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Original article

Involvement of proteases in porcine reproductive and respiratory syndrome virus uncoating upon internalization in primary macrophages

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Abstract – Porcine reproductive and respiratory syndrome virus (PRRSV) replicates in differentiated macrophages. In macrophages, heparan sulphate glycosaminoglycans mediate the initial PRRSV attachment and the receptor sialoadhesin mediates both PRRSV attachment and internalization into endosomes. Upon a pH drop, PRRSV is uncoated and its genome is released from the endosomes into the cytoplasm, which allows virus replication. However, expression of heparan sulphate and sialoadhesin in non-susceptible cells only allows virus internalization, but no virus uncoating and infection, indicating that other factors are involved. In the present study, it is shown that treatment of macrophages with serum (mainly the alpha-globulin fraction) inhibited PRRSV infection without affecting attachment and internalization. Because alpha-globulins contain several protease inhibitors, macrophages were treated with different protease inhibitors to investigate the involvement of proteases in PRRSV uncoating. Treatment of macrophages with broadly active inhibitors of serine or aspartic proteases, but not cysteine- or metallo-proteases, inhibited PRRSV uncoating and infection. Further investigation using specific inhibitors indicated that the aspartic protease cathepsin E is involved during PRRSV uncoating, but did not allow identification of the serine protease involved. The involvement of cathepsin E during PRRSV uncoating was confirmed by partial co-localization of internalized PRRSV with cathepsin E. Furthermore, cathepsin E expression increased with macrophage cultivation, which was positively correlated with an increased susceptibility to PRRSV infection. Together, these data show that, in macrophages, both the aspartic protease cathepsin E and an unidentified trypsin-like serine protease are involved in uncoating of internalized PRRSV and subsequent infection.

porcine arterivirus / macrophage / virus entry / protease / receptor

1. INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) belongs to the family *Arteriviridae* in the order *Nidovirales* [7, 20]. The nidovirus order comprises a group of evolutionarily related enveloped, positive-stranded RNA virus families (arteri-, corona-, and roniviruses) [7, 10, 12, 33]. PRRSV infection is characterized by reproductive failure in sows and by respiratory problems in pigs of all ages, in combination with other pathogens [7, 8, 21, 28]. In vivo, PRRSV has a predilection for differentiated macrophages [18, 44]. In vitro, primary porcine macrophages such as alveolar macrophages and the non-macrophage African green monkey kidney cell line MA-104, and cells derived from MA-104 (Marc-145 and CL-2621) support PRRSV infection [5, 24, 40, 46, 47].

Two PRRSV receptors on primary macrophages have already been identified. The glycosaminoglycan heparan sulphate is a PRRSV receptor that is involved in

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PRRSV attachment via its interaction with structural matrix protein and the matrix-glycoprotein 5 complex [13,43]. Sialoadhesin, a sialic acid binding immunoglobulin-like lectin [11], also mediates PRRSV attachment to macrophages [15, 44]. Initial attachment of PRRSV to macrophages is mediated mainly via an interaction with heparan sulphate, followed by a gradual increase in interaction with sialoadhesin [15]. Sialoadhesin is the PRRSV internalization receptor [15, 19, 44]. Heparan sulphate is not necessary for sialoadhesin to function as a PRRSV internalization receptor, but enhances the interaction of the virus with sialoadhesin. Attachment of PRRSV to sialoadhesin is mediated by sialic acids present on the surface of PRRS virions [14, 16]. CD163, a cellular protein in the scavenger receptor cysteine-rich superfamily, has recently been shown also to function as a PRRSV receptor in Marc-145 cells, but its role in PRRSV infection of primary macrophages has not been studied [6].

Other putative PRRSV receptors have been described both on macrophages and Marc-145 cells. Monoclonal antibodies against macrophage cell surface N-glycosylated proteins (220 kDa and 150 kDa doublet) block or reduce infection of macrophages [48]. A monoclonal antibody against a cytoskeletal filament complex (vimentin, cytokeratin 8, cytokeratin 18, actin, and hair type II basic keratin) blocks PRRSV infection of Marc-145 cells [25], and CD151, a member of the tetraspanin superfamily, also plays a critical role in PRRSV infection of Marc-145 cells [38].

Expression of a recombinant porcine sialoadhesin in porcine kidney (PK-15) cells, which are non-permissive to PRRSV entry and infection, renders these cells capable of internalizing PRRSV, but infection does not occur [44]. In PK-15 cells expressing recombinant porcine sialoadhesin, PRRSV nucleocapsid protein disintegration, as a result of virus uncoating does not occur after virus internalization [44]. Internalized PRRSV remains trapped within endosomes of PK-15 cells expressing recombinant porcine sialoadhesin, suggesting a block in the virus uncoating due to absence of a macrophage-specific factor. In Marc-145 cells and macrophages, the nucleocapsid of internalized PRRSV gradually disintegrates and is almost completely absent from three hours after internalization [44]. The disintegration is followed by expression of viral proteins starting from approximately 6 h after internalization.

In the present study, it was the purpose to search for macrophage-specific factors that are important during early events of PRRSV infection.

