Involvement of CD16 in antibody-dependent enhancement of porcine reproductive and respiratory syndrome virus infection

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The immunological effect of porcine reproductive and respiratory syndrome disease virus (PRRSV) vaccines is thought to be influenced by a variety of host factors, in which antibody-dependent enhancement (ADE) of infection is one crucial factor. Here, we assessed the mechanism of ADE of PRRSV infection. First, we found that subneutralizing serum could induce ADE of PRRSV infection in porcine alveolar macrophages (PAMs). Quantitative PCR, Western blotting and flow cytometry revealed that CD16 is the most abundant Fc receptor (FcR) expressed on the surface of PAMs; thus, the role of CD16 in ADE of PRRSV infection was examined in PAMs. By using functional blocking antibodies, we demonstrated that CD16 is involved in enhanced virus production in PRRSV–antibody immune complex-infected PAMs. Because PAMs co-express different FcR isoforms, we evaluated the effects of CD16 in FcR-non-bearing cells by transfection. Using these engineered cells, we found that CD16 could specifically bind to the PRRSV–antibody immune complex and subsequently mediate internalization of the virus, resulting in the generation of progeny virus. We also showed that efficient expression of CD16 required association of the FcγRIIa chain. Together, our findings provide significant new insights into PRRSV infection, which can be enhanced by CD16-mediated PRRSV–antibody immune complexes. This CD16-mediated ADE may induce a shift in PRRSV tropism towards CD16-expressing cells, distributing virus to more organs during virus infection.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-stranded RNA virus in the family Arteriviridae and order Nidovirales (Cavanagh, 1997). Based on genetic differences, PRRSV isolates have been divided into European groups (EU genotype, represented by the Lelystad strain) and North American groups (NA genotype, represented by strain VR2332) (Meulenberg et al., 1993; Nelsen et al., 1999). PRRSV is the causative agent of porcine reproductive and respiratory syndrome (PRRS), which is characterized by respiratory problems in growing pigs and reproductive failure in sows. PRRS has caused substantial economic losses to the swine industry worldwide. To control this disease, researchers have developed a number of vaccines (Christopher-Hennings et al., 1997; Dee & Joo, 1997; Plana-Durán et al., 1997; Tian et al., 2009). The immunological effect and progression of PRRS, however, are influenced by a variety of host factors, and one crucial factor is antibody-dependent enhancement (ADE) of infection (Christianson et al., 1993; Yoon et al., 1996, 1997). Vaccine-induced enhancement has also been identified as a major problem in the development of certain flavivirus, coronavirus, paramyxovirus and lentivirus vaccines (Huisman et al., 2009).

In ADE of virus infection, immune serum can enhance infection when diluted to a non-neutralizing concentration (Tirado & Yoon, 2003). The antiviral serum binds to antibody Fcγ receptors expressed on the surface of certain cells, including monocytes/macrophages and other immune cells (Fridman, 1991; Raghavan & Bjorkman, 1996). In humans, there are three classes of Fcγ receptors (FcγRs): FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16) (Ravetch & Kinet, 1991). All of these Fcγ receptors have been reported to mediate ADE of infection for various viruses including dengue virus (Balsitis et al., 2010; Chotiwan et al., 2014; Goncalvez et al., 2007; Mady et al., 1993), human immuno-deficiency virus type 1 (HIV-1) (Haubrich et al., 1992; Laurence et al., 1990; Trischmann et al., 1995), Ross river virus (Lidbury & Mahalingam, 2000), enterovirus (Wang et al., 2010) and West Nile virus (He et al., 2014). In swine, three FcγRs have been cloned (Halloran et al., 1994b; Qiao
et al., 2006; Zhang et al., 2006), and CD16 is the best characterized (Jie et al., 2009; Kacskovics, 2004; Sweeney & Kim, 2004; Sweeney et al., 1996). Qiao et al. (2011) recently reported that CD32 mediates ADE of PRRSV infection. However, the role of porcine CD16 in ADE of PRRSV infection is not well understood.

In the current study, we tested the effects of CD16 in an antibody blocking experiment and using genetic modification methods. We demonstrated that CD16 facilitates ADE of PRRSV infection and that this facilitation depends on the FcRγ-chain. We also reported that two cell lines that are normally not permissive to PRRSV became permissive to the PRRSV–antibody immune complex if they were engineered to express porcine CD16. Furthermore, these engineered cells were capable of producing functional PRRSV progeny. Our findings documented a potential pathogenic mechanism for the persistent infection of PRRSV in pigs.

