Differential innate immune response of endometrial cells to porcine reproductive and respiratory syndrome virus type 1 versus type 2

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Abstract

Modification of cellular and immunological events due to porcine reproductive and respiratory syndrome virus (PRRSV) infection is associated with pathogenesis in lungs. PRRSV also causes female reproductive dysfunction and persistent infection which can spread to fetus, stillbirth, and offspring. In this study, alterations in cellular and innate immune responses to PRRSV type 1 or type 2 infection, including expression of PRRSV mediators, mRNA expression of toll-like receptor (TLRs) and cytokine, and cytokine secretion, were examined in primary porcine glandular endometrial cells (PGE). Cell infectivity as observed by cytopathic effect (CPE), PRRSV nucleocapsid proteins, and viral nucleic acids was early detected at two days post-infection (2 dpi) and persisted to 6 dpi. Higher percentage of CPE and PRRSV positive cells were detected in type 2 infection. PRRSV mediator proteins, CD151, CD163, sialoadhesin (Sn), integrin and vimentin, were upregulated following type 1 and type 2 infection. *CD151*, *CD163* and *Sn* were upregulated by type 2. Both PRRSV types upregulated *TLR1* and *TLR6.* Only type 2 infection upregulated *TLR3*, but downregulated *TLR4* and *TLR8*. By contrast, both types upregulated TLR4 and downregulated TLR6 protein expression. *Interleukin* (*IL*)*-1β, IL-6* and *tumor necrotic factor* (*TNF*)*-α* were upregulated by type 2, but *IL-8* was upregulated by type 1. Both PRRSV type 1 and 2 stimulated IL-6 but suppressed TNF-α secretion. In addition, IL-1β secretion was suppressed by type 2. These findings reveal one of the important mechanisms underlying the strategy of PRRSV on innate immune evasion in endometrium which is associated with the viral persistence.

Keywords: innate immune response, glandular endometrium, porcine reproductive and respiratory syndrome virus, PRRSV mediators, toll-like receptors

Author Summary

Widely prevalence of porcine reproductive and respiratory syndrome virus (PRRSV) remains the leading cause of huge economic losses to the global swine industry. Due to an infection of macrophages, PRRSV can persist in animals for extended periods of time associated with long-lasting reproductive disorders. Modification of cellular and immunological responses to PRRSV infection which may be related with the pathogenesis of reproductive disorders remains unclear. Herein, direct PRRSV infection of primary porcine glandular endometrial epithelial cell culture (PGE) demonstrated that PRRSV type 1 and 2 upregulated the protein expression of PRRSV mediators correlated with cell persistence of PRRSV. However, TLR and cytokines gene expression, and cytokine secretion were differentially modulated in response to PRRSV type 1 vs. type 2. Our study provides new insights into the cellular mechanism associating with PRRSV persistence in the endometrial cells and the underlying interaction of virus with the host.

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) disease is one of the most economic issue that affects the swine industry [1]. Massive reproductive disorders in sows causing perinatal losses and respiratory distress in piglets is the main impact of PRRSV outbreak [2]. Increased expenses associated with recirculation of PRRSV infection, i.e., confirming PRRSV status and treatment of secondary infections have an indirect impact on the cost of production [1].

PRRSV is a positive single-stranded RNA virus belonging to the *Arteriviridae* family. Two distinct strains, PRRSV type 1 and type 2, are identified and share approximately 60%

67 genetic identity [3]. The virulence, clinical severity, antigenic and immunological diversity are
68 relevant to genetic variability of PRRSV strains [3, 4].

69 In reproductive organs, PRRSV can be transmitted via transplacental viral shedding
70 leading to aborted fetus and/or weak born piglet [5]. It has been suggested that PRRSV
71 replication can occur at the implantation site [6], although PRRSV is highly restricted to porcine
72 alveolar macrophages (PAMs) [7, 8].

73 PRRSV mediators are the major determinant of cellular susceptibility. At least five
74 molecules have been described as PRRSV mediators, such as CD163, Sn, CD151, integrin and
75 vimentin [9, 10]. These receptors play significant roles in PRRSV infection, including virus
76 binding, internalization, uncoating and replication [11]. Previous study in primary porcine
77 glandular endometrial cells (PGE) demonstrates a low expression level of PRRSV receptors,
78 CD163 and Sn; however, PRRSV can induce CPE and damage PGE [12]. Moreover, high
79 existence and release of PRRSV from PGE was gradually increased with time suggesting the
80 susceptibility or recirculation of PRRSV in the infected PGE [12]. Overexpression or
81 modification of PRRSV receptor cDNA in PRRSV non-permissive cell lines can generate the
82 infectious progeny virus from those cells [13]. This raised the possibility that PGE may express
83 alternative PRRSV receptors, in which PRRSV can infect and process host cellular response.
84 The modification of PRRSV receptor expression associated with the increased PRRSV
85 susceptibility and replication remains to be investigated.

86 Innate immune response is primarily responsible for an immediate protection against
87 pathogen invasion and subsequently activates the adaptive immune response. The innate immune
88 response is mediated through host pattern recognition receptors (PRRs), i.e., toll-like receptors

(TLRs). The TLRs 1-9 are expressed in immune cells and non-immune cells like endometrial epithelial cells [14]. Endosomal TLRs, TLR3, TLR7, TLR8, and TLR9 detect internalized viral nucleic acid and promote the production of anti-viral cytokines such as type I interferons (IFN), IFN-α and IFN-β [15]. Membranous TLRs, TLR2 and TLR4 generally recognize bacterial structural proteins but not the invading viral particles. TLR and cell signaling system mediate host-viral PRRSV interaction by inducing the production of pro-inflammatory cytokines, such as IL-6, IL-8 and TNF-α in MARC-145 and PAMs [16]. PRRSV infections are characterized by prolonged viremia and complication from immunosuppressive effects because of the upregulation of *IL-10* and *IL-1β* expression and reduction of *IFN-γ* in porcine polymorphonuclear cells [17, 18]. Thus, the cellular modification of host immune system is relevant to both pathogenesis and host protection of PRRSV. The effect of PRRSV infection on the local innate immunological responses of the reproductive system is associated with reproductive failure is still in question.

In the present study, we employed the PGE infected with PRRSV from our previous study [12] to determine an *in vitro* cellular response in association with cytopathic effect (CPE) and viral replication pertaining to the expression of aforementioned PRRSV mediators, TLRs and pro-inflammatory cytokines secretion following PRRSV type 1 or type 2 infection.

Results

PRRSV type 2 induced CPE and viral existent in PGE

As shown in Fig. 1, PGE infected with PRRSV type 1 or type 2 showed CPE, including syncytial formation (Sc; Fig. 1A) and vacuolization (Vc; Fig. 1A) as early as 2 dpi. The CPE

area at 2 dpi were significantly extended to 20-30% in both type 1 and type 2 inoculated PGE as compared to mock and uninfected PGE (Fig. 1B). A greater extent of CPE area (40%) was found in type 2 inoculated PGE at 4 dpi, but it was recovered at 6 dpi (Fig. 1B). By contrast, the CPE area produced by type 1 were not significantly different from that by the mock and uninfected groups at 6 dpi (Fig. 1B). Corresponding to CPE formation, immunoreactivity of PRRSV envelop protein (GP5; Fig. 1C) was early observed at 2 dpi and remained up to 6 dpi. Type 2 inoculation increased the PGE immunoreactive cells about 3-4 times higher than type 1 inoculation (Fig 1C, $p < 0.05$).

The PRRSV existing in type 2-inoculated PGE was 10^9 $TCID_{50}$/mL which were a ten-fold higher than type 1 at 2 and 6 dpi (Fig. 1D; $p < 0.05$). However, the PRRSV titers were equivalent in both type 1 and type 2 infected PGE at 4 dpi (Fig. 1D; $p > 0.05$).

