Immunolocalization of Antibacterial Peptide S100A7 in Goat Mammary Gland and Lipopolysaccharide Induces the Expression and Secretion of S100A7 in Goat Mammary Gland Epithelial Cells via TLR4/NFκB Signal Pathway

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Research Article

Keywords: S100A7, Antimicrobial peptides, Toll-like receptor 4, NF-κB, Mammary epithelial cells, Goat.

DOI: https://doi.org/10.21203/rs.3.rs-722547/v1

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Abstract

**Background:** The antimicrobial peptide (AMP) S100A7, with antimicrobial activities for a broad spectrum of bacteria, have attracted more and more attention for the prevention and treatment of mastitis. However, in goat mastitis, there is little information about the expression and regulation mechanism of S100A7. In present study, the immunolocalization of S100A7 in healthy and mastitis goat udder were compared. In order to further explore the regulatory mechanism of S100A7 expression in mammary epithelial cells (MECs), goat MECs were isolated and treated by 2.5, 5, 10 and 20 μg/mL lipopolysaccharide (LPS) respectively for different time.

**Results:** Both in healthy and mastitis goat teat, S100A7 was mainly expressed in stratified squamous epithelium of teat skin and streak canal. In healthy goat mammary gland, weakly S100A7 immunoreactivity was present in the alveolus. But in the collapsed alveolus of mastitis goat mammary gland, densely S100A7 immunoreactivity could be observed. The goat MECs were treated by 2.5, 5, 10 and 20 μg/mL LPS respectively for different time. For all of these four groups, after treatment for 3 h, increase in S100A7 mRNA expression and protein secretion were detected compared to control (p<0.05). For 10 and 20 μg/mL LPS groups, after treatment for 6 h, the mRNA and secreted protein levels of S100A7 were remarkably up-regulated compared to control(p<0.01). For all of these four groups, the secretion level of S100A7 descended after 48 h treatment. Moreover, after treatment with LPS, the mRNA levels of Toll-like receptor 4(TLR4) and MyD88 were up-regulated, and the phosphorylation of p65 was up-regulated markedly compared to control. However, adding TLR4 inhibitor TAK-242 or/and NF-κB inhibitor QNZ significantly suppressed the phosphorylation of p-65 and then inhibited the expression and secretion of S100A7 induced by LPS treatment.

**Conclusions:** S100A7 was mainly expressed in stratified squamous epithelium of teat skin and streak canal. In mastitis goat mammary gland alveolus, the expression level of S100A7 was up-regulated compared to that in healthy goat. LPS induced the expression and secretion of S100A7 in goat MECs depended on concentration and treatment duration. Moreover, LPS induced the expression and secretion of S100A7 in goat MECs via TLR4/NF-κB signaling pathway.

**Background**

Mastitis is an inflammatory reaction of breast tissue commonly caused by microbial infection [1]. Pathogens pass through the teat canal and then enter the cistern and alveolus, resulting in intramammary infection. The tightly closed teat canal regulated by sphincter muscles and keratin lined with squamous epithelium of teat canal constitute physical barrier to limit pathogens invasion [2, 3]. On other hand, the antimicrobial peptide (AMP), with direct antimicrobial and immunomodulatory activities, are critical component of the animal innate immune system and play important roles in host mammary gland defense system [4, 5]. Recently, many studies have indicated that diverse type AMP, such as lingual AMP, lactoferrin and cathelicidins, secreted by epithelial cells of the cistern and alveolus help to resist infection of pathogens [6, 7, 8, 9, 10].
S100A7 (Psoriasin), which is firstly identified in the epithelial cells of human psoriasis skin [11, 12], is a member of the S100 family of calcium binding proteins [13]. S100A7 is one of the AMPs with a strong antimicrobial activity especially against E. coli in humans [14] and bovine [15]. In bovine, S100A7 could be detected in milk [16, 17] and serum [18]. In bovine udder, the expression of S100A7 is common in teat skin and streak canal. But, the expression of S100A7 was limited to bacteria infected teat cistern rather than epithelial cells of the alveolus [19, 20]. However, in goat, the information about the expression and regulation mechanism of S100A7 is fewer than bovine. It is only reported that S100A7 was expressed in stratified squamous epithelium of the teat and epithelial cells of the alveolus and gland cistern in healthy goat [21]. Moreover, the expression of S100A7 in mastitis goat mammary gland has not been reported. Additionally, whether alveolus epithelial cells can be induced by invading bacteria to produce S100A7 to participate in innate immunity in mammary gland remain poorly understood.

