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Research paper

Effect of *Mycoplasma bovis* on expression of inflammatory cytokines and matrix metalloproteinases mRNA in bovine synovial cells

Koji Nishi[a], Satoshi Gondaira[a], Mariko Okamoto[a,1], Takanori Nebu[a], Masateru Koiwa[b,2], Hiromichi Ohtsuka[b], Kiyokazu Murai[c], Kazuya Matsuda[d], Jumpei Fujiki[e], Hidetomo Iwano[e], Hajime Nagahata[a], Hidetoshi Higuchi[a,*]

[a] Animal Health Laboratory, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[b] Department of Veterinary Internal Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[c] Food Safety and Consumer Affairs Bureau, Ministry of Agriculture Forestry and Fisheries, Kasumigaseki, Tokyo, 100-8950, Japan
[d] Department of Veterinary Pathology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan
[e] Laboratory of Veterinary Biochemistry, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

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ABSTRACT

Mycoplasma bovis causes chronic arthritis in calves. Mycoplasma arthritis shows severe inflammatory reactions in joints that is commonly treated with antibiotics and results in significant economic losses in the calf industry. A previous study showed that inflammatory cytokines and matrix metalloproteinases (MMPs) produced by synovial cells promote progression of the pathophysiology of bacterial arthritis. However, the mechanism underlying the pathogenesis of bovine Mycoplasma arthritis has not been fully clarified. In this study, we examined the immunologic response of bovine synovial tissue to *M. bovis*. We observed significant increases in expression of interleukin (IL)-1β, IL-6, IL-8, MMP-1, and MMP-3 mRNA in synovial tissue from Mycoplasma arthritis calves compared with tissues from normal calves. Expression of IL-6, IL-8, and MMP-1 mRNA was also induced in cultured synovial cells stimulated with *M. bovis*, but not expression of IL-1β and MMP-3 mRNA. In contrast, the culture supernatant of peripheral blood mononuclear cells stimulated with *M. bovis* induced marked increases in the expression of IL-1β, IL-6, IL-8, MMP-1, and MMP-3 mRNA in synovial cells. Our results indicate that inflammatory cytokines and MMPs produced by synovial cells play a key role in the pathogenesis of Mycoplasma arthritis. We suggest that interactions between synovial cells and mononuclear cells in the presence of *M. bovis* induce expression of these cytokines and MMPs in synovial cells, resulting in severe inflammatory reactions in the joints.

1. Introduction

Mycoplasmas are bacteria of the class Mollicutes which do not have a cell wall and cause widespread infections in eukaryotes (Razin et al., 1998). *Mycoplasma bovis* is a significant pathogen of cattle, causing mastitis (Fox, 2012), pneumonia (Maunsell et al., 2011), and arthritis (Gagea et al., 2006). Mycoplasma arthritis (MA) is a group of intractable diseases commonly treated with antibiotics and associated with significant economic losses in the calf industry (Nicholas, 2011). MA shows synovial hyperplasia and osteolysis caused by severe inflammatory reactions in the joints (Gagea et al., 2006; Mahmood et al., 2017). However, the mechanism of the immune response to *M. bovis* in bovine synovial tissue has not been elucidated.

Synovial cells play an important role in the immune response in joints (Bartok and Firestein, 2010; Haerdi-Landerer et al., 2011). Inflammatory cytokines induce severe inflammatory reactions and contribute to progression of the pathophysiology of bacterial arthritis (Abdelnour et al., 1994; Nair et al., 1996). Matrix metalloproteinases

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Abbreviations: MA, Mycoplasma arthritis; MMPs, matrix metalloproteinases; TIMPs, tissue inhibitors of metalloproteinases; ECM, extracellular matrix; SF, synovial fluid; stm-supernatant, supernatant of PBMCs stimulated with *M. bovis*; unstm-supernatant, culture supernatant of unstimulated PBMCs

[*] Corresponding author.

E-mail address: higuchi@rakuno.ac.jp (H. Higuchi).

[1] Present address: Division of Bacterial and Parasitic Disease, National Institute of Animal Health, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan.

[2] Present address: Cattle Research Center, Ebetsu, Hokkaido 069-0804, Japan.

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(MMPs) are a major group of enzymes that degrade the extracellular matrix (ECM), including collagen, proteoglycan, and elastin (Visse and Nagase, 2003). MMP activity is regulated by tissue inhibitors of metalloproteinases (TIMPs), which are specific inhibitors that bind MMPs in a 1:1 stoichiometry (Visse and Nagase, 2003).

Synovial cells produce inflammatory cytokines, MMPs, and TIMPs, each of which play key roles in the pathogenesis of human and murine bacterial arthritis (Behera et al., 2005; Kanangat et al., 2006). As indicated above, however, the immune responses in bovine synovial tissue infected with *M. bovis* have not been fully elucidated. Therefore, in this study, we examined the immunologic characteristics of bovine synovial tissue to *M. bovis*.

2. Material and methods

2.1. Synovial fluid (SF) and synovial tissue

We obtained 11 clinically healthy (control) calves and 6 calves with chronic spontaneous MA (1 to 4 months of age) from different commercial dairy farms between 2015 and 2017. SF (from 11 control and 6 MA calves) and synovial tissue (from 4 control and 4 MA calves) were isolated from the knee joints of dissected calves. MA calves were diagnosed based on the results of PCR analyses using *M. bovis*–specific primers, as previously described (Higuchi et al., 2011). Briefly, SF was inoculated into modified PPLO medium (Kanto Kagaku, Tokyo, Japan) and incubated at 37 °C for 48 h. After incubation, the culture medium was analyzed using *M. bovis*–specific PCR. No other bacteria were detected in the joints of control or MA calves using blood agar plates (Eiken Kagaku, Tokyo, Japan) incubated for 24 h at 37 °C. SF isolated from control and MA calves was immediately analyzed in terms of pH (pH meter; Horiba, Kyoto, Japan), protein concentration (Lowry protein assay kit; Nacalai Tesque, Kyoto, Japan), and number of cells (LUNA-FL™ Dual Fluorescence Cell Counter; Logos Biosystems, Anyang, Korea). A total of 1×10^5 SF cells were stained using a Diff-Quick staining kit (Sysmex, Hyogo, Japan), and the percentages of neutrophils, lymphocytes, macrophages, and synovial cells were then determined.