RESULTS

Anti-PRRSV immune serum enhances PRRSV infection in porcine alveolar macrophages (PAMs)

Seven serum samples were collected from four pigs 21 days after intramuscular inoculation with attenuated PRRSV vaccine strain HuN4-F112 (Tian et al., 2009) and from three specific-pathogen-free (SPF) pig sera. ELISA showed that all four PRRSV HuN4-F112-immunized pigs produced PRRSV-specific antibodies; the three SPF pig sera had no PRRSV-specific antibodies (Table 1). Infection-enhancement experiments were conducted using these serially diluted sera. Each serum was assayed for ADE activity using real-time quantitative reverse transcription (qRT)-PCR to compare their ability to mediate increases in virus yield in PAMs. Virus reproduction was reduced by the four PRRSV-positive sera at high antibody titres (from 1/50 to 1/200), indicating that these sera were neutralization antibodies, but was enhanced by these four sera at lower antibody titres (1/3200 to 1/12 800) (Fig. 1a). Compared with inoculation with PRRSV alone, inoculation with the PRRSV–antibody immune complex increased virus reproduction by as much as 2.3-fold. However, the three SPF sera did not affect virus yield at any dilution (Fig. 1b). To determine whether the higher number of genome copies of PRRSV following treatment with subneutralizing antibodies was due to a higher number of infected cells, flow cytometry was performed to detect PRRSV-positive cells using FITC-conjugated anti-PRRSV nucleocapsid (N) protein mAb SDOW17. As shown in Fig. 1(c), the serum at a higher antibody titre (1/200) partially neutralized PRRSV infection, whilst the serum at lower antibody titre (1/3200) enhanced the percentage of PRRSV-positive cells. These results indicated that the four sera from PRRSV-immunized pigs exhibited ADE of PRRSV infection at a subneutralizing level, which was the result of a higher number of infected cells. These four sera were used for the ADE experiments described in the following sections.

CD16 mAb inhibits ADE of PRRSV infection in PAMs

Initial ADE research determined that ADE activity is mediated by the Fcγ receptors of target cells (Halstead & O’Rourke, 1977; Laurence et al., 1990). To determine which Fcγ receptor is responsible for mediating enhancement of PRRSV infection in PAMs, we first compared the mRNA level of three types of FcγRI: FcγRI (CD64), FcγRII (CD32) and FcγRIII (CD16). qRT-PCR revealed that CD16 transcripts were the most abundant (Fig. 2a). Thus, CD16 protein expression in PAMs was assessed by Western blotting and flow cytometry. A specific CD16 protein band was detected in the lysate prepared from freshly isolated PAMs (Fig. 2b). Flow cytometry data confirmed that a high level of CD16 protein was expressed on the surface of PAMs (Fig. 2c). Therefore, we determined whether CD16 was responsible for mediating enhancement of virus infection in the presence of PRRSV subneutralization serum. PAMs were first treated with mouse anti-porcine CD16 mAb G7, which can block the function of porcine CD16 (Halloran et al., 1994b), and were then incubated with the virus–antibody immune complex. Purified mouse IgG1 was used as a negative control. Because the current study had determined that PRRSV immune serum at a 1/3200 dilution had high ADE activity in PAMs, PRRSV at an m.o.i. of 1 was incubated with PRRSV immune serum at a 1/3200 dilution to prepare the immune complex. At 12 h after incubation with the immune complex, PAMs were washed five times, and total RNA was extracted to evaluate viral load. The results showed that treatment with anti-CD16 mAb significantly inhibited ADE of virus infection in

Table 1. Profile of the sera tested

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<th>Property</th>
<th>Serum</th>
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<td>#1</td>
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<tr>
<td>ELISA (absorbance at 650 nm)</td>
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CD16 is involved in PRRSV–antibody immune complex attachment and internalization

