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Innate Immune Response to Intramammary *Mycoplasma bovis* Infection

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ABSTRACT

The objective of the current study was to characterize the systemic and local innate immune response of dairy cows to IMI with *Mycoplasma bovis*, a pathogen of growing concern to the dairy industry. Ten Holstein cows were each infused in 1 quarter with *M. bovis* and studied for a 10-d period. Acute phase protein synthesis, which reflects 1 parameter of the systemic response to infection, was induced within 108 h of infection, as evidenced by increased circulating concentrations of lipopolysaccharide binding protein and serum amyloid A. Transient neutropenia was observed from 84 to 168 h postinfection, whereas a constant state of lymphopenia and thrombocytopenia was observed from 84 h until the end of the study. Milk somatic cell counts initially increased within 66 h of *M. bovis* infusion and remained elevated, relative to control (time 0) concentrations, for the remainder of study. Increased milk concentrations of BSA, which reflect increased permeability of the mammary epithelial-endothelial barrier, were evident within 78 h of infection and were sustained from 90 h until the end of the study. Milk concentrations of several cytokines, including IFN-γ, IL-1β, IL-10, IL-12, tumor growth factor-α, and tumor necrosis factor-α, were elevated in response to infection over a period of several days, whereas increases in milk IL-8 were of a more limited duration. Complement activation, reflected by increased milk concentrations of complement factor 5a, was also observed over several days. Despite the indication by these observed changes that the cows mounted a prolonged inflammatory response to *M. bovis* intramammary infection, all quarters remained infected throughout the study with persistently high concentrations of this bacterium. Thus, a sustained inflammatory response is not sufficient to eradicate *M. bovis* from the mammary gland and may reflect the ongoing struggle of the host to clear this persistent pathogen.

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INTRODUCTION

Mycoplasmas are the smallest self-replicating bacteria. They have a very small genome and their cell envelope consists of a single plasma membrane (Razin et al., 1998). Despite their relatively simplistic composition, mycoplasmas have the capacity to infect both humans and animals, and to cause debilitating disease (Foy, 1993; Ruffin, 2001). Mastitis caused by *Mycoplasma* is a worldwide problem (Nicholas and Ayling, 2003; Fox et al., 2005). Among the different species of mycoplasma that infect cattle, *Mycoplasma bovis* is the most pathogenic and common cause of mastitis. The prevalence of mycoplasma-induced mastitis is believed to be underestimated because of its long incubation period before the onset of clinical symptoms (Jasper, 1981) and its persistence after cessation of clinical signs (Pfutzner and Sachse, 1996), both of which hinder its identification as the causative agent in a given case of mastitis. According to the most recent USDA National Animal Health Monitoring System Dairy Study in 2002, 7.9% of dairy bulk tank milk samples tested positive for mycoplasma, with *M. bovis* accounting for 86% of the positive samples (USDA-APHIS, 2002). The highest percentage (9.4%) of dairies testing positive for mycoplasma were in the western regions of the United States. The distribution of mycoplasma was not limited to this region, however, because positive samples were recovered from 76% of the states surveyed. Because the herds in this survey were sampled only once, the reported prevalence of mycoplasma was likely underestimated.

Mycoplasma bovis causes substantial economic losses to the dairy industry, primarily though the causation of an intractable, untreatable mastitis (Brown et al., 1990; Gonzalez et al., 1992). *Mycoplasma bovis* mastitis results in decreased milk production (Brown et al., 1990), diminished milk quality, and lost quarters (Jain et al., 1969; Bennett and Jasper, 1978a; Horvath et al., 1981). Economic losses have been estimated to approach $450 in lost milk value alone per

case of clinical mastitis caused by mycoplasma (Wilson et al., 1997). Because no efficacious antibiotics or vaccines have been approved for the treatment or prevention of IMI caused by *M. bovis*, culling is recommended for controlling the disease; however, this results in considerable animal replacement costs to the producer (Bushnell, 1984; Nicholas and Ayling, 2003).

Mycoplasma is a highly contagious pathogen that may be spread from cow to cow by the hands of milkers and fomites, such as the milk claw, in the milking parlor. The contagious nature of this pathogen is noted by its high prevalence in herds with a history of *M. bovis* mastitis (Bennett and Jasper, 1977). Within an infected cow, mycoplasma can spread hematogenously to distal sites (Jasper, 1982). In addition to the mammary gland, *M. bovis* is known to colonize other sites in cattle and to induce arthritis, pneumonia, and genital disorders (Pfutzner and Sachse, 1996). Effects on the host vary widely from animal to animal in terms of severity, number of quarters infected, and duration of infection, with subclinical to mild infections predominating (Boughton and Wilson, 1978; Bushnell, 1984). Few cows ever completely clear the organism.

Establishment of infection is governed, in part, by the nature of the host response to the invading organism. It is well established that *Escherichia coli* IMI follows a distinct clinical course compared with that of *Staphylococcus aureus* or *M. bovis*. Intramammary infection by *E. coli* is acute in nature and generally clears within a few days (Smith and Hogan, 1993). In contrast, IMI by *Staph. aureus* or *M. bovis* is often less acute, but results in a chronic infection that can persist for the life of the animal (Bushnell, 1984; Sutra and Poutrel, 1994). We and other researchers have established that the differential inflammatory response elicited during *E. coli* and *Staph. aureus* IMI corresponds with resolution of infection (Riollet et al., 2000; Bannerman et al., 2004c). Compared with *Staph. aureus*, IMI by *E. coli* elicits a more acute inflammatory cytokine response and enhanced activation of complement. Of particular note, *Staph. aureus* IMI is characterized by the complete absence of IL-8 or tumor necrosis factor (**TNF**)-α production, and the overall diminished inflammatory response characteristic of *Staph. aureus* IMI correlates with its ability to persist in the gland. These data indicate that there is pathogen-dependent variability in the host innate immune response to IMI and that a limited inflammatory response may contribute to the development of a chronic IMI.

The ability to recognize highly conserved motifs shared by diverse pathogens enables the innate immune system to respond to multitudes of pathogens. These motifs, known as pathogen-associated molecular patterns, include the bacterial cell membrane and wall components, LPS, peptidoglycan, lipoteichoic acid, and macrophage-activating lipopeptide 2 kDa (Aderem and Ulevitch, 2000). Lipopolysaccharide, a highly proinflammatory component of all gram-negative bacteria including *E. coli*, is recognized by Toll-like receptor (**TLR**)-4 (Poltorak et al., 1998). Peptidoglycan (Yoshimura et al., 1999) and lipoteichoic acid (Schroder et al., 2003) within the cell wall of *Staph. aureus* and other bacteria, and lipopeptides within the cell membrane of mycoplasma (Nishiguchi et al., 2001; Seya and Matsumoto, 2002), are recognized by TLR-2 in concert with TLR-1 or TLR-6 (Omueti et al., 2005). Thus, the ability to recognize conserved elements expressed by an array of bacteria enables the innate immune system to respond to vast numbers of bacteria with just a limited repertoire of host recognition elements.

