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Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus

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The porcine reproductive and respiratory syndrome virus (PRRSV) shows a restricted tropism for subsets of porcine macrophages *in vivo*. To date, two PRRSV receptors have been identified on primary macrophages, heparan sulphate for binding and sialoadhesin for binding and internalization. However, additional factors are needed because the expression of both receptors in non-permissive cells results in virus internalization but not in virus uncoating and productive infection. Recently, CD163 was described as a PRRSV receptor on Marc-145 cells that renders non-permissive cells susceptible to PRRSV. Therefore, the potential role of CD163 in PRRSV entry in macrophages and its potential interplay with sialoadhesin were studied. Incubation of macrophages at 37 °C with either sialoadhesin- or CD163-specific antibodies reduced PRRSV infection by up to 75 %, while infection was completely blocked by a combination of both antibodies. When incubated at 4 °C, only sialoadhesin- and not CD163-specific antibodies reduced PRRSV infection. In addition, confocal analysis of PRRSV entry in non-permissive cells expressing only sialoadhesin showed PRRSV internalization but no uncoating. In contrast, when both sialoadhesin and CD163 were expressed, PRRSV was uncoated upon internalization, resulting in productive infection. Virus internalization was not observed when only CD163 was expressed; although, cells became productively infected. Thus, sialoadhesin is confirmed as a PRRSV internalization receptor and CD163 is shown to be involved in PRRSV entry, probably during uncoating. Co-expression of recombinant sialoadhesin and CD163 in non-permissive cells increased virus production 10–100 times compared with cells expressing only CD163, sustaining the requirement of both for efficient PRRSV infection.

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INTRODUCTION

A ‘mystery swine disease’ appeared in the 1980s and has been present ever since in the pig industry, causing important economical losses worldwide (Neumann *et al.*, 2005). The causative agent, designated porcine reproductive and respiratory syndrome virus (PRRSV), was isolated in the Netherlands in 1991 and shortly after in the USA (Collins *et al.*, 1992; Neumann *et al.*, 2005; Wensvoort *et al.*, 1991). PRRSV, a small enveloped positive-stranded RNA virus, is classified in the order *Nidovirales*, family *Arteriviridae*, genus *Arterivirus* together with equine arteritis virus, lactate dehydrogenase-elavating virus and simian hemorrhagic fever virus based on similar morphology, genomic organization, replication strategy and protein composition (Cavanagh, 1997). In addition, they share a very narrow host tropism and a marked preference for cells of the monocyte–macrophage lineage. More specifically, *in vivo*, PRRSV infects subpopulations of differentiated macrophages, with alveolar macrophages being the major target cells during acute infection (Duan *et al.*, 1997b; Teifke *et al.*, 2001). *In vitro*, PRRSV replicates in primary cultures of alveolar macrophages and to some extent in peripheral blood monocytes, with pre-treatment of alpha interferon in monocytes enhancing PRRSV infection (Delputte *et al.*, 2007a; Duan *et al.*, 1997a; Voicu *et al.*, 1994). Furthermore, the African green monkey kidney cells MA-104 and cells derived thereof (Marc-145 and CL2621) are shown to sustain PRRSV infection, although they are not from porcine origin, they do not belong to the monocyte–macrophage lineage and they do not express sialoadhesin (Duan *et al.*, 1998b; Kim *et al.*, 1993; Mengeling *et al.*, 1995; Wissink *et al.*, 2003). Despite this very restricted cell tropism of PRRSV, the virus is able to replicate in several non-

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permissive cell lines upon transfection of its viral RNA, indicating that cell tropism is determined by the presence or absence of specific receptors on the cell surface or other proteins involved in virus entry (Kreutz, 1998; Meulenberg *et al.*, 1998).

So far, two PRRSV receptors have been identified on macrophages, heparan sulphate (Delputte *et al.*, 2002; Vanderheijden *et al.*, 2001) and sialoadhesin (Vanderheijden *et al.*, 2003). In addition, a yet unidentified 150 kDa protein doublet and a 220 kDa protein, which can be speculated to be sialoadhesin, were found to be involved in PRRSV infection of macrophages (Wissink *et al.*, 2003). In the current model for PRRSV infection of macrophages, PRRSV first binds to heparan sulphate. Subsequently, PRRSV will engage sialoadhesin in a more stable interaction involving sialic acids present on the virion and the N-terminal sialic acid-binding domain of sialoadhesin, followed by internalization (Delputte *et al.*, 2004, 2005, 2007b; Delputte & Nauwynck, 2004). Upon internalization, the virus is transported towards an endosomal compartment where a drop in pH is required for proper virus replication (Kreutz & Ackermann, 1996; Nauwynck *et al.*, 1999). Despite extensive research on PRRSV entry in macrophages, the model is incomplete. In fact, transient expression of sialoadhesin in non-permissive PK-15 cells results in binding and internalization of the virus, but uncoating of the virus particles and productive infection was not observed (Vanderheijden *et al.*, 2003), indicating that other macrophage-specific factors are needed for virus uncoating and replication.

PRRSV infection of Marc-145 cells involves binding to a heparin-like molecule on the surface of Marc-145 cells (Jusa *et al.*, 1997), resembling the initial step of PRRSV infection of macrophages. However, since sialoadhesin is absent from Marc-145 cells, and since sialic acids on the virion are not essential for infectivity on Marc-145 cells (Delputte & Nauwynck, 2004), virus entry clearly differs between the two cell types. In Marc-145 cells, the intermediate filament vimentin was described to bind to the PRRSV nucleocapsid protein and was suggested to interact with other cytoskeletal filaments to mediate transport of the virus in the cytosol (Kim *et al.*, 2006). CD151 was found to interact specifically with the PRRSV 3′ untranslated region RNA and was proposed to be involved in the fusion between the viral envelope and the endosome or to relocalize the ribonucleoprotein complexes to promote viral replication (Shanmukhappa *et al.*, 2007). Still, further research is needed to elucidate their precise molecular modes of action during PRRSV infection.