2. MATERIALS AND METHODS

2.1. Viruses and cells

A 13th passage on macrophages of the European prototype PRRSV strain Lelystad virus (LV; kindly provided by G. Wensvoort, Institute of Animal Science and Health, Lelystad, The Netherlands) was used. Virus was semi-purified by ultracentrifugation at 100 000 × *g* for 3 h through a 30% sucrose cushion in an SW41 Ti rotor (Beckman Coulter Inc., Palo Alto, California, USA). Virus pellets were resuspended in phosphate-buffered saline and stored at −70 °C until use. Primary porcine alveolar macrophages were isolated from 4- to 6-week-old conventional Belgian landrace pigs from a PRRSV-negative herd using the method previously described by Wensvoort et al. [46]. Macrophages were cultivated in macrophage medium consisting of Earle's minimal essential medium (Invitrogen, Grand Island, NY, USA) supplemented with 10% (v/v) foetal bovine serum (FBS), 2 mM *L*-glutamine (BDH Chemicals Ltd., Poole, England), 1% non-essential amino acids (100×, Invitrogen), 1 mM sodium pyruvate (Invitrogen) and antibiotics in a humidified 5% CO_2 atmosphere at 37 °C.

2.2. Sera, globulins and protease inhibitors

During PRRSV titration of sera obtained from PRRSV infected pigs on macrophages, it was observed that lower serum dilutions gave false negative results and only became positive at subsequent higher serum dilutions. This observation indicated that high concentrations of serum inhibited PRRSV infection of macrophages. To investigate the effect of serum on PRRSV infection, macrophages were treated with heat-inactivated sera from different animal species.

Cohn fraction IV-1 (predominantly α-globulins), Cohn fraction IV-4 (predominantly α- and β-globulins), Cohn fraction II and III (predominantly β- and γ-globulins), gamma (γ)-globulins, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 6-aminohexanoic acid, antipain hydrochloride, aprotinin, benzamidine hydrochloride hydrate, bestatin hydrochloride, chymostatin, E-64, leupeptin hemisulphate, pepstatin A, phosphoramidon disodium salt, and trypsin inhibitor type I-S were purchased from Sigma (Bornem, Belgium). Complete™ EDTA-free protease inhibitor cocktail was purchased from Roche Diagnostics (Mannheim, Germany). Cathepsin D inhibitor (H-Gly-Glu-Gly-Phe-Leu-Gly-D-Phe-Leu-OH) was obtained from Bachem (Bubendorf, Switzerland). Recombinant *Ascaris* pepsin (cathepsin E) inhibitor [23] was kindly provided by Prof. Dr Takashi Kageyama (Department of Cellular and Molecular Biology, Primate Research Institute, Kyoto University, Aichi, Japan).

2.3. Virus infection and inhibition experiments

Macrophages were cultivated in adhesion for 24 h before infection experiments were performed. Cultivated macrophages were inoculated with PRRSV at a multiplicity of infection that resulted in an infection rate of approximately 30% of macrophages at ten hours post-inoculation (hpi). For inhibition experiments, macrophages were washed and pre-incubated for 1 h with inhibitors (sera, globulins and protease inhibitors) at 4 °C in RPMI 1640. Next, macrophages were inoculated with PRRSV for 1 h at 37 °C in the presence of the inhibitors. Untreated macrophages were pre-incubated and inoculated with PRRSV in the absence of the inhibitors. Treated and untreated macrophages were then washed and incubated in a humidified 5% CO_2 atmosphere at 37 °C in macrophage medium without inhibitors. Inhibitors were withdrawn from the cells at 1 hpi in order not to block (i) virus-encoded proteases that play a role in the processing of the replicase gene; and (ii) cellular proteases that process viral envelope proteins during virus exocytosis [50]. Cells were then fixed with methanol (−20 °C) at 10 hpi.

2.4. Immunoperoxidase staining of infected macrophages

PRRSV infected macrophages were detected following an immunoperoxidase staining using a mouse monoclonal antibody (mAb) P3/27 directed against the nucleocapsid protein [45] and horseradish peroxidase-labeled goat anti-mouse antibodies (DakoCytomation, Glostrup, Denmark). Substrate was added to stain PRRSV infected macrophages which were counted under a light microscope (Olympus Optical Co., Hamburg, Germany). The percentage of infected macrophages was obtained from three randomly selected microscopic fields, each containing approximately 300 cells.

2.5. PRRSV internalization and infection kinetics on macrophages

For the detection of PRRSV during the course of PRRSV infection, macrophages were pre-incubated with or without protease inhibitors or FBS for 1 h. Macrophages were then inoculated with PRRSV for 1 h at 37 °C with or without protease inhibitors or FBS prior to fixation at 1, 3, 5 and 10 hpi with methanol at −20 °C. PRRS virions were then stained using mAb P3/27 and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (Invitrogen). Cells were washed, mounted and analyzed using a TCS SP2 laser scanning spectrum confocal system (Leica Microsystems GmbH, Heidelberg, Germany). The numbers of bound and internalized PRRS virions were counted from the acquired images.

2.6. Analysis of cathepsin D and E expression

Cathepsin D and E were detected by immunofluorescence staining using goat polyclonal anti-cathepsin D and anti-cathepsin E antibodies (Santa Cruz Biotechnology, Inc., California, USA), respectively. Macrophages were afterwards incubated with Alexa Fluor 594-labelled rabbit anti-goat antibodies (Invitrogen), washed, mounted and analyzed using confocal microscopy.