Relative to other major mastitis pathogens, little is known about the nature of the innate immune response to intramammary *M. bovis* infection. Because *M. bovis* establishes a chronic infection similar to *Staph. aureus* and contains immunostimulatory components that activate the same immune receptor as components found on *Staph. aureus*, one may hypothesize that the inflammatory response elicited by *M. bovis* may closely resemble that evoked by *Staph. aureus*. The objective of the current study was to characterize the innate immune response to IMI with *M. bovis* in dairy cows.

MATERIALS AND METHODS

Animals

Ten first-lactation Holstein cows were selected on the basis of milk SCC of <200,000 cells/mL and the absence of detectable bacterial growth in 2 aseptically collected milk samples obtained 2 d apart. Screening for bacterial growth was performed by plating milk samples on blood and Friis agar plates. The experiment was performed with 2 replicate groups of animals containing 5 cows each. All cows within a replicate group were challenged on the same day and with the same preparation of *M. bovis* inoculum. The mean (±SE) DIM of each replicate were 271 ± 4 and 288 ± 3. For the 7-d period before initiation of the study, the mean (±SE) daily milk production of cows in each replicate was 20.49 ± 3.04 and 23.99 ± 2.23 kg/d. Throughout the study, cows were fed TMR, given access to pasture, and individually milked twice daily by bucket machine. The milking unit was cleaned and sanitized between animals to limit cross-contamination. The use and care of all animals in this study were approved by the Beltsville Agricultural Research Center's Animal Care and Use Committee.

Mycoplasma Growth Media

Modified Friis broth and agar (Friis, 1975; Knudtson et al., 1986) were used for the culture and detection of *M. bovis*, respectively. Friis base media was first prepared as a 2× stock solution, with 1 L containing 12.32 g of brain-heart infusion, 13.07 g of mycoplasma broth base, 7.35 g of Hanks' balanced salt solution (without bicarbonate, calcium, and magnesium), 4 mL of 1% calf thymus DNA, 112.5 mg of L-Arg, 168.75 mg of L-Gln, 1.25 mL of 0.5% phenol red, and 988.75 mL of H_2O. A second solution, prepared by mixing 3.75 mL of 1% L-Cys with 187.5 mg of NAD and 184.5 mg of $MgSO_4 \cdot 7H_2O$, was added to the first solution. Last, 7.5 mL of 1.85% $CaCl_2 \cdot 2H_2O$ was added. The resulting 2× Friis base media was sequentially filtered through Whatman #1 filter paper, a 0.45-μm filter, and a 0.22 μm filter.

One liter of Friis broth was prepared by mixing 500 mL of 2× Friis base media, 200 mL of fetal calf serum, 400 μL of 50× yeast extract, 10 mL of 2% bacitracin, 10 mL of 1% thallium acetate, and 279.6 mL of sterile H_2O. The pH was adjusted to 7.5 with 2 mL of 1 *N* NaOH.

To prepare Friis agar, 50 mL of 2× Friis base media was added to a solution containing 20 mL of fetal calf serum, 40 μL of 50× yeast extract, 1 mL of 2% bacitracin, 1 mL of 7.5% sodium bicarbonate, and 1.96 mL of sterile H_2O. The pH was adjusted to 7.5 with 0.5 mL of 1 *N* NaOH. The solution was heated to 56°C and combined with an autoclaved agar solution of 800 mg of Oxoid agar no. 1 and 10 mg of DEAE dextran in 25 mL of H_2O. A 5-mL quantity of the resulting mixture was poured into individual 60-mm petri dishes and the agar was allowed to solidify at room temperature.

M. bovis Stock and Intramammary Infusion Challenge Preparation

Lyophilized *M. bovis* strain IA St Cs499, originally isolated from a clinical case of mastitis, was rehydrated in 200 μL of sterile water. The reconstituted bacteria were inoculated into 10 mL of modified Friis broth, and the culture was grown overnight at 37°C in 2% CO_2. The overnight stock culture was passed 3× through a 25-gauge needle, added to an equal volume of fresh broth, aliquoted, and stored at –80°C.

To prepare the intramammary bacterial challenge stock, 200 μL of the *M. bovis* stock culture was inoculated into 10 mL of modified Friis broth and incubated overnight at 37°C in 2% CO_2. Cultures were passed 3× through a 25-gauge needle, harvested by centrifugation at 16,000 × *g* for 40 min at 4°C, and the bacterial pellet was washed 3× in sterile PBS. After the final wash, cells were resuspended in PBS containing 10% fetal calf serum and 0.02% bacitracin, and passed through a 25-gauge needle once more. Intramammary bacterial infusate stocks were aliquoted in a 750-μL volume and stored at –80°C. Viability of the challenge stocks was assessed after 2 wk of storage and the concentration was determined to be 8×10^6 cfu/mL. Prior to intramammary infusion, challenge stocks were thawed and serially diluted to 3×10^4 cfu in 5 mL of PBS. Immediately following the morning milking, 1 quarter of each cow was infused with the prepared challenge dose. The number of colony-forming units infused was confirmed by serial dilution and enumeration on Friis agar plates.

Determination of Viable M. bovis Counts

To determine viable bacterial concentrations, cultures and milk samples were serially diluted 1:10 in sterile PBS. A total of 8 serial dilutions were performed (i.e., dilutions up to and including 1×10^{-8}). A 5-μL quantity of the resulting dilutions was spotted on Friis agar plates. Plating of 5 μL of undiluted sample resulted in a minimum detection limit of 200 cfu/mL of milk. All plates were incubated at 37°C in 2% CO_2 for 48 h. Colonies were identified by the classic "fried egg" shape and enumerated under 25× magnification using a standard stereomicroscope. Plates were returned to the incubator for an additional 5 d and reexamined for the presence of colonies. Any quarter from which a plated milk sample had one or more detectable colonies of *M. bovis* was considered to be infected. Further definitive identification of *M. bovis* was performed by immunostaining of colony lifts. Briefly, nitrocellulose membranes (0.45 μ*M*; Millipore, Billerica, MA) were overlain on plates with prospective colonies, gently lifted off, and blocked with 5% skim milk in 0.1% Tween-PBS for 1 h at room temperature. Membranes were then rinsed 3× in 0.1% Tween-PBS and incubated overnight at 4°C with mouse anti-*M. bovis* monoclonal Myb163 (#MAB970) antibody (Chemicon International, Inc., Temecula, CA) diluted 1:4,000 (Adegboye et al., 1995). Membranes were rinsed 3× with 0.1% Tween-PBS and incubated for 2 h at room temperature with 1:4,000 diluted goat antimouse IgG polyclonal antibody conjugated to horseradish peroxidase (BD Transduction Laboratories, San Jose, CA). Membranes were rinsed 3× with 0.1% Tween-PBS and developed with a solution of 4-chloro-1-napthol, the latter of which was prepared as a 3 mg/mL solution in distilled H_2O that was subsequently added to 20 mL of PBS. Immediately before use, 20 μL of 30% H_2O_2 was added to the developer solution.

Milk and Blood Sample Processing

Aseptic milk samples were collected before and at varying intervals up to 10 d postinfection. Milk samples were plated on both Friis and blood agar plates, the latter of which were used to screen for infection with other mastitis pathogens. Milk SCC were determined with a Bentley Somacount 150 instrument (Bentley Instruments Inc., Chaska, MN) following heating of samples at 60°C for 15 min. Blood samples were collected from the coccygeal vein into Vacutainer glass tubes containing K_3 EDTA (Becton Dickinson Corp., Franklin, Lakes, NJ) and inverted 10×. Differential white blood cell enumeration and whey and plasma preparation were performed as previously described (Bannerman et al., 2004c).

