Lipopolysaccharide enhances the inhibition of NF-κB expression in NNK-mediated peritoneal macrophages

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Abstract   *Objective:* The aim of the study was to investigate the effect of lipopolysaccharide (LPS) on the expression of nuclear factor kappa B (NF-κB) in 4-(methylitrosamino)-1-(3-pyridyl)-1-butanone (NNK)-mediated primary mouse peritoneal macrophages *in vitro*. *Methods:* The activity of peritoneal macrophages treated with different concentrations of LPS was detected by MTT assay in rider to find the optimal concentration. Peritoneal macrophages were also treated with NNK (100–500 μM), with or without LPS for 9 h. The expression of NF-κB was demonstrated via immunocytochemistry (ICC) and Western-blot, respectively. *Results:* The concentration of LPS at 25 μg/mL was found to be the optimal concentration to improve the activity of peritoneal macrophages ($P < 0.01$). Simultaneously, LPS (25 μg/mL) increased the expression of NF-κB in both the nucleus and cytoplasm and facilitated transfer of NF-κB to the nucleus. NNK treatment significantly inhibited the expression of NF-κB in a concentration-dependent manner, among the LPS-stimulated or unstimulated peritoneal macrophages, especially when cotreated with LPS (25 μg/mL, $P < 0.01$). Furthermore, NNK treatment (500 μM) with LPS yielded a significant decrease in NF-κB translocation to nucleus and inhibited the expression of NF-κB ($P < 0.005$). *Conclusion:* LPS enhances the suppression of NF-κB expression in NNK-mediated mouse peritoneal macrophages, which may provide a theoretical basis for the inhibition of cancer.

Key words   lipopolysaccharide (LPS); 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK); peritoneal macrophages; mouse; nuclear factor kappa B (NF-κB)

Lung cancer is one of the most commonly diagnosed cancers with the highest mortality worldwide [1–3]. Many factors mediated lung tumorigenesis and pulmonary inflammation has been associated with lung cancer. Some researches suggested that lipopolysaccharide (LPS) significantly increased the incidence of carcinogens 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) induced lung tumor, although pulmonary inflammation induced by LPS alone was not tumorigenic in mice [4, 5]. However, the mechanism of LPS-elicited pulmonary inflammation leading to an increased lung tumorigenesis was still not clear. Alveolar macrophages (AMs), a type of inflammatory cells, play a critical role in promoting lung tumorigenesis and LPS is a potent inducer of macrophage activation [6]. The previous studies showed that treatment of mice with NNK + LPS led to increased recruitment of macrophages and neutrophils [7]. Furthermore, AMs may play an indirect role in the anti-tumour immune response through the recruitment and activation of leukocytes to the tumour site. Other study reported that NNK reduced the production of proinflammatory mediators and increases the production of anti-inflammatory mediators from AMs [8], suggesting that NNK contributes to the lung immunosuppression observed in smokers.

Nuclear factor kappa B (NF-κB), a key regulator of inflammation, was involved in various physiological and pathological processes, such as innate and adaptive immune responses, cell proliferation and cell death [9], especially in the growth, and metastasis of lung tumors [10–13]. Yu *et al* [14] confirmed that LPS can promote tumor growth through inflammatory response, proving that NF-κB is involved in this process. Moreover, targeting the NF-κB and MAPK signaling pathways is considered as an attractive therapeutic strategy for the development of anti-inflammatory drugs [15]. To shed some light on the potential mechanisms that LPS-elicited chronic lung inflammation enhanced NNK-mediated lung tumorigenesis, the present study explored the expression of NF-κB in NNK-mediated primary mouse peritoneal macrophages treated with or without LPS.

Materials and methods

Female Kunming mice (22–25 g, 10–12 weeks of age) were obtained from the Center of Experimental Animals of Medical College of Qingdao University, China. Mice were housed under constant temperature and humidity with 12-hour light-dark cycle and given free access to food and water, which maintained in accordance with the current guidelines for animal welfare in the Guide for the Care and Use of Laboratory Animals.

