Anti-Toll-like receptor 2 antibody, T2.1, reduces tumor necrosis factor α and interleukin-6 release and inhibits nuclear factor κB expression in bacterial lipoprotein stimulated THP-1 cells

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Abstract

This study analyzed the effects of a blocking anti-Toll-like receptor 2 (TLR2) antibody (T2.1) on TLR2 signaling and inflammatory cytokine production by bacterial lipoprotein (BLP) stimulated THP-1 cells. Cells were treated sequentially with anti-TLR2 antibody and BLP (A + B) or in the reverse order (B + A), or directly stimulated with BLP (B). Blank and isotype control groups were included. Tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6) concentrations were measured by ELISA. Expression of nuclear factor κB (NF-κB), TLR2 and myeloid differentiation factor 88 (MyD88) was analyzed by Western Blot. Significantly higher expression of TNF-α, IL-6 and NF-κB was detected in all groups compared with the blank control (P < 0.05), although expression was significantly lower in A + B and B + A compared with the isotype control and B (P > 0.05). However, no significant differences were detected in TLR2 and MyD88 expression among these groups (P > 0.05). These data demonstrated that anti-TLR2 antibody significantly reduced TNF-α, IL-6 and NF-κB expression by BLP-stimulated THP-1 cells, thus indicating the protective value of this antibody in vivo.

Key words: *anti-TLR2 antibody, BLP, NF-κB, TNF-α, IL-6.*

Introduction

Toll-like receptors (TLRs), which are homologs of the Drosophila Toll protein, play an important role in recognition of microorganisms by the innate immune system. These receptors are broadly distributed on cells of the immune system, such as endothelial cells, monocytes, neutrophils and dendritic cells. Toll-like receptors are type 1 transmembrane proteins characterized by an ectodomain composed of multiple copies of leucine-rich motifs and a Toll/interleukin-1 (IL-1) receptor (TIR) motif in the cytoplasmic domain [1, 2]. The extracellular domain discriminates between a variety of pathogen-associated molecular patterns (PAMPs), including lipopolysaccharide (LPS), bacterial lipoprotein (BLP), peptidoglycans and lipoteichoic acids [3]. The TIR domain mediates interactions between TLRs and TIR-containing adaptors and stimulates a series of inflammatory responses through myeloid differentiation factor 88 (MyD88), TRIF (TIR-domain-containing adapter-inducing interferon-β) and other downstream junction proteins [1]. Of the 10 TLRs identified so far, TLR2 and TLR4 are the most extensively studied. Toll-like receptor 4 has been confirmed as the major recognition receptor for LPS, a component of Gram-negative organisms, whereas TLR2 responds to bacterial lipoproteins from Gram-positive organisms, as well as yeasts and mycobacteria [4]. Toll-like receptor signaling pathways play a vital role in the inflammatory response of sepsis, a systemic inflammatory

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response syndrome caused by infection that is associated with mortality rate as high as 50% to 68% in severe cases [5-8]. Many studies have shown that the sensitivity of secondary responses to high levels of PAMPs is downregulated in a phenomenon known as immune tolerance [9-11]. Previous studies have confirmed that pretreatment of THP-1 cells with a low dose of BLP induced tolerance to a second BLP challenge characterized by diminished production of tumor necrosis factor α (TNF-α) and other inflammatory cytokines [12]. However, in clinical practice, tolerance induction through the prophylactic administration of pathogens is unethical. The mechanism of BLP-induced septic shock has been shown to be associated with TLR2 [13]. This study investigated the use of a blocking anti-TLR2 antibody (T2.1) for reduction of inflammatory cytokine production and analyzed associated changes in the TLR2 signaling pathway in BLP-stimulated THP-1 cells *in vitro*.

Material and methods

Reagents

Synthetic BLP (Pam3-Cys-Ser-Lys4-OH) was purchased from Alexis (USA). Penicillin, streptomycin and RPMI 1640 medium were obtained from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum (FBS) was obtained from Hyclone (USA). Rabbit polyclonal antibodies (pAb) against human TLR2 and mouse monoclonal anti-human TLR2 blocking antibody (mAb, T2.1) were obtained from eBioscience (USA). Rabbit pAbs against human MyD88 were obtained from Abcam (UK). Rabbit pAbs against human NF-κB (p65) were obtained from Cell Signaling Technology (USA) and anti-Histone H3 was purchased from Signalway Antibody (USA). β-actin antibody was obtained from BOSTER Company (WuHan, China). Human TNF-α and IL-6 ELISA kits were obtained from Jingmei Biotech Co, Ltd. (China).

