

# Development of a Monoclonal Antibody Against Bovine $\alpha$ -Casein to Evaluate Functional Status of Mammary Epithelial Cells During Mastitis

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## Abstract

Mastitis is a widespread disease in dairy animals and causes huge economic losses around the globe. Mastitis affects the health of animals and also reduces the protein content in the milk. In lactating animals, the surface of mammary gland is coated and lined with mammary epithelial cells (MECs). The functional status of MECs can be evaluated by determining the expression of caseins. The total content of caseins can largely reflect the quality of milk. In the previous literature, there is no general information about the milk quality and expressions of casein in mastitis. The current study was designed to determine if there is any correlations between mastitis and  $\alpha$ -casein expression in MECs. We prepared a hybridoma cell line that produces antibody against bovine  $\alpha$ -casein to evaluate the casein expression status *in vitro* on the MECs during lipopolysaccharide (LPS)-induced mastitis. The results showed that the expression of  $\alpha$ -casein has not changed significantly under the stimulation of LPS. Our study established a useful tool to determine the expression profile of  $\alpha$ -casein in bovine MECs. Furthermore, the result indicated that the expression level of  $\alpha$ -casein remained stable during mastitis.

**Keywords:**  *$\alpha$ -casein, Monoclonal antibody, Mammary epithelial cells, Mastitis*

## Mastitis Süresince Meme Epitel Hücrelerinin Fonksiyonel Durumunu Değerlendirmek Amacıyla Sığır $\alpha$ -Kazein Proteinine Karşı Monoklonal Antikor Geliştirilmesi

## Öz

Mastitis sütçü hayvanlarda yaygın bir hastalıktır ve dünya çapında büyük ekonomik kayıplara neden olmaktadır. Mastitis hayvanların sağlığını etkilemekte ve süttteki protein miktarını düşürmektedir. Laktasyondaki hayvanlarda, meme bezlerinin yüzeyleri meme epitel hücreleri ile döşelidir. Meme epitelyum hücrelerinin fonksiyonel durumu kazein ekspresyonu belirlenerek değerlendirilebilir. Total kazein miktarı büyük ölçüde sütün kalitesini yansıtmaktadır. Literatürde süt kalitesi ve mastitiste kazein ekspresyonu hakkında bir bilgi bulunmamaktadır. Bu çalışma, mastitis ile meme epitelyum hücrelerinde  $\alpha$ -kazein ekspresyonu arasında bir ilişki bulunup bulunmadığını belirlemek amacıyla yapılmıştır. Lipopolisakkarit (LPS) ile oluşturulmuş mastitis süresince meme epitelyum hücrelerinde *in vitro* kazein ekspresyonunu değerlendirebilmek amacıyla sığır  $\alpha$ -kazeinine karşı antikor üreten hibridoma hücre hattı hazırlanmıştır. Elde edilen sonuçlar,  $\alpha$ -kazein ekspresyonunun LPS stimülasyonu altında anlamlı derecede değişmediğini göstermiştir. Bu çalışma sığır meme epitel hücrelerinde  $\alpha$ -kazein ekspresyonunu belirlemek amacıyla yararlı olmuştur. Aynı zamanda, sonuçlar mastitis süresince  $\alpha$ -kazein ekspresyon seviyesinin stabil olduğunu göstermiştir.

**Anahtar sözcükler:**  *$\alpha$ -kazein, Monoklonal antikor, Meme epitel hücreleri, Mastitis*

## INTRODUCTION

Casein is one of the most important components of bovine milk and constitute for more than 80% of total milk protein<sup>[1]</sup>. It is mainly composed of four components:  $\alpha$ -casein ( $\alpha$ 1-

and  $\alpha$ 2-),  $\beta$ -casein, and  $\kappa$ -casein. These four components respectively account for 38%, 10%, 35%, and 13% of the total caseins in the milk<sup>[2]</sup>. The  $\alpha$ -casein is consisted of ( $\alpha$ 1- and  $\alpha$ 2)-parts that are the most important part of caseins. The contents of caseins can largely reflect the quality



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of milk. Several methods have been reported in order to detect certain types of casein. An immunomagnetic bead-based enzyme-linked immunosorbent assay with monoclonal antibodies (mAbs) against  $\beta$ -casein has been established to determine the content of  $\beta$ -casein in bovine milk [3,4]. In addition, mass spectrometry method has been used to determine the content of  $\alpha$ -casein in milk by using an mAb against  $\alpha$ 1-casein [5]. The level of caseins in the milk is not always the same under different feeding conditions, environmental stress and lactation cycles.