PRRSV type 2 upregulated *CD151*, *CD163* and *Sn* mRNA expression

The mRNA expression of PRRSV mediators, *CD151*, *CD163*, *Sn*, *integrin*, and *vimentin*, was determined at 4 dpi to examine whether they might be a target involved in PRRSV infection and existent. The normal uninfected PGE expressed a relatively higher level of *vimentin* (3-fold) and low level of *CD151*, *CD163*, *Sn* and *integrin* (0.5-fold) as normalized to *GAPDH* (Fig 2A). At 4 dpi, only type 2 infection was found to upregulate the expression of *CD151*, *CD163* and *Sn* by 4-60-fold ($p < 0.05$, Fig 2B) with no effects on *integrin* or *vimentin*. However, both mock and type 1 infection could not produce any effects on the mRNA expression of PRRSV mediators tested ($p > 0.05$; Fig 2B).

PRRSV type 1 and type 2 upregulated cellular expression of PRRSV mediators

Apart from mRNA expression, cellular localization of PRRSV mediator proteins at 4 dpi was further evaluated using immunohistochemistry in PGE. In uninfected group, at 0 dpi, the immunoreactivity of CD151, CD163, Sn and integrin was rarely detected, whereas that of vimentin was demonstrated about 10% (Fig. 3A). The immunoreactive CD151, CD163, Sn or integrin was distributed in the cytoplasm of mock or PRRSV infected PGE (Fig. 3A). In contrast, the vimentin immunoreactivity which had fiber-like characteristics was observed in the uninfected and mock PGE. Analysis of expression area of PRRSV mediators was not difference between the uninfected and mock PGE ($p > 0.05$; Fig 3A and 3B). Both type 1 and type 2 markedly upregulated the expression of all PRRSV mediators. In this result, the increases in CD151, CD163, Sn, and integrin expression induced by type 2 was higher than type 1 ($p < 0.05$; Fig 3B).

PRRSV type 1 and type 2 upregulated TLR6 and downregulated TLR4 expression

Changes in mRNA and protein expressions of TLRs 1-10 by PGE were also determined following 4 dpi. RT-PCR results of *TLR1-10* gene expression in Fig 4A showed that PRSSV type 1 and type 2 infection upregulated *TLR1* and *TLR6*, while only type 1 upregulated *TLR3* expression ($p < 0.05$; Fig 4A). In contrast, *TLR4* and *TLR8* was downregulated in type 2 infected group ($p < 0.05$; Fig 4A). Most consistent with mRNA expression, the protein expression of TLR1, TLR3, TLR4, TLR6 and TLR10 in uninfected and mock infected groups was not

different ($p > 0.05$, Fig 4B and 4C). Both PRRSV type 1 and type 2 upregulated TLR6 by 2-fold from uninfected group whereas they suppressed TLR4 expression ($p < 0.05$; Fig 4B).

PRRSV type 1 and type 2 upregulated IL-6 and downregulated TNF-α

The mRNA expression of cytokines at 4 dpi and accumulated cytokine secretion at 6 dpi were determined following PRRSV inoculation in PGE. The mRNA expression of cytokines was not different between uninfected and mock infected groups ($p > 0.05$, Fig 5A). *IL-8* was upregulated by type 1, and *IL-1β*, *IL-6* and *TNF-α* were upregulated by type 2 infection ($p < 0.05$; Fig 5A).

Both PRRSV type 1 and 2 stimulated IL-6 but suppressed TNF-α secretion ($p < 0.05$; Fig 5B). The accumulated secretion of IL-1β was additionally suppressed after infection with type 2 ($p < 0.05$; Fig 5B). CCL2, IL-8 and IFN-γ secretions were not different among groups ($p > 0.05$; Fig 5B). Moreover, there was no difference in cytokine secretion between mock and uninfected groups ($p > 0.05$; Fig 5B).

Discussion

Besides alveolar macrophages, the natural cell trophism of PRRSV, PGE has been recently reported as an alternative target of PRRSV, which was preferentially infected via the apical side of PGE monolayer [12]. In relation to the previous study, PGE infectivity as observed by increases in CPE, PRRSV mediators and viral titers were found to be associated with changes in cellular innate immune system, toll-like receptor expression, and cytokine expression and

172 secretion following the apical PRRSV infection in the present study. Both PRRSV type 1 and
173 type 2 infection not only produced the cytotoxic effects but also persisted in PGE up to 6 dpi.
174 However, different cellular responses of PGE to PRRSV type 1 and type 2 infection were
175 observed.

176 In the current study, PRRSV type 2 infected PGE displayed more CPE and higher
177 percentage of PRRSV positive cells than type 1. Thus, more cellular destruction and PRRSV
178 existence caused by type 2 infection indicated the higher virulence to PGE than that by type 1.
179 This finding was consistent with natural infection, which PRRSV type 2 revealed more severity
180 of respiratory distress than type 1 [3]. However, the natural infection or *in vivo* intranasal
181 inoculation with type 1 or type 2 could not demonstrate the different virulence in reproductive
182 signs, i.e., viral load in fetus or maternal-fetal interface, the number of embryonic death or
183 PRRSV-positive litters [19]. In our previous studies, apical route of PRRSV infection alters the
184 permeability and viability of PGE [12, 20]. However, the basolateral route of PRRSV infection
185 at endometrium occurred following viremia also causes reproductive disorders [21]. Therefore,
186 the different route of PRRSV infection which appears to produce severity in reproductive tissue
187 dysfunction should be taken into consideration and confirmed by further study.

188 Our study is the first report to indicate the upregulated cellular expression of PRRSV
189 mediators in response to PRRSV infection in porcine endometrial cells. PGE infected with
190 PRRSV type 2 induced higher degree of upregulated mRNA expressions of *CD151*, *CD163* and
191 *Sn* which were associated with the high titers of PRRSV persistence and release from infected
192 cells. The increased PRRSV mediators in PGE may play a substantial role in the sensitivity and
193 persistence to the consecutive PRRSV infection.

Besides, CD151 expression was found to be significant higher in endometrioid carcinoma, sarcoma or carcinosarcoma [22]. Interaction between CD151 and integrin promoted outgrowth in human embryonic carcinoma cell line NT2N [23]. Therefore, upregulated CD151 may explain the proliferative effects of PRRSV type 2 infection in PGE [20]. Moreover, the infected PGE was associated with severe membrane integrity dysfunction [20]. Thus, modulation of PRRSV mediators following PRRSV infection may be the underlying pathogenesis of endometrial function leading to the reproductive failure in sows.

TLRs and cytokines are the key components of mucosal innate immune responses in female reproductive tract. Upregulated TLR3, TLR7, and TLR8 expressions were correlated to PRRSV virulence and clinical signs in highly pathogenic PRRSV-infected pigs [24]. Differential regulation on *TLR1-10* expression was also observed following type 1 or type PRRSV infection in PGE. Type 1 infection produced stimulatory effects on *TLRs*, *TLR1*, *TLR3* and *TLR6* which seems not to be correlated with virulent characteristics found in the present study. Although the upregulated *TLR1* and *TLR6* are not described as virulent characteristics [24], our findings that PRRSV type 2 upregulation of *TLR1* and *TLR6* and downregulation of *TLR4* and *TLR8* are likely to produce high infectivity, virulence, and persistence in the reproductive epithelial cells PGE. Among the target genes of PRRSV infected PGE including *TLR1*, *TLR3*, *TLR4*, *TLR6* and *TLR8* were indicated, only TLR4 and TLR6 protein expression were changed by PRRSV infection.

Upregulated TLR protein expression in PRRSV infected pigs contributes to disease progression or the severity of clinical respiratory signs because of an excessive response of pro-inflammatory cytokine via PAMPs/TLR signaling system [25]. Constitutive secretion of IL1β, IL-6, IL-8 and TNF-α which is mediated through TLRs activation by TLRs ligand, including

poly I:C dsRNA simulating viral nucleic acids has been previously demonstrated in PGE [26].