Because we and others (Guilliams et al., 2014) have shown that PAMs have more than one type of FcγR, we investigated the effect of CD16 alone by transfecting non-FcγR-bearing cell lines (COS-7 and HEK293-T) so that they expressed CD16. Researchers have also reported previously that the expression of CD16 on porcine neutrophils and macrophages is associated with the FcγR γ-chain (Sweeney et al., 1996). Therefore, we transfected COS-7 cells with CD16 alone or with CD16 plus the γ-chain, and verified CD16 expression by Western blotting and flow cytometry. On the Western blotting, a specific CD16 protein band was obtained with COS-7 cells transfected with CD16 alone or with CD16 plus the γ-chain (Fig. 3a). As indicated by Western blotting (Fig. 3a) and flow cytometry (Fig. 3b), expression of CD16 protein was greater in COS-7 cells transfected with CD16 plus the γ-chain than with CD16 alone. Similar results were obtained with HEK293-T cells (Fig. 3a, b). These data indicated that the efficient expression of porcine CD16 depends on the FcγR γ-chain.

It is important to note that only the well-characterized cell line MA-104 and its derivative Marc-145 are permissive to PRRSV infection; COS-7 cells are not permissive to PRRSV infection because they lack the PRRSV putative receptors CD163 and CD169 (Calvert et al., 2007; Van Gorp et al., 2008). We hypothesized that CD16 might mediate attachment of the PRRSV–antibody immune complex to CD16-bearing target cells and that such attachment would permit the virus to enter the cells and reproduce. Thus, COS-7 cells transfected with CD16 as described above were incubated with PRRSV–antibody immune complex for 1 h. After the cells had been washed five times, total RNA was extracted and used to evaluate the RNA level of PRRSV. As expected, no PRRSV RNA was detected in the WT COS-7 cells transfected with the empty vector pcDNA3.1 under any condition (Fig. 3c). However, CD16-transfected COS-7 cells were susceptible to the PRRSV–antibody immune complex. Moreover, PRRSV RNA was more abundant in COS/CD16/γ-chain cells than in COS/CD16 cells when these cells were incubated with the immune complex. In addition, CD16-mediated PRRSV attachment was greater at a higher serum titre (1/100) than at a lower serum titre (1/3200). Hence, serum diluted to 1/100 was used for

Fig. 1. Effects of PRRSV serum on ADE of virus infection. (a, b) PAMs were exposed to PRRSV–serum complex, which was prepared by mixing each dilution of the serum with an equal volume of medium containing the virulent PRRSV strain HuN4 for 1 h. After five washes, the PAMs were resuspended in fresh medium and cultured for another 12 h. Production of PRRSV progeny was quantified by qRT-PCR using the primers listed in Table 2. Three independent experiments for each serum with four PRRSV-positive pig sera (a) and three SPF pig sera (b) were performed in triplicate, and values are means ± SD for all three experiments. (c) The number of PRRSV-infected PAMs was examined by flow cytometry using FITC-conjugated anti-PRRSV N protein mAb SDOW17. *P < 0.05 according to Student's t-test.
subsequent experiments. CD16-mediated ADE activity was also assessed using HEK293-T cells. As was the case with COS-7, PRRSV RNA levels were greater when HEK293-T was transfected with CD16/γ-chain than with CD16 alone or with pCDNA3.1 vector (Fig. 3c). These data suggested that CD16 can mediate attachment of the PRRSV–antibody immune complex to its target cells, and that this might contribute to virus internalization and subsequent replication.

We then determined whether the immune complex could mediate ADE of PRRSV entry into CD16-expressing target cells. COS-7 cells transfected with CD16 plus the γ-chain were incubated with the PRRSV–antibody immune complex, and COS-7 cells transfected with pCDNA3.1 was used as a control. After 12 h of incubation, the cells were examined by confocal microscopy. A diffuse staining of PRRSV N protein was observed exclusively in transfected COS-7 cells that also expressed the recombinant porcine CD16 (Fig. 3d), whilst COS-7 cells transfected with pCDNA3.1 had no staining of CD16 or PRRSV N protein. Similarly, N protein of PRRSV was detected exclusively in CD16-positive HEK293-T cells (Fig. 3d) but not in pCDNA3.1-transfected cells. In addition, we determined the number of PRRSV-infected cells by gating CD16-positive cells. As shown in Fig. 3(e), around 1.9% of COS-7 cells transfected with CD16/γ-chain were infected by virus–antibody immune complexes, and around 2.5% of 293-T cells transfected with CD16/γ-chain were positive. The control COS-7 and 293-T cells showed no staining for CD16 or PRRSV N protein. These data indicated that CD16 is involved in the attachment of the PRRSV–antibody immune complex to cells and in the entry of the virus into cells.
ADE of virus reproduction in COS-7 cells expressing CD16

