S. Aureus* Lipoteichoic Acid Failed to Activate NLRP3, but Downregulated the Transduction of NLRP3/MAPK and NF-κB Signaling Pathway in Mouse Mastitis Induced by *S. Aureus

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Research

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Abstract

Background It was confirmed that *S. aureus* infection activated the expression of NLRP3. As a major component of *S. aureus* cell wall, what about LTA? Firstly, in this study a mouse model was built following LTA intramammary infection for 0, 12, 24, 48 and 72 h, the characterization of mammary inflammatory response induced by LTA was observed. Subsequently, mouse mammary preconditioned with LTA was modelling. The effect of LTA treatment on the activation of NLRP3/ MAPK and NF- κ B signaling pathways induced by *S. aureus* were detected.

Results Results shown that the LTA intramammary infection induce mild but rapid recovery inflammation in mammary gland whereas failed to stimulate NLRP3. LTA treatment protected mammary gland against *S. aureus* infection by suppressed the activation of NLRP3/ MAPK and NF- κ B signaling pathways. Meanwhile it is noteworthy that the expression of NLRP3/MAPK JNK induced by *S. aureus* was not regulated by LTA treatment.

Conclusions These results suggested LTA induced mammary gland inflammation was mild and self-limiting. LTA treatment revealed a good anti-inflammation effect through downregulating the transduction of NLRP3/MAPK and NF- κ B signaling pathways in mouse mastitis induced by *S. aureus*. Our research highlights the importance of an LTA during the innate immune response of the mammary gland and offer novel insights for new approaches concerning effective immunomodulation against a local bacterial infection.

Background

Mastitis is a serious problem in dairy industry worldwide which has a profound impact on animal health [1, 2]. *S. aureus* is a major representative pathogenic bacterium causing bovine mastitis [3, 4]. A number of studies have performed to explore the host/*S. aureus* interactions in the mammary gland. Jully et al. have demonstrated that *S. aureus* used complex regulatory networks to sense diverse signals that enable it to internalize into host cell and adapt to different environments [5]. As a result, mastitis induced by *S. aureus* is usually characterized by persistent infection. It was proved that *S. aureus* mastitis was extremely difficult to control by treatment alone. Up to 40% of mastitis cases induced by *S. aureus* may be recurrences of previous infections after therapy [6]. With further research, more and more mechanisms were found involved in the process of *S. aureus* survival in host cells and adaptive immune responses such as autophagy, pyroptosis, etc. [7, 8]. Thus, prevention and control of the *S. aureus* mastitis is facing very serious challenges and further study on the underlying mechanisms of *S. aureus* infection will be helpful.

LTA can be found at the cell surface of most gram-positive. For a long time, LTA is thought to act as an adhesion. For example, LTA secreted by *Streptococcus pyogenes* will act as an adapter by binding the M protein to receptors on host tissues. The attachment of bacteria to host tissues allows colonization of those tissues. As the progress in the knowledge and the availability of pathogen-associated molecular

patterns (PAMP) for gram-positive bacteria and in particular *S. aureus* makes it feasible to research the effects of LTA on immune and inflammatory response [9]. *S. aureus* LTA was proved to trigger inflammation in the lactating bovine mammary gland [10]. This makes LTA a potential substitute for *S. aureus* mastitis in scientific research. Until recently, LTA was proposed as an immunostimulatory component of *S. aureus* which was involved in the activation of TLRs and release of inflammatory cytokines [11]. However, in spite of some certain immunostimulatory effects, the biological role of LTA is still unclear and even controversial. For example, it has been shown that, bovine mammary epithelial cells could express TLR2, however NF- κ B is not activated by LTA in these cells [12]. Even more in some cases live bacteria and bacterial soluble factors might induce different transcriptional [13]. So, *S. aureus* has been considered a major pathogenic bacterium of bovine mastitis, it was thus far been difficult to identify the exact role of the individual bacterial cell components in relation to the onset of mastitis.