ELISA

Enzyme-linked immunosorbent assays for BSA, complement cleavage product 5a (**C5a**), IFN-γ, IL-1β, IL-8, IL-10, IL-12, LPS-binding protein (**LBP**), serum amyloid A (**SAA**), transforming growth factor (**TGF**)-α, TGF-β_1, TGF-β_2, and TNF-α were all performed as previously described (Bannerman et al., 2004c, 2006).

Statistical Methods

Repeated-measures ANOVA was performed using SAS PROC MIXED (SAS Version 9.1.3., SAS Institute, Cary, NC) to compare the mean responses of the variables with control (time 0) values. Milk SCC and bacteriological counts were transformed to $\log_{10}$ values to satisfy distributional requirements of ANOVA. Correlations among repeated measurements across time within cows were modeled using appropriate covariance structures for each parameter analyzed. A P-value of <0.05 was considered significant.

RESULTS

Recovery of M. bovis from the Milk of Experimentally Infected Mammary Glands

Following infusion of 3×10^4 cfu of *M. bovis* into 1 quarter on each of 10 cows, establishment of IMI was determined by screening aseptically collected milk samples for the recovery of viable *M. bovis* (Figure 1). Within 84 h of infusion, *M. bovis* was recovered from all 10 experimentally challenged quarters, all of which remained infected throughout the study. Maximal numbers of *M. bovis* (9.61 ± 0.16 $\log_{10}$ cfu/mL) were recovered 192 h (8 d) postinfusion. Milk samples from all 3 noninfused quarters on each animal were periodically collected and screened for the presence of *M. bovis* (data not shown). Milk samples collected from these quarters 120 h (5 d) postinfusion were all free of *M. bovis*. By d 10 of the study (240 h postinfusion), however, *M. bovis* was recovered from 3 nonchallenged quarters, each from a different animal.

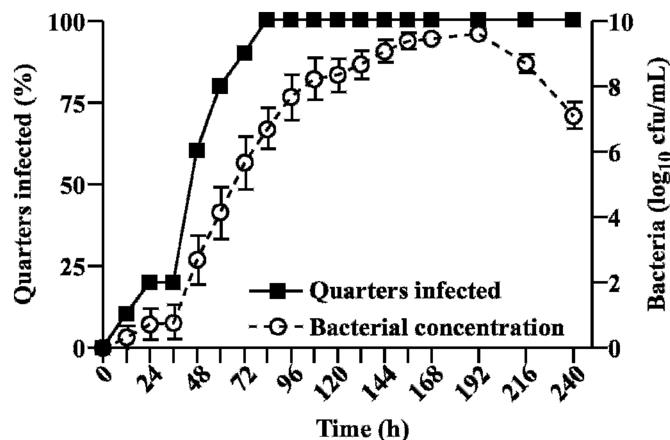

Figure 1. Intramammary growth of *Mycoplasma bovis* following experimental infection. Immediately before (time 0) and at various time points following intramammary infusion of 3×10^4 cfu of *M. bovis* into 1 quarter of each of 10 cows, milk samples were aseptically collected and plated. The percentage of quarters from which viable *M. bovis* was recovered is indicated (solid line; left y-axis). In those quarters from which *M. bovis* was recovered, the mean (±SE) $\log_{10}$ milk bacterial concentration in colony-forming units per milliliter is shown (dotted line; right y-axis).

Systemic Responses to M. bovis IMI

To determine whether *M. bovis* IMI could elicit systemic alterations, changes in body temperature, differential white blood cell counts, and acute phase protein synthesis were evaluated. Mean (±SE) basal body temperature immediately before infection (time 0) was 38.45 ± 0.08°C (data not shown). Following infection, mean body temperature did not fluctuate throughout the study by ≥1°C from control (time 0) measurements. The highest mean (±SE) body temperature recorded was 39.42 ± 0.20°C and was observed at 156 h postinfection. Thus, *M. bovis* failed to elicit a febrile response, which is generally defined as an increase of >1 to 1.5°C in body temperature (Ryan and Levy, 2003) and in cattle has been characteristically defined as temperatures >39.5°C (Drillich et al., 2006).

Transient neutropenia and sustained lymphopenia and thrombocytopenia were observed within 84 h of intramammary infusion of *M. bovis* (Figure 2A and 2B). Relative to control (time 0) neutrophil counts of 5,202 ± 467 cells/μL, decreased concentrations of circulating neutrophils were observed from 84 to 168 h postinfusion. Circulating neutrophils reached a nadir

