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SHORT COMMUNICATION

NLRP12 negatively modulates inducible nitric oxide synthase (iNOS) expression and tumor necrosis factor-α production in *Porphyromonas gingivalis* LPS-treated mouse macrophage cell line (RAW264.7)

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Abstract

Objective The aim of the present study is to investigate the participation of NLRP12 in *Porphyromonas gingivalis* LPS-activated mouse macrophages.

Methods NLRP12-depleted mouse macrophages were stimulated with *P. gingivalis* LPS (1 μg/ml.). At indicated time points, the treated cells were lysed and the supernatant from treated cells was collected. Gene and protein expression of NLRP12 and iNOS were determined by RT-PCR and immunoblotting, respectively. The level of TNF-α production in the supernatant of the activated cells was determined by ELISA.

Results and conclusion NLRP12 was upregulated in response to stimulation with *P. gingivalis* LPS. In addition, when NLRP12 was depleted in *P. gingivalis* LPS-treated macrophages, an increase in TNF-α production and iNOS expression were observed when compared to those of the control cells, indicating that NLRP12 downregulates the inflammatory cytokine and antimicrobial molecule production in the macrophages.

Keywords iNOS · NLRP12 · *Porphyromonas gingivalis* LPS · RAW264.7

Introduction

Porphyromonas gingivalis, a Gram-negative anaerobic pathogen, is a putative keystone species in chronic periodontitis [1]. This disease is characterized by the destruction of tooth-supporting tissues and untreated infections can subsequently result in the formation of tooth pockets and eventually the loss of teeth. To survive and replicate in a host, *P. gingivalis* has the ability to produce a variety of virulence factors such as fimbriae, capsules, lipopolysaccharide (LPS), lipoteichoic acids, gingipains, outer membrane proteins, and outer membrane vesicles, thus leading to the destruction of periodontal tissue, bone resorption, induction of inflammation and subversion of host immune responses [2]. Among the aforementioned virulence factors, LPS is known to be an important pathogenic factor for stimulation of host immune cells and disrupting the host defense mechanisms. In response to *P. gingivalis* LPS, the innate immune cells, particularly monocytes/macrophages can regulate the release of a large number of bioactive substances such as reactive oxygen species, nitric oxide, lysosomal enzymes and cytokines [3]. The secretion of these bioactive substances can lead to tissue damage and inflammation.

Nucleotide-binding oligomerization domain (NOD)-like receptor (NLR) family is composed of a large number of intracellular pathogen recognition receptors that function as sensors for microbial-derived and danger-associated molecules in the cytoplasm of host cells [4]. Among the members of NLR family, NLRP12 (NALP12, MONARCH-1, or PYPAF7) contributes to suppressing the production of proinflammatory cytokines and chemokines by interfering with canonical and noncanonical NF-κB signaling pathways [5]. Recently, several reports have revealed a potential role of NLRP12 during infectious diseases. NLRP12-deficient mice showed higher mortality and bacterial burden after infection with *Yersinia pestis,* whereby NLRP12 inflammasome was shown to drive the activation

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of caspase-1, IL-1β and IL-18 secretion [6]. Moreover, the functional role of NLRP12 as a negative regulator of proinflammatory cytokine was also observed in response to *Salmonella typhimurium, Klebsiella pneumoniae* LPS and TDP ligand from *Mycobacterium tuberculosis* [5, 7]. The reduction of proinflammatory cytokine production in *Pseudomonas aeruginosa*-infected mouse corneas and macrophages was observed when NLRP12 was overexpressed in both in vivo and in vitro studies [8]. However, another in vivo study demonstrated that *Nlrp12*$^{-/-}$ mice exhibit similar host immune response in lung infections stimulated by *Mycobacterium tuberculosis* or *Klebsiella pneumonia* when compared to that of the wild-type mice [5]. As a result, the functional role of NLRP12 in bacterial infection remains controversial and the involvement of this NLR is not fully elucidated, particularly in periodontitis. Since periodontitis is known to associate with *P. gingivalis*, therefore, the aim of this study is to investigate the participation of NLRP12 in *P. gingivalis* LPS-activated macrophages.

