Toll-like receptor-4, but not toll-like receptor-2 mediates secretion of tumour necrosis factor α and interleukin-8 in lipopolysaccharide-stimulated mouse mammary epithelial cells

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Abstract

Mammary epithelial cells (MECs) from Kunming mice were isolated and stimulated in vitro with 10 μg/mL of Escherichia coli lipopolysaccharide (LPS). The release of tumour necrosis factor α (TNF-α) and interleukin-8 (IL-8) into culture supernatants was measured by ELISA. Furthermore, blocking experiments with Toll-like receptor 2 (TLR2) and TLR4 antibodies were performed to verify whether cytokine secretion depended on LPS-induced activation of TLR2 or TLR4. The results revealed that LPS-stimulated mouse MECs significantly secreted TNF-α and IL-8. Blocking of the TLR4 pathway inhibited the secretion of TNF-α and IL-8, while inhibition of LPS-induced TNF-α and IL-8 production was not observed when TLR2 was blocked. Thus, TLR4 can mediate the LPS-induced expression of cytokines such as TNF-α and IL-8 in mouse MECs.

Key words: mouse, mammary epithelial cells, cytokine, TLR2, TLR4.

Introduction

Mastitis is an inflammation of the mammary gland resulting from bacterial infection. One of the most effective ways to decrease the occurrence of mastitis is to enhance acquired or innate immune responses in the animal which is the predominant defence strategy during bacterial invasion. In addition to innate immune cells including neutrophils and macrophages, mammary epithelial cells (MECs) are involved in the first line of defence against microorganisms (24). Therefore, MECs are prominent participants of innate immune responses in the development of mastitis (22). Unlike the phagocytosis of neutrophils or macrophages, MECs mainly depend on attracting circulating immune effector cells through the secretion of cytokines and chemokines to eliminate invading pathogens (7).

Cytokines, released by various cell types such as monocytes, neutrophils, macrophages, and epithelial cells (6, 13, 18, 21), play a crucial role in the innate immune system. In most infectious diseases, the key host defence mechanism is the up-regulation of cytokines production (9), including tumour necrosis factor α (TNF-α), interleukin-1 (IL-1), IL-10, and IL-12. During initial mammary inflammation, MECs together with other immune cells participate in inflammatory responses by secreting pro-inflammatory cytokines and chemokines to recruit immune cells to the site of infection (29). The previous study has indicated the strong capacity of MECs in cows to produce pro-inflammatory cytokines/chemokines such as TNF-α, IL-1β, IL-8, and chemokine (C-X-C motif) ligand 6 (CXCL6) (22). However, similar functions of MECs have not been reported in mice.

The innate immune system senses invasion of microbial pathogens via pattern-recognition receptors (PRRs) (10). Toll-like receptors (TLRs) are a large class of PRRs that mediate pathogen recognition through pathogen-associated molecule patterns (PAMPs) such as lipopolysaccharides (LPS) from Gram-negative bacteria and lipoteichoic acid (LTA) from Gram-positive bacteria (2). After recognition of PAMPs, TLRs initiate innate immune responses and
regulate the expression of TLR-related molecules (4). TLRs include 13 family members in mammals and each has its own functional characteristics (15). One of the best characterised TLRs is TLR4, which is mainly activated by LPS, a component of Gram-negative bacteria. TLR2 recognises bacterial cell wall components including those found on Gram-positive bacteria (peptidoglycan, lipoteichoic acid, lipoproteins) and also LPS from *Leptospira interrogans* and *Porphyromonas gingivalis* (16, 23).

Currently, the knowledge of MEC innate immune responses has been focused on humans and cattle. The release of cytokines from mouse MECs and their precise mechanism remain obscure. The aim of this study was to determine the secretion of TNF-α and chemotactic factor IL-8 during stimulation of mouse MECs with *Escherichia coli* LPS. Further experiments were designed to determine whether the production of these two cytokines depended on TLR2 or TLR4 in combination with LPS.

**Material and Methods**

**Animals.** Ten to 12-weeks old, mid-pregnant Kunming mice were used. The mice were purchased from the Medical Experimental Animal Center of Daping Hospital, Research Institute of Surgery of the Third Military Medical University, Chongqing. They were housed in micro isolator cages, receiving standard laboratory animal feed and water *ad libitum*. The experiments followed the guidelines of the regional Animal Ethics Committee.

