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Reduction of porcine reproductive and respiratory syndrome virus (PRRSV) infection in swine alveolar macrophages by porcine circovirus 2 (PCV2)-induced interferon-alpha

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Abstract

Two common viral pathogens of swine, namely, porcine circovirus type 2 (PCV2) and porcine reproductive and respiratory syndrome virus (PRRSV), were investigated in regard to their effects on monolayer cultures of swine alveolar macrophages (AMs). The purpose was to identify selected cellular changes and responses potentially associated with the clinical reactions of pigs infected with either or both of these viruses. Measurements included the (1) absolute and relative numbers of infected, viable, and apoptotic cells; (2) distribution of viral antigens; (3) levels of interferon-alpha (IFN- α) and tumor necrosis factor-alpha (TNF- α) produced and their association with the extent of virus-induced cytopathology. Four groups of AMs were studied, including mock-infected, PCV2 alone-infected (PCV2-A), PRRSV alone-infected (PRRSV-A), and PCV2 and PRRSV dually infected (PCV2/PRRSV) groups. The AMs of PCV2-A group had high antigen-containing rate without cell death. There was a marked increase in cell death and apoptosis in PRRSV-A group. However, a lower PRRSV-induced infectious rate, cell death, and apoptosis were seen in PCV2/PRRSV group. High levels of IFN- α production were detected in PCV2-infected groups, but not in mock-infected and PRRSV-A groups. The PRRSV-induced cytopathic effect (CPE) on MARC-145 cells or swine AMs was markedly reduced by pre-incubation of the cells with UV-treated or non-UV-treated supernatants of PCV2-infected AMs. In addition, the reduction in CPE was abolished when the supernatants of PCV2-infected AMs were pre-treated with a mouse anti-recombinant porcine IFN- α antibody. The results suggest that swine AMs were an important reservoir of PCV2; PCV2 infection reduced PRRSV infection and PRRSV-associated CPE in PCV2/PRRSV AMs; the reduction of PRRSV infection in AMs was mediated by IFN- α generated by PCV2 infection. The reduced

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PRRSV-associated CPE in AMs and increased pro-inflammatory cytokine production may lead to a more severe pneumonic lesion in those dually infected pigs.

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1. Introduction

Post-weaning multisystemic wasting syndrome (PMWS) is a worldwide disease that debilitates nursery and fattening pigs with systemic lymphadenopathy and interstitial pneumonia and has contributed to significant economic loss to swine industry (Allan et al., 1998; Ellis et al., 1998). Porcine circovirus 2 (PCV2) alone has been demonstrated to induce PMWS in gnotobiotic (Ellis et al., 1999), cesarean-derived colostrum-deprived (CD/CD) (Bolin et al., 2001), and specific pathogen-free (SPF) (Magar et al., 2000) pigs. However, experimental infection with PCV2 alone only produced minimal symptoms and mild bronchiolitis and interstitial pneumonia (Allan et al., 2000; Bolin et al., 2001; Ellis et al., 1999; Magar et al., 2000). Pigs with PMWS often had concurrent infection with porcine parvovirus (PPV) (Choi and Chae, 2000), porcine reproductive and respiratory virus (PRRSV) (Segalés et al., 2002), or Aujeszky's disease virus (PRV) (Quintana et al., 2001). Experimental dual infection of PCV2 and PRRSV-induced PMWS in CD/CD pigs that had more severe interstitial pneumonia and enhanced replication and distribution of PCV2 in the tissues (Allan et al., 2000; Harms et al., 2001). These findings, therefore, suggest that the development of full-blown PMWS-induced clinical symptoms and pathological lesions requires other porcine pathogens as the co-factors.

Monocyte/macrophage lineage cells are the major target cells, both in vivo and in vitro, for PCV2 and PRRSV (Allan and Ellis, 2000; Chiou et al., 2000). Alveolar macrophages (AMs) are essential defense cells in the lungs. However, there is no study addressing the effects of dual infection of PCV2 and PRRSV on swine AMs. Interferon-alpha (IFN- α) and/or tumor necrosis factor-alpha (TNF- α) have been shown to possess anti-viral activity (Vilcek and Sen, 1996; Wong and Goeddel, 1986). Porcine respiratory coronavirus (PRCV)-induced IFN- α was shown to interfere with PRRSV replication in the lungs of pigs,

but PRRSV infection has little or no effect on the replication of PRCV in porcine lungs (Buddaert et al., 1998). Currently, the interaction between PCV2 and PRRSV in swine AMs and the production of cytokines associated with individual or dual infection are unclear. The purpose of the present study was to determine the effects of PCV2 and/or PRRSV infection on swine AMs regarding infectious rate, cell viability, apoptosis, production of IFN- α and TNF- α , and the role of IFN- α and TNF- α in the interaction.