Recently, scavenger receptor CD163 has been described to be essential in PRRSV infection of Marc-145 cells and to render non-permissive cells susceptible to PRRSV upon expression; however, without addressing the mechanism involved (Calvert *et al.*, 2007). Thus far, the best characterized function of CD163 is related to its binding of haemoglobin–haptoglobin (HbHp) complexes. Upon binding, these complexes are internalized, thereby protecting tissues from free Hb-mediated oxidative damage (Kristiansen *et al.*, 2001; Schaer *et al.*, 2006). Scavenger receptor CD163 [also known as RM3/1, haemoglobin scavenger receptor (HbSR), M130 or p155] is a member of the scavenger receptor cystein-rich family class B (Hogger *et al.*, 1998; Sarrias *et al.*, 2004) and is expressed selectively on monocytes and subpopulations of mature tissue macrophages (Fabriek *et al.*, 2005; Van den Heuvel *et al.*, 1999). Although CD163 is known to be expressed in porcine macrophages (Sanchez *et al.*, 1999), no role for CD163 in PRRSV infection of the primary target cells has been described so far. Therefore, the role of CD163 and its interaction with sialoadhesin during PRRSV infection of macrophages were investigated in this study.

METHODS

Cell culture and transfection. Primary alveolar macrophages were obtained from 4- to 6-week-old conventional Belgian Landrace pigs from a PRRSV-negative herd as described by Wensvoort *et al.* (1991), and cultivated in RPMI 1640 supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 1 % non-essential amino acids, 1 mM sodium pyruvate and a mixture of antibiotics. Marc-145 cells were cultivated in minimum essential medium (MEM) with Earle's salts supplemented with 5 % FBS. PK-15 cells were grown in MEM supplemented with 10 % FBS. BHK-21 cells were cultivated in MEM supplemented with 10 % FBS, 1 % non-essential amino acids and 1 mM sodium pyruvate. CHO-K1 cells were cultivated in F-12 medium supplemented with 10 % FBS and 1 mM sodium pyruvate. All continuous cell lines were grown in their specific medium supplemented with 2 mM L-glutamine and a mixture of antibiotics in a humidified 5 % CO_2 atmosphere at 37 °C. PK-15, BHK-21 and CHO-K1 cells were transfected with lipofectamine (Invitrogen), lipofectamine 2000 (Invitrogen) and FuGENE 6 (Roche), respectively, according to the manufacturer's instructions. A control experiment showed that 95 % of the double-transfected cells expressed both recombinant proteins.

Viruses. The European prototype PRRSV strain, Lelystad virus (LV) (kindly provided by G. Wensvoort), was passaged 13 times on macrophages and subsequently four times on Marc-145 cells (Wensvoort *et al.*, 1991). The American prototype PRRSV strain, VR-2332, was cultivated on Marc-145 cells for four passages but never passaged on macrophages (Collins *et al.*, 1992). The Belgian strain 94V360, was passaged two times on macrophages and subsequently five times on Marc-145 cells (Duan *et al.*, 1997a).

Antibodies. CD163 was detected via the mouse monoclonal antibody (mAb) anti-porcine CD163 2A10 (Ab-Direct) (Bullido *et al.*, 1997; Sanchez *et al.*, 1999) or a goat polyclonal antibody (pAb) anti-human CD163 (R&D Systems). For porcine sialoadhesin detection, mAb 41D3 was used (Duan *et al.*, 1998b; Vanderheijden *et al.*, 2003). Isotype-matched (IgG_1) irrelevant mAb 13D12 directed against gD of pseudorabies virus (Nauwynck & Pensaert, 1995) and purified goat antibodies were used as negative controls. For some experiments, mAb were purified using protein G chromatography following the manufacturer's instructions (GE Healthcare). PRRSV was visualized via the nucleocapsid protein-specific mAb P3/27 (Wieczorek-Krohmer *et al.*, 1996) or a polyclonal swine serum obtained from PRRSV-infected pigs (Delputte *et al.*, 2004). An affinity purified goat pAb was used to visualize early endosome antigen 1 (EEA1) (Santa Cruz Biotechnology).

Plasmids. CD163 variants differing in their cytoplasmic tail were described previously (Nielsen *et al.*, 2006). Since these variations do not appear to determine PRRSV receptor function (Calvert *et al.*, 2007), only one variant, corresponding with susCD163v2 (Calvert *et al.*, 2007), was cloned. Therefore, total cellular RNA was isolated from porcine macrophages using the RNeasy Mini kit (Qiagen) and subsequently converted into cDNA via oligo(dT) primers (Invitrogen) and SuperScript II reverse transcriptase (Invitrogen) followed by RNase H (Gibco) treatment. The obtained single-stranded cDNA served as template for PCR amplification of the CD163 sequence via the Platinum *Pfx* polymerase (Invitrogen) and the following primers: forward primer 5′-CACCATGGACAAACTC-AGAATGGTGCTACATGAAAACTCT-3′ and reverse primer 5′-TCATTGTACTTCAGAGTGGTCTCCTGAGGGATT-3′ (Invitrogen). The PCR fragment was then finally cloned into the pcDNA3.1D/V5-His-TOPO vector (Invitrogen). Sialoadhesin was previously cloned into the same vector as described by Vanderheijden *et al.* (2003). Constructs were verified via restriction digestion and sequencing. The cDNA sequence of the porcine CD163 used in this study is available under the GenBank accession number EU016226.

