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Original Article

Protective effect of a novel antibody against TLR2 on zymosan-induced acute peritonitis in NF-κB transgenic mice

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Abstract: In addition to antibiotic therapy for treatment of peritonitis, biologics have also been found to exhibit both anti-inflammatory and inflammation-resolving properties. Here, we first developed NF-κB transgenic mice with zymosan-induced acute peritonitis to investigate the effects of a novel anti-Toll-like receptor (TLR)2 antibody (anti-T20). In this mouse model, anti-T20 treatment significantly attenuated the increase of peritoneal NF-κB activity and serum levels of inflammatory cytokines, including monocyte chemoattractant protein (MCP)-1, interleukin (IL)-6 and tumor necrosis factor (TNF)-α, in a dose-dependent manner compared to mice treated with isotype control antibody. Additionally, anti-T20 treatment significantly reduced MCP-1 levels as well as the leukocyte and total protein concentrations in the peritoneal exudates of peritonitis mice. Moreover, anti-T20 treatment significantly reduced TLR2 signal transduction in the leukocytes in peritoneal exudates from the experimental peritonitis mice. In conclusion, we developed a zymosan-induced acute peritonitis mouse model that facilitated visualization of NF-κB activity and demonstrated that anti-T20 treatment plays a protective role in this model concomitant with the inhibition of the zymosan-induced inflammatory response.

Keywords: Toll-like receptor 2, NF-κB, zymosan, peritonitis

Introduction

Acute inflammation and its timely resolution are important in the host response to danger signals. Unresolved inflammation is associated with various recurrent diseases. Mechanistically, the resolution of inflammation involves active biochemical programs that enable inflamed tissues to return to homeostasis [1, 2].

Peritonitis is a common and serious complication that occurs especially in patients with end-stage renal disease treated by peritoneal dialysis (PD). Although less than 5% of peritonitis episodes result in death, peritonitis is the direct or major contributing cause of death in approximately 16% of PD patients [3]. A variety of micro-organisms, including gram-positive and gram-negative bacteria and fungi, cause peritonitis [4]. Antibiotic treatment, even if non-specific, is essential for the rapid resolution of inflammation and preservation of peritoneal membrane function [3], as unresolved inflammation exacerbates tissue injury and causes functional damage in the form of abscess or scar formation [5]; however, the side effects of antibiotic treatment, including drug resistance, are becoming a serious concern. Thus, specific treatment for peritonitis is of particular urgency.

Peritonitis in animal models is usually initiated by microbial agents, such as zymosan, which is a ligand found on the surface of fungi. Zymosan is a glucan that contains repeating glucose units connected by β-1,3-glycosidic linkages, which bind to Toll-like receptor 2 (TLR2) and induce an inflammatory response cascade [6]. Therefore, specific blockade of TLR2-mediated inflammatory signaling and hypersensitivity reactions may offer a novel therapeutic strategy for prevention of a variety of TLR2 agonist-induced inflammatory conditions. We produced a novel TLR2 antibody (named anti-T20) against a 20-mer peptide that encodes the amino acid sequence of the predicted B cell-dominant epi-

tope (DSQS LKSIRDIH HLTL HLSE), which is located in the extracellular specific domain of mouse TLR2. We then demonstrated that anti-T20 specifically binds to TLR2 and significantly inhibited tumor necrosis factor (TNF)-α and interleukin (IL)-6 production induced by the TLR2 ligands peptidoglycan (PGN), lipoteichoic acid (LTA), and the synthetic triacylated lipoprotein Pam3CSK4 in mouse monocyte RAW264.7 cells [7].

In the current study, we established a novel mouse model of zymosan-induced acute peritonitis in nuclear factor (NF)-κB transgenic mice and then investigated the role of anti-T20 on the inflammatory response in an effort to illuminate potential strategies for the prevention of peritonitis.