2.7. Co-localization of cathepsin D and E with PRRSV

Macrophages were cultivated for 24 h before they were inoculated with PRRSV at 37 °C. At 1 hpi, unbound PRRSV was washed off and cells were fixed with methanol at −20 °C. A double immunofluorescence staining against cathepsin D or E with PRRSV was performed. Cathepsin D and E were detected using goat polyclonal anti-cathepsin D and E antibodies,

respectively, followed by Alexa Fluor 594 rabbit anti-goat antibodies. PRRSV was then detected using mAb P3/27 and FITC-conjugated goat anti-mouse antibodies.

3. RESULTS

3.1. Serum and alpha globulins inhibit PRRSV infection of macrophages without affecting virus attachment and internalization

Treatment of macrophages with two-fold serial dilutions of heat-inactivated FBS resulted in a dose-dependent inhibition of virus infection (Fig. 1A). The relative percentages of infected macrophages compared to untreated macrophages were 19.6 ± 7.2 and $20.4 \pm 5.2\%$ when cells were treated with 10 and 20% (v/v) FBS, respectively. All sera from the different animal species tested reduced PRRSV infection of macrophages (Fig. 1B). Rat, bovine, goat and sheep sera exerted relatively higher inhibitory effects compared to porcine, horse, human and donkey sera. These data indicate that serum contains a component that is inhibitory to PRRSV infection regardless of animal species origin.

Serum proteins consists of albumin, α-, β- and γ-globulins [31]. Macrophages were treated with these fractions in order to investigate their effect on PRRSV infection. The highest concentrations used in this study were similar to those present in serum. The relative percentages of infected cells compared to untreated macrophages were $7.9 \pm 1.5\%$, $4.3 \pm 0.7\%$, $40.9 \pm 3.9\%$, and $83.4 \pm 12.2\%$ when macrophages were treated with 5 mg/mL of α-, αβ-, βγ- and γ-globulin fractions, respectively (Fig. 1C). From these results, it can be concluded that α-globulins are most effective in inhibiting PRRSV infection.

To investigate the effect of serum on PRRSV attachment and internalization, macrophages were treated with FBS one hour before and during PRRSV inoculation at 37 °C. The number of bound and internalized PRRSV virions in FBS-treated and untreated macrophages did not differ significantly (Fig. 1D). These results indicate that serum affects neither PRRSV attachment nor internalization in macrophages.

3.2. The aspartic protease cathepsin E and a serine protease are involved in PRRSV uncoating in macrophages

The α-globulins consist of protease inhibitors (antiproteases) e.g. α1-antichymotrypsin, α1-antitrypsin, α1-macroglobulin, α2-macroglobulin, antiplasmin, antithrombin III, ceruloplasmin, haptoglobins and heparin cofactor II [1, 32]. To investigate if the antiproteolytic property of α-globulins caused the inhibition of PRRSV infection, macrophages were treated with a commercial broad-spectrum protease inhibitor cocktail (Complete™ EDTA-free protease inhibitor cocktail) at a concentration recommended by the manufacturer. PRRSV infection of macrophages was reduced by 99.5% at this recommended concentration of the protease inhibitor cocktail. Two-fold dilutions starting from the recommended dilution of this protease inhibitor cocktail resulted in a dose dependent inhibition of PRRSV infection. A 1:2, 1:4, 1:8, 1:16 and 1:32 dilution resulted in 98, 91, 68, 58 and 26% inhibition of PRRSV infection, respectively. This indicates that the inhibition of PRRSV infection by α-globulins results from the antiproteases present in this serum fraction.

Proteases are classified into serine, cysteine, aspartic and metalloproteases [4]. To identify the class(es) of proteases involved in PRRSV infection, macrophages were treated with different protease inhibitors (Tab. I). Their effect on PRRSV disassembly and infection was analysed (Tab. I and Fig. 2). AEBSF (inhibits serine proteases) and pepstatin A (inhibits aspartic proteases) inhibited PRRSV disassembly in macrophages without affecting virus internalization (Tab. I). PRRSV disassembly in macrophages treated with either E-64 (inhibits cysteine proteases) or phosphoramidon (inhibits metalloproteases) was not significantly affected (Tab. I). Similarly, AEBSF and pepstatin A reduced PRRSV infection while E-64 and phosphoramidon had no effect (Tab. I). These results suggest that a serine and an aspartic protease are involved during PRRSV uncoating in macrophages.