Mammary epithelial cells (MECs) are the functional unit for lactation in animals and produce caseins [6,7]. The amount of casein secretion is closely related to the functional state of the MECs; therefore, casein content is a useful indicator of the functional state of MECs [6]. However, there are no previous reports which analyzed the change of caseins during mastitis. Therefore, it is reasonable to study the level of casein expression in order to determine the functional status of MECs, especially during mastitis.

The amount of  $\alpha$ -casein is the highest among the four caseins secreted by MECs [8]. Some studies have shown that the hydrolysis-derived peptide Met-Lys-Pro of  $\alpha$ -casein has strong angiotensin-converting enzyme-inhibitory activity and can significantly reduce the systolic blood pressure of hypertensive rats [9]. Anastasia et al. [10] found that  $\alpha$ -casein of different species can inhibit the proliferation of human breast cancer cell line T47D. Therefore, the preparation of mAbs against  $\alpha$ -casein can not only measure its expression level in MECs but also evaluate the functional status of MECs. In this study, mice were immunized with  $\alpha$ -casein to prepare mAbs against bovine  $\alpha$ -casein, and the expression of  $\alpha$ -casein in MECs was measured under different conditions.

## MATERIAL and METHODS

### Cells and Antigen

The SP2/0 cells were preserved at the laboratory and cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, Massachusetts, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (ExCell Bio, Shanghai, China) and 100 units/mL penicillin-streptomycin. Bovine mammary epithelial cells (BMECs) were isolated from fresh raw milk and cultured in Dulbecco's Modified Eagle's Medium/F-12 (DMEM/F-12) (Thermo Fisher Scientific) supplemented with 10% FBS (ExCell Bio), 100 units mL<sup>-1</sup> penicillin-streptomycin, 10  $\mu$ g mL<sup>-1</sup> bovine insulin (Sigma Aldrich, St. Louis, Missouri, USA), 10 ng mL<sup>-1</sup> of human epidermal growth factor (hEGF Sigma Aldrich), and 5  $\mu$ g mL<sup>-1</sup> of hydrocortisone. Bovine alpha-casein was also utilized in this study (Sigma Aldrich).

### Ethical Approval

The animal-related protocols in this study were reviewed and

approved by the Research Ethics Committee of Northwest A&F University according to the guidelines of the Ministry of Health in China for the care and use of laboratory animals.

### Animals

Female 6- to 7-week-old BALB/c mice were purchased from the Laboratory Animal Center of the Fourth Military Medical University.

### Animal Immunization

Six-week-old mice were immunized subcutaneously with 100  $\mu$ g  $\alpha$ -casein (Sigma Aldrich) emulsified in complete Freund's adjuvant (Sigma Aldrich) for the first immunization. Three weeks later, the second immunization was injected subcutaneously and was comprised of 100  $\mu$ g  $\alpha$ -casein (Sigma Aldrich) emulsified in incomplete Freund's adjuvant (Sigma Aldrich). Three weeks later, the third immunization was conducted according to the dose and method of the second immunization. Two weeks following this, the fourth immunization was conducted intraperitoneally with 100  $\mu$ g  $\alpha$ -casein (Sigma Aldrich). After one week, the fifth immunization was conducted intraperitoneally with 100  $\mu$ g  $\alpha$ -casein (Sigma Aldrich). To test the antibody titers, an indirect-enzyme-linked immunosorbent assay (ELISA) was conducted by using the peripheral blood of the immunized mice. Mice with the highest antibody titers were selected as the donors of splenocytes.

### Hybridoma Generation

Splenocytes of the mice with the highest antibody titers were collected and fused with SP2/0 cells in the presence of PEG 1500 (Roche, Basel, Schweizer). Hybridoma cells were cultured in RPMI 1640 medium (Thermo Fisher Scientific) with hypoxanthine-aminopterin-thymidine (HAT) selection medium (Sigma Aldrich), supplied with 10% FBS (ExCell Bio) and 100 units mL<sup>-1</sup> of penicillin and streptomycin. Culture supernatants were screened by ELISA. The specific mAbs were selected using western blot and immunofluorescence techniques.