In present study, the expression of S100A7 was compared in healthy and mastitis goat udder. The goat mammary epithelial cells (MECs) were isolated and treated with lipopolysaccharide (LPS). The results indicated that LPS could promote the expression and secretion of S100A7, and this promotion effect is depended on Toll-like receptor 4 (TLR-4) /NF-κB signaling pathway.

**Methods**

**Goat mammary tissue sample**

This study was approved by the Institutional Animal Ethical and welfare Committee, Northwest A&F University, Shaanxi, China (Approval No. 2020039, Date. 2020.03.09). All animal procedures were performed under the control of the Guidelines for Animal Ethical and welfare Committee, Northwest A&F University. Goat mammary tissue samples including teat and mammary gland of health and mastitis goats were collected from lactating Guanzhong dairy goats in Shaanxi, China. The mastitis goats’ udders were swollen and hard, there are floccules, clots or yellow color in the milk, even stop lactation. Both of samples from health and mastitis goats were used to make paraffin sections. Only mammary gland tissues of health goats were used to isolate MECs.

**Goat mammary epithelial cells isolation and culture**

Goat MECs were isolated and cultured using methods previously reported with minor modifications [36]. Briefly, the mammary gland tissues were trimmed of visible fat and connective tissues and washed with PBS several times until the solution became pellucid and devoid of milk. The mammary tissues were then minced into about 1 mm3 cubes and soaked in culture medium for 10 min at room temperature. The tissue pieces were implanted onto the 35-mm Petri dish and were incubated at 37°C with saturated humidity and 5% CO2. After 6 hours, 2 mL culture medium was added to the culture dish. The culture medium consisted of DMEM-F12 (Invitrogen Corporation, Waltham, MA) supplemented with 10 % fetal bovine serum (Invitrogen Corporation, Waltham, MA) and 100 I.U./mL penicillin, 100µg/mL streptomycin (Invitrogen Corporation, Waltham, MA). The medium was replaced with fresh medium every 48 h until the cells migrated out of the tissue. About 10 days later, the cells spread across the bottom of
the dish were passaged by digestion with 0.25 % trypsin/EDTA. The cells were reseeded in a new 35-mm Petri dish at a density of 2×10^5 cells/cm^2 and cultured at 37°C under 5% CO_2. In present experiments, cells passaged within 10 times were used.

**Experimental design**

Goat MECs were seeded in 35-mm Petri dish at a density of 2×10^5 cells/cm^2 and cultured for 2 days until cell confluence reached 80%. Goat MECs were treated by 2.5, 5, 10 and 20 µg/mL LPS respectively for different time in serum-free medium. The control group was treated by equivalent PBS.

**Immunofluorescence**

Cells were fixed for 30 min in 4% paraformaldehyde (Solarbio, Beijing, China), permeabilized for 15 min in 0.2% TritonX-100 (Sigma, St. Louis, MO), incubated for 2 h in blocking buffer (3% BSA in PBS), and treated with primary antibody against CK18 (1:100, Abcam, Cambridge, MA), CK14 (1:100, Abcam, Cambridge, MA) or p-p65(S536)(1:100, Abcam, Cambridge, MA) overnight at 4°C. The secondary antibody used was Goat Anti-Rabbit IgG-Alexa Fluor® 488 (1:100, Abcam, Cambridge, MA). The nuclei were stained with DAPI (Beyotime, Shanghai, China) for 5 min at room temperature. Images were captured using a fluorescence microscope (Olympus, Tokyo, Japan).

