Interferon gamma, lipopolysaccharide, and modified-live viral vaccines stimulation alter the mRNA expression of tumor necrosis factor α, inducible nitric oxide synthase, and interferon β in bovine alveolar macrophages

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Corresponding Author:	Seiji Hobo, Ph.D. Kagoshima Daigaku Kagoshima, Kagoshima JAPAN
First Author:	Shingo Ishikawa
Order of Authors:	Shingo Ishikawa
	Masataka Miyazawa
	Chiho Tanaka
	Ryoma Uesawa
	Juri Nishizawa
	Ryoko Uemura
	Ikuo Kobayashi
	Seiji Hobo, Ph.D.
Abstract:	To understand the pathogenesis of bovine respiratory disease (BRD), it is necessary to elucidate the mechanisms of alveolar macrophage regulation by cytokines and pathogen-associated molecular patterns (PAMPs). Moreover, "trained immunity," an innate immune regulatory mechanism in response to vaccines containing PAMPs, has recently attracted attention. It may be applied to BRD control, but there is limited knowledge in bovine. To investigate this, we stimulated alveolar macrophages in vitro with lipopolysaccharide (LPS), polyinosinic-polycytidylic acid sodium salt (Poly I:C), interferon gamma (IFN-γ), and modified-live viral (MLV) vaccines, respectively, and analyzed changes in interferon beta (IFN-β), tumor necrosis factor alpha (TNF- α), and inducible nitric oxide synthase (iNOS) mRNA expression levels. mRNA expression levels of TNF-α, iNOS, and IFN-β were significantly increased in bovine alveolar macrophages stimulated by IFN-γ and MLV vaccine; LPS, IFN-γ, and MLV vaccine; and MLV vaccine only, respectively. Additionally, all MLV vaccine-stimulated mRNA expression increases were observed in a concentration-dependent manner. These results revealed in part, the mechanism of bovine alveolar macrophage regulation by cytokines and PAMPs. Understanding the regulatory mechanisms of alveolar macrophages will contribute to understanding the pathogenesis of BRD and preventive and therapeutic BRD management based on trained immunity.
Response to Reviewers:	

Highlights

- IFN-γ and PAMPs alter bovine alveolar macrophage mRNA expression
- MLV vaccine alter bovine alveolar macrophage mRNA expression
- The effects of PAMPs on bovine alveolar macrophages vary with the type of PAMP
- PAMPs in modified-live viral vaccines directly stimulate alveolar macrophages