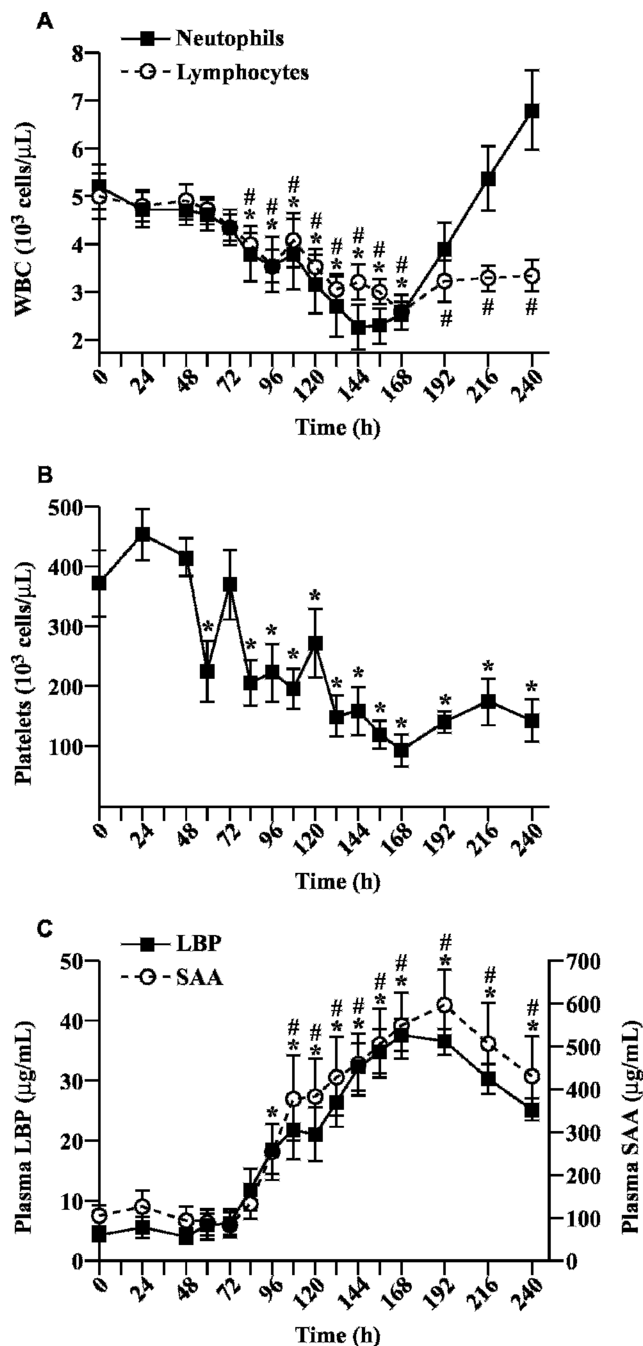

Figure 2. Systemic responses to intramammary *Mycoplasma bovis* infection. Blood samples were collected immediately before (time 0) and at various time points following *M. bovis* intramammary infusion. Whole blood was analyzed for total and differential white blood cell (WBC; A) and platelet (B) counts, and plasma was assayed by ELISA for LPS-binding protein (LBP) and serum amyloid A (SAA; C). Mean (±SE) cell and platelet counts are reported in thousands per microliter. *Decreased ($P < 0.05$) circulating neutrophils or platelets relative to control (time 0) counts (A, B); #decreased ($P < 0.05$) circulating lymphocytes relative to control (time 0) counts (A). Mean (±SE) concentrations of plasma LBP (solid line; left y-axis) and SAA (dotted line; right y-axis) are reported in micrograms per milliliter (C). *,#Increased ($P < 0.05$) LBP and SAA concentrations, respectively, relative to control (time 0) concentrations.

of 2,251 ± 463 cells/μL at 144 h. Relative to control (time 0) lymphocyte and platelet counts of 5,007 ± 463 and 372,400 ± 54,482 cells/μL, respectively, significant and sustained decreases in circulating concentrations of lymphocytes and platelets were observed from 84 h postinfection until the end of the study. Lymphocyte and platelet counts reached nadirs of 2,585 ± 185.47 and 94,500 ± 25,902.92 cells/μL, respectively, at 168 h postinfusion.

The systemic response to *M. bovis* IMI was also characterized by the induction of acute phase protein synthesis of LBP and SAA (Figure 2C). Relative to control (time 0) concentrations of 4.32 ± 0.90 μg/mL, blood LBP concentrations increased within 96 h of *M. bovis* intramammary infusion and remained elevated throughout the study. Maximal blood concentrations of LBP were observed at 168 h postinfection and reached a peak of 37.43 ± 2.71 μg/mL. Blood SAA concentrations were increased, relative to control (time 0) concentrations of 104.22 ± 24.50 μg/mL, within 108 h of *M. bovis* administration and reached a peak of 593.12 ± 83.92 μg/mL 84 h later. Similar to LBP, blood SAA concentrations remained elevated throughout the study.

Changes in Milk SCC and Blood-Mammary Gland Barrier Function During M. bovis IMI

As a local sign of inflammation, milk was screened for increases in SCC (Figure 3A). Relative to control (time 0) counts, increased milk SCC were evident within 66 h of *M. bovis* infection and remained elevated throughout the study. Maximal elevations in milk SCC were observed 90 h postinfusion, reaching a concentration of $119.82 \times 10^6 \pm 29.36 \times 10^6$ cells/mL.

To determine whether IMI with *M. bovis* could alter the integrity of the blood-mammary gland barrier, milk concentrations of 2 blood-derived proteins, BSA and LBP, were assayed by ELISA. Relative to control (time 0) concentrations, increases in milk BSA concentrations were initially detected 78 h postinfection and were sustained from 90 h until the end of the study (Figure 3B). Maximal concentrations of milk BSA were detected on the final day of the study (240 h postinfection) and reached a mean (±SE) concentration of 3,542.67 ± 607.58 μg/mL. Increases in milk LBP were initially detected at 90 h postinfection and were sustained from 102 h until the end of the study (Figure 3C). Maximal milk LBP concentrations were detected 192 h after *M. bovis* administration and reached a peak of 35.80 ± 3.81 μg/mL. Increases in milk LBP were highly correlated with increases in milk BSA (r = 0.9589; $P < 0.0001$) and circulating concentrations of LBP (r = 0.9631; $P < 0.0001$).

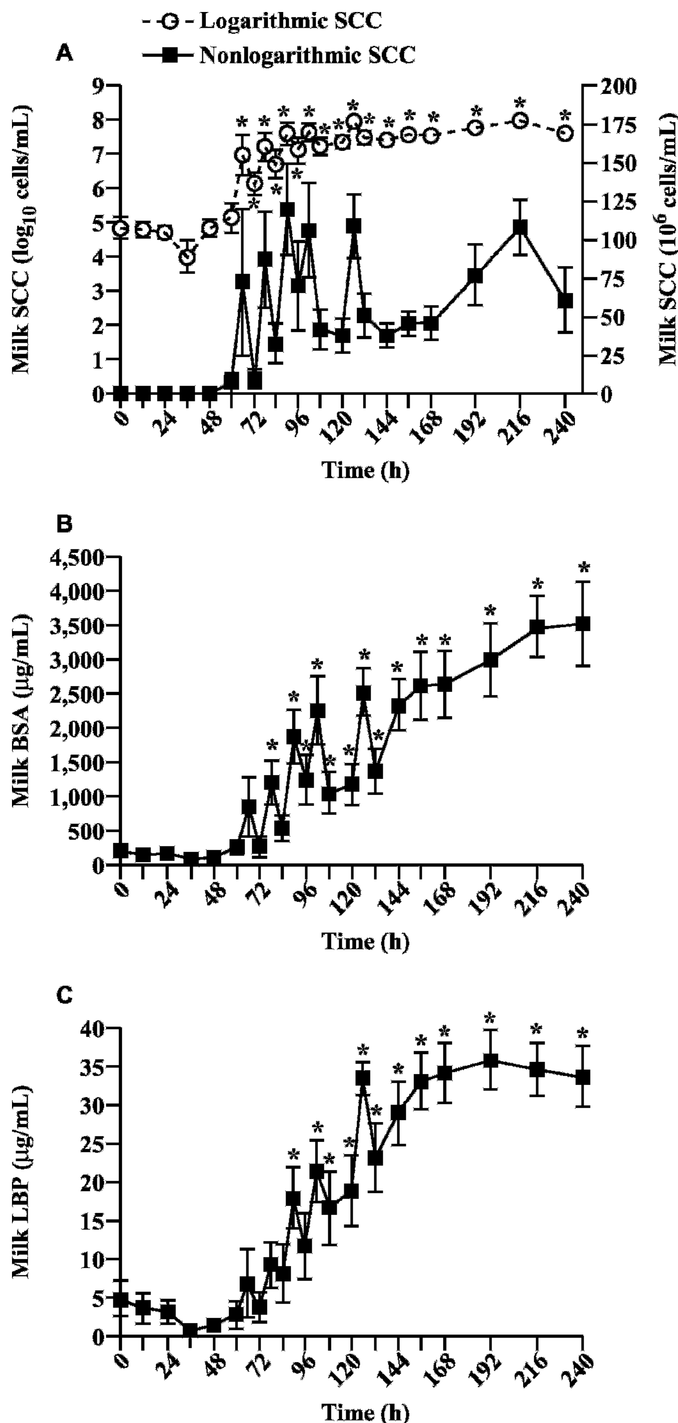

Figure 3. Effect of *Mycoplasma bovis* IMI on milk SCC and mammary influx of BSA and LPS-binding protein (LBP). Milk samples were collected immediately before (time 0) and at various time points following *M. bovis* intramammary infusion. Whole milk was analyzed for SCC (A) and milk whey was assayed by ELISA for BSA (B) and LBP (C). Mean (±SE) milk SCC (A) are reported in millions of cells per milliliter (solid squares) and $\log_{10}$ cells per milliliter (open circles). Mean (±SE) BSA (B) and LBP (C) concentrations are reported in micrograms per milliliter. *Increased ($P < 0.05$) relative to control (time 0) concentrations.