Nod-like receptors (NLRs) are critical for the host immune response [14]. As intracellular pattern recognition receptors (PRRs), NLRs are capable to form inflammasomes [15]. NLRP3 is one of the most researched NLRs. The NLRP3 inflammasome is assembled by NLRP3, apoptosis-associated speck-like protein containing (ASC) and caspase-1 [16]. Moreover, the NLRP3 inflammasome was proven to be activated by *S. aureus* in monocytes/macrophages [17]. The activation of the NLRP3 inflammasome promotes the maturation of caspase-1, which then processes pro-IL-1 β into IL-1 β [18, 19]. On the other hand, NF- κ B and MAPK are involved in the activation of NLRP3 inflammasome, furthermore activated NF- κ B and MAPK can recruit a series of cytokines and chemokines, including TNF- α and IL-1 α [20]. Although these molecules have the potential to protect against inflammation, however overreaction of pro-inflammatory cytokines will also injure cells and tissues [21]. Given the fact that LTA or *S. aureus* might have different effects on NLRP3/MAPK and NF- κ B signaling pathways. LTA was defined as different functions: infection function or treatment function, the characterization of mammary inflammatory response induced by LTA and the regulation effect of LTA on the NLRP3/MAPK and NF- κ B signaling pathways induced by *S. aureus* were studied.

Methods

Chemicals

LTA from *S. aureus* was purchased from Sigma-Aldrich Inc. (St. Louis, MO, US). *S. aureus* (ATCC29213) was purchased from Micro biologics Inc. (St. cloud, MN, US). Mouse IL-1 α , IL-1 β and TNF- α enzyme-linked immunosorbent assay (ELISA) kit was purchased from Cusabio Biotech (Wuhan, Hubei, China). Trizol was purchased from Invitrogen (Carlsbad, CA, US). The reverse transcriptase synthesis kit was purchased from Takara Biotech (Dalian, Liaoning, China). SYBR Premix Ex TaqTM II was purchased from Sheng gong Biotech (Shanghai, China). Bicinchoninic acid (BCA) protein assay kit was purchased from BioChain Institute Inc (Silicon Valley, CA, US). Polyvinylidene fluoride (PVDF) membrane was purchased from Merck KGaA (Darmstadt Germany). NLRP3 rabbit monoclonal antibody (mAb) was purchased from Abcam (Cambridge, UK). Caspase-1, P65, P-P65, I κ B α , P-I κ B α , P38, P-P38, Jnk, P-Jnk, Erk, P-Erk and β -

actin rabbit mAb and HRP-conjugated goat anti-rabbit antibodies were purchased from Cell Signaling Technology (CST; Danvers, MA, US).

Experimental design

The schematic of experimental design is shown in Fig. 1.

Characterization of inflammation induced by LTA

Eighty SPF BALB/c female mice and ten male mice (60 days old) were purchased from the Center of Experimental Animals of Linyi University and the protocol was approved by the Animal Care and Ethics Committee of the Linyi University. The mice were fed separately in an air-conditioned room maintained at a temperature of 24 ± 1 °C for 2 weeks to adapt to the environment. The diet was prepared consulting the standard of AIN-93 purified diets for laboratory rodent in Animal Nutrition and Feed Science Laboratory of Linyi University. Pregnancy is determined by vaginal plugs. Pregnant mice were randomly divided into two groups: Control group and Experimental group. Control group (CG, n = 40) were infected with 0.1 mL physiological saline from milk ducts; Experimental group (EG; n = 40) were infected with 0.1 mL LTA (5 µg). At each time point (0, 12, 24, 48 and 72 h), twenty mammary glands (CG = 10, EG = 10; R4, L4) were collected.

Characterization of LTA treatment on the activation of NLRP3/NF-κB and NLRP3/MAPK signaling pathway induced by *S. aureus*

Forty SPF pregnant mice were randomly divided into four group: Control group (CG, n = 10) were infected with 0.1 mL physiological saline from milk ducts; Model group (MG, n = 10) was infected with 0.1 mL *S. aureus* (10^5 CFU/mL); LTA control group (LCG, n = 10) was infected with 0.1 mL LTA (5 µg) for 72 h; LTA treatment group (LTG, n = 10) was infected with 0.1 mL LTA (5 µg) for 72 h and then infected with 0.1 mL *S. aureus* (10^5 CFU/mL). 12 h after stimulus, mammary gland (R4, L4) in each group were collected.