Figure

Fig. 1

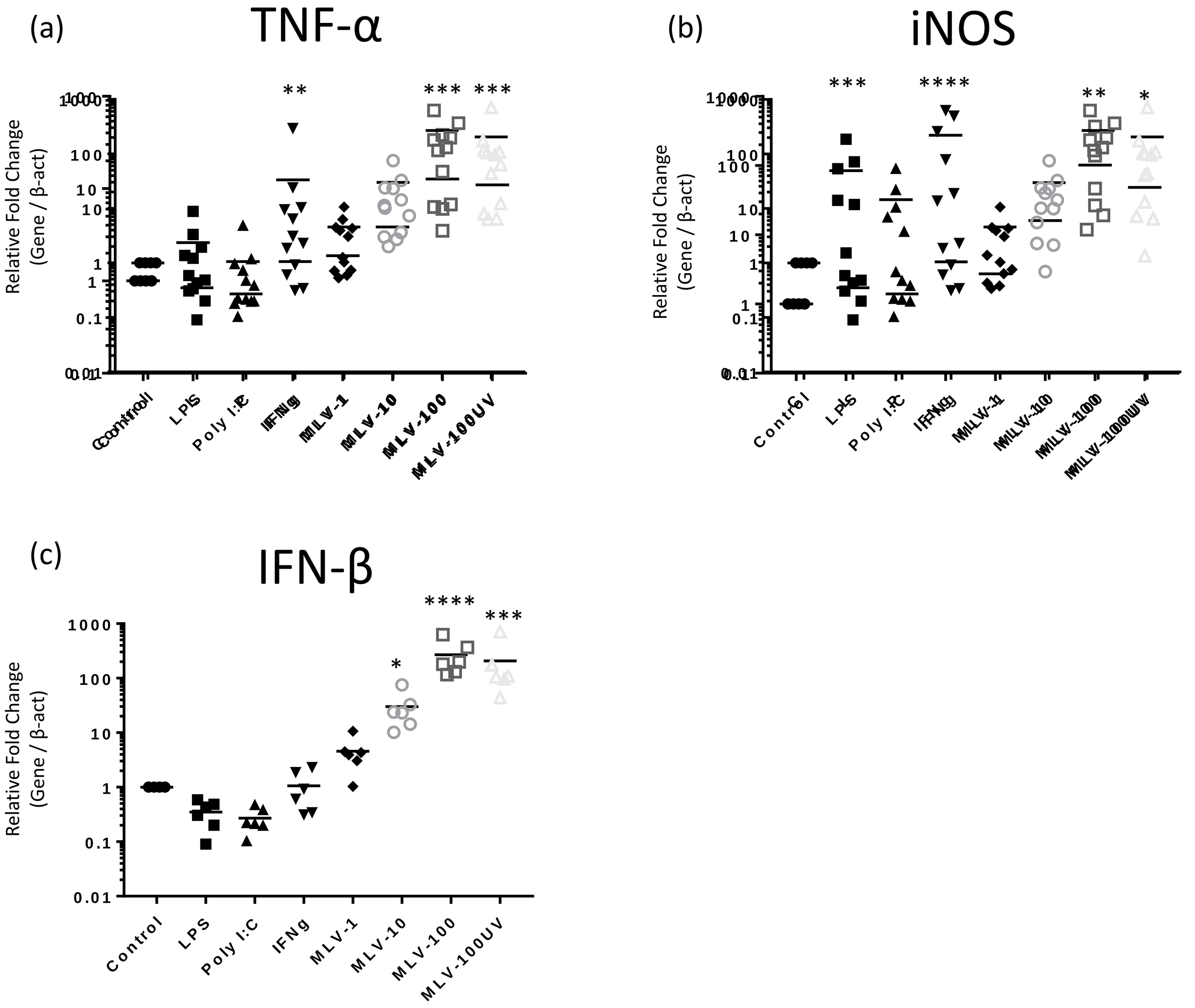

Table 1. Sequences of primers used for PCR

Primer	Kind	Sequence (5'—3')	Accession Number
TNF-α	Sense	TTGCTTGTGCCTCAGCCTCT	NM_173966.3
	Antisense	GGGACTGCTCTTCCCTCTGG	
iNOS	Sense	AAAACCCACGTCTGGCAGGA	NM_001076799.1
	Antisense	GGCGAAGAACACGGCTTTGA	
IFN-β	Sense	GAGGAGATGAAGCAAGAACAGCA	NM_174350.1
	Antisense	TCTGGTGAGAATGCCGAAGA	
β-actin	Sense	CCCAGATCATGTTCGAGACC	NM_173979.3
	Antisense	GAGGCATACAGGGACAGCAC	

Conflict of Interest

The authors declare the following financial interests/personal relationships that may be considered potential conflict of interests: SI received research funding from Zoetis Japan Inc. None of the other authors have conflicting financial interests.

Short communication

Interferon gamma, lipopolysaccharide, and modified-live viral vaccines stimulation alter the mRNA expression of tumor necrosis factor α, inducible nitric oxide synthase, and interferon β in bovine alveolar macrophages

Shingo Ishikawa[a,b], Masataka Miyazawa[b], Chiho Tanaka[b], Ryoma Uesawa[b], Juri Nishizawa[b], Ryoko Uemura[c], Ikuo Kobayashi[c], and Seiji Hobo[b,*]

[a]Division of Veterinary Science, Graduate School of Life and Environmental Biosciences, Osaka Prefecture University, Izumi-Sano, Osaka, 598-8531, Japan

[b]Joint Faculty of Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima, 890-0065, Japan.

[c]Center for Animal Disease Control, University of Miyazaki, 1-1 Gakuenkibanadai-nishi, Miyazaki 889-2192, Japan