**RNA extraction and reverse transcription and polymerase chain reaction**

Total RNA was extracted using RNAiso Plus (Takara Bio Inc, Otsu, Japan) according to the manufacturer's instructions. cDNA was synthesized using PrimeScript™ RT Reagent Kit (Takara Corporation, Dalian). The primer sequences are shown in Table 1. The amplified products were separated and analyzed by electrophoresis on a 1% agarose gel. The quantitative RT-PCR reactions were performed with the Fast SYBR Green Master Mix (Genstar, Beijing, China), data collection and data analysis were performed on the QuantStudio 6 Flex machine (Invitrogen Corporation, Waltham, MA) by using the GraphPad Prism 6 software. The quantitative RT-PCR parameters were as follows: 95°C for 2 min, followed by 40 cycles each of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. Relative gene expression was obtained by normalizing with GAPDH expression, calculating differences in mRNA expression as fold changes relative to expression in Control group.
Table 1
Primer sequences for the RT-PCR and q-PCR analyses

| Gene  | Primer sequences (5' → 3') | Product size, bp | Anneal T, °C | Genbank accession |
|-------|---------------------------|------------------|--------------|-------------------|
| GAPDH | F:TGCCCGTTCGACAGATAGC     | 145              | 60           | XM_005680968.3    |
|       | R: ACGATGTCCTTTGCCAGTA    |                  |              |                   |
| S100A7| F:CCAGCAAGGACAGGAACTCA    | 140              | 60           | XM_005677510.2    |
|       | R: GCAGCTGCTGAAGGAAACT    |                  |              |                   |
| TLR4  | F:TTCAACCGTATCACGGCCTC    | 127              | 60           | NM_001285574.1    |
|       | R:TGACCCACTGCAGGAAACTC    |                  |              |                   |
| MYD88 | F:TTGAGAAGAGGTGCCGTCG     | 187              | 60           | XM_013973392.2    |
|       | R:CAGACAGTGAAGCGCGCAG     |                  |              |                   |

**Western blot analysis**

Cells were lysed with RIPA buffer (Solarbio, Beijing, China) supplemented with 1 mM phenylmethanesulfonyl fluoride (Solarbio, Beijing, China) on ice for 30 min. Western blot was carried out with equal amounts of protein. The primary antibodies were as follows: anti-β-casein (1:1000, CST, Danvers, MA), anti-p65(1:1000, Abcam, Cambridge, MA), anti-p-p65(S536)(1:1000, Abcam, Cambridge, MA). The secondary antibodies used were goat anti-rabbit IgG-HRP(1:2000, Abcam, Cambridge, MA). The protein bands were detected with a chemiluminescence kit (Tanon, Shanghai, China).

**ELISA**

Secretion of S100A7 from goat MECs was measured using ELISA. The cell culture medium were collected after LPS or inhibitors treatment and stored at -20°C. The ELISA was performed using Goat S100A7 ELISA kit (Jianglaibio, Shanghai, China) according to manufacturer’s protocol. The absorbance was measured at 450 nm using the microplate reader (Tecan)

**Immunohistochemistry**

Paraffin sections were deparaffinized and hydrated in graded ethanol series before staining with the streptavidin-peroxidase method. Antigens were retrieved by boiling for 15 min in Citrate Antigen Retrieval solution (Solarbio, China). Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide. Then followed the instructions of the universal SP staining kit, in which the primary antibody anti-S100A7 (1:100, Bioss, Beijing, China) is incubated overnight at 4°C, and the second antibody is incubated at 37°C for 1 hour. After DAB incubation, sections were lightly counterstained with hematoxylin and were dehydrated and cover slipped.

**Statistics**
All quantitative data are presented as mean ± standard deviation (SD) with no less than three replicates for each experimental condition. Comparison between experimental and control groups were performed with Factorial ANOVA with the use of SPSS 10.0. A value of $p < 0.05$ was considered significant difference, a value of $p < 0.01$ was considered extremely significant difference.

**Results**

**Immunolocalization of S100A7 in healthy and mastitis goat udder**

Firstly, pathological examination results of teat and mammary gland in healthy and mastitis goat were compared. Hematoxylin-Eosin staining showed that the keratin and stratum corneum of healthy goat streak canal (Fig. 1. A) were significantly thicker than those of mastitis goat (Fig. 1. B). In addition, compared with the healthy goat streak canal, the epidermis and dermis of streak canal in goats with mastitis were more disordered, and a large number of exfoliated keratinocytes could be seen in the streak canal lumen (Fig. 1. A, B). In healthy goat mammary gland, the lobule and alveolus were well developed and completed (Fig. 1. C), while in mastitis goat, the alveolus collapsed, and the infiltration of blood cells can be seen in collapsed alveolus (Fig. 1. D).

