Research Article

The Influence of Porcine Reproductive and Respiratory Syndrome Virus Infection on the Expression of Cellular Prion Protein in Marc-145 Cells

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Abstract

Cellular prion protein (PrP^C) represents a potential regulator of cellular immunity and has been confirmed to be an invasive receptor exploited by bacteria such as Brucella abortus to facilitate the infection in host cells. Up to now, there are few informations about the role of PrP^C in virus replication in host cells. Porcine reproductive and respiratory syndrome virus (PRRSV), a single positive-stranded RNA virus, prefers to infect African green monkey kidney cell line MA-104 and its derivatives, such as Marc-145. We found that PrP^C is expressed on the surfaces of Marc-145 cells and its expression profiles greatly changed after PRRSV incubation with these cells. PrPC expression reached the peak at 12 hrs after PRRSV inoculation and then decreased sharply to recover to the original status while CD163, a PRRSV receptor in Marc-145 cells, achieved the climax at 48 hrs after virus infection and then slowly lowered its expression level. Here, we firstly suggest that PRRSV infection has an influence on the expression of PrP^C on the surfaces of Marc-145 cells and PrP^C may be involved in PRRSV invasion of host cells.

Keywords

Cellular prion protein; CD163; Porcine reproductive and respiratory syndrome virus; Marc-145 cells; Receptor

Introduction

Cellular prion protein (PrP^C) is a glycosylphosphatidylinositol (GPI)-anchored glycoprotein concentrated in lipid rafts of the plasma membrane. PrPC is widely expressed in various of cells including haematopoietic stem cells and immune cells in addition to cells of the central nervous system [1]. PrPC plays a complex role in the regulation of cell signalling and protein trafficking. Post-translational modification of the PrPC is intimately associated with the pathogenesis of prion disease, yet the normal function of the protein remains unclear [2-4] The highly conserved nature of PrP^C during evolution suggests that it may serve as a link between innate and adaptive immunity [5]. PrP^C has been proposed to represent a potential regulator of cellular immunity [6]. Accumulating evidence confirmed that many infectious agents and their toxins use GPI-anchored proteins to gain entry into host cells [7]. PrP^C binds a wide range of molecules [8] and its localization in the plasma membrane indicates a role in cellular interactions, such as adhesion, recognition, and ligand capture. PrPC can modulate phagocytosis and inflammatory response of cells [9]. Some results show that PrP^C is a generalized, negative modulator of phagocytosis with down-regulation of the cellular phagocytic activity [6,10] . For example, PrP^C knock-out mice showed more efficient phagocytosis of zymosan particles leading to acute peritonitis than wild-type mice [10]. However, other findings suggest that PrP^C is a positive modulator of phagocytosis with enhancement of cellular phagocytic activity [11]. For example, the PrPC deficiency prevented the internalization and intracellular replication of Brucella abortus, with the result that phagosomes bearing the bacteria were targeted into the endocytic network [12]. In addition, PrP^C was confirmed to promote brucella infection into intestinal M cells by serving as a major uptake receptor [13].

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of porcine reproductive and respiratory syndrome (PRRS). PRRSV is an enveloped positive stranded RNA virus, belonging to the genus Arterivirus, the family Arteriviridae, the order Nidovirales [14]. Based on genetic differences, PRRSV has been divided into two major genotypes: the European and the American [15]. Gene sequence of PRRSV has much difference in different strains, especially, type I and type II, share only about 60 % genome [16] . The virus genome is approximately 15.4 kb in length, containing ten open reading frames (ORFs), designated ORF1a, ORF1b, ORF2a,ORF2b and ORFs 3 through 7, including ORF5a [17,18]. In China, the highly pathogenic North American PRRSV (HP-NA-PRRSV), characterized by a 30-amino-acid depletion in nonstructural protein 2 (NSP2), appeared and caused great economic losses for the swine industry since 2006 [19,20]. However, the pathogenesis of HP-NA-PRRSV is still unclear.

PRRSV has a very restricted cell tropism for cells of the monocytic lineage, especially the differentiated macrophages. The primary target cells for PRRSV infection are the fully differentiated porcine alveolar macrophages (PAMs) [21]. *In vitro* condition, among non-macrophage cells, the MA-104 cell line derived from African green monkey kidney and its derivative including Marc-145, were susceptible to PRRSV and were found to fully support PRRSV propagation because of the expression of PRRSV receptors [22,23]. To date, several important PRRSV receptors, such as heparin sulfate (HS), sialoadhesin (Sn), CD163, CD151 and vimentin, have been identified. These receptors play significant roles during PRRSV infection of cells since they can involve in the virus binding, internalization or uncoating [24,25]. CD163 was confirmed to be responsible for uncoating virus particles and releasing the viral genome after early adhesion during PRRSV infection [26]. Vimentin is thought to opsonize and endocytose PRRSV.