### Indirect Enzyme-Linked Immunosorbent Assay

Polystyrene microtiter 96-well plates (Corning Costar, Corning, New York, USA) were coated with 1  $\mu$ g/well of bovine  $\alpha$ -casein (at the concentration of 1 mg mL<sup>-1</sup> in 0.1 M sodium carbonate buffer with a pH of 9.6), fresh raw cow milk, or fresh raw goat milk overnight (14 h at 4°C). After saturating the residual sites with 2% cold fish gelatin in PBS at 37°C for 1 h, the plates were washed three times with PBS containing 0.05% Tween-20 (PBST). The cell culture supernatant of the mAbs was incubated with the plates at 37°C for 1 h and then washed with PBST. Furthermore, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG secondary antibody (Bioss, Beijing, China) was diluted to 1:5000 in PBST and incubated at 37°C for 1 h. The substrate (0.3% H<sub>2</sub>O<sub>2</sub>, 0.1% TMB in 0.1M citric acid, and

pH=5) (TIANGEN, Beijing, China) was incubated for 18 min at 37°C, and the reaction was stopped with 0.2 M H<sub>2</sub>SO<sub>4</sub> [3]. The absorbance at 450nm was determined (BIO-RAD, Model 680).

### Isotype Identification of mAbs

Two clones (1-C3 and 2-F1) of strong response were further cloned and propagated. They were given additional isotype identification using the Mouse Monoclonal Antibody Isotyping Kit (Roche) according to the manufacturer's instructions.

### Western Blot Analysis

Samples of fresh raw cow milk (50 µg), goat milk (50 µg), and bovine α-casein (2 µg) were boiled in sodium dodecyl sulfate (SDS) (Sigma Aldrich) sample buffer supplied with β-mercaptoethanol for 15 min, respectively. The proteins were electrophoresed on 6%-12% polyacrylamide gels and were transferred onto a polyvinylidene difluoride (PVDF) membrane under 85 V for 50 min. After blocking with 3.5% fish gelatin in PBST at room temperature for 2 h, the PVDF membrane was incubated with the supernatant of anti-bovine α-casein (1-C3 and 2-F1 mAbs) diluted by 20 times in 1% blocking buffer at 4°C overnight. Bound antibodies were recognized with an HRP-conjugated goat anti-mouse IgG secondary antibody (Sigma Aldrich) at a 1:6000 dilution in blocking buffer. The signal was visualized with enhanced chemiluminescence reagent (ECL) and ChemiDoc™MP Imaging System (BIO-RAD).

### BMECs Isolated From Cow Milk

Bovine milk was analyzed with a mastitis diagnosis kit developed by our laboratory. Milk from healthy teat was sterilely collected and diluted 1:1 with phosphate buffered saline (with 1000 units mL<sup>-1</sup> of penicillin-streptomycin) and centrifuged at 1500 rpm for 20 min at room temperature. Layer of fat and whey in the milk were discarded and the cell pellet was washed twice with PBS by centrifugation at 1000 rpm for 10 min. Subsequently, the pellets were resuspended in proliferation medium consisting of DMEM/F12 (Thermo Fisher Scientific) supplemented with 10% FBS, 10 µg mL<sup>-1</sup> bovine insulin (Sigma Aldrich), 10 ng mL<sup>-1</sup> human epidermal growth factor (hEGF) (Sigma Aldrich) [11], 5 µg mL<sup>-1</sup> of hydrocortisone, and 100 units mL<sup>-1</sup> of penicillin-streptomycin. Cells were cultured at 37°C in 5% CO<sub>2</sub> and the culture medium was refreshed after every 24 h. Cells were passaged at a ratio of 1:2 until they had a 70%-90% confluence.