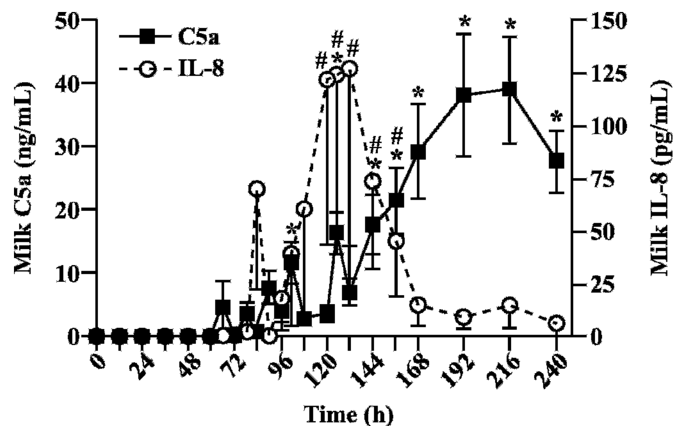

Figure 4. Effect of *Mycoplasma bovis* IMI on milk concentrations of the chemoattractants complement factor 5a (C5a) and IL-8. Milk samples were collected immediately before (time 0) and at various time points following *M. bovis* intramammary infusion. Concentrations of C5a (solid line; left y-axis) and IL-8 (dotted line; right y-axis) in milk whey were determined by ELISA and are expressed as mean (±SE) nanograms per milliliter and picograms per milliliter, respectively. *,#Increased ($P < 0.05$) concentrations of C5a or IL-8, respectively, relative to control (time 0) concentrations.

Complement Activation and IL-8 Production During M. bovis IMI

To determine whether *M. bovis* could evoke localized complement activation and IL-8 production, milk samples were collected before (time 0) and following experimental infection and were assayed by ELISA for C5a and IL-8 (Figure 4). Milk C5a concentrations were transiently elevated at 102 and 126 h postinfection and were consistently elevated from 144 h until the end of the study. Maximal concentrations of C5a were detected at 216 h postinfection and reached a peak concentration of 38.63 ± 8.33 ng/mL. Milk IL-8 concentrations initially increased 120 h after *M. bovis* infection and remained elevated for >36 h. Peak milk IL-8 concentrations of 126.07 ± 83.17 pg/mL were detected at 132 h postinfection.

M. bovis IMI Elicits Production of the Proinflammatory Cytokines TNF-α, IL-1β, and TGF-α

To assess the ability of *M. bovis* to evoke a proinflammatory cytokine response, TNF-α, IL-1β, and TGF-α concentrations were determined in milk samples collected before (time 0) and during the course of *M. bovis* IMI (Figure 5). There were no detectable concentrations of TNF-α or IL-1β in control (time 0) samples (Figure 5A and 5B). Initial elevations in TNF-α and IL-1β were observed between 96 and 102 h and 90 and 102 h postinfection, respectively, and sustained

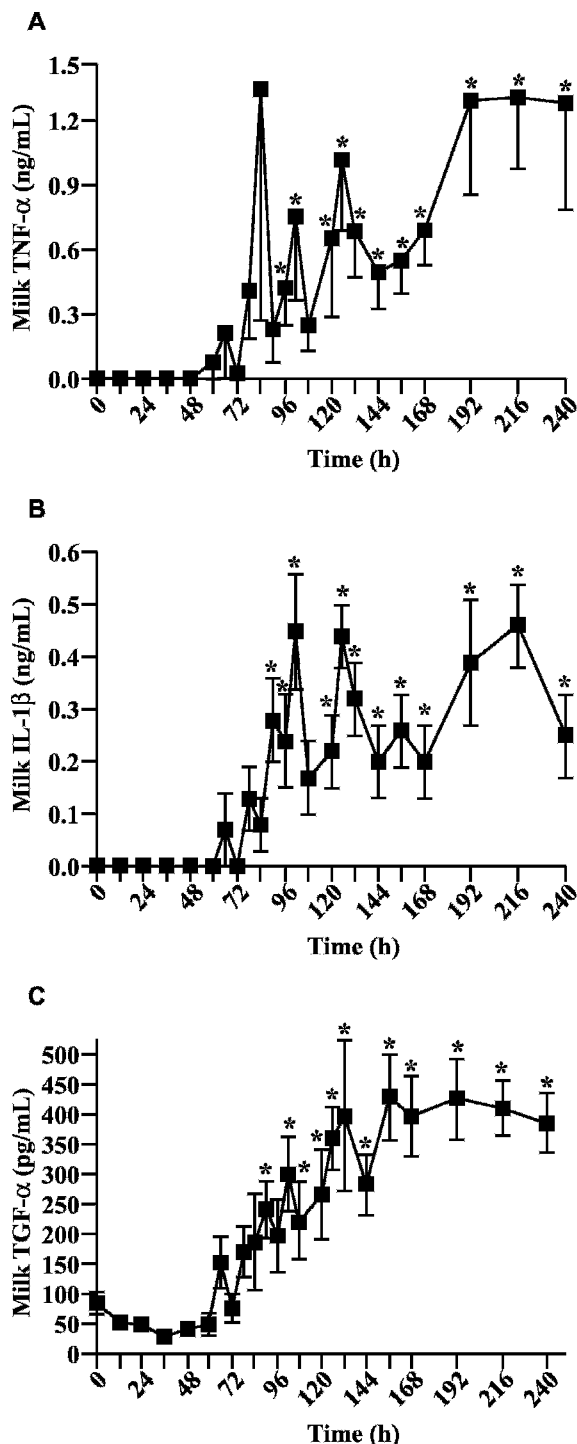

Figure 5. Effect of *Mycoplasma bovis* IMI on milk concentrations of the proinflammatory cytokines tumor necrosis factor (TNF)-α, IL-1β, and tumor growth factor (TGF)-α. Enzyme-linked immunosorbent assays were used to quantify the concentrations of TNF-α (A), IL-1β (B), and TGF-α (C) in whey obtained from quarter milk samples collected immediately before (time 0) and at various time points following intramammary infusion of *M. bovis*. Mean (±SE) TNF-α and IL-1β concentrations are reported in nanograms per milliliter; mean (±SE) TGF-α concentrations are reported in picograms per milliliter. *Increased ($P < 0.05$) relative to control (time 0) concentrations.

increases in both cytokines were detected from 120 h until the end of the study. Peak concentrations of TNF-α and IL-1β were both detected at 216 h postinfection and reached mean (±SE) maximal concentrations of 1.35 ± 0.34 and 0.46 ± 0.08 ng/mL, respectively. In contrast to TNF-α and IL-1β, TGF-α was present in control (time 0) samples (Figure 5C). The mean (±SE) basal concentration of TGF-α in these samples was 84.96 ± 18.46 pg/mL. Increases in milk TGF-α concentrations were initially observed 90 h after infection and were sustained from 102 h until the end of the study. Maximal TGF-α concentrations of 429.01 ± 72.13 pg/mL were observed at 156 h postinfection.