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To our knowledge, there are no reports about the role of PrP^C in virus infection of host cells up to now. In this study, we firstly examined the expression of PrP^C on Marc-145 cell surface and compared the expression profiles between PrP^C and CD163 during PRRSV infection of these cells. Our date suggested that PRRSV infection impacts PrP^C cell surface expression in Marc-145 and PrP^C maybe plays an important role during PRRSV entry into the host cells. These results provide a new sight of the physiological function of PrP^C related to virus infection.

Materials and Methods

Cells culture and reagents

Marc-145 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (Gibco, USA) and antibiotics (100U/ml penicillin and 100μg/ml streptomycin), and grown at 37°C in a humidified incubator containing 5% CO_2. Anti PRRSV nucleocapsid monoclonal antibody (MAb) conjugated with fluorescein isothiocyanate (SR30-F) was obtained from Rural Technologies Inc (Brookings, SD). Anti prion protein MAb SAF32 was bought from Bertinpharma and MAb against CD163 was from AbD Serotech (Raleigh, NC). APC (Allophycocyanin) conjugated goat anti-mouse IgG antibody was from biolegand company. Isotype control MAb (IgG2b) of PrP^C were used to evaluate levels of nonspecifics staing (biolegand company).

PRRSV culture and virus titration

A HP-NA-PRRSV strain, named QH-08, was isolated from a pig farm suffered from HP-NA-PRRSV outbreak in 2008 in Qinghai province, China and propagated in Marc-145 cells (The virus identification and the whole genome sequence will be published in the future). The 50% tissue culture infected dose ($TCID_{50}$) assay was used to measure PRRSV titration. Marc-145 cells were seeded into 96-well plates 24 hrs before infection. Virus supernatants were diluted 10-fold serially. Cells were inoculated by 100 μl/well of the serial diluted supernatants. Five days post-infection, $TCID_{50}$ was determined by the Reed-Muench method.

PRRSV RNA extraction and cDNA synthesis

Total RNA was extracted from the collected QH-08 supernatants. RNA concentration was analyzed using a NanoDrop spectrophotometer (NanoDrop, USA). cDNA was prepared using Reverse Transcriptase M-MLV kit (Invitrogen) according to the manufacturer's instructions and stored at - 40? until further use.

Quantitative Real-Time PCR (qPCR) of PRRSV, PrPC and CD163

The fragments of PRRSV, PrPC and CD163 target genes were amplified by the following primer pairs: PrPC primers (forward 5 '-atgaagcacatggctggtgc-3'; reverse 5'-ctgatccacaggcctgtagtaca-3'),CD163 primers (forward 5'-atgggctaattccagtgcag-3'; reverse5'-gatccatctgagcaagtcactcca-3') and PRRSV primers (forward5'-aaccacgcatttgtcgtc-3'; reverse 5'-tggcacagctgattgactgg-3'). The amplified gene fragments were inserted into pMD18-T vector. Then the corresponding recombinant plasmids were constructed and used to establish the standard curve after quantification. Reactions were run on Stratagene Mx3005P thermocycler. The threshold cycle (Ct) values were determined at the fluorescence threshold line and the Ct value of each sample was obtained by calculating the arithmetic mean of the triplicate values.

Flow cytometry analysis of the expression of PrPC and CD163 and PRRSV infection in Marc-145 cells

Flow cytometry analysis was performed to measure the expression level changes of PrPC and CD163 with PRRSV infection of Marc-145. The cells were inoculated with QH-08 at a MOI of 0.1 and collected at different time points for flow cytometry analysis as described below. The collected cells were fixed with a 4% paraformaldehyde solution for 1 hr on ice and kept at 4°C for further use after washing with PBS. For PrPC or CD163 cell surface expression detection, then the cells were incubated with corresponding MAb in PBS with 1 % BSA at 4 for 1 hr. 100 μl MAb SAF32 for PrPC detection or CD163 MAb was added at the final concentration of 2 μg/ml. After the incubation, APC conjugated anti-mouse IgG antibody was added and incubated with cells for 1 hr at 4°C. As for intracellular PRRSV detection, 100 μl SR30-F was added and incubated with Marc-145 after cells were pretreated with BD Cytofix/Cytoperm Fixation/Permeabilization Kit following the handbook instruction. Isotype control MAb were used to evaluate the levels of nonspecifics staining.