2.2. Bacterial strain

Mycoplasma bovis (PG45; ATCC 25523) cells were grown in 300 mL of modified PPLO medium for 48 h at 37 °C. The bacteria were obtained by centrifugation (6500 × *g* for 20 min) and then suspended in 3 mL of PPLO medium at a concentration of 10^{11} colony forming units/mL. The bacterial concentration in the suspension was determined by bacterial counts using PPLO agar plates (Kanto Kagaku), and cells were stored at −80 °C until use.

2.3. Peripheral blood mononuclear cells (PBMCs)

Blood samples were collected from 5 clinically healthy Holstein cows in mid-lactation with no history of *M. bovis* infection. PBMCs were isolated by centrifugation on Lympholyte® H cell separation medium (Cosmo Bio, Tokyo, Japan). Collected PBMCs were suspended in RPMI 1640 medium with L-glutamine and phenol red (Wako, Osaka, Japan) containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 ng/mL amphotericin B (Wako), and the number of cells was determined using a LUNA-FL™ Dual Fluorescence Cell Counter. PBMCs (4×10^6) were then incubated in the presence of live *M. bovis* at a multiplicity of infection (MOI) of 1000:1 for 24 h at 37 °C with 5% CO_2 in 60-mm dishes (Iwaki, Chiba, Japan). The culture medium of PBMCs stimulated with *M. bovis* was transferred to a sterile tube and then centrifuged (10,000 × *g* for 20 min). The supernatant was filtered using a 0.1-μm membrane filter (Merck Millipore, Billerica, Mexico) to remove *M. bovis*. We confirmed that *M. bovis* cells were completely removed from the supernatant using PPLO agar plates incubated for 5 days at 37 °C with 5% CO_2.

2.4. Synovial cells culture

Synovial cells were isolated from the carpal joints of 5 clinically healthy calves obtained from *Mycoplasma*-free farms (1 to 2 months of age). Isolation and culture techniques were performed as described previously (Haerdi-Landerer et al., 2008). Briefly, the synovial membrane was collected from the carpal joints and cut into small pieces of 1 × 1 × 0.5 cm. The synovial membrane was digested with 100 U/mL collagenase (Wako) for 1 h at 37 °C with 5% CO_2. Collected synovial cells were filtered using a 70-μm nylon cell strainer (Falcon, NY, USA). Synovial cells were suspended in Dulbecco's modified Eagle's medium (low glucose) with L-glutamine and phenol red (Wako) containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 25 ng/mL amphotericin B and then seeded at 7000 cells/cm^2 in a collagen-coated 6-well plate (Iwaki) and cultured at 37 °C with 5% CO_2. Synovial cells were cultured with live *M. bovis* at a MOI of 1000:1 or the supernatant of PBMCs stimulated with *M. bovis* (stm-supernatant) for 24, 48, and 72 h at 37 °C with 5% CO_2.

2.5. Total RNA purification and cDNA synthesis

Total RNA (tRNA) extracted from synovial tissue, PBMCs, and synovial cells was prepared using a Total RNA Purification kit (Jena Bioscience, Jena, Germany). DNAse digestion was performed using a TURBO DNA-free DNAse system (Ambion, TX, USA). The tRNA was precipitated with sodium acetate and 100% ethanol and then washed with 70% ethanol. The tRNA was dried using a Mini Vacuum-Centrifugal Evaporator (TOMY, Tokyo, Japan) at 55 °C for 20 min, and then the tRNA pellet was dissolved in water (Millipore). tRNA was quantified using a BioSpecnano spectrophotometer (Shimadzu, Osaka, Japan). cDNA was synthesized from 500 ng of tRNA using ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). For each reaction, a parallel negative control reaction was performed in the absence of reverse transcriptase, and we confirmed that no DNA was detectable in the negative control using β-actin–specific PCR.

2.6. Quantitative RT-PCR (qRT-PCR) analysis

Information regarding the primers used in this study is shown in Table 1. We used the melting curve analysis method to evaluate each primer pair for specificity to ascertain that only one product was amplified. We performed a BLAST search to confirm that the primer sequences amplified only the target gene of interest. We performed qRT-PCR analyses as previously described (Gondaira et al., 2015) using Thunderbird SYBR qPCR mix (Toyobo) and a CFX Connect Real-time system (BIO-RAD, CA, USA). 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. Expression of each gene was assessed using a standard curve constructed from dilution series of quantified PCR products (obtained with the same primers as those used in the mRNA quantification analysis). The fold-change ratio between stimulated and unstimulated samples for each gene was calculated in reference to the following housekeeping genes: β-actin, tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.7. Apoptosis assay

We evaluated the effect of *M. bovis* and stm-supernatant on apoptosis of synovial cells. Synovial cells were incubated in the presence of *M. bovis* and stm-supernatant for 24, 48, and 72 h at 37 °C with 5% CO_2.

Table 1
Information regarding the primers used in this study.