[*]Corresponding author: Seiji Hobo

Joint Faculty of Veterinary Medicine, Kagoshima University

1-21-24 Korimoto, Kagoshima 890-0065, Japan

E-mail address: k2088185@kadai.jp, Fax: +81-992853538

Abstract

To understand the pathogenesis of bovine respiratory disease (BRD), it is necessary to elucidate the mechanisms of alveolar macrophage regulation by cytokines and pathogen-associated molecular patterns (PAMPs). Moreover, "non-specific effects (NSEs)" an innate immune regulatory mechanism in response to vaccines containing PAMPs, has recently attracted attention. It may be applied to BRD control, but there is limited knowledge in bovine. To investigate this, we stimulated alveolar macrophages *in vitro* with lipopolysaccharide (LPS), polyinosinic-polycytidylic acid sodium salt (Poly I:C), interferon gamma (IFN-γ), and modified-live viral (MLV) vaccines, respectively, and analyzed changes in tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS), and interferon beta (IFN-β) mRNA expression levels. mRNA expression levels of TNF-α, iNOS, and IFN-β were significantly increased in bovine alveolar macrophages stimulated by IFN-γ and MLV vaccine; LPS, IFN-γ, and MLV vaccine; and MLV vaccine only, respectively. Additionally, all MLV vaccine-stimulated mRNA expression increases were observed in a concentration-dependent manner. These results revealed in part, the mechanism of bovine alveolar macrophage regulation by cytokines and PAMPs. Understanding the regulatory mechanisms of alveolar macrophages will contribute to understanding the pathogenesis of BRD and preventive

and therapeutic BRD management based on NSEs.

Keywords: bovine; alveolar macrophage; modified-live viral vaccine; innate immunity; cytokines; PAMPs

Abbreviations

BRDC: Bovine respiratory disease complex; MLV: Modified-live viral; PAMPs: Pathogen-related molecular patterns; PRRs: Pattern recognition receptors; LPS: Lipopolysaccharide; Poly I:C: Polyinosinic-polycytidylic acid sodium salt; IFN: Interferon; IL: Interleukin; TNF: Tumor necrosis factor; iNOS: Inducible nitric oxide synthase; BALF: bronchoalveolar lavage fluid; RPMI: Roswell Park Memorial Institute; FBS: Fetal bovine serum

1. Introduction

Bovine respiratory disease (BRD), a complex infection caused by the intertwining of various pathogens and stress (Griffin et al., 2010), is the most common disease in cattle, and it causes great economic losses (Edwards, 2010). Some of the causative pathogens of BRD, such as *Mycoplasma bovis*, do not have a vaccine and are difficult to treat with antibiotics; thus, complicating respiratory disease countermeasures (Maunsell et al., 2011). The internal respiratory organ is in contact with the outside world; hence, there is always a risk of infection by various pathogens in the outside world. Therefore, the respiratory system develops various immune mechanisms in mucosal tissues, particularly, innate immunity (Ackermann et al., 2010), which is mostly dependent on alveolar macrophages (Batista et al., 2012; Bertagnon et al., 2019). In experimental models of humans and animals, alveolar macrophages exhibit plasticity and maintain lung homeostasis by altering their function (Hussell and Bell, 2014). The maintenance and alteration of these functions are regulated by the alveolar microenvironment, particularly by fluid factors, including cytokines and pathogen-associated molecular patterns (PAMPs).

When pathogens invade a host, pattern recognition receptors (PRRs) expressed by alveolar macrophages are activated by recognizing bacterial and viral PAMPs and

produce type I interferons (IFNs) and proinflammatory cytokines, thus, changing the immune dynamics from a steady state to an unbalanced state (Aggarwal et al., 2014). Subsequently, the production of chemokines and the collection of neutrophils and lymphocytes are enhanced. Lymphocytes produce IFN-γ (type II IFN), or IFN-β (type I IFN) which polarizes alveolar macrophages to classically activated (M1)-like inflammatory states which improve pathogen clearance (Herold et al., 2011), or to alternatively activated (M2)-like phenotypes which resolve inflammation and repair the alveolar environment (Hussell and Bell, 2014; Kumaran Satyanarayanan et al., 2019), respectively. Thus, the function of alveolar macrophages is regulated by various humoral factors and PAMPs, but all the above findings are from humans and experimental animals, and there are few reports in bovine.

Epidemiological studies showed that the tuberculosis vaccine, BCG, protects against childhood mortality caused by non-antigenic pathogens (Levine and Sackett, 1946). Subsequent numerous studies have shown its ability to induce a strong protective effect against other infectious diseases, due to "non-specific effects (NSEs)" (Netea et al., 2020) which is induced by the recognition of PAMPs in BCG by various immune cells (Covian et al., 2019). Studies in mice have shown that intranasal BCG directly affects alveolar macrophages and influences secondary viral infections in the respiratory mucosa

(Mukherjee et al., 2017). A report from Japan suggests that nasal cavity-administered modified-live viral (MLV) vaccines also protect against infection by non-antigenic pathogens in bovines (Aoki, 2019; Kano, 2018), but the immunological basis is unknown.

