGACGTTGACATCCGT
	reverse primer	GCAGCTCAGTAACAGTCCGC
human TNF-α	forward primer	CCTCTCTCTAATCAGCCCTCTG
	reverse primer	GAGGACCTGGGAGTAGATGAG
human IFN-β	forward primer	ATGACCAACAAGTGTCTCCTCC
	reverse primer	GGAATCCAAGCAAGTTGTAGCTC
human IL-1	forward primer	ATGATGGCTTATTACAGTGGCAA
	reverse primer	GTCGGAGATTCGTAGCTGGA
human IL-6	forward primer	ACTCACCTCTTCAGAACGAATTG
	reverse primer	CCATCTTTGGAAGGTTCAGGTTG
GAPDH	forward primer	GGAGCGAGATCCCTCCAAAAT
	reverse primer	GGCTGTTGTCATACTTCTCATGG

100 mM NaCl, and 1 mM EDTA), supplemented with a protease and phosphatase inhibitor cocktail. Following a 10-minute incubation on ice, the cell lysates were centrifuged at 10,000 × g for 10 minutes at 4 °C. Protein concentrations were determined using a BCA (Bicinchoninic acid) protein assay kit (Thermo, Waltham, MA, USA). Equal amounts of proteins were loaded onto a 10% SDS-polyacrylamide gel and separated by electrophoresis (SDS-PAGE) before being transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk or 5 % BSA (Bovine serum albumin) in TBS (10 mM Tris, pH 7.5, 1%, 50 mM NaCl) containing 0.1% Tween 20 (TBST) for one hour at 4°C. Immunoblotting was performed using primary antibodies specific for phosphor-(p38, p-65, IRF3), diluted to a ratio of 1:1000, and detected with HRP-conjugated goat anti-rabbit or anti-mouse IgG antibodies from Sigma. Visualization was achieved using a chemiluminescent substrate from Millipore. All reagents utilized in the phosphor-flow and western blot experiments were free of endotoxins.