2. Materials and methods

2.1. Experimental animals

Four to 6-week-old, crossbred, male and female, non-vaccinated, SPF pigs were used for collection of AMs from the lungs. All pigs were tested negative for PRRSV, PCV1, and PCV2 antibodies and nucleic acids by indirect immunofluorescence assay (IFA) and RT-PCR or multiplex-PCR, respectively.

2.2. Collection of AMs

Bronchoalveolar lavage was performed as previously described (Chiou et al., 2000). The cells collected by lavage were suspended in RPMI-1640 medium (Gibco Laboratories, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (HIFBS) (Gibco Laboratories), 2 mM L-glutamine (Sigma, St. Louis, MO, USA), 100 U/ml of penicillin (Sigma), and 100 μ g/ml of streptomycin (Sigma) (RPMI-C). The overall cell viability determined by trypan blue dye exclusion assay was greater than 95% and more than 95% of the cells were AMs. The cells were adjusted to 2×10^7 cells/ml in a pre-cooled medium containing 60% RPMI-1640, 20% HIFBS, and 20% dimethylsulfoxide (DMSO) (Merck,

Darmstadt, Germany) and stored in liquid nitrogen until used.

2.3. *Viruses*

A stock of the 8th passage of PRRSV tw91, a Taiwan field isolate obtained in 1991, at a titer of 10^7 TCID₅₀/ml was prepared and titrated on MARC-145 by cytopathic effect (CPE) (Chiou et al., 2000). The PCV2 was isolated from the pooled spleen and lymph nodes of a PMWS pig using a PCV1/PCV2/PRV-free PK-15 cell line and propagated as reported by IFA at a titer of 5×10^6 TCID₅₀/ml (Tischer et al., 1987).

2.4. *Preparation of AMs*

Prior to each experiment, AMs were quickly thawed and washed with RPMI-C. The cell viability was re-evaluated and the AMs concentration was adjusted to 5×10^5 cells/ml. Only those cells with viability above 95% after freezing and thawing were used. Three millilitres or 0.5 ml/well of AMs at 5×10^5 cells/ml were placed in Teflon flasks (Nalgene Company, Rochester, USA) or 24-well culture plates (Costar, Cambridge, MA, USA), respectively, and immediately exposed to one or both viruses or equal volume of RPMI-C. Infected or mock-infected cultures were subsequently evaluated for the extent of infection, the presence and distribution of viral antigen, cell viability, apoptosis, and the production of IFN- α and TNF- α . Values associated with cultures infected with one or both viruses and with mock-infected control were compared.

2.5. *Experimental design*

For each assay, four groups of AMs, including mock-infected, PCV2 alone-infected (PCV2-A), PRRSV alone-infected (PRRSV-A), and PCV2 and PRRSV dually infected (PCV2/PRRSV) groups, were inoculated with one or both viruses at an m.o.i. of 0.1 each or with an equal volume of medium. At 18, 36, 54, 72, 90, and 108 h post-infection (HPI), AMs or culture supernatants from each group were obtained and used for various assays.

2.6. *Antigen-containing rate or infectious rate*

The PCV2 or nucleocapsid protein of PRRSV was detected by IFA. At each time point, 100 μ l of PCV2 and/or PRRSV-infected or mock-infected AMs were taken from each flask, spun onto glass slides by cytospin centrifuge (Shandon, Pittsburgh, PA, USA), fixed in acetone at 4 °C for 10 min, and incubated with a hyperimmune rabbit anti-PCV2 anti-serum followed by a goat anti-rabbit IgG-Texas Red (Rockland, Gilbertsville, PA, USA) or with the anti-PRRSV monoclonal antibody SDOW17 (South Dakota State University, South Dakota, USA) followed by a goat anti-mouse IgG-FITC (Leinco, St. Louis, MO, USA). Cells with light red or green fluorescence were counted and the percentages of positivity were calculated.

2.7. *Viability assay*

The direct cytotoxic effect of PCV2 and/or PRRSV on AMs was determined using the trypan blue dye exclusion assay as previously described (Chiou et al., 2000). The percentage of viable cells out of 100 randomly selected AMs was calculated.

2.8. *Apoptosis*

The rate of apoptosis was analyzed by TUNEL assay. At each time point, an aliquot of 100 μ l of AMs was taken from each experimental group and spun onto glass slides by cytospin centrifuge (Shandon), and fixed in 4% paraformaldehyde (PFA) (Merck) in PBS (pH 7.4) at 15–20 °C for 1 h. Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end-labeling was then applied according to the manufacturer's instructions (in situ cell death detection kit, fluorescein; Boehringer Mannheim, Mannheim, Germany). After 1 h of incubation and PBS wash, the cells were counterstained with 1% Hoechst 33258 (Sigma) and mounted with coverslips. Apoptotic cells had green fluorescent nuclei. The percentage of AMs with apoptosis from each group was counted on a fluorescent microscope.

