academicJournals

Vol. 8(10), pp. 256-262, 15 March, 2014
DOI 10.5897/AJPP2013.3682
ISSN 1996-0816

http://www.academicjournals.org/AJPP

African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Protective effect of 2,2,2-trichloroethanol on peptidoglycan-induced inflammation in murine macrophages

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Received 3 May, 2013: Accepted 27 February, 2014

The aim of this study was to investigate the effect of 2,2,2-trichloroethanol on peptidoglycan-induced murine macrophages inflammation. The effect of 2,2,2-trichloroethanol on the production of TNF-α and IL-6 by murine peritoneal macrophages with peptidoglycan-stimulation were investigated. Also, RAW264.7 macrophages transfected with a NF-κB luciferase reporter plasmid with the stimulation of peptidoglycan were used to test the effect of 2,2,2-trichloroethanol on NF-κB activity. Flow cytometry and Western blotting were used to check the expression levels of toll-like receptor 2 on treated RAW264.7 macrophages. 2,2,2-Trichloroethanol decreased the unregulated levels of IL-6 and TNF-α produced by the peritoneal macrophages stimulation with peptidoglycan. The NF-κB activities of the RAW264.7 macrophages stimulated by peptidoglycan were decreased. 2,2,2-Trichloroethanol decreased the peptidoglycan-induced murine macrophage inflammation response.

Key words: 2,2,2-Trichloroethanol, peptidoglycan, inflammation, macrophage.

INTRODUCTION

Peptidoglycan is one of the major conserved components of Gram-positive bacteria walls and can be detected in the blood of 80% of the serious bacterial infected patients (Merdink et al., 2008). The mononuclear phagocyte represents an important cell type of the innate immune system and plays a key role against infection in the innate immune system, and murine macrophages are important models for studying response of immune system to infection. Many studies reported that after stimulation with peptidoglycan, nuclear factor kappa-light-chain-enhancer of activated B cells (NK-κB) which has long been considered a prototypical proinflammatory signaling pathway factor becomes activated and then macrophages release many of the proinflammatory mediators, such as IL-6 and TNF-α. At the same time, levels of toll-like receptor 2 expression on macrophages become upregulated (Chen et al., 2009; Lin et al., 2011).

We have shown that chloral hydrate, a well-known sedative and anesthetic that is used in pediatric procedures, can attenuate the inflammation and improve the survival of lipopolysaccharide/D-galactosamine-induced acute lethal liver injury mice. These protective function of chloral hydrate on acute lethal liver injury mice was related to the inhibitory effects on NF-κB activity and serum levels of

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MCP-1, IL-6 and TNF-α (Pan et al., 2010).

Based on chloral hydrate being rapidly and extensively metabolized in the liver and erythrocytes by alcohol dehydrogenase to its major active metabolite, 2,2,2-trichloroethanol *in vivo* (Ann et al., 2001) is responsible for its physiological and psychological effects (Reinhard et al., 2007). The active metabolite of 2,2,2-trichloroethanol is 2,2,2-trichloroethanol (TCE). So, its necessary and very important to use 2,2,2-trichloroethanol instead of chloral hydrate to study its effect on inflammatory response of macrophages *in vitro*. So, here, we investigate the effect of 2,2,2-trichloroethanol on the production of the inflammatory cytokines and NF-κB activity in murine peritoneal macrophages and RAW264.7 macrophages stimulated with peptidoglycan. Also, the effects of 2,2,2-trichloroethanol treatment on toll-like receptor 2 expression in murine peritoneal macrophages and RAW264.7 macrophages stimulated with peptidoglycan were explored.

MATERIALS AND METHODS

Reagents

Peptidoglycan (*Staphylococcus aureus*, strain DSM346) and 2,2,2-trichloroethanol were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A). Fetal bovine serum and Iscove's Modified Dulbecco's Medium (IMDM) medium were obtained from Gibco (NY, U.S.A). IL-6 and TNF-α enzyme linked immunosorbent assay (ELISA) kits were from R&D Systems, Inc. (Minneapolis, USA). The fluorescein isothiocyanate (FITC)-anti- toll-like receptor 2 antibody (clone TL2.5), FITC-mouse IgG1, anti-mouse toll-like receptor 2 antibody (clone TL2.5), HRP-anti-mouse secondary antibody were obtained from Ebioscience (San Diego, CA, USA) and Biolegend (San Diego, CA, USA). The NF-κB-luciferase, β-galactosidase reporter vectors and the Dual-Luciferase reporter assay system were purchased from Promega Corporation (Madison, WI, USA). The immobilon membrane and the Electrochemiluminescence (ECL) were from Millipore (Watford, UK) and Amersham Life Science Ltd (Little Chalfont, UK), respectively.