Histopathologic examination

The tissues were collected and fixed in 10% formaldehyde solution, and then embedded in paraffin wax. The mammary tissue sections were stained with hematoxylin eosin (H&E) staining and the pathological changes were observed under a microscope (Olympus DX45, Japan).

Immunohistochemistry analysis

The mammary tissues were collected and fixed in 10% form-aldehyde solution, and then embedded in paraffin wax. For the immunohistochemistry staining, the mammary section was routinely processed. Tissue sections were deparaffinised, rehydrated and then antigen retrieval was applied by microwave heat for 10 minutes at medium voltage in a 10 mM citrate buffer, pH 6.0. After cooling at room temperature, the sections were incubated in 3% hydrogen peroxide (H₂O₂) for 30 minutes to block endogenous peroxidase. Nonspecific staining was eliminated by 10 minutes incubation with normal goat serum at the room temperature. Excess normal serum was removed and slides were then incubated with

primary antibody (1:100) at 4°C overnight. After washing the slides, the sections were incubated with biotinylated secondary antibody for 15 minutes and replaced in the streptavidin, horseradish peroxidase (HRP) conjugate for 15 minutes at the room temperature. The colour was developed with DAB in PBS for 5 minutes. Slides were counterstained with Harris haematoxylin, dehydrated and mounted with Entellan. IPP6.0 software was used to analyze the optical density of immunohistochemical photos. Four 400-fold photos were selected from each section for optical density analysis.

Western blot analysis

Total protein was extracted by using a total protein extraction kit and the protein concentrations were determined by BCA protein assay kit. 50 µg (5 µL) proteins were loaded into a 10% sodium dodecyl sulphate (SDS) polyacrylamide gel for electrophoresis and transferred to PVDF membranes. The membranes were blocked in 5% nonfat milk for 1 h to 2 h and incubated overnight with the primary monoclonal antibodies, including Caspase-1, NF-κB, MAPK and β-actin. Then, all of the membranes were subsequently incubated with HRP-conjugated goat anti-rabbit antibodies. The blots were washed with PBS-T and processed with supersignal west pico chemiluminescent substrate.

qRT-PCR analysis

The total RNA of tissue was extracted according to the manufacturer's instructions by using Trizol reagent. The quantity and quality of the extracted RNA was evaluated with a Nanodrop 2000 spectrophotometer (Thermo, USA). The ratio of absorption (OD 260 nm/OD 280 nm) was between 1.8 and 2.2, and the RNA integrity was verified with electrophoresis using a 1% agarose gel. Then, RNA was converted to cDNA using a reverse transcriptase synthesis kit. qRT-PCR primers were designed using "Primer 3 web" (<http://bioinfo.ut.ee/primer3/>) to detect the genes encoding *IL-1α* forward (5'-TCTCAGATTCACAACTGTTCTGTG-3') and reverse (5'-AGAAAATGAGGTCGGTCTCACTA-3'); *IL-1β* forward (5'-ACCTGTGTCTTTCCCGTGG-3') and reverse (5'-TCATCTCGGAGCCTGTAGTG-3'); *TNF-α* forward (5'-TGCTGTCCCTGTATGCCTCT-3') and reverse (5'-GGCAGCCTTGTCCTTG-3'); *β-actin* forward (5'-TGCTGTCCCTGTATGCCTCT-3') and reverse (5'-TTTGATGTCACGCACGATTT-3'). The PCR reaction system contained 10 µL of SYBR Green PCR mix, 0.8 µL of each primer (both 10 µmol/L), and 2 µL of cDNA template in a final volume of 20 µL per reaction. The cycling parameters for real-time PCR were 95 °C for 2 min, 40 cycles of 95 °C for 10 s, 60 °C for 30 s, and 72 °C for 30 s using a CFX connect real-time PCR system (BIO-RAD, US). The real-time quantitative PCR reaction was performed in triplicate for each sample, and the mean value was used to calculate the mRNA expression levels. The fold change (n-fold) for gene expression was calculated using the relative quantification method as previously described [22].

ELISA analysis

Mammary tissue samples were weighed and grinded with RIPA lysis buffer until homogenized. Then the homogenate was centrifuged at 12000 rpm for 15 min at 4 °C, and the supernatant was collected in – 20 °C until measurement. The expression of IL-1α, IL-1β and TNF-α were measured by ELISA and according to the manufacturer.