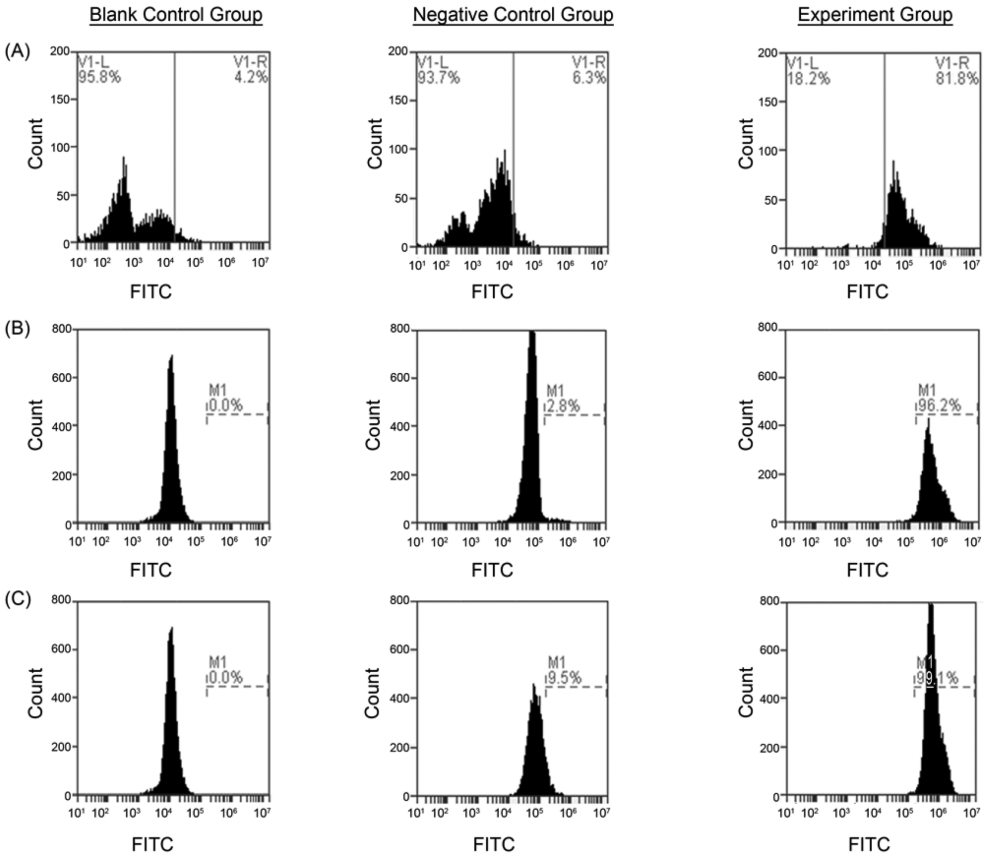
In vivo experiments

C57BL/J mice were pre-treated with an intraperitoneal injection of hTLR4-Fab for 15 minutes, followed by an LPS injection (1mg/kg) for 90 minutes. The doses of hTLR4-Fab were divided into three groups (0.5 mg/kg, 1 mg/kg, 2 mg/kg) according to the body weight of the mice. Whole blood was collected via terminal cardiac puncture and allowed to coagulate at room temperature for 2 hours. Coagulated blood was then centrifuged at 12,000 × g for 10 minutes at 4 °C to separate the serum. The cytokine levels in the serum were analyzed by ELISA.

Statistical Analysis

Experiments were conducted with at least three replicates. Results are presented as mean ± standard deviation. Statistical analyses were performed using SPSS 26.0 software, with a $p < 0.05$ considered as statistically significant.

Figure 1. Flow cytometry was utilized to detect the specific binding of anti-TLR4 Fab segment to cells expressing TLR4 (A: mouse dendritic cells, B: human macrophages, C: human dendritic cells).



Results

hTLR4-Fab exhibits specific binding affinity towards TLR4 expressed on the surfaces of diverse immune cell types

In our previous study, we observed that hTLR4-Fab exhibited specific binding to TLR4 on the surface of mouse macrophages, with a binding rate of 64.43%. In the current study, we extended our investigation to the specific binding affinity of hTLR4-Fab to TLR4 on a diverse array of cell types. Our results show that the binding rate of hTLR4-Fab to TLR4 is approximately 81.8% on murine bone marrow-derived dendritic cells (DCs) (Figure 1A), and exceeded 90% on human blood monocyte-derived macrophages and DCs (Figure 1B and Figure 1C).

