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Original Article

The phenotypical induction and damage effect of peptidoglycan on microglia

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Abstract: This study is to investigate the effect of peptidoglycan (PGN) on the inflammatory phenotypes of microglia. BV-2 cells were treated with different concentrations of *Staphylococcus aureus* PGN (1 μg/mL, 10 μg/mL and 50 μg/mL), and the expressions of pro-inflammatory factors TNF-α and IL-6, the anti-inflammatory factors IL-10 and ARG-1, and the M1 phenotype specific receptor CD86 and the M2 phenotype specific receptor CD206 were measured by ELISA. PGN (1 μg, 10 μg and 50 μg) was injected into lateral ventricle of mice for 48 hours. Immunohistochemical staining and Nissl's staining of brain tissues were performed to detect microglia death rate. PGN of 1 μg/mL had little effect on glial inflammatory mediators and inflammatory phenotypes, while PGN of 10 μg/mL and 50 μg/mL had significant effects. In the presence of 50 μg/mL PGN, the proinflammatory phenotype CD86 showed the highest expression, while the expression of CD206 was significantly inhibited. It was obvious that cell aging and apoptosis were obviously increased. Immunohistochemistry and Nissl's staining also showed that the death rate of microglia increased with increasing PGN concentration. PGN was positively correlated with the inflammatory phenotypic changes of microglia. High dose of PGN caused obvious increase of neuronal cell death.

Keywords: Microglia, peptidoglycan, phenotype induction, apoptosis

Introduction

Microglia are innate immune cells in the nervous system, which express TOLL-like receptors (TLRs) on the surface to recognize pathogens, thereby activating host immune response [1]. So far, there are more than ten types of TLRs, which recognize different microbial structures. TLR1, TLR2, TLR4, TLR5, TLR6 and TLR10 are expressed on the cell surface, mainly identifying bacterial components. TLR2 recognizes bacterial lipoprotein, peptidoglycan and lipoteichoic acid [2, 3], TLR4 recognizes lipopolysaccharide [4], and TLR5 recognizes bacterial flagella [5]. TLR3, TLR7 and TLR8, together with TLR9, are located within endosomal compartments, and are specialized primarily in viral detection and more generally, in recognition of RNA [6].

Microglia, which act as a phagocytic cell of the central nervous system, participates in the inflammatory processes in brain [7, 8]. M1 and M2 are two known microglia phenotypes. M1 has pro-inflammatory effect whereas M2 is involved in the anti-inflammatory immune process [9]. M1 microglia produce a large number of inflammatory factors, such as TNF-α and IL-6, whereas M2 microglia express anti-inflammatory factors like IL-10 and Arg-1 [10-12]. The expression of CD86 and CD40 on cell surface is increased in M1 phenotype, while the expression of CD206 on the cell surface is increased in M2 phenotype [13-15]. These two phenotypes of microglia can be converted under certain conditions. Under moderate infection and stress, microglia mainly express M2 type to inhibit excessive inflammatory expression and play a protective role [10]. However, when infection and stress are further enhanced, microglia transform from M2 to M1, over-expressing pro-inflammatory factors and promoting cell death [10].

PGN is a major component of the cell wall of Gram-positive bacteria. It is a specific activa-

tion ligand for the TLR2 pathway and has been used as a stimulator to study the function of the pathway, and the relationship between the pathway and autophagy [16]. In this study, the stimulatory effects of peptidoglycan (PGN) on microglia at different concentrations were investigated.

Materials and methods

Reagents

PGN from *Staphylococcus aureus* were purchased from Sigma Co. (St. Louis, MO, USA). Cytokine detection kits were purchased from MultiSciences Co. (Hangzhou, China). Detection kits for CD206 and CD86 were purchased from USCN life sciences Inc. (Wuhan, China). Dulbecco's Modified Eagle Medium (DMEM) and Gibco fetal bovine serum (FBS) were purchased from Thermo Fisher Scientific Co. (Waltham, MA, USA).

Cell culture and treatment

Mouse microglial cell line BV2 cells were provided by the central laboratory of Medical School, Shandong University. The cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C in an atmosphere containing 5% CO_2. BV-2 cells were stimulated by different concentrations of PGN (1 μg/mL, 10 μg/mL and 50 μg/mL) for 24 hours. Cell medium was used as blank control. Three repeats were set for each group.

Enzyme-linked immunosorbent assay (ELISA)

The amount of TNF-α, IL-6, CD86, IL-10, Arg-1 and CD206 in the supernatant of cell lysates was measured by corresponding ELISA kits.