We next investigated the production of viral particles by virus that had entered COS-7 cells via CD16. The intracellular virus was first analysed by treating the cells with Pronase to cleave cell-surface proteins; this treatment should eliminate the attachment of virus mediated by the virus–antibody immune complex. As shown in Fig. 4(a), CD16 on the cell surface was removed, indicating that the virus attachment to COS-7 cells was also eliminated. qRT-PCR showed that the intracellular virus RNA level was not enhanced at early time points (1–6 h post-incubation), that it increased significantly at 12 h post-incubation and that it reached a peak at 24 h post-incubation (Fig. 4b). These results suggested that the virus had replicated in the CD16-transfected cells after virus entry into cells, which had been facilitated by the attachment of the virus to the immune complex. The production of viral particles that had entered the HEK293-T cells via CD16 was also assessed. In contrast to COS-7 cells, although a detectable level PRSSV RNA was present in the cells, virus RNA levels did not increase over the time course (1–48 h post-incubation; data not shown), indicating that the virus did not replicate in the CD16-transfected HEK293-T cells after virus entry into cells. In addition, the intracellular virus RNA level in COS-7/HEK293-T cells transfected with pcDNA3.1 was analysed, and no detectable PRSSV RNA was present at any of the time points (data not shown).

We then characterized the production and infectivity of PRSSV virions released into the fluid used to culture COS-7 cells expressing CD16. qRT-PCR showed that PRSSV virions were first released at 24 h after the immune complex had been added, and that the release increased gradually over time (Fig. 4b). The production of PRSSV virions released into fluid in HEK293-T cells expressing CD16 was also examined, and there was no detectable level of PRSSV RNA present in the fluid at any time point (1–48 h post-incubation; data not shown). The infectivity of the released virus from COS/CD16/γ-chain cells was also tested in the PRSSV-permissive cell line Marc-145 by immunofluorescence assay to detect PRSSV N protein. The COS-7 cells containing CD16 plus the γ-chain showed ADE-mediated infectivity and virus production; in contrast, the pcDNA3.1 vector control-transfected cells did not generate PRSSV virions (Fig. 4c). In addition, we evaluated the kinetics of virus production from the supernatants at different time points by a plaque assay. As shown in Fig. 4(d), the virus titre increased significantly with time, confirming that PRSSV was replicated and released over time. These data provided the evidence that ADE of PRSSV infectivity and output can be mediated by CD16.

DISCUSSION

PRRS is one of the most severe diseases of pigs worldwide, and vaccines against PRRSV have been developed and broadly used. Antibodies elicited by the vaccine can block infection via antibody neutralization. However, the vaccine-induced antibodies may enhance virus infection when the antibodies are at subneutralizing concentrations via the phenomenon referred to as ADE. As early as 1993, researchers reported that PRRSV replication was enhanced by the addition of virus plus antibody (Christianson et al., 1993). Later, direct evidence was provided of ADE of PRRSV infection in vivo by injecting pigs with subneutralizing amounts of PRRSV-specific IgG (Yoon et al., 1996, 1997). These authors also revealed that infection of PAMs by PRRSV was significantly enhanced in vitro by antibody raised against PRRSV. Consistent with these previously published findings, we observed that PRSSV-specific neutralization serum blocked virus infection of PAMs at a high titre but enhanced infection of PAMs when diluted to a subneutralizing level.