### α-Casein Secretion by BMECs After Stimulation of LPS In Vitro

Bovine mammary epithelial cells were seeded into 6-well plates at 3 generations. The cells were preincubated with medium containing 0.5% FBS for 6 h prior to lipopolysaccharide (LPS) treatment (Sigma Aldrich) at 10 ng µL<sup>-1</sup>

(n=5) or 100 ng µL<sup>-1</sup> (n=5) for 3 h after they grew up to 70%. The control group was treated with medium containing 0.5% FBS and simultaneously refreshed with new medium (n=5). Goat MECs were also used as a control (n=5). All cells were cultured for an additional 12 h. Immunofluorescence assays were carried out to analyze the secretion of bovine α-casein by BMECs with the 1-C3 mAb. All the experimental and control groups were repeated three times.

### Immunofluorescence Analysis of Bovine α-casein Secretion by BMECs In Vitro

Immunofluorescence assays were performed to analyze the secretion of bovine α-casein from BMECs with the 1-C3 mAb. BMECs were passaged to 3 generations and cultured for an additional 12 h. After that they were harvested for immunofluorescence analysis. Cells were fixed with 4% polyoxymethylene at room temperature for 30 min and briefly washed with filtered PBS for three times. After the cells were infused with 0.5% triton-X 100 for 15 min, the cells were blocked with 3.5% fish gelatin (Sigma Aldrich) at room temperature for 30 min. The primary antibody of mAb 1-C3 (diluted in 1% gelatin) was reacted with BMECs overnight at 4°C and the bound antibody was detected with FITC-conjugated goat anti-mouse IgG (San Ying, Wuhan, China). Finally, 5 µg mL<sup>-1</sup> DAPI (Sigma Aldrich) was added for 5 min. Images were observed under a microscope (Leica, Model DMI3000B, Germany) and analyzed by an image pro 6.0 software [12].

## RESULTS

In order to obtain mAbs that specifically react against bovine α-casein, we screened out the splenocytes and SP2/0 myeloma cell hybridoma supernatant by indirect ELISA. Plates were coated with bovine α-casein, raw cow milk, and raw goat milk separately. Four positive hybridoma clones were obtained which were named as 1-C3, 2-H1, 3-H4, and 4-G6 (Fig. 1). The results showed that all of these antibodies could react with cow milk and bovine α-casein. In addition, 2-F1, 1-H4, and 3-G4 reacted with goat milk, while 1-C3 showed no reactivity (Fig. 1).

Subsequently, we selected 1-C3 and 2-F1 for further identification via western blot analysis. We tested the subtype of 1-C3 and 2-F1 in order to obtain a broad-spectrum IgG subtype antibody. The results showed that both 1-C3 and 2-F1 mAbs were IgG1 subtype and had κ-type light chains (Fig. 2A).

To examine the availability of the mAbs, we performed a western blot analysis using bovine α-casein, cow milk, and goat milk. The results showed that the 2-F1 mAb reacted with α-casein from both goat milk and cow milk (Fig 2B). In comparison, 1-C3 did not exhibit reactivity to goat milk, cow milk, or purified bovine α-casein. These results may be because 2-F1 mAb recognized a linear epitope which existed in both bovine and goat caseins, even though