2.9. *Interferon-alpha bioassay*

The IFN- α bioassay was modified from that reported by Rubenstein et al. (1981). Before the measurement,

the culture supernatants collected at various incubation intervals were thawed and irradiated with UV light at a distance of 15 cm for 20 min by using a GL-15 UV lamp (15 W, Toshiba, Tokyo, Japan) to inactivate the remaining PCV2 and PRRSV. Complete inactivation was further confirmed in PK-15 or MARC-145 by IFA. The IFN titers were then determined by the CPE reduction bioassay using Madin–Darby bovine kidney (MDBK) cells (kindly provided by National Veterinary Research Institute, Tamsui, Taiwan) and vesicular stomatitis virus (VSV) (kindly provided by Dr. Tse-Wen Chang, Department of Life Sciences, National Tsing Hua University, Hsin-Chu, Taiwan). MDBK cells were seeded at 5×10^5 cells/well in 96-well culture plates in 100 μ l of DMEM-C. After overnight incubation and medium removal, 100 μ l of serially two-fold diluted culture supernatants or recombinant porcine IFN- α (Chemicon, Temecula, California, USA) used as a standard were added. Following another 18 h of incubation, 50 μ l/well of VSV at an m.o.i. of 0.1 or DMEM-C were then added. Twenty-four hours later, CPE was examined by light microscopy. After removing the medium, the cells were washed with PBS and fixed with 4% PFA in PBS (pH 7.4) at room temperature (RT) for 10 min. After PBS wash, 100 μ l/well of a 0.05% crystal violet solution in 20% ethanol were added and reacted at RT for 10 min. Following tap water rinse and drying, 100 μ l/well of absolute methanol were added. The plates were gently agitated, and the optical densities (ODs) were read on an ELISA reader (Model EL311sx, Bio-Tek, Winooski, VT, USA) at 595 nm. All samples were tested in triplicate. Data were expressed as mean unit (U)/ml, where 1 unit of IFN- α activity was defined as the reciprocal of the dilution producing 50% inhibition of CPE. To verify the anti-viral activity of IFN- α , culture supernatants were mixed with an equal volume of a mouse anti-recombinant porcine (rp) IFN- α antibody at 1 μ g/ml (Serotec, Oxford, England) before subjected to IFN- α bioassay.

2.10. Titration of tumor necrosis factor-alpha

The levels of TNF- α in the culture supernatants were determined using a commercialized ELISA kit (Endogen, Woburn, MA, USA). The plates were read on the ELISA reader (Bio-Tek) at 550 nm. All samples were tested in triplicate. Data were expressed as mean

concentration at pg/ml, where it was obtained by converting the OD value of each sample to the corresponding concentration based on the standard curve obtained from the serially 10-fold diluted known concentration of rpTNF- α .

2.11. Protection of MARC-145 cells or swine AMs from PRRSV infection by PCV2-induced IFN- α and/or TNF- α

To determine the role of PCV2-induced IFN- α and/or TNF- α on PRRSV infection, a protection assay using the supernatant of PCV2-A AMs collected at 54 HPI was performed in PRRSV-infected MARC-145 cells or swine AMs. MARC-145 cells or swine AMs at 5×10^5 cells/well were seeded in 96-well culture plates in 100 μ l of DMEM-C. Following overnight incubation and medium removal, 100 μ l of PCV2-infected AM supernatant or supernatant pre-mixed with an equal volume of a mouse anti-rpIFN- α (Serotec) or rpTNF- α (Endogen) antibody at 1 μ g/ml each were added. Fifty microlitres per well of PRRSV at an m.o.i. of 0.1 were added and CPE was evaluated 5 days later. After PBS wash and fixing with 4% PFA, 100 μ l/well of 0.05% crystal violet in 20% ethanol were added and reacted at RT for 10 min. After adding 100 μ l/well of absolute methanol, the plates were gently agitated and the ODs were read on the ELISA reader (Bio-Tek) at 595 nm.

2.12. Statistical analysis

The data were analyzed by the analysis of variance (ANOVA) (Statistical Analysis System; SAS for windows 6.12; SAS Institute Inc., Cary, NC, USA) and Duncan's multiple range test. A *P*-value of less than 0.05 was considered significant.

3. Results

3.1. Antigen-containing or infectious rate of infected swine AMs

No PCV2 or PRRSV antigen was detected in the mock-infected group (Fig. 1A). In the PCV2-infected groups, the positive cells displayed a pinpoint to small granular, intracytoplasmic bright red fluorescence (Fig. 1B). Greater than 95% of the AMs from either