Statistical analysis

All data is shown as the mean $\pm$ *S.E.M*, and experiments were independently repeated at least 3 times. One-way ANOVA and Dunnett's test were used for statistical analyses. Values of $p < 0.05$ were considered statistically significant.

Results

LTA stimulus induce mild inflammation in mammary gland

Histopathologic examination and white blood cell count were used to detect the pathological changes of mammary gland. The results are shown in Fig. 2. There were no obvious pathological changes in control group (0 h, Fig. 2A); after 12 h challenge, the acinar contour became unclear (Fig. 2B blue rectangular contour mark) and there was a small amount of inflammatory cell infiltration in the non-lactation acinar (Fig. 2B blue arrow mark); after 24 h challenge, the acinar contour was still unclear (Fig. 2C blue rectangular mark) and the inflammatory cells infiltrated into both non-lactation acinar (Fig. 2C blue arrow mark) and lactation acinar (Fig. 2C red arrow mark); after 48 h challenge, the acinar contour became clear (Fig. 2D blue rectangular contour mark) but there was still a small amount of inflammatory cell infiltration in the non-lactation acinar (Fig. 2D blue arrow mark); after 72 h challenge, no obvious pathological changes were observed (Fig. 2E); White blood cell count shown that the WBC were above normal at 12, 24 and 48 h but at 72 h the WBC returns to normal (Fig. 2F).

The expression of IL-1 α , IL-1 β and TNF- α in serum and mammary gland induced by LTA back to normal within 72 h

The qRT-PCR analysis and ELISA assay were used to detect the mRNA and release of IL-1 α , IL-1 β and TNF- α . The results are shown in Fig. 3. Compared with 0 h, the IL-1 α mRNA expression in mammary gland increased significantly from 12 h to 24 h ($p < 0.05$), compared with 12 h and 24 h, the IL-1 α mRNA expression decreased significantly ($p < 0.05$) at 48 h, there were not statistically difference in the IL-1 α mRNA expression between 0 h and 72 h (Fig. 3A); compared with 0 h the mRNA expression of IL-1 β increased significantly from 12 h to 48 h ($p < 0.05$), at 72 h the IL-1 β mRNA expression subside and drop back to a normal level (Fig. 3B); compared with 0 h and 72 h, the TNF- α mRNA expression increased significantly from 12 h to 48 h ($p < 0.05$) (Fig. 3C); compared with 0 h the release of IL-1 α in mammary gland increased significantly from 12 h to 48 h ($p < 0.05$) and at 72 h the release of IL-1 α was back to normal level (Fig. 3D); the release level of IL-1 β and TNF- α in mammary gland were both increased significantly from 12 h to 24 h ($p < 0.05$) and compared with 0 h no significant increase was detected at 72 h (Fig. 3E and F); the release of IL-1 α , IL-1 β and TNF- α in serum were not as much as those in mammary gland (Fig. 3D, E, F and G, H, I), No significant changes in the release level of IL-1 α was detected in serum from 0 h to 72 h (Fig. 3G); compared with 0 h the release of IL-1 β increase significantly at 12 h and 48 h ($p < 0.05$) in serum and at 72 h the release of IL-1 β was back to normal level (Fig. 3H), compared with 0 h the release of TNF- α increase significantly from 12 h to 24 h ($p < 0.05$) in serum and from 48 h to 72 h the release of TNF- α was back to normal level (Fig. 3I).

LTA stimulus activated the expression of Caspase-1 but failed to activate NLRP3 in mammary gland

The expression of NLRP3 and Caspase-1 were detected by using immunohistochemistry and western blot analysis. The results are shown in Fig. 4. Compared with 0 h no statistically significant difference was observed on the expression of NLRP3 from 12 h to 72 h (Fig. 4A, B, C, D, E and F); compared with 0 h the expression of Caspase-1 increased significantly at 12 h and 48 h ($p < 0.05$) and reach peak at 48 h, compared with 48 h the expression of Caspase-1 decreased significantly at 72 h ($p < 0.05$), however compared with 0 h the expression of Caspase-1 was still increased significantly at 72 h ($p < 0.05$) (Fig. 4G and H).

