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Dynamics of CD4+ Lymphocytes in Mouse Mammary Gland Challenged with *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is one of the major pathogen which can cause mastitis in dairy animal. CD4+ lymphocytes play a significant role in clearing this type of bacteria. To our knowledge, CD4+ lymphocytes change in mouse model of *S. aureus* mastitis is poorly understood. To investigate the response of CD4+ lymphocytes in mammary gland, the mice mastitis was induced with *S. aureus* in this study. New protocol was used to separate lymphocytes in mammary gland. The results showed that CD4+ lymphocytes increased significantly comparing with blank and saline mice ($p = 0.0019$, $p < 0.01$) at 72 h post Intramammary Inoculation (IMI). At the same time, the infection mice mammary gland tissues were swelled and were suffering from hyperemia. From histological observation, massive inflammatory cells are infiltrated in the tissue. The results suggest that CD4+ lymphocyte is one of important cells in against *S. aureus* infection in mammary gland.

Key words: C57BL/6J mouse, mastitis, *S. aureus*, CD4+ lymphatic cells, mammary glands

INTRODUCTION

Mastitis is one of the major diseases in dairy animals which can cause the decrease of the milk in the yield and quality (Leitner *et al.*, 2002). Over a long time antibiotic is the main treatment and prevention for this disease and has worked effectively in the past years. However, pathogenic bacteria are becoming progressively less susceptible to the available therapy (Myllys *et al.*, 1994), milk quality and food safety were affected by antibiotic residues (Bushnell, 1984; Shitandi and Kihumbu, 2004; Shitandi and Sternesjo, 2004).

Staphylococcus aureus is one of the major pathogen which can cause mastitis in dairy animals. Most importantly, it also resist against multiple antimicrobials which makes people always crucial concern (Osteras *et al.*, 1999; Barkema *et al.*, 2006; Haltia *et al.*, 2006; Seyffert *et al.*, 2012). To avoid these situations, one of effective way is came up that is using immunotherapy to prevent and treat bacterial mastitis (Kehrli and Harp, 2001; Salman *et al.*, 2009; Bharathan and Mullarky, 2011). CD4 T lymphocytes as a vital immunocytes play an important role in adapting immune response, especially in bacteria and virus cleaning (Shafer-Weaver *et al.*, 1999). On the one hand, when host was infected by *S. aureus*, CD4 T cells will control local CXC chemokine production, neutrophil recruitment to the site of infection and clean the bacteria (McLoughlin *et al.*, 2006). On the other hand, these types of cells' families, such as Th 1 and Th 17 can produce pro-inflammatory cytokines that enhanced phagocytic killing of both organisms (Lin *et al.*, 2009). In the recent research shows that CD4 T lymphocytes not only can enhance adapt immunity but also are

required for promoting IFN- γ mediated immunoglobulin isotype switching in B lymphocytes to produce high affinity IgG antibodies and for IFN-gamma mediated phagocyte activation for an effective resolution of bacterial infection (Lawrence *et al.*, 2012). In dairy cow research, CD4+ lymphocytes protein was tested and they suggested that CD4+ could protect mammary tissue in the early stage in *S. aureus*-induced mastitis (Rivas *et al.*, 2000). During mastitis inflammatory reaction, T cells can be recruited in milk and CD4+ cells become the predominant phenotype after infection by bacteria (Riollet *et al.*, 2000). It is obvious that both CD4+ lymphocytes and their subsets play important roles during bacteria infection in mastitis, however, the change of CD4+ lymphocytes in mammary gland during the pathological period of mastitis have rarely been reported .

The aim of this study was to investigate the change of CD4+ lymphocytes in mammary gland after *S. aureus* infection in mastitis of C57BL/6 mouse model. The result of this study will lay foundations for the research of cellular immune response in mastitis of dairy animal and human breast cancer.

MATERIALS AND METHODS

Bacteria: *S. aureus* strains were isolated from the milk of the clinical mastitis of Guanzhong dairy goats in Guanzhong area of Shaanxi province in May 2009, Northwest of The People's Republic of China (PRC). The bacterial inoculum was grown overnight in nutrient broth at 37°C. The bacteria were washed twice in Pyrogen Free Saline (PFS) and diluted in PFS. The number of Colony-Forming Units (CFU) was determined after serial dilution and plate counting.