Immunohistochemical analysis showed that S100A7 was mainly expressed in stratified squamous epithelium of teat skin(Fig. 2. A, B), stratified squamous epithelium of streak canal(Fig. 2. A, C) and skin gland duct(Fig. 2. A, D) in healthy goat teat. In mastitis goat teat, S100A7 was mainly expressed in stratified squamous epithelium of teat skin(Fig. 2. F, G), stratified squamous epithelium of streak canal(Fig. 2. F, H) and sebaceous gland (Fig. 2. F, I). In healthy goat mammary gland, weakly S100A7 immunoreactivity was present in the alveolus, but not in the connective tissue(Fig. 3. A, B). However, the densely S100A7 immunoreactivity could be observed in collapsed alveolus of mastitis goat mammary gland(Fig. 3. D, F).

**Characterization of goat MECs**

In order to further analyze the regulatory mechanism of S100A7 expression in mammary epithelial cells, goat MECs were isolated. After about 5 days culture, cells migrated out of the mammary tissue (Fig. 4. A) and then spread across the bottom of the dish after another 5 days later. The cells were dissociated by 0.25 % trypsin/EDTA and reseeded in a new 35-mm Petri dish. The isolated cells possessed typical epithelial cell morphology, including colony forming and cobblestone-like shape (Fig. 4. B). If the isolated cells cultured at a high density, dome-like structure could be observed (Fig. 4. C). Immunofluorescence results showed that the isolated cells expressed cytokeratin typical of differentiated luminal epithelial cells (CK18) (Fig. 4. D, E, F) but not myoepithelial cells (CK14) (Fig. 4. G, H, I). Furthermore, the mRNA and protein of β-casein could be detected by RT-PCR (Fig. 4. J) and western-blotting (Fig. 4. K) respectively in isolated cells. These results indicated that the isolated cells were goat mammary epithelial cells.
LPS induced the mRNA expression of S100A7 in cultured goat MECs

In order to determine whether LPS can induce the expression of S100A7 in goat MECs cultured in vitro, the isolated goat MECs were treated by 2.5, 5, 10 and 20 µg/mL LPS respectively for different time. For 2.5 and 5µg/mL LPS groups, after treatment for 3–6 h, an increase in S100A7 mRNA expression was detected compared to control (p < 0.05). After treatment for 12 h, the mRNA levels of S100A7 were remarkably up-regulated compared to control (p < 0.01). And this up-regulation lasted for treatment for 48 h (Fig. 5. A, B). For 10 and 20µg/mL LPS group, S100A7 mRNA expression were also increased after treatment for 3 h compared to control (p < 0.05). However, after treatment for 6 h in 10 and 20µg/mL LPS groups rather than 12 h in 2.5 and 5µg/mL LPS groups, the mRNA levels of S100A7 were markedly up-regulated compared to control(p < 0.01) (Fig. 5. C, D). In 20µg/mL LPS group, after treatment for 48 h, S100A7 mRNA expression were decreased compared to 6–24 h (p < 0.01) (Fig. 5. D). These results indicated that LPS induced the mRNA expression of S100A7 in goat MECs depended on concentration and treatment duration.

LPS induced the secretion of S100A7 in cultured goat MECs

As a secretion protein, although LPS could induced the expression of S100A7 mRNA, it is necessary to test the secretion of S100A7 into medium in which goat MECs were cultured. For 2.5, 5 and 10 µg/mL LPS groups, after treatment for 3 h, the secreted S100A7 protein in medium increased compared to control (p < 0.05), and after treatment for 6 h, the S100A7 protein level secreted in medium were substantially up-regulated compared to control(p < 0.01) (Fig. 6. A, B, C). However, for 20 µg/mL LPS group, even treatment for 3 h, the secreted S100A7 protein in medium were extremely increased(p < 0.01) (Fig. 6. D). Except for 10 µg/mL LPS group, the peak of secreted S100A7 protein in medium in other groups appeared at treatment for 12 h (Fig. 6. D). For all of these four groups, the secretion level of S100A7 descended after 48 h treatment (Fig. 6). These results indicated that LPS induced the secretion of S100A7 from goat MECs depended on concentration and treatment duration before 12h treatment and the secretion of S100A7 is not sustained.