Materials and methods

Animals

Six-to-eight-week-old NF-κB-RE-luc (Oslo) transgenic mice (NF-κB transgenic mice) were purchased from Xenogen Corporation (Alameda, California, USA) and maintained in the specific pathogen-free facility of the experimental animal center at Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences with the approval of the Ethics Committee for Experimental Animals. Peritonitis in NF-κB transgenic mice was induced by i.p. injection of 1 mg zymosan per mouse [8, 9]. The experimental protocol was approved by the Committee for the Ethical Care and Use of Laboratory Animals of the Guangzhou Institute of Biomedicine and Health, Chinese Academy of Sciences.

Reagents

Zymosan A was purchased from Sigma-Aldrich (St. Louis., MO, USA). The Mouse Inflammation Cytometric Bead Array (CBA) kit was purchased from Bender Medsystems (Burlington, CA, Germany). Antibodies against phospho-p38 mitogen-activated protein kinase (MAPK), p38 MAPK, phospho-extracellular signal-regulated kinase (ERK)1/2, ERK1/2, phospho-IκBα, IκBα, phospho-Akt, Akt, and β-actin were obtained from Cell Signaling Technology, Inc. (Waltham, MA, USA). The NF-κB-luciferase reporter assay system was obtained from Promega Corporation (Madison, WI, USA). The Immobilon-P membrane was obtained from Millipore UK Ltd. (Watford, UK), and the enhanced chemiluminescence (ECL) kit was purchased from Amersham Life Science Ltd. (Little Chalfont, UK). Evans blue was purchased from the Laboratory Reagents Center of Southern Medical University and prepared as a working solution with normal saline.

Establishment and treatment of acute peritonitis model

Acute peritonitis was established by i.p. injection of 1 mg zymosan per individual NF-κB transgenic mouse [8, 9]. Animals were treated with anti-T20 or isotype control antibody 0.5 h post-injection with zymosan. The animals were then observed for 8 h. After experiments, all animals were sacrificed by cervical dislocation under appropriate anesthesia.

Measurement of NF-κB activity

Approximately 0, 4, and 8 h after i.p. injection of zymosan, NF-κB transgenic mice also received an i.p. injection of the luciferase substrate D-amino phenol (150 mg/kg in 250 μL sterile saline) and immediately transferred into a dark chamber of the IVIS Imaging System 200 (Xenogen Corporation, Alameda, CA, USA) for image acquisition. Also, control mice received an i.p. injection of luciferase substrate, and 3 min later, luminous signals were counted as the number of collected released photons for 5 min. Control mice were used a baseline reference. The normalized luciferase activity is presented as fold-change of relative light units (RLU).

Measurement of monocyte chemoattractant protein (MCP)-1, IL-6, and TNF-α

Serum levels of MCP-1, IL-6, TNF-α, and MCP-1 in peritoneal exudates were analyzed using the mouse inflammation CBA kit according to the manufacturer's instructions. The data were analyzed using CBA software (Bender Medsystems), and standard curves were generated for each cytokine using the mixed cytokine/chemokine standard.

Measurement of leukocyte numbers and total protein concentration in peritoneal exudates

Peritoneal exudates were collected by lavage using 3 ml of sterile saline at 0, 4, and 8 h after establishment of acute peritonitis. Cells and