Flow cytometry. Twenty-four hours after seeding, macrophages were lifted from the cell culture plate by incubation with ice-cold PBS for 30 min at 4 °C immediately prior to immunostaining and flow-cytometric analysis. Cells were first fixed with 3 % paraformaldehyde in PBS followed by washing and incubation at 4 °C with primary mAb 41D3, 2A10 or isotype-matched control antibody 13D12 diluted in PBS containing 10 % heat-inactivated goat serum (PBS-G). Afterwards, cells were washed three times and subsequently incubated with fluorescein isothiocyanate-labelled goat-anti-mouse antibody (Molecular Probes) diluted in PBS-G. Finally, cells were washed three times, resuspended in PBS and analysed with a Becton Dickinson FACScalibur. Twenty thousand cells were analysed for each sample and three parameters were stored for further analysis: forward light scatter, sideward light scatter and green fluorescence.

Virus titration. To determine the titre of extracellular virus, supernatant was collected and centrifuged to remove cell debris. To determine the titre of intracellular virus, cells were washed, collected and lysed by three cycles of freeze–thaw. For titration on Marc-145 cells, cells were planted 3 days before inoculation. Then, they were inoculated with a 10-fold dilution series of the samples and incubated for 7 days at 37 °C. For titration on macrophages, cells were planted 1 day before inoculation followed by inoculation with a 10-fold dilution series of the samples and incubation for 3 days at 37 °C. Infected cells were then visualized via an immunoperoxidase monolayer assay (Wensvoort *et al.*, 1991). Finally, virus titres were calculated as $TCID_{50}$ ml^{-1} (Reed & Muench, 1938).

Immunofluorescence staining and (confocal) microscopy. Transfected and/or infected cells were fixed with ice-cold methanol for the detection of infected cells. For localization studies, cells were fixed with 3 % paraformaldehyde and if needed, permeabilized with 0.1 % Triton X-100. Cells were washed and incubated with primary antibodies for 1 h at 37 °C, washed three times with PBS and further incubated with secondary antibodies for 1 h at 37 °C. Finally, cells were washed three times, embedded in a glycerine–PBS solution (0.9/0.1, v/v) containing 2.5 % 1,4-diazabicyclo(2,2,2)octane, mounted and analysed via a Leica DM RBE fluorescence microscope (Leica Microsystems) or via a TCS SP2 laser scanning spectrum confocal system (Leica Microsystems) using an Argon 488 nm and a Gre/Ne 543 nm laser for excitation.

Treatment of macrophages with sialoadhesin- and CD163-specific ligands. Macrophages were seeded in 96-wells 24 h before the experiment was performed. A threefold dilution series was prepared for different antibodies (2A10, pAb CD163, 41D3, 13D12, purified control goat antibodies) and the HbHp complex [Hb A_o (H0267), Hp type2-2 (H9762) from Sigma-Aldrich], which was assembled via 15 min incubation of both components at room temperature. For treatment at 37 °C, macrophages were incubated for 1 h at 37 °C with the ligands followed by inoculation with PRRSV in the presence of a new dilution series of ligands for 1 h at 37 °C. After inoculation, cells were washed, further incubated for 9 h at 37 °C and then fixed with methanol. For treatment at 4 °C, a similar protocol as described above for 37 °C was used, except for the preincubation of cells for 30 min at 4 °C on ice prior to adding the ligands and after washing the ligands and the virus, cells were incubated for 10 instead of 9 h at 37 °C. Infected cells were visualized via immunoperoxidase staining with mAb P3/27 or the polyclonal swine serum as primary antibodies and horseradish peroxidase-labelled goat anti-mouse or rabbit anti-swine (Dako) as secondary antibodies, respectively. No difference in the percentage of infected cells was observed for the two different PRRSV-recognizing antibodies. Cells without ligand treatment are represented as control. For these untreated cells the average percentage of infected cells was calculated from six replicates. This average percentage was used as reference value in the calculation of the relative percentages of infection.

Infection experiments on non-target cells expressing recombinant receptors. For different infection experiments, a similar protocol was used as will be described here. Twenty-four hours post-transfection, non-target cells were washed once with RPMI 1640 followed by inoculation with PRRSV-containing supernatant that was cleared from cell debris via centrifugation. Inoculated cells were incubated for 1 h at 37 °C in the presence of the virus. After virus removal, non-target cells were washed five times with RPMI 1640 before incubation in medium. The final wash solution was collected and titrated to determine the amount of background virus still present after removal of the inoculum. At different time points after inoculation, cells were fixed with ice-cold methanol or paraformaldehyde, and intra- and extracellular virus were collected as described in virus titration.

RESULTS

Analysis of sialoadhesin and CD163 expression in primary macrophages, the *in vivo* target cells of PRRSV

Both sialoadhesin and CD163 expression are restricted to the monocyte–macrophage lineage (Duan *et al.*, 1998b; Sanchez *et al.*, 1999). To allow further investigation of their role in PRRSV infection, the expression pattern of both proteins was analysed in a population of macrophages via flow cytometry and confocal microscopy. The flow cytometric data (Fig. 1a) showed that 95 % of the macrophages were positive for both proteins on the cell surface, showing that alveolar macrophages represent a homogeneous population with respect to the expression of both sialoadhesin and CD163. Immunofluorescence staining followed by confocal analysis (Fig. 1b) revealed an almost exclusive expression of sialoadhesin on the cell membrane, while CD163 was present both on the cell surface and in what appeared intracellular, endosomal vesicles. Double-immunofluorescent staining with the early endosomal marker EEA1 confirmed the localization of intracellular CD163 to early endosomes (data not shown).