In the present study, we investigated the regulatory role of MLV vaccines in bovine alveolar macrophages. As an NSEs effect of MLV vaccines, we hypothesized the activation of non-specific innate immune mechanisms in alveolar macrophages. Common nonspecific innate immune mechanisms of macrophages include production of inflammatory cytokines (tumor necrosis factor alpha: TNF-α) induced by the nuclear factor kappa B pathway, inducible NO synthase (iNOS), which synthesizes NO, type I interferon (IFN-α, β) induced by the interferon regulatory factor pathway. In the above-mentioned processes, PRRs such as Toll like receptors, RIG-I like receptors, and NOD like receptors activate signal transduction circuits when PAMPs are recognized, and increase mRNA (Akira et al., 2006). We stimulated alveolar macrophages *in vitro* with lipopolysaccharide (LPS), a bacterial PAMP, polyinosinic-polycytidylic acid sodium salt (Poly I:C), a synthetic RNA analog mimicking viral PAMPs, IFN-γ, and MLV vaccine, respectively, and analyzed the changes in tumor necrosis factor alpha (TNF-α), inducible nitric oxide synthase (iNOS), and IFN-β mRNA expression levels.

2. Materials and methods

2.1. Animals

This study was approved by the Animal Care and Use Committee of the Feed Research Institute of the University of Miyazaki (No. 2017-016-1). All experimental procedures were approved and carried out according to the guidelines of the committee. We used six Japanese Black cattle (aged 30 – 42 months old), born between December 8, 2018, and March 7, 2019, and raised them according to standard guidelines at the Sumiyoshi Livestock Science Station (Miyazaki, Japan) which had Global G.A.P. certification (GGN: 4052852881722). BALF samples were collected from cattle that were systemically healthy, which did not have a chronic or immediate history of respiratory disease.

2.2. Bronchoalveolar lavage fluid (BALF)

While holding the cattle, a flexible electronic endoscope (VQ TYPE 5112 B, Olympus, Tokyo, Japan) was inserted through the nasal passage to the carina, and its distal end was then passed into a main stem bronchus. The flexible electronic endoscope was then inserted into a subsegment of each lobe. Three 30 mL aliquots of sterile 0.9 % normal saline solution were infused into the lobes and immediately aspirated. All

aspirations were pooled, and the volume of the BALF was measured and recorded. Additionally, BALF was collected from the right cranial lobe, and a part of the BALF was used for microbiological tests and was confirmed to be negative. No secondary symptoms were observed after BALF collection.

2.3. Alveolar macrophage processing

After measuring their volumes, the BALF samples were filtered using sterilized gauze and centrifuged (400 × *g* for 10 min at 4 °C) to separate their cellular components. The number of total nucleated cells was counted using a Countess Ii FL automated cell counter (Thermo Fisher Scientific, Waltham, MA, USA). Sample was diluted with equal volume of PBS and carefully layered 20 ml of diluted sample over the two 4ml Lympholyte-H (Cederlane Lab., Ontario, Canada) in 15 ml tube. Centrifuge for 60 minutes at 800 × g at room temperature. The interface layer was placed into a new 15ml tube, washed three times in PBS. After additional wash, cells were resuspended and cryopreserved in CELLBANKER 2 (Takara Bio Inc., Shiga, Japan) and kept frozen at -80 ° C for later use.

2.4. Stimulated culture

141 Alveolar macrophages (2×10^5 cells) were cultured in 1mL of Roswell Park
142 Memorial Institute 1640 medium (Fujifilm Wako Pure Chemical Corporation, Osaka,
143 Japan) containing 10 % FBS (Japan Bioserum Co. Ltd., Hiroshima, Japan) and 1 %
144 penicillin-streptomycin-amphotericin B suspension (×100 solution; Fujifilm Wako Pure
145 Chemical Corporation) in 12-well plates in 5 % CO_2 at 37 ℃ with stimulation. Cultures
146 were stimulated with 1 μg/mL LPS, 25 μg/mL Poly I:C (Sigma Aldrich, St. Louis, MO,
147 USA), 1 μL/mL MLV vaccine-1, 10 μL/mL MLV vaccine-10, 100 μL/mL MLV vaccine-
148 100, 100 μL/mL ultraviolet (UV)-irradiated MLV vaccine-100, or 5 ng/mL IFN-γ
149 (Thermo Fisher Scientific) for 24 h. The MLV vaccine (Inforce-3; Zoetis, Kalamazoo, MI,
150 USA) containing temperature-sensitive variants of bovine herpesvirus type 1 (BoHV-1),
151 bovine parainfluenza virus type 3 (BPIV-3), and bovine respiratory syncytial virus
152 (BRSV) were used for stimulation. Approximately 2.86×10^7 copies/mL of BoHV-1
153 DNA were used. UV irradiation using the UV cross-linker FS-1500 for 5 min kills the
154 virus particles which are then used in the MLV vaccine (Rajan et al., 2011).
155
156 2.5. Quantitative reverse transcription PCR (RT q-PCR)
157 Total RNA was isolated using RNAiso Plus Total RNA extraction reagent
158 (Takara Bio Inc.) and Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA)