Cell lines and culture

The human monocytic cell line, THP-1, was obtained from the National Institute of Cells (Shanghai, China). Cells were cultured at 37°C in a 5% CO_2 atmosphere in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 U/ml streptomycin.

Grouping and treatment of THP-1 cells

Five experimental groups of THP-1 cells were treated as follows: blank control group, no treatment; isotype control group, incubated with an antibody of irrelevant specificity that was isotype-matched with the anti-TLR2 antibody (10 μg/ml) for 30 min at room temperature and then stimulated with BLP (1000 ng/ml) for 14 h at 37°C in a 5% CO_2 atmosphere; group B, directly stimulated with BLP (1000 ng/ml) for 14 h at 37°C in a 5% CO_2 atmosphere, group A + B, incubated with anti-TLR2 antibody (10 μg/ml)

at room temperature for 30 min followed by stimulation with BLP (1000 ng/ml) for 14 h at 37°C in a 5% CO_2 atmosphere; group B + A, stimulated with BLP (1000 ng/ml) for 30 min followed by incubation with anti-TLR2 antibody (10 μg/ml) for 14 h at 37°C in a 5% CO_2 atmosphere.

Tumor necrosis factor α and interleukin-6 ELISA assays

THP-1 cells (1.0×10^6/ml) were treated in 24-well plates as described above. Cell-free supernatants were harvested by centrifugation and stored at –70°C prior to analysis. Interleukin-6 and TNF-α production was assayed using ELISA kits (Jingmei Biotech Co.) according to the instructions provided by the manufacturer.

Western blot analysis

The effect of anti-TLR2 antibody on the expression of TLR2 and MyD88 and p65 translocation, cytoplasmic and nuclear proteins were prepared from THP-1 cells and analyzed by Western blot. The following buffers were used for sample preparation: Buffer A (5 mM Tris·HCl pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 10 mM EGTA pH 8.0, 1 mM dithiothreitol (DTT), protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and phosphatase inhibitors: 50 mM NaF, 30 mM β-glycerophosphate, 1 mM Na_3VO_4, and 20 Mm ρ-nitrophenol-Na).

Buffer B (5 mM Tris·HCl pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 10 mM EGTA pH 8.0, 1 mM DTT, protease inhibitors, phosphatase inhibitors, 10% Nonidet P-40, 10% BriJ-35, 10% deoxycholic acid sodium salt). Cells from each experimental group were washed three times with ice-cold PBS and suspended in 200 μl buffer A. Cells were lysed by ultrasonic disruption using a Microtip Probe for 10 pulses of 5 s each. Lysates were centrifuged at 10,000 rpm for 20 min at 4°C. The supernatants containing cytoplasmic proteins were collected and stored at –80°C. Cell pellets were suspended in 50 μl ice-cold buffer B. Suspensions were incubated on ice for 60 min with occasional mixing, and then centrifuged for 10 minutes at 7900 g in a precooled (4°C) microcentrifuge. The supernatants (nuclear fraction) were transferred into a pre-chilled microcentrifuge tube and stored at –80°C. Protein concentrations were determined using the Pierce protein assay reagent kit (Pierce Chemical, Rockford, IL). Proteins were denatured at 100°C for 5 min in loading buffer (60 mM Tris, 2.5% sodium dodecyl sulfate, 10% glycerol, 5% mercaptoethanol, 0.01% bromphenol blue). Aliquots containing an equal amount of total proteins from each sample were separated by SDS-PAGE (10% gel) and transferred onto Immobilon-P membranes (Millipore, Bedford, MA, USA) (100 V, 1.0 h, 4°C). After blocking for 2 h at 4°C in TBST (20 mM Tris-HCl, 150 Mm NaCl, 0.1% Tween 20) containing 5% non-fat milk, membranes were washed three times in TBST and probed overnight at 4°C with appropriate primary pAbs (anti-TLR2, anti-MyD88 and anti-NF-κB [p65]) in TBST

containing 1% bovine serum albumin. Blots were washed three times in TBST, incubated for 2 h with appropriate horseradish peroxidase-conjugated secondary antibodies, developed with the SuperSignal chemiluminescent substrate (Pierce Chemical, Rockford, IL) and exposed to X-Omat BT films (Kodak). Bands were quantified using Quantity One software (Bio-Rad, USA).

Statistical analysis

Data were presented as the mean ± SD. Parameters between groups were compared by one-way ANOVA. A probability value < 0.05 was considered statistically significant.