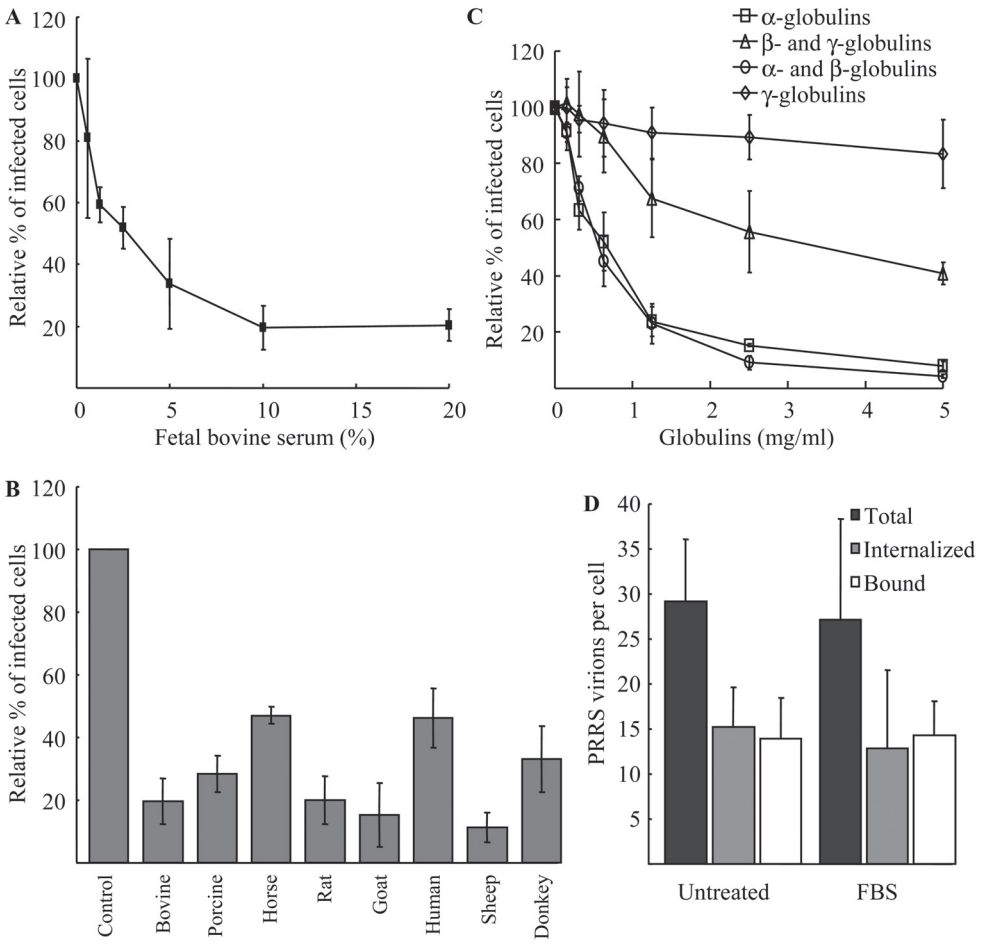

Figure 1. Effect of serum and serum globulins on PRRSV infection and internalization in macrophages. Macrophages were treated with two-fold serial dilutions of foetal bovine serum (FBS) (A), 10% serum from different animal species (B), or two-fold serial dilutions of serum globulins (C). Control macrophages were left untreated. Treated and untreated macrophages were then inoculated with an equal dose of PRRSV in the presence or absence of sera or serum globulins, respectively. The number of PRRSV infected macrophages was evaluated at 10 hpi. Data represent means ± standard deviation of triplicate assays. In (D), the number of bound and/or internalized PRRSV is shown in macrophages treated with or without 10% FBS as visualized by confocal, 1 h after inoculation.

Further analysis using serine protease inhibitors with a more restricted activity compared to AEBSF (antipain hydrochloride, leupeptin, 6-aminohexanoic acid, aprotinin, benzamidine hydrochloride, bestatin, chymostatin and trypsin inhibitor type I-S) did not allow to pinpoint the exact serine protease potentially involved in PRRSV uncoating (Tab. I).

The number of aspartic proteases is limited. Pepsins are secreted into the stomach, renin is secreted into blood, while cathepsin D and E remain intracellular as they are secreted into intracellular compartments [41, 49]. Macrophages were treated with inhibitors for cathepsin D (the inhibitory peptide H-Gly-Glu-Gly-Phe-Leu-Gly-D-Phe-Leu-OH) and

Table I. Effect of different protease inhibitors on PRRSV internalization, uncoating and infection of macrophages.

Protease inhibitor	Protease class(es) or protease(s) inhibited*	Concentration	PRRSV internalization (1 hpi)	PRRSV uncoating (5 hpi)	Relative % infected cells (10 hpi)
AEBSF HCl	Serine proteases	1 mM	Yes	No	0.9 ± 0.3
E-64	Cysteine proteases	10 μM	Yes	Yes	86.4 ± 9.7
Pepstatin A	Aspartic proteases	1 μg/mL	Yes	No	6.5 ± 1.3
Phosphoramidon	Metalloproteases	10 μM	Yes	Yes	68.7 ± 3.2
Antipain HCl	Some serine and cysteine proteases (e.g. trypsin-like serine proteases, trypsin, papain, cathepsin A, B and D, plasmin, chymotrypsin, pepsin, calpain I	100 μM	Yes	Yes	66.8 ± 6.8
Leupeptin	Some serine and cysteine proteases (e.g. trypsin, trypsin-like serine proteases, chymotrypsin, cathepsin B, H and L)	100 μM	Yes	No	1.7 ± 1.0
6-Aminohexanoic acid	Some serine proteases (e.g. chymotrypsin, factor VIIa, plasmin and plasminogen activator, and the metalloprotease lysine carboxypeptidase	5 mg/mL	Yes	Yes	80.5 ± 8.9
Aprotinin	Some serine proteases e.g. trypsin, tryptase, kallikrein, plasmin, chymostrypsin, elastase, urokinase, and thrombin	800 nM	Yes	Yes	89.5 ± 5.6
Benzamidine HCl	Some serine proteases e.g. trypsin, trypsin-like proteases, factor Xa and thrombin	4 mM	Yes	Partial	21.7 ± 8.3
Bestatin	Some serine proteases e.g. aminopeptidases, trypsin, chymotrypsin, elastase, papain, pepsin and themolysin	40 μM	Yes	Yes	93.2 ± 6.6
Chymostatin	Serine proteases (e.g. α-β-γ-δ-chymotrypsin, cathepsin G), most cysteine proteases (e.g. cathepsin B, H and L)	100 μM	Yes	Yes	89.6 ± 6.2
Trypsin inhibitor type I-S	Serine proteases e.g. trypsin, chymotrypsin, kallikrein, factor Xa and plasmin	5 mg/mL	Yes	Partial	21.6 ± 14.8

*List not exhaustive.

