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HOST RESPONSE AND INFLAMMATION

Immunosuppression in Cows following Intramammary Infusion of *Mycoplasma bovis*

Satoshi Gondaira,[a] Koji Nishi,[a] Takahiro Tanaka,[a] Takashi Yamamoto,[a] Takanori Nebu,[a] Reina Watanabe,[a] Satoru Konnai,[b] Tomohito Hayashi,[c] Yoshio Kiku,[c] Mariko Okamoto,[c] Kazuya Matsuda,[d] Masateru Koiwa,[e] Hidetomo Iwano,[f] Hajime Nagahata,[a,g] Hidetoshi Higuchi[a]

[a]Animal Health Laboratory, Graduate School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan
[b]Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[c]National Institute of Animal Health, National Agriculture and Food Research Organization, Sapporo, Hokkaido, Japan
[d]Department of Veterinary Pathology, Graduate School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan
[e]Department of Large Animal Sciences, Graduate School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan
[f]Department of Veterinary Biochemistry, Graduate School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido, Japan
[g]Farm Animal Veterinary Nursing Laboratory, Department of Veterinary Associated Science, Faculty of Veterinary Medicine, Okayama University Of Science, Imabari, Aichi, Japan

ABSTRACT *Mycoplasma bovis* is a destructive pathogen that causes large economic losses in rearing cattle for beef and dairy worldwide. *M. bovis* causes suppression of and evades the host immune response; however, the mechanisms of host immune function involved in *M. bovis* mastitis have not been elucidated. The purpose of this study was to elucidate the characteristics of the bovine immune response to mycoplasmal mastitis. We evaluated the responsiveness of the bovine mammary gland following infusion of *M. bovis*. Somatic cell counts and bacterial counts in milk from the infected quarter were increased. However, the proliferation of peripheral blood mononuclear cells (blood MNCs) and mononuclear cells isolated from *M. bovis*-stimulated mammary lymph nodes (lymph node MNCs) did not differ from that in the unstimulated cells. Transcriptome analysis revealed that the mRNA levels of innate immune system-related genes in blood MNCs, complement factor D (CFD), ficolin 1 (FCN1), and tumor necrosis factor superfamily member 13 (TNFSF13) decreased following intramammary infusion of *M. bovis*. The mRNA levels of immune exhaustion-related genes, programmed cell death 1 (PD-1), programmed cell death-ligand 1 (PD-L1), lymphocyte activation gene 3 (LAG3), and cytotoxic T-lymphocyte-associated protein 4 (CTLA4) of milk mononuclear cells (milk MNCs) in the infected quarter were increased compared with those before infusion. Increase in immune exhaustion-related gene expression and decrease in innate immune response-related genes of MNCs in quarters from cows were newly characterized by *M. bovis*-induced mastitis. These results suggested that *M. bovis*-induced mastitis affected the immune function of bovine MNCs, which is associated with prolonged duration of infection with *M. bovis*.

KEYWORDS mycoplasma, cattle, veterinary immunology

Mycoplasmas, bacteria of the *Mollicutes* class, do not have a cell wall, are widespread in nature, and infect eukaryotes (1). *Mycoplasma bovis* is a destructive pathogen of beef and dairy cattle worldwide (2, 3) that is known to be a major contributing factor to the occurrence of mastitis, pneumonia, and arthritis (2, 4), which all contribute to large economic losses on dairy farms (2, 5). *M. bovis* infection leads to calf mortality, weight loss in surviving calves, and a decline in milk production in dairy cows (2, 6). Mycoplasma mastitis, particularly intramammary infection with *M. bovis*,

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Address correspondence to Hidetoshi Higuchi, higuchi@rakuno.ac.jp.

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increases somatic cell counts (SCCs; 10^7 to 10^9 cells/ml) in milk, as do other pathogens causing mastitis (7–9). *M. bovis*-induced mastitis causes swelling and induration of the udder following a marked decrease of milk yield (3). *M. bovis*-induced mastitis is also known to have a long infection period and shows a subclinical type of mammary infection (10). It has been reported that *M. bovis*-induced mastitis causes a sustained increase in SCCs and proinflammatory cytokines in milk (9). However, the sustained inflammatory response is not strong enough to eradicate *M. bovis* from the mammary gland (9). The major cell population present in mastitis comprises neutrophils (>90%), and the influx of neutrophils into the mammary gland is mediated by mononuclear cells (MNCs) (11). Although subpopulations of MNCs are of various types and have various functions (12), the trafficking of different lymphocyte subpopulations in *M. bovis*-induced mastitis is not fully understood.

M. bovis is thought to evade the host immune response, and our previous study showed that *M. bovis* evades bovine neutrophil extracellular traps (13). *M. bovis* exhibits immunosuppressive characteristics that inhibit the proliferation of lymphocytes in response to mitogens (14–16). In a long-period infection, immunosuppressive factors, including programmed cell death 1 (PD-1), lymphocyte activation gene 3 (LAG3), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), T cell immunoglobulin, and mucin-domain containing-3 (Tim3), are expressed on lymphocytes (17). These proteins bind to their respective ligands to induce immune exhaustion of the effector cells (17). These immune exhaustion-related proteins induce a marked reduction in cell proliferation and cytotoxic activity (18, 19). However, the underlying mechanisms of the immunosuppressive host immune response of *M. bovis*-induced mastitis have not been elucidated.