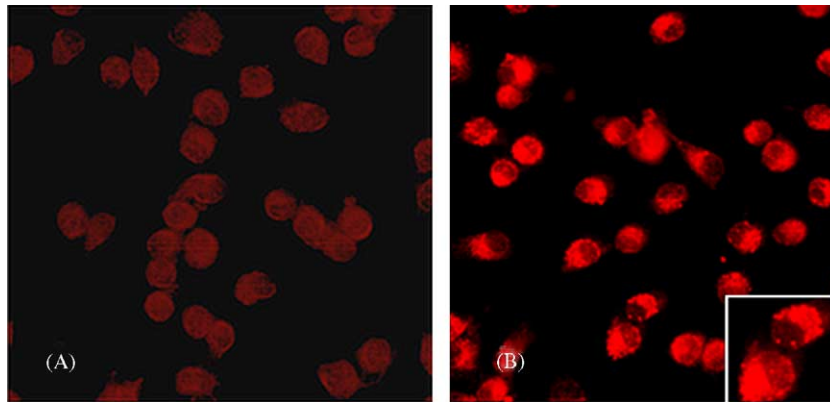


Fig. 1. The results of immunofluorescence antibody staining for mock-infected and porcine circovirus 2 (PCV2)-infected swine alveolar macrophages (AMs). (A) No PCV2 antigen is present in mock-infected group; $\times 450$. (B) Approximately 97% of the AMs in PCV2 alone-infected group have positive signal in the cytoplasm at 18 h post-infection (HPI); $\times 450$. *Inset*: a pinpoint to small granular, intracytoplasmic bright red fluorescence (Texas-red) is seen in PCV2-infected AMs; $\times 1250$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PCV2-A or PCV2/PRRSV group were PCV2-positive at 18–108 HPI (data not shown). On the contrary, the PRRSV-infected AMs showed a diffuse bright green fluorescence in the cytoplasm; however, the non-PRRSV-infected but PRRSV-positive apoptotic body-containing AMs had small, scattered, spotted, instead of diffuse, green fluorescence in the cytoplasm (Fig. 2A). Only those AMs with diffuse bright green fluorescence in the cytoplasm were counted as PRRSV-infected AMs. A low but constant infectious rate about 5–10% was noted in PRRSV-A group at 18–

108 HPI (data not shown). The PRRSV infectious rate was about 9.3% at 18 HPI in PCV2/PRRSV group, but the infectious rate gradually reduced and became 1.2% by 108 HPI (Fig. 2B).

3.2. Viability of infected swine AMs

The viability of swine AMs from the four experimental groups is summarized in Fig. 3. The average survival rate of AMs was $96.1 \pm 1.3\%$ before viral inoculation. It dropped to $83.7 \pm 1.5\%$ at 18 HPI

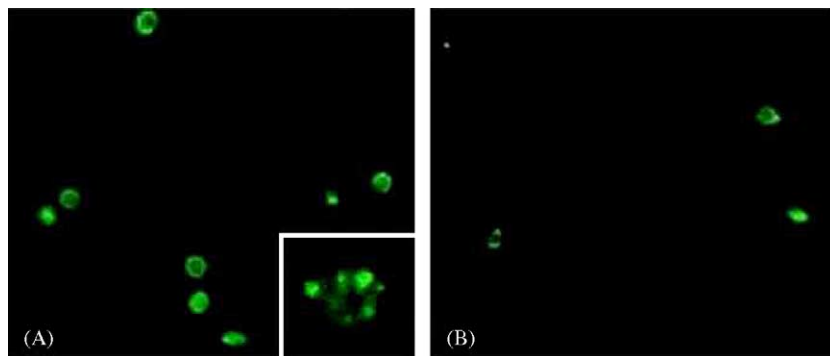


Fig. 2. The results of immunofluorescence antibody staining for porcine reproductive and respiratory syndrome virus (PRRSV)-infected swine alveolar macrophages (AMs). A diffuse, intracytoplasmic bright green fluorescence (fluorescein isothiocyanate, FITC) is seen in PRRSV-infected AMs. (A) Only approximately 9.3% of AMs have positive signal in PRRSV alone-infected group at 18 h post-infection (HPI); $\times 450$. *Inset*: a non-PRRSV-infected but PRRSV-positive apoptotic body-containing AM. Note the scattered intracytoplasmic granular to globular fluorescence; $\times 1250$. (B) In porcine circovirus 2 and PRRSV dually infected group, the rate of PRRSV-positive AMs was only 1.2% by 108 HPI; $\times 450$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

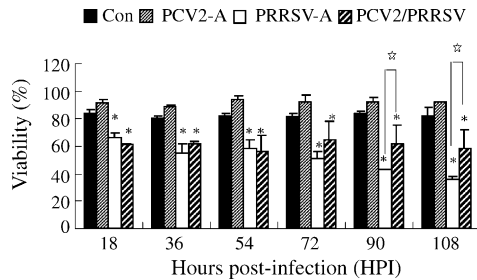


Fig. 3. Changes in cell viability of swine alveolar macrophages (AMs) over time following infection with porcine circovirus 2 (PCV2) and/or porcine reproductive and respiratory syndrome virus (PRRSV) as determined by trypan blue dye exclusion assay. Data are expressed as percentage and shown as mean \pm S.D. of three independent experiments. Con: mock-infected group; PCV2-A: PCV2 alone-infected group; PRRSV-A: PRRSV alone-infected group; PCV2/PRRSV: PCV2 and PRRSV dually infected group. The differences between PCV2 and/or PRRSV-infected and mock-infected groups at the same hour post-infection (HPI) are significant ($*P < 0.05$). The two values labeled at the same HPI differ significantly ($^{**}P < 0.05$).