Results

Anti-TLR2 antibody (T2.1)-mediated reduction of TNF-α and interleukin-6 expression

Significant differences in TNF-α and IL-6 expression in cell-free supernatants were detected between the groups ($P < 0.05$). Significantly higher expression of TNF-α and IL-6 was detected in the isotype control group, groups B, A + B and B + A compared with the blank control group ($P < 0.05$), with the highest levels detected in the isotype control group and group B. Expression of TNF-α and IL-6 in groups A + B and B + A was significantly lower than in the isotype control group and the directly stimulated group B ($P < 0.05$) although no significant difference was observed between groups A + B and B + A (Fig. 1).

Anti-TLR2 antibody (T2.1) suppressed NF-κB activation in BLP-stimulated THP-1 cells

Western blot analysis of nuclear proteins showed that BLP induced NF-κB activation in THP-1 cells. Incubation with anti-TLR2 antibody T2.1 decreased expression of the NF-κB p65 subunit although there was no significant difference between groups A + B and B + A (Fig. 2A). Furthermore, these results indicated that NF-κB activation in BLP-stimulated THP-1 cells was inhibited to a similar extent in both groups A + B and B + A (Fig. 2A).

Anti-TLR2 antibody (T2.1) had no effect on TLR2 and MyD88 expression in THP-1 cells

Toll-like receptor 2 serves as the primary receptor for BLP. Myeloid differentiation factor 88 is the central adapter protein for signal transduction of all TLRs, with the exception of TLR3. In this study, no effects on TLR2 and MyD88 expression were observed in THP-1 cells following BLP stimulation. Furthermore, the anti-TLR2 antibody T2.1 did not attenuate the expression of TLR2 and MyD88 in this system (Fig. 2B).

Discussion

Sepsis and septic shock is the leading cause of death in critically ill patients. Statistics provided by the American Centers for Disease Control (CDC) show that there are approximately 750,000 cases of sepsis reported every year, approximately 210,000 of which die, while in Europe, it is estimated that approximately 15 million people die of sep-
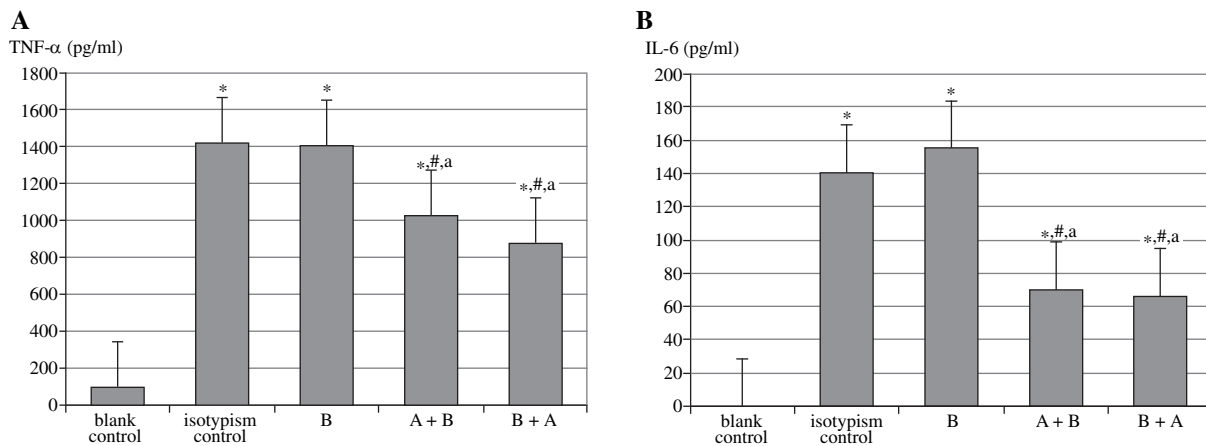