The mechanism underlying ADE of virus infection is generally assumed to involve several cell-surface molecules, including the FcR, complement receptors, CD4/15/33, and β2-microglobulin (Tirado & Yoon, 2003). Porter et al. (1972) pointed out that phagocytosis of virus–antibody immune complexes by macrophages could lead to an increase of virus infection, whilst Halstead & O’Rourke (1977) first reported that FcR facilitates the uptake of virus–antibody immune complexes and mediates mechanism of ADE of dengue virus infection. In humans, the FcγR family consists of at least one high-affinity receptor (FcγRI or CD64) and two low-affinity receptors (FcγRII or CD32, and FcγRII or CD16) (Indik et al., 1995). The best-characterized receptors involved in ADE of dengue virus are FcγRI and FcγRII (Boonkang et al., 2013; Kontny et al., 1988; Littaua et al., 1990; Mady et al., 1993). Other investigators found that FcγRII is involved in ADE of infection by severe acute respiratory syndrome coronavirus, dengue virus, and coxsackievirus B (Hoher et al., 2012; Kam et al., 2007; Sun et al., 2011; Yip et al., 2014). Recently, Qiao et al. (2011) reported that FcγRII is involved in the ADE of PRSSV infection in Marc-145 cells expressing FcγRII. In characterizing the role of FcγR in ADE activity, we observed that CD16 is the most abundant FcγR expressed in PAMs. By using a specific functional blocking mAb against porcine CD16 in PAMs, we inhibited the enhancement of virus infection, suggesting that CD16 influences the ADE of PRRSV infection. Similarly, other investigators used functional blocking antibodies to determine that ADE of HIV-1 infection in human syncytiotrophoblast cells is mediated by CD16 and other FcγRs (Tóth et al., 1994).

Because multiple FcγRs are expressed in macrophages (Nimmerjahn & Ravetch, 2008), we transfected FcγR-non-bearing cells so that they expressed CD16 but no other FcR. In pigs, CD16 is the first FcγR to be cloned and characterized in natural killer cells and polymorphonuclear cells; along with several other proteins, CD16 is associated with the FcγRII γ-chain on the surface of porcine polymorphonuclear cells and macrophages (Halloran et al., 1994a, b; Sweeney & Kim, 2004; Sweeney et al., 1996). In
**Fig. 3.** CD16 is involved in PRRSV–antibody immune complex attachment and internalization in COS-7 and HEK293-T cells. (a) CD16 expression in COS-7 and HEK293-T cells as determined by Western blotting. COS-7 and HEK293-T cells were transfected with pcDNA3.1 vector control (lane 2), pcDNA3.1/CD16/γ-chain (lane 3), pcDNA3.1/CD16 (lane 4) or a mock control (lane 1) for 24 h. Cell lysates were subjected to reducing SDS-PAGE before Western blotting with antibodies to Flag.

(b) (d) Merged

(c) 293T with 1/100 SPF serum 293T with 1/100 serum 293T with 1/3200 serum

(d) DAPI PRRSV CD16

(e) PRRSV-positive cells (%)
addition, the γ-chain is responsible for the assembly of, and the signal transduction by, the functional cell-surface complex (Wirthmueller et al., 1992). To determine whether the γ-chain contributes to CD16 expression, we transfected cells with CD16 cDNA in the presence or absence of γ-chain cDNA. As expected, CD16 was not expressed efficiently on the cell surface without the γ-chain.

COS-7 cells, which are non-FcγR-bearing and non-permissive to PRRSV, were found previously to be useful for determining the function of FcγRs in mediating endocytosis (Davis et al., 1995). We therefore hypothesized that, if expressed on the surface of COS-7 cells, CD16 would initiate the attachment and internalization of the virus–antibody immune complex. When we transfected COS-7 cells, which do not express the essential PRRSV receptors CD163 and CD169 (Calvert et al., 2007; Vanderheijden et al., 2003), with porcine CD16, we found that the COS-7 cells became permissive to PRRSV coupled with antibodies. We obtained similar results with a human kidney fibroblast cell line, HEK293-T, which was transfected to express CD16. These data indicated that CD16 facilitates the attachment of the virus–antibody immune complex to the cell surface, which consequently might contribute to the internalization of virus. Similarly, other researchers have found that the complex of foot-and-mouth disease virus and antibody resulted in virus infection of a cell line (Chinese hamster ovary) that was transfected to express FcγR (Mason et al., 1993).

Based on our results, the PRRSV–antibody immune complex can be internalized into cells. The fate of internalized PRRSV can be replicated and did not produce infective virions (Vanderheijden et al., 2003). Here, we observed that the internalized PRRSV replicated in COS-7 but not in HEK293-T cells, and that the progeny from COS-7 cells were released efficiently. These findings suggest that internalized PRRSV in HEK293-T cells, similar to the situation for poliovirus (Mason et al., 1993), cannot undergo the subsequent steps of replication such as uncoating. However, the internalized viruses might contribute to developing latent infection and periodic reactivation. In addition, we found that the virus only entered and replicated in the CD16-positive cells, which further supported the inference that CD16 mediates ADE of PRRSV infection in non-permissive cells.