The purpose of this study was to elucidate the characteristics of the host immune response to mycoplasmal mastitis. Furthermore, we aimed to evaluate the response of the bovine mammary gland after infusion of *M. bovis*.

RESULTS

Quantification of the SCC and bacterial counts in milk. The SCCs of each cow following intramammary infusion of *M. bovis* are shown in Fig. 1a. The SCCs of the infected quarter at day 2 to 5 increased sharply in comparison with the SCCs of day 1 in three cows. The SCCs of the infected quarter of each cow at days 8 to 11 peaked at $>10^7$ cells/ml. The SCCs of B quarter at days 9 to 11 increased compared with that at day 1, and the SCCs of B quarter at day 14 (cow1), day 12 (cow2), and day 13 (cow3) peaked at $>10^5$ (cow1), $>10^7$ (cow2), and $>10^6$ (cow3) cells/ml, respectively. The SCCs of C and D quarters tended to increase until day 14 postinfusion of *M. bovis*, but the SCC was $<3.0 \times 10^5$ cells/ml (except for quarter C in cow3 at day 7 and days 12 to 14). The bacterial counts following intramammary infusion of *M. bovis* are shown in Fig. 1b. The bacterial counts of the infusion quarter at day 7 to 11 peaked at 10^8 to 10^9 CFUs/ml in three cows. *M. bovis* was detected in all B quarters in three cows, and the bacterial counts of *M. bovis* were $>10^3$ (cow1 and cow3) and $>10^7$ (cow2) CFU/ml.

Surface marker analysis of blood MNCs and milk MNCs. The surface marker analysis of blood MNCs and milk MNCs is shown in Fig. 2. The average ratios of CD4-, CD8-, CD21-, WC1-, and CD14-positive cells in the blood MNCs were not significantly altered. However, the average ratio of CD4-positive cells in the blood MNCs was increased until day 14 compared with that at day 0. The average ratios of CD4-, CD8-, and CD21-positive cells in the milk of the infected quarter at days 7 to 14 were higher than those of the noninfusion quarter (D quarter). The average ratios of WC1 (days 1, 3, and 7)- and CD14 (day 7)-positive cells in the milk of the infected quarter were higher than those of the noninfusion quarter, D quarter.

Proliferation of MNCs stimulated with *M. bovis*. The proliferation of blood MNCs and lymph node MNCs stimulated with concanavalin A (ConA) and *M. bovis* at day 0 (only blood MNCs) and day 14 is shown in Fig. 3. The proliferation of blood MNCs stimulated with ConA at day 14 was higher than that at day 0; however, *M. bovis* did not induce changes in cell proliferation (both PG45 and five wild-type strains). The

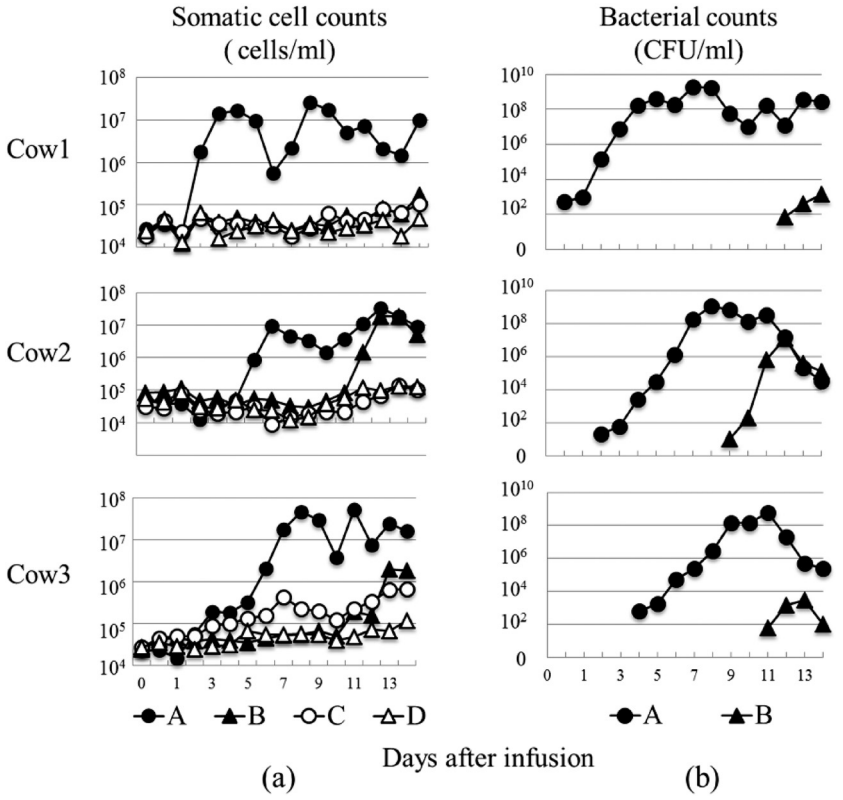

FIG 1 SCC and bacterial counts following intramammary infusion of *M. bovis*. (a) SCC ($\times 10^4$ cells/ml) and (b) bacterial counts (CFU/ml) evaluated at days 0, 0.5, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, and 14 postinoculation in the *M. bovis* inoculation quarter (A, left forequarter), PBS inoculation quarter (D, right rear quarter), and other quarters (B, left rear quarter; C, right forequarter) in three cows.

lymph node MNCs stimulated with *M. bovis* did not exhibit a change in proliferation compared with that of the nonstimulated cells.

