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Bovine respiratory syncytial virus infection enhances *Pasteurella multocida* adherence on respiratory epithelial cells

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ABSTRACT

Primary infection with bovine respiratory syncytial virus (BRSV) predisposes cattle to secondary infection with bacteria that cause bovine respiratory disease complex (BRDC). However, the interaction between BRSV and bacteria is unclear. This *in vitro* study examined the adherence of *Pasteurella multocida* (PM) to BRSV-infected cells was assessed in colony forming unit assays, by flow cytometry analysis, and by indirect immunofluorescence analysis (IFA) of epithelial cells (A549, HEp-2, and MDBK). An *in vitro* model based on infection of BRSV-infected epithelial cells revealed that PM adherence to BRSV-infected cells was 2- to 8-fold higher than uninfected cells. This was confirmed by flow cytometry analysis and IFA. Epithelial cell expression of mRNA encoding cytokines and chemokines increased after exposure to PM, but increased further after co-infection with BRSV and PM. BRSV-mediated adherence of PM to epithelial cells may underlie the serious symptoms of BRDC.

1. Introduction

Bovine respiratory syncytial virus (BRSV) is a single negative-stranded RNA virus belonging to the *Paramyxoviridae* family and shows a close genetic relationship with human respiratory syncytial virus (HRSV). The genome is translated into 11 proteins by 10 mRNAs (Gershwin et al., 2005; Meyer et al., 2008). BRSV is the primary etiological agent of respiratory disease in calves aged up to 12 months (Gershwin et al., 2005); indeed, it is a major cause of respiratory disease in beef and dairy calves worldwide (Beaudeau et al., 2010; Klima et al., 2014). BRSV is capable of infecting feedlot cattle and cattle on dairy farms, resulting in clinical disease in the absence of bacteria (Larsen et al., 2001; Tjønehøj et al., 2003); however, initial infection by BRSV suppresses the bovine immune system and facilitates secondary infection of the lower respiratory tract by bacteria (Larsen et al., 2001; Gershwin et al., 2005). Therefore, BRSV is considered to be a causative agent of bovine respiratory disease complex (BRDC), which results in economic losses to farmers because of the morbidity and mortality in cattle (Beaudeau et al., 2010; Klima et al., 2014).

BRDC is caused by an interaction between viral and bacterial pathogens; infectious agents include bovine herpesvirus, bovine viral diarrhea virus, bovine parainfluenza-3 virus, *Pasteurella multocida* (PM), *Mannheimia haemolytica*, *Mycoplasma bovis*, and *Histophilus somni* (Larsen et al., 2001; Tizioto et al., 2015). Each pathogen harbors unique features that enable it to act as either a primary source of infection or as a partner in cases of multi-pathogen infection; such infections compromise the protective barrier function of the respiratory epithelium (Härtel et al., 2004; Gershwin et al., 2005). It was reported that the adherences of these bacteria were increased to virus infected respiratory epithelia cells (Galdiero et al., 2002).

According to our preliminary findings based on the BRDC gene detection from respiratory samples, paired virus and bacteria were detected and PM was the most common bacterial agent (unpublished data). PM is common in the nasopharynx of cattle (Timsit et al., 2016; Holman et al., 2017), but not all calves harboring the bacterium develop pneumonia (Autio et al., 2007; Holman et al., 2017). However, although PM appears to be part of the normal flora, it can contribute to pneumonia when cattle become stressed and/or infected by a respiratory virus (Dabo et al., 2007). To act as a pathogen, PM must adhere to and invade epithelial cells (Dabo et al., 2005). Disruption of

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the epithelial lining of the lower respiratory tract allows bacteria to invade the lungs and cause a secondary bacterial infection (Tjønehøj et al., 2003; Gershwin et al., 2005). Treatment of BRSV-infected bovine alveolar cells with *Histophilus somni* concentrated culture supernatant results in morphological changes (cell rounding, retraction, and paracellular migration) (Agnes et al., 2013). Studies in humans demonstrate that infection by HRSV increases adherence of *Streptococcus pneumonia* to respiratory epithelial cells (Yokota et al., 2010) and increases biofilm formation by *Pseudomonas aeruginosa* (Hendricks et al., 2016).