Animals

Six to eight weeks old male C57BL/6J mice (n=12) were provided by the central laboratory of Medical School, Shandong University. They were kept in standard conditions. All animal experiments were conducted according to the ethical guidelines of Qilu Hospital of Shandong University.

Animal treatment and sampling

The mice were anesthetized with pentobarbital sodium (0.75 mg/10 g body weight) and fixed on the stereotaxic instrument. After disinfection, the top skin was cut in the middle to expose anterior fontanelle. The lateral ventricle was injected with various amounts of PGN (1 μg, 10 μg and 50 μg). The mice were sacrificed by cervical decapitation after 48 hours of treatment, and the brains were collected and fixed in 4% paraformaldehyde.

Immunohistochemistry

After fixation for 24 h, the brain was embedded in paraffin and sliced into 5 μm sections. The sections were dehydrated and the antigens were retrieved in a microwave oven in citrate buffer (pH 6.0), incubated in a 3% hydrogen peroxide solution at room temperature for 15 min in the dark, and washed in PBS (pH 7.4) for 3 times for 5 min each time. CD11b primary antibody (Cat# PAB685HU, Cloud-Clone Co., Wuhan, China) was added and incubated at 4°C overnight, and then incubated with secondary antibody (Cat# PAE001, Cloud-Clone Co., Wuhan, China) at 37°C for 50 min. The sections were colored with DAB and counterstained with hematoxylin, and then dehydrated and mounted. The images were photographed at a high magnification and analyzed for the percentage of positive cells.

Nissl's staining

The prepared paraffin sections were sequentially placed in xylene I for 30 min, xylene II for 30 min, absolute ethanol I for 10 min, absolute ethanol II for 10 min, 95% alcohol for 5 min, 90% alcohol for 5 min, 80% alcohol for 5 min, 70% alcohol for 5 min and distilled water. The Nissl dyeing solution was added and stained for 10 minutes, and then the sections were differentiated with 95% alcohol. After dehydration, the sections were sealed with neutral gum. The image was collected and analyzed under a microscope. Three fields of view were randomly selected for each slice in each group. Image-Pro Plus 6.0 software was used to count the positive cells. The number of necrotic neurons was counted and averaged.

Statistical analysis

SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. All experiments were repeated three times. Data were expressed as mean ± standard deviation (SD). Student's *t* test was used for the