**Isolation, culture, and identification of mouse MECs.** The procedures for cell culture were previously described (20). In brief, mammary tissue was harvested from mid-pregnancy mice, minced directly with ophthalmic scissors, and incubated with 8 mL of sterile digestive solution containing 0.2% collagenase I; 0.2% collagenase II (Sigma); and 0.2% trypsin (Sigma) at 37°C for 1 h with shaking. After incubation, the digested tissue was centrifuged at 1000 rpm for 5 min and resuspended with DMEM/F12 containing 10% foetal bovine serum (FBS, Hyclone). Then, the epithelial organoid suspension was filtered through 80-μm mesh. The organoids were separated from fibroblasts by differential adhesion method and suspended in DMEM/F12 containing: 10% FBS, 100 IU/mL of penicillin, 100 IU/mL of streptomycin (Gibco), 4 mM L-glutamine (Sigma), 5 μg/mL of bovine pancreatic insulin (Sigma), 10 ng/mL of cholera toxin (Sigma), and 10 ng/mL of epidermal growth factor (Sigma). Medium was changed once every 24 h.

Purity of MECs was determined by observing the morphological characteristics under inverted microscope and detection of Keratin 18 (Novocastra), a specific marker for epithelial cells, using indirect immunocytochemistry. MECs were used for the following experiments after three passages. To determine whether TLR2 and TLR4 proteins were expressed by normal mouse MECs, cells were cultured on glass coverslips and fixed with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature, followed by washing three times with phosphate-buffered saline (PBS). After blocking with the solution and washing three times with PBS, coverslips were incubated overnight at 4°C with rabbit anti-TLR2 and anti-TLR4 monoclonal antibodies (1/100 dilution; Abcam) followed by chromoes™488-conjugated sheep anti-rabbit secondary antibodies (specificly binding anti-TLR2) or chromoes™633-conjugated sheep anti-rabbit secondary antibodies (specificly binding anti-TLR4) for 1 h at room temperature, in the dark. Finally, coverslips were sealed by nail polish. The proteins were visualised with enhanced fluorescence using confocal laser scanning fluorescence microscope (Olympus).

**LPS treatment of mouse MECs.** To assess innate immune responses of mouse MECs, they were grown to 80% confluence in 6-well dishes, and medium was replaced with fresh challenge medium, in the presence or absence of 10 μg/mL of LPS. After challenge for 8 h, the supernatant was harvested and immediately centrifuged at 2500 rpm for 10 min. The resulting supernatant was stored at −80°C until used. Concentrations of TNF-α and IL-8 were determined using an enzyme-linked immunosorbent assay (ELISA) (Jingwei Biological Technology Co. Ltd., Hong Kong). Samples were measured in duplicate.

**Immunocytochemistry analysis.** To verify whether TNF-α and IL-8 were generated by activation of TLR2 or TLR4, the following experiment was designed. When cells reached 80% confluent state in 24-well plates, they were randomly divided into four equal groups (n = 8 per group), as follows: control group (A), TLR2 inhibitor group (B), TLR4 inhibitor group (C), and LPS group (D). To assess the involvement of TLR, cells in B and C groups were preincubated with polyclonal rabbit anti-rat TLR2 and TLR4 antibody (10 μg/mL), respectively, and cells in A and D groups were preincubated with rabbit IgG (10 μg/mL) as a negative control. After 45 min, cell supernatants were discarded, replaced with fresh challenge media with 10 μg/mL of LPS for 8 h in B, C, and D groups. Cells in A group (negative control) were not treated with LPS. Then, concentrations of TNF-α and IL-8 in supernatants were examined by ELISA.

**Statistical analysis.** Data was expressed as the mean ± standard deviation (SD). TNF-α and IL-8 concentrations were assessed by one-way ANOVA (Dunnett’s t-test) and Student’s t-test. A value of P<0.05 indicated statistical significance.
Fig. 1. A. Culture and characterisation of mouse MECs. Epithelial organoid attached to the plastic dish after 12 h (100×). B. Epithelial organoid was spreading out (100×). C. Cells formed confluent monolayer of cobblestone (100×). D. Single mouse MECs after passaged (200×). E. Cells spread out completely and confluent monolayer of cobblestone forms (200×). F. Cells were stained with antibodies against cytokeratin 18 (yellow), nuclei were stained with PI (red) (600×). High power double immunofluorescence confocal imaging demonstrated the presences of TLR2 (G) and TLR4 (H).

Fig. 2. Effect of LPS on the expressions of TNF-α and IL-8 in mouse MECs. MECs were cultured with or without 10 μg/mL of LPS for 8 h, respectively. Contents of TNF-α and IL-8 in each group were measured (n = 8) by ELISA. Data are presented as mean ± SD of two independent experiments. ** P < 0.01, in comparison with control group.