However, the interactions between multiple agents associated with BRDC are not clear. Therefore, the aim of this study was to investigate the effect of BRSV infection on PM adherence to respiratory epithelial cells.

2. Materials and methods

2.1. Cell culture

Adenocarcinoma human alveolar basal epithelial cell type II (A549) cells, human nasopharyngeal carcinoma epithelial (HEp-2) cells, and Madin-Darby Bovine Kidney (MDBK) cells were used. Cells were seeded at 1×10^5 cells/ml and cultured as a monolayer in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biowest, Nuaillé, France), 100 U/ml penicillin, 100 µg/ml streptomycin (Wako, Mie, Japan), and 1 µg/ml amphotericin-B (Wako, Mie, Japan). Cultures were maintained in a humidified atmosphere at 37 °C/5% CO_2.

2.2. Virus and bacteria

BRSV RS-52 strain, isolated from a BRSV vaccine (Kyoto Biken, Kyoto, Japan), was propagated for 7 days in HEp-2 cells cultured in DMEM/2% FBS. BRSV was harvested and kept at −80 °C. Virus titer was checked in a plaque assay using MDBK cells overlaid with 0.8% carboxymethyl-cellulose in Modified Eagle's Medium (Gibco, MD, USA); 5% FBS; 100 U/ml penicillin; 100 µg/ml streptomycin; 1 µg/ml amphotericin-B. Cells were stained with crystal violet in paraformaldehyde. Culture supernatant from BRSV-infected HEp-2 cells was inactivated by UV treatment for 1 h. Inactivation of BRSV was confirmed in a plaque assay.

PM 2368 strain, capsular type B isolated from nasal swab of cattle, was grown at 37 °C in Brucella broth (BD, CA, USA) and shaken at 120 rpm for 24 h. Bacteria were diluted in horse serum (1:1), aliquoted, and kept at −80 °C. PM was then diluted in FBS-free and antibiotic-free DMEM to achieve a multiplicity of infection (MOI) of 100; this was used for co-infection experiments.

2.3. Adherence assay

A549, HEp-2, and MDBK cells were seeded as a monolayer (> 80% confluence) and then inoculated with BRSV (MOI = 0.1 or 1), UV-inactivated BRSV (MOI = 1), and culture medium as a negative control. After 2 h, the inoculation medium was replaced with culture medium containing 2% FBS. After 24, 48 and 72 h post infection (hpi), BRSV-infected cells were exposed to a PM suspension for 2 h at 37 °C. Cells were dissociated by trypsinization and collected by centrifugation at 300 × *g* for 5 min. Cells were then lysed for 10 min at 37 °C with 0.1% Triton-X (Wako, Mie, Japan) in PBS. The resulting bacteria were serially diluted and plated on Brucella agar. The number of colony forming units (CFU) per ml was counted 24 h later. Adherence of bacteria per cell was calculated from total counting of CFU with the total number of monolayer cell.

2.4. Fluorescence assay

For the fluorescence assay, PM (10^9 CFU/ml) was suspended in 0.1 M sodium bicarbonate buffer (pH 9.0) and incubated for 30 min at room temperature with 2 µg/ml FITC (Dojindo, Tokyo, Japan) saturated in DMSO (Nacalai Tesque, Kyoto, Japan). FITC-labeled PM cells were washed with PBS and adjusted to a MOI of 100 in antibiotic-free DMEM.

For immunofluorescence analysis (IFA), BRSV-infected A549 cells were treated with FITC-labeled PM for 2 h and washed with PBS-tween 0.02% (PBST). The cells were then fixed for 30 min in 4% paraformaldehyde (Wako, Mie, Japan). Fixed cells were incubated at 37 °C for 30 min with a rabbit-anti-BRSV antibody (1:500 dilution; Thermo Scientific, MA, USA) and washed three times with PBST. Cells were then incubated with phycoerythrin-conjugated mouse anti-rabbit IgG (1 µg/10^6 cells; Santa Cruz Biotechnology, CA, USA). After washing three times with PBST, cells were examined under a confocal microscope (KEYENCE BZ-900; Keyence, Osaka, Japan).