Microarray analysis. Statistical analysis revealed that at day 7, following intramammary infusion of *M. bovis*, the expression levels of 47 genes in the blood MNCs were significantly altered ($P < 0.025$ with a fold increase of >2) compared with those at day 0 (Fig. 4a; Table S1 in the supplemental material). Only the gene expression of transglutaminase 3 (TGM3) in blood MNCs was significantly increased, whereas the expression levels of the remaining 46 genes were significantly decreased. Gene ontology (GO) terms were identified as cell morphogenesis and immune system process (Fig. 4b; Table S2 in the supplemental material). For validation of this result, genes associated with the immune system were quantified using real-time PCR (Fig. 4c). The mRNA expression levels of complement factor D (CFD), ficolin 1 (FCN1), and tumor necrosis factor superfamily member 13 (TNFSF13) of the blood MNCs at day 7 were significantly ($P < 0.01$) decreased compared with those at day 0.

Immune exhaustion-related gene expression. The mRNA expression levels of PD-1, PD-1 ligand 1 (PD-L1), lymphocyte activation gene 3 (LAG3), cytotoxic T-lymphocyte-associated protein 4 (CTLA4), and T cell immunoglobulin and mucin-domain containing-3 (Tim3) in blood MNCs and milk MNCs following intramammary infusion of *M. bovis* were evaluated (Fig. 5). The mRNA expression levels of PD-1, LAG3, and CTLA4 in the blood MNCs following intramammary infusion of *M. bovis* were higher than those at day 0. The mRNA expression levels of PD-1, PD-L1, LAG3, and CTLA4 of milk MNCs in the *M. bovis*-infected quarter were increased compared with those at day 0; however, the mRNA expression levels of PD-1, LAG3, and CTLA4 in the milk MNCs of the noninfusion quarter (D quarter) were unchanged. The mRNA expression levels of PD-1 (at days 3 and 14), PD-L1 (at day 7), LAG3 (at day 14), and CTLA4 (at days 7 and 14) of milk MNCs were significantly ($P < 0.05$) increased compared with those at day 0.