Gene	Primer sequence	Amplicon size (bp)	Reference	Efficiency (%)
β-actin	F: AGC AAG CAG GAG TAC GAT GAG R: ATC CAA CCG ACT GCT GTC A	241	Robinson et al. (2007)	105
YWHAZ	F: GCA TCC CAC AGA CTA TTT CC R: GCA AAG ACA ATG ACA GAC CA	120	Spalenza et al. (2011)	102.5
GAPDH	F: GGC GTG AAC CAC GAG AAG TAT AA R: CCC TCC ACG ATG CCA AAG T	118	Sander et al. (2005)	116.3
IL-1β	F: AGT GCC TAC GCA CAT GTC TTC R: TGC GTC ACA CAG AAA CTC GTC	114	Griesbeck-Zlich et al. (2009)	105.2
IL-2	F: CCA GAG AGA TCA AGG ATT CAA TGG R: CAG CGT TTA CTG TTG CAT CAT CA	108	O'Gorman et al. (2006)	111.1
IL-6	F: ATC AGA ACA CTG ATC CAG ATC C R: CAA GGT TTC TCA GGA TGA GG	145	O'Gorman et al. (2006)	101.8
IL-8	F: GAA GAG AGC TGA GAA GCA AGA TCC R: ACC CAC ACA GAA CAT GAG GC	142	O'Gorman et al. (2006)	98.8
IL-10	F: AAG GTG AAG AGA GTC TTC AGT GAG C R: TGC ATC TTC GTT GTC ATG TAG G	110	O'Gorman et al. (2006)	101
IL-12p40	F: CAT CAG GGA CAT CAT CAA AC R: AAC GTC AGG GAG AAG TAG GA	135	Xu et al. (2007)	109.5
IL-17A	F: TGG TGG CTC TTG TGA AGG CAG G R: TCA GGG TCC TCA TTG CGG TGG A	193	Gondaira et al. (2015)	104.4
IFN-γ	F: TCA AAT TCC GGT GGA TGA TCT GC R: GAC CAT TAC GTT GAT GCT CTC CG	150	O'Gorman et al. (2006)	100
TNF-α	F: TCT TCT CAA GCC TCA AGT AAC AAG C R: CCA TGA GGG CAT TGG CAT AC	418	Lee et al. (2006)	112.4
MMP-1	F: ACG TGG CTC CGT TTG TTC TT R: AGG GTG TGA CAT TGC TCC AG	153		96.8
MMP-2	F: TTT GGA CTG CCC CAG ACA GG R: GCT GCG GCC AGT ATC AGT GC	518	Puyraimond et al. (1999)	91
MMP-3	F: AGA GCA AGC CAT TAA GAC CAC A R: CTA GAT ATT GCT GAA CAA GCT CCA T	161		105.3
MMP-14	F: GTT CTA TGG TCT GCG AGT GAC R: CCA AAT GTG GCA TAC TCG CCC	215		99.4
TIMP-1	F: GGT TCA GTG CCT TGA GGG AT R: CCA GCA GCA TAG GTC TTG GT	226		101.4
TIMP-2	F: TGG GCT GTG AGT GCA AGA TC R: AGC CGT CGC TTC TCT TGA TG	150	Kasimanickam et al. (2012)	106.3
TIMP-3	F: ACT TTG GAG ACT CGA GCA GC R: TGG GGC ATC TTG GTG AAT CC	275		105

The primer names, sequences, size of amplicons, references, and PCR amplification efficiency are shown.

After incubation, the cells were treated with Muse Annexin V & Dead Cell reagent (Merck Millipore), and percentage of apoptotic cells (annexin V+) was calculated using a MUSE Cell Analyzer (Merck Millipore). For each sample, at least 2000 events were analyzed.

2.8. Statistical analyses

Data are expressed as the mean ± standard error (SE). The Mann-Whitney *U* test and steal method were used for comparisons of medians or means between different groups using the statistical analysis program MEPHAS (http://www.gen-info.osaka-u.ac.jp/MEPHAS/). In all cases, a *p* value of < 0.05 was considered to indicate statistically significant differences.

3. Results

3.1. SF pH, protein concentration, and number of SF cells isolated from control and MA calves

The pH, protein concentration, and number of SF cells are shown in Fig. 1. The pH of SF from MA calves was 7.3 ± 0.1, which was significantly ($p < 0.05$) lower than that of control calves (7.9 ± 0.1). The concentration of protein in SF was significantly ($p < 0.01$) higher in MA calves (8.1 ± 0.9 g/dL) than in control calves (1.9 ± 0.1 g/dL). The SF of MA calves contained $2.9 \pm 0.8 \times 10^7$ cells/mL, which was significantly ($p < 0.01$) higher than the number of cells in the SF of control calves ($4.0 \pm 1.2 \times 10^5$ cells/mL).

3.2. Cell populations in SF isolated from control and MA calves

The percentages of different cell types in SF from control and MA calves are shown in Table 2. The percentages of macrophages and synovial cells in SF from MA calves were significantly ($p < 0.05$ and $p < 0.01$) lower than those of control calves. The percentage of neutrophils in SF from MA calves was 80.4%, which was significantly ($p < 0.01$) higher than that of control calves (4.5%).

3.3. Expression of cytokines, MMPs, and TIMPs mRNA in synovial tissue isolated from control and MA calves

Profiles of cytokines, MMPs, and TIMPs mRNA expression in synovial tissue from MA calves are shown in Fig. 2. Expression of IL-1β, IL-6, IL-8, and IL-17 mRNA in synovial tissue from MA calves was approximately 24- to 365-fold higher ($p < 0.05$) compared with control calves (Fig. 2A). No significant increase in TNF-α, IL-10, or IL-12 mRNA expression was observed in synovial tissue from MA calves compared with control calves (Fig. 2A). Expression of MMP-1 and MMP-3 mRNA in synovial tissue from MA calves was approximately 1500- and 150-fold higher ($p < 0.05$), respectively, compared with control calves (Fig. 2B). No significant differences between MA and control calves were observed in the expression of MMP-2, MMP-14, TIMP-1, TIMP-2, and TIMP-3 mRNA in synovial tissue (Fig. 2B and C).