according to the manufacturers' protocols. RNA concentration and purity were measured using a nanophotometer (Wako, Kyoto, Japan). The total RNA was used as a template for complementary DNA synthesis using the PrimeScript RT reagent kit (Takara) according to the manufacturer's protocol. Real-time quantitative PCR reactions were performed using TB Green Premix Ex Taq II (Takara). Cycling conditions were as follows: 95 °C for 30 s; 40×: 95 °C for 5 s, 60 °C for 30 s; 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Quantitative evaluation of mRNA was performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Values were normalized to those of β-actin. Changes in gene expression were calculated using the ΔΔCt method. The oligonucleotide primer sequences used are shown in Table 1. All experiments were independently replicated in duplicates.

2.6. Date statistics

Statistical data for unstimulated and stimulated samples were obtained using the Kruskal-Wallis and Dunn's multiple comparison tests using GraphPad Prism Software (version 7.05; GraphPad Software Inc., San Diego, CA, USA).

3. Results and discussion

177 This study revealed that mRNA expression levels in bovine alveolar
178 macrophages were altered by stimulation with PAMPs, cytokines, and vaccines, and that
179 these changes were different depending on the type of stimulus. We analyzed TNF-α,
180 iNOS, and IFN-β which cause changes in alveolar macrophage function due to changes
181 in mRNA expression in humans and experimental animals (Aggarwal et al., 2014).
182 TNF-α expression in bovine alveolar macrophages was significantly upregulated
183 by IFN-γ and MLV vaccines (Fig. 1a), and iNOS expression was significantly upregulated
184 by LPS, IFN-γ, and MLV vaccine stimulation (Fig. 1b). Inflammation, which activates
185 and induces immune cells to eliminate pathogens, is essential for the healing of infectious
186 pneumonia, and TNF-α and nitric oxide induced by iNOS are important for the initiation
187 of inflammation. However, cytokine storms can cause excessive inflammation, which can
188 lead to more severe symptoms (Fajgenbaum and June 2020). In acute respiratory
189 syndrome coronavirus 2 (SARS-COV-2) pneumonia, a feedback loop of infected
190 macrophages and IFN-γ produced by T cells leads to excessive alveolar inflammation
191 (Grant et al., 2021). Importantly, these modulations may increase anti-pathogen activity
192 and increase the risk of symptom exacerbation.
193 IFN-β is a type I IFN which provides antiviral immunity, and when produced by
194 alveolar macrophages, acts on autocrine cells and promotes the resolution of

195 inflammation in the respiratory tract (Connolly and Hussell, 2020). Additionally,
196 individuals with severe SARS-COV-2 pneumonia have impaired responses to IFN-β,
197 which may be the reason for the difference in host immune responses (Hadjadj et al.,
198 2020). In the present study, only the MLV vaccine significantly increased the IFN-β
199 expression (Fig. 1c). We believe that elucidation of the mechanism by which IFN-β
200 expression is increased in alveolar macrophages will be an important key point in the
201 development of preventive and therapeutic measures against BRD, and we plan to
202 conduct further analyses.