In summary, we found that CD16 is involved in the ADE of PRRSV infection, indicating that PRRSV-specific antibodies can induce a shift in the cell tropism of PRRSV towards porcine cells expressing CD16. Antibody- and CD16-dependent, but CD163/CD169-independent, virus entry represents a novel mechanism by which PRRSV can enter target cells. More research is needed to elucidate further the in vivo functional consequences of this phenomenon. It follows that the efficient control of PRRSV infection will require the development of safer PRRSV vaccines, i.e., vaccines that maintain high serum virus-neutralizing activity and that do not result in ADE of infection.

METHODS

Cells and viruses. Marc-145, COS-7 and HEK293-T cell lines were maintained in Dulbecco’s minimum essential medium (DMEM) supplemented with 10% heat-inactivated FBS (Thermo Fisher Scientific). Primary PAMs were freshly harvested from 6-week-old SPF pigs and maintained in DMEM with 10% heat-inactivated FBS and 100 U/mL penicillin/100 U/mL streptomycin. The animal experiment was approved by the Harbin Veterinary Research Institute and performed in accordance with animal ethics guidelines and approved protocols. The animal ethics committee approval number is Heilongjiang-SYXK-2006-032. All cells were cultured in a humidified atmosphere at 37°C and 5% CO2. The virulent PRRSV strain HuN4 (GenBank accession no. EF635006) and the attenuated vaccine strain HuN4-F112 were maintained and titrated in Marc-145 cells as described previously (Tian et al., 2009; Tong et al., 2007) and were stored at −80°C.

Preparation of PRRSV immune sera. PRRSV-positive sera from four HuN4-F112-immunized pigs were used in the current study; these sera were obtained in a previous study in our laboratory (Yang et al., 2012). Three SPF pig sera were used as PRRSV-negative sera in the current study. Individual sera were heat-inactivated at 56°C for 30 min. The anti-PRRSV responses of these seven sera were analysed using a commercial ELISA kit (IDEXX Laboratories) according to the
CD16 influences ADE of PRRSV

Fig. 4. CD16 is critical for ADE of PRRSV infection. (a) Effect of Pronase on cell-surface proteins. Transfected COS-7 cells were incubated with virus–antibody immune complex and treated with Pronase (5 mg ml⁻¹) at 37 °C for 30 min. CD16 expression on the cell surface was determined by flow cytometry. (b, c) Production of virus from ADE of PRRSV in COS/CD16/γ-chain cells. PRRSV was recovered from culture fluids of transfected cells at different time points after incubation with virus–antibody immune complex. To detect intracellular PRRSV, after supernatants were collected, transfected cells were treated with Pronase (5 mg ml⁻¹) to remove the binding immune complex and were collected. Total RNA was extracted separately from cells and supernatants, and the PRRSV level was assessed by qRT-PCR. Each value represents the mean ± SD of six separate experiments. (c) Infectivity of virions collected from the supernatants of ADE of PRRSV infection in COS/CD16/γ-chain or COS/cDNA3.1 cells. Marc-145, a well-characterized PRRSV-permissive cell line, was inoculated with the recovered virus or control. At 24 h post-incubation, cells were fixed, stained with FITC-labelled anti-PRRSV N protein mAb SDOW17 and analysed by fluorescence microscopy. Bar, 400 μm. (d) Kinetics of virus supernatants of ADE of PRRSV infection in COS/CD16/γ-chain cells. Viruses were recovered from culture fluids of transfected cells at different time points after incubation with virus–antibody immune complex. The virus titre was determined by a plaque assay. *P<0.05 according to Student's t-test.