Statistical analysis

All experiments were done with at least three independent experiments and statistical analyses were performed by GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Data were expressed as the mean ± standard deviation (SD). A value of $P<0.05$ was considered as statistically significant.

Results

Effects of PRRSV infection on PrPC expression

PrP^C expression in Marc-145 cells infected by PRRSV was analyzed by flow cytometry. Surface PrP^C expression was measured using MAb SAF-32 and APC-labeled antibody, and virus replication in individual cells was detected by the presence of FITC. For control Marc-145 cells, only 10% cells were PrP^C positive (Figure 1E and Figure 2C). After virus infection, over 30% of Marc-145 cells were PrPC positive at 12 hr. While at 36 hr, 48 hr, 60 hr and 72 hr, the percentage of the positive cells returned to 10% (Figure 1E and Figure 2C). In qPCR assay, there was an increase in Prnp mRNA from Marc-145 cells. Prnp expression was 2-fold higher after 12 hrs infection compared with the uninfected cells (Figure 1F). qPCR assay along with the flow cytometry results , confirmed that surface PrP^C expression levels reached the peak at 12 hr after PRRSV inoculation (Figure 1E and Figure 2C).

CD163 expression correlates with PRRSV infectivity in Marc-145 cells

PRRSV replication and CD163 expression in Marc-145 cells during virus infection were measured by flow cytometry assay. For these experiments, CD163 surface expression was measured using APC-labeled antibody, and virus replication in individual cells was detected by the presence of FITC. Infected cells were collected at different times after incubation 1 hr of virus at MOI=0.1, and flow cytometry was performed prior to lysis of infected cells. For Marc-145 cells , a majority of cells expressed high levels of CD163 from 0 hr to 72 hr (Figure 1C and Figure 2A). At day 1, over 50% of Marc-145 cells were CD163 positive. By 36 hr, 48 hr, 60 hr and 72 hr, the percentage of CD163 positive cells increased to more than 60% (Figure 1C and Figure 2A). While there were few PRRSVs in Marc-145 cells at day 1, over 20% of cells were PRRSV positive when the viruses were
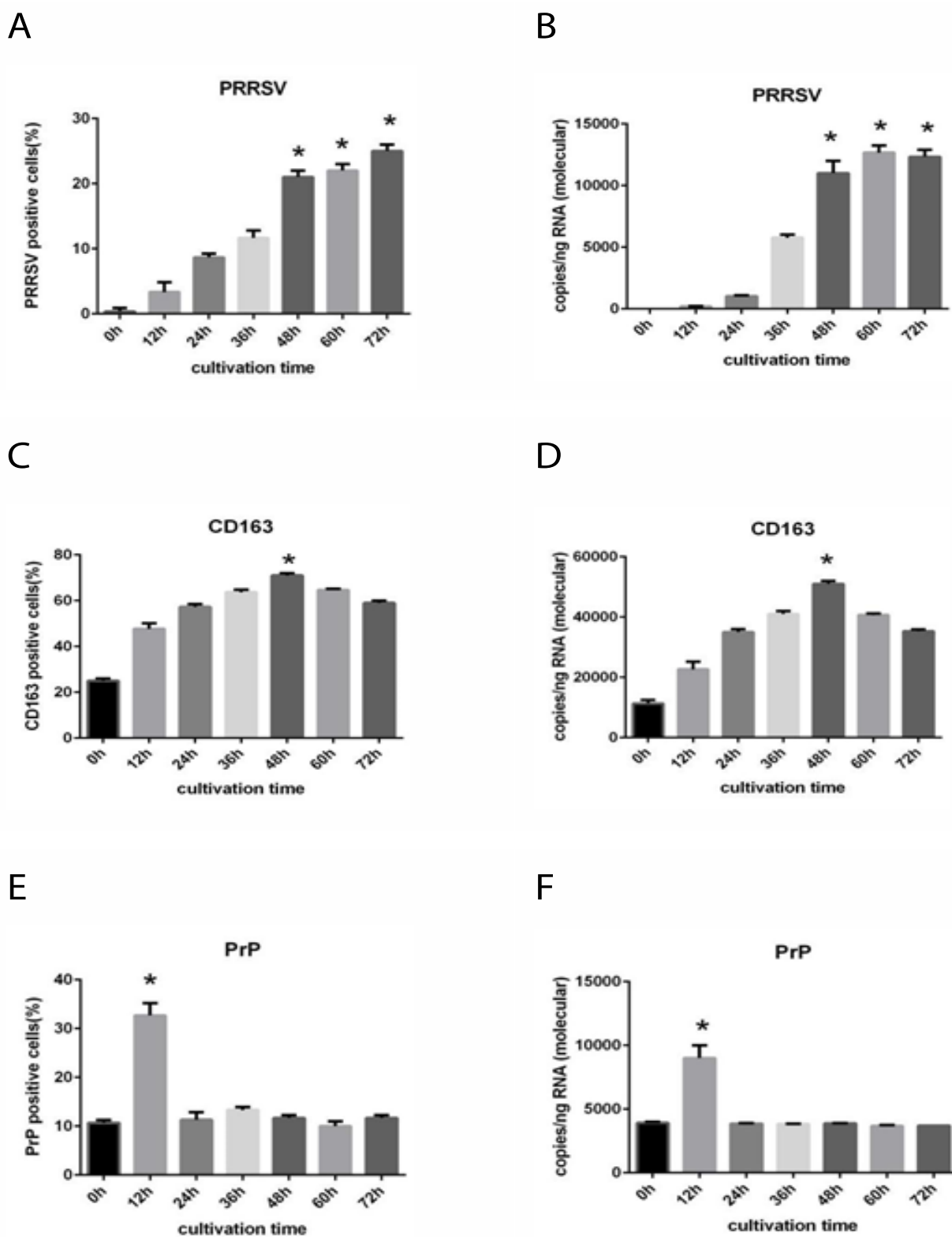
Figure 1: Effect of PRRSV infection time on CD163 and surface PrP^C expression. Marc-145 cells were cultured for the indicated time; all data were presented as the mean ± standard deviation (SD). *p* value less than 0.05 was considered statistically significant correlation. Experiments were replicated three times using independent samples at each time point. (C and D) *Significantly enhanced when compare to other time points (P<0.05). (A and B) *Significantly enhanced when compare to 0 h, 12 h, 24 h, 36 h (P<0.05). (E and F) *Significantly enhanced when compare to other time points (P<0.05).
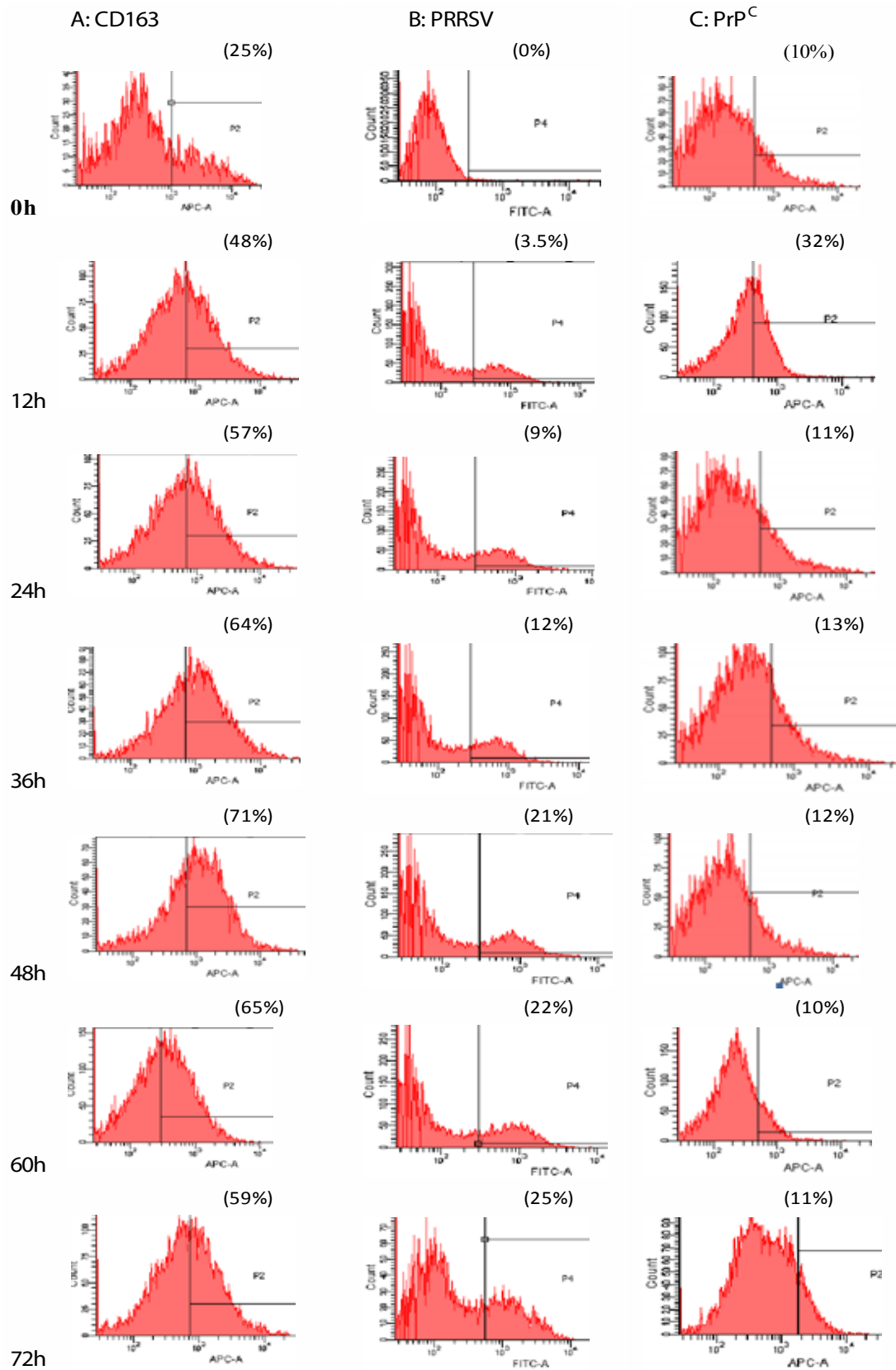
Figure 2: The flow cytometry results of PRRSV (column B), CD163 (column A) and PrPC (column C)at different time points.