For the flow cytometry assay, BRSV-infected A549 cells were treated with FITC-labeled PM as described above. The cells were harvested from culture flasks using a cell scraper and fixed with 4% paraformaldehyde. All washing steps after scraping were performed using PBST. Fixed cells were incubated with antibodies as described above. Antibody-stained cells were examined using a BD FACSCanto II (BD Biosciences, CA, USA). Data was analyzed with BD FACSDIVA software (BD Biosciences, CA, USA).

2.5. Quantitative RT-PCR (qRT-PCR) to examine expression of mRNA encoding cytokines and chemokines

A549 cells were infected with BRSV for 3 days and then exposed to PM for 60 min. Total RNA was extracted from the cell lysates using a NucleoSpin kit (TaKaRa, Kyoto, Japan). Quantitative RT-PCR (qRT-PCR) was performed using a One Step SYBR PrimeScript plus RT-PCR kit (TaKaRa, Kyoto, Japan). The primer sets and amplification conditions for qRT-PCR of IL-1β, IL-6, IL-8, MCP-1, and RANTES mRNA were described previously (Lau et al., 2013). Data were normalized to expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Amplification was carried out in a LightCycler 96 system (Roche, CT, USA). All experiments were performed in duplicate. Relative expression of mRNA between infected samples and uninfected controls was calculated using the $2^{-\Delta\Delta CT}$ method and expressed as a -fold change.

2.6. Statistical analysis

All experiments were performed in triplicate unless stated otherwise. Data were expressed as the mean ± standard error of the mean (SEM). Statistical analysis of data in these experiments were performed using a one-way analysis of variance (ANOVA) and Tukey's Multiple Comparison Test, and a p value < 0.05 was considered significant. Data analysis was done using RStudio version 1.0.143.

3. Results

3.1. Adherence of PM to epithelial cells infected with BRSV is time-dependent

We calculated the number of PM cells adhered to each cultured epithelial cell and expressed the results in terms of CFU (Fig. 1A). We did not see a marked increase in PM adherence to A549 and HEp-2 cells during the first 24 hpi with BRSV (MOI = 1). However, there was a marked increase in PM adherence after 48 and 72 hpi with BRSV. The number of PM cells (expressed as bacteria/cell) adhered to BRSV-infected and uninfected A549 cells was increased significantly (73.6 *vs.* 5.8, respectively, at 48 hpi, and 78.7 *vs.*16.1, respectively, at 72 hpi). The number of PM cells adhered to BRSV-infected HEp-2 cells was increased significantly rather than uninfected cells at 48 hpi (32.5 *vs.* 2.85, respectively), and at 72 hpi (32.6 *vs* 12.1, respectively). Similar results were observed for BRSV-infected and uninfected MDBK cells