Secretion of CCL2 and IFN-γ by PGE was additionally demonstrated in this study; however, secretion of IL-10 and IFN-α was absence in all groups (data not shown). Thus, the presence of these innate immunity-related molecules indicates the ability of PGE to interact with the viral pathogens and establish a major part of the innate immune response of endometrial cells.

In PGE, PRRSV type 2, but not type 1 infection, induced *IL-1β*, *IL-6*, *IL-8* and *TNF-α* mRNA expression at 4 dpi, which revealed the highest CPE area of infected PGE. The recent findings agree with the virulent effects of PRRSV type 2 in *in vitro* studies of PAMs and peripheral blood mononuclear cell (PBMCs) [18, 25, 27].

In general, pro-inflammatory cytokines IL-1β and TNF-*α* are released by host to encounter invasive pathogens including viruses. The downregulated TNF-α synthesis and secretion by PRRSV infection in PGE were consistent to the study of PAMs infected with PRRSV type 2 [28]. Incubation with recombinant TNF-α was reported to reduce existent of PRRSV [28]. In PRRSV-infected PGE, particularly type 2, the increased viral existence at 2-6 dpi (Fig. 1D) was concurrent to the decreased IL-1β and TNF-α.

The suppressed TNF-α induced by PRRSV has been indicated to carry out by Nsp1 by inhibiting the activation of NF-κB and Sp1 transcription factors on the promoter region of *TNF-α* [29], whereas upregulated IL-β production in PAMs induced by PRRSV was mediated via TLR4/MyD88 pathway [30]. Therefore, the reduced IL-1β secretion in PGE by type 2 may be due to the suppressive effect of PRRSV on TLR expression. This was evidenced by the highest degree of TLR4 suppression and IL-1β inhibition in type 2 infected PGE. As the expression level of IL-1β was related to PRRSV clearance [31], the reduced TLR4/IL-1β system and TNF-α

238 production by PRRSV might be the strategy of virus to escape the obliteration of host cells.
239 Herein, the decreased CPE with persistence of PRRSV type 2 at 6 dpi may support these
240 assumptions. Taken together, the downregulated TNF-α and IL-1β secretion by PRRSV in
241 immune and PGE cells reflects a poor innate immune response which leads to secondary
242 infection by other microbial pathogens [32].

243 Additionally, the largely increased IL-6 secretion was found in response to PRRSV
244 infection. Excessive expression of IL-6 followed by the increased expressions of pro-
245 inflammatory cytokines and chemokines, including IL-1β, IL-12, IL-8, and TNF-α was relevant
246 to the severe lung injury and damages of lymphoid organs in highly pathogenic PRRSV (HP-
247 PRRSV) [33]. It is likely that type 2 induced higher damages of PGE in our previous [20] and
248 current studies may be explained by the increase in *IL-1β*, *IL-6* and *TNF-α* mRNA expression.

249 Apart from immune function, pro-inflammatory cytokines play roles in pregnancy and
250 parturition. The locally increased IL-1β and TNF-α from neutrophil infiltrated uterine are
251 required for muscle contraction and cervical ripening during parturition [34]. However,
252 application of IL-1β and/or TNF-α caused the dissolution of collagen fibers, stromal edema, and
253 severe inflammation in the cervix of guinea pigs [35]. Exposure or infusion of IL-1β and TNF-α
254 caused the defects associated with peripartum intrauterine inflammation, abnormal lung
255 development associated with bronchopulmonary dysplasia, and brain injury [34]. Thus, the
256 modulation of cytokine synthesis and release by PRRSV infection in PGE affecting the
257 reproductive infertility needs a further study.

258 # Materials and Methods

Reagents and materials

Cell culture-grade reagents of Ringer's solution, ethanol, isopropanol, H_2O_2 and methanol were purchased from Sigma Chemical Co., (St Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's PBS, fetal bovine serum (FBS), collagenase type I, 0.05% trypsin-0.53 mM ethylenediaminetetraacetic acid (EDTA), kanamycin, penicillin-streptomycin and fungizone were purchased from Gibco BRL (Grand Island, NY, USA).

Antibodies

Rabbit polyclonal antibody (pAb) against PRRSV envelop glycoprotein GP5 (Biorbyt Ltd., Cambridge, UK; dilution 1:100) was used to detect PRRSV protein. To localize PRRSV mediators, antibodies with the final dilution including rabbit pAb anti-human CD151 (Abcam, Waltham, MA, USA; 1:250), goat pAb-anti-porcine CD163 (Santa Cruz biotechnology, Dallas, TX, USA; 1:25), mouse monoclonal Ab (mAb)-anti-sialoadhesin (Bio-rad, Inc., Hercules, CA, USA; 1:250), goat pAb anti-integrin-α3 (Santa Cruz biotechnology, Dallas, TX, USA; 1:25) and mAb anti-vimentin (Santa Cruz biotechnology, Dallas, TX, USA; 1:250) were used for immunostaining. To assess the expression of toll-like receptors, mAb anti-TLR1, mAb anti-TLR3, mAb anti-TLR4, mAb anti-TLR6, and mAb anti-TLR10 were purchased from Santa Cruz biotechnology (Dallas, TX, USA). Other antibodies including mAb anti-β-actin, HRP-conjugated anti-mouse IgG, and mAb anti-goat IgG were purchased from Bio-rad (Hercules, CA, USA).

PGE cell culture

PGE were isolated from Thai crossbred commercial 4–6 months old gilts provided by a government-qualified slaughterhouse in Bangkok, Thailand, following a previous protocol [36]. Briefly, the uterine horn was opened along the longitudinal line and washed in Ca^{2+}- and Mg^{2+}-free PBS. The mucosal layer was stripped off, minced, and digested overnight with 0.2% collagenase. Endometrial glands were isolated from the digested tissues by filtration (40 µm pore size) followed by a gravitational sedimentation. The sedimented glands were collected and cultured in DMEM containing 5% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 100 µg/mL kanamycin, 1% non-essential amino acids, and 10 µg/mL insulin) at 37°C in 5% CO_2. The culture media were refreshed every 2 days. The contamination of immune cells was removed, and the attached epithelial cells became confluent monolayers within 3-4 days. The remaining stromal cells were removed by adding 0.02 % collagenase in serum-free medium for 24 h. After reaching 90% confluence, the cells were trypsinized and sub-cultured to an appropriate cell culture vessel for each experiment.

Following our previous study [36], PGE purity over 98% was determined by the immunocytochemistry staining of anti-pan cytokeratin antibody and transepithelial electrical resistance (TER) of greater than 400 $\Omega.cm^2$. PGE monolayers with TER of 400-800 $\Omega.cm^2$ which were considered as high tight junction integrity for studying ion transport were chosen for inoculation. The contamination of PGE with *Mycoplasma spp*., swine fever or PRRSV was assessed by a multiplex reverse transcription quantitative polymerase chain reaction (RT-qPCR) detection kit (Microplasma 16 s Ribosomal RNA Gene Genesig® Standard kit, [Primerdesign, Camberley, UK]; Virotype® CSFV RT-PCR kit, [QiagenIAGEN, Leipzig, Germany]) [12]. The mentioned pathogen-negative PGE was chosen for PRRSV inoculation.

Preparation of PRRSV inoculum

PRRSV was isolated from the lungs of pigs with respiratory and reproductive illness and positive PRRSV sera at the Farm Animal Hospital (Faculty of Veterinary Science, Chulalongkorn University, Nakorn Pathom, Thailand). To confirm and prepare PRRSV inoculum, 2.3 g of the infected lung tissues was minced, homogenized in 15 mL of cold FBS-free DMEM, and centrifuged at 10,000 × g and 4°C for 10 min following the previous protocol [37]. The supernatants were collected and filtered through a 0.2-µm syringe, diluted with FBS-free DMEM at a 1:1 ratio, and freshly proceeded to RT-qPCR using primers specific to ORF7 of type 1/type 2, ORF 7 of type 1, and ORF 7 of type 2, as previously described [12, 38].