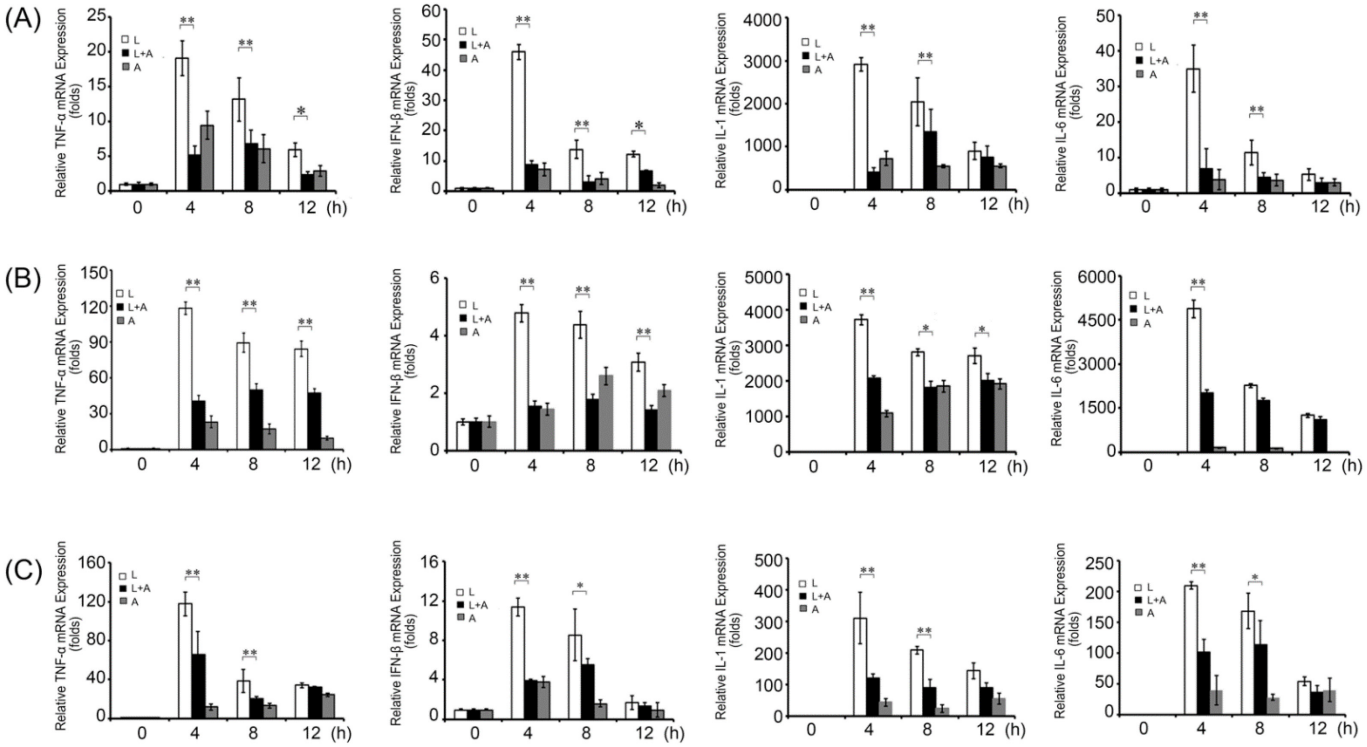
Inhibitory effects of hTLR4-Fab on LPS-induced up-regulation of cytokine mRNA expression in immune cells

To evaluate the inhibitory effect of hTLR4-Fab on LPS-induced pro-inflammatory cytokine expression at various incubation intervals, we quantified mRNA levels of TNF-α, IFN-β, IL-1, and IL-6. These cytokines are pivotal in mediating inflammatory responses. Following stimulation with LPS (1000 ng/ml), in the presence or absence of hTLR4-Fab (1 ug/ml), cytokine mRNA expression was quantified by q-PCR. LPS stimulation significantly upregulated the mRNA levels of TNF-α, IFN-β, IL-1, and IL-6 in murine bone marrow-derived DCs as well as human blood monocyte-derived macrophages and DCs (Figure 2). Treatment with hTLR4-Fab exhibited a time-dependent inhibition of expressed cytokine mRNA levels compared to untreated control groups. The inhibitory effects were most pronounced following 4 hours of LPS incubation, gradually attenuating over time. These findings demonstrate that hTLR4-Fab possesses the capacity to modulate LPS-induced inflammatory responses at the mRNA level in three distinct types of immune cells.

hTLR4-Fab attenuates LPS-mediated cytokine secretion in cell culture supernatants

While Figure 2 shows that hTLR4-Fab effectively inhibits the upregulation of cytokine mRNA production induced by LPS, it is important to note that changes in protein expression do not always correlate with mRNA levels. Therefore, we examined whether the humanized

Figure 2. The Fab segment of the anti-TLR4 antibody exerts an inhibitory effect on the transcription of inflammatory factors in LPS-stimulated mouse dendritic cells (A), human macrophages (B) and human dendritic cells (C).