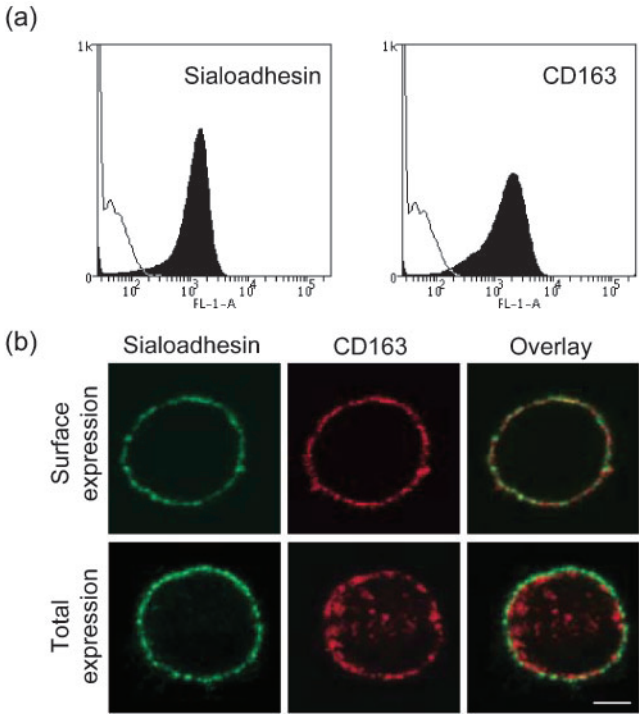

Fig. 1. Expression of sialoadhesin and CD163 in primary alveolar macrophages. (a) Flow cytometric analysis of macrophages stained with mAb 41D3 for porcine sialoadhesin (black curve) or mAb 2A10 for porcine CD163 (black curve). In both experiments, the isotype-matched antibody 13D12 (white curve) was used as control. (b) Representative confocal microscopical images of non-permeabilized (surface expression) and permeabilized (total expression) macrophages stained with mAb 41D3 for sialoadhesin (green) and the pAb for CD163 (red). Images represent one confocal *z*-section through the middle of the cell. Bar, 5 μm.

Effect of sialoadhesin- and CD163-specific antibodies on PRRSV infection of macrophages

To examine the potential role of CD163 in PRRSV infection of primary macrophages, macrophages were incubated with different concentrations of sialoadhesin- and CD163-specific ligands at 37 °C to evaluate their effect on PRRSV infection. Relative percentages of infected macrophages are given in Fig. 2(a). mAb 41D3, directed against porcine sialoadhesin, strongly reduced PRRSV infection in a dose-dependent manner, as was described previously (Duan *et al.*, 1998a). Interestingly, the CD163-specific pAb also reduced PRRSV infection up to 75 % in a dose-dependent manner. In contrast, no inhibition was observed with the porcine CD163-specific mAb 2A10 or with the HbHp complex, a well-characterized biological ligand of CD163 (data not shown). When the sialoadhesin-specific mAb 41D3 and the CD163-specific pAb were combined, PRRSV infection was completely blocked at the highest concentrations used. Negative-control antibodies had no effect on PRRSV infection (data not shown). These results show that both CD163 and sialoadhesin are involved in PRRSV infection of macrophages.

PRRSV displays remarkable genetic, antigenic and clinical variability, resulting in distinct groups of strains within the same viral family (Goldberg *et al.*, 2003; Nelson *et al.*, 1993). Therefore, different strains were tested for their infectivity on macrophages in the presence of sialoadhesin- and CD163-specific antibodies at 37 °C as described above. Infection with either the European prototype PRRSV strain LV, the American prototype strain VR-2332 or the Belgian strain 94V360 was significantly reduced in the presence of 41D3 or the CD163-specific pAb and infection was almost completely blocked when both antibodies were combined (Fig. 2b–c). Furthermore, both Marc-145- and macrophage-grown viruses use sialoadhesin and CD163 as receptors. In conclusion, different PRRSV strains use both sialoadhesin and CD163 to infect macrophages, independent of the cells in which the virus was produced.

To investigate whether CD163 is involved in PRRSV attachment, as is already known for sialoadhesin, macrophages were incubated with sialoadhesin- and CD163-specific ligands as described above; however, treatments were performed at 4 instead of 37 °C as described previously (Delputte *et al.*, 2005). mAb 41D3 reduced PRRSV infection, contrasting with the pAb directed against CD163, which did not reduce PRRSV infection when administered at 4 °C (data not shown). These data confirm the role of sialoadhesin as PRRSV attachment receptor and suggest that CD163 is not involved in PRRSV attachment.

PRRSV non-target cells expressing both sialoadhesin and CD163 support productive PRRSV infection more efficiently compared with cells expressing CD163 alone

To allow discrimination between the respective roles of sialoadhesin and CD163 during PRRSV infection, non-permissive CHO-K1, BHK-21 and PK-15 cells transiently expressing recombinant sialoadhesin and CD163, either separately or combined, were inoculated with the European or the American prototype PRRSV strain and analysed for expression of viral nucleocapsid protein and production of infectious virus (Fig. 3).

Immunofluorescence experiments showed for all three cell lines that cells expressing only sialoadhesin internalized virus particles, but this did not result in productive infection, as was described previously (Vanderheijden *et al.*, 2003). Cells expressing only CD163 were infected as described by Calvert *et al.* (2007), although for CHO-K1 cells only very few infected cells were observed. In contrast, when sialoadhesin and CD163 were combined, clearly more infected cells were observed, 20–200 times more depending on the cell line (data not shown).