Cell culture and cell viability assay

Mice were anesthetized by an intramuscular injection of Zoletil 50 (75 mg/kg) after they were maintained for 2 weeks in our lab. Peritoneal macrophages were obtained by sterile lavage of the peritoneal cavity with cold PBS medium. The lavage fluid was centrifuged at 1000 rpm for 5 min and the supernatant was decanted and the cells were resuspended in Hyclone's medium (1640), supplemented with 10% (v/v) heat-inactivated fetal calf serum.

The viability of peritoneal macrophages was assessed using the microculture tetrazolium (MTT)-based colorimetric assay. Primary peritoneal macrophages were seeded in a 96-well plate at density of 2×10^5 cells for 2 h and then the cells were cultured in new medium for a further 6 h. Whereafter, peritoneal macrophages were stimulated with LPS (6–50 μg/mL) for 9 h. MTT was added to each well (final concentration was 0.5 mg/mL). After incubation for 4 h at 37 ℃ and 5% CO_2, the supernatant was removed and the formed formazan crystals in viable cells were solubilized with 150 mL of DMSO. The absorbance of each well was then measured at a wavelength of 490 nm. The percentage of viable cells represented the mean ± SD from three replicate experiments. Results were representative of three independent experiments.

Immunohistochemistry (ICC)

Peritoneal macrophages were plated on coverslips in 6-well plates and treated with NNK (100 μM, 250 μM, and 500 μM) with or without LPS for 9 h. The slides of cells were washed with PBS, fixed for 15 min with 4% paraformaldehyde in PBS (pH 7.4) at room temperature. After that, the slides (PBS) were incubated with 0.1% Triton X-100 for 20 min. Then blocked endogenous peroxidase activity with 3% hydrogen peroxide for 10 min and rinsed in phosphate-buffered saline afterwards. Rabbit antihuman polyclona NF-κB antibody (1:100 in PBS, Santa Cruz Biotechnology, USA) were incubated with peritoneal macrophages at 37 ℃ for 4 h, using PBS instead of antibody served as negative control and the secondary antibodies 2 h at 37 ℃ in a humid chamber. Finally, reaction products were visualized using DAB, and the sections were then counterstained with hematoxylin.

Western-blot analysis

The peritoneal macrophages treated with NNK (100–500 μM), with and without LPS, for 9 h were washed with phosphate buffered saline (PBS) three times and scraped off and re-suspended in RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing 5 mM EDTA and a protease inhibitor cocktail. Lysates were kept on ice for 10–15 min, vortexed, and centrifuged at 4–8 ℃. Protein concentration of lysates was determined using Bradford reagent and equal amounts of protein were separated electrophoretically using 10% SDS-PAGE, and then the gel was transferred to 0.45 mm PVDF membranes (Millipore, Bedford, USA) using a mini trans-blot (Bio-Rad Laboratories, Hercules, USA) at 4 ℃. Blocking of membranes was performed with 10% nonfat dry milk in PBS containing 0.1% Tween-20 (PBST) over a 2 h period. After a minimal wash with PBST, membranes were probed with rabbit antihuman NF-κB polyclonal antibody (1:1000) for 2 h, followed by three washes with PBST. Membranes were incubated with horseradish peroxidase-conjugated secondary (1:1000 dilution in blocking solution) for 1 h. Antibody-specific proteins were visualized using the enhanced chemiluminescence (ECL) detection system as directed by the manufacturer and the chemiluminescent ECL detection reagent .

Statistical analysis

A one-way ANOVA was used to determine whether means differed significantly $(P < 0.05)$. All results were expressed as means ± SD. Each experiment was repeated at least three times. Statistical significances were compared between each treated group and analyzed by the Student's t-test.