L: LPS (1000 ng/mL) group, L+A: LPS (1000 ng/mL) + antibody group (1 ug/mL), A: antibody group (1 ug/mL), The independent-samples t test was used between two groups. *$p < 0.05$, **$p < 0.01$.

anti-TLR4 antibody could suppress the expression of cytokine proteins induced by LPS. Cells were pretreated with hTLR4-Fab (1 μg/mL) for 2 hours before being stimulated with LPS for 8 hours. Pro-inflammatory cytokines were then measured in the cell supernatants. ELISA results indicated that cytokine concentrations of TNF-α, IFN-β, IL-1, and IL-6 were significantly reduced in the hTLR4-Fab pre-treatment group juxtaposed to the LPS-only group (Figure 3). These findings suggest that hTLR4-Fab may also influence LPS-induced inflammatory responses at the protein level in these three types of immune cells.

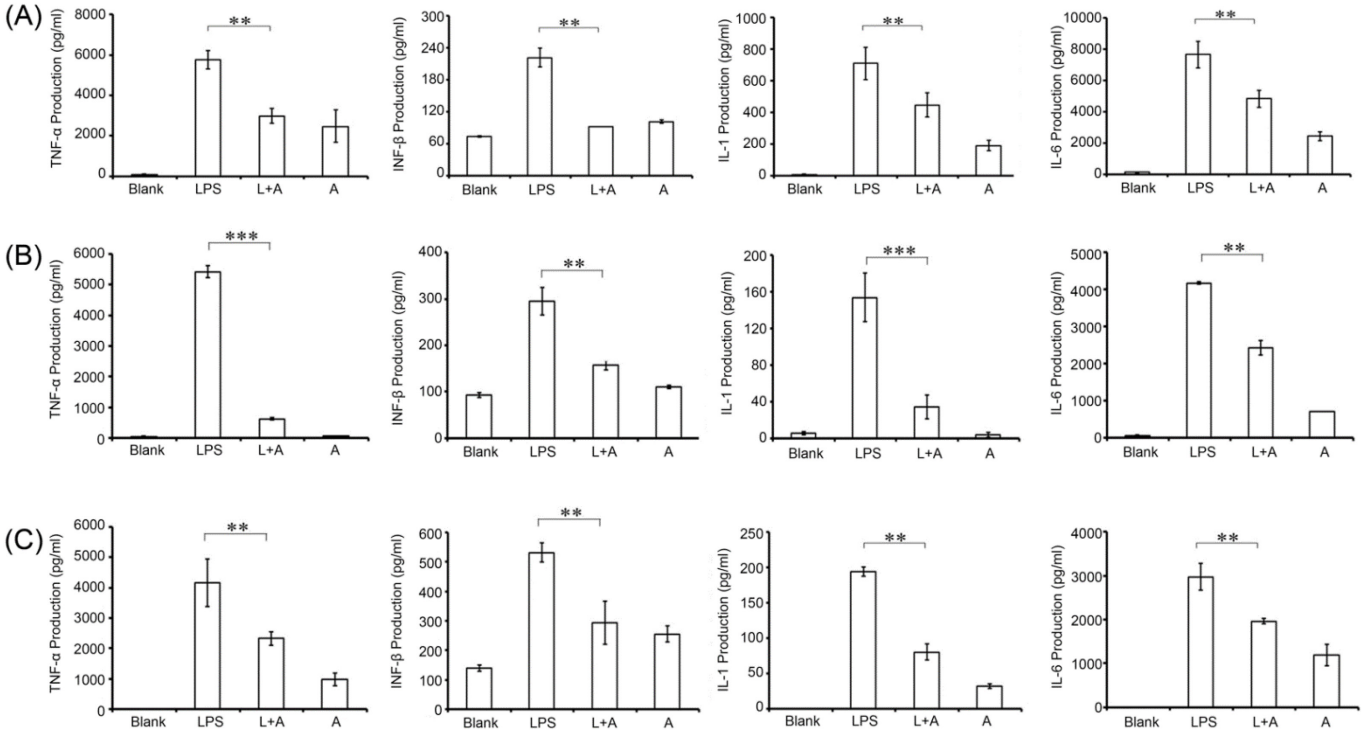
Impact of hTLR4-Fab on MAPK and NF-κB signaling pathways in LPS-induced immune cells