and remained $80.1 \pm 1.9\%$ by 108 HPI in the mock-infected group. In the PCV2-A group, the average survival rates of AMs ranged from 88.8 ± 0.9 to $93.5 \pm 2.5\%$ at 18–108 HPI; they were 8.1 ± 6.0 to $11.7 \pm 0.7\%$ higher, although not statistically significant, than those of mock-infected group. The average survival rates of AMs in both PRRSV-A and PCV2/PRRSV groups were consistently and significantly lower than those of mock-infected and PCV2-A groups throughout the study. The average survival rate of AMs in PRRSV-A group reduced shortly after viral inoculation and gradually decreased in a time-dependent manner, dropping from $66.0 \pm 3.4\%$ at 18 HPI to $35.1 \pm 3.2\%$ by 108 HPI. A low but steady survival rate, ranging from 55.6 ± 12.1 to $58.2 \pm 13.8\%$, was found in PCV2/PRRSV group at 18–108 HPI. The average survival rates of AMs in PCV2/PRRSV group were significantly higher than those in PRRSV-A group at 90–108 HPI.

3.3. Apoptosis of infected swine AMs

The viability of swine AMs from the four experimental groups is summarized in Fig. 4. The incidence of apoptosis was constantly below 3% in both mock-infected and PCV2-A groups at 18–108 HPI. A significantly increased apoptotic rate, ranging

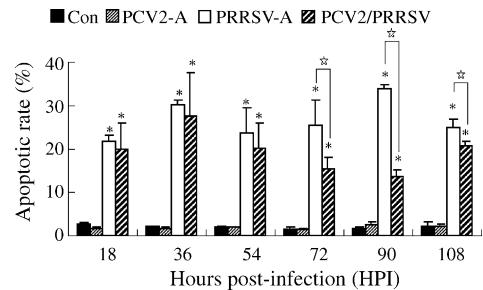


Fig. 4. Changes in apoptotic rate of swine alveolar macrophages (AMs) over time following infection with porcine circovirus 2 (PCV2) and/or porcine reproductive and respiratory syndrome virus (PRRSV) determined by TUNEL assay. Data are expressed as percentage and shown as mean \pm S.D. of three independent experiments. Con: mock-infected group; PCV2-A: PCV2 alone-infected group; PRRSV-A: PRRSV alone-infected group; PCV2/PRRSV: PCV2 and PRRSV dually infected group. The differences between PCV2 and/or PRRSV-infected and mock-infected groups at the same hour post-infection (HPI) are significant ($*P < 0.05$). The two values labeled at the same HPI differ significantly ($^{**}P < 0.05$).

from 21.8 ± 1.1 to $33.9 \pm 0.7\%$, was seen in PRRSV-A group. In PCV2/PRRSV group, instead of increase, the average apoptotic rate reduced, ranging from 13.4 ± 1.6 to $27.5 \pm 9.9\%$. Although the values were still consistently and significantly higher than those of mock-infected and PCV2-A groups, they became significantly lower than those of PRRSV-A groups by 72 HPI and thereafter.

3.4. IFN- α and TNF- α levels in infected swine AMs

Marked inhibition of VSV-induced CPE in MDBK cells was seen with the culture supernatants from PCV2-A and PCV2/PRRSV groups at 18 HPI and thereafter (Fig. 5A and B). The titers of IFN- α in both groups ranged from 1077.7 ± 431.0 to 4815.8 ± 211.6 U/ml and were significantly higher than those of mock-infected group, ranging from 0 to 77.0 ± 12.6 U/ml (Fig. 6). The PRRSV-A group had a similar low IFN- α titer, 25.0 ± 20.3 to 75.6 ± 65.5 U/ml, as the mock-infected group. There was no protection from VSV-induced CPE in MDBK cells by the culture supernatants of either PRRSV-A or mock-infected group (Fig. 5C).

There was only a low level of TNF- α , 22.4 ± 5.5 to 55.1 ± 8.2 pg/ml, in the culture supernatants of