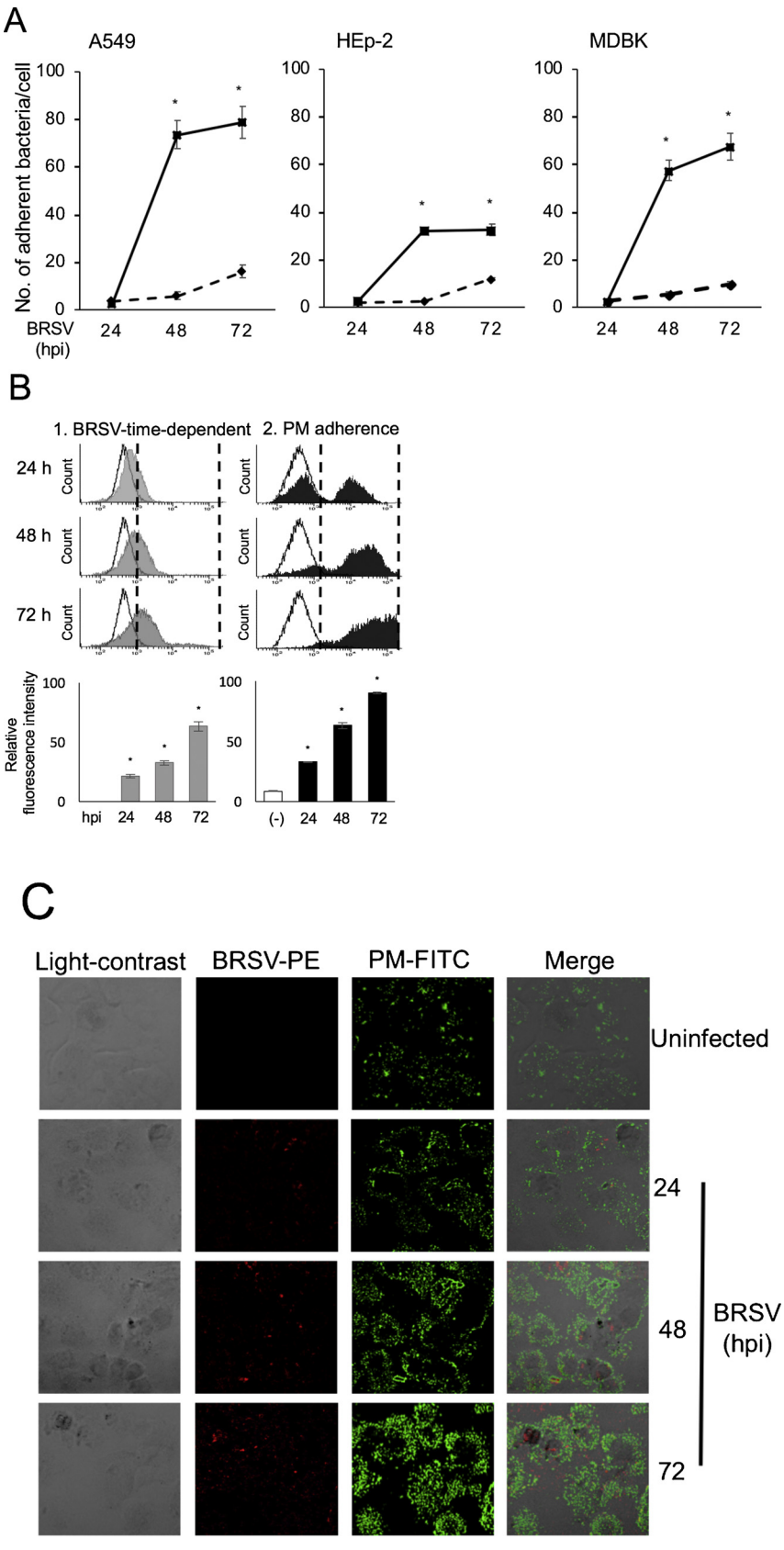

Fig. 1. Adhesion of *Pasteurella multocida* (PM) to respiratory epithelial cells depends on the time for which cells are exposed to the bovine respiratory syncytial virus (BRSV) at MOI 1. (A) The number of PM cells adhering to BRSV-infected A549 HEp-2 and MDBK at 48 and 72 h (*straight line*) post-infection was significantly higher than the number adhering to uninfected cells (*dash line*) at the same times. Data are expressed as the mean ± SEM (n = 4); * $p < 0.01$. (B) Flow cytometry analysis confirmed that the number of PM cells adhering to BRSV-infected A549 cells increased with virus infection time: [1] *gray layer*, number of BRSV-infected cells; [2] *black layer*, number of PM adhering to infected cells significantly enhance from uninfected cell (*open layer*). The graph shows mean relative fluorescence intensity ± SEM (n = 3); * $p < 0.05$. (C) Fluorescence microscopy analysis of FITC-labeled PM (PM-FITC). The results confirm the BRSV-staining with phycoerythrin (BRSV-PE) infection time-dependent increase in adherence of PM-FITC cells to BRSV-infected A549 cells (compared with uninfected A549 cells).

($p < 0.01$). We next used flow cytometry (Fig. 1B) and IFA (Fig. 1C) to examine adherence of FITC-labeled PM cells to A549 cells. Again, the number of FITC-labeled PM cells adhering to BRSV-infected A549 cells increased in a manner dependent on the virus infection time.