To elucidate the molecular mechanism behind hTLR4-Fab's inhibition of LPS-induced inflammatory responses, we examined its effects on MAPK activation in human blood monocyte-derived macrophages. As downstream signaling cascades of LPS/TLR4, MAPK pathways are critical components of the innate immune response that activate alternative factors involved in cytokine transcription (e.g., AP-1). Therefore, we explored the effect of hTLR4-Fab pre-treatment on the phosphorylation levels of p38 following LPS stimulation. As depicted in Figure 4, there was a significant reduction in the phosphorylation of these targets in the hTLR4-Fab pre-treated groups after LPS stimulation compared to the LPS-only group. Additionally, we investigated the influence of hTLR4-Fab on the activation of the NF-κB pathway, a downstream signaling pathway of LPS/TLR4. Our findings demonstrate that, in human blood monocyte-derived macrophages stimulated with LPS and hTLR4-Fab, there was a significant reduction in the level of phosphorylated p65 (Figure 4B). Comparable results were observed in murine bone marrow-derived DCs and human blood monocyte-derived DCs following LPS stimulation (Figure 4A).

The effect of hTLR4-Fab on the IRF3 signaling pathway in LPS-induced immune cells.

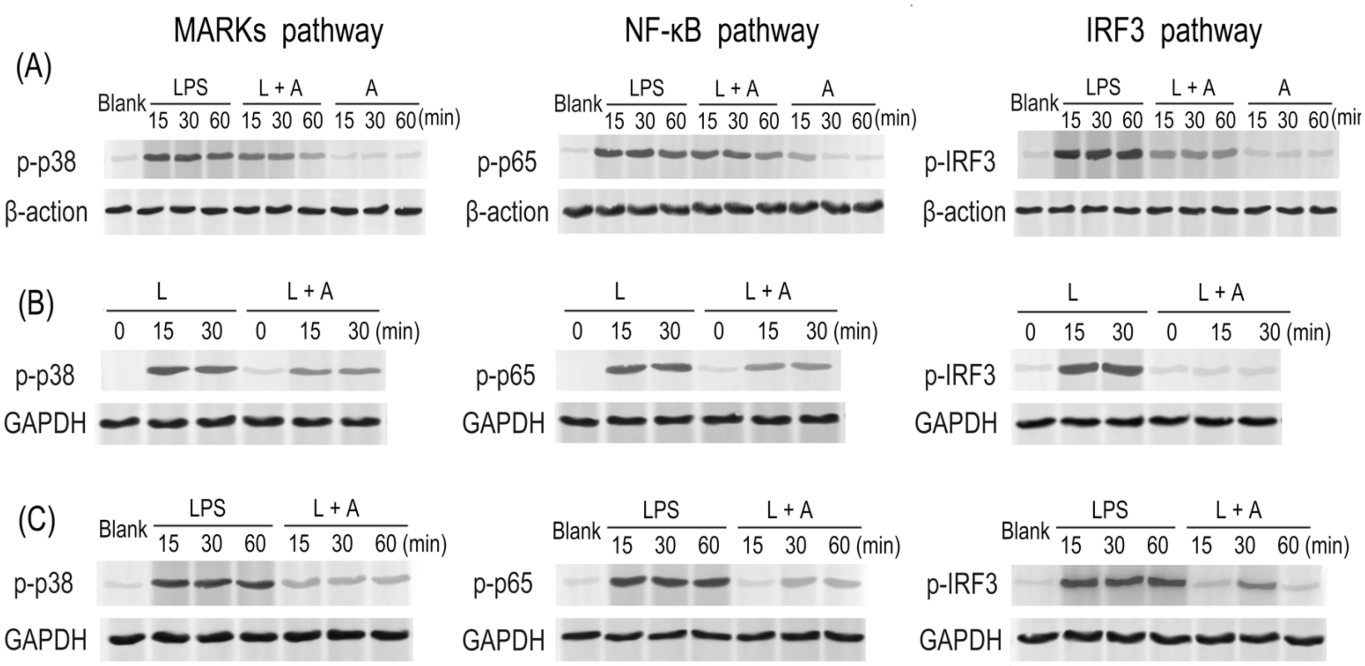
In the next phase of our study, we investigated the impact of hTLR4-Fab on the LPS-activated IRF3 signaling pathway. This pathway is initiated by LPS-TLR4 endocytosis through adaptors distinct from MyD88, leading to signaling via the transcription factor IFN, which in turn induces expression of the IFN-β gene. IFN-β is crucial in antigen presentation and adaptive immunity. Our findings reveal that the