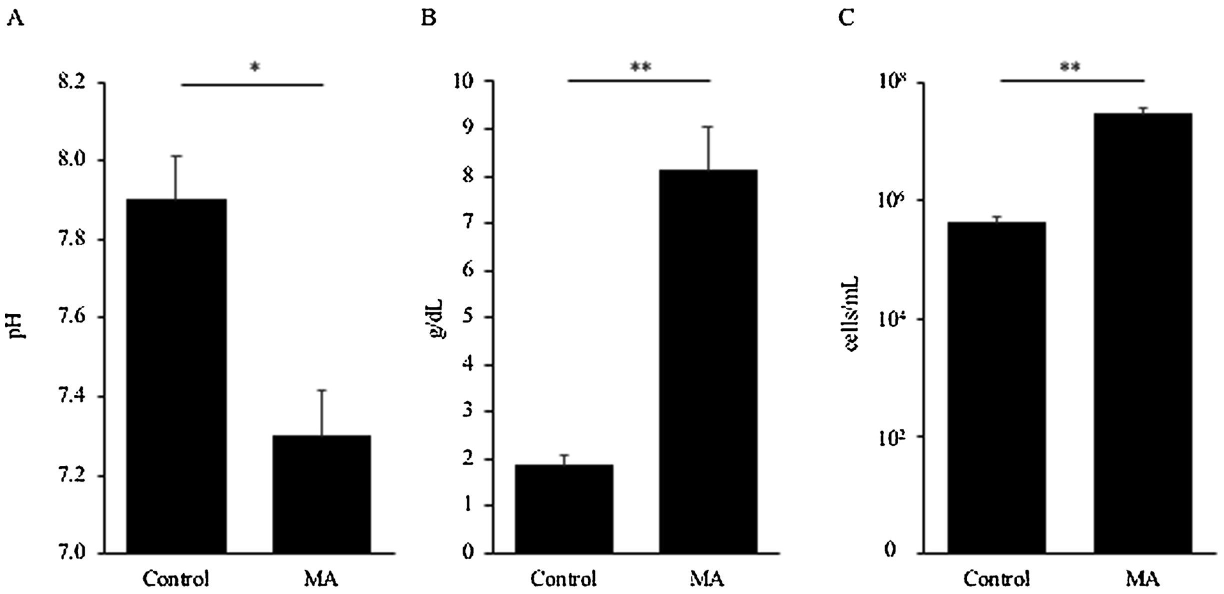

Fig. 1. Synovial fluid (SF) pH, protein concentration, and number of cells in SF isolated from control and *Mycoplasma* arthritis (MA) calves.
The pH (A), protein concentration (B), and number of cells (C) in SF were determined for 11 clinically healthy (control) and 6 MA calves. Values are mean ± SE. Significant difference at $^{*}p < 0.05$ and $^{**}p < 0.01$ versus control calves.

Table 2
The percentages of different cell types in SF from control and MA calves.

	Neutrophil (%)	Lymphocyte (%)	Macrophage (%)	Synovial cell (%)
Control	4.5 ± 2.3	20.9 ± 4.4	33.6 ± 10.3	41.0 ± 9.8
Mycoplasma arthritis	80.2 ± 10.8 **	9.9 ± 7.2	7.0 ± 2.9 *	2.9 ± 1.4 **

Control (n = 5) and mycoplasma arthritis calves (n = 5).
* : $p < 0.05$.
** : $p < 0.01$, compared with control calves.

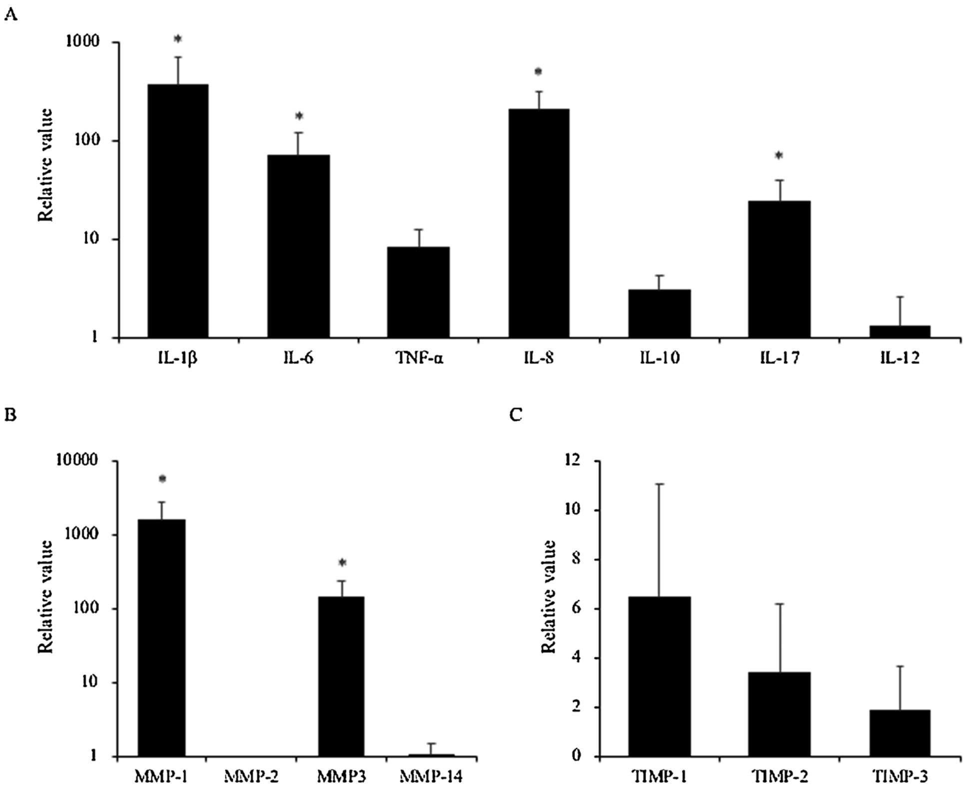

Fig. 2. Expression of cytokines, MMPs, and TIMPs mRNA in synovial tissue isolated from control and *Mycoplasma* arthritis (MA) calves.
Expression of cytokines (A), MMPs (B), and TIMPs (C) mRNA in synovial tissue from MA calves expressed as relative values compared with control calves. Data are expressed as mean ± SE for 4 control and 4 MA calves. Significant difference at $^{*}p < 0.05$ compared with control calves.