3.2. Adherence of PM cells to BRSV-infected epithelial cells is MOI-dependent

Next, we infected epithelial cell lines with different concentrations

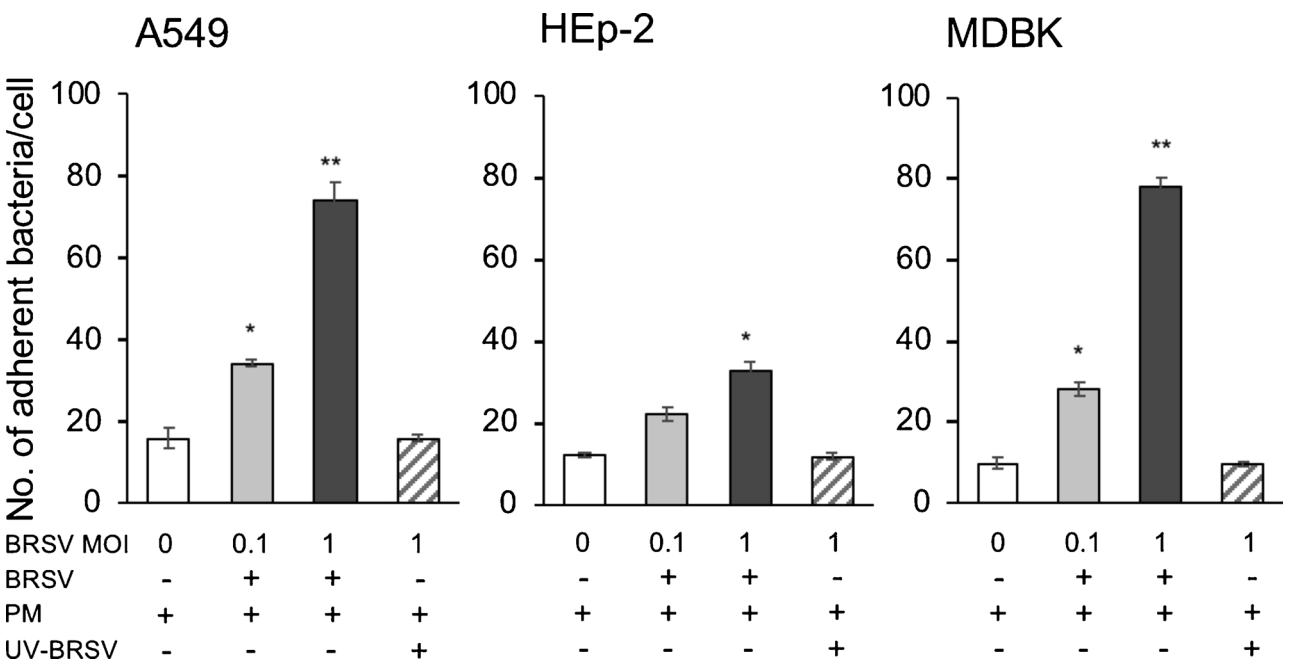

Fig. 2. Adherence of *Pasteurella multocida* (PM) to bovine respiratory syncytial virus (BRSV)-infected epithelial cells is MOI-dependent (*closed bars*). A549, HEp-2 and MDBK cells (were infected with virus at a MOI of 0.1 and 1). This was not the case for uninfected cells (*open bars*) and cells infected with UV-inactivated BRSV (UV-BRSV; *patterned bars*). Data are expressed as the mean ± SEM (n = 4); * $p < 0.05$ and ** $p < 0.01$.

of BRSV (MOI = 0.1 and 1) or UV-inactivated BRSV (MOI = 1) for 3 days and then exposed them to PM cells (Fig. 2). PM adherence to epithelial cells infected with non-UV-inactivated BRSV was significantly higher than that to uninfected cells ($p < 0.01$). BRSV increased adherence of PM cells in a manner dependent on the MOI of virus. Similar results were observed for HEp-2 ($p < 0.05$) and MDBK ($p < 0.01$) cells. UV inactivation of BRSV did not affect PM adherence. Thus, PM adherence to respiratory epithelial cells infected with BRSV is dependent on the MOI of the virus.

