Veterinary Immunology and Immunopathology 155 (2013) 245–252

Contents lists available at ScienceDirect

Veterinary Immunology and Immunopathology

journal homepage: www.elsevier.com/locate/vetimm

Research paper

Staphylococcus aureus and *Escherichia coli* elicit different innate immune responses from bovine mammary epithelial cells

Fu Yunhe[a], Zhou Ershun[a], Liu Zhicheng[a], Li Fenyang[a], Liang Dejie[a], Liu Bo[a], Song Xiaojing[a], Zhao Fuyi[a], Fen Xiaosheng[b], Li Depeng[a], Cao Yongguo[a], Zhang Xichen[a], Zhang Naisheng[a], Yang Zhengtao[a,*]

[a] *Department of Clinical Veterinary Medicine, College of Veterinary Medicine, Jilin University, Changchun, Jilin Province 130062, People's Republic of China*
[b] *Guangdong Institute of Modern Agricultural Group, Guangzhou, Guangdong Province, People's Republic of China*

ARTICLE INFO

Article history:
Received 10 October 2012
Received in revised form 7 August 2013
Accepted 13 August 2013

Keywords:
Escherichia coli
Staphylococcus aureus
Bovine mammary epithelial cells
TLR4
TLR2
NF-κB

ABSTRACT

Escherichia coli and *Staphylococcus aureus* are the most important pathogenic bacteria causing bovine clinical mastitis and subclinical mastitis, respectively. However, little is known about the molecular mechanisms underlying the different host response patterns caused by these bacteria. The aim of this study was to characterize the different innate immune responses of bovine mammary epithelium cells (MECs) to heat-inactivated *E. coli* and *S. aureus*. Gene expression of Toll-like receptor 2 (TLR2) and TLR4 was compared. The activation of nuclear factor kappa B (NF-κB) and the kinetics and levels of cytokine production were analyzed. The results show that the mRNA for TLR2 and TLR4 was up-regulated when the bovine MECs were stimulated with heat-inactivated *E. coli*, while only TLR2 mRNA was up-regulated when the bovine MECs were stimulated with heat-inactivated *S. aureus*. The expression of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6 and IL-8 increased more rapidly and higher when the bovine MECs were stimulated with heat-inactivated *E. coli* than when they were stimulated with heat-inactivated *S. aureus*. *E. coli* strongly activated NF-κB in the bovine MECs, while *S. aureus* failed to activate NF-κB. Heat-inactivated *S. aureus* could induce NF-κB activation when bovine MECs cultured in medium without fetal calf serum. These results were confirmed using TLR2- and TLR4/MD2-transfected HEK293 cells and suggested that differential TLR recognition and the lack of NF-κB activation account for the impaired immune response elicited by heat-inactivated *S. aureus*.

1. Introduction

Bovine mastitis, an infection of the bovine mammary gland, is a highly prevalent and important infectious disease of dairy cattle (Blosser, 1979; Seegers et al., 2003). It can cause a decline in milk production and quality and result in great losses to the dairy industry worldwide. Mastitis can be caused by more than 150 different types of pathogens. In most cases, an infection with Gram-negative bacteria, such as *Escherichia coli* (*E. coli*), can often cause clinical mastitis, which is characterized as an acute and severe infection that can be cleared within a few days (Vangroenweghe et al., 2005). In contrast, an infection with Gram-positive bacteria, such as *Staphylococcus aureus* (*S. aureus*), often causes a chronic and persistent subclinical mastitis (Taponen and Pyorala, 2009). The molecular

* Corresponding author at: Department of Clinical Veterinary Medicine, College of Animal Science and Veterinary Medicine, Jilin University, Changchun, Jilin Province 130062, People's Republic of China.
Tel.: +86 431 87981688; fax: +86 431 87981688.
E-mail address: yzt@jluhp.edu.cn (Z. Yang).

0165-2427/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.vetimm.2013.08.003

mechanisms by which different pathogens induce different responses during mammary gland infections are poorly understood.

The innate immune system and the immunological functions it mediates are the host's first line of defense against the invading pathogens during mastitis (Blosser, 1979; Hoffmann et al., 1999). It plays an important role during the early stages of infection. The innate immune system recognizes highly conserved motifs shared by diverse pathogens, which are called pathogen-associated molecular patterns (PAMPs), via pattern recognition receptors (PRR). Toll-like receptors (TLRs) are one of the PRRs that recognize the conserved components of pathogens, or pathogen-associated molecular patterns, and initiate the innate immune response (Akira et al., 2001; Beutler et al., 2003). Reports have recently identified 13 different Toll-like receptors (TLRs) (Akira et al., 2006; Alexopoulou et al., 2001; Takeda and Akira, 2005), and 10 different bovine TLRs have been described (McGuire et al., 2006). Each of the TLRs has their own ligand and functional characteristics. For example, TLR2 recognizes lipoteichoic acid (LTA) and peptidoglycan (PGN) from Gram-positive bacteria, e.g., *S. aureus* (Schroder et al., 2003; Takeuchi et al., 2000). TLR4 recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, e.g., *E. coli*.