Effects of 2,2,2-trichloroethanol on IL-6 and TNF-α production by murine peritoneal macrophages with the stimulation of peptidoglycan (PGN)

After peritoneal macrophages separated from mice (Yin et al., 2006), the cells were seeded at the levels of 10^5 cells/well in IMDM media (5% fetal bovine serum) at 37°C in 5% CO_2. At the indicated time, cells and culture supernatants were collected after stimulated with PGN (1.5 μg/ml) or PGN (1.5 μg/ml) plus 2,2,2-trichloroethanol (0.1 and 0.5 mg/ml) and measured with ELISA kits.

Effects of 2,2,2-trichloroethanol on the activity of NF-κB of PGN-stimulated RAW264.7 macrophages

RAW264.7 macrophages were cultured in IMDM media (5% fetal bovine serum) and maintained at 37°C in 5% CO_2. These cells were digested with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (2.5 g/L trypsin, 1 g/L EDTA) at 37°C for five minutes, and 8.2×10^6 RAW 264.7 macrophages were transiently transfected with 8 μg NF-κB-luciferase and 8 μg β-galactosidase reporter vectors which was used for normalization of the efficiency of the transfection in a volume of 640 μl by electroporation at 250 V and 960 μF of capacitance pulse[21]. Then, RAW264.7 macrophages were subsequently washed two times in IMDM media and divided into 40 wells (150 μl/well) and cultured for 24 h in IMDM media (5% fetal bovine serum) before being stimulated with PGN (1.5 μg/ml) for 12 or 24 h with or without 2,2,2-trichloroethanol (0.1 or 0.5 mg/ml). For the luciferase activity assays, the transfeced RAW264.7 macrophages were stimulated for 6 or 12 h and subsequently harvested, and luciferase activity in extracts were analyzed with the dual-luciferase reporter assay system following manufacturer's instructions.

Effects of 2,2,2-trichloroethanol on PGN-induced toll-like receptor 2 expression in RAW264.7 macrophages

Flow cytometry was performed to investigate levels of toll-like receptor 2 expression on RAW264.7 macrophages. RAW 264.7 macrophages were cultured in IMDM media (5% fetal bovine serum) for 12 h with PGN (1.5 μg/ml) with or without 2,2,2-trichloroethanol (0.1 mg/ml). Then, RAW264.7 macrophages were incubated with FITC-anti-mouse-toll-like receptor 2 (1.0 μg) for thirty minutes at room temperature after harvested, and FITC-mouse IgG1 was used as isotype control, the cells were washed two times with cell staining buffer. Finally, a six-parameter flow cytometer (FACScan; BD Biosciences, San Jose, CA) was used for data acquisition and analysis using CellQuest software (BD Bioscienc, San Jose, CA). Also, toll-like receptor 2 expression in the extracts of RAW264.7 macrophages stimulated with PGN (1.5 μg/ml) for 12 h with or without 2,2,2-trichloroethanol (0.1 mg/ml) were semi-quantitatively analyzed by Western blotting using the anti-mouse-toll-like receptor 2 antibody by band scan software 5.0 (Glyko Inc). First, the extracts of RAW264.7 macrophages were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Then, the immunoblotting was performed with a standard protocol onto the immobilon membrane. HRP-anti-mouse IgG as secondary antibody, followed by ECL chemiluminescence which was used to detect toll-like receptor 2 expression.

Statistical analysis

Data were expressed as the means ± standard deviation (SD) and statistical analysis with statistical package for social sciences (SPSS) version 15.0 statistical software. The statistical significance between two groups was determined by the unpaired Student's t-test. A value of $P < 0.05$ was considered statistically significant.

RESULTS

2,2,2-Trichloroethanol-treatment decreased the production of IL-6 and TNF-α by peritoneal macrophages with the stimulation of peptidoglycan

The production of IL-6 (Figure 1A) and TNF-α (Figure 1B) increased significantly after stimulation with peptidoglycan (1.5 μg/ml) for 12 and 24 h and decreased significantly post 2,2,2-trichloroethanol treatment (0.1 and 0.5 mg/ml) (all $P < 0.01$). Also, the higher concentration of 2,2,2-trichloroethanol decreased the production of IL-6 significantly compared to the lower concentration (0.1 mg/ml) at 12 h and 24 h time points (all $P < 0.05$) (Figure 1A), but not for TNF-α.