According to our previous study [12], 1 µg of cDNA template was mixed with qPCR SYBR master mix in the presence of forward and reverse primers. All reactions were subjected to CFX96™ Real-Time PCR Detection System (Bio-rad, Hercules, CA, USA) using the following cycle: 95°C for 3 min to activate the reaction, followed by 40 cycles of amplification steps, including denaturation at 95°C for 20 s, annealing at 60℃ for 30 s and extension at 72℃ for 30 s, respectively. The specificity of amplified products was confirmed using 1.5% agarose gel electrophoresis and melting curve analysis. The lungs of PRRSV-negative pigs were isolated and used for mock infection. No amplicons were produced in the mock control.

PRRSV inoculum quantification

All PRRSV inoculum were quantitated using MARC-145 cells (ATCC American Type Culture Collection, VA, USA) cultivating in a 25-cm^2 flask (Costar®, Corning, MA, USA) supplemented with DMEM containing 10% FBS. To quantitate the virus concentration as

described previously [12], PRRSV inoculum at 1 mL of 10-fold serial dilutions (10^{-1} to 10^{-6}) were incubated with the confluent MARC-145 cells at 37°C in 5% CO_2 for 1 h. After viral inoculation, the cells were washed and replaced with the fresh media. CPEs in each MARC-145 cell culture well were observed microscopically at 2-, 4- and 6-days post-infection (dpi). Following the Reed-Muench method [39], the dilution that produced CPEs by 50% was considered the tissue culture infective dose 50% ($TCID_{50}$)/mL PRRSV inoculum stock at the endpoint dilution of 10^5 $TCID_{50}$/mL to produce the pathological changes was used in this study [12].

PRRSV inoculation

PGE ($1x10^6$ cells/mL) were seeded into 24 mm membrane cell culture inserts (Transwell, MA, USA) or 25 cm^2 flasks (Costar, MA, USA), and maintained in the culture medium for 7 days to become confluence. PGE were then allocated to Mock-, PRRSV type-1 or type-2 group (n = 5 pigs each group). According to our previous protocol [12], the PGE monolayer in 24 mm culture inserts or 25 cm^2 flasks was respectively inoculated with 1 mL or 5 mL of PRRSV 10^5 $TCID_{50}$/mL inoculum in 5% CO_2 at 37°C for 1 h. Inoculation with solution extracted from PRRSV-negative lungs was used for the mock group. After 1 h of viral adsorption, the cells were washed and replaced with fresh medium for 2-6 days. Each inoculation was performed in duplicate.

Immunohistochemistry, detection, and quantitation of PRRSV infection and mediators

All PRRSV-infected PGE were confirmed by the presence of CPE at 2, 4 and 6 dpi. All CPEs were observed and measured in filter-grown PGE under light microscope with digital camera (BX50F and UC50, Olympus, Tokyo, Japan). The total area of CPE was normalized with the membrane filter area and reported as a percentage. To detect and quantitate PRRSV existent, PRRSV isolated from PGE and culture medium were assessed by RT-qPCR with the inoculation to MARC-145 cells following the Reed-Muench method [39].

Following the detection of CPE in PRRSV-infected PGE, immunostaining with primary antibodies (Ab) to recognize viral protein PRRSV-GP5, and PRRSV mediators CD151, CD163, sialoadhesin (Sn), integrin, and vimentin was performed. Following our previous protocol [12], filter-grown PGE were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 10 min at 25°C. Fixed PGEs were treated with the non-specific blocking solution, 10% H_2O_2 in methanol, followed by 4% goat serum in PBS for 4 h. The treated samples were incubated with primary antibodies at 4°C overnight followed by universal HRP-conjugated secondary antibodies (Vectastain® Elite ABC-HRP kit, Vector Laboratories, Inc., Burlingame, CA, USA). Immunoreactivity was developed by incubating membrane filter with DAB (3,3-diaminobenzidine tetrahydrochloride) substrate (Sigma Aldrich, MO, USA) and counter-stained with hematoxylin (Invitrogen, Waltham, MA, USA). Immunoreactive area was visualized under light microscope connected to a digital camera (BX50F and UC50, Olympus, Tokyo, Japan) with 20x magnification. All positive cells were captured by the digital images at the magnification of 20x and analyzed by ImageJ software (National Institutes of Health, Bethesda, MD, USA). The area in pixel numbers of immunoreactive cells (dark-brownish staining) was measured and calculated as the percentage of the total area of PGE, which comprised immunoreactive and non-

363 immunoreactive cells per fields. The means of all percentages of positive cells in each group
364 were compared.

365 Total RNA isolation and reverse transcription

366 Total RNA was collected from PGE (uninfected, mock, type 1, and type 2 groups)
367 grown in T25 flask using TRIzol® reagent (Invitrogen, Waltham, MA, USA). The concentration
368 of total RNA was measured for optical density at 260 nm (OD_{260}) using Nanodrop (NanoDrop
369 2000, Thermo Fisher Scientific, Waltham, MA USA). The purity of RNA was acceptable if the
370 OD_{260}/OD_{280} ratio was at 1.8-2.0. Reverse transcription was done by using iScript® Select
371 cDNA synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, a 20 µl of reaction
372 mixture consisting of total RNA 3 µg, oligo dT primer and iScript reaction was prepared. First-
373 strand DNA was synthesized in thermocycler (Biometra GmbH, Göttingen, Germany) using the
374 following cycle: 25°C for 3 min, 46°C for 20 min, and 95°C for 1 min. cDNA concentration was
375 measured using NanoDrop and product stored at -20°C until performing real-time PCR.

376 Real-time PCR

377 The mRNA expression of PRRSV mediators, TLRs, and cytokines was carried out
378 using a GeneOn SYBR green based qPCR kit (GeneOn, Deutschland, Germany) following the
379 previous protocol [12, 20]. Briefly, a 20 µl of PCR reaction containing 1 µg of cDNA template,
380 2x qPCR SYBR master mix, and a pair of forward and reverse primers for each gene (Table 1)
381 was prepared. Forty cycles of reaction at 95°C, 60°C, and 72°C for 20, 30, and 30 s, respectively,
382 were carried out in a DNA Thermal Cycler (CFX96^{TM}, Bio-rad, Hercules, CA, USA). The
383 amplicons were evaluated for the specificity of product by running 1.5% agarose gel

384 electrophoresis and analyzing melting curve. The relative mRNA expression of interested genes
385 was determined by normalization with GAPDH mRNA expression and reported as fold change
386 compared to those of uninfected PGE using $2^{-\Delta\Delta Ct}$ calculation [40].

387 Semiquantitative Western blot analysis

388 PGE proteins were harvested using lysis buffer containing 50 mM tris, 150 mM NaCl,
389 1mM EGTA, 1mM PMSF, 1% NP-40, 6.02 mM sodium deoxycholate, 0.01 mg/ml aprotinin, 1
390 mM NaF and a cocktail protease inhibitor. Cell lysate was centrifuged at 12000 rpm for 15 min
391 at 4°C. The supernatant was collected and measured for a protein concentration using the BCA™
392 protein assay (Thermo Fisher Scientific, MA, USA). The protein sample was diluted with
393 Laemmli buffer containing β-mercaptoethanol (Bio-rad, Hercules, CA, USA) and incubated at
394 65°C for 5 min. The obtained denatured protein (30 μg) was separated by 10% sodium dodecyl
395 sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to a PVDF membrane
396 (Millipore®, St Louis, MO, USA). After blocking non-specific protein with 2% bovine serum
397 albumin, the blotted membrane was probed with primary antibodies at 4°C for 12 h and further
398 stained with HRP-conjugated secondary antibodies for 1 h at room temperature. Dilution of all
399 antibodies was used following the manufacturer's instruction. An ECL substrate (Santacruz
400 Biotechnology, Dallas, TX, USA) was used to develop immunoreactive bands, which were
401 visualized by exposure to an X-ray film (GE healthcare, Bloomington, IL, USA). The protein
402 expression of TLRs (n = 5 pigs each group) was analyzed by Scion image software and
403 normalized to β-actin. The results were represented as relative fold changes of protein expression
404 over those of mock group.