Figure 3. The inhibitory effect of anti-TLR4 antibody Fab fragment on the expression of inflammatory cytokines in LPS-stimulated (8h) mouse DCs' cell supernatant (A), human macrophage cell supernatant (B) and human dendritic cells (C).



L: LPS (1000 ng/mL) group, L + A: LPS (1000 ng/mL) + antibody group (1 ug/mL), A: antibody group (1 ug/mL), The independent-samples t test was used between two groups. *$p < 0.05$, **$p < 0.01$.

Figure 4. The Fab segment of the anti-TLR4 antibody inhibits activation of the TLR4 signaling pathway in LPS-stimulated (15min, 30min, 60min) mouse DCs (A), human macrophages stimulated by LPS (B), and human dendritic cells (C).



L: LPS (1000 ng/mL) group, L + A: LPS (1000 ng/mL) + antibody group (1 ug/mL), A: antibody group (1 ug/mL).

presence of hTLR4-Fab during LPS stimulation resulted in decreased IRF3 phosphorylation levels in human blood monocyte-derived macrophages (Figure 4). Similar results were found in LPS-stimulated murine bone marrow-derived DCs and human blood monocyte-derived DCs.

In vivo administration of hTLR4-Fab reduces LPS-induced production of pro-inflammatory cytokines

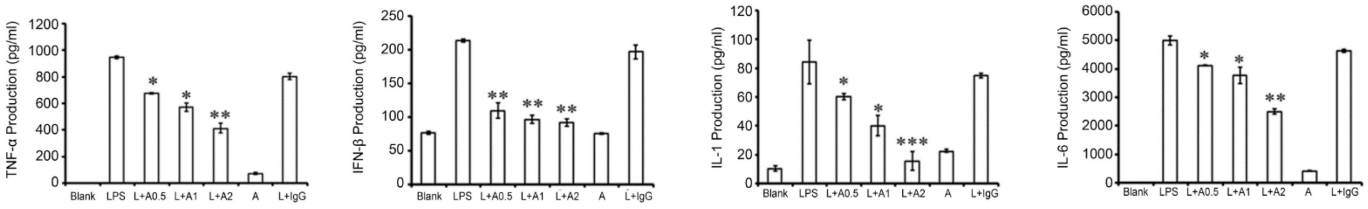
To evaluate the inhibitory effects of hTLR4-Fab on LPS-induced pro-inflammatory cytokine production in vivo, we established an endotoxemia mouse model. Our findings demonstrate that pre-treatment with hTLR4-Fab significantly attenuated cytokine production compared to the LPS-only group, as evidenced by reduced serum cytokine levels (Figure 5).

Discussion

Lipid A, a constituent of LPS, is highly immunogenic and can elicit septic severe immune reactions such as septic shock or sepsis—an uncontrolled immune response potentially leading to organ failure and mortality [13]. Currently, there are no specific pharmacotherapies available for managing LPS-induced inflammatory reaction. Therefore, our study aimed to evaluate the effectiveness of hTLR4-Fab, our anti-TLR4 antibody, in mitigating inflammation responses induced by LPS both in vitro and in vivo.

Toll-like receptor 4 (TLR4) is primarily expressed

Figure 5. The Fab segment of anti-TLR4 antibody effectively inhibits the expression of inflammatory factors in mouse serum induced by LPS.