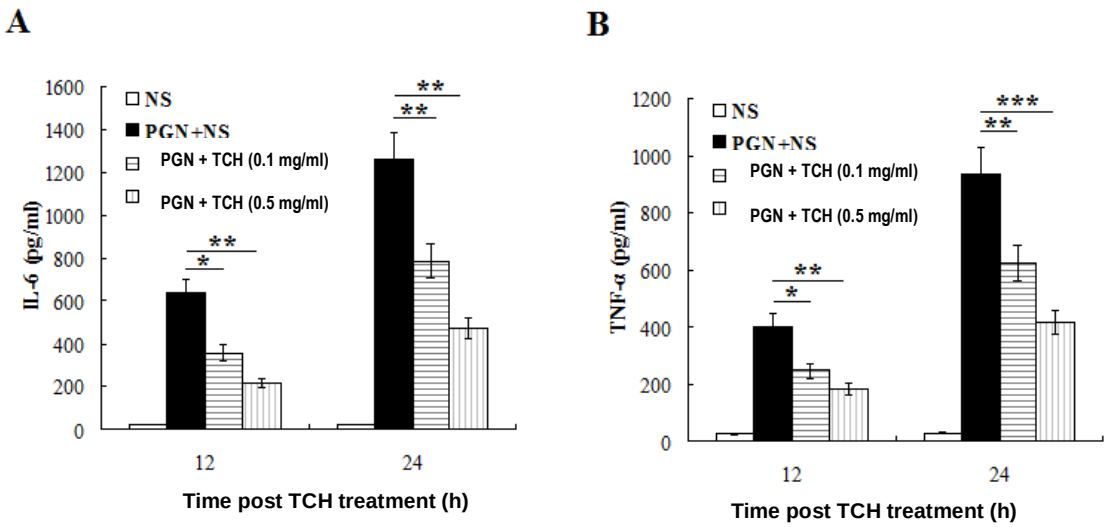

Figure 1. 2,2,2-trichloroethanol (TCH) decreased the production of IL-6 and TNF-α by peritoneal macrophages with stimulation of peptidoglycan (PGN). Effects of 2,2,2-trichloroethanol (0.1 and 0.5 mg/ml) on the levels of IL-6 (A) and TNF-α (B) produced by peritoneal macrophages (10^6 cell/well) with PGN stimulation for 12 and 24 h were shown.
Data were expressed as the means ± standard deviation (SD) of three independent experiments. **P*<0.05, ***P*<0.01 and ****P*<0.001.

2,2,2-trichloroethanol-treatment decreased the NF-κB activity of peptidoglycan-stimulated RAW264.7 macrophages

Luciferase activities in the transfected RAW264.7 macrophages were measured after peptidoglycan stimulation for 6 and 12 h (Figure 2). The results showed about 3- and 5-fold increase of the luciferase activity post peptidoglycan stimulated for 6 and 12 h, respectively. But this increase of luciferase activity was significantly decreased by 2,2,2-trichloroethanol treatment (0.1 and 0.5 mg/ml) (all $P < 0.01$). A higher concentration of 2,2,2-trichloroethanol (0.5 mg/ml) led to a significantly larger decrease in the activity of luciferase after 12 h when compared to the lower concentration of 2,2,2-trichloroethanol (0.1 mg/ml) ($P < 0.01$).

2,2,2-Trichloroethanol-treatment decreased the increase of toll-like receptor 2 expression in peptidoglycan-treated RAW264.7 macrophages

Based on both concentrations (0.1 and 0.5 mg/ml) of 2,2,2-trichloroethanol treatment, it significantly decreased the inflammatory response of peritoneal macrophages and RAW264.7 macrophages after PGN-stimulation, so we tested the effect of 2,2,2-trichloroethanol treatment on the expression of toll-like receptor 2, the receptor for PGN, using the lowest effective concentration of 2,2,2-trichloroethanol (0.1 mg/ml). RAW264.7 macrophages were cultured in the IMDM media (5% fetal bovine serum) for 12 h with or without peptidoglycan (1.5 μg/ml) in the presence or absence of 2,2,2-trichloroethanol (0.1 mg/ml) (Figure 3A) and harvested, and the levels of toll-like receptor 2 were measured. In RAW264.7 macrophages stimulated with PGN (1.5 μg/ml) for 12 h, toll-like receptor 2 expression was significantly increased (Figure 3B), and treatment of 2,2,2-trichloroethanol (0.1 mg/ml) significantly decreased the upregulation of toll-like receptor 2 expression stimulated with peptidoglycan ($P < 0.05$). Also, the results of semi quantitative analysis of toll-like receptor 2 expression in the extracts of RAW264.7 macrophages by Western blotting showed a similar pattern (Figure 3C). It showed that if the related amounts of toll-like receptor 2 signal to the signal of a house-keeping proteins β-actin were similar, the increase and the reduction of the toll-like receptor 2 signal were not caused by cell death or variations in the number of cells in the samples.