Measurement of cytokine secretion

Media collected from the apical and basolateral compartment of filter-grown PGE at 0, 2, 4 and 6 dpi were determined for cytokine secretion, which consisted of chemokine (C-C motif) ligand 2 (CCL-2), IL-1β, IL-6, IL-8, IL-10, TNF-α, IFN-α and IFN-γ by enzyme-linked immunosorbent assay (ELISA) using commercial kits (R&D System, Minneapolis, MN, USA). The ELISA processes were performed according to the instruction of commercial kits (n = 5 pigs for each group). Cytokine concentration was measured at OD_{450}, by a microplate reader (Biotek, Winooski, VT, USA), in which a background OD_{620} was subtracted from all data. The concentration was calculated from a standard curve. The accumulated concentrations of each cytokine from 0-6 dpi were normalized by dividing with those of uninfected cells and displayed as fold changes of uninfected cells.

Statistical analysis

All PGE data were obtained from 5 pigs per group and shown as the mean ± SEM. Statistical analysis was done by GraphPad Prism 9.0 (GraphPad software Inc., San Diego, CA, USA). The percentages of CPE and PRRSV positive cells among different dpi were analyzed by two-way ANOVA, and the mRNA expression of interested genes, protein expression, and cytokine secretion were analyzed by one-way ANOVA. The multiple comparison with the uninfected group was performed by Bonferroni's post hoc test. A p value of less than 0.05 was considered as significant differences.

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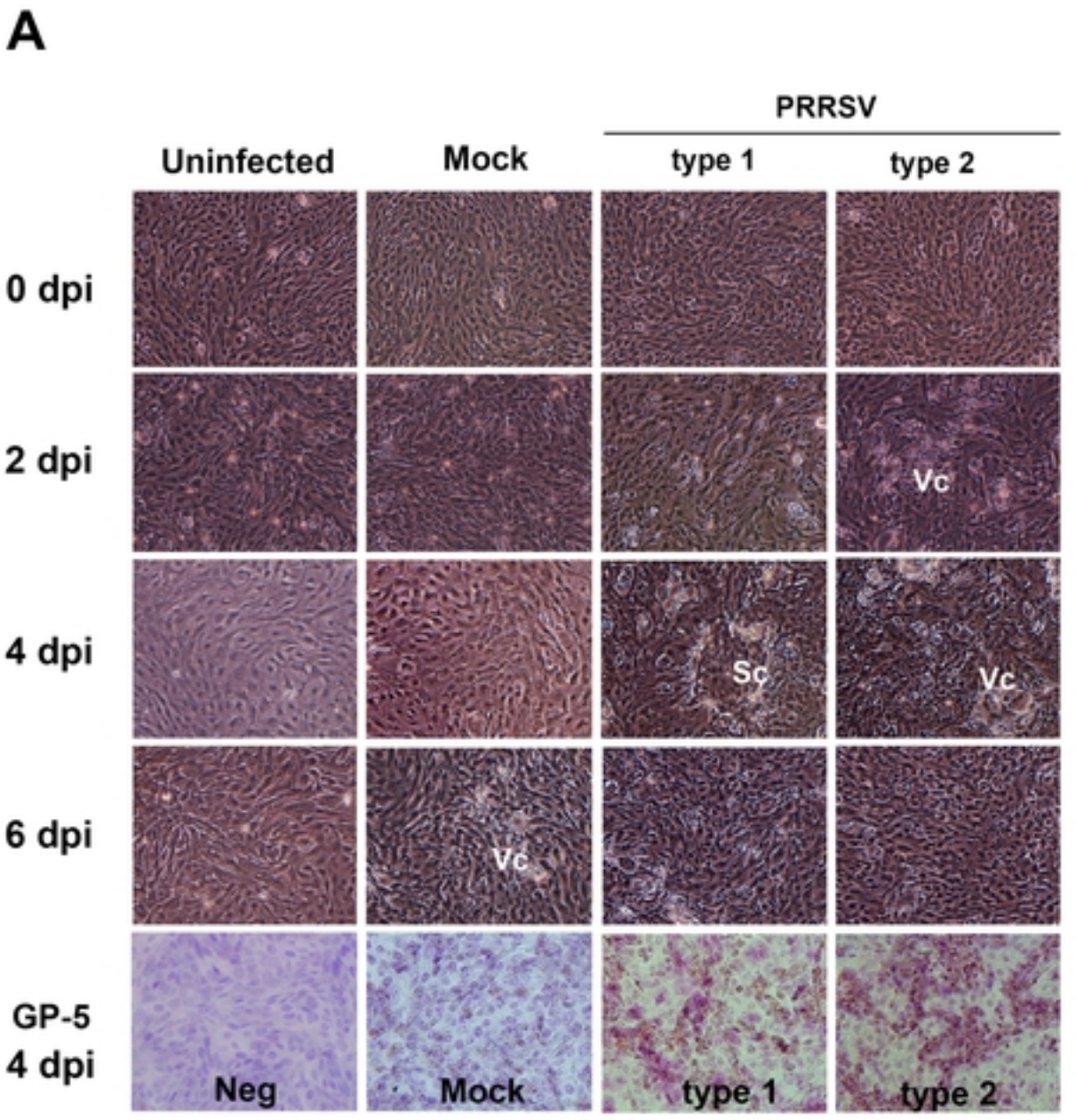
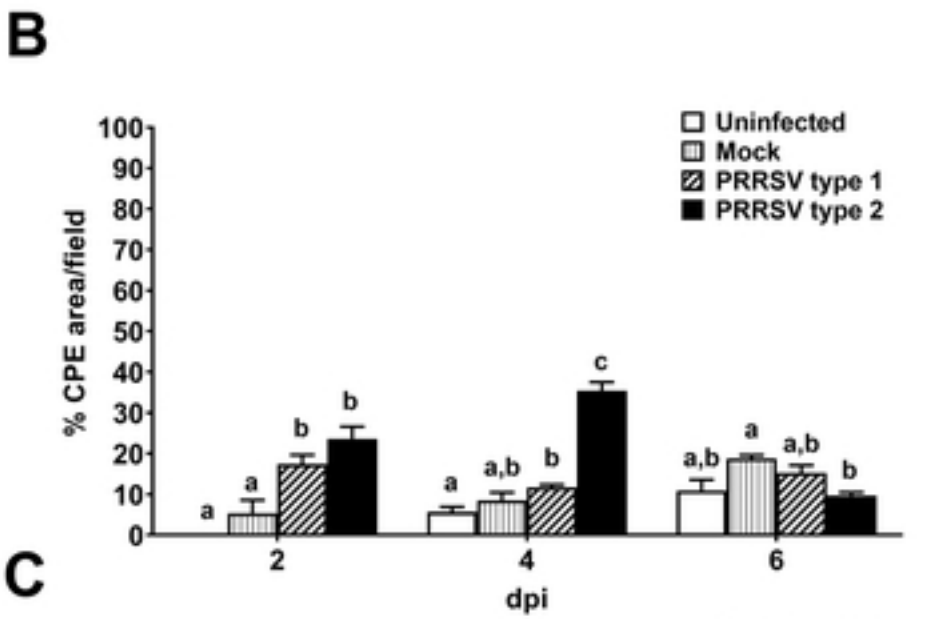
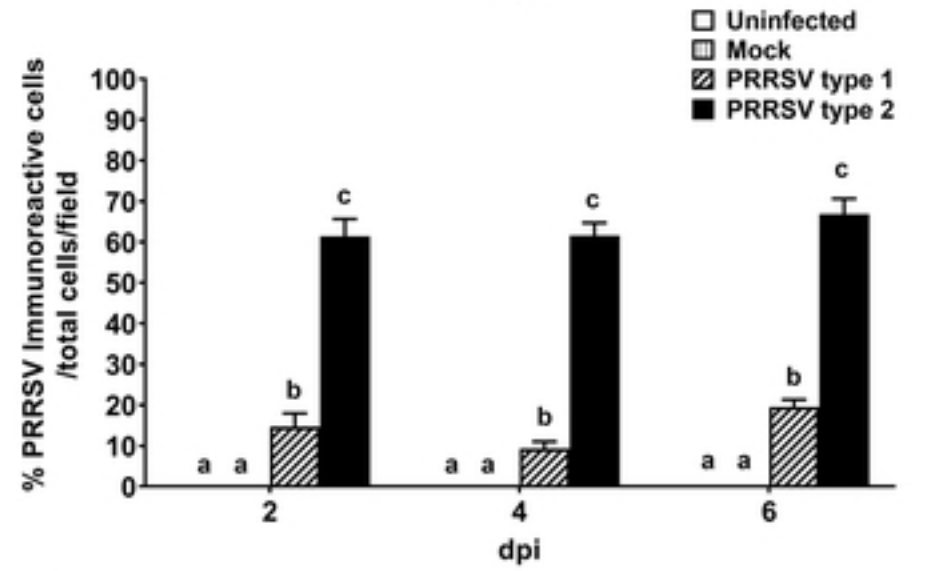