Fig. 1. Effect of T2.1 on TNF-α and IL-6 expression in human THP-1 cells. Cells were pretreated with culture medium (blank control, direct stimulation group B), 10 µg/ml T2.1 (A + B), 1000 ng/ml BLP (B + A) or 10 µg/ml homologous immunoglobulin of T2.1 (isotype control) for 30 min. Cells were then incubated with culture medium (blank control), 1000 ng/ml BLP (A + B, isotype control, group B) or 10 µg/ml T2.1 (B + A) for 14 h. Concentrations of TNF-α and IL-6 in culture supernatants were measured by ELISA. Data are expressed as the mean ±SD, and are representative of eight separate experiments
$P < 0.05$ isotype control group, group B, A + B and B + A vs. the blank control group; #$P < 0.05$ group A + B and B + A vs. the isotype control group; a$P < 0.05$ group A + B and B + A vs. group B
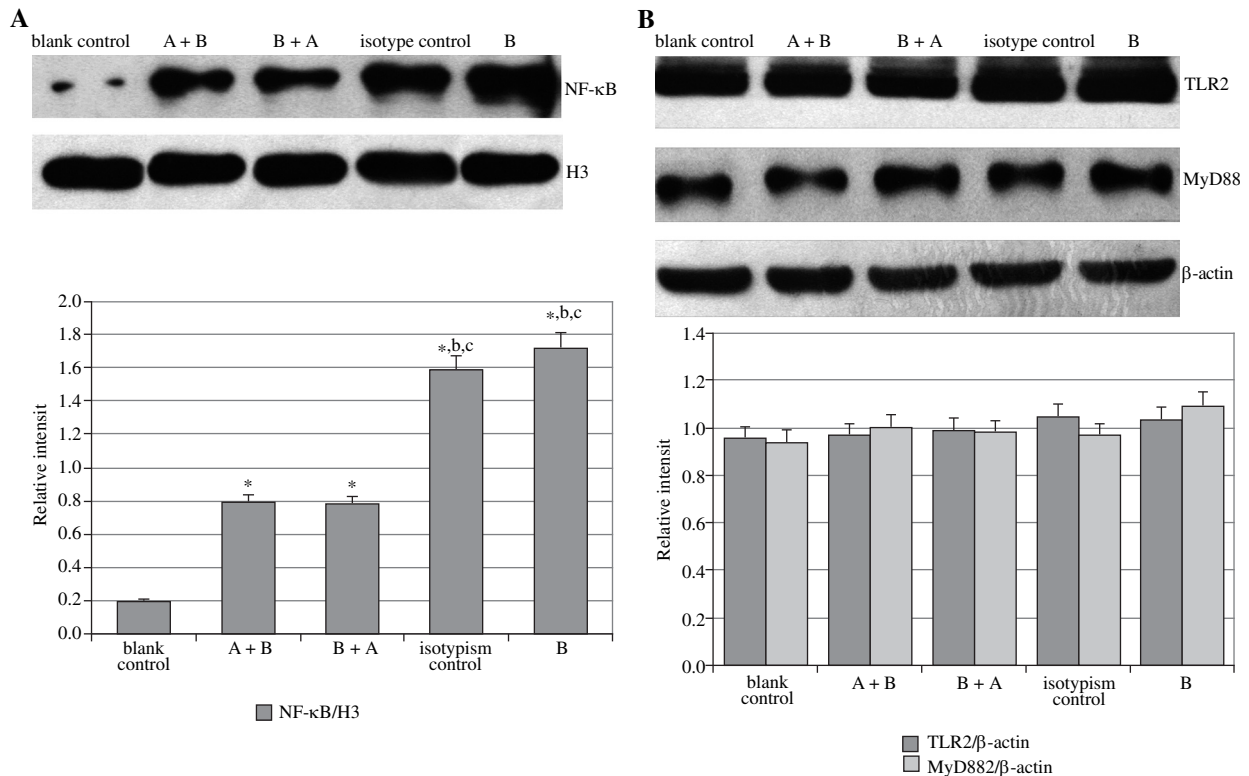
Fig. 2. Effect of T2.1 on cytoplasmic TLR2 and MyD88 expression and nuclear NF-κB expression in human THP-1 cells
Cells were pretreated with culture medium (blank control, direct stimulation group B), 10 μg/ml T2.1 (A + B), 1000 ng/ml BLP (B + A) or 10 μg/ml homologous immunoglobulin of T2.1 (isotype control) for 30 min. Cells were then incubated with culture medium (blank control), 1000 ng/ml BLP (A + B, isotype control, group B) or 10 μg/ml T2.1 (B + A) for 14 h. Nuclear and cytoplasmic extracts were then prepared for analysis of (A) NF-κB (p65) and (B) TLR2 and MyD88, respectively by Western-blot as described previously. All the samples were probed with H3 or β-actin to show equal protein loading. Results shown are representative of three independent experiments
*P < 0.05 isotype control group, group B, A + B and B + A vs. the blank control group; #P < 0.05 group A + B and B + A vs. the isotype control group; aP < 0.05 group A + B and B + A vs. group B

sis every year [6, 14]. In recent decades, the mortality of sepsis and septic shock has remained high despite active monitoring and the availability of effective support care for critically ill patients [15]. The pathogenesis of sepsis involves a complex multi-factorial process. Recent studies have indicated that anti-inflammatory responses and immune paralysis are major factors involved in the pathogenesis of sepsis [16, 17]. A variety of immune cells including monocytes and neutrophils, release large quantities of cytokines and other inflammatory mediators in response to pathogenic and non-pathogenic stimulation. Furthermore, immune cells are involved in the pro-inflammatory reaction stage responsible for the development of sepsis and induction of the subsequent systemic inflammatory response, severe sepsis and septic shock, which can result in multiple organ failure.