Transfection efficiency differed between cell lines, with 5, 10 and 30 % transfected cells for CHO-K1, BHK-21 and PK-15 cells, respectively. Titration of extracellular virus

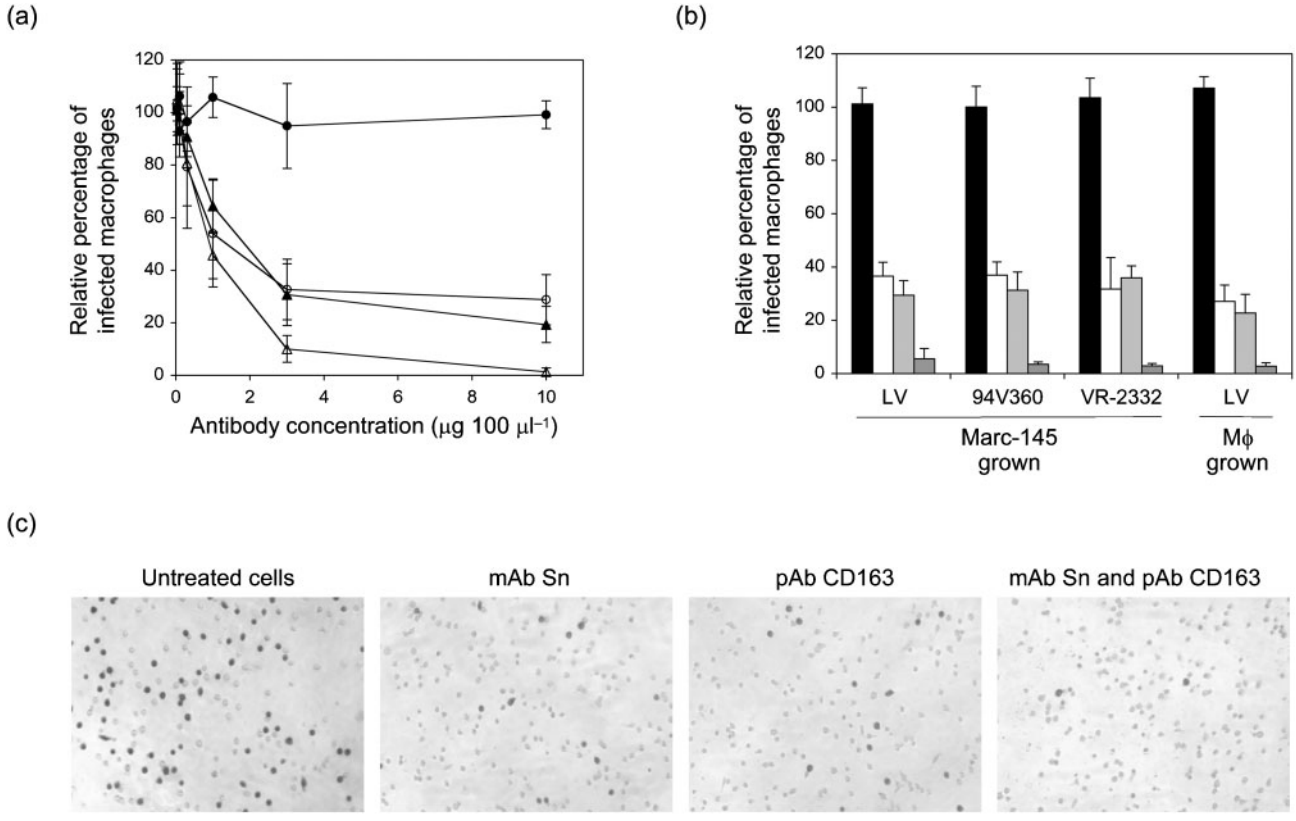

Fig. 2. Effect of sialoadhesin- and CD163-specific antibodies on PRRSV infection of macrophages. (a) Macrophages were treated with different concentrations of sialoadhesin- (▲) or CD163- (○) specific antibodies or a combination of both (△). After inoculation with LV, the relative percentage of infected macrophages was calculated, with untreated cells (●) as reference. Each value represents the mean ± SD of three experiments. (b) Macrophages were treated with 3.3 μg 100 μl^{-1} of sialoadhesin- (white bars) or CD163- (grey bars) specific antibodies or a combination of both (dark grey bars). After inoculation with different PRRSV strains, the relative percentage of infected macrophages was calculated, with untreated cells (black bars) as reference. Each value represents the mean ± SD of three experiments. (c) Representative light microscopic images of macrophages infected with LV and stained with PRRSV nucleocapsid protein-specific mAb for data represented in (b).

showed no production of infectious virus in cells expressing only sialoadhesin. When only CD163 was expressed, all three cell lines produced infectious virus. Interestingly, when both sialoadhesin and CD163 were present, virus production was 10–100 times higher compared with cells expressing only CD163, especially for PK-15 and CHO-K1 cells. Comparison between the European and the American prototype PRRSV strains showed higher virus titres for the VR-2332 strain in PK-15 and CHO-K1 cells but not in BHK-21 cells.

Kinetics of PRRSV infection in PK-15 cells expressing both sialoadhesin and CD163

Because the combination of sialoadhesin and CD163 efficiently supports PRRSV infection in non-permissive PK-15 cells, the kinetics of PRRSV infection in these cells was analysed. Therefore, PK-15 cells expressing recombinant sialoadhesin and CD163 were inoculated with LV (m.o.i. of 0.1 or 1) and at different time points after inoculation, intra- and extracellular virus were titrated (Fig. 4).

The amount of internalized virus remained constant or declined for the first 6 h post-inoculation (p.i.), while from 6 h p.i. onwards the amount of intracellular virus increased to reach a maximum around 24 h p.i. Starting from 12 h p.i., an increase in the extracellular virus titre was detected, reaching a maximum level around 48 h p.i., showing that the produced virus is infectious for PK-15 cells expressing sialoadhesin and CD163.