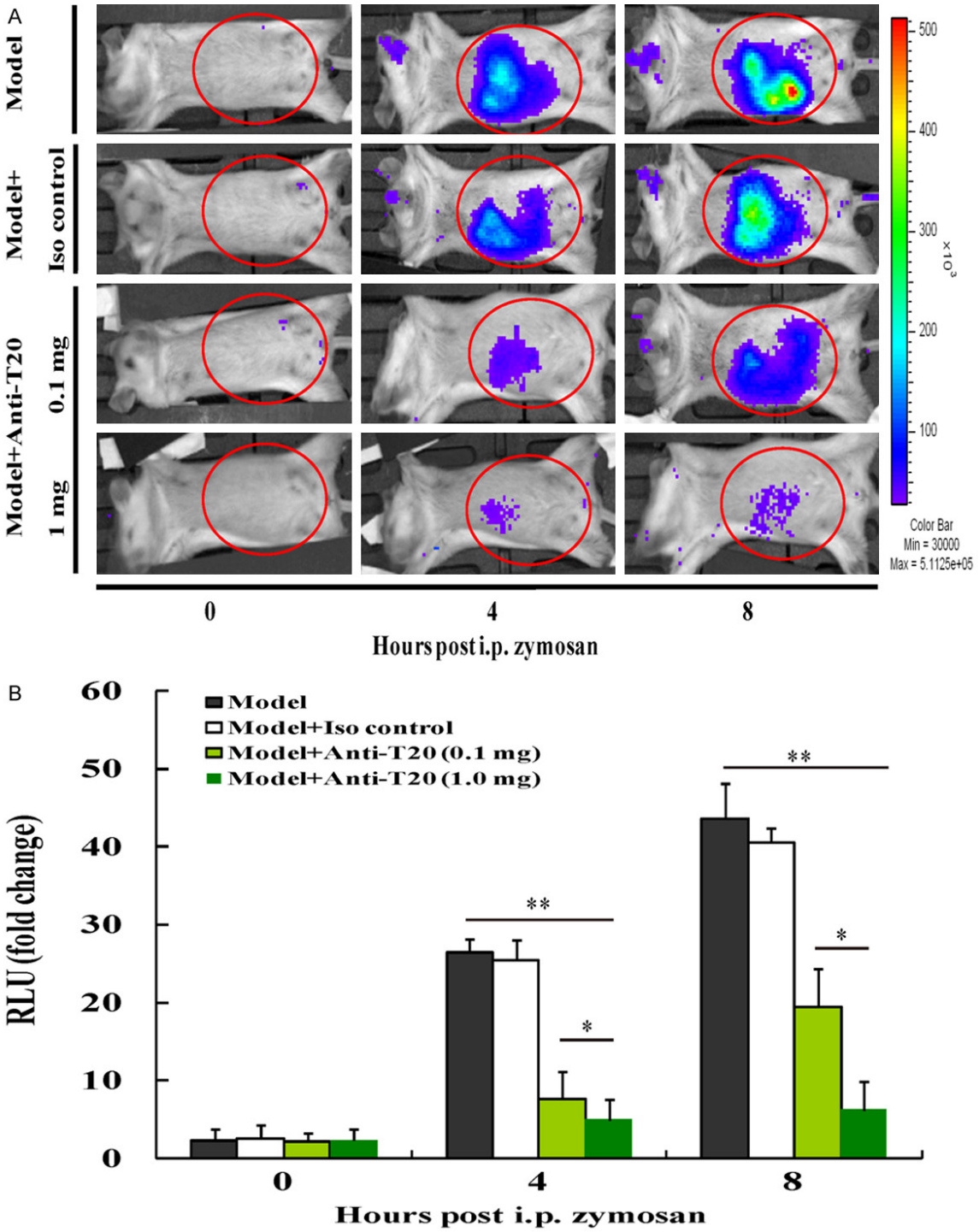

Figure 1. Effects of anti-T20 treatment on NF-kB activity during zymosan-induced peritonitis. NF-kB-RE-luc (Oslo) luciferase reporter transgenic mice were treated with zymosan (1 mg/mouse) followed immediately by treatment with anti-T20 (0.1 mg/mouse), anti-T20 (1.0 mg/mouse), or with isotype control (1.0 mg/mouse) antibodies. One group of peritonitis mice was not treated with antibody. A: In vivo images were taken at 0, 4, and 8 h after challenge with zymosan. B: Representative samples showing NF-κB activities measured as described in Methods. Data are expressed as means ± SD (n=5). *P<0.05, **P<0.01.

supernatants from exudates were obtained for analyses as previously described [8, 9]. Briefly, 0.5% Evans blue (150 μl/mouse) was administered via the tail vein of the experimental mice,