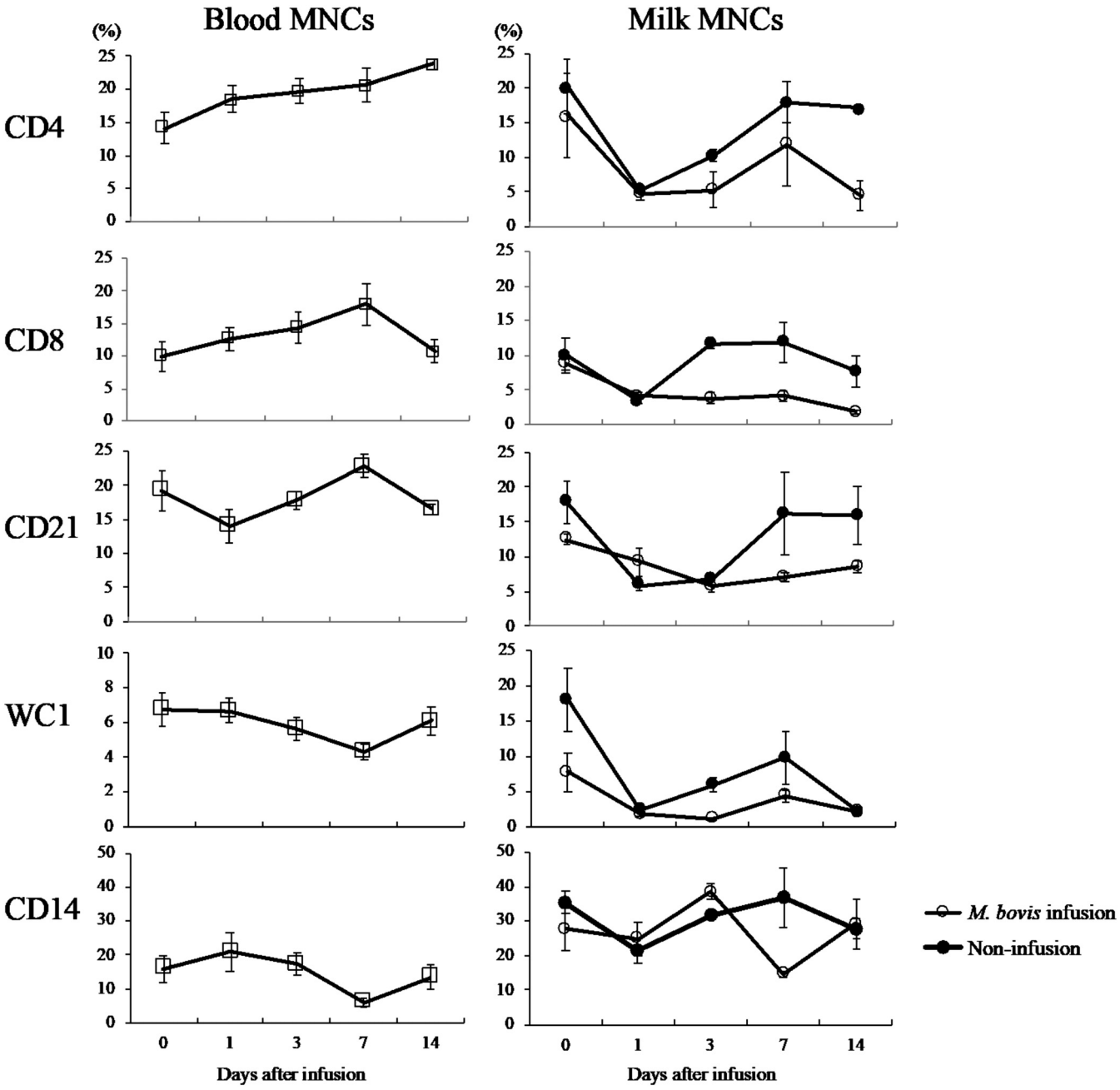

FIG 2 Surface marker analysis of blood MNCs and milk MNCs following intramammary infusion of *M. bovis*. Effect at days 0, 1, 3, 7, and 14 following intramammary infusion of *M. bovis* on the populations of blood MNCs and milk MNCs in the *M. bovis* inoculation quarter (left forequarter) and noninfusion quarter (right rear quarter) as determined by flow cytometry. Data are presented as the mean ± SEM of the results from three cows. Percentages of cells in blood MNCs and milk MNCs gate positive for CD4, CD8 CD21, WC1, and CD14.

DISCUSSION

The present study characterized the response of the immune system to *M. bovis*-induced mastitis, particularly the effects on immune function in MNCs during *M. bovis*-induced mastitis. Following intramammary infusion of *M. bovis*, the maximal bacterial counts of *M. bovis* in milk were 10^8 to 10^9 CFU/ml. *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* induce counts of 10^3 to 10^5 CFU/ml in milk following their intramammary infusion (7, 8). The results of this study showed that bacterial counts in milk from the *M. bovis*-infected quarter were 10^3 to 10^6 times higher than those from other pathogens. Although the specific mechanisms for this increased

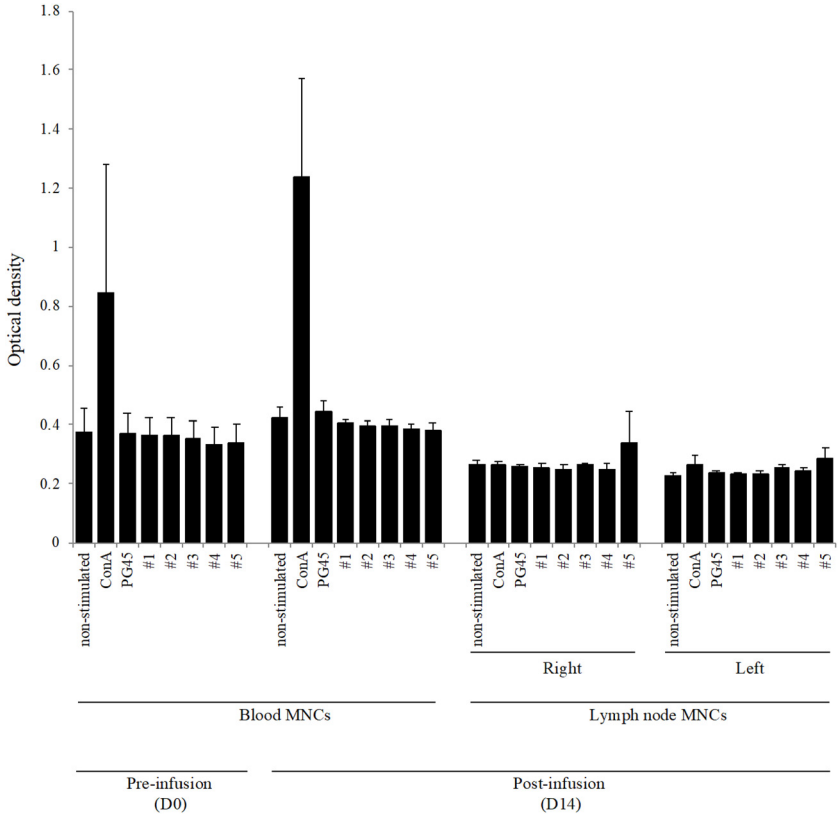