Induction of the Type 1 Helper T Cell Cytokines, IFN-γ and IL-12, During M. bovis IMI

Milk samples collected before (time 0) and following *M. bovis* IMI were assayed by ELISA for IFN-γ and IL-12 (Figure 6). There were no detectable concentrations of either cytokine in control (time 0) samples. Transient elevations in IFN-γ were detected at 90 and 102 h postinfection and sustained increases were detected from 126 h until the end of the study (Figure 6A). Peak concentrations of IFN-γ were detected 168 h after infusion of *M. bovis* and reached a concentration of 1,105.32 ± 249.08 pg/mL. Milk IL-12 concentrations increased within 78 h of infection and remained elevated throughout the study (Figure 6B). Interleukin-12 concentrations reached a maximum of 621.62 ± 112.23 U/mL at 126 h.

M. bovis IMI Elicits Production of the Anti-inflammatory Cytokines TGF-β1, TGF-β2, and IL-10

To assess the ability of *M. bovis* to evoke a counter-regulatory anti-inflammatory cytokine response, TGF-β1, TGF-β2, and IL-10 concentrations were determined in milk samples collected before (time 0) and during the course of *M. bovis* IMI (Figure 7). The mean (±SE) basal concentrations of TGF-β1 and TGF-β2 in control (time 0) samples were 4.84 ± 0.52 and 57.09 ± 9.14 ng/mL, respectively (Figure 7A). Relative to control concentrations, significant increases in TGF-β1 were observed at 216 h (d 9) and 240 h (d 10) postinfection and mean (±SE) concentrations on these days were 7.43 ± 1.29 and 10.30 ± 2.17 ng/mL, respectively. Significant increases in TGF-β2 were detected only in milk from the final sampling on d 10 (240 h postinfection), and the mean (±SE) concentration of TGF-β2 in these samples was 115.42 ± 29.29 ng/mL. The other anti-inflammatory cytokine assayed, IL-10, was completely absent from control (time 0) samples (Figure

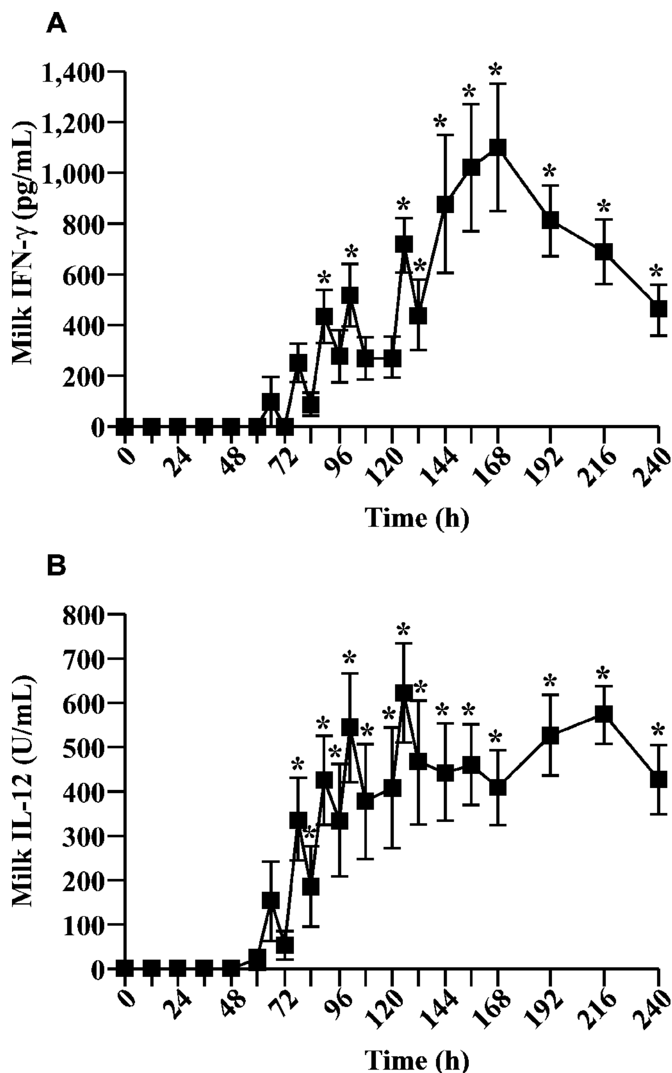

Figure 6. Effect of *Mycoplasma bovis* IMI on milk concentrations of IFN-γ and IL-12. Milk samples were collected immediately before (time 0) and at various time points following *M. bovis* intramammary infusion. Concentrations of IFN-γ (A) and IL-12 (B) in milk whey were determined by ELISA and expressed as mean (±SE) picograms per milliliter and units per milliliter, respectively. *Increased (P < 0.05) relative to control (time 0) concentrations.

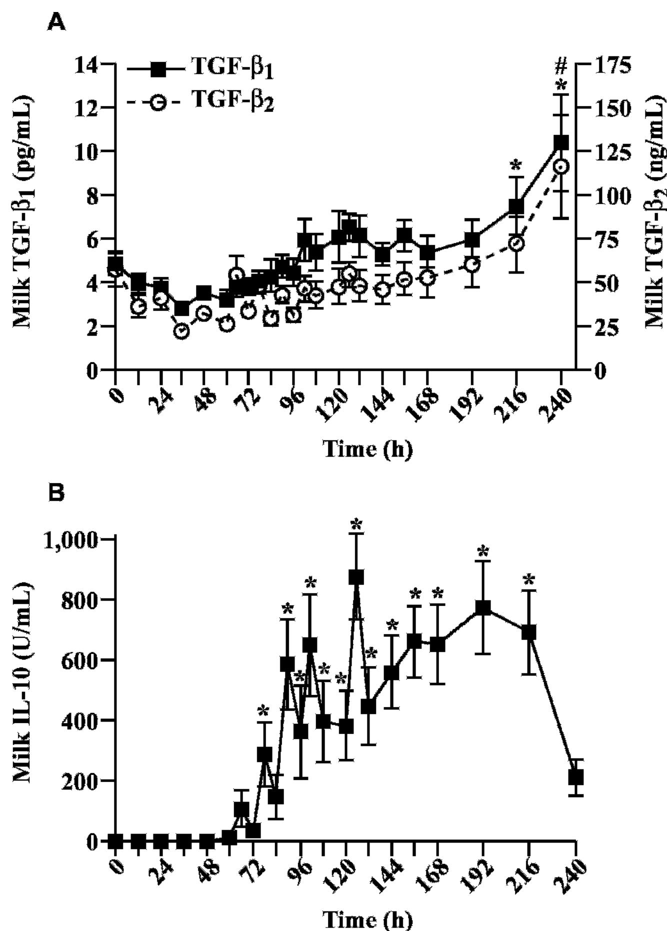

Figure 7. Effect of *Mycoplasma bovis* IMI on milk concentrations of the anti-inflammatory cytokines tumor growth factor (TGF)-β_1, TGF-β_2, and IL-10. Enzyme-linked immunosorbent assays were used to quantify the concentrations of TGF-β_1 (A), TGF-β_2 (A), and IL-10 (B) in whey obtained from quarter milk samples collected immediately before (time 0) and at various time points following intramammary infusion of *M. bovis*. Mean (±SE) TGF-β_1 (solid line; left y-axis) and TGF-β_2 (dotted line; right y-axis) concentrations are reported in nanograms per milliliter; mean (±SE) IL-10 concentrations are reported in units per milliliter. *Increased (P < 0.05) TGF-β_1 (A) or IL-10 (B) concentrations relative to control (time 0) concentrations; #increased (P < 0.05) TGF-β_2 (A) concentrations relative to control (time 0) concentrations.