PRRSV infection of non-permissive PK-15 cells expressing recombinant sialoadhesin, CD163 or a combination of both

To investigate further the role of sialoadhesin and CD163 during PRRSV infection, confocal microscopical analysis was performed on transfected PK-15 cells at different time points p.i. (Fig. 5). Sialoadhesin-expressing cells clearly

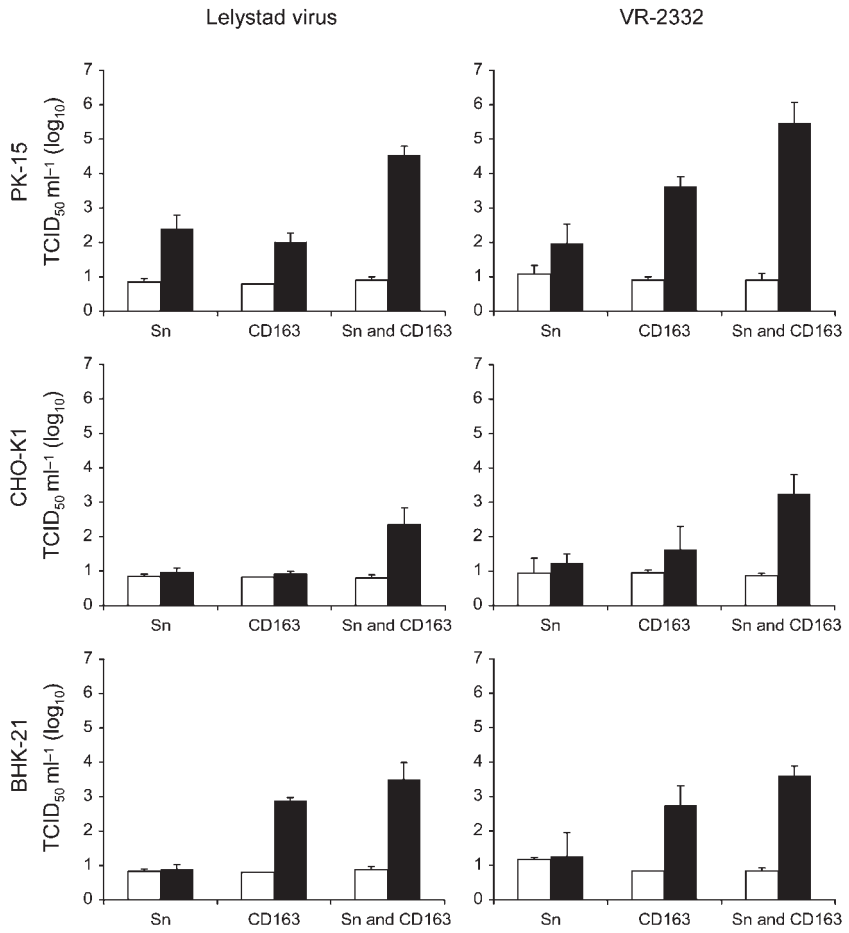

Fig. 3. Virus production in non-target cells expressing either recombinant sialoadhesin (Sn), recombinant CD163 or a combination of both, after inoculation with the European prototype PRRSV strain Lelystad or the American prototype PRRSV strain VR-2332. Transfected PK-15, CHO-K1 and BHK-21 cells were inoculated with either LV or VR-2332. At 1 h (white bars) and 24 h (black bars) p.i., representing the residual virus after removal of the inoculum and newly produced virus, respectively, supernatant was collected and infectious extracellular virus was determined via titration [detection limit 0.8 $TCID_{50}$ ml^{-1} (log_{10})]. Values represent mean ± SD of three experiments.

internalized PRRSV particles. However, virus uncoating and productive infection were not detected as described previously (Vanderheijden *et al.*, 2003). In CD163-expressing cells, surface bound or internalized virus particles were not detected. However, infected cells producing infectious virus particles were observed. PK-15 cells expressing both sialoadhesin and CD163 internalized virus particles similar to PK-15 cells expressing only sialoadhesin. Interestingly, at 6 h p.i., the PRRSV-specific immunofluorescence signal almost completely disappeared, suggesting that PRRS virions were uncoated. An increasing number of infected cells was detected from 12 h p.i. onwards. In addition, the number of infected cells was higher in cells expressing both sialoadhesin and CD163 compared with cells expressing only CD163. These observations confirm the role of sialoadhesin as an internalization receptor and suggest that CD163 functions during PRRSV uncoating, i.e. virus disassembly and release of the viral genome.

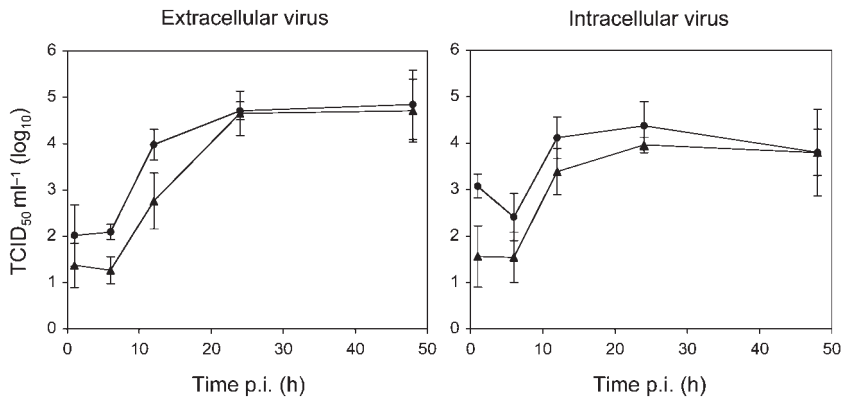

Fig. 4. Kinetics of PRRSV infection in PK-15 cells expressing sialoadhesin and CD163. PK-15 cells expressing recombinant sialoadhesin and CD163 were inoculated with the European prototype PRRSV strain Lelystad at an m.o.i. of 0.1 (▲) or 1 (●). At 1, 6, 12, 24 and 48 h p.i., extra- and intracellular virus were collected for titration [detection limit 0.8 $TCID_{50}$ ml^{-1} (log_{10})]. Values represent mean ± SD of three experiments.