Mice: Ten pregnant C57BL/6J mice (6-8 weeks old) were purchased from Laboratory Animal Center of the Fourth Military Medical University (Xi'an, Shaanxi, China). Animals were housed in the animal facilities of the Department Veterinary Medicine Northwest A and F University (Yangling, Shaanxi, China). All mice were fed with special food, free water and they were given enough natural light. The room temperature was kept in 18~22°C during the experiments. The animal experimental protocols are in accordance with the animal care and use committee of Northwest A and F University and have been approved by the Animal Ethics Committee of the University.

***Staphylococcus aureus* mastitis:** Following the rules as described by Chandler (Chandler 1970), C57BL/6J mice were infected with *S. aureus* by intramammary inoculation (IMI) on the 7~10th day of lactation. Ten mice were separated into three groups. In the blank group (3), no treatment was introduced. In the saline group (3), 0.9% sterile saline was injected in fourth teats, 50 μ L teat⁻¹. In the infection group (4), 1 \times 10⁷ CFU mL⁻¹ *S. aureus* was injected in fourth teats, 50 μ L teat⁻¹. The lactating mice were removed from their pups 1-2 h before bacterial inoculation of the mammary gland. The mice were anaesthetized with ketamine (0.1 mg g⁻¹). Using a stereoscopic microscope, *S. aureus* were injected in the left and right fourth mammary gland, respectively and each teat was inoculated with 50 μ L of a suspension containing 1 \times 10⁷ CFU. The bacteria through the teat canal using a 30-gauge blunt needle. The contrary group with 0.9% Sterile Saline. Ten lactating mice were euthanized at 72 h infection.

Histological analysis: Three groups of the mammary gland tissues were collected .The tissues was put in 4% paraformaldehyde at room temperature for 24 h and embedded in paraffin. Sections

(6 μm thick) were stained with haematoxylin and eosin (H and E) for histological examination. Light microscopy was used for exam the sections and photoed by Nikon DS-U2/L2 Controler and NIS-Elements F 3.22 (Nikon Corporation, Tokyo, Japan).

Separating lymphocytes from mammary gland: The fourth mammary gland tissues were removed from the dead mice. One side was cut into pieces and washed by sterilized Phosphate Buffered Saline (PBS). The tissues were through 100 mesh and were added 5 mL of predigestion solution which combined with 1 \times HBSS containing 5 mM EDTA and 1 mM DTT. It was put in the table concentrator 150 rpm, 37°C, 30 min after that 2000 rpm centrifuge. The production through 200 mesh and added 5 mL digestion solution which was dissolved 0.05 g of collagenase D (Sigma), 0.05 g of DNase I (Roche) in 100 mL of 1 \times PBS (Weigmann *et al.*, 2007) it was put in the table concentrator 150 rpm, 37°C, 30 min; 1200 rpm centrifuge. Here is something else to consider that a fresh solution must be put into an incubator at 37°C for a few minutes before using it. Repeat the steps until the tissues were dissolved in the solution. After that, the suspension was centrifuged 1200 rpm, 10 min. At the bottom lied some cell debris and lymphocytes. To test the viability and the number of extracted cells perform a quick Trypan Blue exclusion test. The cell suspension was transferred into each well of 24-well plates. The culture medium (RPMI-1640 supplemented with 100 U mL⁻¹ penicillin and 100 g mL⁻¹ streptomycin, 2 mM glutamine and 5% fetal calf serum) was added into the plates. The final cell density was made sure 1 \times 10⁶ cells in 1 mL. Then, it was cultured 4 h in an incubator which was set at 37°C under a 5% CO₂ environment.

Flow cytometry: After 4 h of culture, using 1.5 mL sterile tube collected the contents of the well. The cells were then centrifuged at 1000 rpm for 10 min. For analysis of CD4+ lymphocytes, 100 μL PBS was adding into the tube and the cells were incubated with APC anti-mouse CD69; phycoerythrin (PE) anti-mouse CD4 (Biolegend, Inc, California, USA) at 4°C for 20-30 min. After that cells were washed twice by PBS and fixed cells with 1% paraformaldehyde. Immunofluorescent cytometric analysis was performed using FACS Aria (Becton Dickinson) with FACS Diva software (Becton Dickinson).