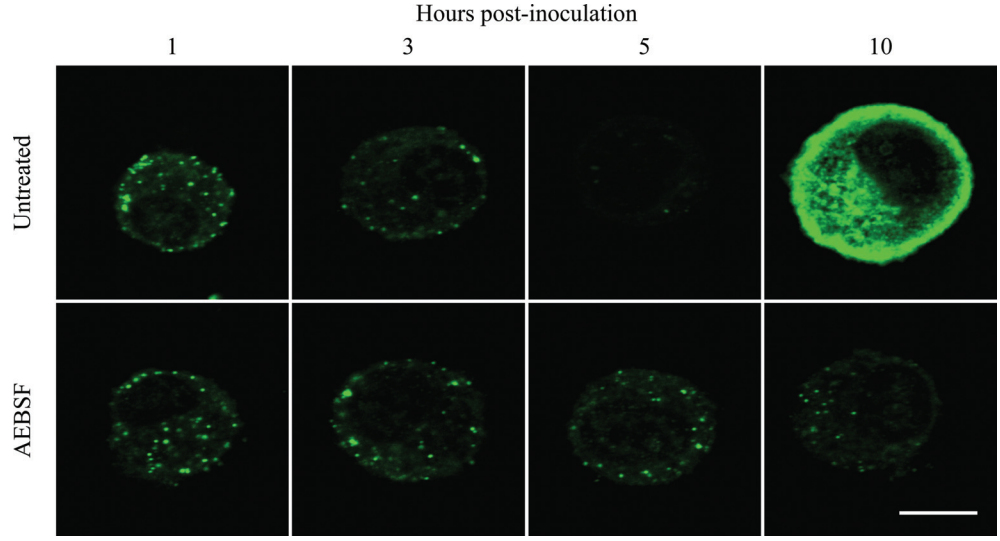

Figure 2. Representative confocal microscopical images of PRRSV entry and infection in the presence or absence of a serine protease inhibitor. Macrophages were treated with or without the serine protease inhibitor AEBSF for 1 h at 4 °C before inoculation. Treated and untreated macrophages were then inoculated with PRRSV at 37 °C in the presence or absence of AEBSF. After 1 h, macrophages were washed and incubated with macrophage medium before fixation at 1, 3, 5 and 10 hpi. An immunofluorescence staining was performed against PRRSV nucleocapsid protein (green fluorescence). Cells were mounted and analyzed by confocal microscopy. Complete PRRSV nucleocapsid disassembly at 5 hpi and full nascent nucleocapsid protein expression at 10 hpi was observed in untreated cells but not in AEBSF-treated macrophages. Bar, 10 µm. (A colour version of this figure is available at www.vetres.org.)

E (Ascaris inhibitor) to investigate the role of the major intracellular aspartic proteases on PRRSV uncoating. PRRSV disassembly and infection were significantly affected by the cathepsin E inhibitor but not the cathepsin D inhibitor (Tab. I and Fig. 3). From these results, it can be concluded that cathepsin E is involved in PRRSV uncoating in macrophages.

3.3. Internalized PRRSV co-localizes with cathepsin E

When a double immunofluorescence staining was performed for PRRSV and cathepsin E, it was observed that internalized PRRSV partially co-localizes with cathepsin E (Fig. 4). No or limited co-localization was observed between cathepsin D and internalizing PRRSV.

3.4. Correlation between cathepsin E expression and PRRSV infection

Macrophages, cultivated in adhesion for 1, 24, 48 and 72 h, were used to assess the presence of cathepsin D and E and to evaluate their susceptibility to PRRSV. Cathepsin E staining was specific and was abolished by a blocking peptide (data not shown). The number of macrophages expressing cathepsin E and the intensity of cathepsin E expression increased with duration of cultivation (Fig. 4). The number of cathepsin E-expressing macrophages positively correlated with the susceptibility of macrophages to PRRSV infection. Macrophages exhibited increased susceptibility to PRRSV with increased cultivation (Fig. 4). In contrast, cathepsin D was expressed by all macrophages and its expression pattern did not change with cultivation (Fig. 5).