Blank: blank control group, L: LPS(1mg/kg) group, L + A0.5: LPS (1mg/kg) + antibody (0.5mg/kg) group, L + A1: LPS(1mg/kg) + antibody (1mg/kg) group, L + A2: LPS (1mg/kg) + antibody (2mg/kg) group, A: antibody (1mg/kg) group, L + IgG: LPS(1mg/kg) + control antibody IgG (1mg/kg) group; The independent-samples t test was used between two groups. *$p < 0.05$, **$p < 0.01$.

on the cytoplasmic membrane of hematopoietic stem cells, including macrophages, monocytes, and dendritic cells. It functions as a pattern recognition receptor (PRR) [14]. TLR4 predominantly interacts with gram-negative bacterial endotoxin lipopolysaccharide (LPS) or lipooligosaccharide (LOS), initiating the activation of the TLR4 signaling pathway [15,16]. Upon internalization into cells, LPS lipid A located on the surface of gram-negative bacteria is recognized by LPS binding proteins (LBP) [17]. Studies have demonstrated that an anti-LBP antibody can inhibit LPS-induced TLR4 signaling pathways [18]. Following recognition by LBP, various components of LPS transfer to CD14 molecules which are glycosylphosphatidylinositol-anchored proteins localized in plasma membrane nanodomains enriched in cholesterol and sphingolipids. The CD14-LPS complex facilitates TLR4 activation along with myeloid differentiation protein 2 (MD2), a crucial adaptor protein in the TLR4 signaling pathway, forming the TLR4/MD2/LPS complex [19]. Subsequently, the homodimerization of two MD2/TLR4/LPS complexes triggers two consecutive signaling pathways: MyD88-dependent and TRIF-dependent signaling pathways [20]. In the MyD88-dependent signaling pathway, initially, membrane-anchored TLR4 receptors interact with Toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP), an adaptor protein downstream from TLR [21]. TLR4-bound TIRAP recruits MyD88 and initiates a signaling cascade, including the phosphorylation of IkBs and nuclear factor kB (NF-kB)-mediated inflammatory responses. Under normal physiological conditions, NF-kB is inhibited by IkBs; however, activation of the MyD88-dependent signaling pathway promotes the phosphorylation of IkBs, leading to their ubiquitination and degradation by proteasomes [22]. This releases their inhibitory effect on NF-kB, allowing it to enter the nucleus and activate the expression of various proinflammatory mediators such as cyclooxygenase 2 (COX-2), interleukin-1b (IL-1 b), interleukin-6 (IL-6), nitric oxide synthase (iNOS), and tumor necrosis factor-a (TNF-a). These mediators further stimulate inflammatory responses [23]. Concurrently, membrane-bound TLR4 dissociates from TIRAP and MyD88 through endocytosis before entering the cell. It then binds to a second group of adaptor proteins TRAM and TRIF in the endosome. Similar to TIRAP in the MyD88-dependent signaling pathway, TRAM supports the stimulation of the TRIF-dependent signaling pathway by TRIF-TLR4. Several studies have shown that TRAM localizes to regions enriched with CD14, which plays a vital role in the TRIF-dependent pathway. Overexpression of TRIF can activate two non-canonical IKK kinases: TANK binding kinase 1 (TBK1) and IKKε [24]. These kinases subsequently activate interferon regulator-3 (IRF3) and IRF7. Finally, activated IRF3 and IRF7 induce the expression of type I interferons resulting in an inflammatory response. This cascade induces degradation of IKK while activating MAPK pathways that lead to NF-κB translocation regulating pro-inflammatory cytokine expression [1]. The MAPK pathway also significantly contributes to pro-inflammatory cytokine production [25].