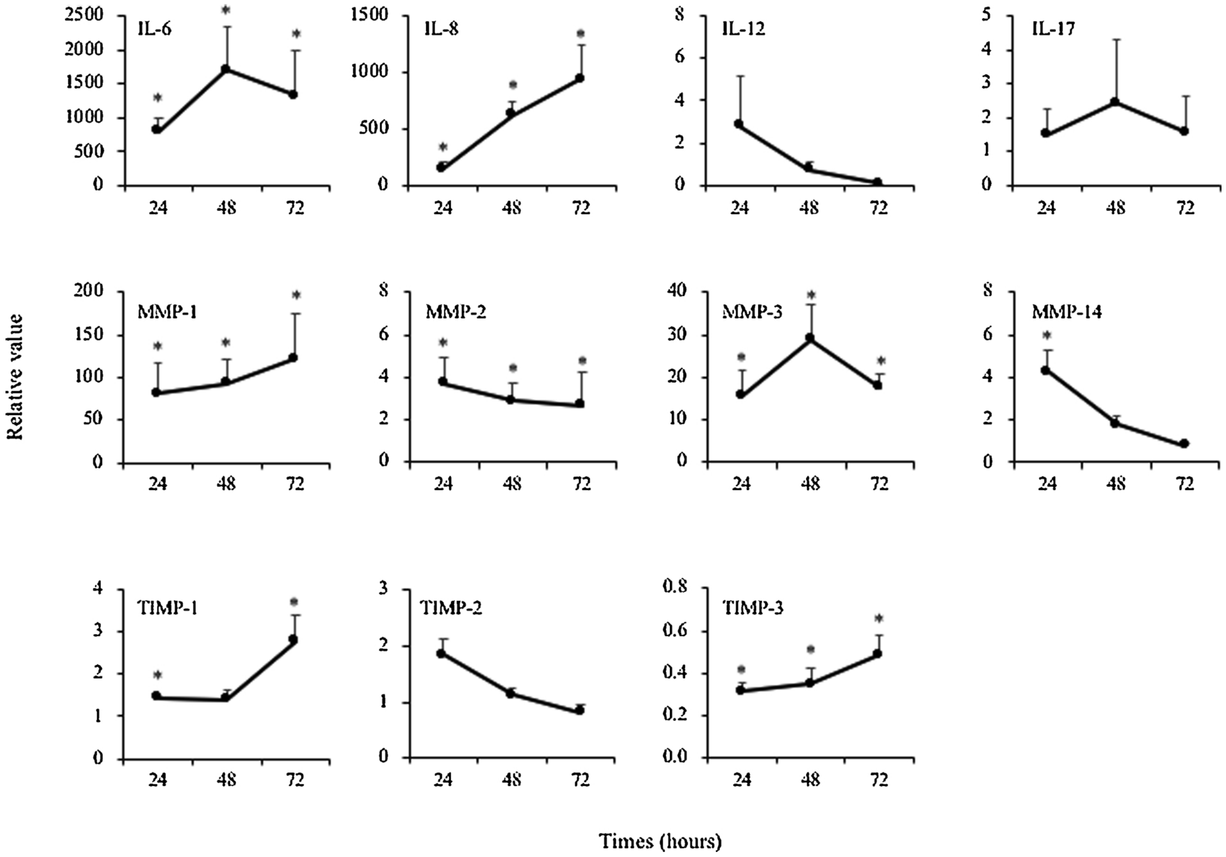

Fig. 3. Expression of cytokines, MMPs, and TIMPs mRNA in synovial cells stimulated with *M. bovis in vitro*.
Synovial cells isolated from 5 control calves were incubated with *M. bovis* for 24, 48, and 72 h. Expression of cytokines, MMPs, and TIMPs mRNA was evaluated using real-time PCR and indicated as relative values compared with unstimulated cells. Expression of IL-1β, TNF-α, and IL-10 mRNA was undetectable by real-time PCR. Data are expressed as mean ± SE (*$p < 0.05$).

3.4. Expression of cytokines, MMPs, and TIMPs mRNA in synovial cells stimulated with M. bovis in vitro

Profiles of cytokines, MMPs, and TIMPs mRNA expression in synovial cells stimulated with *M. bovis* are shown in Fig. 3. Expression of IL-6, IL-8, MMP-1, MMP-2, and MMP-3 mRNA in synovial cells stimulated with *M. bovis* was significantly ($p < 0.05$) higher at 24, 48, and 72 h compared with unstimulated cells. Expression of MMP-14 mRNA in synovial cells stimulated with *M. bovis* was significantly ($p < 0.05$) higher at 24 h compared with unstimulated cells. A significant ($p < 0.05$) increase in TIMP-1 mRNA expression was observed at 24 and 72 h in synovial cells stimulated with *M. bovis* compared with unstimulated cells. Expression of TIMP-3 mRNA in synovial cells stimulated with *M. bovis* was significantly ($p < 0.05$) lower than that of unstimulated cells. No significant increase in the expression of IL-12, IL-17, or TIMP-2 mRNA was observed in synovial cells stimulated with *M. bovis*.

3.5. Effect of culture supernatant of PBMCs stimulated with M. bovis on expression of cytokines, MMPs, and TIMPs mRNA in synovial cells

We evaluated the effect of culture supernatant of PBMCs stimulated with *M. bovis* (stm-supernatant) on cytokine mRNA expression in synovial cells (Fig. 4). After 24, 48, and 72 h, significant ($p < 0.05$) increases in the expression of IL-1β, IL-6, IL-8, IL-12, MMP-3, and TIMP-3 mRNA were observed in synovial cells treated with stm-supernatant compared with synovial cells treated with the culture supernatant of unstimulated PBMCs (unstm-supernatant). A significant decrease ($p < 0.05$) in the expression of MMP-14, TIMP-1, and TIMP-2 mRNA was observed in synovial cells treated with stm-supernatant compared with cells treated with unstm-supernatant. No significant differences in the expression of TNF-α, IL-17, IL-10, MMP-1, or MMP-2 mRNA in synovial cells were observed between cells treated with stm-supernatant and those treated with unstm-supernatant.

3.6. Cytokine mRNA expression in PBMCs stimulated with M. bovis

Profiles of cytokine mRNA expression in PBMCs stimulated with *M. bovis* are summarized in Fig. 5. Expression of IL-17, IFN-γ, and IL-12 mRNA in PBMCs stimulated with *M. bovis* was significantly higher ($p < 0.05$) compared with unstimulated PBMCs. The expression of IL-1β, IL-6, TNF-α, IL-8, and IL-2 mRNA in PBMCs stimulated with *M. bovis* tended to be higher compared with unstimulated PBMCs.