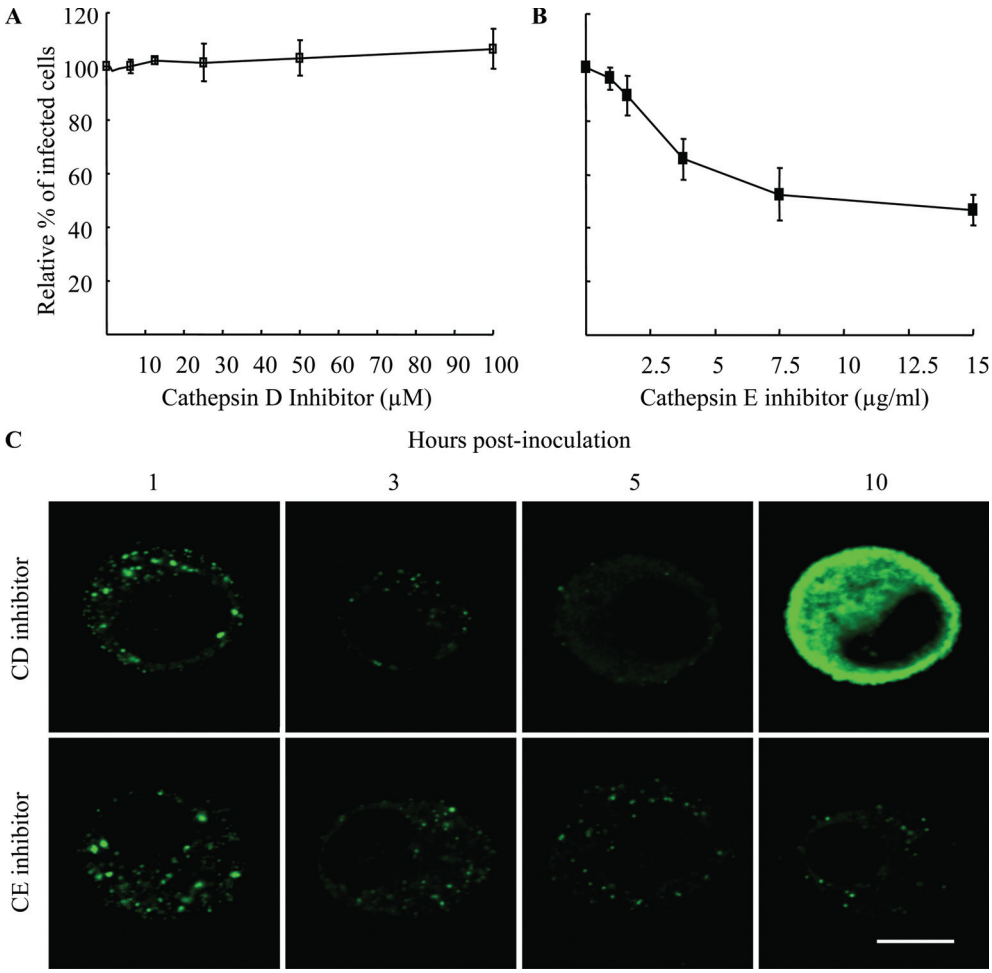

Figure 3. Effect of aspartic protease inhibitors on PRRSV disassembly and infection of macrophages. Macrophages were treated with cathepsin D (CD) inhibitor (A) or cathepsin E (CE) inhibitor (B) as described in the methods before fixation at 10 hpi. The proportion of PRRSV infected cells was determined by light microscopy. Data represent means ± standard deviation of triplicate assays. (C) Representative images of macrophages treated with cathepsin D or E inhibitors. Macrophages were stained to detect PRRSV nucleocapsid protein before they were mounted and analysed by confocal microscopy. Bar 10 μm. (A colour version of this figure is available at www.vetres.org.)

4. DISCUSSION

In this study, the involvement of cellular proteases in PRRSV uncoating during PRRSV replication in macrophages was investigated. It was shown that PRRSV infection of macrophages requires a serine and an aspartic protease. Cathepsin E was identified to be the aspartic protease involved during PRRSV uncoating in macrophages. Cathepsin E partially co-localized with internalized PRRSV. Further, the expression of cathepsin E increased with the duration that macrophages were kept in culture, which was positively correlated with the susceptibility of macrophages to PRRSV infection. The requirement for proteases during the replication of other viruses classified together with