7B). A transient increase in IL-10 was observed at 78 h postinfection and sustained increases were detected from 90 h until the end of the study. A mean (±SE) maximal IL-10 concentration of 878.94 ± 143.22 U/mL was detected 126 h after intramammary infusion of *M. bovis*.

DISCUSSION

In the present study, an inoculating dose of 3×10^4 cfu of *M. bovis* was used successfully to establish an IMI in all 10 cows. The range of intramammary inoculating doses of *M. bovis* used in previous studies has varied greatly and has ranged from 70 cfu (Bennett and Jasper, 1980) to 1.5×10^{11} cfu (Bennett and Jasper, 1978b). The most common inoculating amounts reported have been in the range of 10^6 to 10^8 cfu (Jain et al., 1969; Horvath et al., 1983; Meszaros et al., 1986; Boothby et al., 1988; Byrne et al., 2005). Across these studies, and regardless of the size of the inoculating dose, maximal intramammary growth of *M. bovis*, clinical symptoms, and milk SCC responses have all been comparable, although the kinetics of the onset of these events has differed slightly. Similarly, a recent study

comparing 2 inoculating doses of *E. coli*, which differed by 2 log-fold, showed that both doses induced comparable inflammatory and cytokine responses, with only slight differences in the kinetics of a limited number of the responses studied (Vangroenweghe et al., 2004).

Experimentally induced IMI with *E. coli* (Shuster et al., 1997; Riollet et al., 2000; Bannerman et al., 2004c; Vangroenweghe et al., 2004), *Pseudomonas aeruginosa* (Schalm et al., 1967; Bannerman et al., 2005), *Streptococcus uberis* (Thomas et al., 1994; Pedersen et al., 2003; Rambeaud et al., 2003), or *Staph. aureus* (Gudding et al., 1984; Riollet et al., 2000; Bannerman et al., 2004c) has typically resulted in milk bacterial growth to concentrations of 10^4 to 10^6 cfu/mL. In the current study with *M. bovis*, bacterial counts reached maximal concentrations of 10^9 cfu/mL of milk (Figure 1). The ability of milk to support growth of this pathogen at such high concentrations has been reported with other strains of *M. bovis* as well (Horvath et al., 1981; Boothby et al., 1986; Byrne et al., 2005).

In addition to differences in maximal intramammary growth concentrations between *M. bovis* and other mastitis-causing pathogens, there is also a profound difference in the lag time between initial intramammary infusion and the earliest time at which bacteria growth reaches concentrations that are detectable in milk. Experimental inoculation of quarters with high amounts of *M. bovis* (10^8 cfu/quarter) has been reported to enable detectable pathogen recovery from milk samples collected within 24 h of infusion (Byrne et al., 2005). In the current study, in which a dose ~10,000-fold smaller was administered, only 20% of the quarters infused with 3×10^4 cfu of *M. bovis* had detectable amounts of *M. bovis* within 36 h of infusion (Figure 1). It was not until 84 h after infusion that all quarters had detectable amounts of *M. bovis*. Similar lag times of 3 to 4 d between intramammary infusion and recovery have been reported following inoculation of 70 cfu of *M. bovis* (Bennett and Jasper, 1978a). Thus, a considerable lag time (>3 d) following intramammary infusion of low amounts of *M. bovis* ($\leq 10^4$ cfu/quarter) is necessary before growth reaches amounts that are detectable in milk. In contrast to *M. bovis*, recovery of *E. coli* (Shuster et al., 1997; Riollet et al., 2000; Bannerman et al., 2004c), *Klebsiella pneumoniae* (Bannerman et al., 2004b), *P. aeruginosa* (Bannerman et al., 2005), *Serratia marcescens* (Bannerman et al., 2004a), *Strep. uberis* (Bannerman et al., 2004a), and *Staph. aureus* (Riollet et al., 2000; Bannerman et al., 2004c) within 24 h of intramammary infusion of low amounts (~50 to 250 cfu) of these bacteria has been reported, indicating that these pathogens are more readily adaptable to the environment within the mammary gland.

Previous studies have typically reported the spread of *M. bovis* to 40 to 100% of nonchallenged quarters following experimental infection of a single quarter (Jain et al., 1969; Bennett and Jasper, 1978a; Boothby et al., 1986; Byrne et al., 2005). In the current study, *M. bovis* was detected in only 1 nonchallenged quarter on 3 of the 10 cows (i.e., 10% of noninfused glands). In both previous studies and the current study, rigorous sanitation methods have been used to prevent fomite spread through the milking unit. These methods included running detergent and sanitizing solutions through the teat cups, milk claw, and milk bucket between milking of each animal. In addition, the same quarter was infused with *M. bovis* within an experimental group of cows that were milked, ensuring that teat cups always came into contact with quarters that were or were not originally infused with *M. bovis*. In addition to spread through fomites, there is evidence that *M. bovis* can spread hematogenously (Jain et al., 1969; Bennett and Jasper, 1978a). Therefore, *M. bovis* strain-dependent differences in the ability to spread through the blood may account for the differential rates of infectivity of nonchallenged quarters reported in the current and previous studies. In addition, the length of time that cows were followed was considerably longer in those studies reporting greater infectivity rates of nonchallenged quarters. For several of the nonchallenged quarters that eventually became infected in previous studies, *M. bovis* was not detected in milk from those quarters until 10 to 15 d after infection of the single experimental quarter (Bennett and Jasper, 1978a; Boothby et al., 1986; Byrne et al., 2005). Thus, the ability of the *M. bovis* strain used in the current study to spread to noninfused quarters may be equivalent to that of other strains, but was not confirmed because of the limited 10-d experimental period used to investigate the immediate inflammatory response to *M. bovis* infection. Finally, stage of lactation and parity have been reported to influence the severity and localized control of infection in cases of mastitis caused by other pathogens (Burvenich et al., 2003). In the current study, only primiparous cows in late lactation were experimentally infected. Therefore, one cannot discount the influences that these factors may have on limiting the spread of *M. bovis* to other quarters.

In the current study, *M. bovis* failed to evoke a febrile response (i.e., mean rectal temperature >39.5°C), a finding that is consistent with previous reports demonstrating that IMI with other strains of *M. bovis* are characterized by the complete absence of fever or induction of a slight and transient elevation in body

temperature in a limited subset of cows within a group of experimentally infected animals (Jasper et al., 1966; Jain et al., 1969; Bennett and Jasper, 1978a; Byrne et al., 2005). Similar to *M. bovis*, different strains of *Staph. aureus*, which establish chronic IMI comparable to that of *M. bovis*, also fail to induce fever (Riollet et al., 2000; Bannerman et al., 2004c; Sladek et al., 2005; Wall et al., 2005). This contrasts with the ability of mastitis-inducing pathogens, such as *E. coli* (Shuster et al., 1997; Bannerman et al., 2004c; Vangroenweghe et al., 2004), *K. pneumoniae* (Rose et al., 1989; Bannerman et al., 2004b), *S. marcescens* (Bannerman et al., 2004a), and *Strep. uberis* (Pedersen et al., 2003; Rambeaud et al., 2003; Bannerman et al., 2004a), to elicit a febrile response.