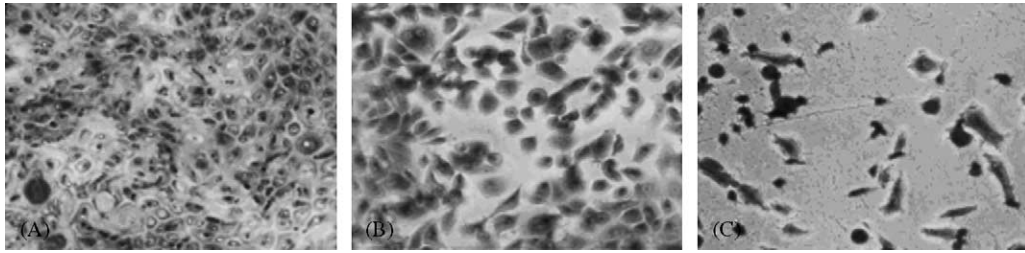


Fig. 5. Protective effect of culture supernatants from mock-infected and porcine circovirus 2 (PCV2) and/or porcine reproductive and respiratory syndrome virus (PRRSV)-infected swine alveolar macrophages (AMs) collected at various time intervals after infection determined by cytopathic effect (CPE) reduction bioassay using Madin–Darby bovine kidney cells and vesicular stomatitis virus (VSV). Crystal violet is used to stain the viable cells. Complete (A) or almost complete (B) protection from VSV-induced CPE is found by adding the supernatant of PCV2 alone-infected or PCV2 and PRRSV dually infected swine AMs collected at 36 h post-infection (HPI), respectively; $\times 115$. No protection from VSV-induced CPE is found by adding the supernatant of PRRSV alone-infected AMs at 36 HPI (C); $\times 115$.

mock-infected group (Fig. 7). The levels of TNF- α in the PCV2-A group ranged from 188.8 ± 9.3 to 297.7 ± 5.3 pg/ml. The peak levels of TNF- α in the PRRSV-A group were detected at 54–72 HPI, ranging from 494.2 ± 25.1 to 878.4 ± 33.3 pg/ml, about four-fold higher than those of PCV2-A group. When compared with PRRSV-A group, the PCV2/PRRSV group showed a lower TNF- α secretion, ranging from 446.9 ± 26.9 to 762.4 ± 20.1 pg/ml, with statistically significant differences at 72–108 HPI.

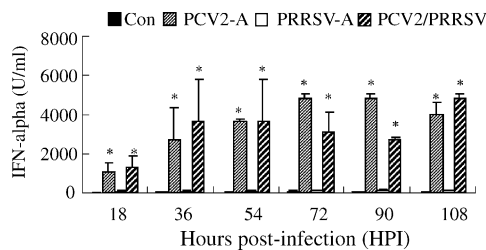


Fig. 6. Changes in the levels of IFN- α in the culture supernatants over time following infection with porcine circovirus 2 (PCV2) and/or porcine reproductive and respiratory syndrome virus (PRRSV) determined by cytopathic effect (CPE) reduction bioassay using Madin–Darby bovine kidney (MDBK) cells and vesicular stomatitis virus (VSV). One unit of IFN- α is defined as the reciprocal of the dilution of the supernatant producing 50% inhibition of CPE. Data are expressed as U/ml and shown as mean \pm S.D. of three independent experiments. Con: mock-infected group; PCV2-A: PCV2 alone-infected group; PRRSV-A: PRRSV alone-infected group; PCV2/PRRSV: PCV2 and PRRSV dually infected group. The differences between PCV2 and/or PRRSV-infected and mock-infected groups at the same hour post-infection (HPI) are significant ($^*P < 0.05$).

3.5. Protection of MARC-145 cells or swine AMs from PRRSV infection by PCV2-induced IFN- α and/or TNF- α

A marked reduction in PRRSV-induced CPE was observed when MARC-145 cells were pre-treated with either UV-treated or non-UV-treated supernatant of PCV2-A AMs (Fig. 8A and B). The reduction in CPE was abolished when the supernatant of PCV2-A AMs was pre-mixed with the mouse anti-rpIFN- α antibody (Fig. 8C), but the reduction in CPE was not

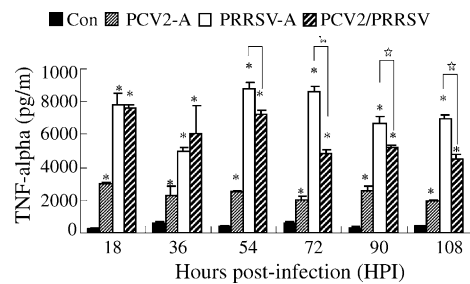


Fig. 7. Changes in the level of TNF- α in the culture supernatants of swine alveolar macrophages (AMs) over time following infection with porcine circovirus 2 (PCV2) and/or porcine reproductive and respiratory syndrome virus (PRRSV) determined by an ELISA kit. Data are expressed as pg/ml and shown as mean \pm S.D. of three independent experiments. Con: mock-infected group; PCV2-A: PCV2 alone-infected group; PRRSV-A: PRRSV alone-infected group; PCV2/PRRSV: PCV2 and PRRSV dually infected group. The differences between PCV2 and/or PRRSV-infected and mock-infected groups at the same hour post-infection (HPI) are significant ($^*P < 0.05$). The two values labeled at the same HPI differ significantly ($^{**}P < 0.05$).