Cytokines, an important group of inflammatory mediators, play an important role in the host innate immune response to infection. Tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β are major pro-inflammatory cytokines that mediate the inflammatory response at both the local and systemic levels (Mueller et al., 2001; Singh et al., 2004). TNF-α is an endogenous pyrogen that causes fever and stimulates endothelial cells and leukocytes to release various inflammatory mediators (e.g., NO and oxygen-free radicals), thereby promoting neutrophil phagocytosis. It plays a crucial role in the pathological damage from inflammation and septic shock and is the key mediator contributing to endotoxin shock (Su et al., 2010). IL-1β acts on target cells to boost the synthesis of acute phase proteins and generate inflammatory responses such as fever (Zheng et al., 1995). In addition, this cytokine induces the expression of cellular adhesion molecules, attracts aggregates of neutrophils, stimulates immune cells and endothelial cells to produce various inflammatory cytokines and chemokines and promotes the generation and release of neurotoxic compounds (Dustin et al., 1986). IL-6 is one of the most common inflammatory cytokines (Hodge et al., 2005; Martin, 1999). Circulating levels of IL-6 have been shown to be excellent predictors of the severity of acute respiratory distress syndrome (ARDS) of different etiologies, such as sepsis and acute pancreatitis (Leser et al., 1991). IL-8 is an important chemokine that recruits neutrophils to the infection site.

Apart from the innate immune cells, the mammary epithelial cells (MECs) play an important role in udder immunity (Griesbeck-Zilch et al., 2008). They can recognize the PAMPs of invading pathogens via PRR, such as TLRs, and induce the secretion of cytokines and chemokines. Additionally, they can express bactericidal β-defensins, acute phase proteins that help fight off pathogens (Isobe et al., 2009). In this study, we used bovine MECs to investigate the immune defense mechanisms in the udder. We compared the key factors produced by these cells in response to treatment with heat-inactivated *S. aureus* and *E. coli*. In addition, we tested if fetal calf serum (FCS) could affect NF-κB activation by heat-killed *S. aureus* and *E. coli* in bovine MECs.

2. Materials and methods

2.1. Cell culture and challenge with mastitis pathogens

Epithelial cells from the bovine mammary gland were separated using procedures that have been described previously (Hu et al., 2009). Animal experiments were done in accordance with the guidelines on animal care and use established by the Jilin University Animal Care and Use Committee. The protocols were reviewed and approved by the committee. Six healthy lactating Chinese Holstein cows were selected on the basis of milk somatic cell counts (SCC) and clinical investigations. Then the cows were killed and the mammary tissues obtained from six Holstein cows were transported on ice to the laboratory within 1–1.5 h of harvesting. The mammary tissue was cut into 1-cm^3 pieces and washed with D-Hank's solution several times, until the solution was pellucid and did not contain any milk. The tissue was then cut into 1-mm^3 pieces and washed with D-Hank's solution until clean. The tissue pieces were transferred into cell culture flasks that were coated with rat-tail collagen. The culture flasks were incubated at 37 °C in 5% CO_2. After 4 h, 3 mL of DMEM/F12 basal media were added to every culture flask. After 12 h, 2 mL of the basal media were added. The media was changed once every 48 h until the cells were spread across the bottom of the culture flask. The fibroblasts were digested with Trypsin (0.25%) supplemented with 0.1% EDTA-2Na. The pure MECs were isolated after 3 passages for subsequent experiments. HEK293-TLR2 and HEK293-TLR4/MD2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS.

We challenged bovine MECs or HEK293-TLRs cells with heat-inactivated mastitis pathogens *E. coli* strain 1303 and *S. aureus* capsular polysaccharide type 5 strain. Heat inactivation of the pathogens was conducted at 80 °C for 1 h. Inactivation efficiency was tested on blood agar plates. The cells were stimulated with 200 μg/mL of heat-inactivated *E. coli* or 200 μg/mL of heat-inactivated *S. aureus* bacteria debris. Bovine MECs were stimulated by LPS (μg/mL) or LTA (μg/mL) as positive controls.

2.2. RNA extraction and qRT-PCR

The total RNA was isolated from the MECs at 1, 3, 6, 12 and 24 h after treatment with heat-inactivated *E. coli*, heat-inactivated *S. aureus*, LPS or LTA. The total RNA was extracted using TRIzol (Invitrogen) by following the manufacturer's instructions. The RNA was reverse-transcribed into cDNA using a Revert Aid First Strand cDNA Synthesis Kit (Thermo). The relative mRNA concentrations were detected by qRT-PCR using a 7500 Fast Real-Time PCR System (Applied Biosystems) and a SYBR green Plus reagent kit (Roche), as has already been described elsewhere (Arms