Results

Effect of LPS on cell viability

An earlier study has shown that LPS-induced inflammatory could activate macrophages. As shown in Fig. 1, the viability of peritoneal macrophages treated with LPS (6–50 μg/mL) for 9 h was enhanced compared with that of control group ($P < 0.05$). And the concentration of LPS at 25 μg/mL was the most effective in improving the activity of peritoneal macrophages ($P < 0.01$), which were used as the optional concentration for later experiment in this study.

The expression of NF-κB protein detected by immunohistochemistry (ICC)

To investigate the effect of NF-κB expression in peritoneal macrophages treated with NNK on LPS-stimulated mouse peritoneal macrophages for 9 h, we conducted an immunohistochemical analysis (Fig. 2). The images
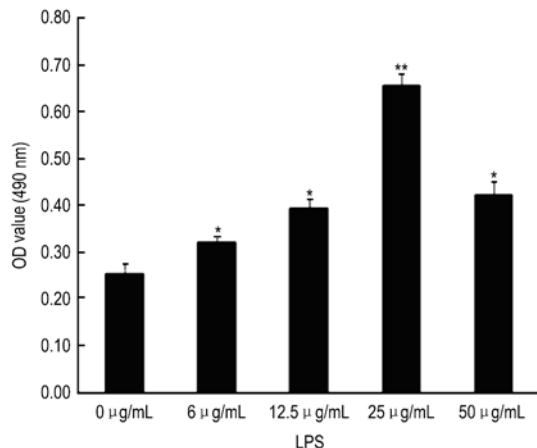
Fig. 1 Effect of LPS on the cell viability of primary mouse peritoneal macrophages. Cells were seeded in a 96-well plate and incubated with LPS (6–50 μg/mL) for 9 h. Cell viability was measured by the MTT assay. Treatment without LPS served as a positive control. $*$ $P < 0.05$; $**$ $P < 0.01$ vs untreated group

shown were representative figures of each group. NNK treatment significantly inhibited, in a concentration-dependent manner (100–500 μM), the production of NF-κB by both LPS-stimulated and unstimulated peritoneal macrophages. In addition, the results were shown in Fig. 2a–2d, NNK treatment yielded a significant and dose-dependent manner decrease in NF-κB translocation to nucleus compared with the treatment with LPS. Meanwhile, the protein expression of NF-κB was markedly decreased in a dose-dependent manner in cytoplasm. A morphological difference also appeared between NNK-treatment and the untreatment. The main morphological of the former peritoneal macrophages (Fig. 2b–2d and 2f–2h) was fusiform with few fine cytoplasmic projections, while the latter cells (Fig. 2a and 2e) were rotundity or oval with many fine cytoplasmic projections.

The expression of NF-κB protein in NNK-mediated primary peritoneal macrophages treated with or without LPS for 9 h detected by Western-blot

As shown in Fig. 3, NF-κB expression was observed in the peritoneal macrophages treated with NNK with or without LPS for 9 h by Western-blot. The NF-κB expression levels were downregulated in the peritoneal macrophages with NNK increased concentration (Fig. 3a). Although NF-κB expression levels were upregulated in the peritoneal macrophages with LPS, LPS could reduce this trend obviously (Fig. 3b). Low expression levels of NF-κB were also observed in the peritoneal macrophages but with obvious difference between the groups. As shown in Fig. 3, the numbers of NF-κB were reduced in the peritoneal macrophages treated with NNK, in spite
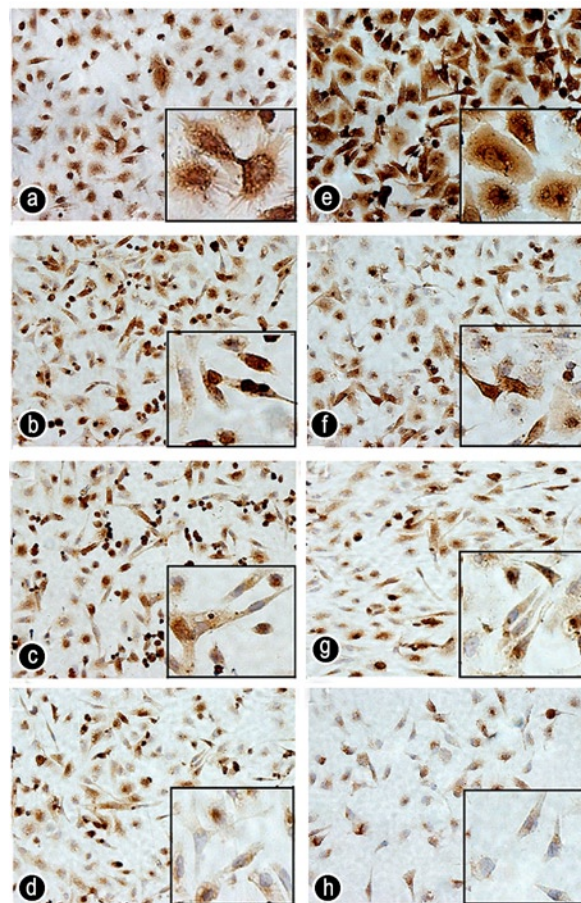