FIG 3 Proliferation of blood MNCs and lymph node MNCs stimulated with *M. bovis* or ConA. Bovine blood MNCs and lymph node MNCs were incubated with live *M. bovis* (multiplicity of infection of 100; PG45 strain and five wild-type strains) or ConA. The optical density (OD) values are expressed as the mean ± SE from the results in three cows.

infection are unknown, one possible explanation is the evasion of *M. bovis* from the immune response of the mammary gland. In this study, *M. bovis* was detected not only in the A quarter (infused quarter) but also in the B quarter (noninfused quarter). These results were consistent with a previous report that *M. bovis* can spread to nonchallenged quarters (9). The maximal SCC reached a peak of $37.0 \times 10^6 \pm 3.8 \times 10^6$ cells/ml in the infused quarter. Bannerman et al. (7, 8) reported that the SCC level of milk obtained from bacterium-infused quarters of *E. coli* was $44.9 \times 10^6 \pm 5.0 \times 10^6$ cells/ml; that from *S. aureus*, $32.1 \times 10^6 \pm 5.9 \times 10^6$ cells/ml; and that from *P. aeruginosa*, $62.9 \times 10^6 \pm 6.4 \times 10^6$ cells/ml. From these results, the highest level of SCCs following the intramammary infusion of *M. bovis* was similar to that of *E. coli* and *S. aureus* infusions.