The results of this study revealed significantly increased expression of TNF-α, and IL-6 in the isotype control group, the direct BLP stimulation group (B), and groups A + B and B + A, compared with the blank control group. This data demonstrated THP-1 cell activation in response to bacteria or bacterial components resulting in production of proin-

flammatory cytokines such as TNF-α and IL-6. Furthermore, TNF-α and IL-6 production by BLP-stimulated THP-1 cells was significantly reduced by incubation with the blocking anti-TLR2 antibody, T2.1. It certified that TLR2 was involved in BLP stimulation. Similar observations have been reported by Meng [18] who showed that systemic administration of anti-TLR2 mAb, T2.5, inhibited lipopeptide stimulated release of inflammatory mediators such as TNF-α and prevented lethal shock-like syndrome in mice. The data obtained in this study also showed that production of inflammatory cytokines by THP-1 cells was attenuated by T2.1 administered either before or after BLP-stimulation. Prevention of *Bacillus subtilis*-induced toxemia has also been demonstrated by application of T2.5 at 2 h, or even 3 h, after shock-like syndrome induction (100% or 75% of survival, respectively) [18]. These results implicate antibody mediated TLR2 blockage on immune cells as a promising strategy for attenuation of potentially fatal host-response amplification in the course of acute infection.

In the TLR2-mediated signal transduction pathway, the NF-κB pathway is closely related to the inflammatory

response. Nuclear factor-κB is the generic name for a family of dimers formed by a several proteins: NF-κB1 (also known as p50/p105), NF-κB2 (also known as p52/p100) and REL, RELA (also known as p65/NF-κB3) and RELB. The different heterodimers bind to specific promoters to initiate transcription of a wide range of genes that influence the inflammatory response as well as cell death and survival and tissue repair [19]. The most common NF-κB dimer comprises a p65 and p50 heterodimer. IκBα binds NF-κB in the cytoplasm to block its nuclear translocation. A variety of stimuli lead to phosphorylation of critical serine residues on IκBα, targeting it for ubiquitination and degradation by the proteasome, thus allowing NF-κB to enter the nucleus and mediate transcription. This study indicated that nuclear expression of NF-κB in THP-1 cells was increased in response to BLP-stimulation and was inhibited by administration of T2.1 30 minutes before or after BLP-stimulation. The pattern of nuclear NF-κB expression was consistent with that of TNF-α and IL-6 expression. These data support that hypothesis that TLR2 induces inflammatory reactions through activation of NF-κB signaling pathways. Furthermore, it can be speculated that the reduction in inflammatory cytokine production by BLP-stimulated THP-1 cells associated with TLR2 blockade by T2.1 is mediated by inhibition of NF-κB nuclear transfer.

In this investigation, no increase in THP-1 cell expression of TLR2 and MyD88 was detected in response to BLP-stimulation. Neither were the levels of these molecules reduced by blockade of TLR2 with T2.1. It has been reported previously that expression of TLR2 is strongly enhanced in macrophages following *in vitro* BLP stimulation for 30 min and returns to basal levels by 2 h after BLP stimulation [20]. It has also been observed that TLR2 expression is strongly enhanced following streptococcal protein stimulation for 6 h followed by decreased expression intensity with time [21]. Therefore, it was postulated that the expression of TLR2 can be increased by BLP stimulation of THP-1 cells. However, no changes were observed in this study although this may be due to the time point investigated. It has become clear that MyD88-dependent and MyD88-independent pathways exist as part of TLR-mediated signaling pathways. In this study, we had not observed the changes of the expression of MyD88. The MyD88-independent pathways may play a major role in TLR2-mediated signaling pathway.

In conclusion, this study demonstrated that T2.1-mediated blockade of TLR2 significantly attenuated BLP-induced TNF-α and IL-6 production by THP-1 and inhibited nuclear transfer of NF-κB in BLP-stimulated THP-1 cells. Furthermore, it was observed that this effect was independent of the sequence of antibody treatment and BLP-stimulation. These data indicate the potential of TLR2 inhibition as an appropriate target for therapeutic intervention in acute infections.

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