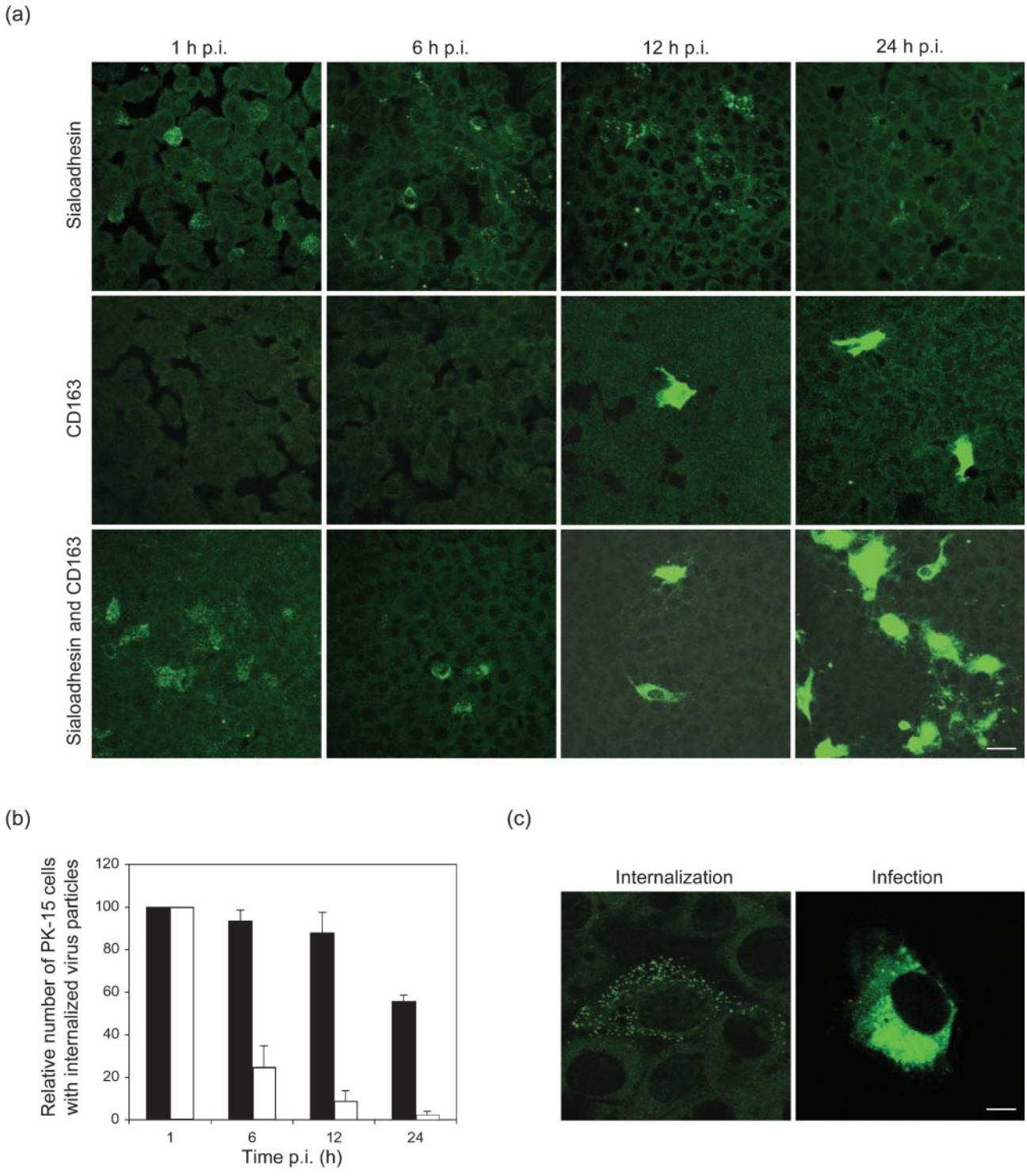

Fig. 5. Confocal microscopical analysis of PRRSV during infection of PK-15 cells expressing recombinant PRRSV receptors sialoadhesin and/or CD163. PK-15 cells expressing sialoadhesin and/or CD163 were inoculated with PRRSV and fixed at different time points after inoculation as indicated above the pictures (h p.i.). PRRSV was visualized via mAb P3/27 recognizing the viral nucleocapsid protein. Images represent an overlay of several *z*-sections throughout whole cells. Bar, 15 μm. (b) The relative number of PK-15 cells with internalized virus particles was calculated with 1 h p.i. as reference point. Data are shown for PK-15 cells expressing sialoadhesin (black bars) and the combination of sialoadhesin and CD163 (white bars), but not for CD163-expressing PK-15 cells since no internalized virus particles were observed. (c) Representative images of a PK-15 cell, expressing sialoadhesin and CD163, with either internalized PRRSV particles or production of viral nucleocapsid protein as indicator of virus replication. Images represent one confocal *z*-section through the middle of the cell. Bar, 5 μm.

DISCUSSION

Enveloped viruses enter into cells via a series of steps involving interactions between viral surface proteins and cell surface molecules followed by activation of cellular pathways that lead either to fusion at the plasma membrane or internalization of the virus particles and release of the viral genome in the cytoplasm. With increased understanding of virus entry, it became apparent that most viruses use multiple attachment factors, receptors and entry mediators in parallel or in succession (Marsh & Helenius, 2006; Smith & Helenius, 2004). So far, one attachment factor and one receptor have been identified for PRRSV on its *in vivo* target cell, the macrophage. As for many other viruses, heparan sulphate is described as a PRRSV attachment factor (Delputte *et al.*, 2002). Unlike attachment factors, virus receptors actively promote entry, with sialoadhesin being established as PRRSV receptor that allows both PRRSV binding and internalization (Delputte *et al.*, 2005; Vanderheijden *et al.*, 2003). The interaction between PRRSV and sialoadhesin was shown to occur via sialic acids present on the virion and the N-terminal sialic acid-binding immunoglobulin domain of sialoadhesin (Delputte & Nauwynck, 2004; Delputte *et al.*, 2007b).