Assay for detecting ADE activity in PAMs. Each serum was assayed in triplicate for ADE activity using a standard ADE assay with a slight modification (Morens & Halstead, 1990). In brief, each serum was serially diluted from 1/50 to 1/12 800. PRRSV HuN4, at an m.o.i. of 1 (Manokaran et al., 2008), was placed in the antibody dilution tubes at equal volumes and incubated for 60 min at 37 °C to allow the immune complex to form. The content of each tube was then added to a PAM monolayer and incubated for 60 min. The exposed cells were washed five times with PBS to remove excess PRRSV–antibody immune complexes and then resuspended in complete medium for an additional 12 h. Subsequently, viral RNA was quantified by qRT-PCR to assess whether ADE of PRRSV infection occurred. The three SPF porcine sera were included as a negative control.

Blocking of CD16 in PAMs. In blocking assays of FcR-mediated ADE, freshly isolated PAMs were pre-treated with mouse mAb G7, which binds to and blocks the functioning of porcine CD16 in neutrophils (Halloran et al., 1994b). A match-up mouse IgG1 (Santa Cruz Biotechnology) was used as a negative control. Mouse IgG1 or anti-porcine CD16 mAb G7 at 10 μg ml⁻¹ was pre-incubated with PAMs for 2 h, before PAMs were inoculated with the PRRSV HuN4–antibody immune complex. At 12 h post-incubation, the production of PRRSV was measured by viral RNA quantification (see below).

RNA quantification. Quantitative RT-PCR analyses were carried out as described previously with a slight modification. Typically, total RNA was extracted from cell cultures and subjected to qRT-PCR using specific primers (Table 2). Relative quantification was performed by the cycle threshold (ΔΔCT) method (Livak & Schmittgen, 2001). Briefly, CT values were normalized to an internal standard β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as CT, which was determined by the formula ΔCT = CT (PRRSV) − CT (beta-actin/GAPDH). Fold changes of viral RNA were determined by 2⁻ΔΔCT, where ΔΔCT = ΔCT (PRRSV–antibody immune complex) − ΔCT (control). Fold changes of different FcRs were also determined by 2⁻ΔΔCT, where ΔΔCT = CT (CD16/CD32/ CD64) − CT (β-actin) and ΔΔCT = ΔCT (CD16) − ΔCT (CD32/CD64).
Expression of CD16 in COS-7 and HEK293-T cells. Porcine CD16 and the FcR γ-chain genes were amplified from PAMs and cloned into the pcDNA3.1 (+) vector (Promega). The Flag tag (DYKDDDDK) was fused to the carboxyl terminal of CD16, and the Myc tag (EQKISEEDL) was fused to the carboxyl terminal of Fcγ-chain; PCR was used in both cases, and the primers are listed in Table 2. The nucleotide sequences of the plasmids encoding CD16 and Fcγ-chain were determined to ensure that the correct clones were used. XtremeGENE transfection reagent (Roche) was used to transfect the Fcγ-R-non-bearing cell lines COS-7 and HEK293-T with 2 μg of the target plasmids pcDNA3.1/CD16 and pcDNA3.1/Fcγ-chain, pcDNA3.1/CD16 alone and pcDNA3.1 control. At 24 h post-transfection, the expression of CD16 was analysed by Western blotting and flow cytometry. In some experiments, the transfected cells were assessed for ADE activity.

ADE of PRRSV infection in COS-7 and HEK293-T cells containing CD16. COS-7 and HEK293-T cells were transfected to express CD16 as described above. The immune complex was prepared with CD16 plus antibody immune complex for 1 h. After the cells had been washed five times, fresh medium was added to the cell monolayer. After 12 h of incubation, the cells were fixed in 4 % paraformaldehyde for 30 min and washed five times with PBS before being permeabilized with 0.3 % Triton X-100 for 20 min. The cells were blocked with 2 % BSA/PBS and then incubated with mouse anti-porcine CD16 mAb G7 (Abcam) for 1 h. After washing, the cells were incubated with FITC-conjugated goat anti-mouse IgG (Zhongshan Biotechnology) for 1 h. The cells were then washed with PBS and stained with FITC-labelled mAb SOW17 (RTI). Finally, the cells were stained with DAPI for 5 min and examined with a Leica SP2 laser-scanning confocal system (Leica Microsystems).

Immunofluorescence assay and viral plaque assay. An immunofluorescence assay was performed as described previously (Guo et al., 2014). Briefly, the culture supernatants were inoculated onto Marc-145 cells. At 24 h post-inoculation, the cells were fixed and stained with FITC-labelled mAb SOW17 to detect PRRSV N protein. Fluorescence was visualized with an Olympus inverted fluorescence microscope equipped with a camera. A plaque assay was carried out as described previously (Wang et al., 2008). The virus titres of cell-culture supernatants collected at different time points were determined by a plaque assay in Marc-145 cells.