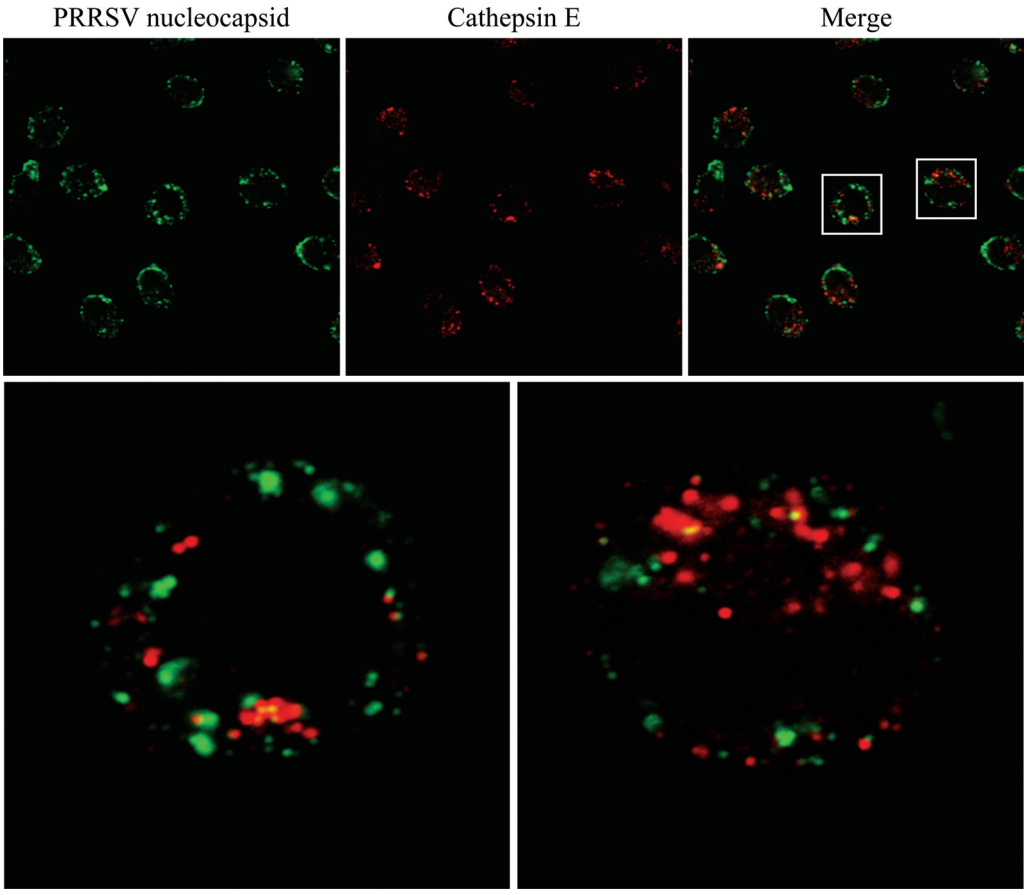

Figure 4. Co-localization between cathepsin E and PRRSV. Macrophages were cultivated for 24 h before they were incubated with PRRSV for 1 h at 37 °C to allow detection of internalized PRRSV. Macrophages were fixed and stained for cathepsin E (red fluorescence) and PRRSV (green fluorescence) as described in the methods. The two inserts marked with squares in the merged image are magnified in the two bottom images. Yellow fluorescence indicates co-localization between PRRSV and cathepsin E.

PRRSV into the nidovirus order has been reported. The serine proteases trypsin, factor Xa and elastase and the metalloprotease thermolysin enhance severe acute respiratory syndrome-coronavirus (SARS-CoV) replication by inducing virus-cell fusion at the cell surface [17, 27] and the cysteine protease cathepsin L mediates SARS-CoV-endosomal membrane fusion within endosomes [39]. Endosomal proteolysis by cysteine proteases cathepsin B and L is necessary for murine coronavirus mouse hepatitis virus type 2 spike-mediated entry [34].

In this study, it was shown that cathepsin E is involved in PRRSV uncoating in macrophages. Cathepsin E is an intracellular aspartic protease homologous to cathepsin D [22, 35]. Cathepsin D is localized within the lysosomes of most cells [9] while cathepsin E is localized in various non-lysosomal compartments such as the endosome, the endoplasmic reticulum and the Golgi complex and has a limited cell and tissue distribution [26, 35, 37, 41]. Cathepsin E has been detected in mouse, rabbit and rat macrophages [2, 9, 30, 36]. This study shows that cathepsin E is also expressed