LTA treatment decreased the expression of NLRP3 inflammasome induced by *S. aureus*

Immunohistochemistry and western blot analysis were used to detect the effect of LTA treatment on the expression of NLRP3 inflammasome induced by *S. aureus*. The results are shown in Fig. 5. *S. aureus* infection significantly upregulate the expression of NLRP3 in mammary gland ($p < 0.05$), LTA treatment significantly decreased the expression of NLRP3 induced by *S. aureus* ($p < 0.05$), however compared with CG group and LCG group the expression of NLRP3 in LTG group was still markedly increased ($p < 0.05$) (Fig. 5A, B, C, D and E); compared with CG and LCG group the expression of Caspase-1 in MG and LTG group increased significantly by *S. aureus* infection ($p < 0.05$), LTA treatment decreased the expression of Caspase-1 but its distinction does not proved a statistical significance (Fig. 5F and G).

LTA treatment inhibited the activation of MAPK signaling pathway induced by *S. aureus*

Western blot analysis was used to detect the effect of LTA on the activation of MAPK signaling pathway induced by *S. aureus*. The results are shown in Fig. 6. Compared with CG and LCG group, *S. aureus* infection significantly increased the phosphorylation level of JNK in MG and LTG group ($p < 0.05$) however there was no statistical difference between MG group and LTG group (Fig. 6A and B); compared with CG and LCG group the phosphorylation level of P38 and ERK were significantly increased by *S. aureus* in MG and LTG group ($p < 0.05$), LTA treatment in LTG group significantly decreased the phosphorylation level of P38 and ERK induced by *S. aureus* ($p < 0.05$) (Fig. 6A, C and D).

LTA treatment inhibited the activation of NF- κ B signaling pathway induced by *S. aureus*

Western blot analysis was performed to detect the effect of LTA on the activation of NF- κ B signaling pathway induced by *S. aureus*. The results are shown in Fig. 7. Compared with CG and LCG group the phosphorylation level of P65 and I κ B α were significantly increased by *S. aureus* in MG and LTG group ($p < 0.05$), LTA treatment significantly decreased the phosphorylation level of P65 and I κ B α induced by *S. aureus* ($p < 0.05$) (Fig. 7A, B and C).

LTA treatment suppressed the expression of cytokines IL-1 α , IL-1 β and TNF- α in serum and mammary

The qRT-PCR and ELISA analysis were used to detect the effect of LTA treatment on the mRNA and release of IL-1 α , IL-1 β and TNF- α . The results are shown in Fig. 8. *S. aureus* infection significantly increased the mRNA expression of IL-1 α , IL-1 β and TNF- α in mammary gland ($p < 0.05$) (Fig. 8A, B and C), LTA treatment significantly decreased the mRNA expression of IL-1 α and TNF- α ($p < 0.05$) (Fig. 8A and C) and the mRNA expression of IL-1 β was also decreased by LTA treatment but its distinction does not proved a statistical significance (Fig. 8B); compared with CG and LCG group the release of IL-1 α , IL-1 β and TNF- α increased significantly in MG group ($p < 0.05$) and LTA treatment significantly decreased the release of these three cytokines in mammary gland ($p < 0.05$) (Fig. 8D, E and F); compared with mammary gland there were less amount of cytokines in serum, *S. aureus* significantly increased the release of these three cytokines in serum ($p < 0.05$), however LTA shown no inhibitory effect on the release of these three cytokines in serum (Fig. 8G, H and I).