Western blotting. Western blotting analysis was done as described previously with a slight modification (Wang et al., 2011). Typically, samples were separated by SDS-PAGE under reducing conditions and transferred onto a PVDF membrane. After blocking, the membranes were incubated with a primary antibody and then incubated with an IRDye-conjugated secondary antibody (Li-Cor Biosciences). The membranes were scanned using an Odyssey instrument (Li-Cor Biosciences) according to the manufacturer’s instructions. The mAb M2 (Sigma-Aldrich) was used to detect the expression of Flag-tagged CD16 proteins. The mAb A7 (Earthox) was used to detect the expression of Myc-tagged Fcγ-chain proteins. The blotting antibody for CD16 was raised in rabbit and stocked in our laboratory. Anti-β-actin mAb was purchased from Santa Cruz Biotechnology.

Table 2. Primers used in construction and qRT-PCR analysis

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<th>Primer</th>
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<td>CAAGCAACCACACGGCACA</td>
<td>FcRII qRT-PCR</td>
</tr>
<tr>
<td>FcRIII-forward</td>
<td>GGGCTTCTGGTGGTGGGACAGA</td>
<td>FcRIII (CD16) qRT-PCR</td>
</tr>
<tr>
<td>FcRIII-reverse</td>
<td>AGCCACATTGTTAAGGTGT</td>
<td>FcRIII (CD16) qRT-PCR</td>
</tr>
<tr>
<td>PRRSV-forward</td>
<td>GCCCTTGCTTGGTGGTGGCcAGA</td>
<td>PRRSV qRT-PCR to N protein</td>
</tr>
<tr>
<td>PRRSV-reverse</td>
<td>CACGGTGCCCTAATTGGAATAGG</td>
<td>PRRSV qRT-PCR to N protein</td>
</tr>
<tr>
<td>β-Actin-forward</td>
<td>CAAGCAACCACACGGCACA</td>
<td>Porcine β-actin qRT-PCR</td>
</tr>
<tr>
<td>β-Actin-reverse</td>
<td>AGATGCGAGGGCCGATC</td>
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</tr>
<tr>
<td>GAPDH-forward</td>
<td>GAGTCACCAGTATGTCGT</td>
<td>Human GAPDH qRT-PCR</td>
</tr>
<tr>
<td>GAPDH-reverse</td>
<td>GGTTGCAGTGGATTGCCAT</td>
<td>Human GAPDH qRT-PCR</td>
</tr>
<tr>
<td>CD16-forward</td>
<td>CCGGAATTCAATGCGAGCTGCTGTAACCAACGG</td>
<td>Porcine CD16 amplification</td>
</tr>
<tr>
<td>CD16-reverse</td>
<td>CTCTCGATGATTTATTGTCGT</td>
<td>Porcine CD16 amplification</td>
</tr>
<tr>
<td>γ Chain-forward</td>
<td>CCGGAATTCAATGCGAGCATTGCCGCTTCTGGTCTT</td>
<td>Porcine FcR γ chain amplification</td>
</tr>
<tr>
<td>γ Chain-reverse</td>
<td>CCGGTCAGATACGATCTCCTCTGAGATAGATT</td>
<td>Porcine FcR γ chain amplification</td>
</tr>
</tbody>
</table>
Flow cytometry. Cells were labelled with particular mAbs and analysed on a FACS Aria instrument (BD Biosciences), as described previously (Wang et al., 2010b). Phycoerythrin-conjugated mouse anti-porcine CD16 mAb (BioLegend) detects CD16 expressed on PAM, HEK293-T and COS-7 cells. Isotype-matched negative-control mAb was used to evaluate levels of non-specific staining. Flow cytometry was also performed to examine PRRSV-positive cells, as described previously (Guo et al., 2014). Typically, 10,000 labelled cells were analysed. All samples were analysed with FlowJo 8.7 (Tree Star).

Statistical analysis. Values are expressed as means ± SD. Data were analysed with Student's t-test in Excel. A P value of <0.05 was considered significant.

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REFERENCES