203 The results of stimulation with the MLV vaccine were of particular interest in
204 this study. The expression levels of TNF-α, iNOS, and IFN-β, increased in a
205 concentration-dependent manner. The MLV vaccine did not contain any adjuvants.
206 BoHV-1 and BPIV-3 are temperature-sensitive strains that cannot infect or be amplified
207 under the present culture conditions. Moreover, BRSV is unstable and grows very poorly
208 in cell culture *in vitro* (Larsen, 2000), and amplification in macrophage-derived cells has
209 not been reported. Based on the above background and the result indicating that the same
210 effect was observed with a vaccine whose infectivity was eliminated by UV irradiation,
211 we believe that viral PAMPs contained in the MLV vaccine directly stimulated alveolar
212 macrophages. Importantly, a detailed examination of the stimulatory or cooperative

213 effects of PAMPs in the three viruses should be conducted. Furthermore, it has been
214 reported that the nasally administered MLV vaccine used in this study does not proliferate
215 in the deep respiratory tract when administered to cattle, but the virus particles themselves
216 do reach the site (Walz et al., 2017). Although it is unclear whether the phenomenon
217 observed *in vitro* is also observed *in vivo*, we hypothesized that virus particles contained
218 in the MLV vaccine administered into the nasal cavity reach the respiratory mucosa and
219 directly stimulate alveolar macrophages. If the interrelationship between alveolar
220 macrophages and PAMPs can be elucidated in detail, it is expected to induce more
221 effective and controlled NSEs.

222 The limitations of this study and future prospects are described below. Whether
223 the upregulation of mRNA expression of IFN-β, TNF-α, and iNOS in bovine alveolar
224 macrophages has the same effect as in humans and experimental animals requires further
225 analysis at the protein and infectivity testing levels. To elucidate the details of the
226 stimulatory response and NSEs induced by PAMPs, including the MLV vaccine, it is
227 important to analyze their relationship with PRRs. However, the RIG-I like receptor
228 family, which has been reported to be particularly important as a viral receptor in humans
229 and mice, has not been reported in cattle (Takeuchi and Akira, 2009), the expression
230 and function of PRRs in bovine alveolar macrophages should be elucidated first.

In summary, this study revealed that mRNA expression levels in bovine alveolar macrophages were altered by stimulation with cytokines and PAMPs. Therefore, it is likely that bovine alveolar macrophages have plasticity, which is regulated by cytokines and PAMPs. In addition, nasal mucosal MLV vaccine stimulation also resulted in large fluctuations in the mRNA expression levels. We believe that this suggests the potential application of NSEs as a candidate for a new preventive strategy against bovine infectious diseases. Further elucidation of the factors and methods that induce NSEs most potently and analyze the plasticity and regulatory mechanisms of alveolar macrophages will contribute to the understanding of the pathogenesis of BRD and the development of preventive and therapeutic measures.

Declarations

Ethics approval

This study was approved by the Animal Care and Use Committee of the Feed Research Institute of the University of Miyazaki which approved all experimental procedures (No. 2017-016-1).

Conflict of Interest

The authors declare the following financial interests/personal relationships that may be considered potential conflict of interests: SI received research funding from Zoetis Japan Inc. None of the other authors have conflicting financial interests.

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345
346 **Figure legend**
347 Fig. 1. Changes in bovine alveolar macrophages. Changes in (a) tumor necrosis factor
348 alpha (TNF-α), (b) inducible nitric oxide synthase (iNOS), and (c) interferon beta (IFN-
349 β) mRNA expression in alveolar macrophages (2×10^5) stimulated by lipopolysaccharide
350 (LPS; 1 μg/mL), polyinosinic-polycytidylic acid sodium salt (Poly I:C; 25 μg/mL),
351 interferon gamma (IFN-γ; 5 ng/mL), modified-live viral (MLV) vaccine-1 (1 μL/mL),
352 MLV vaccine-10 (10 μL/mL), MLV vaccine-100 (100 μL/mL), UV-irradiated MLV
353 vaccine-100UV (100 μL/mL), or non-stimulated (control). Alveolar macrophages were
354 collected 24 h after stimulation. Analysis of relative gene expression data using real-time
355 quantitative PCR and the ΔΔCt method was assessed. Each value was normalized to that
356 of β-actin mRNA and fold-changes in the gene of each stimulation were calculated by

357 referring to the value of the control. Dot plots of individual stimulations: control (circle),
358 LPS (square), Poly I:C (triangle), IFN-γ (inverted triangle), MLV-1 (rhombus), MLV-10
359 (white circle), MLV-100 (white square), MLV-100UV (white triangle), and mean are
360 shown (n=6). Data are representative of two independent experiments. Significant
361 differences between the control and stimulated cells are denoted as *$p<0.05$, **$p<0.01$,
362 ***$p<0.001$, or ****$p<0.0001$.