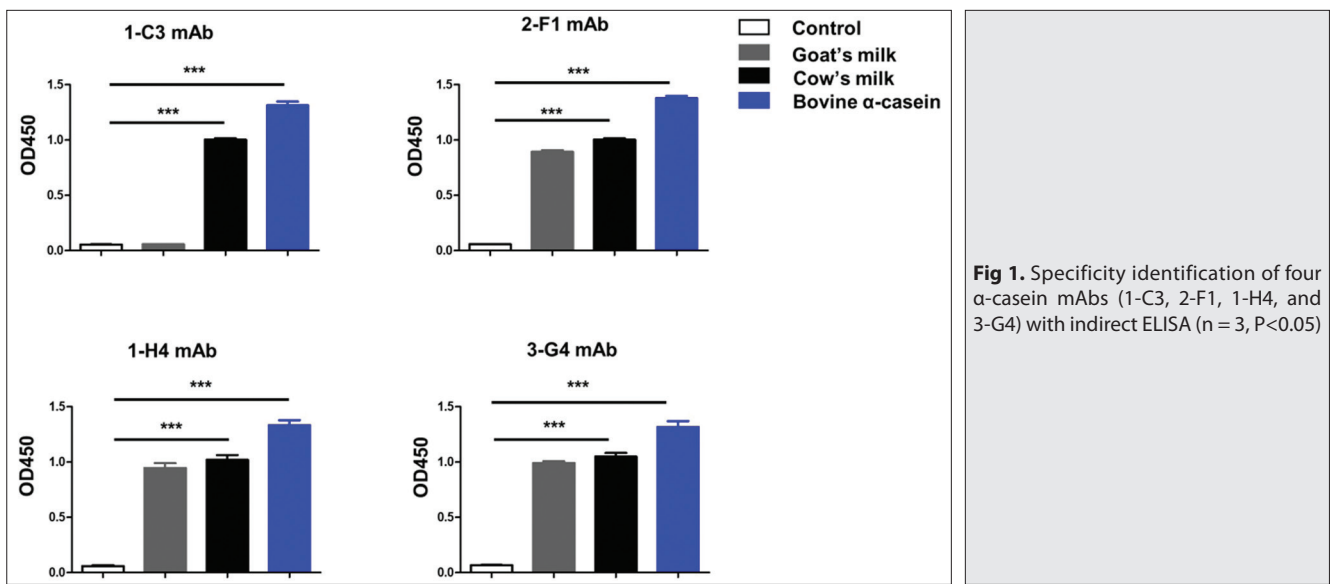
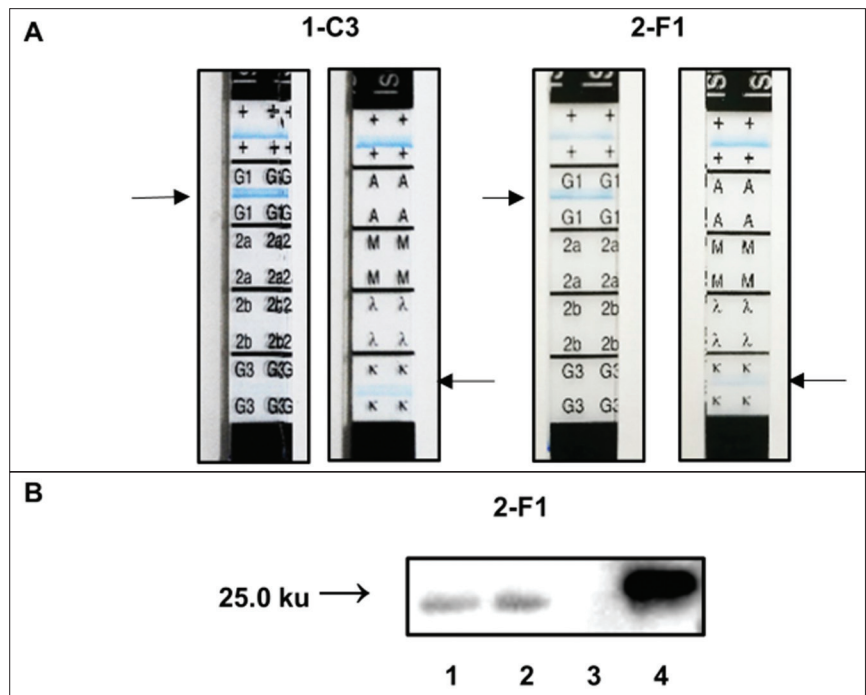


Fig 1. Specificity identification of four α-casein mAbs (1-C3, 2-F1, 1-H4, and 3-G4) with indirect ELISA (n = 3, P<0.05)

**Fig 2.** Subtype identification of 1-C3 and 2-F1. (A) The band on the top of the strip was the positive control. Subtype of anti-bovine α-casein mAb was IgG1 (the left strip), the light-chain was κ-type (the right strip). The blue bands on the top of each band indicate a positive result. (B) Western blot analysis of 2-F1 supernatants against α-casein was carried out. In lane 1, the loaded sample was 50 μg of goat milk; in lane 2, the loaded sample was 50 μg of cow milk; in lane 3, the loaded sample was buffer only; and in lane 4, the loaded sample was 2 μg of bovine α-casein. The molecular weight of α-casein is about 25 kDa