Fig. 2 NF-κB expression in NNK-mediated primary peritoneal macrophages treated with or without LPS for 9 h by ICC (400 ×). (a) Control; (b) NNK (100 μM); (c) NNK (250 μM); (d) NNK (500 μM); (e) LPS (25 μg/mL); (f) LPS (25 μg/mL) + NNK (100 μM); (g) LPS (25 μg/mL) + NNK (250 μM); (h) LPS (25 μg/mL) + NNK (500 μM)

of with LPS, which may result in the relief of antitumor immune suppression.

Discussion

Macrophages are crucial cellular ingredients of innate immunity. They detect and respond to pathogenic and tissue-derived signals to clear pathogens, remodel injured tissue and restore tissue homeostasis [16]. Their diverse but polarized functional phenotypes, driven by micro-environmental cues, allow them to adjust promptly to changing conditions within tissues [10]. The extent of macrophage heterogeneity and the polarizing stimuli that exist *in vivo* are not clear but two main functional phenotypes have been well characterized experimentally [17]. Classical activated (or M1) macrophages, induced by microbial products are tumoricidal and pro-inflammatory. Alternative (or M2) macrophages activated by IL-4 or
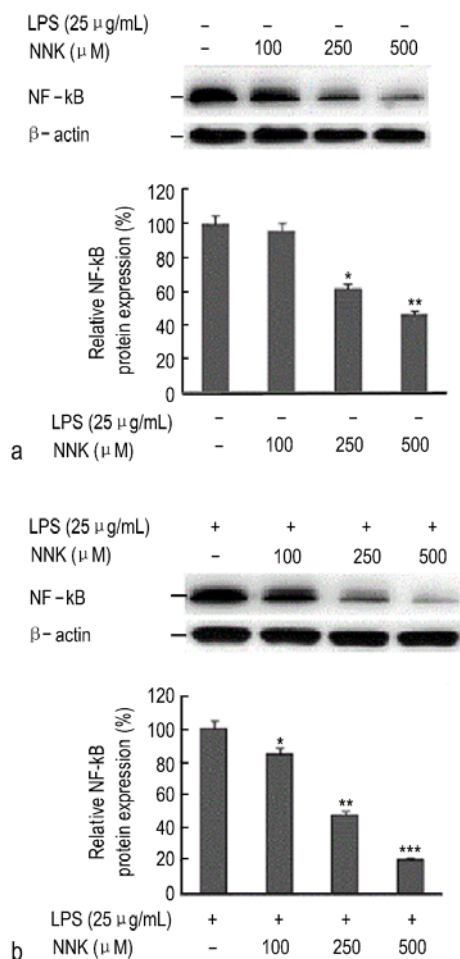
Fig. 3 Inhibition of production of NF-κB by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Peritoneal macrophages were treated for 9 h with NNK (100–500 μM), without (a) or with (b) 25 μg/mL lipopolysaccharide (LPS). The expression of NF-κB was significantly inhibited (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$) in a concentration-dependent manner. Furthermore, NNK (500 μM) significantly (*** $P < 0.005$) inhibited LPS-stimulated NF-κB production. Error bars indicated the means ± SD of three independent experiments

