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Cow-to-cow variation in fibroblast response to a toll-like receptor 2/6 agonist and its relation to mastitis caused by intramammary challenge with *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is a common cause of chronic mammary gland infections in dairy cattle. However, the inflammatory response and duration of infection following pathogen exposure is variable between individual animals. To investigate interanimal differences in immune response, dermal fibroblast cultures were established from skin biopsies collected from 50 early lactation Holstein cows. The fibroblasts ability to produce IL-8 in response to a 24-h treatment with a synthetic toll-like receptor 2/6 agonist (Pam2CSK4) was used to assign a response phenotype to the animals. Five high-responding and 5 low-responding animals were then selected for an intramammary challenge with *S. aureus* to evaluate differences in the inflammatory response, chronicity of infection, and development of antibodies to the pathogen. All animals exhibited clinical symptoms of mastitis at 24 h postchallenge. Animals previously classified as high responders experienced a greater inflammatory response characterized by elevated levels of milk somatic cell count, IL-8, and BSA following the challenge compared with low responders. In addition, antibodies toward the challenge strain of *S. aureus* reached higher levels in whey from the challenged gland of high responders compared with low responders. Despite the antibody response, all 5 high responders were chronically infected for the 6-wk duration of the study, whereas 2 of the low responders cleared the infection, although 1 of these did become reinfected. The observed differences between animals classified as low and high responders based on their fibroblast responsiveness suggests that this cell type can be used to further examine the causes of interanimal variation in response to mammary infection.

Key words: experimental mastitis, Pam2CSK4, interanimal variation, dairy cow

INTRODUCTION

Bovine mastitis can be caused by a variety of pathogens, resulting in variable degrees of inflammation of the infected mammary gland and subsequent decreases in milk production and quality (Ballou, 2012). Infections caused by *Staphylococcus aureus* typically result in a relatively mild inflammatory response with a sustained elevation in SCC; however, some animals will experience a more severe response, leading to collateral damage of the host (Atalla et al., 2009). These infections can develop into chronic, subclinical cases, in which infected animals maintain a reservoir of *S. aureus* on a farm (Bannerman et al., 2008; Schukken et al., 2011). Rates of mastitis due to *S. aureus* have decreased since the implementation of the 10-point plan from the National Mastitis Council (NMC, 2011), which recommends teat dipping and dry cow therapy as ways to minimize the spread of contagious pathogens. However, *S. aureus* is still present on most commercial herds, with incident rates ranging from 0.1 to 10% (Olde Riekerink et al., 2008; Barlow et al., 2013; Hertl et al., 2014). Treatment of long-term infections with antibiotics commonly yields an unsatisfactory cure rate (Sol et al., 2000; Barkema et al., 2006), and these animals are typically removed from the herd to limit spread of the pathogen.

Pathogens entering the mammary gland are recognized by toll-like-receptors (**TLR**), which are found on multiple cell types within the gland and play a pivotal role in the innate immune response. These membrane-bound receptors are highly specific and identify conserved motifs known as pathogen-associated-molecular-patterns from various pathogens, including bacteria, fungi, and viruses (Takeuchi and Akira, 2010). For instance, LPS, a component of the *Escherichia coli* outer membrane, activates TLR4 (Park et al., 2009), and lipoproteins from the *S. aureus* cell wall activate TLR2 (Zähringer et al., 2008). Toll-like receptor signaling pathways lead to the activation of the transcription factor nuclear factor-kappa B (**NF-κB**), which regulates expression of various proinflammatory genes (Kawai and Akira, 2007). Increased expression of chemokines,

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such as IL-8, following NF-κB activation promotes recruitment of neutrophils to the site of infection. Neutrophils can account for up to 90% of the increased cell population within the infected gland and are the main cause of the rise in SCC following infection (Sordillo and Streicher, 2002).