Statistical analysis: The data were analyzed by using a standard t-test by GraphPad prism 5.0 for Windows (GraphPad Software Inc, San Diego CA, USA).

RESULTS

Clinical observation: No clinical change was shown in the normal group and saline group. So they can merge into a control group. The mice were healthy and alive. Compared with the control group, the mice in the infected group were hard to move, listlessness and poor appetite after 72 h bacterial injection. Furthermore, autopsy found that the mammary gland tissue became red, swelling and hyperemia (Fig. 1).

Histological observation: The change of mammary gland was evaluated by microscopy following H and E staining. Microscopic examination showed that lots of inflammatory cells were infiltrated in interstitial. Compared with the control group, the infection group's acinar lumina and the acinar structure were disintegration after 72 h injection. Also, the epithelial tight junction was destroyed by the bacteria (Fig. 2).

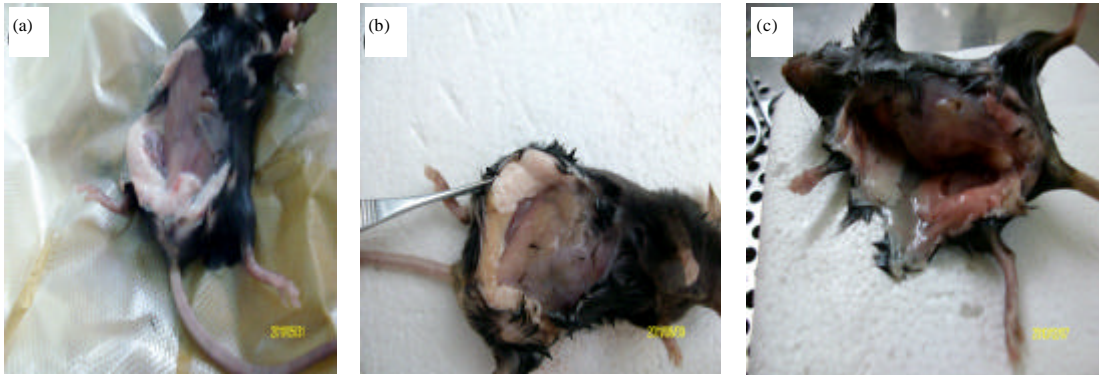


Fig. 1(a-c): Comparing the normal and infection mammary gland in clinical, (a) Blank group, there was no swelling in mouse mammary gland tissue, (b) Saline group, mouse was also same as the blank group and (c) After 72 h IMI with *S. aureus*, the tissue was swelled, red and with bleeding symptoms

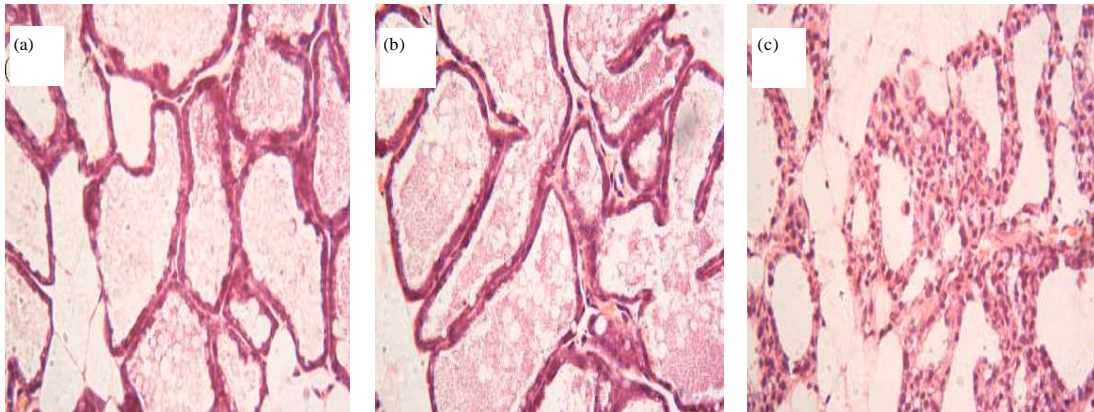


Fig. 2(a-c): Histology of mouse mammary gland at 72 h after IMI with *S. aureus*, (a) Blank control: Mammary gland tissue, epithelial tight junction, intact acinar structure and no infiltration of inflammatory cells in mammary tissue, (b) Saline group: There was no histology change in mammary gland, the tissue is same as blank control and (c) After 72 h *S. aureus* infection: lots of inflammatory cells were infiltrated in mammary tissue, acinar structure and acinar lumina were also disrupted