Methods

Stimulation of mouse macrophages with *P. gingivalis* LPS

Mouse macrophages (0.5×10^6 cells/well) were treated with *P. gingivalis* LPS (Invivogen, CA, USA) at a concentration of 1 μg/ml. For depletion of NLRP12, mouse macrophages (2.5×10^5 cells/well) were transfected with scramble or NLRP12 siRNAs (Dharmacon, CO, USA) according to the manufacturer's protocol [9]. At indicated time intervals, the treated cells were lysed and the expression of gene and protein was determined by RT-PCR and immunoblotting, respectively.

Reverse transcriptase PCR

RNA extraction was performed as previously described [9]. PCR was then performed using primer pairs specific for *nlrp12*, *inos* and β-*actin.*

Immunoblotting

The immunoblotting was performed as previously described [9]. Following primary antibodies were used: antibody against NLRP12 (Abcam), iNOS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ACTIN (Merck Millipore, NJ, USA).

Determination of TNF-α production

The supernatant from activated cells was collected and the level of TNF-α production was measured by mouse TNF-α ELISA kit (BD Bioscience, San Diego, USA) following manufacturer's instructions. The statistical analysis was performed using SigmaPlot 10.0.

Results

To demonstrate the involvement of NLRP12 in *P. gingivalis* LPS activation, mouse macrophages were treated with *P. gingivalis* LPS at a concentration of 1 μg/ml. At different time intervals, the treated cells were lysed and the expression of *nlrp12* gene and protein were determined by RT-PCR and immunoblotting, respectively. The results showed that *P. gingivalis* LPS upregulated the *nlrp12* mRNA (Fig. 1a) and protein expression (Fig. 1b) at 1 h after activation before reaching the maximum level in 6 h, indicating that the mediation of NLRP12 in *P. gingivalis* LPS may involve the regulation of macrophage innate immune responses. To elucidate this hypothesis, mouse macrophages were transfected with siRNAs against NLRP12 or scramble siRNAs for 24 h prior to stimulation with *P. gingivalis* LPS. Since inducible nitric oxide synthase (iNOS) is an enzyme that produces by macrophages and plays an essential role in controlling of microbial growth [10], we examined whether there is an alteration in iNOS level in these cells. Our results showed a markedly increase in both *inos* gene (Fig. 1c) and protein expression (Fig. 1d) in *P. gingivalis* LPS-treated NLRP12-depleted cells when compared to that of the control siRNA-transfected cells. Furthermore, we extended our experiment to explore the role of NLRP12 in the regulation of proinflammatory cytokines such as TNF-α after challenged with *P. gingivalis* LPS. As expected, the level of TNF-α production was significantly increased in *P. gingivalis* LPS-treated NLRP12-depleted cells when compared to that of the control cells (Fig. 1e).

Discussion

Recognition of invading pathogens by innate immune cells relies on their ability to detect microbial components by TLRs or NLRs [11]. Several studies have demonstrated the involvement of TLR signaling pathway in response to *P. gingivalis* infection. However, the participation of NLRs, particularly NLRP12 is not extensively studied in periodontitis. *P. gingivalis* stimulates immune cells to produce several proinflammatory cytokines such as IL-1β, IL-6, IL-12