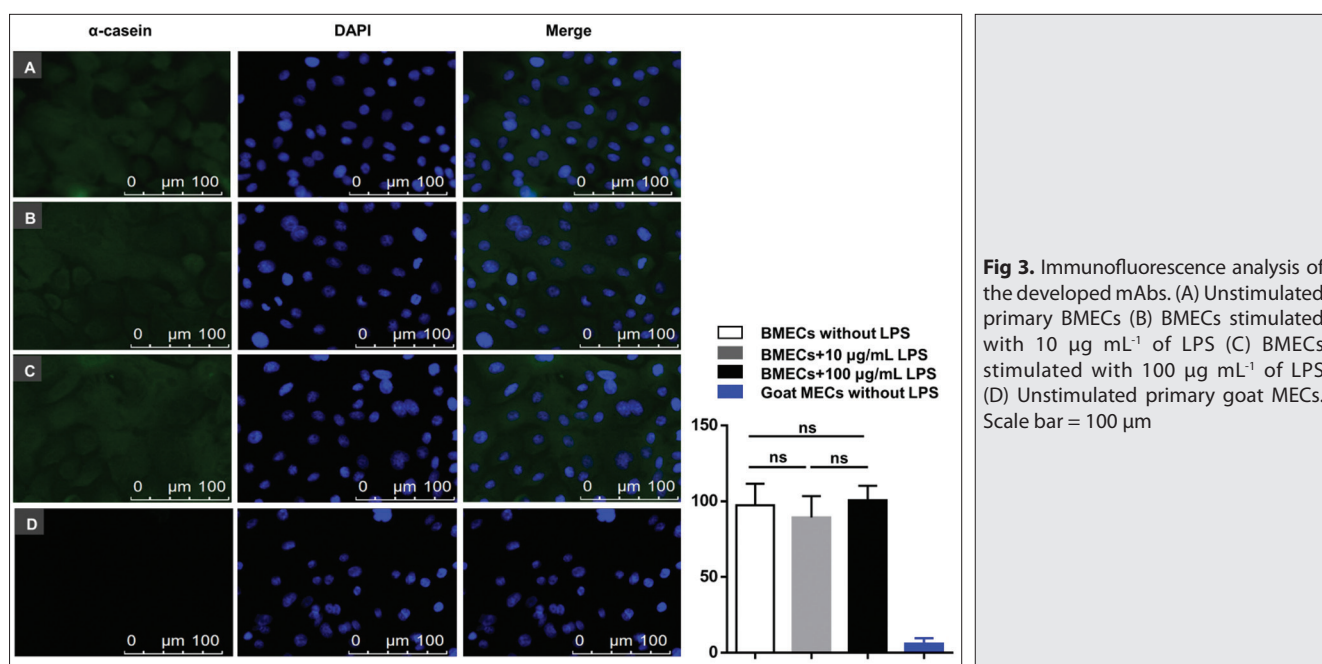
these epitopes were denatured. However, the lack of the reactivity with the mAb1C3 in western blot analysis suggested that the mAb was most likely directed against a conformational epitope in bovine α casein.

In this study, we used a described *in vitro* method to establish a mastitis model of MECs *in vitro* via stimulation with 10 ng μL<sup>-1</sup> or 100 ng μL<sup>-1</sup> of LPS [13,14]. Both the experimental and control group were analyzed by an immunofluorescence assay after culture of an additional 12 h. The results showed that α-casein synthesized and secreted by BMECs could be detected by the 1-C3 mAb at a steady level (Fig. 3A). In addition, LPS (10 ng/μL or 100 ng/μL) stimulation did not affect the expression of α-casein (Fig. 3B,C). Compared with the bovine MECs,

the goat MECs could not be detected by any bovine α-casein antibodies (Fig. 3D). Our results are consistent with previous studies and showed that the expression of caseins were not significantly changed or even decreased when an animal is in mastitis [15,16].

## DISCUSSION

Mastitis is a worldwide problem in dairy animals that can result in huge economic losses around the globe. During mastitis, MECs play a critical role in initiating the inflammatory responses in the mammary gland and sequentially the function of MECs may also be affected by the immune response [17,18]. However, there have not been any relevant studies reporting the expressions of



**Fig 3.** Immunofluorescence analysis of the developed mAbs. (A) Unstimulated primary BMECs (B) BMECs stimulated with  $10 \mu\text{g mL}^{-1}$  of LPS (C) BMECs stimulated with  $100 \mu\text{g mL}^{-1}$  of LPS (D) Unstimulated primary goat MECs. Scale bar =  $100 \mu\text{m}$

caseins by MECs during mastitis. In this study, we aimed at determining the expression of caseins for the evaluation of MEC function. Using the antibody specifically directed against bovine  $\alpha$ -casein, we found that the level of  $\alpha$ -casein did not change significantly in the *in vitro* mastitis model. Our results may provide an efficient tool for the determination of  $\alpha$ -casein expression.