LPS activated TLR4 and NF-κB signaling pathway in cultured goat MECs

Goat MECs were treated with LPS at a concentration of 10 µg/mL for 3 h, 6 h and 12 h. The mRNA levels of TLR4, MyD88 were tested. As the results showed, the mRNA level of TLR4 was up-regulated after LPS treatment (p < 0.05) (Fig. 7. A). But, the increase of MyD88 mRNA was observed only in LPS treatment for 6 h group (p < 0.01) (Fig. 7. B). These results suggested that LPS could promote the expression of genes involving in TLR4 signal pathway. On the other hand, after LPS treatment for 3 h, 6 h, 12 h, the phosphorylated p65 had been up-regulated markedly at 3 h (p < 0.05), peaked at 6 h (p < 0.01), and also increased at 12 h (p < 0.01) compared to control (Fig. 7. C). Meanwhile, after LPS treatment for 6 h,
immunofluorescence staining showed that p65 was mainly found in cell nucleus rather than cytoplasm (Fig. 7. D), suggesting that phosphorylated p65 enter the nucleus to regulate the transcription of target genes. These results indicated that LPS could activate TLR4 and NF-κB signaling pathway in cultured goat MECs.

*LPS induced the expression and secretion of S100A7 in cultured goat MECs depending on TLR4 and NF-κB signaling pathway*

Since LPS could activate TLR4 and NF-κB signaling pathway in cultured goat MECs, the expression and secretion of S100A7 were detected when TLR4 and NF-κB signaling were inhibited. As the results showed in Fig. 8, LPS treatment could induce the phosphorylation of p-65, improve the expression and secretion of S100A7. However, adding TLR4 inhibitor TAK-242 or/and NF-κB inhibitor QNZ significantly suppressed the phosphorylation of p-65 (Fig. 8. A), and then inhibited the expression and secretion of S100A7 (Fig. 8. B, C) induced by LPS treatment. These results indicated that LPS induced the expression and secretion of S100A7 in cultured goat MECs depending on TLR4 and NF-κB signaling pathway.

**Discussion**

Mastitis causes huge economic losses in the milk industry in worldwide. One of the popular methods for prevention and treatment of mastitis is antibiotics. However, the use of antibiotics reduces the quality of milk and sometimes causes resistant microbes. Therefore, an alternative strategy to prevent and treat mastitis is necessary for the healthy development of dairy industry. The AMP, with antimicrobial activities for a broad spectrum of bacteria, becomes valuable substitutes for antibiotics in prevention and treatment of mastitis [22].

S100A7 is one of the AMPs with a strong antimicrobial activity against E. coli. In present study, the expression sites of S100A7 in healthy and mastitis goat udder were analyzed. In both healthy and mastitis goats, S100A7 was expressed in stratified squamous epithelium of teat skin and streak canal, sebaceous gland cells, skin gland duct. This result supports the findings of previous reports in goat [21] and bovine [8]. The teat skin is consistently exposed to external environment, and streak canal may remain open for short time after milking [23]. Considering that E. coli is one of the most important environmental pathogens [24], the constitutive expression of S100A7 and other AMP, for example S100A8 [25] in teat suggested their critical roles in innate immunity in teat which as a first barrier to limit pathogens invasion.

In healthy goat mammary gland, compared with teat, S100A7 was only slightly expressed in epithelial cell of alveolus. This result is consistent with previous report that the expression level of S100A7 mRNA in alveolus is fewer than other udder regions [21]. In collapsed alveolus of mastitis goat mammary gland, the densely S100A7 immunoreactivity could be observed. As a member of AMP, predictably, the expression of S100A7 in epithelial cell of alveolus took part in local host defenses against pathogens invasion. However, in mastitis collapsed alveolus, S100A7 might not only play anti-pathogens function, but also played an important role in the fibrosis of alveolus and the destruction of blood-milk barrier. The
role of S100A7 in fibrosis and epithelial barrier destruction has been reported in other pathological processes [26, 27], therefore, the relationship between S100A7 and alveolus degeneration needs to be investigated in more detail in further studies.