3.3. Expression of genes encoding proinflammatory cytokines and chemokines in respiratory epithelial cells co-infected with BRSV and PM

The results so far show that infection with BRSV increases adherence of PM cells to respiratory epithelial cells. Next, we examined changes in expression of mRNA encoding IL-1β, IL-6, IL-8, MCP-1, and RANTES in respiratory epithelial cells (A549) co-infected with BRSV and PM (Fig. 3). Treatment of uninfected cells with PM led to marked upregulation of IL-1β, IL-6, and IL-8 mRNA ($p < 0.01$); however, upregulation of IL-1β, IL-6, and IL-8 mRNA was more significant in cells infected with BRSV prior to exposure to PM ($p < 0.01$). PM treatment of uninfected cells did not affect expression of MCP-1 and RANTES mRNA. However, we noted significant upregulation of MCP-1 and RANTES mRNA in BRSV-infected cells exposed to PM ($p < 0.01$). Thus, BRSV infection leads to a significant increase in expression of genes encoding proinflammatory cytokines and chemokines.

4. Discussion

In vitro co-infection models showed that BRSV infection increased adherence of PM to epithelial cell lines. In addition, we noted a significant increase in expression of mRNA encoding proinflammatory cytokines/chemokines in cases co-infected with BRSV and PM.

BRSV infection led to a significant increase in the number of PM cells adhering to respiratory and/or bovine epithelial cells: adherence was both time- and MOI-dependent (Figs. 1 and 2). BRSV is an important pathogen that can predispose calves to respiratory disease (Gershwin et al., 2005); indeed, BRSV infection often leads to a secondary bacterial infection, which causes BRDC (Larsen et al., 2001; Brodersen, 2010). BAV-7, PIV-3, BAV-3, BCV, and BRSV are common in Finland, and both PM and *Mycoplasma dispar* have been isolated from cattle along with BRSV (Härtel et al., 2004). *Pasteurella* spp. were recovered from BRSV-single inoculated calves made over the BRDC signal (Tjønehøj et al., 2003). Galdiero et al. (2002) reported that increased adherence of PM and *Mannheimia haemolytica* to BHV-infected MDBK cells, but had not tested it on respiratory cells. In humans, infection of airway epithelial cells with HRSV (Hament et al., 2004; Yokota et al., 2010) and metapneumovirus (Lai et al., 2016) increases adhesion of *S. pneumonia*.

Here, our *in vitro* co-infection models showed that adherences of PM cells to BRSV-infected both cell types (A549 and HEp-2) were significantly higher than that to uninfected epithelial cells, this effect was most noticeable in A549 cells. Increased adhesion may be related to virus-induced changes in expression of receptors for bacteria depend on types of cell. Previously, we confirmed that HRSV infection upregulates expression of platelet-activating factor receptor (PAF-R), a receptor for *S. pneumonia*, on A549 (Yokota et al., 2010). Similar phenomena are observed during infection with rhinovirus and coronavirus, which upregulate expression of fibronectin, carcinoembryonic antigen-related cell adhesion molecule, and PAF-R on respiratory epithelial cells (Ishizuka et al., 2003; Golda et al., 2011). However, little is known about how PM attaches to virus-infected respiratory cells. Further experiments are needed to identify receptors for PM on these cells.

BRDC often occurs in cattle that are stressed; this is often coincident with natural exposure to viral and bacterial agents and results in clinical signs and lung pathology (Tizioto et al., 2015). In addition, the presence of more than one microorganism in the respiratory tract can lead to more severe pneumonia (Larsen et al., 2001; Klima et al., 2014). Here, we found that epithelial cells exposed to PM showed increased expression of genes encoding IL-1β, IL-6, IL-8, MCP-1, and RANTES; however, expression increased further upon co-infection with BRSV. We assume that upregulation of these cytokines depends on the number of PM cells adhering to the respiratory epithelial cells. These cytokines play important roles in BRDC-related pneumonia. Cytokines and other mediators released by bovine cells and virus-infected cells contribute to the pathogenesis of BRDC by inducing leukocyte recruitment,