DISCUSSION

Over recent years, the mechanisms of bacteria-induced acute inflammation, anti-cytokines and anti-inflammatory therapies as therapeutic agents for the treatment of acute inflammation have been re-evaluated. Large quantities of reported cases of sepsis-related anti-cytokine therapeutic trials were unpromising or disappointing (Hall and Muszynski, 2009; Zeni et al., 1997; Ratsimandresey et al., 2009). So, recently, the developments of new strategies for modulating severe sepsis and acute inflammation have been investigated intensively.

As we all know, the new drug discovery, development

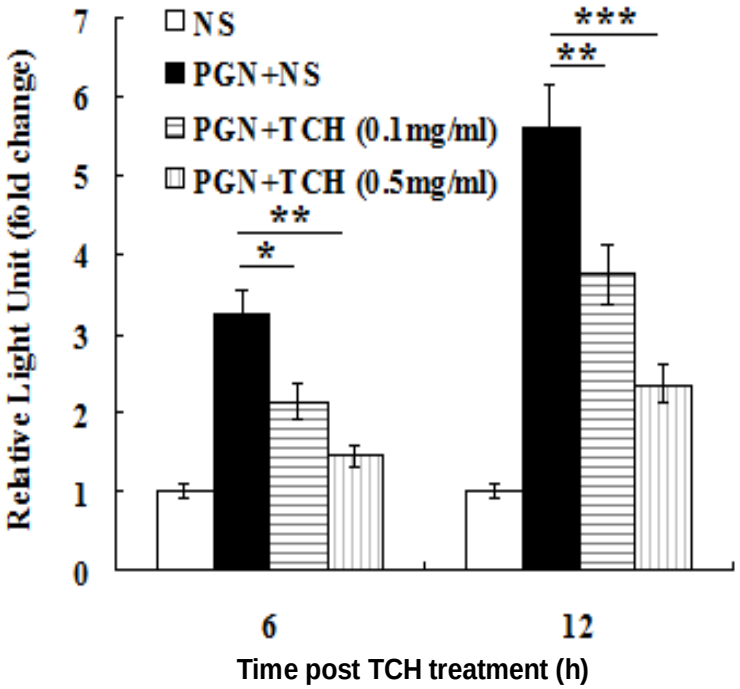

Figure 2. 2,2,2-trichloroethanol (TCH) treatment decreased the upregulation of NF-kB activity of RAW264.7 macrophages with peptidoglycan (PGN) stimulation. 24 h after the RAW264.7 macrophages were co-transfected with NF-κB-luciferase- and β-galactosidase- reporter vectors by electroporation, PGN (1.5 μg/ml) or PGN (1.5 μg/ml) plus 2,2,2-trichloroethanol (0.1 or 0.5 mg/ml) were added into the medium and incubated for 6 and 12 h. The luciferase activity of the cell extracts was expressed as the folds of luciferase-induction over saline-treated controls of three independent experiments. RLU stands for "Relative Light Unit", * P<0.05, ** P<0.01 and *** P<0.001.

and approval process of therapeutic programs requires investing significant amounts of time and effort. However, discovering new uses for the old drugs may accelerate the development and application of new therapies, take for example, the bisphosphonate zoledronic acid can decrease breast cancer metastasis beside treatment of osteoporosis and similar diseases (Coleman and Gnant, 2009) and thalidomide can treat multiple myeloma in addition to alleviate nausea and morning sickness (Cavo et al., 2009). Importantly, protective effect of some local and gerenral anesthetics and sedatives have reported against infection, and inflammations (Plachinta et al., 2003; Gallos et al., 2004; Fuentes et al., 2006; de Klaver et al., 2002; Liu et al., 2009) are likely to yield new insights into anti-inflammatory therapies.