The expression of S100A7 is a responding to pathogens infection. The teat epithelium is considered as the main source of S100A7 in bovine [20] and goat [28]. But S100A7 concentration in milk was increased after the intramammary infusion of LPS [21], suggesting that MECs can synthesize and secrete S100A7 into the milk. In present study, goat MECs were isolated and treated with LPS. The results indicated that LPS induced the expression and secretion of S100A7 in goat MECs depended on concentration and treatment duration, providing a directed evidence to support the view that MECs is also an important source to synthesize and secrete S100A7. After LPS treatment for 3–6 h, the mRNA and secreted protein of S100A7 were significantly increased. These results suggested that the transcription, translation and secretion of S100A7 were a rapid response to infection in goat mammary gland. This rapid response played an important role in the clearance of early intramammary infection. On the other hand, these results also help to explain the previous reports in which after LPS intramammary infusion, the increased concentration of S100A7 was 48 h earlier than S100A8 [21, 25]. When bacteria invasion or LPS infusion in mammary alveolus, they firstly stimulated mammary epithelial cells to secrete S100A7, then leukocytes infiltrated mammary alveolus and secreted S100A8 into milk. Therefore, the concentration of S100A7 in milk rises earlier than S100A8. However, LPS treatment alone could not induce the continuous secretion of S100A7 in goat MECs. It is reported that sustained secretion of S100A7 is dependent on the downregulation of Caspase-8 [29]. Although LPS was reported to induce the expression and secretion of S100A7 in human breast cancer [30], its mechanism is still unclear. LPS is a cell wall component of Gram-negative bacteria, its main receptor is Toll-like receptor 4(TLR4) [31, 32]. In bovine, TLR4 was expressed in MECs [33] and its expression was up-regulated after stimulation with heat-inactivated E. coli [34]. In goat MECs, LPS treatment could increase the mRNAs level of TLR4 and MYDD88, this result is consistent with previous report [35]. Moreover, LPS treatment promoted the phosphorylation of p-65 and transfer location from cytoplasm into nuclear. If the TLR4 and NF-κB signaling pathways were inhibited by special inhibitors, the phosphorylation of p-65 was suppressed, and then the expression and secretion of S100A7 induced by LPS treatment were also inhibited. These results indicated that LPS induced the expression and secretion of S100A7 in cultured goat MECs depending on TLR4 and NF-κB signaling pathway.

**Conclusion**

In conclusion, antimicrobial peptide S100A7 was mainly expressed in stratified squamous epithelium of teat skin and streak canal. In mastitis goat mammary gland alveolus, the expression level of S100A7 was up-regulated compared to that in healthy goat. LPS induced the expression and secretion of S100A7 in goat MECs depended on concentration and treatment duration. Moreover, LPS induced the expression and secretion of S100A7 in goat MECs via TLR4/NF-κB signaling pathway.
Abbreviations

AMP: antimicrobial peptide
MECs: mammary epithelial cells
LPS: lipopolysaccharide
TLR4: Toll-like receptor 4

Declarations

- Ethics approval and consent to participate

This study was approved by the Institutional Animal Ethical and welfare Committee, Northwest A&F University, Shaanxi, China (Approval No. 2020039, Date. 2020.03.09). All animal procedures were performed under the control of the Guidelines for Animal Ethical and welfare Committee, Northwest A&F University.

- Consent for publication

Not applicable

- Availability of data and materials

The datasets generated and/or analysed during the current study are available in the figshare repository, 10.6084/m9.figshare.15049479.

- Competing interests

The authors declare that they have no competing interests.

- Funding

This research was funded by National Natural Science Foundation of China, grant number 31772818.

- Authors' contributions

Yan Y, Fan M and Miao Y performed the experiment. Yan Y, Zhu K and Wei Q analyzed and interpreted the data. Wei Q, Pan M and Zhao X were major contributor in writing the manuscript. Wei Q and Ma B support to the financial cost throughout the experiment and generate the ideas. All authors read and approved the final manuscript.