incubated in culture longer than 2 days (Figure 1A and Figure 2B). At 72 hr, PRRSV positive cells reached 25% (Figure 1A and Figure 2B), and the majority of virus-infected Marc-145 cells were lysed (data not shown). The increased percentage of CD163 positive cells was not the result of the insufficient CD163 negative cells, since cell numbers remained relatively constant over the culture period. At the same time, qPCR was performed, results in accordance with flow cytometry (Figure 1B and D). These studies suggested that the overall expression levels of CD163 on Marc-145 cells may determine the levels of PRRSV replication and pathogenicity in vivo. It would be interesting to examine the expression levels of CD163 on the macrophages and the correlation with PRRSV infection.

The relationship of PRRSV, PrPC and CD163

In qPCR and flow cytometry assay, PRRSV infection has influence on the expression of CD163 and surface PrPC in Marc-145 cells (Figures 2 and 3). The trend of CD163 changes was consistent with PRRSV infection level from 12 hrs to 48 hrs. CD163 expression increased with PRRSV infection over time during this period. Then CD163 slowly lowered its expression and restored to the original level while PRRSV maintained stable infection (Figure 3 A and B). Comparison with the expression profile of CD163, PrPC possesses a distinctive model during PRRSV infection in Marc 145 cells. PrPC expression appeared a peak at 12 hr after PRRSV inoculation and then decreased sharply to recover to the original status (Figure 3 A and B). The results suggest that PrPC is maybe involved in the early stage of PRRSV infection of Marc-145 cells.

Discussion

PrP^C, widely expressed on the surfaces of almost all kinds of cells [2,26], was confirmed to be present on Marc-145 cell surfaces by flow cytometry and real-time PCR in our study for the first time. The nonspecific staining was excluded by using Isotype negative control MAb. Marc-145 is derived from African green monkey kidney cell line MA-104 and serves as the preferable cells for PRRSV propagation *in vitro* for vaccine production while PAMs are the primary cells for PRRSV replication [27]. PRRSV infection of the host cells actually is a receptor-mediated endocytosis and replication process [28]. PRRSV receptors including HS [29], CD163 [30] CD151 [31] and vimentin [32] have been proved to be responsible for the virus entry into Marc-145 cells. Sn, another PRRSV receptor restrictedly expressed on PAMs surface, is not expressed in MA-104 and its derivatives [33,34]. PrP^C plays an important role in cellular immunity by modulating phagocytosis and inflammatory response of macrophages, influencing T cell activation and effector function or promoting immune cells recruitment in pathogen-causing infections [27,35] . Recently, PrP^C was confirmed to promote brucella infection into intestinal M cells by serving as a major uptake receptor[9,12]. Therefore, it is very interesting to investigate the role of PrPC in virus infection of host cells because the related reports are hardly found until now.