In the present study, the major cells of recruitment in the milk of *M. bovis*-induced mastitis were neutrophils, as with other causes of mastitis (11). Although MNCs serve an important role in the influx of neutrophils to the mammary gland or are associated with excessive inflammatory response (11), characterization of the surface marker of MNCs has not been fully clarified in *M. bovis*-induced mastitis. In the present study, it was first shown that CD4- and CD8-positive cells at day 3, 7, and 14 postinfusion of *M. bovis* were marginally increased compared with those in the D quarter (noninfusion quarter). CD4-positive T cells enhance humoral and cell-mediated immunity. In addition, CD8-positive T cells are associated with cell-mediated immunity and the T cell-mediated recruitment of neutrophils during bacterial infection (20). We showed that the numbers of CD21-positive cells in milk at days 3, 7, and 14 postinfusion of *M. bovis* tended to be higher than those in the D quarter. CD21-positive cells are capable of antigen presentation (21), secretion of cytokines (22), and differentiation into plasma cells that produce immunoglobulins (23). It has

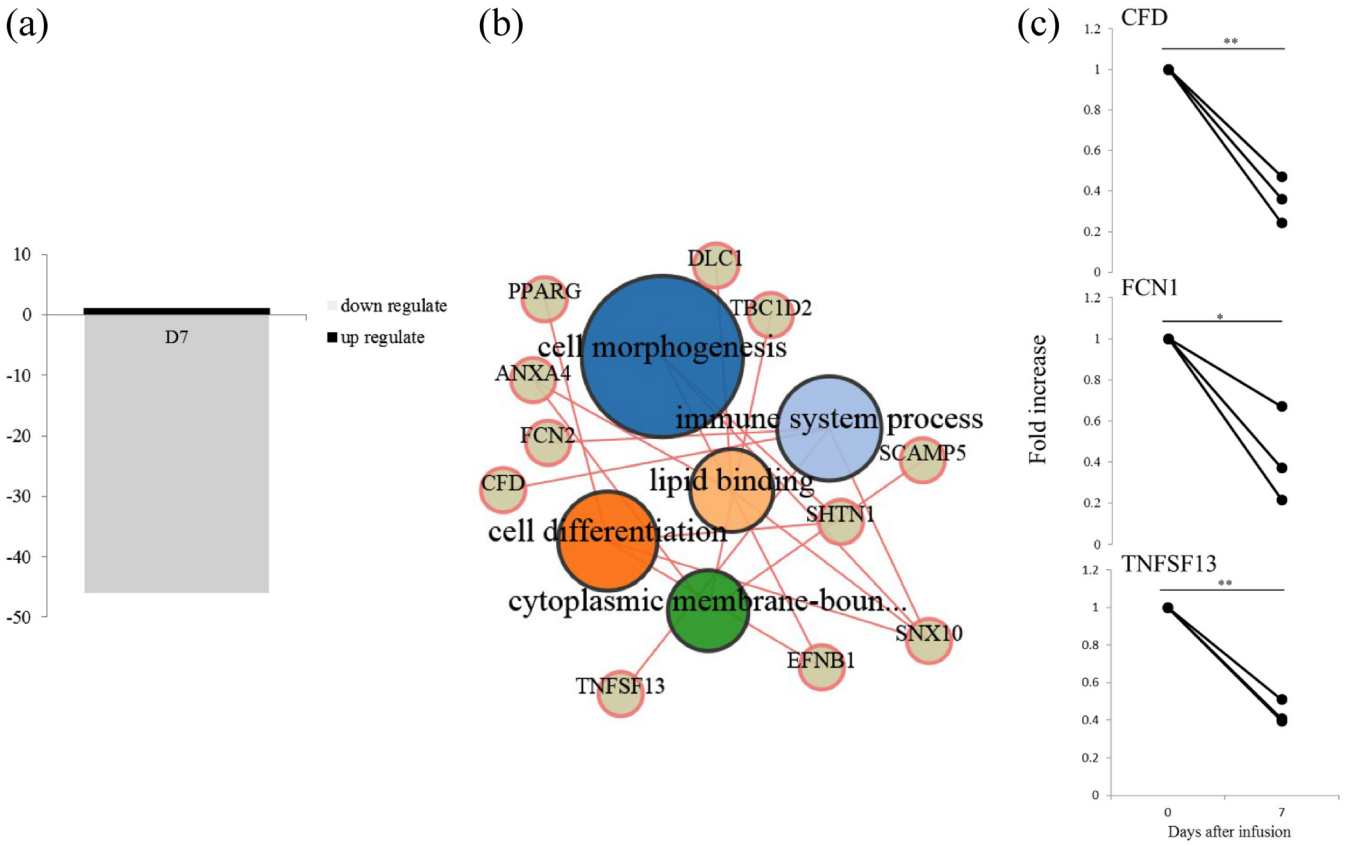

FIG 4 Microarray analysis in blood MNCs following intramammary infusion of *M. bovis*. Blood MNCs evaluated at days 0 and 7 postintramammary infusion of *M. bovis* in three cows. (a) Number of significantly downregulated or upregulated mRNAs; (b) gene ontology term enrichment analysis of genes recognized as significantly different in blood MNCs following intramammary infusion of *M. bovis*; (c) quantification of mRNA expression by microarray analysis; significant difference at $*P < 0.05$ and $**P < 0.01$ compared with day 0.

been reported that CD4-, CD8-, and CD21-positive cells in milk are significantly increased in *S. aureus*- or *E. coli*-induced mastitis (24–26). Our results suggest that the CD4-, CD8-, and CD21-positive cells in the milk MNCs are weakly affected by *M. bovis*-induced mastitis compared with *E. coli*- or *S. aureus*-induced mastitis.

Of note, the stimulation of *M. bovis* in MNCs isolated from mammary lymph nodes and blood MNCs following intramammary infusion of *M. bovis* did not induce cell proliferation. It has been reported that blood MNC proliferation is inhibited by *M. bovis* infection (14–16) and that *M. bovis* induces the colonization of other pathogenic bacteria (2, 27). Our results suggest that effects on immune function in *M. bovis* infection may prevent proliferation.

The expression levels of genes associated with the innate immune response were significantly decreased in the transcriptome analysis of blood MNCs at day 7. GO enrichment analysis suggests that *M. bovis*-induced mastitis influences the suppression of cell morphogenesis, which is related to the cell migration and immune system process. Two genes associated with complement activation were significantly decreased; FCN1 is associated with the lectin pathway and CFD is associated with another pathway (28, 29). TNFSF13, associated with the proliferation of B cells, was also significantly decreased (30). Although further studies are required, our results indicate that *M. bovis*-induced mastitis influenced inhibition of the host innate immune response. In addition, the mRNA expression levels of PD-1, LAG3, and CTLA4 of blood MNCs were higher following the intramammary infusion of *M. bovis*. The mRNA expression levels of PD-1, PD-L1, LAG3, and CTLA4 in milk MNCs were also increased. These immune checkpoint-related genes are known to be associated with T cell exhaustion (17), inducing marked reductions in cell proliferation and cytotoxic activity

