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NOTE

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Inflammatory cytokine mRNA and protein levels in the synovial fluid of *Mycoplasma* arthritis calves

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ABSTRACT. Bovine *Mycoplasma* arthritis (MA) is caused by *Mycoplasma bovis* and exhibits severe clinical symptoms. However, the pathophysiology of bovine MA is incompletely understood. In this study, we examined the cytokine mRNA expression of synovial fluid (SF) cells and cytokine concentrations in the SF of MA calves. The SF was isolated from five clinically healthy (control) and seven MA calves. mRNA and protein levels of interleukin (IL)-1β, IL-6, IL-8, IL-12, and IL-17 in the SF from MA calves were significantly higher than those from control calves. Our results indicate that SF cells produce inflammatory cytokines, which mainly contribute to the severe inflammatory response in the joints of the MA calves.

KEY WORDS: bovine bacterial arthritis, inflammatory cytokine, *Mycoplasma* arthritis, *Mycoplasma bovis*, synovial fluid

Mycoplasma bovis (*M. bovis*) is a destructive pathogen of beef and dairy cattle worldwide, and causes mastitis, pneumonia, and arthritis in cattle [5, 6, 14]. *Mycoplasma* arthritis (MA) calves exhibit severe clinically symptoms such as lameness and swelling of joints [4]. In human infectious arthritis and rheumatoid arthritis, high concentrations of inflammatory cytokines were detected in synovial fluid (SF) of affected joints. These cytokines play crucial role in the immune response to eliminate pathogens and immunogens from the host [3], but excessive production of inflammatory cytokines induces severe inflammatory reaction and can result in tissue destruction [15]. It is known that cytokines can be produced by synovial cells and SF cells in joints [2, 18]. The immune reaction of these cells is closely related to pathophysiology of arthritis. We previously reported that excessive immune reaction of synovial cells induced the development and progression of MA pathophysiology in calves [16, 17]. However, the role of SF cells in the pathophysiology of bovine MA is not fully clarified. In this study, we examined the mRNA expression of inflammatory cytokines in SF cells and protein levels of that in SF of MA calves.

We obtained SF sample from the joints (stifle or carpal) of dissected five clinically healthy (control) Holstein calves (1 to 2 months of age) and seven calves (six Holstein and one Jersey) with chronic spontaneous MA (1 to 4 months of age) from different commercial dairy farms between 2017 to 2020. All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the School of Veterinary Medicine at Rakuno Gakuen University. MA calves were diagnosed based on the PCR analyses using *M. bovis*–specific primers, as previously described [10, 16]. We confirmed that no other bacteria were detected in SF from control and MA calves using specific primers of *Mycoplasma* spp. [10] and blood agar plates (Eiken Kagaku, Tokyo, Japan) incubated for 24 hr at 37°C. The cells number and percentages of neutrophils, lymphocytes, macrophages, and synovial cells in SF were determined as previous described [16]. Total RNA (tRNA) purification and cDNA synthesized of SF cells were performed using Total RNA Purification kit (Jena Bioscience, Jena, Germany) and ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). The primers information is shown in Supplementary Table 1. qRT-PCR analyses were performed as

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previously described [8]. β-Actin and tryptophan 5-monooxygenase activation protein, zeta polypeptide (YWHAZ) were used as housekeeping genes. The protein level of interleukin (IL)-1β (ESS0027: Invitrogen, Carlsbad, CA, USA), IL-6 (ESS0029: Invitrogen), IL-8 (D8000C: R&D Systems, Minneapolis, MN, USA), IL-12 (OKEH03774: AVIVA SYSTEMS SCIENCE, San Diego, CA, USA), IL-17 (VS0284B-002: Kingfisher Biotech, Saint Paul, MN, USA), and interferon (IFN)-γ (ESS0026B: Invitrogen) in the SF was determined using an ELISA kit. The Mann-Whitney *U* test was used for comparisons between control and MA calves data using the statistical analysis program MEPHAS (http://www.gen-info.osaka-u.ac.jp/MEPHAS/). In all cases, a *P* value of <0.05 and <0.01 were considered to indicate a statistically significant difference.