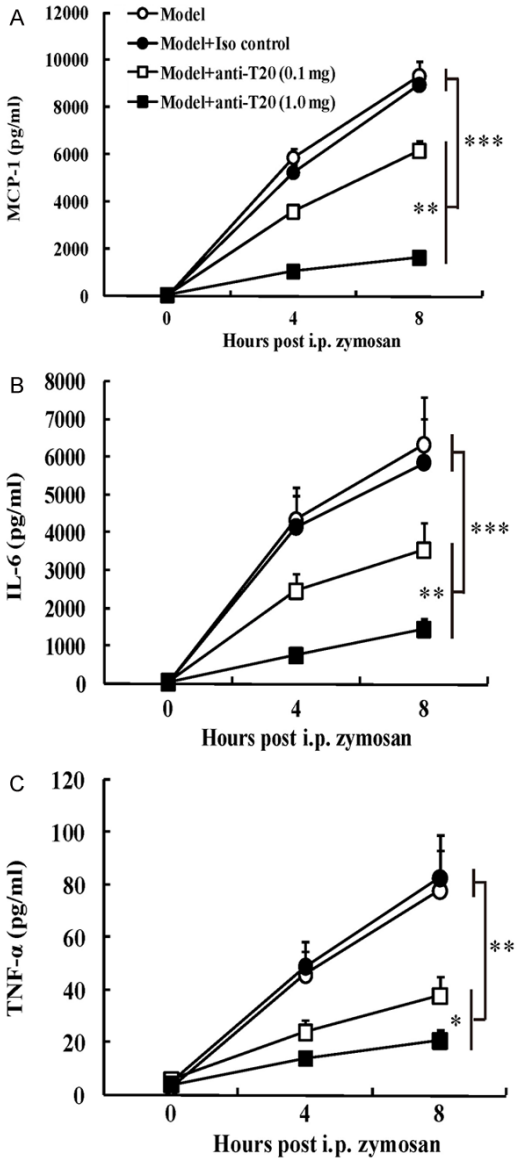

Figure 2. Effects of anti-T20 treatment on serum MCP-1, IL-6, and TNF-α levels in mice with peritonitis. Peritonitis was induced by i.p. injection of 1 mg zymosan followed immediately by treatment with anti-T20 (0.1 mg/mouse), anti-T20 (1.0 mg/mouse). Or isotype control (1.0 mg/mouse) antibodies. Additionally, one group of peritonitis mice was not treated with antibody. Serum samples were collected for measurement of MCP-1, IL-6, and TNF-α levels using a specific CBA kit. Data are expressed as means ± SD (n=5). *P<0.05, **P<0.01, and ***P<0.001.

and then animals were sacrificed at the indicated time points. The peritoneal cavity was irrigated, exudative fluid was collected, and cells were separated by centrifugation. Cell precipitates were then stained with Turk's solution containing 0.01% crystal violet and 3% acetic acid and then counted. The absorption value of the supernatant was measured at 650 nm using a Beckmann 520 spectrophotometer.

Western blotting

Expression of TLR2 signal transduction proteins in peritoneal leukocytes from experimental peritonitis mice were semi-quantitatively analyzed by western blotting using antibodies against phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2, ERK1/2, phospho-IκBα, IκBα, phospho-Akt, Akt, and β-actin. Analysis of the western blotting results was conducted using BandScan software.

Statistical analysis

Data are presented as means ± S.D. Multiple group comparison was performed using one-way ANOVA, followed by Bonferroni or Dunnett post-hoc tests. If significance was reached, an unpaired two-tailed Student's *t*-test was performed between each compared population, unless otherwise indicated. P<0.05 was considered statistically significant. Statistical analysis was performed using SPSS 15.0 software.

Results

Anti-T20 treatment attenuates the increase of NF-κB activity in the acute peritonitis mouse model

After injection of zymosan, NF-κB activity was increased in a time-dependent manner in peritonitis model mice according to luciferase expression (**Figure 1A**). Thus, this acute peritonitis model in NF-κB transgenic mice allows the visualization of inflammatory NF-κB activity. Compared with the untreated and isotype control-treated peritonitis mice, anti-T20 treatment significantly attenuated the increase of NF-κB activity in peritonitis model mice in a time- and dose-dependent manner (all comparisons, P<0.05) (**Figure 1**). No difference in NF-κB activity was detected between the untreated and isotype control-treated peritonitis mice (**Figure 1**).