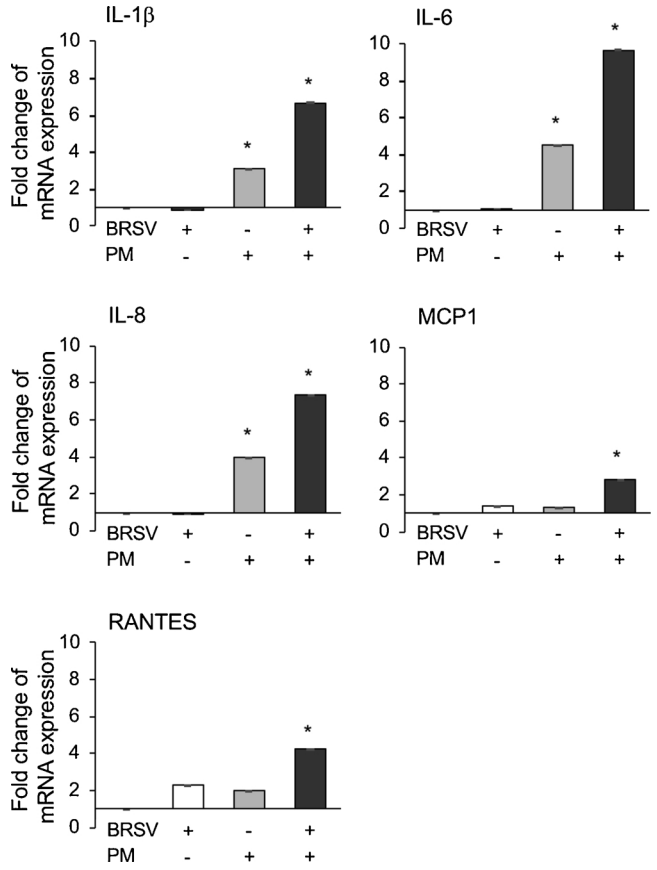

Fig. 3. Expression of mRNA encoding proinflammatory cytokines and chemokines by respiratory epithelial cells co-infected with bovine respiratory syncytial virus (BRSV) and *Pasteurella multocida* (PM). A549 cells were infected with BRSV (MOI = 1) and then exposed to PM at a MOI of 100. The cells were then washed, and total mRNA was analyzed by quantitative RT-PCR. Expression of IL-1β, IL-6, and IL-8 by PM-infected or BRSV plus PM-infected A549 cells is shown. Expression of MCP-1 and RANTES increased only upon co-infection with BRSV and PM. Data are expressed as mean total mRNA expression normalized to GAPDH (± SEM) (n = 4); * $p < 0.01$.

attachment, and activation (N'jai et al., 2013; McGill et al., 2016). BHV-1 infection of bovine bronchial epithelial cells (Rivera-Rivas et al., 2009) and mononuclear cells (Leite et al., 2004) triggers cytokine expression; these cytokines then recruit and activate neutrophils and exacerbate the detrimental effects of MH. Thus, it might be that upregulated production of proinflammatory cytokines, triggered by co-infection with BRSV and PM, initiates an acute and local inflammatory response in the lungs, thereby contributing to development of BRDC.

We and others have used experimental adherence assays based on respiratory and/or bovine epithelial cell lines (Galdiero et al., 2002; N'jai et al., 2013). Galdiero et al. (2002) point out that an *in vitro* model is essential to clarify the relationship between virus and bacterium. Our previous report shows that innate immune responses to HRSV infection of A549 cells are different from those in telomerase reverse transcriptase-transfected human primary respiratory cells (Okabayashi et al., 2011). Thus, establishing an *in vitro* co-infection model based on primary bovine respiratory epithelial cells will not only help identify the origin of inflammatory responses in BRDC but also help us to strengthen the first line of defense against BRDC-related pathogens.

In conclusion, we showed here that BRSV infection of respiratory epithelial cells increases adherence of PM, leading to increased expression of genes encoding proinflammatory cytokines. The *in vitro* data suggest that initial infection by BRSV increases PM adherence, which in turn induces a local acute inflammatory response during the early stages of BRDC.

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