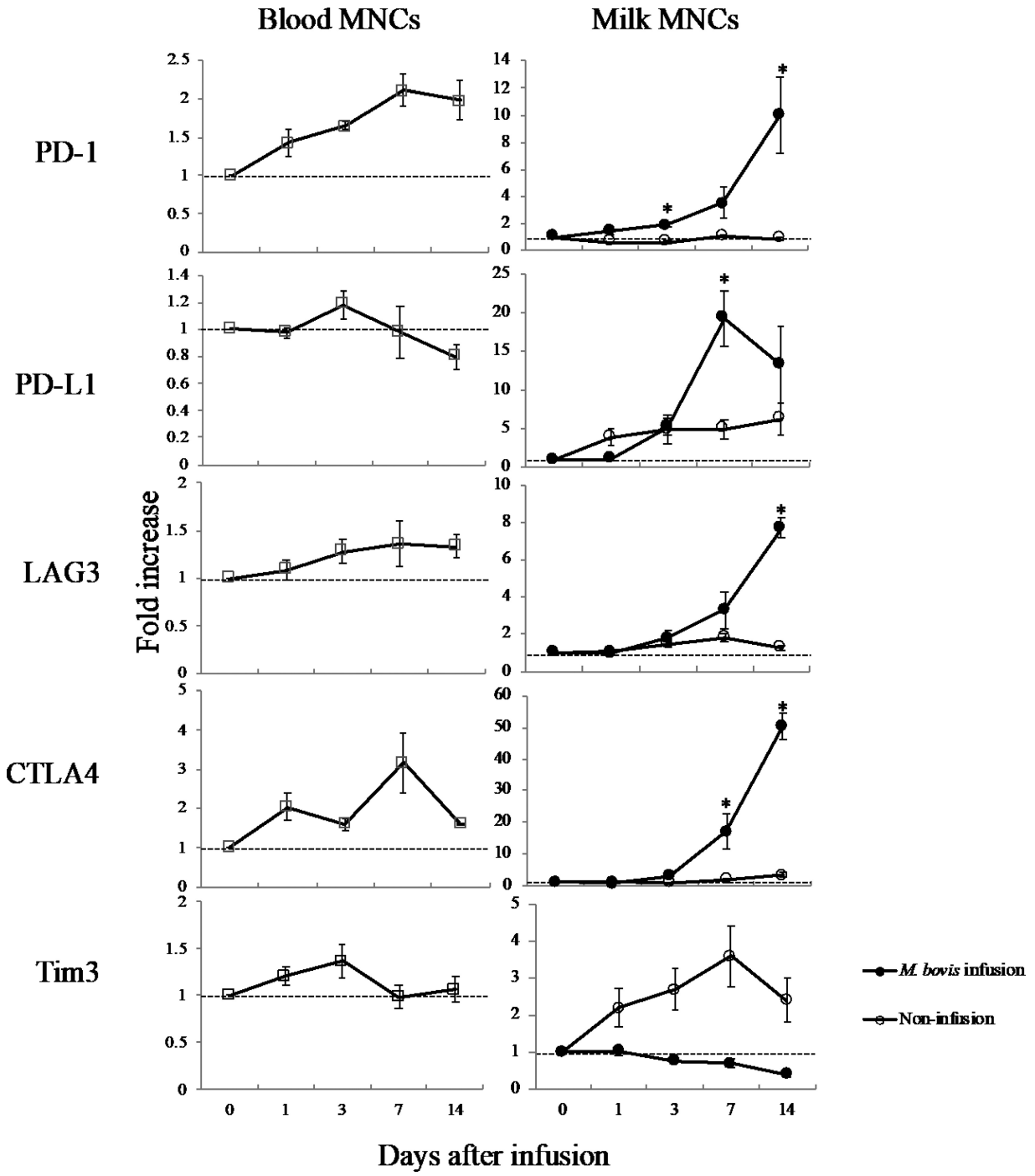

FIG 5 mRNA expression of immune exhaustion-related genes following intramammary infusion of *M. bovis*. Blood MNCs and milk MNCs from the *M. bovis* inoculation quarter (A, left forequarter) and noninfusion quarter (D, right rear quarter), following intramammary infusion of *M. bovis*, were evaluated on days 0, 1, 3, 7, and 14. The mRNA expression levels of PD-1, PD-L1, LAG3, CTLA4, and Tim3 were determined by real-time PCR and expressed as a fold increase. The data are expressed as the mean ± SE from the results in three cows; significant difference at $^*P < 0.05$ compared with day 0.

(18, 19). Our results suggest that *M. bovis* induced immune exhaustion following the downregulation of cell proliferation. This is the first report to demonstrate that *M. bovis* can trigger immune exhaustion in *M. bovis*-induced mastitis. *M. bovis* affects the cell function and gene expression of bovine MNCs in *M. bovis*-induced mastitis, which may contribute to a long period of infection and lead to severe mastitis.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study were *M. bovis* (PG45, ATCC 25523 and five wild-type strains). *M. bovis* was grown in modified PPLO medium (Kanto Kagaku, Tokyo, Japan) at 37°C for 48 h. *M. bovis* bacteria were obtained by centrifugation (16,000 × *g* for 40 min) and were then washed with phosphate-buffered saline (PBS). The bacteria were then suspended in PBS to a cell density of 10^8 CFU/ml, and the suspension was stored at −70°C until used.

Animals. Three Holstein cows with negative bacteriological culture in milk and SCCs of <200,000 cells/ml at quarter level were used in this study. The SCC was evaluated by the Fossomatic 90 instrument (Foss Electric, Denmark).

Intramammary challenge of *M. bovis*. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Rakuno Gakuen University. The left forequarter (A quarter) was infused with ~50,000 CFUs (10,000 CFUs/ml in 5 ml of sterile PBS) of *M. bovis*. PBS was infused in the right rear D quarter (as noninfusion quarter). A remnant of the 5-ml intramammary challenge solution was added to modified PPLO agar plates (Kanto Kagaku) for counting the CFU/ml of the *M. bovis* culture.

Milk and blood sampling. Milk samples from all four quarters (A, left fore; B, left rear; C, right fore; D, right rear) were tested for SCC on day 0 preinfection control and day 0.5 and days 1 to 14 postinfection from three cows. These milk samples were collected using aseptic techniques and cultured for bacteria, including *Mycoplasma* spp., at day 0 preinfection control and day 0.5 and days 1 to 14 postinfection. *M. bovis* in milk was determined as CFU/ml by plating 100 μl of milk onto PPLO modified agar plates (Kanto Kagaku). Milk from highly infected quarters was serially diluted (10-fold) in PBS prior to plating. Following incubation of 1 week at 37°C and 5% CO_2, CFU/ml was calculated. PCR for the detection of *M. bovis* in cultured broth was performed according to the method described by Higuchi et al. (31). For assessing the condition of the cows, rectal temperature, heart rate, and respiratory rate were measured (see Fig. S1 in the supplemental material), and blood was collected for hematological examination, biochemical examination, and peripheral white blood cell counts at day 0 preinfection control and days 1, 3, 7, and 14 postinfection from three cows (see Table S3a to c in the supplemental material).

Blood MNCs and milk MNCs. Blood samples of 70 ml each were collected from cows using evacuation tubes containing sodium heparin (Terumo, Tokyo, Japan). Milk samples of 1,000 ml (~10^7 cells as SCC) were collected into tubes from the A and D quarters. The cells were isolated by centrifugation on a Ficoll-Conray gradient, with specific gravity of 1.080, as previously described (32). The cells were separated by centrifugation (300 × *g* for 30 min) and were transferred to a sterilized tube (Becton, Dickinson, Tokyo, Japan). The viability of blood MNCs was assessed using an AO/PI cell viability kit (Logos Biosystems, Gyunggi, South Korea) and Luna-FLTM (Logos Biosystems) according to the manufacturer's protocols.