Chloral hydrate has been long used as a safe sedative and hypnotic drug in patients and animal models. Also, different from isoflurane, which is an inhalation agents and must be inhaled continuously, chloral hydrate can be used without control in many countries, but unlike other sedatives, for example, ketamine is under strictly regulated conditions in China and other countries, also, and is not approved by US Food and Drug Administration (USFDA) for patients lower than 16 years old (Mellon et al., 2007).

In this study, we used 2,2,2-trichloroethanol, which is a major active metabolite *in vivo* after chloral hydrate rapidly and extensively metabolized in the liver and erythrocytes by alcohol dehydrogenase, to study its function on inflammatory response with macrophages *in vitro*. Here, our studies showed that 2,2,2-trichloroethanol treatment can decrease the rise of the inflammatory cytokine levels produced after peptidoglycan stimulation in murine peritoneal macrophages (Figure 1), indicating that the effect of 2,2,2-trichloroethanol on inflammation could be attributed to its inhibition of function of macrophages. The TNF-α and IL-6 levels sharply increased at 12 and 24 h after the peptidoglycan-challenge. Similar studies using peptidoglycan (10 or 25 μg/ml) were performed by others (Shirasawa et al., 2004; Wang et al., 2004), but they only tested 24 h after one challenge. Also, the treatments with 2,2,2-trichloroethanol significantly decreased the rise of inflammatory cytokines production (Figure 1).