IL-13 gave rise to anti-inflammatory tissue-remodelling cells, while immunoregulatory or M2-like macrophages up-regulated IL-10 to dampen immune responses. M1 macrophages, was commonly described as iNOS+ cells on account of high iNOS expression, activated in response to LPS. LPS is ubiquitously present in the environment around us, including water, soil, plant, dust, and ambient air. Especially in cigarette smoke, the level of LPS is high [18], showing that LPS may play a significant role in tobacco smoke-induced pulmonary inflammation.

Keohavong *et al* [7] reported that the NNK-treated group showed no inflammation and macrophages were not increased. This was consistent with our experiment that NNK inhibited the activity of peritoneal macrophages

(Fig. 2b–2d) *in vitro*. Mice treated with LPS alone and that treated with LPS + NNK showed recruitment of a large number of macrophages, although inflammatory treated with LPS + NNK was less severe. The same function of LPS alone *in vitro* was found in our study. However, Our research showed that the function of LPS was reversed to increase the inhibition of NNK in macrophages treated with LPS + NNK (Fig. 2f–2h). Its specific mechanism remains to be elucidated. Therriault *et al* [19] indicated that NNK had the immunosuppressive effects (in a concentration-dependent and time-dependent manner) on AM, especially on AM pretreated with LPS. Furthermore, this observation needed at least 20 h. Similar trend was observed in peritoneal treated for 9 h in our study. However, it obviously reduced the time. The leading cause may be mediated by the concentration of LPS, which also led to the result that the inhibition of macrophages treated for 12 h was not modulated by NNK (data not shown). Moreover, since the smoke often contains LPS and NNK two ingredients, once inhaled smoke, these two substances were inhaled. Instead of LPS pretreatment, our study used NNK with LPS simultaneously.

NF-κB plays a central role in immune and inflammatory responses. It is a family of dimeric transcription factors and central components of innate and adaptive immunity, responsible for the activation of many genes required in infection, stress and injury. The NF-κB family of transcription factors comprises five members: p50 (NF-κB1), p52 (NF-κB2), p65 (RelA), RelB, and c-Rel [20]. In the absence of inflammatory stimuli, NF-κB is maintained in an inactive form through binding to an inhibitor of NF-κB (IκB). Once the cells are stimulated, IκB is rapidly phosphorylated, ubiquitinated, and degraded, facilitating the subsequent translocation of NF-κB to the nucleus to induce a wide array of genes critical in the immune response and inflammation [21]. As shown, NF-κB/p65 is central in driving M1 macrophage activation and expression of pro-inflammatory genes [22]. As expected, NF-κB nuclear translocation and activity was up-regulated in LPS-induced macrophages (Fig. 2e). Rioux *et al* [23] observed that NNK induced NF-κB activation in U937 human macrophages. The induction of NF-κB by NNK, is delayed compared with more common activators such as LPS. In contrast, the expression of NF-kB was inhibited (Fig. 2) and the numbers of NF-κB were reduced (Fig. 3) in the peritoneal macrophages treated with NNK, in spite of with LPS.

Conclusion

All results suggested that NNK exhibited immunomodulatory effect via decreasing the expression of NF-κB and inhibiting nuclear translocation of NF-κB in mouse peritoneal macrophages, while LPS can enhance this trend. The immunosuppression caused by NNK provides

some basis for the increased risk of pulmonary infections and lung cancer in cigarette smokers. Nevertheless, further investigations are needed to elucidate the mechanism of NNK immunomodulatory effects on peritoneal macrophages functions. A better understanding of NNK-mediated and LPS-enhenced immunomodulation may contribute to the prevention of lung cancer.

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