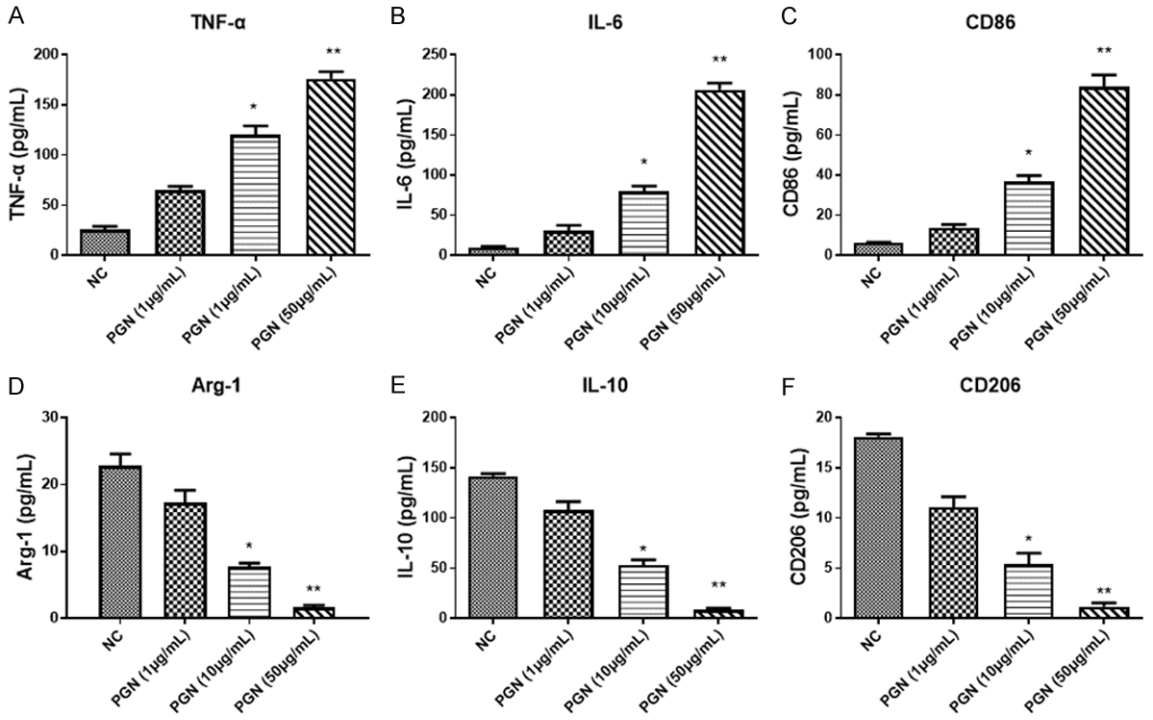

Figure 1. The effect of PGN on cytokine levels in BV-2 cells. BV-2 cells were treated with various concentrations of PGN (1 μg/mL, 10 μg/mL, 50 μg/mL) for 24 hours, and (A) TNF-α, (B) IL-6, (C) CD86, (D) Arg-1, (E) IL-10 and (F) CD206 were measured by ELISA. All data were averaged from three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group.

comparison between two groups. One-way analysis of variance (ANOVA) was performed for comparisons between multiple groups. A *P* value less than 0.05 was considered statistically significant.

Results

PGN induces the changes in the phenotypes of microglia

In order to study the effect of PGN on the *in vitro* culture of glial cells, BV-2 cells were treated with PGN for 24 hours, and ELISA was used to detect the intracellular concentration of cytokines closely related to the cell phenotype (**Figure 1**). After PGN treatment, the levels of TNF-α (**Figure 1A**), IL-6 (**Figure 1B**) and CD86 (**Figure 1C**) were significantly increased than those in control group ($P < 0.05$), and the increase was dose-dependent. On the contrary, the levels of Arg-1 (**Figure 1D**), IL-10 (**Figure 1E**) and CD206 (**Figure 1F**) in PGN groups were significantly decreased than those in control group ($P < 0.05$) and also in a dose-dependent manner. Specifically, at low concentrations (1 μg/mL), the phenotypic changes were not obvious. At the concentration of 50 μg/mL and 10 μg/mL PGN, CD206 expression (M2 phenotype) was significantly lower, and CD86 expression (M1 phenotype) was significantly higher than that of control groups ($P < 0.01$). The results showed that PGN stimulated phenotypic changes of microglia in a dose-dependent manner.

PGN induces apoptosis of microglia

To observe the survival status of microglia under the action of PGN, cell morphology was observed. After 24 hours of treatment with various concentrations of PGN, BV-2 cells gradually showed apoptosis and aging (**Figure 2**). At 50 μg/mL PGN, microglia showed the most obvious aging and apoptosis. This indicated that PGN caused microglia apoptosis in a dose-dependent manner.

PGN causes microglia activation in the brain of mice

To observe the activation of microglia by the treatment of PGN, immunohistochemistry was performed. The microglia labeled with CD11b showed clear brownish yellow color in the tis-

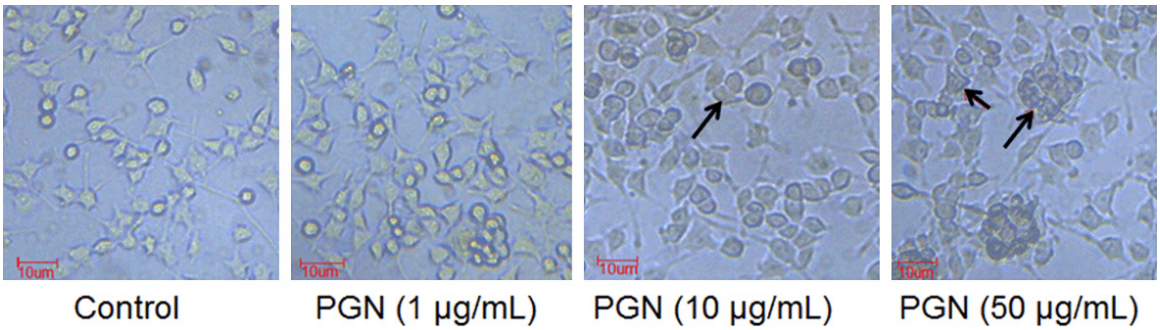

Figure 2. Cell morphology analysis. After incubated with various concentrations of PGN for 24 hours, the state of BV-2 cells was observed under a microscope. Scale bar: 10 μm. Arrows indicate aging and apoptotic microglia.