Although *M. bovis* did not induce fever, other systemic responses were elicited, including neutropenia, lymphopenia, and thrombocytopenia (Figure 2A and 2B). The observed transient neutropenia is consistent with the previous finding of a study involving 3 cows (Jain et al., 1969). Maximal increases in milk SCC (Figure 3A) occurred within 6 h of initial decreases in circulating neutrophils (Figure 2A). Because >90% of milk somatic cells during acute mastitis are neutrophils (Saad and Ostensson, 1990), this finding is compatible with neutrophil migration from the vascular compartment to the infected quarter. Although observed neutropenia and lymphopenia following IMI are not unique to this pathogen and have been reported in response to other pathogens (Bannerman et al., 2004a,b,c, 2005), the duration of these responses was profoundly prolonged following *M. bovis* infection. The prolonged decrease in circulating concentrations of these cells and sustained increase in milk SCC may reflect the continual attempt of the host to recruit effector cells to control and eradicate *M. bovis*, which persisted in the gland at high concentrations throughout the study (Figure 1). In addition to neutropenia and lymphopenia, a persistent state of thrombocytopenia was observed (Figure 2B). To our knowledge, this is the first study to evaluate changes in circulating platelets in response to IMI with *M. bovis*. There are several reports of thrombocytopenia, platelet aggregation, or thrombus formation during the course of Mycoplasma infection in cows, goats, and humans (Lloyd et al., 1975; Rosendal, 1981; Ryan et al., 1983; Chiou et al., 1997; Chen et al., 2004). Although the definitive cause of decreased circulating platelets during Mycoplasma infections remains unknown, disseminated intravascular coagulation, decreases in megakaryocytes, and the generation of autoantibodies that promote platelet destruction have all been implicated.

The finding that *M. bovis* IMI resulted in increased milk BSA and LBP concentrations (Figure 3B and C) indicates the ability of this pathogen to induce an inflammatory response that alters the mammary vascular barrier function. Because changes in milk BSA concentrations have been shown to parallel those of IgG (Rainard and Caffin, 1983), the increase in milk BSA concentrations is consistent with previous studies demonstrating increases in milk IgG concentrations following *M. bovis* infection (Bennett and Jasper, 1980; Boothby et al., 1987; Byrne et al., 2005). The duration of increased milk BSA concentrations over several days is comparable with that of IMI caused by other pathogens not readily cleared from the mammary gland, including *K. pneumoniae* (Bannerman et al., 2004b), *Strep. uberis* (Bannerman et al., 2004a), and *P. aeruginosa* (Bannerman et al., 2005). In contrast to these pathogens and *M. bovis*, IMI with *Staph. aureus* is characterized by transient or less-pronounced alterations in mammary vascular barrier function, or both (Riollet et al., 2000; Bannerman et al., 2004c).

Mycoplasma bovis IMI was also characterized by the sustained activation of complement and production of the cytokines TNF-α, IL-1β, TGF-α, IFN-γ, IL-12, IL-10, TGF-β1, and TGF-β2 (Figures 4 to 7). Similarly, *E. coli* IMI evokes production of these cytokines as well as complement activation (Riollet et al., 2000; Bannerman et al., 2004c; Chockalingam et al., 2005). Although *Staph. aureus* elicits TGF-α, IFN-γ, IL-12, TGF-β1, and TGF-β2 production, IMI with this pathogen is marked by a complete absence of TNF-α, IL-8, and IL-10 production and variable production of IL-1β and complement activation (Riollet et al., 2000; Bannerman et al., 2004c, 2006). Thus, in contrast to our stated hypothesis, the inflammatory mediators that are operative during the course of *M. bovis* IMI more closely reflect those that are involved in the host response to *E. coli* rather than to *Staph. aureus*.

There was a marked difference in the TNF-α response and the duration of complement activation between *M. bovis* and other intramammary pathogens that evoke these responses. Compared with *E. coli* (Riollet et al., 2000; Bannerman et al., 2004c), *K. pneumoniae* (Bannerman et al., 2004b), *S. marcescens* (Bannerman et al., 2004a), and *P. aeruginosa* (Bannerman et al., 2005), the maximal concentration of TNF-α detected in milk samples from *M. bovis* infection was ~10-fold less. The TNF-α concentrations reported here are comparable with those reported following IMI with strains of *Strep. uberis* that establish chronic infection (Rambeaud et al., 2003; Bannerman et al., 2004a). Elevated milk C5a concentrations, which reflect complement activation, were observed over a 138-h period during the course of *M. bovis* IMI (Figure 4). This duration was similar to that observed in response to *Strep. uberis*, but was markedly longer

than the ≤80-h duration reported following IMI with *E. coli* (Riollet et al., 2000; Bannerman et al., 2004c), *K. pneumoniae* (Bannerman et al., 2004b), *S. marcescens* (Bannerman et al., 2004a), and *P. aeruginosa* (Bannerman et al., 2005). The sustained increase in the neutrophil chemoattractant C5a in response to *M. bovis* may be responsible, in part, for the sustained elevation of milk SCC throughout the 10-d study (Figure 3A).

Relative to other intramammary pathogens studied, a unique feature of the inflammatory response to *M. bovis* was the delay in its induction relative to initial infusion of the bacteria. Inflammatory cytokine responses and complement activation elicited by *E. coli* (Riollet et al., 2000; Bannerman et al., 2004c), *K. pneumoniae* (Bannerman et al., 2004b), *S. marcescens* (Bannerman et al., 2004a), *P. aeruginosa* (Bannerman et al., 2005), and *Strep. uberis* (Rambeaud et al., 2003; Bannerman et al., 2004a) have all been reported to occur within 66 h, and more typically within 36 h, of initial infusion. Further, initial increases in milk SCC following infusion of these pathogens is observed within 30 h. In contrast, increases in milk SCC, cytokine production, and complement activation were all detected >66 h after *M. bovis* infusion. The delay in induction of these inflammatory responses may be attributed, in part, to the delayed growth of *M. bovis* within the gland (Figure 1). Therefore, only when *M. bovis* reaches a critical amount does the innate immune system seemingly respond by mounting an inflammatory response. Once induced, however, a prolonged inflammatory response was observed and may represent persistent, but futile, attempts of the innate immune system to control *M. bovis*.

Previous studies that have examined hematological, mammary vascular barrier function, and milk SCC responses to *M. bovis* IMI have been conducted following experimental challenge of only a few cows (Hale et al., 1962; Jain et al., 1969; Bennett and Jasper, 1978a,b, 1980; Horvath et al., 1981; Byrne et al., 2005). The low numbers in these studies has precluded statistical analysis of these parameters and has resulted in published reports on individual cow responses. In the current study, statistically appropriate numbers of animals were infected to enable analysis of these parameters. More importantly, this is the first report to elucidate the cytokines that are operative during *M. bovis* IMI.

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