Pathogen-specific differences in infection outcomes following experimentally induced mastitis and the resulting inflammatory responses have been observed (Bannerman, 2009). However, even in well-controlled situations considerable variation in response exists between individual animals. At the farm level, animals in similar physiological states under common environmental conditions also exhibit variable susceptibility to development of clinical mastitis. Various cell models have been used to investigate potential sources for these interanimal differences, including neutrophils (Sohn et al., 2007; Revelo and Waldron, 2012), monocyte-derived macrophages (Taraktsoglou et al., 2011), and mammary epithelial cells (Lahouassa et al., 2007; Brand et al., 2011). As model cells, neutrophils and macrophages are difficult to cryopreserve for future studies and can become activated during isolation and culturing processes, potentially adding to experimental variation. Mammary epithelial cells show a robust response to immunostimulants such as LPS, and these cells can successfully be cryopreserved (Wellnitz and Kerr, 2004; Pareek et al., 2005). However, although these cells can easily be isolated from tissue collected at euthanasia, procedures to isolate sufficient mammary tissue from live animals are relatively invasive and may compromise the ability of the gland to return to full production. We have previously examined the autologous dermal fibroblast as a model cell to predict an animal's responsiveness to experimental *E. coli* mastitis (Kandasamy et al., 2011). The fibroblast is easily obtainable and, following isolation, the cells can be cultured quickly and cryopreserved. Cultured fibroblasts also demonstrate a robust response following stimulation with various TLR agonists (Kandasamy and Kerr, 2012), suggesting that it may be a useful cell type to explore the underlying causes for interanimal variation in response to mastitis. In the current study we extend these findings to include experimentally induced *S. aureus* mastitis.

MATERIALS AND METHODS

Animal and Experimental Procedures

The University of Vermont's Institutional Animal Care and Use Committee approved all animal procedures before commencement of the study. Dermal fibroblast cultures were established from skin samples collected from 50 lactating Holstein cows housed at a collaborating dairy farm. Animals were randomly enrolled in the study, and at the time of biopsy were in early to mid lactation (117 ± 30 DIM). The average lactation number of the 50 enrolled cows was 2.8 (±0.9).

Skin samples were collected as described previously (Kandasamy et al., 2011) with slight modifications. Briefly, a 6-cm^2 skin sample was taken from the shoulder area following standard surgical preparation of the site and transported back to the laboratory on ice in Dulbecco's PBS (**DPBS**; Hyclone Laboratories, Logan, UT) supplemented with a 1× antibiotic cocktail (100 U/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B; Hyclone Laboratories). After removing subcutaneous fat and connective tissue, a 3-cm^2 piece of skin was minced into smaller pieces with opposing scalpel blades and subsequently washed 4 times with fresh DPBS. After washing, 10 mL of 0.5% collagenase type I enzyme (Life Technologies, Grand Island, NY) diluted in Dulbecco's modified Eagle medium (**DMEM**; Hyclone) containing 1× antibiotic cocktail was added to the minced skin pieces and incubated at 37°C for 6 h with orbital shaking. The collagenase-digested tissue was then filtered through a 70-μm nylon mesh filter (Fisher Scientific, Pittsburgh, PA), and the filtrate was centrifuged at 1,100 × *g* for 5 min at 20°C. The cell pellet was reconstituted with DMEM containing 10% fetal bovine serum (**FBS**; Hyclone Laboratories), 1× insulin-transferrin-selenium (Mediatech Inc., Herndon, VA), and 1× antibiotic cocktail and cultured in a 25-cm^2 flask (Corning Inc., Corning, NY) in a humidified 37°C incubator with 5% CO_2 until 70% confluency was reached. Cells were then detached with 0.25% trypsin (MP Biomedical, Santa Ana, CA) and seeded in a 75-cm^2 flask (Corning Inc.) with DMEM supplemented with 5% FBS, 1× insulin-transferrin-selenium, and 1× antibiotic cocktail. After approximately 4 d, cells were trypsinized and split into three 75-cm^2 flasks. Once cells reached confluency, they were lifted with trypsin and aliquots of the third passage were diluted in DMEM supplemented with 20% FBS and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO) and cryopreserved in liquid nitrogen for future challenges.

Dermal Fibroblast Challenges with IL-1β, LPS, and Pam2CSK4

Aliquots of cells were revived from cryopreservation in duplicate and cultured in a 75-cm^2 flask until confluent. Cells were lifted with trypsin and fourth-passage cells were seeded into a 6-well plate (Corning Inc.) at 1×10^5 cells/mL in a total volume of 2 mL. Following a 24-h incubation, media was removed and replaced with 2 mL of fresh media (negative control), or media