Anti-T20 treatment attenuates the increase of serum levels of MCP-1, IL-6 and TNF-α in the acute peritonitis mouse model

Because MCP-1, IL-6, and TNF-α are downstream of TLR2 signaling, we assessed the lev-

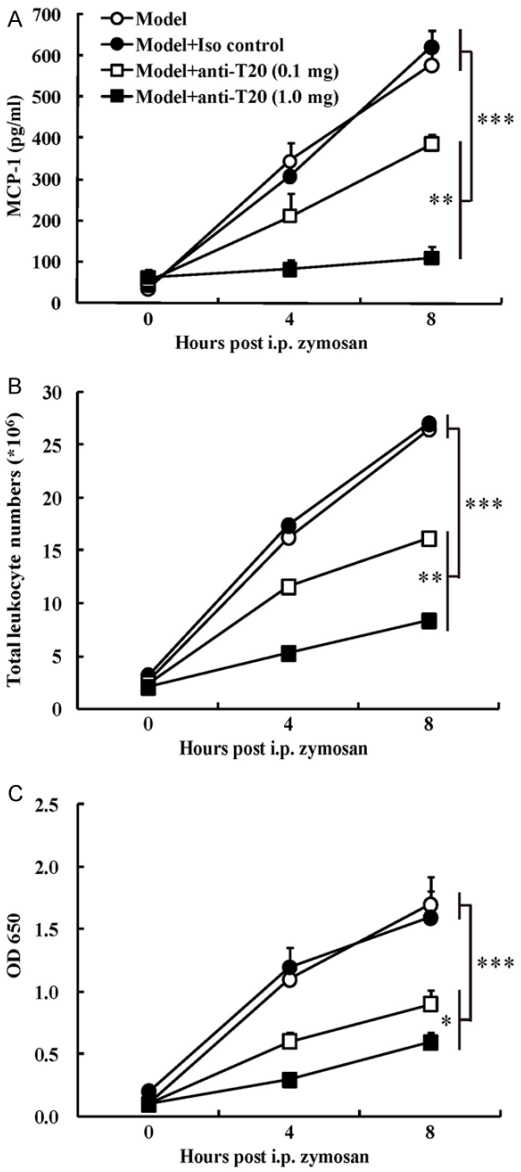

Figure 3. Effects of anti-T20 treatment on leukocyte number and total protein content in peritoneal exudates of mice with peritonitis. Peritonitis was induced by i.p. injection of 1 mg zymosan followed immediately by treatment with anti-T20 (0.1 mg/mouse), anti-T20 (1.0 mg/mouse), or isotype control (1.0 mg/mouse) antibody. One group of peritonitis mice was not treated with antibody. Peritoneal exudates from mice were collected at 0, 4, and 8 h after zymosan challenge to measure total leukocyte counts and total protein content. A: MCP-1, B: Leukocyte counts, and C: Total protein content in peritoneal exudates are shown for the four treatment groups. Data are expressed as means ± SD of three independent experiments (n=5). *P<0.05 and **P<0.01.

els of these inflammatory cytokines in our peritonitis mouse model in NF-kB transgenic animals. Levels of MCP-1, IL-6, and TNF-α were increased in the peritonitis model animals.

Compared with no treatment and isotype control antibody treatment, anti-T20 treatment significantly attenuated the increase in serum levels of MCP-1 (**Figure 2A**), IL-6 (**Figure 2B**), and TNF-α (**Figure 2C**) in peritonitis model mice in a time and dose-dependent manner (all comparisons, P<0.05). No differences in these three parameters were found between the untreated and isotype control antibody-treated groups (**Figure 2**).

Anti-T20 treatment attenuates the increase of MCP-1, leukocyte number, and total protein concentration in peritoneal exudates of peritonitis mice

Followed, we analyzed MCP-1, leukocyte number, and total protein concentration in the exudates of peritonitis mice for comparison with the results of the anti-T20 group.