A
PRRSV
Uninfected
Mock
type 1
type 2
0 dpi
2 dpi
4 dpi
6 dpi
GP-5
4 dpi
Vc
Sc
Neg
Mock
type 1
type 2


B
Uninfected
Mock
PRRSV type 1
PRRSV type 2
% CPE area/field
dpi


C
Uninfected
Mock
PRRSV type 1
PRRSV type 2
% PRRSV Immunoreactive cells /total cells/field
dpi

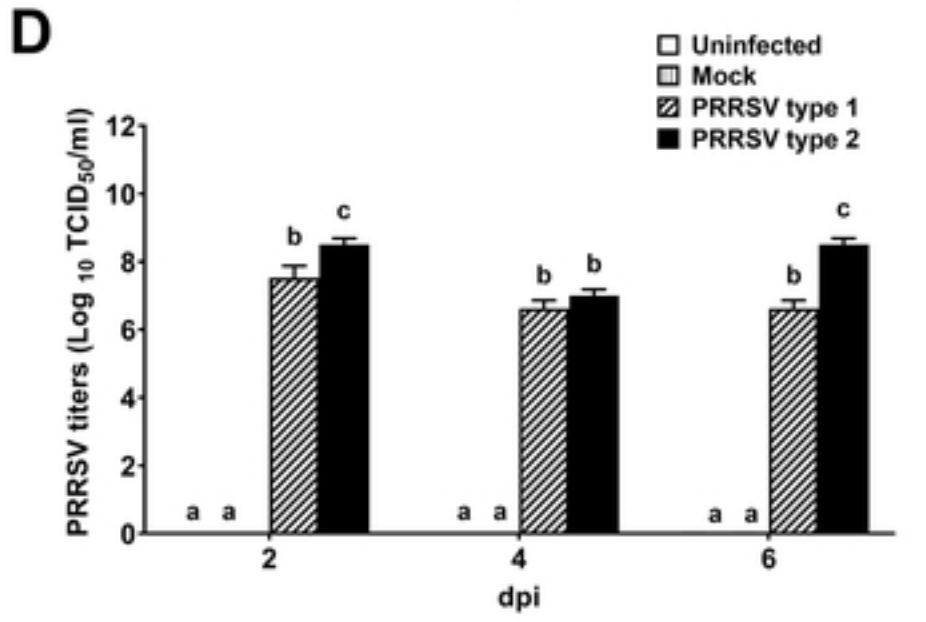

D
Uninfected
Mock
PRRSV type 1
PRRSV type 2
PRRSV titers (Log 10 TCID50/ml)
dpi

Figure 1

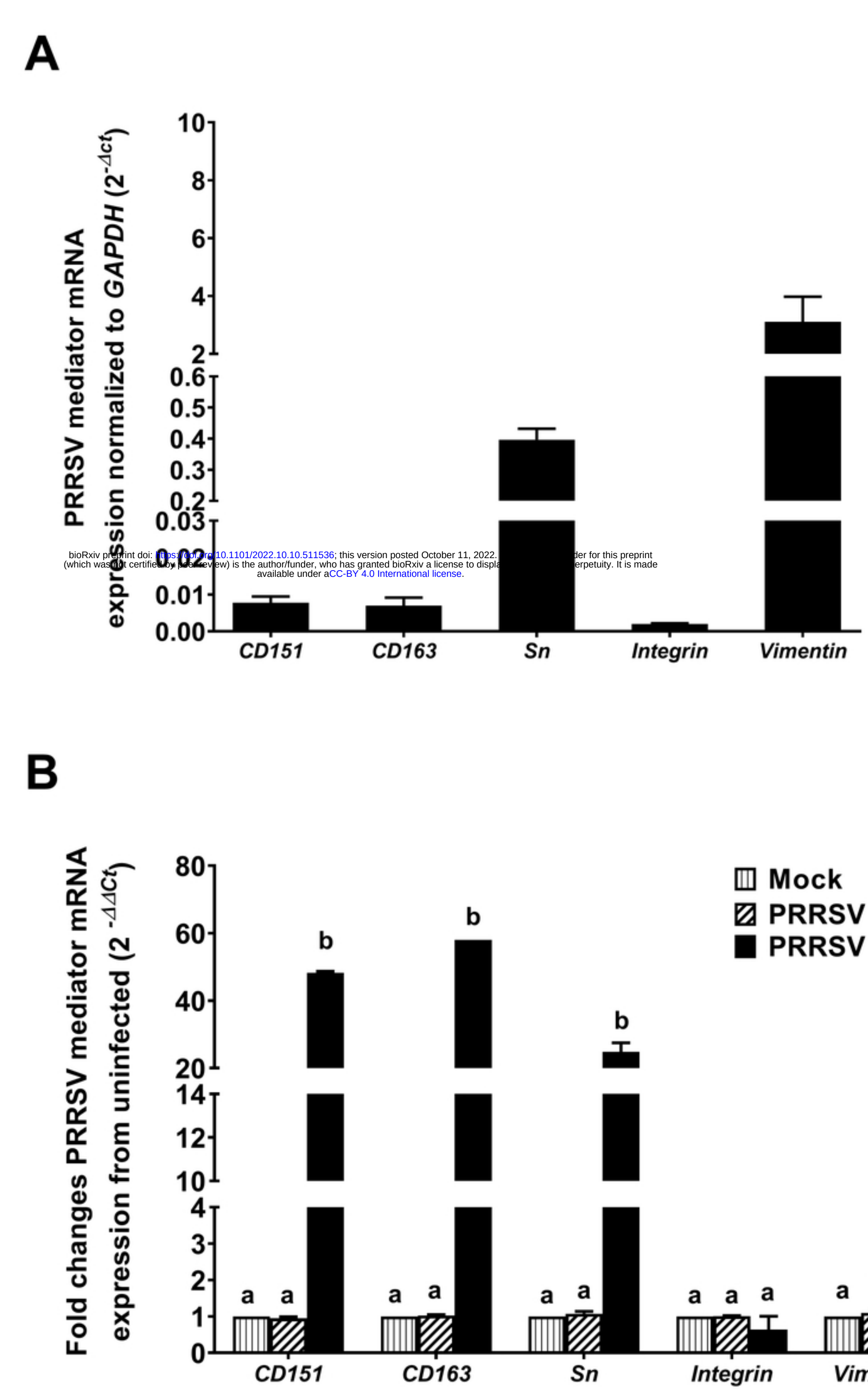
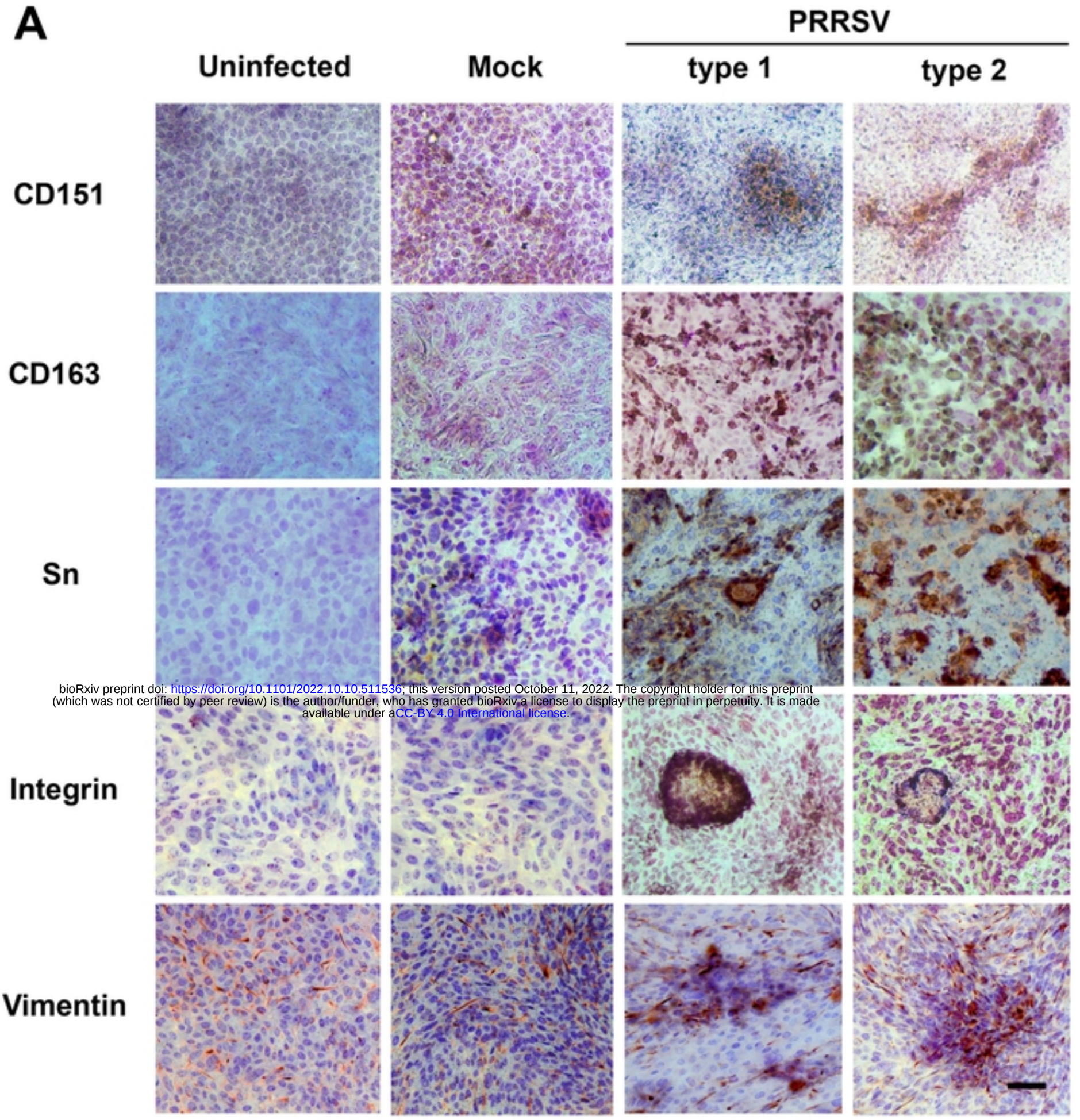