Surface marker analysis of blood MNCs and milk MNCs. The cells were adjusted to 10^6 cells/ml in PBS and surface stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD4 (Bio-Rad clone CC8; for CD4-positive T cell marker), CD8 (Bio-Rad clone CC63; for CD8-positive T cell marker), CD14 (Bio-Rad clone M-M9; for monocyte/macrophage marker), CD21 (Bio-Rad clone CC21; for B cell marker), and WC1 (Bio-Rad clone CC15; for γδ T cell marker) antibody. Following incubation for 15 min in a dark room, the cells were centrifuged (500 × *g* for 5 min at 4°C), then washed in PBS, and fixed and permeabilized with 1% paraformaldehyde (Polysciences, USA). Data were acquired using the BD FACSVerse (BD Biosciences, USA) system and analyzed on a FACScalibur flow cytometer using CellQuest software (BD Biosciences). Ten thousand events per sample were collected for blood MNCs and 5,000 events per sample were collected for milk MNCs. Data were analyzed for gated MNCs based on the forward- and side-light scatter characteristics, as previously reported (33).

Proliferative response. Following sacrifice, the bovine mammary lymph nodes (left and right) were immediately collected in cold RPMI 1640 medium (Sigma-Aldrich, Tokyo, Japan), cut using a surgical knife, pulverized, and washed with RPMI 1640 medium. The viability of MNCs was assessed using an AO/PI cell viability kit (Logos Biosystems, Gyunggi, South Korea) and Luna-FLTM (Logos Biosystems) according to the manufacturer's protocol. The subpopulations of MNCs were analyzed by flow cytometry, as described above. The blood MNCs and lymph node MNCs stimulated with mitogen, i.e., concanavalin A (ConA; Wako Pure Chemical Industries, Osaka, Japan), or *M. bovis* (PG45 and five wild strains) were seeded on 96-well tissue culture plates at a concentration of 2×10^5 live MNCs/well. The bovine MNCs were incubated at 37°C in 5% CO_2 in the presence of 5 μg of mitogen/well or live *M. bovis* at a multiplicity of infection (MOI) of 100 for 72 h in triplicate. The cellular proliferative response was measured using a cell counting kit-8 (CCK-8) assay (Dojindo, Osaka, Japan), according to the manufacturer's protocol.

RNA extraction. Total RNA (tRNA) extracted from blood MNCs and milk MNCs was obtained using the PureLink RNA mini kit (Ambion, TX, USA). DNase digestion was performed using Turbo DNA-free DNase (Ambion). tRNA was quantified via spectrophotometry using a BioSpec-nano instrument (Shimadzu, Kyoto, Japan). cDNA was synthesized from 1 μg of tRNA using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan) and oligo(dt)18 Primer (Toyobo). For each reaction, a parallel negative-control reaction was performed in the absence of reverse transcriptase and analyzed by the β-actin band using PCR and 1.5% agarose gel stained with ethidium bromide, which was visualized on a UV transilluminator.

Microarray experiment and analysis. Data from six microarrays (three from day 0 and three from day 7) of blood MNCs following intramammary infusion of *M. bovis* were provided by TaKaRa Bio Inc. (Shiga, Japan). The gene expression data set was obtained on the Agilent one-color microarray platform (4x44K bovine gene expression array, grid ID 023647). The samples were processed for Agilent microarrays, and data were normalized as previously described (34). A *t* test was used to identify significant gene expression differences ($P < 0.025$) between samples. In a further filtering step, only genes with a fold

change of ≥2 were selected. bioDBnet was used for gene annotation (35), and GO enrichment analysis was performed using the BioMart enrichment tool (36).

qRT-PCR analysis. Reverse transcription-quantitative PCR (qRT-PCR) was performed using Thunderbird SYBR qPCR mix (Toyobo) and a MyiQ-icycler (Bio-Rad Laboratories, Hercules, CA, USA). Information on the primers is shown in Table S4 in the supplemental material. Melting curve analysis was used to evaluate each primer pair for specificity to ascertain that only one product was amplified. A BLAST (NCBI platform) search was performed to confirm that the primer sequences amplified only the target gene of interest. Thermal cycling consisted of initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The melting temperature of the PCR product was determined by melting curve analysis, which was performed by heating the PCR product from 55°C to 95°C and monitoring the fluorescence change every 0.5°C. The fold-change ratio between preinfection and postinfection samples for each gene was calculated by the $\Delta\Delta C_T$ method which is based on the following housekeeping genes: β-actin, tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (37, 38).

Statistical analysis. Data from the three cows are expressed as the mean ± standard error (SE). Kruskal-Wallis test was used for comparison between groups, Steel test was used for multiple comparisons, and Welch's *t* test was used for paired groups, performed using Ekuseru-Toukei 2010 for Windows (Social Survey Research Information, Tokyo, Japan). In all cases, a *P* value of <0.05 was considered to indicate a statistically significant difference.

Data availability. The complete microarray data set is publicly available from the ArrayExpress database (accession number E-MTAB-7905).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 3, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 4, PDF file, 0.1 MB.

SUPPLEMENTAL FILE 5, PDF file, 0.1 MB.

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