Discussion

S. aureus induced mastitis have become a serious threat to human health and the stockbreeding [23, 24]. Thus, it is particular important to identify the underlying mechanism of *S. aureus* mastitis. A number of studies have performed to explore the host/pathogen interactions in the mammary gland by using animal model [25, 26]. However, the experimental infections are costly furthermore the interactions between host defense and bacterial virulence factors are complex which make it difficult to analysis. As a result, a surrogate for live bacterial infection would be helpful. Studies have proved that LPS was a suitable substitute for mastitis [27]. However, the *Escherichia coli* and *S. aureus* would elicit differential innate immune responses whether in vivo or in vitro [28, 29]. Recently, LTA has been suggested to contribute to *S. aureus* virulence and LTA induced mastitis has been reported by several studies also [30, 31]. Data indicated that that LTA usually induced a weak inflammatory response in mammary epithelial cells. In this study characterization of LTA induced inflammatory response following intramammary infection was observed. Result shown at the early stage of infection (within 48 h), LTA would induce mild inflammation in mammary gland which highlights the morphologic changes of the acinar contour, the increase of white blood cell in peripheral blood, and the release of inflammatory cytokines mainly in mammary gland. However, as the immune response progressed, all of these indicators were back to normal which indicated that the inflammatory reaction disappeared. These results suggested that to some extent LTA used as a surrogate for *S. aureus* mastitis was suitable, however the inflammatory reaction induced by LTA were mild and rapid recovery. The results were interesting and further research is needed to understand the underling mechanisms. Here we hypothesized two main mechanisms may explain: "Mechanism one": LTA infection enhanced the pathogen recognition of innate immune system; "mechanism two": LTA treatment upregulated the host immune response. However which mechanism is a more sensitive one is not clear. Thus, a follow-up experiment was performed.

Innate immune system plays an important role in defending against infection. Pattern recognition receptors (PRRs) act as the first line in innate immune system [32]. There are two PRRs mainly response for *S. aureus* infection: Toll-like receptors family (TLRs) and Nod-like receptors family (NLRs) [33, 34]. Most TLRs are located at the cell surface, which serve as important links between innate and adaptive immunity primarily by responding to extracellular bacteria including LTA [35]. Unlike TLRs, NLRs were capable to form inflammasomes in response to their specific stimulators, which mainly mediate cytosolic recognition of intracellular stimulate. Research about NLRs are incomplete and whether LTA acts as a specific ligand for NLRs remains to be further studied. Thus, in this study the LTA induced NLRP3 (one of the most characterized members of the NLRs) expression was detected. Result shown that LTA failed to active the expression of NLRP3 in mammary gland which indicated that LTA was not an excellent intracellular stimulator or there were others related mechanism to clear LTA in mammary gland.

MAPK and NF- κ B are two main pathways that associated to inflammatory immune response. Experiments have verified that NF- κ B and MAPK involved in the regulation of *S. aureus* mastitis [36, 37]. Based on the important roles of MAPK and NF- κ B in inflammation, another follow-up experiment was designed to investigate the regulation effect of LTA treatment on the NF- κ B and MAPK signaling pathways during *S. aureus* mammary infection. Results shown that LTA preconditioning decreased the expression of NLRP3 and Caspase-1 induced by *S. aureus* and the protective effects of LTA treatment were achieved mainly through NF- κ B, MAPK P38 and MAPK ERK signaling pathway. However, the inhibition effects of LTA treatment on MAPK JNK pathway was not obvious. During inflammation the release of cytokines such as IL-1 α , IL-1 β and TNF- α were regulated by NLRP3-Caspase-1, NF- κ B and MAPK pathways [38, 39]. Subsequently, the inhibitory effects of LTA treatment on the expression of IL-1 α , IL-1 β and TNF- α induced by *S. aureus* were examined. Result shown LTA played an inhibitory effect of LTA treatment on the mRNA and protein expression of cytokines induced by *S. aureus* in mammary gland however the inhibitory effects for serum cytokines were not obvious. These results revealed that the innate immune response was upregulated by LTA preconditioning prior to *S. aureus* infection.

Conclusion

In conclusion, the experiment proved LTA mouse intramammary stimulation failed to activate the expression of NLRP3 but induce mild and rapidly recovering inflammation, LTA treatment inhibited the *S. aureus* induced mastitis by suppressing the NLRP3/Caspase-1/NF- κ B and MAPK P38/ERK signaling pathways.

Declarations

Ethical approval and consent to participate

The protocol was approved by the Animal Care and Ethics Committee of Linyi University.

Consent for publication

Written informed consent for publication was obtained from all participants.

Availability of date and materials

All data used during the study appear in the submitted article.

Competing interests

The authors declare that they have no conflict of interest.

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Authors' contributions

Chong-Liang Bi contributed to the overall study design and performed the experiment. Hui Li analyzed the data and prepared Figures. Yi-Zhao Shen contributed partly to writing. Mirielle Pauline finally revising the manuscript. All the authors reviewed and finally approved the manuscript.

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Not Applicable.