Figure 2



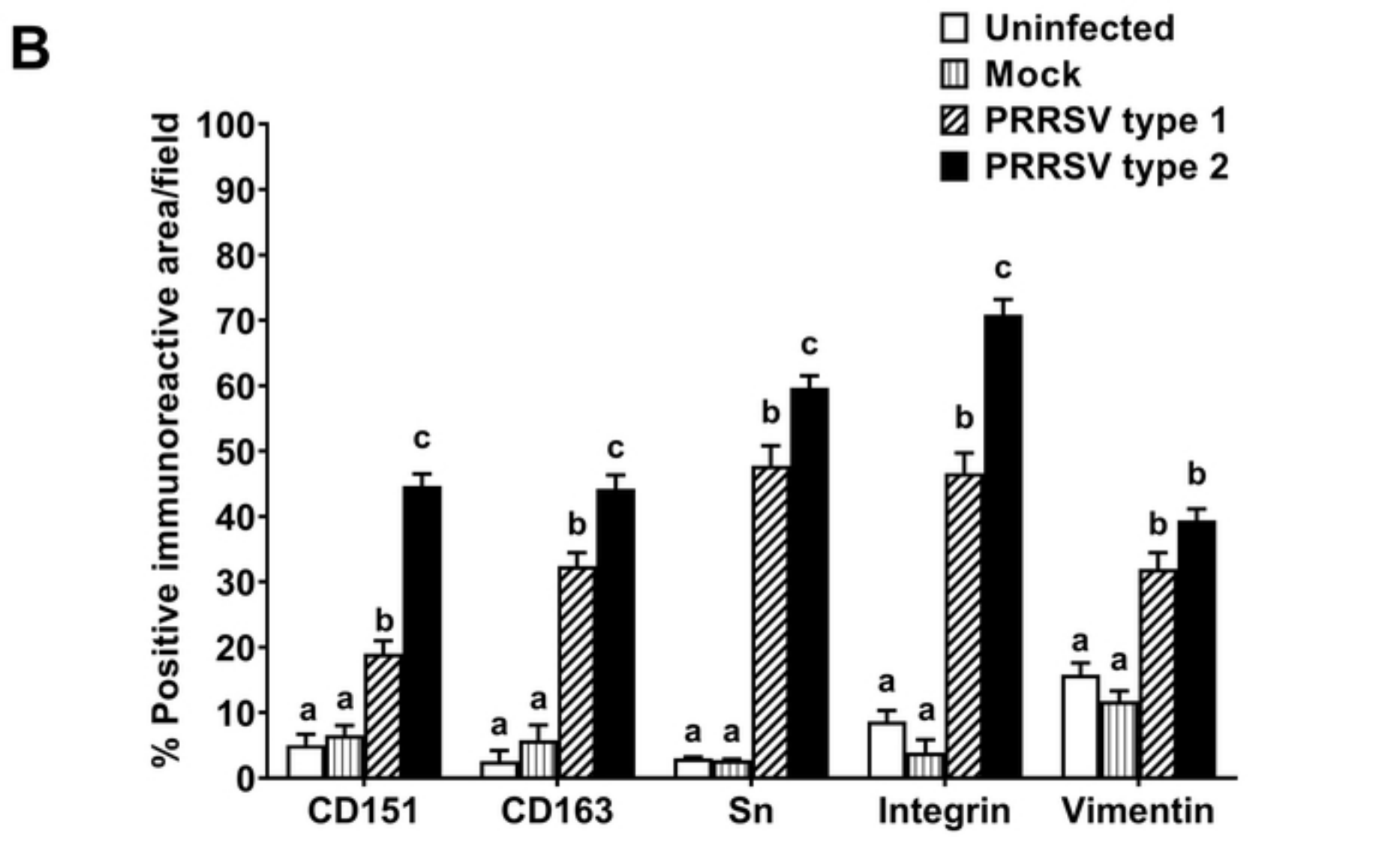


Figure 3