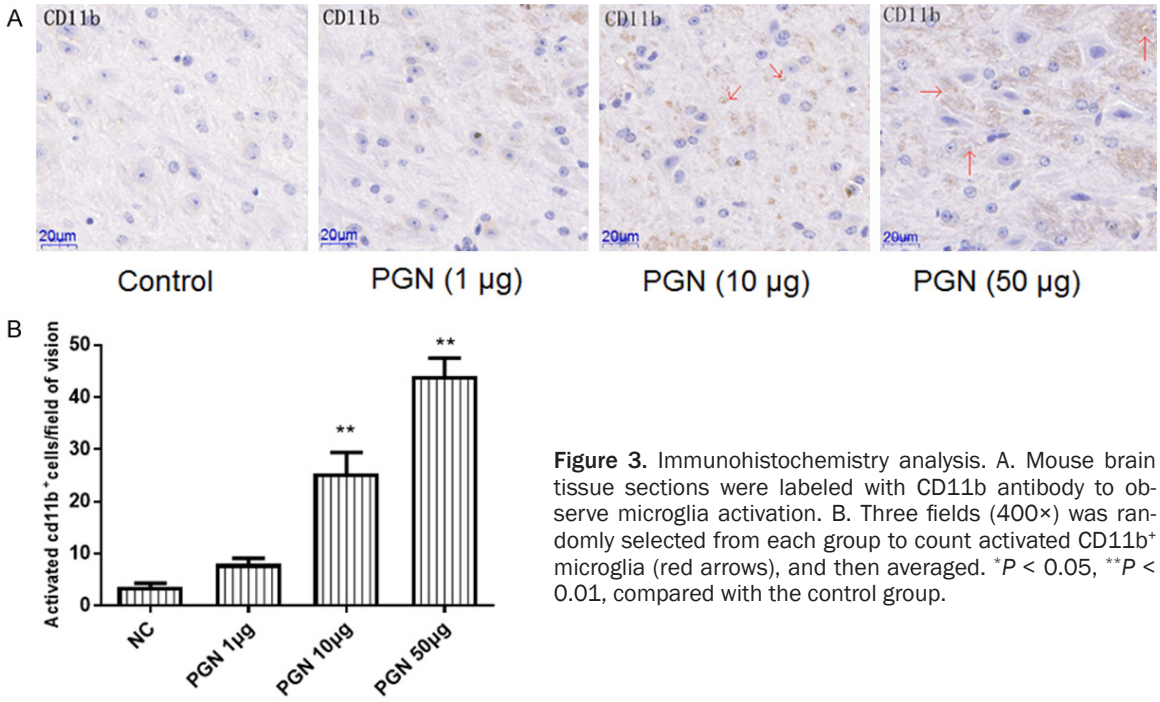

Figure 3. Immunohistochemistry analysis. A. Mouse brain tissue sections were labeled with CD11b antibody to observe microglia activation. B. Three fields (400×) was randomly selected from each group to count activated $CD11b^+$ microglia (red arrows), and then averaged. $^*P < 0.05$, $^{**}P < 0.01$, compared with the control group.

sue sections (**Figure 3A**). Different degrees of microglia aggregation occurred in the brain tissue area injected with PGN, which was related to the dose of PGN at the injection site. When the dose of PGN was increased, the number of activated microglia was increased, and the cells became larger, the protrusions became thicker and the small spines were visible on the protrusions. At the dose of 50 μg of PGN, the number of activated microglia was the highest, which was significantly different from that of the control group ($P < 0.05$) (**Figure 3B**).

PGN causes neuron death in the brain of mice

Nissl's staining was performed on mouse brain tissue sections to observe the survival of neuronal cells. Under the treatment of PGN, the arrangement of neuronal cells was disordered, and the Nissl body was partially disappeared. The higher the concentration of PGN, the greater the cell damage was (**Figure 4A**). The results showed that the number of dead neuronal cells increased with the increase of PGN concentration, and the difference between groups was statistically significant ($P < 0.05$) (**Figure 4B**). These results suggest that PGN induced neuronal cell death in a dose-dependent manner.

Discussion

Microglia are immune cells inherent in the central nervous system that can recognize a large number of pathogens [17], through a series of