Caseins are the most important nutritional components in bovine milk. Generally, there are four types of bovine caseins and classified as  $\alpha$ s1-casein,  $\alpha$ s2-casein,  $\beta$ -casein and  $\kappa$ -caseins [2]. Among these four types of caseins,  $\alpha$ -caseins (including  $\alpha$ s1-casein and  $\alpha$ s2-casein) are the most abundant form of casein in bovine milk [1]. Importantly,  $\alpha$ -caseins have been reported to be involved in a variety of physical or pathological conditions, with  $\alpha$ s1- and  $\alpha$ s2-subtypes playing redundant but usually distinct roles [19,20]. However, in the present study, although we developed mAbs directed against bovine  $\alpha$ -caseins, the antibody cannot distinguish  $\alpha$ s1- from  $\alpha$ s2-caseins, because we cannot separate these two proteins during the development of the mAbs. Thus, the mAbs developed by this study need further optimization in the future.

As an important part of the mammary gland, MECs not only have important lactation function, but also cooperate with immune cells to defend against microbial infection. In most of the studies, MECs serve as a key element in the study of the physiological functions of mammary gland. However, it is difficult to obtain primary MECs. Alternatively, immortalized mammary epithelial cell lines have been generally used in many studies [21-23]. Accumulating evidence shows that the level of casein transcriptome changes greatly during the *in vitro* culture of MECs, while primary MECs with high casein synthesis and secretion ability yield results closer to the real condition of mammary gland

tissues [6,7]. Thus, the primary MECs used in the present study are more appropriate in studying casein expression profile to reflect a real *in vivo* condition.

At present, there have been several technical methods developed to access expression profiles of casein. Reverse transcription polymerase chain reaction (RT-PCR) analysis has been used by a few studies to determine the mRNA expression level of casein in MECs. However, drawbacks of this method are that mRNA expression level may not always truly reflect the level of the protein, and this method requires high-quality templates, and the process is complicated and time-consuming [24]. In addition, liquid chromatography tandem-mass spectrometry (LC-MS) has been used to determine the casein contents at different times after *E. coli* infection [25]. However, this is also time-consuming and labor-intensive. In the present study, a regular immunofluorescence assay using mAbs against bovine  $\alpha$ -casein were used to determine casein levels in MECs. Compared with the above mentioned methods, our method is easier to conduct and the yield results are more accurate.

Plenty of evidence exists that confirms the ability of MECs to synthesize and secrete casein changes under different conditions [15,26], and it is very important to measure these changes for the evaluation of cellular function. The total casein content showed a decreasing trend in an *in vitro* mastitis model. However, the changes of different caseins are different. Using an ELISA method, it was found that  $\beta$ -casein decreased significantly during mastitis, while the content of  $\alpha$ -casein did not change significantly [15], which was in compliance with the results of several other studies [25,27]. In this study, we found that the content of  $\alpha$ -casein of primary MECs did not change significantly under the stimulation of various concentrations of LPS, indicating that

the expression level of  $\alpha$ -casein does not alter significantly during mastitis. Thus,  $\alpha$ -casein alone is not suitable for the evaluation of the function and state of MECs. However, as discussed above, the mAbs developed in the present study cannot distinguish  $\alpha$ s1- from  $\alpha$ s2-casein. It is possible that if the expression levels of these two casein subtypes show an opposite trend, the stable expression profile of  $\alpha$ -casein during mastitis will also be generated. Therefore, further investigations into developing mAbs with better specificity to  $\alpha$ s1- and  $\alpha$ s2-caseins are needed. In addition, a more appropriate biomarker for MEC function evaluation is also needed in the future.

## CONFLICT OF INTEREST

The authors declare that no conflict of interest exists.

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