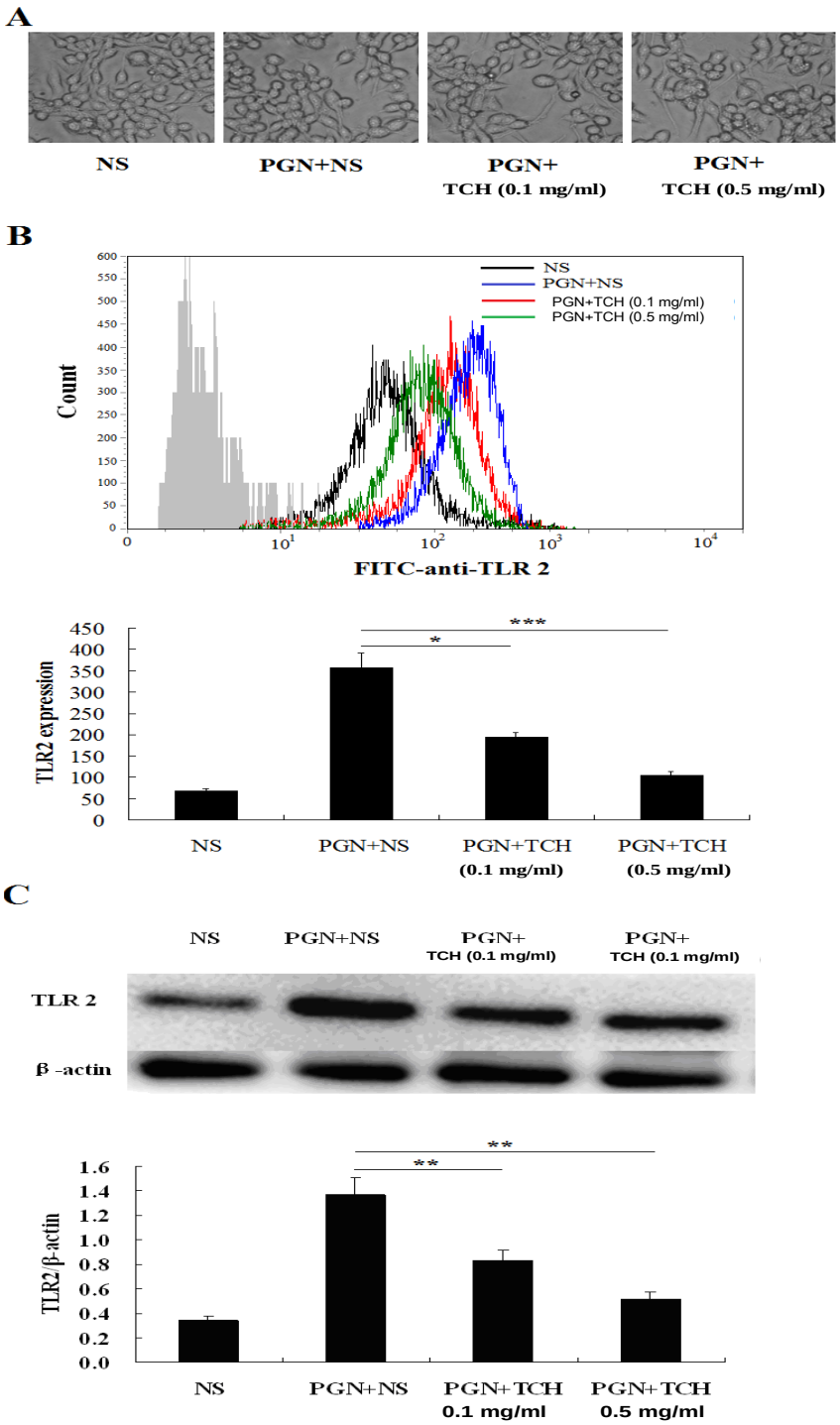

Figure 3. 2,2,2-trichloroethanol (TCH) treatment reduced the expression of toll-like receptor 2 (TLR 2) in peptidoglycan (PGN)-stimulated RAW264.7 macrophages. RAW 264.7 macrophages were cultured in IMDM media (5% FBS) for 12 h with PGN (1 µg/ml) with or withought 2,2,2-trichloroethanol (0.1 mg/ml) (A). Flow cytometry was performed to investigate the expression levels of TLR 2 on the treated RAW264.7 macrophages represented as dot plots and represented as histograms. The mean channel fluorescence intensity (MFI) was calculated (B). The semi-quantitative analysis of the extracts from RAW264.7 macrophages by band scan and the ratio of TLR 2 to β–actin was shown, showed a western blot of β–actin and TLR 2 of Normal, PGN+NS and PGN+TCH group and showed a quantitation of them (C). The data were expressed as the means ± SD of three independent experiments.
* P<0.05, ** P<0.01 and *** P<0.001.

Next, we examined whether peptidoglycan-induced NF-κB-dependent gene transcription is regulated by 2,2,2-trichloroethanol in RAW264.7 macrophages. The results of RAW264.7 macrophages transfected with a NF-κB luciferase reporter vector showed that 2,2,2-trichloroethanol treatment can significantly reduce NF-κB activity (Figure 2). Because NF-κB is a key mediator for inducible transcription of proinflammatory cytokine expression, these results suggested that 2,2,2-trichloroethanol could affect cytokine expression maybe by influencing the activity of NF-κB. After 6 and 12 h with the stimulation of peptidoglycan, the respective increase of NF-κB-induced luciferase activity in RAW264.7 macrophages was approximately 3- and 5-fold compared to control levels. A similar study using peptidoglycan (1.5 μg/ml) was performed by others (Ito et al., 2005), and they observed the same trend.

Following this, we investigated whether peptidoglycan-induced upregulation of toll-like receptor 2 expression is regulated by 2,2,2-trichloroethanol. Flow cytometry was used to analyze the effect of 2,2,2-trichloroethanol on the expression of toll-like receptor 2 in RAW264.7 macrophages stimulated with peptidoglycan. The results showed that the expression of toll-like receptor 2 in RAW264.7 macrophages is significantly upregulated after PGN stimulation. Similar results have been found by others (Chen, 2009). After 2,2,2-trichloroethanol treatment, the highly upregulation of the toll-like receptor 2 expression in response to peptidoglycan exposure was remarkably decreased (Figure 3), which is consistent with the effect of 2,2,2-trichloroethanol on NF-κB activity and inflammatory cytokine production by RAW264.7 macrophages stimulated with peptidoglycan.

Taken together, this is the first time to report that 2,2,2-trichloroethanol can decrease the upregulation of peptidoglycan-induced inflammatory response by macrophages in a concentration and time-dependent manner. Also, this decrease was associated with a decreased upregulation of the peptidoglycan-induced-toll-like receptor 2 expression in macrophages. By knowing the effect and mechanisms of 2,2,2-trichloroethanol treatment in inflammatory response of macrophages may provide new opportunities to design new therapeutic strategies to reduce inflammation caused by Gram-positive bacteria.

Conflict of interest

No competing interests were disclosed.

ACKNOWLEDGEMENTS

We thank NingFu, Yiguo Chen and Daqing Yang for their technical assistance. The work was supported by Zhanjiang Planning Project of Science and Technology (2012C3101028 and 2013B01086), Medical Scientific Research Foundation of Guangdong Province, China (B2012284) and Doctoral Fund of Guangdong Medical College and Affiliated Hospital of Guangdong Medical College to Qingjun Pan.

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