The dissected MA calves showed increases in SF, synovial hyperplasia, and osteolysis in the affected joints (Supplementary Fig. 1). SF cells of control and MA calves were observed with a light microscope (Supplementary Fig. 2). The cells number of SF from MA calves was 1.6×10^7 cells/ml which showed 45-fold increase compared to that from control calves (3.6×10^5 cells/ml). The proportion of neutrophils in SF of MA calves was significantly higher (P<0.01); that of lymphocytes, macrophages, and synovial cells were significantly lower than (P<0.05) those of control calves (Table 1). The copy number (target/housekeeping) of IL-1β, IL-8, and IL-12 mRNA in SF cells from MA calves were significantly higher (IL-1β: P<0.05; IL-8 and IL-12: P<0.01) than those of control calves (Fig. 1A). The copy number of IL-6 and IL-17 mRNA in SF cells from MA calves were tended to be higher than those of control calves. The mean IL-1β, IL-6, IL-12, and IL-17 concentration in SF of MA calves were 358.8 ± 180.3 pg/ml, 57.7 ± 8.3 ng/ml, 11.8 ± 3.3 ng/ml, and 110.4 ± 68.8 pg/ml which were significantly higher (IL-1β and IL-12: P<0.05; IL-6 and IL-17: P<0.01) than those from control calves (did not detect) (Fig. 1B). The mean IL-8 concentration in SF of MA calves was 36.2 ± 34.2 pg/ml, and, which was slightly higher than those of control calves (did not detect). IFN-γ was not detected in SF of control and MA calves.

All MA calves exhibited increases in SF, synovial hyperplasia, and osteolysis in the affected joint, which were consistent with previous reports [6, 13]. The proportion of lymphocytes and macrophages in SF of MA calves were decreased compared to control calves, but the cells number of SF from MA calves was markedly increased. This data shows that the number of lymphocytes and macrophages in SF of MA calves were increased. It has been reported that lymphocytes, monocytes, and neutrophils promote excessive immune reaction of synovial cells [2, 16]. Our data suggests that the interaction between these cells and synovial cells are related to pathology of MA.

Protein levels of IL-1β, IL-6, IL-8, IL-12, and IL-17 in SF of MA calves were higher than those of control calves. IL-1β, IL-8, and IL-12 mRNA expression in SF cells from MA calves were significantly higher than those of control calves, and IL-6 and IL-17 were tended to be higher. We have already reported that synovial cells infected with *M. bovis* showed increasing in IL-1β, IL-6, IL-8, and IL-17 mRNA expression [16]. These findings indicated that not only synovial cells but also lymphocytes, macrophages, and neutrophils in SF also produce these inflammatory cytokines in joints. IL-1β induce the production of cartilage degradation enzymes e.g., matrix metalloproteinases (MMPs) in synovial cells [11, 17]. IL-6 activate osteoclast that destructs bone in joints [19]. Thus, IL-1β and IL-6 are key factors of cartilage and bone destruction in joints of arthritis. IL-8 and IL-17 are important mediator causing migration of neutrophils to joints [1, 7]. Our data indicated that SF cells produced inflammatory cytokines which played the key role of progression of pathology in MA calves. The pathological mechanism in MA is shown as schematic summarizing (Fig. 2).

Interestingly, high concentration IL-12 was detected, but IFN-γ was not detected in SF of MA calves. IFN-γ is induced by IL-12 stimulation that can promote Th1 responses to eliminate the pathogens [12]. It has been reported that *M. bovis* reduce the IFN-γ production via induction of program death-1 molecule in mononuclear cells to escape from host immunity [9]. We speculate that *M. bovis* might reduce the production of IFN-γ in SF cells nevertheless stimulated with IL-12, thereby *M. bovis* cannot be eliminated from the joint of calves.