3.7. Apoptosis of synovial cells treated with M. bovis and supernatant from M. bovis–stimulated PBMCs

The effect of *M. bovis* and stm-supernatant on the apoptosis of synovial cells is shown in Fig. 6. After 48 and 72 h of incubation, an average of 16.2% and 24.8% of synovial cells treated with stm-supernatant had undergone apoptosis, which differed significantly ($p < 0.05$) from cells treated with unstm-supernatant. Direct stimulation with *M. bovis* did not induce apoptosis of synovial cells at any of the time points examined.

4. Discussion

Infection with *M. bovis* causes chronic arthritis in calves that typically responds poorly to treatment with antimicrobial agents, resulting in significant economic losses in the calf industry (Nicholas et al.,

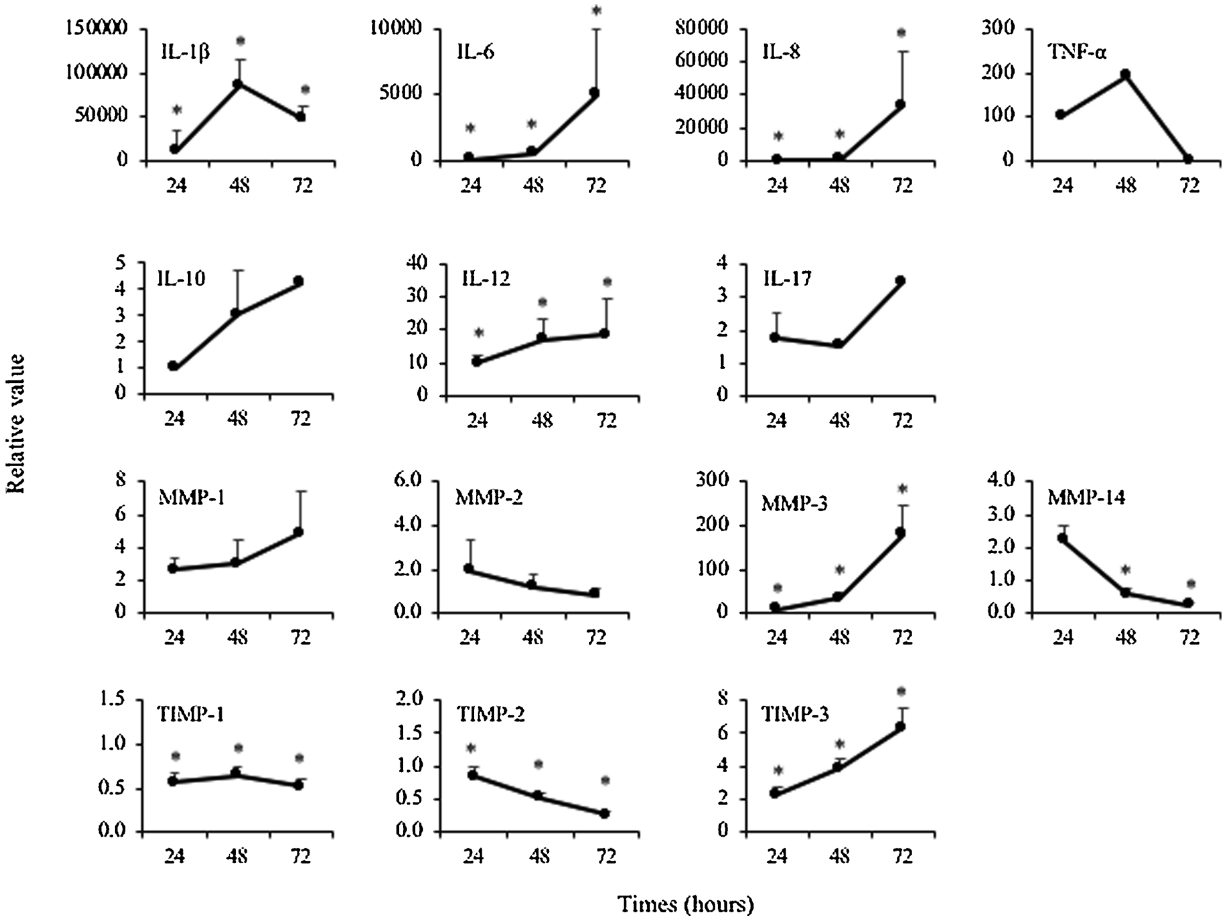

Fig. 4. Effect of culture supernatant of PBMCs stimulated with *M. bovis* on the expression of cytokines, MMPs, and TIMPs mRNA in synovial cells.
Synovial cells were treated with culture supernatant from *M. bovis*–stimulated PBMCs for 24, 48, and 72 h. Expression of cytokines, MMPs, and TIMPs mRNA was evaluated using real-time PCR and indicated as relative values compared to treatment with culture supernatant of unstimulated PBMCs. Expression of TNF-α, IL-17, and IL-10 mRNA in synovial cells was nearly undetectable by real-time PCR. Data are expressed as mean ± SE of 5 calves. *$p < 0.05$.

2011). Chronic MA exhibits with synovial hyperplasia and osteolysis caused by severe inflammatory reactions in the joints (Gagea et al., 2006; Mahmood et al., 2017). Previous studies showed that inflammatory cytokines and MMPs produced by synovial cells induce the development of bacterial arthritis (Bremell et al., 1992; Abdelnour et al., 1994). However, the mechanism underlying the development of bovine MA has not been fully elucidated. In this study, we examined changes in the immunologic characteristics of bovine synovial tissues in response to *M. bovis* infection.

We observed a decrease in pH and increases in protein concentration and the number of cells in the SF of MA calves. In addition, the percentage of neutrophils in SF from MA calves was markedly higher than that in SF from control calves. These results were consistent with other reports describing bacterial arthritis in calves (Rohde et al., 2000; Francoz et al., 2005; Desrochers and Francoz, 2014). Our results demonstrated that *M. bovis* also causes inflammatory reactions in joints.