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Figures
Figure 1

Representative images of Hematoxylin-Eosin staining in healthy and mastitis goat mammary tissues. A, Healthy goat teat; B, Mastitis goat teat; C, Healthy goat mammary gland; D, Mastitis goat mammary gland. TS=teat skin; SC=streak canal; MA=mammary alveolus; Bar=100μm.
Figure 2

Representative images of S100A7 Immunolocalization in healthy and mastitis goat teat. A-E, Healthy goat teat; B, Healthy goat teat skin; C, Healthy goat streak canal; D, Healthy goat teat gland duct; E, Healthy goat teat negative control; F-J, Mastitis goat teat; G, Mastitis goat teat skin; H, Mastitis goat streak canal; I, Mastitis goat teat sebaceous gland; J, Mastitis goat teat negative control; TS=teat skin; SC=streak canal; GD=gland duct; SG=sebaceous gland; Bar=100μm.
Figure 3

Representative images of S100A7 Immunolocalization in healthy and mastitis goat mammary gland. A-C, Healthy goat mammary gland; B, Healthy goat mammary alveolus; C, Healthy goat mammary gland negative control; D-F, Mastitis goat mammary gland; E, Mastitis goat mammary alveolus; F, Mastitis goat mammary gland negative control; MA=mammary alveolus; Bar=100μm.
Characterization of goat MECs. A, Goat MECs migrated out of the mammary tissue after 5 days culture. B, Morphology of the isolated goat MECs (passage 5); C, Dome-like structure could be observed in cells cultured at a high density. D-F, Representative images of cytokeratin 18 (CK-18) detected by immunofluorescence; G-I, Representative images of cytokeratin 14 (CK-14) detected by immunofluorescence; J, Goat MECs at passage 3, 6, 9 expressed β-casein detected by RT-PCR; K, Goat MECs at passage 3, 6, 9 expressed β-casein detected by western-blotting; GMF= goat mammary fibroblast; Bar=100μm. Full-length blots/gels are presented in Supplementary Figure S1.
Figure 5

The S100A7 mRNA expression level in goat MECs treated with different concentration of LPS. A, 2.5 μg/mL LPS group. B, 5 μg/mL LPS group. C, 10 μg/mL LPS group. D, 20 μg/mL LPS group. *: p < 0.05; **: p < 0.01.
Figure 6

The S100A7 secretion level in goat MECs treated with different concentration of LPS. A, 2.5 μg/mL LPS group. B, 5 μg/mL LPS group. C, 10 μg/mL LPS group. D, 20 μg/mL LPS group. *: p < 0.05; **: p < 0.01.
Figure 7

LPS activated TLR4 and NF-κB signaling pathway in cultured goat MECs. A, The TLR4 mRNA expression level in goat MECs treated with 10 μg/mL LPS. B, The MYD88 mRNA expression level in goat MECs treated with 10 μg/mL LPS. C, The phosphorylation of p65 in goat MECs treated with 10 μg/mL LPS. D, The immunofluorescence results showed that phosphorylated p65 was translocated from cytoplasm into
nucleus after treatment with 10 μg/mL LPS for 6 h. *: p < 0.05; **: p < 0.01. Full-length blots are presented in Supplementary Figure S1.

Figure 8

LPS induced the expression and secretion of S100A7 depending on TLR4 and NF-κB signaling pathways. A, The phosphorylation of p65 in goat MECs treated with 10 μg/mL LPS alone, or 10 μg/mL LPS in combination with TLR4 inhibitor TAK-242 or/and NF-κB inhibitor QNZ for 6 h. B, The S100A7 mRNA expression level in goat MECs treated with 10 μg/mL LPS alone, or 10 μg/mL LPS in combination with TLR4 inhibitor TAK-242 or/and NF-κB inhibitor QNZ for 6 h. C, The S100A7 secretion level in goat MECs treated with 10 μg/mL LPS alone, or 10 μg/mL LPS in combination with TLR4 inhibitor TAK-242 or/and NF-κB inhibitor QNZ for 6 h. *: p < 0.05; **: p < 0.01. Full-length blots are presented in Supplementary Figure S1.

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