Intramammary Inoculation with *S. aureus* cause an increase in CD4+ lymphocytes in the mammary gland tissue: To investigate the CD4+ lymphocytes involved in *S. aureus* infection, lymphocytes in mammary gland tissue were collected from blank, saline and infected group which were evaluated by flow cytometry. As shown in Fig. 3, the number of lymphocytes in infection group have increased remarkably in 72 h injection comparing the blank and saline group. The results illustrated that the CD4+ lymphocytes increased significantly ($p = 0.0019$, $p < 0.01$) after IMI at 72 h (Fig. 3) which were inconsistent with studies in a mouse model of *Streptococcus agalactiae* mastitis (Trigo *et al.*, 2009). Figure 4 represents histograms of the CD4+

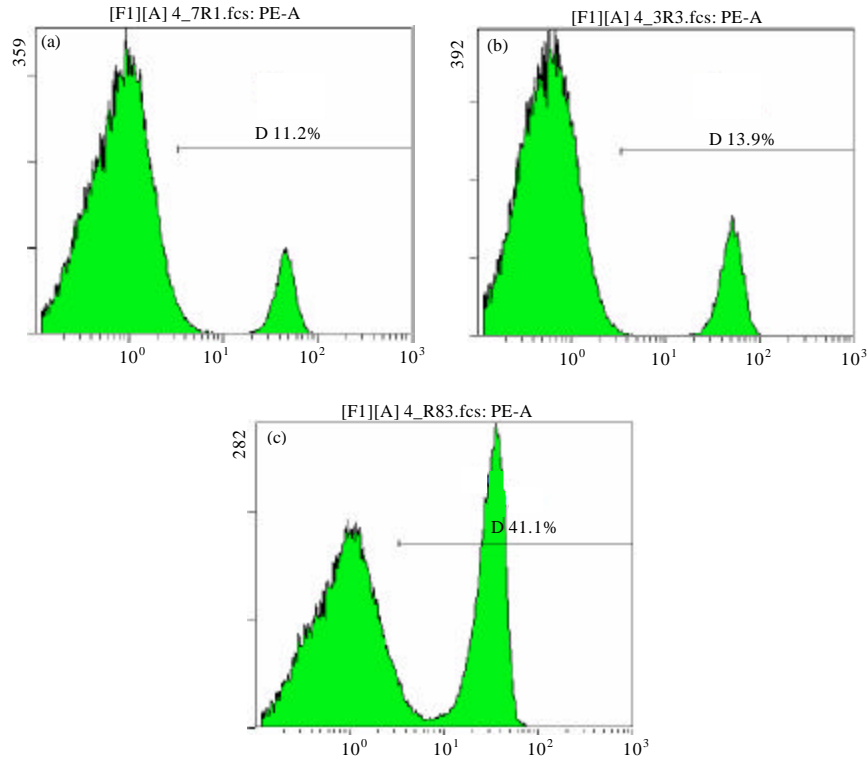


Fig. 3(a-c): Flow cytometric analysis of the expression of the marker CD4 on mammary gland lymph cells of female C57BL/6 mice after 72 h intramammary infection with *S. aureus*, (a) Normal mouse, (b) Saline mouse and (c) *S. aureus* infected mouse

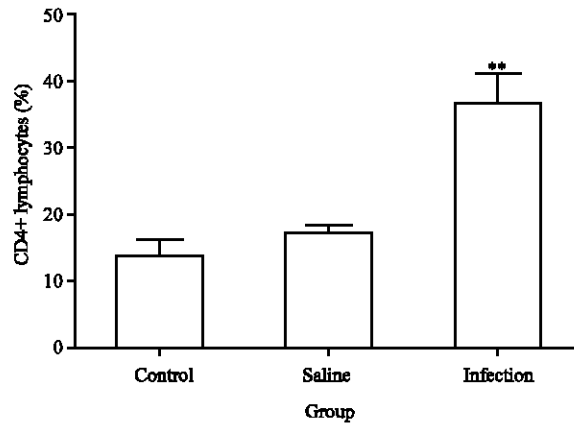


Fig. 4: Comparing the control group and infection group after 72 h IMI, The results are expressed as Means \pm SD of three mice per group, **Extremely significant at p<0.01

lymphocytes percentage in mammary gland of normal mouse, saline mouse and infected *S. aureus* mouse. It shows that CD4+ lymphocytes have increased significantly after 72 h infection (p<0.01).