We found that the expression of IL-1β, IL-6, IL-8, IL-17, MMP-1, and MMP-3 mRNA in synovial tissue from MA calves was significantly higher compared with control calves. It has been reported that IL-1β, IL-6, IL-8, and IL-17 contribute to the development of bacterial arthritis (Abdelnour et al., 1994; Nair et al., 1996; Blauvelt et al., 2018). IL-1β is an important mediator of the acute inflammatory response and bone resorption via stimulation of osteoclasts and cartilage (Nair et al., 1996). Our results suggest that production of IL-1β in synovial tissue causes inflammatory reactions in the joints and bone resorption in MA calves by activating osteoclasts. IL-6 induces the production of chemokines and adhesion factors by endothelial cells, which leads to recruitment of lymphocytes into the joints (Romano et al., 1997; Alonzi et al., 1998). These results suggest that increased expression of IL-6 mRNA in synovial tissue in MA calves promotes the progression of synovial hyperplasia. IL-8 induces the recruitment of bovine neutrophils (Galligan and Coomber, 2000). A previous study showed that inoculation of joints with recombinant IL-8 promotes neutrophil infiltration (Endo et al., 1991). IL-17 induces synovial cells to produce chemokines that induce leukocyte migration in joints (Blauvelt and Chiricozzi, 2018). These results suggest that IL-8 and IL-17 induce the recruitment of neutrophils and/or mononuclear cells in the SF of MA calves.

MMPs are zinc endopeptidases reportedly involved in degradation of the ECM under pathologic conditions (Nagase et al., 2006). MMP-1, MMP-2, MMP-3, and MMP-14 are produced by synovial cells and cause joint destruction in arthritis (Hu et al., 2001; Lin et al., 2001; Scian et al., 2011). MMP-1 cleaves interstitial collagen I, II, and III (Woessner et al., 2000). MMP-3 degrades various matrix proteins, including proteoglycan (Lin et al., 2004). MMP-1 and MMP-3 degrade joint cartilage composed of collagen II and proteoglycan (Saito et al., 1998). The activation of MMPs is regulated by the balance between MMPs and TIMPs expression (Visse and Nagase, 2003). In the present study, the expression of MMP-1 and MMP-3 mRNA in synovial tissue from MA calves was significantly higher compared with control calves, but the expression of TIMP-1, TIMP-2, and TIMP-3 mRNA was not. Our results suggest that the balance between MMPs and TIMPs expression is disrupted in MA calves, resulting in osteolysis mediated by MMP-1 and MMP-3.

We observed a significant increase in the expression of IL-6, IL-8, MMP-1, and MMP-3 mRNA in cultured synovial cells following *M. bovis*

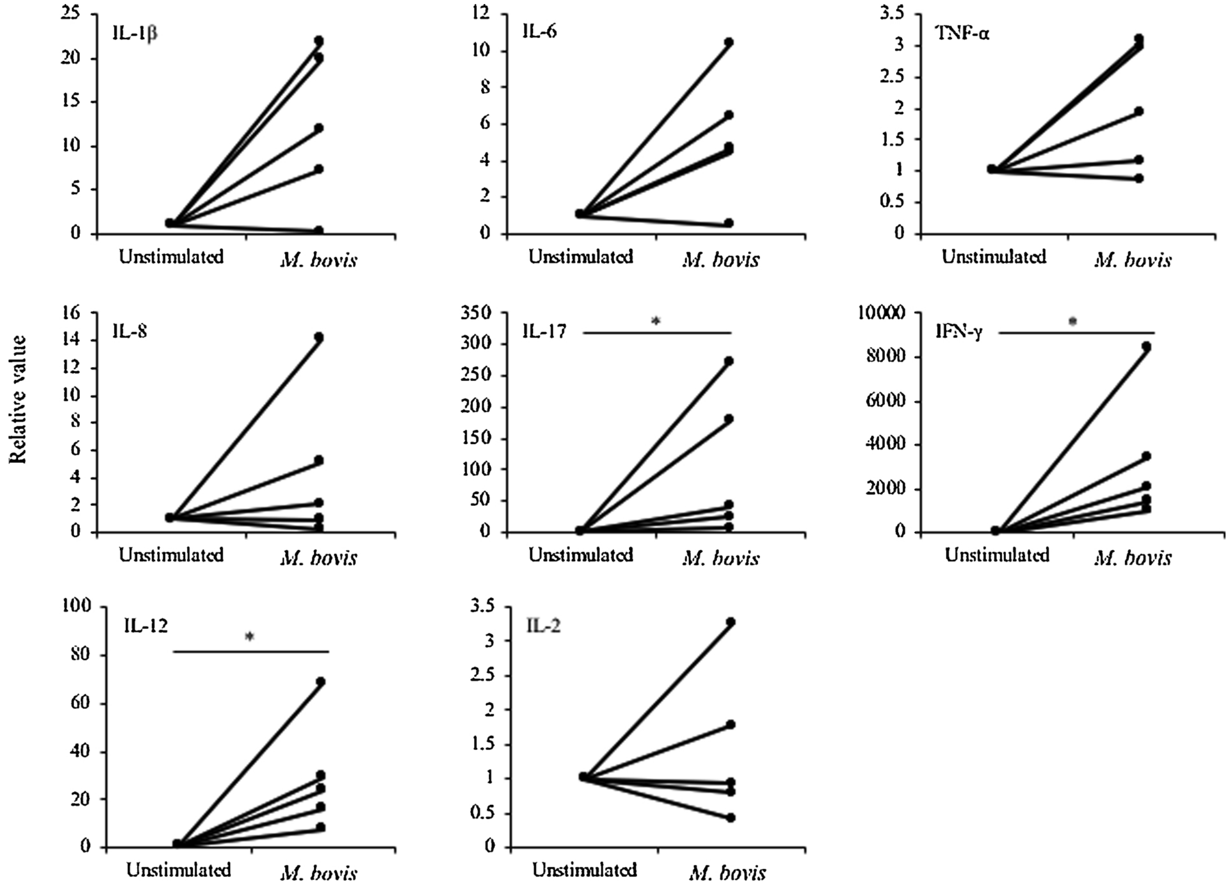

Fig. 5. Expression of cytokine mRNA in PBMCs stimulated with *M. bovis*.
PBMCs isolated from 5 clinically healthy cows were incubated with *M. bovis* for 24 h. Expression of cytokine mRNA was evaluated using real-time PCR and indicated as relative values. $^*p < 0.01$.