Our results showed that there is close relationship between PrPC expression and PRRSV infection in Marc-145 cells. PrPC expression level began to greatly increase and reached the peak at 12hr after PRRSV inoculation. Then, after virus infection of 12 hrs, PrP^C level sharply decreased and restored to the original status quickly. The data demonstrated that PRRSV infection impacts PrP^C expression on Marc-145 cell surface and also suggested that PrPC maybe takes a role in PRRSV replication in Marc-145 cells.

In this study, we also found that the expression profiles of PrPC are greatly different from that of CD163. PrP^C peaked its expression level at 12 hr while the highest expression of CD163 was at 48 hr after PRRSV inoculation in Marc-145 cells. PrP^C expression quickly restored to the level of naive situation from the peak while CD163 expression level decrease slowly from the climax. The great differences of the expression profiles between PrPC and CD163 supported that PrP^C and CD163 play roles in different process during PRRSV replication in Marc-145 cells. It was reported that CD163 is involved in uncoating of PRRSV particles, but not internalization. Our results were well in agreement with this conclusion because CD163 was found to increase its expression level at the late stage of PRRSV infection of Marc-145 cells. In comparison to CD163 expression profiles, PrP^C expression began to largely increase at the early stage of PRRSV infection, indicating that PrP^C is probably implicated in PRRV early infection process.

To clarify the role of PrP^C in PRRSV infection of host cells, more experiments including the influence of the deficiency and enhancement of PrP^C expression level on PRRSV propagation need to be done. For example, knock-out PrP^C gene or inhibiting PrP^C expression by RNA interference or blocking its function via monoclonal antibody can be taken to down-regulate the function. In contrast, PrP^C function can be up-regulated by introduction of PrP^C gene into host cells. Here, our results provide a novel aspect of PrP^C function in virus infection of host cells. It is worth to reveal PrP^C roles in cellular immunity for better understanding the microbial pathogenesis.

Conclusion

It was the first report that PrP^C has a role in PRRSV infection of host cells. PrP^C possesses a distinct expression profile during PRRSV infection of Marc-145 cell in comparison with CD163 which is implicated in the uncoating process of the virus replication. PrPC appeared a peak at 12 hr after PRRSV inoculation in Marc-145 cells
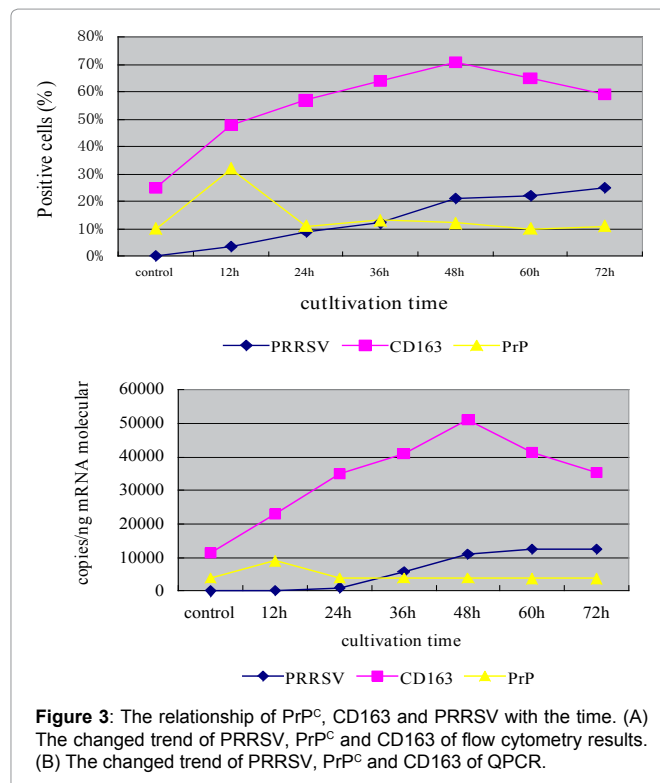

Figure 3: The relationship of PrP^C, CD163 and PRRSV with the time. (A) The changed trend of PRRSV, PrP^C and CD163 of flow cytometry results. (B) The changed trend of PRRSV, PrP^C and CD163 of QPCR.

and restored the original expression level in a short time. The results show that PrP^C maybe plays a role in PRRSV early infection and more evidences are needed to support the novel conclusion.

Acknowledgments

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