DISCUSSION

Some reports show that T help cells are important immunocytes which can clear viruses, parasites, intracellular and extracellular bacteria. CD4 T cell as an important immunocyte in adaptive immune response. T helper cells (CD4+ cells) which will recognize processed foreign peptide complexed with MHC class molecules on the surface of Antigen Presenting Cells (APC) (Corradin and Lanzavecchia, 1991). They can elicit immune response and differentiate many sorts of cells, such as Th 1, Th 2, Treg and Th 17 cells, etc. Th 1 cell as one of the family member has strong ability to eliminate bacteria, especially in *S. aureus* infection. When animal was inoculated Protein A (PA) of *S. aureus*, Th 1 would increased immediately after injection (Sinha *et al.*, 1999). In the recent studies, T-helper 17 (Th 17) cells are characterized by their production of interleukin-17 (IL-17) and have a role in the protection against infections and in certain inflammatory diseases. It can strongly clear away *S. aureus* from the tissues or establish barriers to prevent the bacteria. Humans who lack Th 17 cells are more susceptible to *S. aureus* infections compared to individuals having Th 17 cells (Islander *et al.*, 2010). In addition, Th 2/Treg responses are mechanisms of protection against chronic *S. aureus* implant infection which may play a role in the development of chronic infection (Prabhakara *et al.*, 2011). In these articles, CD4+ lymphocytes and their family cells number probably increased after infection which were the same as our results.

When Naïve CD4+ T cells were stimulated by pathogens, the cells secreted lots of cytokines such as IFN- γ , IL-17A and IL-17F, etc. These cytokines can clean bacteria directly or recruit macrophages, neutrophils and monocytes to the inflammation sites for eliminating pathogens. Interferon- (IFN-) gamma the secretion of Th 1 which is known to be important to elicit the acute phase response and allow the accumulation of leukocytes at the site of infection (Riollet *et al.*, 2000). When patients were infected by Methicillin-Resistant *S. aureus* (MRSA), IFN-gamma enhanced rapid intracellular killing of MRSA with triple-drug treatment or daptomycin alone (Smith *et al.*, 2010). IL-17 the secretion of Th 17 is important for mobilization and activation of neutrophils (Proctor, 2011). This cytokine play a vital role in mucosal immunity. People were infected by *Candida albicans*, IL-17A and IL-17F would be essential for mucocutaneous immunity against the bacteria (Puel *et al.*, 2011). Some reports in dairy cow or goat mastitis found that IL-17A and IL-17F had a potential to modulate the mammary gland immune response to mastitis-causing pathogens (Bougarn *et al.*, 2011; Jing *et al.*, 2012). In our study, through histological observation we could see lots of inflammatory cells were infiltrated in interstitial and CD4+ T cells increased significantly. It indicated that CD4 T cell-derived cytokines should responsible for the cells recruitment which can provide protection and clean the bacteria.

For development of *S. aureus* mastitis vaccine, lack of known CD4+ T cell antigens from *S. aureus* has made it difficult to design effective vaccines (Lawrence *et al.*, 2012). The families of CD4+ T cells are also important for *S. aureus* vaccines research. Th 17 cells play an essential role in IsdB vaccine-mediated defense against invasive *S. aureus* infection in mice (Joshi *et al.*, 2012). Mice were immunized with rAls3p-N (recombinant N-terminus of Als3p), the vaccination induced a Th 1/Th 17 response resulting in recruitment and activation of phagocytes at sites of infection and more effective clearance of *S. aureus* from tissue (Lin *et al.*, 2009). In *S. aureus* mastitis vaccines research, we should consider that CD4+ T cells and their families are essential for developing new vaccines.

In conclusion, the present study indicates that CD4+ lymphocytes in mouse mammary gland have increased remarkably at 72 h post IMI *S. aureus*. It provides information about the CD4

cellular immune response in mammary gland after infection with *S. aureus*. For further research, the T-helper cells must be discussed and investigated in order to find out which type of the cells and cytokines contribute to clean and defense bacterial in mouse model of mastitis.

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