The secretion of multiple inflammatory cytokines is upregulated by lipopolysaccharide (LPS), which triggers TLR4-mediated phosphorylation of NF-κB, MAPK, and IRF3 pathways to enhance the production of proinflammatory cytokines [26]. Therefore, this study evaluated the efficacy of human anti-TLR4 Fab fragment by quantifying levels of TNF-α, iFn-β, and IL-6 involved in the MyD88 pathway following LPS stimulation. The results demonstrated a significant reduction in various inflammatory factors after incubation with LPS, indicating effective blockade of TLR4 on the MPM surface by the anti-TLR4 Fab fragment (Figure 3). Furthermore, RT-qPCR analysis revealed a substantial increase in mRNA expression levels of TNF-α, iFn-β, and IL-6 induced by LPS; however, these levels decreased upon retreatment with human anti-TLR4 Fab. Western blotting results showed that LPS-induced phosphorylation of p65, p38 JnK erK iκBα iKKα/β, and IRF3 was observed; nevertheless, pre-incubation with human anti-TLR4 reversed these effects as evidenced by Fab binding and reduced expression of proinflammatory cytokines. Furthermore, due to the significant homology between mice and humans, in vivo tests were conducted using a mouse CLP model with intraperitoneal injection of LPS. The research demonstrated that human anti-TLR4 Fab effectively shielded mice from LPS attack and suppressed LPS-induced sepsis by reducing serum pro-inflammatory cytokine levels. As a result, it is hypothesized that human anti-TLR4 Fab may be beneficial in treating severe sepsis in mice. However, the use of this mouse model has limitations, as the activation of individual human genes may not be accurately predicted by the corresponding gene in mice. Additionally, experiments on mouse dendritic cells do not fully replicate the inflammatory response observed in humans [1,27-29]. Therefore, further investigations are necessary to assess the inhibitory effects of human anti-TLR4 Fab in treating infection-associated immune dysfunction in humans.

The TLR4 inflammatory signaling pathway is implicated in a diverse array of clinical conditions, encompassing acute lung injury, acute kidney injury, rheumatoid arthritis, intestinal inflammation, heart disease, diabetic hypertension, pregnancy-related diseases, acute hepatitis, severe lung infection, sepsis and novel coronavirus lung infection [30]. The therapeutic potential of TLR4 antagonists for the treatment of LPS-induced clinical syndromes has been acknowledged. Modulation of pro-inflammatory cytokines and inhibition of associated signaling pathways may confer therapeutic benefits for inflammatory diseases [31]. Several antibodies targeting TLR4 have been documented and can be categorized into two classes [32,33]. The first class comprises agonistic monoclonal antibodies that induce NF-κB activation and confer protection against lethal LPS stimulation in mice (known as LPS tolerance). Antagonistic monoclonal inhibitors such as MTS510 suppress LPS-induced NF-κB activation in TLR4-expressing cells; however, most exhibit low activity or some cytotoxicity [12,28]. The humanized TLR4 Fab described herein belongs to the second class. Furthermore, the application of humanized TLR4 Fab offers certain advantages: it is a fully human TLR4 precursor Fab fragment generated using phage display technology and devoid of non-human components that may elicit antigenic reactions; it exhibits high binding affinity with the TLR4 receptor and demonstrates robust activity.

Based on previous research, it has been established that LBP initially binds to LPS and presents it to CD14, which then transfers LPS to the TLR4/MD2 complex, forming the M-type TLR4/MD2/LPS complex dimer [34]. This activates downstream signaling molecules and promotes the secretion of inflammatory cytokines. Therefore, we postulate that human anti-TLR4 Fab may disrupt MD2's interaction with TLR4, thereby interfering with the LPS-induced TLR4 signaling pathway. However, further investigation is required to elucidate the protective mechanism of human anti-TLR4 Fab.

Conclusions

Our results indicate that hTLR4-Fab has demonstrated efficacy in reducing LPS-induced inflammation both in vivo and in vitro, suggesting its potential clinical therapeutic value for treating syndromes associated with LPS-induction and offering prospects for future treatment of infection-related diseases.

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Conflict of interests

No conflict of interests is declared.

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