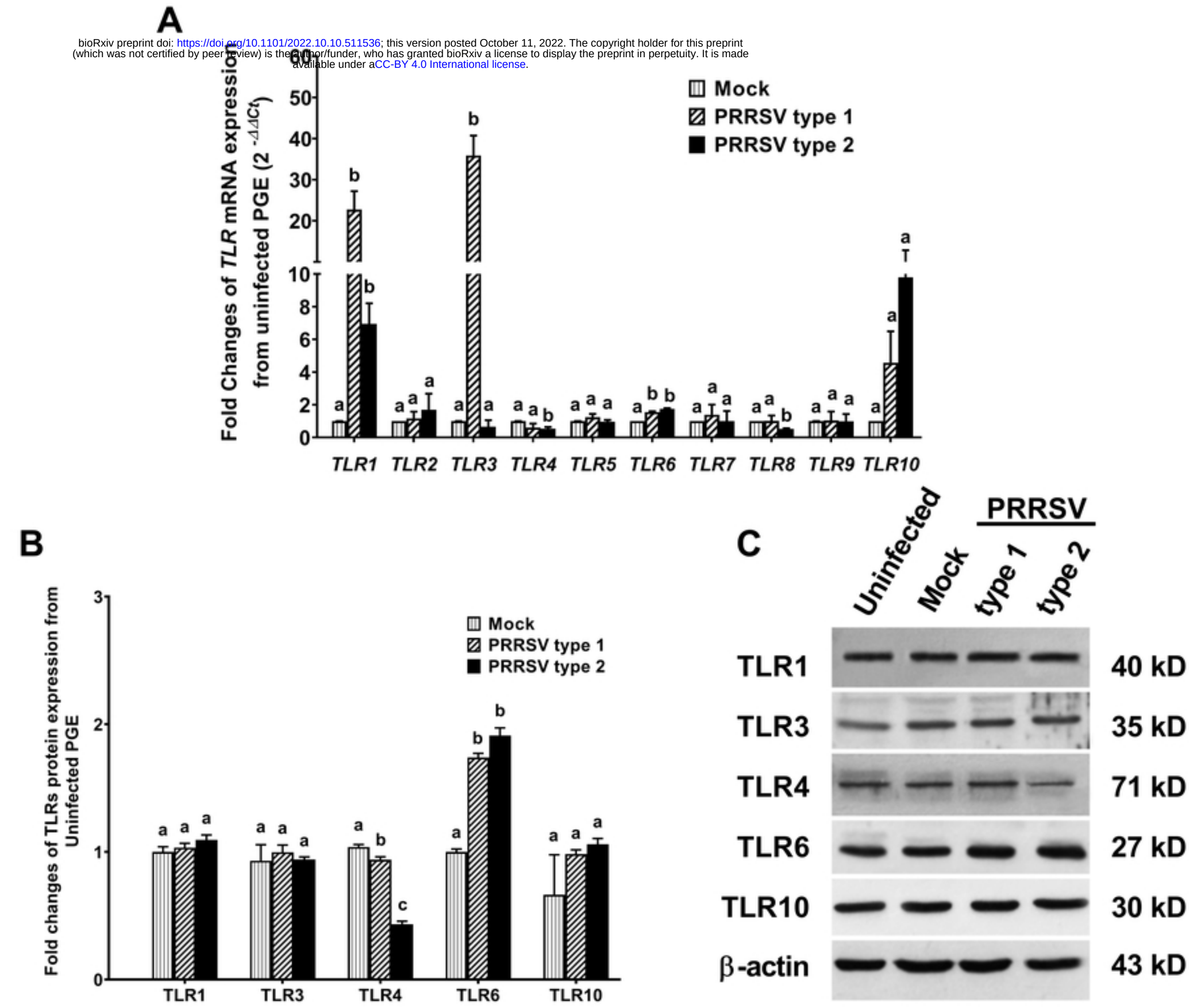


Figure 4

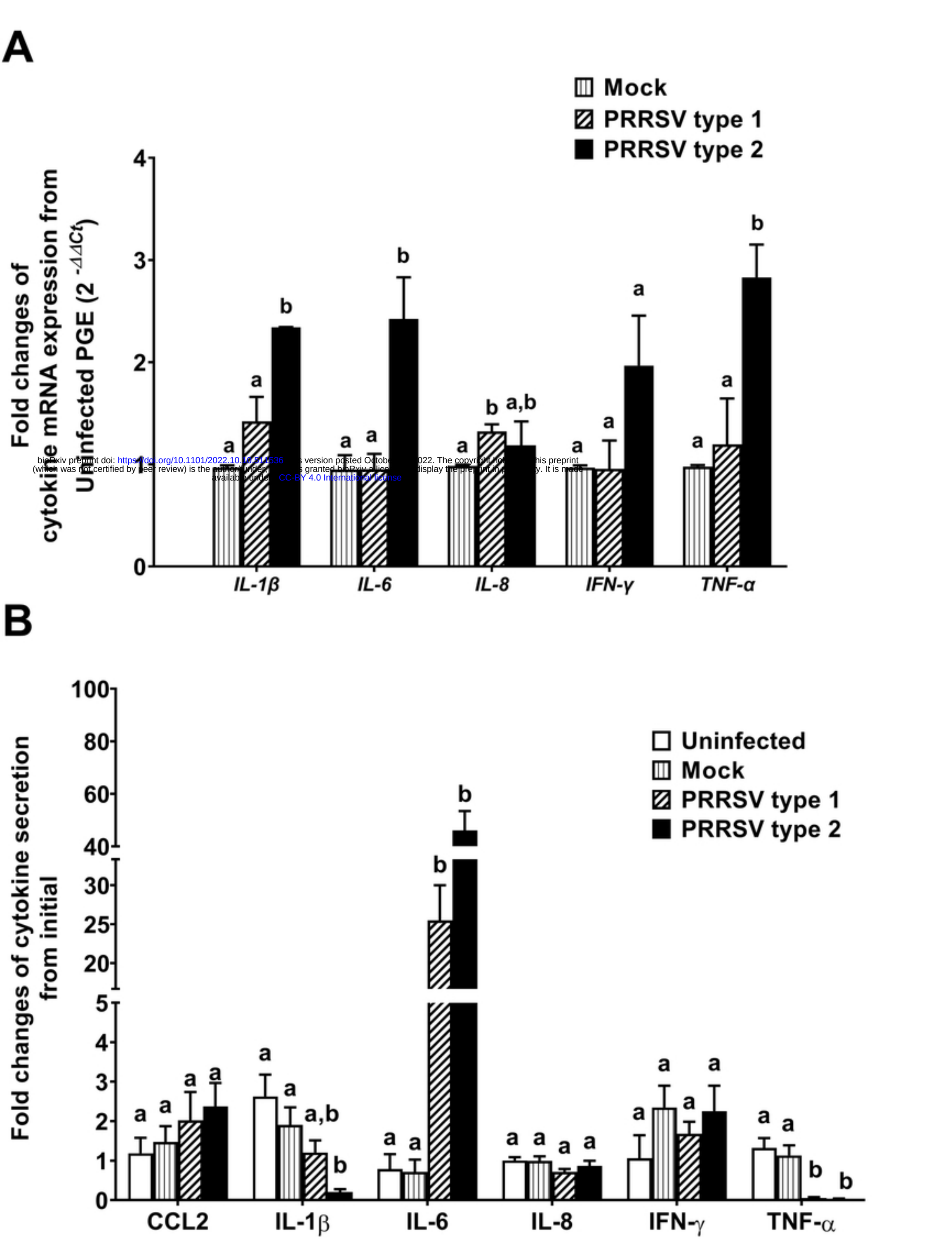


Figure 5