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Figures

Figure 1

The schematic of experimental design

Figure 2

Pathological changes in mammary gland (HE staining, × 200) and white blood cell count changes in peripheral blood. A: LTA intramammary infection for 0 h; B: LTA intramammary infection for 12 h; C: LTA intramammary infection for 24 h; D: LTA intramammary infection for 48 h; E: LTA intramammary infection for 72 h; F: White blood cell count in different time. Blue rectangular contour mark: acinar contour was not clear; blue arrow mark: inflammatory cell infiltration in the non-lactation acinar; red arrow mark: inflammatory cell infiltration in the lactation acinar. Means with different letters (a, b) are significantly different ($p < 0.05$) from each other.

Figure 3

The cytokines mRNA expression changes in mammary gland and cytokines release changes in mammary gland and serum. A: the mRNA expression changes of IL-1 α in mammary gland from 0 h to 72 h after LTA intramammary infection; B: the mRNA expression changes of IL-1 β in mammary gland from 0 h to 72 h after LTA intramammary infection; C: the mRNA expression changes of TNF- α in mammary gland from 0 h to 72 h after LTA intramammary infection; D: the release of IL-1 α in mammary gland from 0 h to 72 h after LTA intramammary infection; E: the release of IL-1 β in mammary gland from 0 h to 72 h after LTA intramammary infection; F: the release of TNF- α in mammary gland from 0 h to 72 h after LTA intramammary infection; G: the release of IL-1 α in serum from 0 h to 72 h after LTA intramammary infection; H: the release of IL-1 β in serum from 0 h to 72 h after LTA intramammary infection; I: the release of TNF- α in serum from 0 h to 72 h after LTA intramammary infection. Means with different letters (a, b, c) are significantly different ($p < 0.05$) from each other.

Figure 4

The expression of NLRP3 and Caspase-1. A: LTA intramammary infection for 0 h; B: LTA intramammary infection for 12 h; C: LTA intramammary infection for 24 h; D: LTA intramammary infection for 48 h; E: LTA intramammary infection for 72 h; F: NLRP3 expression analysis results with IPP6.0 software; G: the expression of Caspase-1 in different time detected by western blot; H: Caspase-1 was measured relative to that of β -actin. Means with different letters (a, b, c) are significantly different ($p < 0.05$) from each other.

Figure 5

The effect of LTA treatment on the expression of NLRP3 and Caspase-1 induced by *S. aureus*. A: Control group (CG, mammary gland were challenged with 0.1 mL physiological saline for 84 h); B: LTA control group (LCG, mammary gland were challenged with 0.1 mL/5 μ g LTA for 84 h); C: Model group (MG, mammary gland were infected with 0.1 mL/105 cfu *S. aureus* for 84 h); D: LTA treatment group (LTG, mammary gland challenged with LTA for 72 h then infected with *S. aureus* for 12 h); E: NLRP3 expression analysis results with IPP6.0 software; F: the expression of Caspase-1 in different time detected by western blot; G: Caspase-1 was measured relative to that of β -actin. Means with different letters (a, b, c) are significantly different ($p < 0.05$) from each other.

Figure 6

The effect of LTA treatment on the expression of MAPK induced by *S. aureus*. A: western blot analysis; B: the phosphorylation of JNK; C: the phosphorylation of ERK; the phosphorylation of P38. Means with different letters (a, b, c) are significantly different ($p < 0.05$) from each other.

Figure 7

The effect of LTA treatment on the expression of NF- κ B induced by *S. aureus*. A: western blot analysis; B: the phosphorylation of I κ B α ; C: the phosphorylation of P65. Means with different letters (a, b, c) are significantly different ($p < 0.05$) from each other.

Figure 8

The effect of LTA on the expression of cytokines in mammary gland and serum induced by *S. aureus*. A, B and C: the effect of LTA on the mRNA expression of IL-1 α (A), IL-1 β (B) and TNF- α (C) in mammary gland induced by *S. aureus*; D, E and F: the effect of LTA on the release of IL-1 α (D), IL-1 β (E) and TNF- α (F) in mammary gland induced by *S. aureus*; G, H and I: the effect of LTA on the release of IL-1 α (G), IL-1 β (H) and TNF- α (I) in serum induced by *S. aureus*. Means with different letters (a, b, c) are significantly different ($p < 0.05$) from each other.