Compared with the no treatment and isotype control antibody-treated groups, the anti-T20 treatment group exhibited significantly attenuated MCP-1 levels (**Figure 3A**), leukocyte numbers (**Figure 3B**), and total protein concentration (**Figure 3C**) in the peritoneal exudates of peritonitis mice in a time- and dose-dependent manner (all comparisons, P<0.05). No differences in these three parameters were found between the untreated and the isotype control-treated peritonitis mice (**Figure 3**).

Anti-T20 treatment attenuates the increase of TLR2 signal transduction in peritoneal leukocytes of peritonitis mice

In the peritonitis mouse model, TLR2 signal transduction factors including phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2, ERK1/2, phospho-IκBα, IκBα, phospho-Akt and Akt in peritoneal leukocytes was analyzed by western blotting.

Following anti-T20 treatment, peritoneal leukocytes from peritonitis mice exhibited significantly attenuated increase in these TLR2 signal transduction factors 8 h post injection of zymosan compared with the untreated and isotype control antibody-treated peritonitis mice (all comparisons, P<0.05) (**Figure 4**). No difference in these parameters was found between

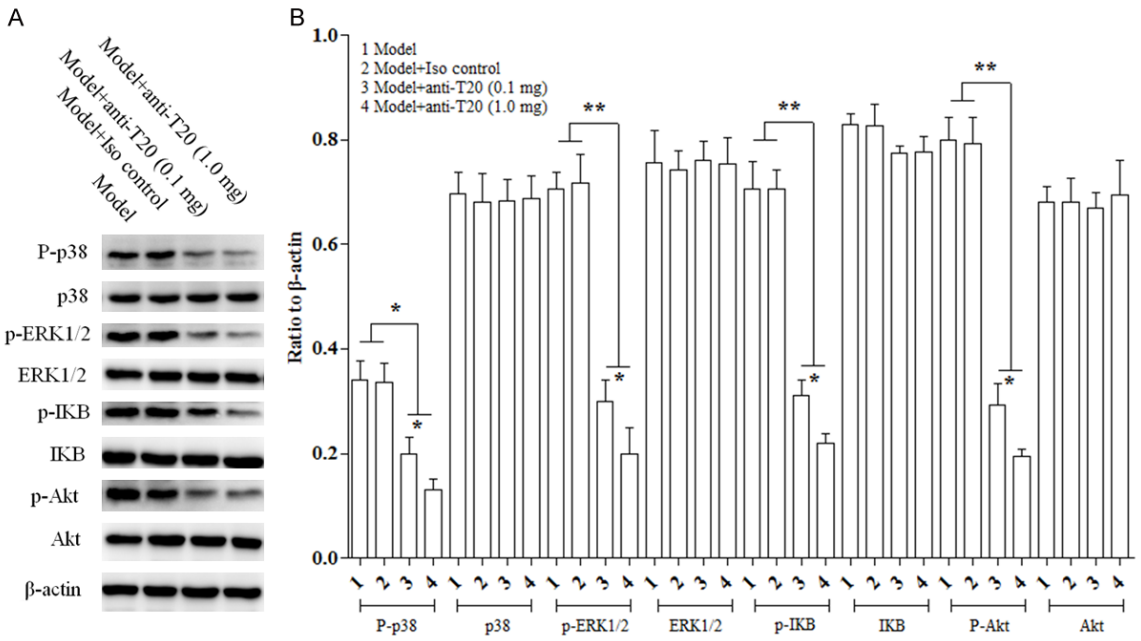

Figure 4. Effects of anti-T20 treatment on TLR2 signal transduction in peritoneal leukocytes from mice with peritonitis. The cell lysates of peritoneal leukocytes from zymosan-induced peritonitis mice that had been left untreated or treated with anti-T20 (0.1 mg/mouse), anti-T20 (1.0 mg/mouse), or isotype control (1.0 mg/mouse) antibody were (A) immunoblotted with antibodies against phospho-p38 MAPK, p38 MAPK, phospho-ERK1/2, ERK1/2, phospho-IκBα, IκBα, phospho-Akt, Akt, or β-actin and (B) subjected to semi-quantitative analysis. The experiments were repeated three times with similar results. MAPK, mitogen-associated protein kinase; ERK, extracellular signal-regulated kinase; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; Akt, serine/threonine kinase. **P*<0.05 and ***P*<0.01.

the untreated and the isotype control antibody-treated groups (**Figure 4**).