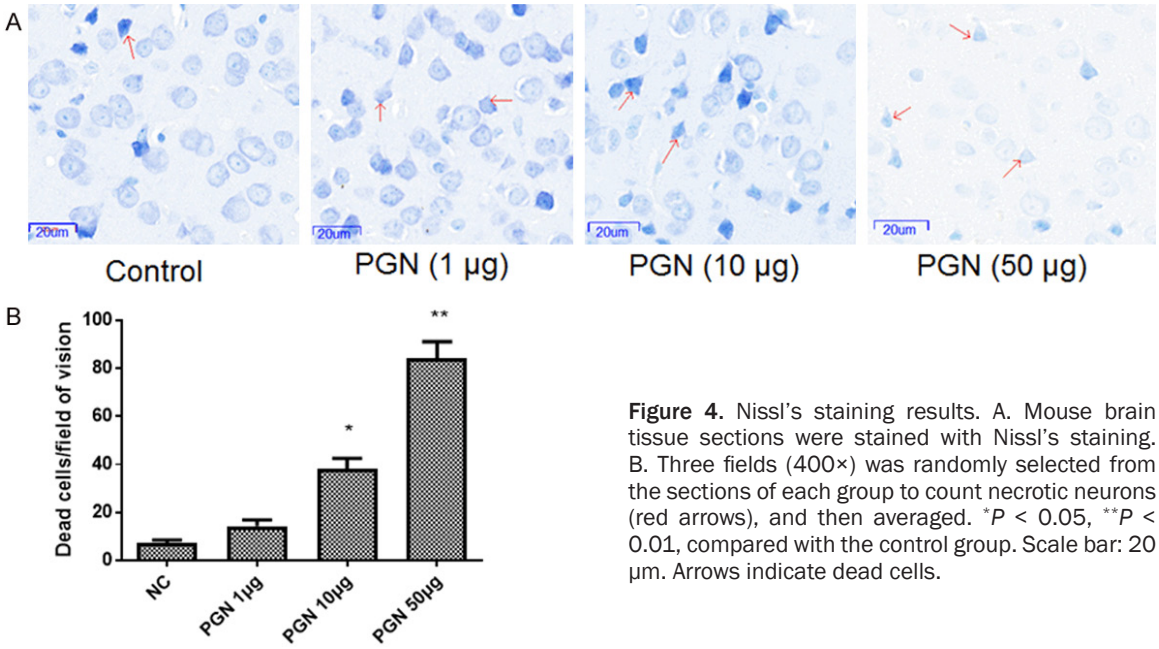

Figure 4. Nissl's staining results. A. Mouse brain tissue sections were stained with Nissl's staining. B. Three fields (400×) was randomly selected from the sections of each group to count necrotic neurons (red arrows), and then averaged. $^{*}P < 0.05$, $^{**}P < 0.01$, compared with the control group. Scale bar: 20 μm. Arrows indicate dead cells.

pattern recognition receptors (PRRs), including TLRs, NOD like receptors (NLRs) and scavenger receptors. Microglia express all known TLRs, including TLR2, which can recognize a large number of TLR2 ligands, such as lipoproteins, lipoteichoicacid (LTA) and peptidoglycans [18-21], and TLR4 [19, 22]. Using microglia from TLR4-KO mice, it was found that deletion of TLR4 led to the loss of response to LPS in microglia [23, 24].

Microglia in the brain have been shown to exhibit high inducibility and can respond to various stimuli by activating the classical phenotypes of M1 and M2 [25]. The M1 phenotype is characterized by the production of pro-inflammatory factors IL-1β, TNF-α and IL-6, and the increase of cell surface markers such as CD16/32, CD86 and CD40, which exacerbate the inflammatory process [26]. M2 microglia can improve phagocytosis and release many protective and trophic factors that trigger anti-inflammatory and immunosuppressive responses [27]. For example, CD206 is a classical M2 marker that functions as a C-type lectin in endocytosis and phagocytosis, and maintains cell function stability by eliminating unwanted mannoproteins [13].

This study investigated the effect of TLR2-specific ligand PGN on microglia by detecting microglia phenotypic changes and cell damage. Three concentrations were used to stimulate microglia in this study. Low-dose PGN (1 μg/mL) had no obvious effect on the phenotypic changes of microglia. The expression of CD86, a marker of proinflammatory phenotype M1, and proinflammatory mediators TNF-α and IL-6 was slightly increased without significant difference. At the PGN concentration of 10 μg/mL and 50 μg/mL, the phenotype of microglia changed significantly, and both M1 and M2 phenotypes were expressed. The pro-inflammatory phenotype was dominant. *In vivo* experiments using CD11b-labeled microglia showed that there were different degrees of microglia aggregation in the brain tissue after PGN injection. The higher the dose of PGN, the more increased aggregation of microglia, and the more death of microglia. When the dose of PGN was 50 μg, the number of dead microglia in the tissue section was the highest. Thus, the effect of PGN on microglia is dose-dependent.

In conclusion, PGN can activate microglia *in vitro* and *in vivo*, promote the transformation of microglia into M1 type, stimulate the activation of microglia, and accelerate the apoptosis of neuronal cells.

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Disclosure of conflict of interest

None.

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