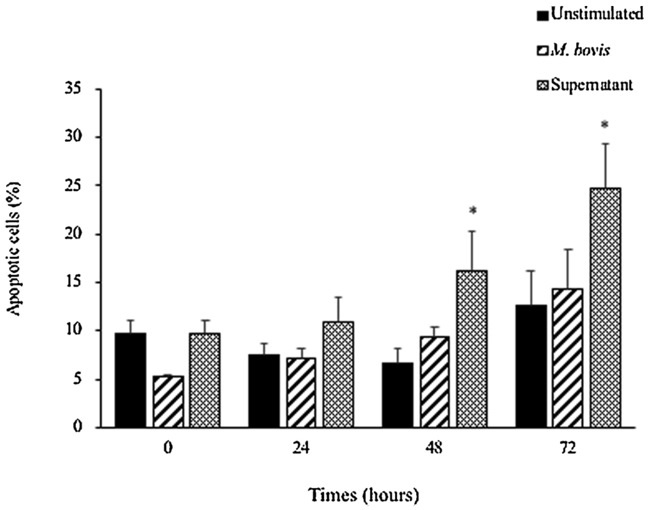

Fig. 6. Apoptosis of synovial cells treated with *M. bovis* and culture supernatant from *M. bovis*–stimulated PBMCs.
Apoptosis of synovial cells was evaluated at 24, 48, and 72 h after stimulation with *M. bovis* or culture supernatant from *M. bovis*–stimulated PBMCs. Data are expressed as mean ± SE of 5 calves. Significant difference at $^*p < 0.05$ compared with treatment with unstimulated synovial cells.

stimulation. These results were consistent with the analysis of synovial tissue from MA calves as described above, which represents the first report that *M. bovis* induces such expression of cytokines and MMPs mRNA in synovial tissue and cells. We also found that expression of MMP-2 and MMP-14 mRNA in synovial cells stimulated with *M. bovis* was upregulated. MMP-2 and MMP-14 degrade various ECM components, leading to infiltration of inflammatory cells (Visse and Nagase, 2003). We speculate that *M. bovis* induces the expression of MMP-2 and MMP-14 mRNA in synovial cells, which induces the migration of inflammatory cells from the blood into synovial tissue. Expression of TIMPs mRNA in synovial cells stimulated with *M. bovis* was approximately 0.5- to 3.0-fold higher than that in unstimulated cells, whereas expression of MMP-1 and MMP-3 was approximately 120- and 30-fold higher, respectively, as described above. These results were similar to those observed with synovial tissue from MA calves. Interestingly, no expression of IL-1β mRNA was detected in synovial cells, and the expression of MMP-1 and MMP-3 mRNA in synovial cells stimulated with *M. bovis* was lower than that observed in synovial tissue from MA calves. A previous study reported that both synovial cells and mononuclear cells mediate immune responses in joints (Hamilton and Slywka, 1981; Bambara et al., 1993). We demonstrated that there is an increase in the number of lymphocytes and macrophages in SF from MA calves. We speculate that mononuclear cells are also involved in the progression of MA.

We also examined the effect of mononuclear cells affect the immunologic status of synovial cells stimulated with *M. bovis*. We observed significant increases in the expression of IL-1β, IL-6, IL-8, IL-12, and MMP-3 mRNA in synovial cells treated with stm-supernatant, and the degree of increased expression was markedly greater than that of synovial cells directly stimulated with *M. bovis*. These characteristic changes in expression of cytokines and MMP-3 mRNA were similar to those observed in synovial tissue from MA calves. We examined the expression of cytokine mRNA in PBMCs stimulated with *M. bovis* to identify the major factors that provide immune enhancement in synovial cells. We also found significant increases in the expression of IL-17, IL-12, and IFN-γ mRNA in PBMCs stimulated with *M. bovis*, and the expression of IL-1β, IL-6, TNF-α, and IL-8 mRNA tended to be higher compared with unstimulated cells. These results were similar to those of a previous study (Gondaira et al., 2015). We speculate that the stm-supernatant contains inflammatory cytokines secreted by *M. bovis*–-stimulated PBMCs and that these cytokines play a role in promoting increased expression of cytokine mRNA in synovial cells. Production of MMP-3 in synovial cells is upregulated by IL-1β and TNF-α (Saito et al., 1998). We speculate that these factors secreted by PBMCs stimulated with *M. bovis* induce increased expression of MMP-3 mRNA in synovial cells. Interestingly, no increase was observed in the expression of MMP-1 mRNA in synovial cells treated with stm-supernatant. We found that the expression of MMP-1 mRNA in synovial cells was induced by direct stimulation with *M. bovis*, as described above. Therefore, direct stimulation with *M. bovis* could play a key role in the increased expression of MMP-1 mRNA in bovine synovial cells. Our results suggest that interactions between synovial cells and mononuclear cells in the presence of *M. bovis* play an important role in the immune response and pathogenesis of MA.

The effect of *M. bovis* and stm-supernatant on the apoptosis of synovial cells was also investigated. We found that *M. bovis* did not induce apoptosis, but stm-supernatant did. A previous study demonstrated that IL-1β induces the apoptosis of peripheral blood cells (Friedlander et al., 1996). Increased expression of IL-1β mRNA in PBMCs stimulated with *M. bovis* could therefore play a role in inducing the apoptosis of synovial cells. Our results suggest that inflammatory cytokines secreted by PBMCs induce the apoptosis of synovial cells in MA calves, leading to a disruption of joint homeostasis.

In conclusion, we clarified the immunologic characteristics of synovial tissue in calves infected with *M. bovis*. Our results indicate that interactions between synovial cells and mononuclear cells following stimulation with *M. bovis* play a key role in the development of MA and are closely related to the characteristic severe clinical symptoms of MA.

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