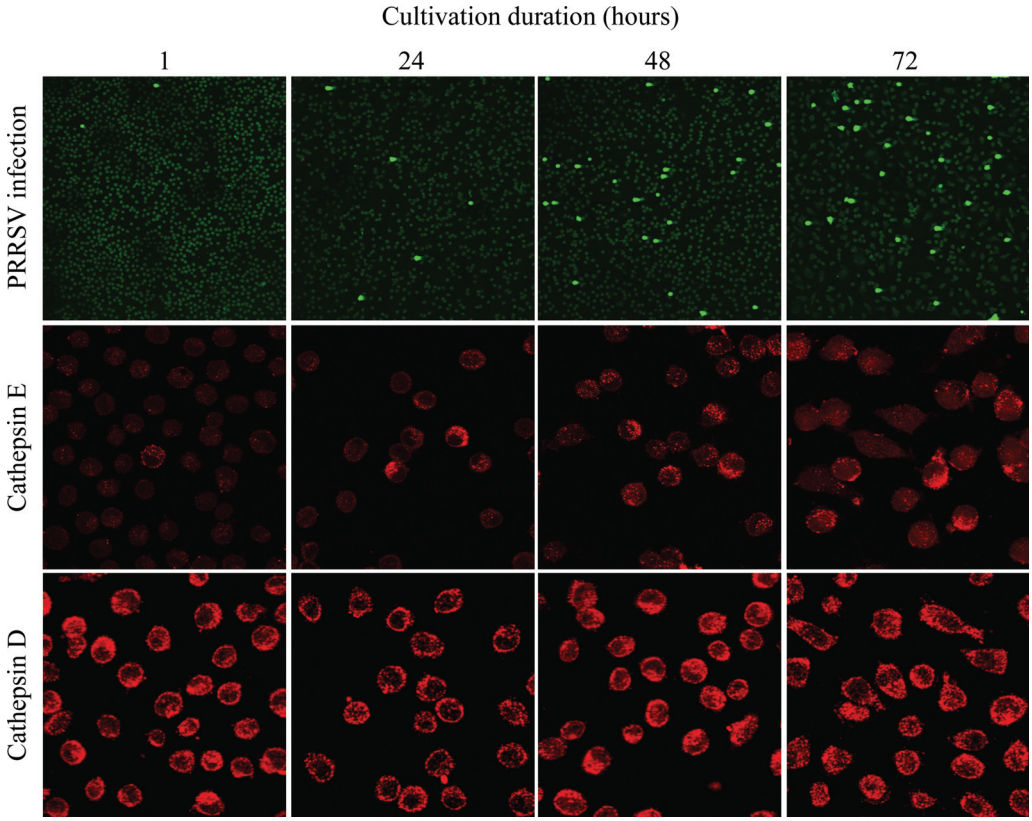

Figure 5. Effect of macrophage cultivation time on cathepsin D and E expression in macrophages and the susceptibility of macrophages to PRRSV infection. Macrophages were isolated and left in culture for 1, 24, 48 and 72 h before being analysed for cathepsin D and E expression. In addition, cells were also inoculated with PRRSV and fixed and stained 10 hpi to analyze the susceptibility of the cells. The susceptibility of macrophages to PRRSV infection and cathepsin E expression increased with duration of cultivation. Cathepsin D expression in macrophages was not influenced by cultivation. (A color version of this figure is available at www.vetres.org.)

by porcine macrophages. Internalizing virions partially co-localized with cathepsin E. This partial co-localization suggests that some PRRSV particles transits through cathepsin E containing intracellular vesicles. Although cathepsin D was expressed by all macrophages at high levels regardless of the duration of cultivation, it very rarely co-localized with PRRSV. This may explain why inhibiting cathepsin E but not cathepsin D affected PRRSV uncoating and infection despite the fact that these two enzymes have similar cleavage specificities.

The replication of PRRSV has been shown to be dependent on the endosomal-lysosomal system acidification [29]. It is very well possible that this pH dependency arises from the low pH-autocatalytic conversion of procathepsin E to the mature active form [37], which cleaves one of the PRRSV structural proteins. Cathepsin E has a cleavage specificity and maximal activity at around pH 6.0 [3, 42]. This corresponds to the pH within early and late endosomes.

Based on this and previous studies, a model for the initial stages of PRRSV infection

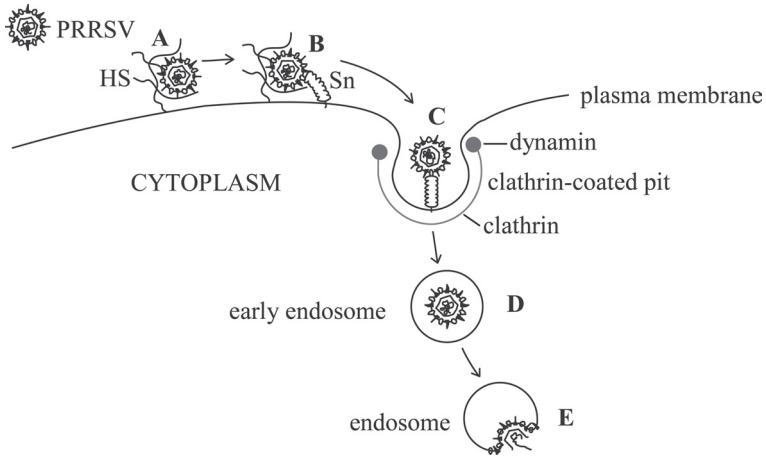

Figure 6. Model for the initial stages of PRRSV infection in macrophages. PRRSV attaches first to heparan sulphate (HS) (A) before gradually binding to porcine sialoadhesin (Sn) (B). PRRSV is then internalized via clathrin-mediated endocytosis by Sn (C). Internalized PRRSV is localized within the endosomes (D) where PRRSV uncoating occurs during the pH drop and is mediated by cellular proteases (cathepsin E and an unidentified serine protease).

on macrophages can be developed (Fig. 6). Initial PRRSV attachment to the surface of macrophages is mediated by a cellular heparan sulphate proteoglycan receptor [13, 43]. PRRSV is then attached and subsequently internalized via clathrin-mediated endocytosis [29] by the macrophage restricted porcine sialoadhesin receptor [44]. Porcine sialoadhesin binds to sialic acids, present on PRRSV viral glycoproteins [14, 16]. Following virus internalization, a subsequent pH drop in the endosome is required for virus replication [29]. Most likely, endosomal acidification activates proteases such as cathepsin E or other serine proteases, which are involved in the uncoating of PRRSV. In addition, transient transfection of CD163 was shown to enable PRRSV replication in non-susceptible cells and CD163 antibodies were shown to interfere with infection of Marc-145 cells [6]. These results suggests that besides a critical role of CD163 in PRRSV entry in Marc-145 cells, this molecule might also be involved in PRRSV entry in macrophages, however this needs to be addressed in more detail in studies with primary macrophages.

Future studies will focus on (i) identification of viral structural proteins that are potentially cleaved by proteases during PRRSV uncoating and (ii) co-expression of proteases, porcine sialoadhesin and/or CD163 in non-permissive to analyse if this makes cells susceptible to PRRSV replication. In addition, it might be interesting to investigate if different PRRSV isolates have the same dependency on proteases during entry and if there is a correlation between virulence and the usage of specific proteases during entry in macrophages. If so, the fact that certain protease inhibitors can block PRRSV replication may merit the design of protease based anti-PRRSV drugs.

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