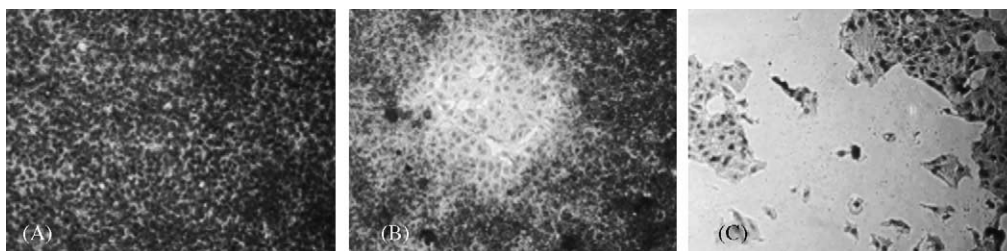


Fig. 8. Protection of MARC-145 cells from porcine reproductive and respiratory syndrome virus (PRRSV) infection by culture supernatants of porcine circovirus 2 (PCV2)-infected swine alveolar macrophages (AMs). Crystal violet is used to stain the survival cells; $\times 115$. Complete (A) or almost complete (B) protection from PRRSV-induced CPE is found by adding UV-treated or non-UV-treated supernatant of PCV2-infected AMs collected at 54 HPI, respectively; $\times 115$. No protection from PRRSV-induced CPE is found by adding the AM supernatant pre-mixed with a mouse anti-recombinant porcine IFN- α antibody (C); $\times 115$.

abolished when the supernatant was pre-mixed with the mouse anti-rpTNF- α antibody (data not shown). A marked reduction in PRRSV-induced CPE was also observed when AMs were pre-treated with UV-treated or non-UV-treated supernatants of PCV2-infected AMs; however, the reduction in CPE was not abolished as that in the MARC-145 cell system when the supernatant of AMs was pre-mixed with the mouse anti-rpIFN- α antibody (data not shown).

4. Discussion

Macrophages and other members of the mononuclear phagocytic system (MPS) play crucial roles in both innate and adoptive immune responses. Alveolar macrophages are essential for the pulmonary defense system against various pathogens and also are one of the major targets for both PCV2 and PRRSV (Chiou et al., 2000; Gilpin et al., 2003). Therefore, it is conceivable that any pathogen that induces direct impairment of AMs may render the host to secondary infection in the lungs.

Our data showed that, unlike PRRSV infection having a low PRRSV antigen-containing rate with prominent cytotoxic and apoptotic effects on swine AMs, PCV2 infection had a high PCV2 antigen-containing rate with no cell death. In addition, PCV2 antigen is predominantly and persistently present in the cytoplasm of infected AMs. Such findings were consistent with others that despite the presence of persistent infectious virus, viral antigen, and viral DNA, there was no detectable viral replication and

cellular injury in PCV2-infected swine AMs (Gilpin et al., 2003) and monocyte- or bone marrow-derived dendritic cells (Vincent et al., 2003). It has been suggested that PCV2 may enter dendritic cells by endocytosis rather than active infection (Vincent et al., 2003). In the present study, scattered pinpoint to small granular positive signals were detected as early as 1 h after the inoculation of either non-UV-treated or UV-inactivated PCV2 and the signal intensity in both groups increased with time in a similar pattern (data not shown). This indicated that PCV2 could indeed passively enter and accumulate in the cytoplasm of AMs. Antigens of PCV2 detected in AMs might result from active infection and passive endocytosis and/or phagocytosis. Thus, MPS lineage cells may function as an important reservoir to help PCV2 evade the immunosurveillance and also assist PCV2 to spread.

In comparison with PCV2, PRRSV alone caused much more adverse effect on AMs, directly or indirectly. As seen in a previous study (Chiou et al., 2000) and the present study, PRRSV-infected AMs had a low but constant infectious rate between 5 and 10%, markedly reduced cell viability, and significantly increased apoptosis and TNF- α production. Currently, available data suggest that the low PRRSV infectious rate but prominent cell injury in AMs might well be the result of a non-viral indirect mechanism due to the bystander effect of the released PRRSV open reading frame 5 gene product (Suarez et al., 1996) or TNF- α (Choi and Chae, 2002) from the infected cells. In the present study, significantly elevated TNF- α production occurred in all PCV2 and/or PRRSV-infected groups, particularly in the PRRSV-A group. Co-

infection of PCV2 and PRRSV is often seen in PMWS-affected pigs (Allan and Ellis, 2000; Segalés et al., 2002). Compared to PRRSV infection alone, simultaneous PCV2 and PRRSV infection caused variable degrees of reduction in the PRRSV-associated infectious rate, cell death, and apoptosis of swine AMs. Such findings might have resulted from decrease in the infectivity and/or replication of PRRSV. A prior PRCV infection could interfere the subsequent swine influenza virus (SIV) replication in the lungs of pigs, characterized by markedly reduced nasal excretion and pulmonary infection of SIV; the PRCV-induced anti-viral activities were suggested due to the IFN- α and TNF- α released in the lungs (Van Reeth and Pensaert, 1994a,b). A similar anti-viral effect may also be present in PCV2-infected groups as evidenced by high levels of IFN- α detected shortly after PCV2 infection in all PCV2-infected groups in the present study. The result indicates that PCV2 is a potent IFN- α inducer for swine AMs and the released IFN- α may contribute to the anti-viral effects to PRRSV in PCV2/PRRSV group.