In conclusion, inflammatory cytokines mRNA and protein levels in SF from MA calves were markedly higher than those of control calves. SF cells produced inflammatory cytokines which were closely related to progression of pathophysiology in MA calves.

Table 1. The percentage of different cell types in synovial fluid from control and *Mycoplasma* arthritis calves

	Neutrophil (%)	Lymphocyte (%)	Macrophage (%)	Synovial cells (%)
Control (n=5)	4.2 ± 2.0	17.6 ± 3.6	35.6 ± 10.3	42.8 ± 0.8
Mycopalsma arthritis (n=7)	89.2 ± 3.4[a)]	3.2 ± 1.4[b)]	6.2 ± 2.0[b)]	1.3 ± 0.8[b)]

a: P<0.01, b: P<0.05, compared to control calves.

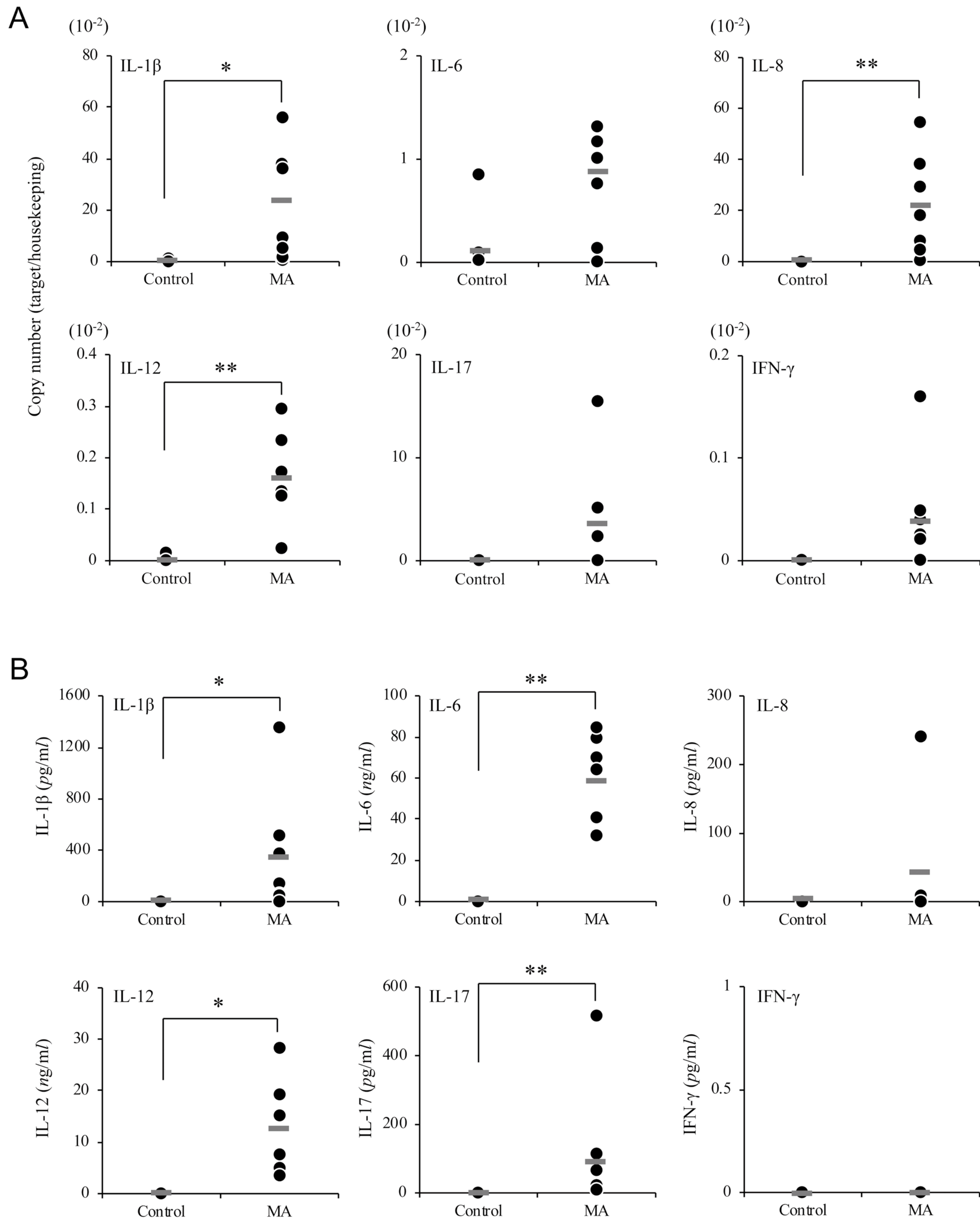

Fig. 1. (A) mRNA expression of inflammatory cytokines in synovial fluid (SF) cells isolated from control and *Mycoplasma* arthritis (MA) calves. The level of mRNA expression of interleukin (IL)-1β, IL-6, IL-8, IL-12, IL-17, and interferon (IFN)-γ in SF cells from MA calves is shown as copy numbers relative to housekeeping genes. The mean values of each cytokines are expressed as gray bar. Data are expressed for control (n=5) and MA calves (n=7). ** $P<0.01$; * $P<0.05$. (B) Protein concentration of inflammatory cytokines in SF of control and MA calves. Protein level of IL-1β, IL-6, IL-8, IL-12, IL-17, and IFN-γ in SF from control (n=5) and MA calves (n=7) is shown as mean ± SE. ** $P<0.01$; * $P<0.05$.

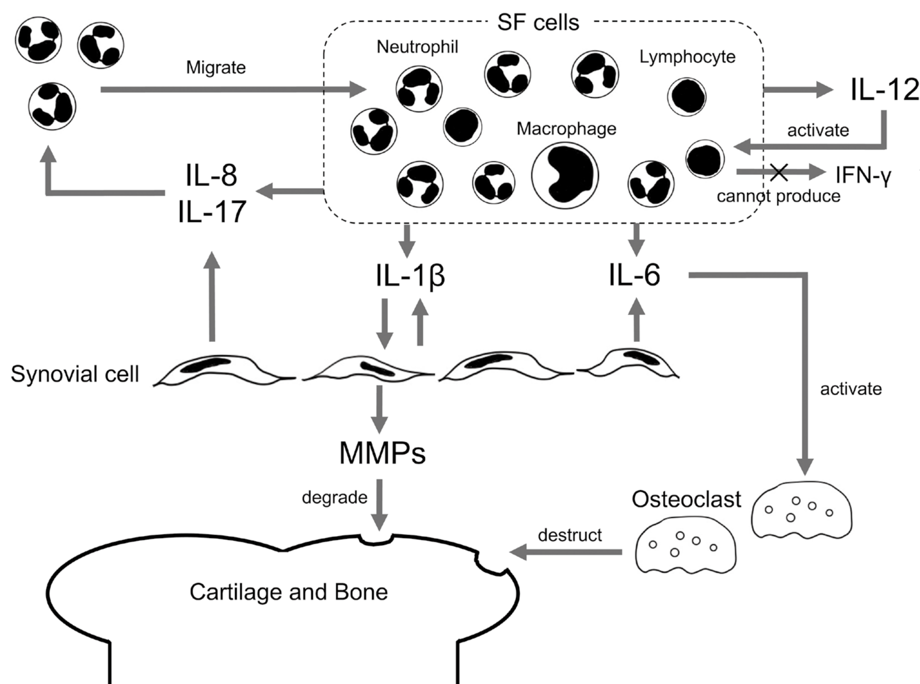

Fig. 2. The schematic summarizing of pathological mechanism in *Mycoplasma* arthritis. Synovial cells and synovial fluid (SF) cells produce inflammatory cytokines which are related to progression of pathology in joints.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

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