Fig. 3. The expressions of TNF-α and IL-8 induced by LPS in each group under the preincubation with TLRs antibodies. After preincubation with antibodies against TLR2 or TLR4 (10 μg/mL) for 45 min, contents of TNF-α and IL-8 in each group were measured (n = 8) by ELISA. Data are presented as mean ± SD of two independent experiments. ** P < 0.01, in comparison with blank control group. ** P < 0.01, in comparison with LPS group.
Results

Characterisation of mouse MECs is presented in Fig. 1. Epithelial organoids were acquired as they attached to the plastic dish after 12 h, and then MECs spread out, forming a confluent cobblestone monolayer after 72 h. In addition, the tissue-specific expression of cytokeratin 18 in MECs was detected (Fig. 1F), which indicated the cells were pure MECs. Thus, pure MECs could be harvested and used for experiments. TLR2 and TLR4 were expressed by normal mouse MECs.

LPS up-regulated expression of TNF-α and IL-8 in mouse MECs is presented in Fig. 2. MECs stimulated with LPS secreted significant amounts of TNF-α and IL-8. After LPS stimulation, the TNF-α concentration increased to 50.78 ng/mL and IL-8 increased to 60.09 pg/mL, which were significantly higher than in control groups (P < 0.01).

TLR4 signalling is involved in TNF-α and IL-8 expression in mice MECs. Preincubation with TLR4 antibody completely inhibited the expression of TNF-α and IL-8 in mouse MECs stimulated with LPS (P < 0.01). In contrast, pretreatment with TLR2 antibody failed to induce inhibition (P > 0.05) (Fig. 3).

Discussion

Recent studies (22, 17, 12, 3) have indicated that bovine mammary epithelial cells play a crucial role in innate immunity, which can respond to bacterial LPS and other microbial products by producing pro-inflammatory cytokines/chemokines (TNF-α, IL-1β, IL-8, β-defensins, and serum amyloid A) to neutralize invading pathogens. However, bovine mammary tissue cannot be harvested easily, and few studies have reported a correlation between epithelial cells and immune mechanisms in the mammary glands. Therefore, in the study, pure MECs from mice were obtained and the innate immune response of mouse MECs to LPS was investigated. It was found that mouse MECs stimulated with LPS could enhance the release of TNF-α and IL-8, two important cytokines in innate immune responses. The molecular mechanisms of these effects were attributed to the activation of TLR4, but not TLR2. These results are similar to the finding of Bocker et al. (5).

TNF-α is situated at the beginning of the pro-inflammatory cascade, and recruits neutrophils and macrophages to sites of infection (11). IL-8 is a chemokine associated with the number of neutrophils migrating to sites of infection. Epithelial cells are considered a major source of TNF-α and IL-8 (21). The obtained data showed that mouse MECs significantly released TNF-α and IL-8 when stimulated with 10 μg/mL of LPS, and were similar to those of bovine MECs (17). However, the sensitivity of mouse MECs to LPS stimulation might be different from that of bovine. Many previous reports have demonstrated that 1 μg/mL of LPS stimulation could produce an infection model of bovine MECs (19, 8). However, in our preliminary experiments, stimulation of mouse MECs with 0.1, 1.0, and 5.0 μg/mL of LPS did not significantly induce the release of TNF-α and IL-8. Only 10 μg/mL of LPS had an effect (data not shown). These findings suggest that mouse MECs may have a specific mechanism of responding to the presence of pathogenic products that is different from bovine MECs.

Studies have established that TLRs participate in LPS-mediated signalling pathways and can activate common inflammatory pathways that induce inflammatory cytokines such as TNF-α, IL-1β, and IL-6 (1). TLR4 is the main recognition receptor for LPS, and combines TIR domain-containing adaptor protein (TIRAP) and myeloid differentiation factor 88 (MyD88) to initiate a downstream cascade resulting in nuclear translocation of nuclear factor (NF)-κB and production of pro-inflammatory cytokines (14). TLR-2 is also a signalling molecule for LPS (26, 27), although previous evidence indicates that TLR2 mainly recognises Gram-positive bacteria cell wall components. TLR4 and TLR2 activate NF-κB through the TIRAP and MyD88 dependent pathway (25). In bovine MECs challenged with E. coli, TLR2 and TLR4 work together to induce TNF-α and IL-8 production (28). However, these events in mouse MECs are not clear. In this study, TLR antibodies were used to inhibit the activity of TLR2 and TLR4 in mouse MECs, revealing the relationship between cytokines such as TNF-α and IL-8, and TLR2 or TLR4. The results showed that mouse MECs releasing TNF-α and IL-8 were dependent on TLR4, but not TLR2. Thus, it seems that in mouse MECs, TLR2 may not initiate a downstream cascade to produce TNF-α and IL-8.

The innate immune response of mouse MECs was assessed using a LPS-stimulated infection model. In conclusion, it was demonstrated that mouse MECs can respond to the presence of LPS (10 μg/mL) and significantly up-regulate the release of TNF-α and IL-8. TLR4 signalling plays a major role in the release of these cytokines.

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References