Induction of type I IFNs by most RNA viruses is initiated by virus-derived double-stranded RNA produced during replication (Vilcek and Sen, 1996). For retro-, DNA-, and some RNA viruses, the interaction of viral envelope glycoproteins with host cell membrane serves as an alternative mechanism of IFN induction (Ankel et al., 1998; Capobianchi et al., 1992; Charley and Laude, 1988). PCV2 is a non-enveloped DNA virus and possesses a small and circular genome, which is quite similar to the plasmid DNA. The relatively high content of unmethylated CpG dinucleotides in plasmid DNA is known for its immunostimulatory activity in vertebrates, including cytokine production (Tighe et al., 1998). A recent study has shown that one out of five oligodeoxynucleotides (ODNs) with central CpG motifs selected from the genome of PCV2 exhibited an inhibitory activity in IFN- α production in porcine peripheral blood mononuclear cells (PBMCs), while the other four had a stimulatory effect; this inhibitory ODN also showed a complete or variable inhibitory action on other stimulatory ODNs or known inducers of IFN- α , respectively, and this particular genomic sequence was speculated to be associated with the immunosuppressive effect of PCV2 (Hasslung et al., 2003). Our data of IFN- α induction by intact infectious PCV2 or UV-

inactivated PCV2 in swine AMs, however, clearly displayed a strong stimulatory activity. The discrepancy may be explained by the observation that the position of the inhibitory sequence in relation to a stimulatory sequence on the same strand of DNA is considered of importance with respect to the net effect of the ODN (Yamada et al., 2002). Further studies are needed to elucidate the mechanisms of IFN- α induction by PCV2.

Our data showed that the UV-treated or non-UV-treated supernatants of PCV2-infected swine AMs markedly reduced the PRRSV-induced CPE in both MARC-145 cells and swine AMs; however, such reduction in CPE could be effectively abolished in MARC-145 cells, but not in swine AMs, when the supernatants were pre-mixed with the mouse anti-rpIFN- α antibody. The surface of macrophages and dendritic cells is known to contain Toll-like receptor 9 and unmethylated CpG ODNs could effectively activate macrophages and dendritic cells after binding with this receptor (Ashkar and Rosenthal, 2002). The DNA of either live or UV-inactivated PCV2 was speculated to continuously stimulate swine AMs to produce IFN- α resulting in the situation that the amount of IFN- α for reduction in CPE overrode that the anti-rpIFN- α antibody for neutralization.

In contrast to PCV2, our data indicated that PRRSV was a rather poor IFN- α inducer, which is in consistent with others' findings (Albina et al., 1998; Buddaert et al., 1998). However, PRRSV is fairly sensitive to the anti-viral effect of IFN- α (Buddaert et al., 1998). This may explain the reduction in the infectious rate, cell death, and apoptosis of swine AMs in the PCV2/PRRSV group when compared with the PRRSV-A group. TNF- α has been shown to synergize with interferons in the inhibition of viral replication (Wong and Goeddel, 1986). Recombinant porcine TNF- α at 100 ng/ml could induce a slight but clear and reproducible inhibition in PRRSV replication in AMs (Lopez-Fuertes et al., 2000); however, the TNF- α level in our testing system was lower than 1 ng/ml (Fig. 5). Such low level of TNF- α may explain the consistent negative result in the protection assay.

PCV2 and/or PRRSV-infected pigs frequently suffer from interstitial pneumonia. IFN- α therapy-induced activation in cellular immunity has been suggested to cause a complication of interstitial pneumonia (Nakamura et al., 1997; Pohl et al.,

2000). TNF- α has strong pro-inflammatory effects, including the release of inflammatory mediators, angiogenic factors, and chemokines (Beutler and Cerami, 1988). Thus, aside from direct viral effect, the increased IFN- α and/or TNF- α level associated with PCV2/PRRSV infection may contribute in part to the development of interstitial pneumonia in PMWS-affected pigs.

In summary, PCV2 accumulated in swine AMs immediately following infection but caused no cell death. PCV2 was a potent IFN- α inducer but a relatively weak TNF- α inducer for swine AMs. PRRSV possessed a low infectivity but high cytopathogenicity in swine AMs, and was a potent TNF- α inducer but a rather poor IFN- α inducer for swine AMs. Simultaneous infection of PCV2 and PRRSV in swine AMs resulted in the reduction of PRRSV infection and PRRSV-associated CPE in swine AMs contributed by PCV2-induced IFN- α . The reduced PRRSV-associated CPE and the increased production of IFN- α and TNF- α may consequently increase the severity of the pneumonic lesion in those dually infected pigs due to the elevated cellularity in the lung tissue secondary to the rescue of AMs and the enhanced pulmonary inflammatory response.

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