Discussion

In addition to antibiotic therapy for treatment of peritonitis, biologics have also been found in both humans and experimental animals to have anti-inflammatory and inflammation-resolving properties [10-12]. To date, specific biologics validated for the treatment of peritonitis are few [13-15], and the cellular and molecular mechanisms for the treatment of peritonitis still require in-depth investigation before these novel treatment strategies can even be considered for clinical use. Animal models of zymosan-induced peritonitis have been established for a long time. In this study, we established a novel acute peritonitis mouse model in NF-κB luciferase transgenic mice to allow visualization of zymosan-induced NF-κB activity. We utilized this novel animal model to demonstrate that anti-T20 treatment attenuated the increase in NF-κB activity in peritonitis mice. As NF-κB plays a key role in the transcriptional regulation of proinflammatory cytokine expression, this result suggests that blockade of TLR2 signaling may affect cytokine expression by influencing the activity of NF-κB. Consistent with this finding, the increase in serum levels of the cytokines MCP-1, IL-6, and TNF-α in peritonitis mice was abrogated following treatment with anti-T20 antibody. Similar tendencies in levels of inflammatory cytokines, including TNF-α, IL-1β, and IL-6, have been previously described in peritonitis mice [8]. MCP-1, also known as monocyte chemotactic and activating factor (MCAF), was characterized as a monocyte-specific chemoattractant that was later shown to attract T lymphocytes and NK cells as well [16-19]. This cytokine is mainly expressed by macrophages in response to a wide range of stimulants; however, this factor is also produced by other cells, such as fibroblasts, endothelial cells, and certain tumor cells [20, 21]. In this study, MCP-1 levels in serum and the peritoneal exudates of peritonitis mice were significantly increased following i.p. injection of zymosan, suggesting that MCP-1 plays an important role in the induction of systemic and local

inflammation in this peritonitis model. Similarly, IL-6 [19] and TNF-α were also induced in zymosan-induced peritonitis inflammation, and the levels of these cytokines were also significantly decreased by anti-T20 treatment in peritonitis mice.

Finally, we investigated the effect of anti-T20 treatment on TLR2 signal transduction in the peritoneal leukocytes of peritonitis mice. The zymosan-induced inflammatory response is based on the binding of this molecule to TLR2, as this binding induces a cascade of well-known inflammatory responses [6]. In this study, anti-T20 treatment attenuated the increase in TLR2 signal transduction in peritoneal leukocytes of zymosan-induced peritonitis mice, as evidenced by the abrogation of levels of downstream inflammatory mediators in the presence of anti-T20 antibody. Thus, this novel anti-TLR2 antibody specifically blocks TLR2-mediated inflammatory signaling induced by zymosan. Additionally, we previously reported that anti-T20 specifically binds to TLR2 and inhibits PGN, LTA, and Pam3CSK4-driven TNF-α and IL-6 production by monocytic RAW264.7 cells [7]. Therefore, anti-T20 may serve as a useful agent to block these stimulant-induced inflammatory responses; however, its effects on other TLR2 agonists, such as lipomannan from *Mycobacterium smegmatis* (LM-MS) and lipopolysaccharide (LPS) from *Porphyromonas gingivalis*, warrant further investigation in the future.

In summary, a novel acute zymosan-induced peritonitis mouse model that allowed visualization of NF-κB activity was developed. Our experiments with these animals demonstrated that anti-TLR2 treatment functions in a protective role via inhibition of the zymosan-induced inflammatory response. Humanized antibodies and preclinical studies of this TLR2-targeting antibody are urgently needed in order to further explore the use of this biologic in the treatment of peritonitis.

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Disclosure of conflict of interest

None.

Authors' contribution

All authors have contributed to, read and approved the final manuscript for submission.

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