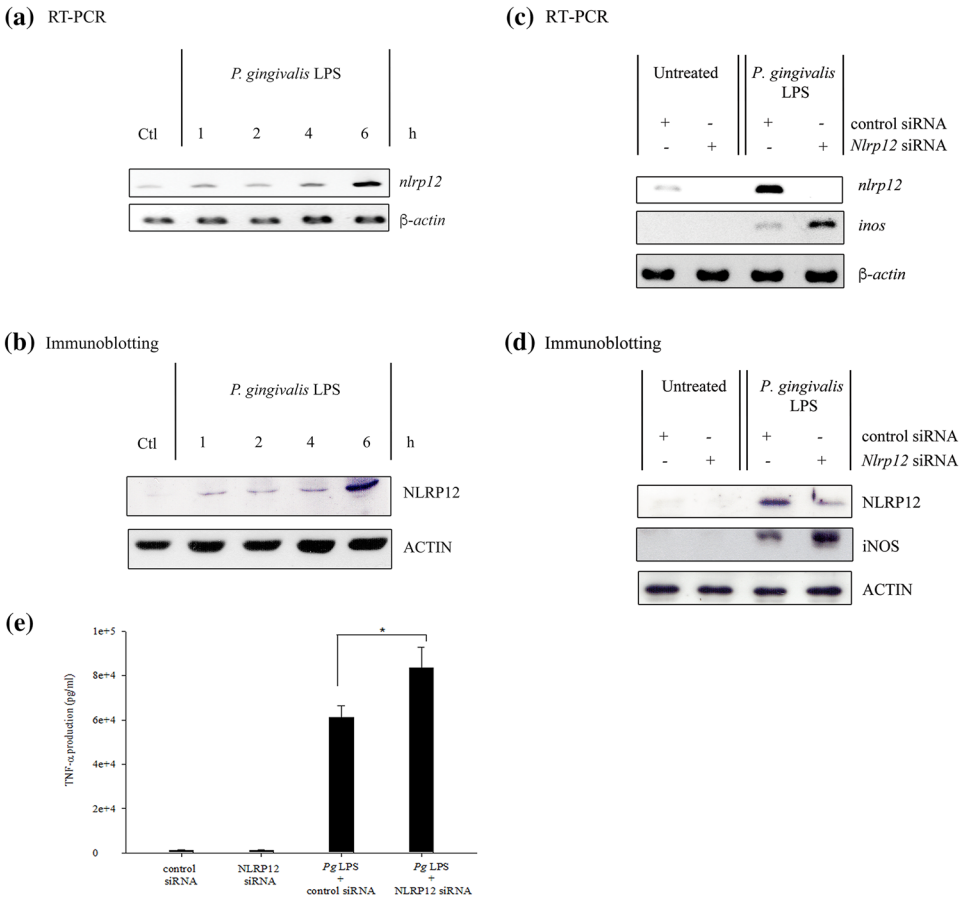

Fig. 1 NLRP12 attenuates iNOS expression and TNF-α secretion. Mouse macrophages were treated with *P. gingivalis* LPS at a concentration of 1 µg/ml. At different time intervals, the activated cells were lysed. The NLRP12 gene (**a**) and protein (**b**) expression were determined by RT-PCR and immunoblotting, respectively. For depletion of NLRP12, the cells were transfected with siRNAs against NLRP12 prior to stimulation with *P. gingivalis* LPS. At 6 h of incubation, the treated cells were lysed and the supernatant was collected. The gene (**c**) and protein (**d**) expression were analyzed by RT-PCR and immunoblotting, respectively. The level of TNF-α production was measured by ELISA assay (**e**). Data are expressed as mean ± SEM of three independent experiments. $*p < 0.05$ (Student's *t* test). All experiments in this study were conducted at least three times

and TNF-α, leading to the amplification and progression of the pathogenesis [11]. NLRP12 exhibits a protective role in periapical bone destruction by attenuating inflammation and reducing alveolar bone loss [12]. At present, our data demonstrated that *P. gingivalis* LPS can mediate host innate immune responses by upregulating NLRP12. The absence of NLRP12 showed the induction of iNOS expression in *P. gingivalis* LPS-treated NLRP12-depleted cells when compared to that of the control siRNA-transfected cells. These data are consistent with an increase nitric oxide production in response to *S. typhimurium*-infected NLRP12$^{-/-}$ BMDMs, suggesting that NLRP12-mediated dampening of host immune responses is used by *S. typhimurium* to persist and survive in the host [7]. Moreover, a higher level of TNF-α production was also observed in *P. gingivalis* LPS-treated NLRP12-depleted cells when compared to that of the control siRNA-transfected cells, suggesting that NLRP12 participates in suppression of macrophage innate immune responses against this periodontal pathogen. In agreement with this result, an increased production of proinflammatory cytokines were also observed in NLRP12$^{-/-}$ BMDMs infected with *S. typhimurium* and *B. abortus* [7, 13]. Collectively, these results suggest that NLRP12 hampers the production of proinflammatory cytokine and antimicrobicidal substance in macrophages treated with LPS from *P. gingivalis*. However, further studies of the regulation of NLRP12 in *P. gingivalis* infection remains to be investigated.

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Compliance with ethical standards

Conflict of interest The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article.

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