Besides heparan sulphate and sialoadhesin, other factors are involved in PRRSV infection since non-target cells expressing only heparan sulphate and recombinant sialoadhesin internalize the virus but no virus uncoating and consequently no productive infection is observed (Vanderheijden *et al.*, 2003). Recently, CD163 was reported to be involved in PRRSV infection of Marc-145 cells and to allow PRRSV infection of some continuous cell lines upon expression of a recombinant form (Calvert *et al.*, 2007). Furthermore, susceptibility of macrophages to PRRSV infection was previously shown to be associated with high expression of CD163 (López-Fuertes *et al.*, 2000).

In the present study, it was shown that both sialoadhesin and CD163 are involved in infection of macrophages with different PRRSV strains based on two findings. (i) In primary macrophages, both sialoadhesin- and CD163-specific antibodies strongly reduce infection and a combination of both completely blocks infectivity. (ii) Cells expressing both sialoadhesin and CD163 are highly susceptible to PRRSV infection, this compared with cells expressing only sialoadhesin or CD163, which are, respectively, not susceptible and partially susceptible depending on the cell line used.

Sialoadhesin was previously shown to mediate both PRSSV attachment and internalization in macrophages (Delputte *et al.*, 2005). In contrast, although CD163 is clearly essential during PRRSV entry, its precise functioning is not known so far. Incubation of macrophages with CD163-specific antibodies at 37 but not at 4 °C reduced PRRSV infection, showing that CD163 is involved in PRRSV infection of macrophages; however, probably not as attachment receptor. This is further sustained by the analysis of PRRSV attachment to and internalization in cells expressing recombinant CD163 revealing no evidence for PRRSV binding and internalization in these cells. This hypothesis is in agreement with previous results showing that PRRSV attachment to macrophages is completely blocked by interfering with PRRSV interaction with heparan sulphate and sialoadhesin, thus showing that only heparan sulphate and sialoadhesin are involved in PRRSV attachment to macrophages (Delputte *et al.*, 2005).

The observation that cells expressing recombinant sialoadhesin internalize PRRSV without uncoating the virus, contrasts with the observation that cells expressing both recombinant sialoadhesin and CD163 show virus uncoating upon internalization. This then results in productive infection thereby resembling the PRRSV entry pathway as observed in macrophages (data not shown) and Marc-145 cells (Vanderheijden *et al.*, 2003). These data suggest that CD163, rather than being a PRRSV receptor, functions as an entry mediator involved in PRRSV uncoating, i.e. virus disassembly and release of the viral genome. So far, no such role has been described for CD163 and a full understanding of the precise molecular events during PRRSV uncoating needs further research.

In this study, non-target cells expressing recombinant sialoadhesin and CD163 are shown to produce infectious extracellular PRRSV with virus titres ranging between 2.4 and 5.5 $TCID_{50}$ ml^{-1} (log_{10}). It should be noted that during infection experiments in non-target cells, only a limited number of cells expressed the recombinant proteins since transiently transfected cells were used. Therefore, higher virus titres can be expected when a cell culture stably expressing both sialoadhesin and CD163 is used. Interestingly, all three non-permissive cell lines responded similarly to PRRSV infection upon expression of sialoadhesin and CD163, either separately or combined. No infection was observed for sialoadhesin-expressing cells, contrasting with CD163-expressing cells that could be infected, and finally highest virus titres were produced for all three cell lines in cells expressing both sialoadhesin and CD163. However, virus titres differed remarkably between the three cell lines. This correlates with differences in transfection efficiency, but may also be the result of cell line-specific characteristics. Such cell line-specific characteristics are also observed in the differential requirement of recombinant sialoadhesin for efficient virus production. For BHK-21 cells, expression of sialoadhesin in addition to CD163 results only in a 0.8 $TCID_{50}$ ml^{-1} (log_{10}) increase in virus titre, whereas in CHO-K1 and PK-15 cells, addition of sialoadhesin to CD163 results in a virus titre increase of 1.6 and 2.2 $TCID_{50}$ ml^{-1} (log_{10}), respectively. These data show that, although the size of the increase in virus titre is dependent upon the cell line used, cells expressing sialoadhesin and CD163 always produce more virus compared with cells expressing only CD163, suggesting that sialoadhesin and CD163 work together efficiently during PRRSV entry. Such a co-operation between sialoadhesin and CD163 is also suggested for infection of

the primary target cells because of the additive effect of sialoadhesin- and CD163-specific antibodies on infection of macrophages, as observed in this study.

More data sustaining the requirement of both sialoadhesin and CD163 during PRRSV infection can be obtained from *in vivo* observations. *In vivo*, PRRSV mainly replicates in subsets of macrophages, cells known to express sialoadhesin and CD163 (Duan *et al.*, 1997b). In contrast, cells lacking sialoadhesin but expressing CD163, like circulating blood monocytes, do not become infected *in vivo* and only at very low levels after *in vitro* cultivation (Duan *et al.*, 1997a, b; Sanchez *et al.*, 1999). However, when *in vitro* sialoadhesin is induced in these monocytes, they do become susceptible to PRRSV infection (Delputte *et al.*, 2007a). In addition to supporting the requirement of both sialoadhesin and CD163, these data also indicate that sialoadhesin is responsible for the specific *in vivo* cell tropism of PRRSV.

To date, only one other virus is described to use CD163 during infection, the African swine fever virus (ASFV) (Sanchez-Torres *et al.*, 2003). In addition to a correlation between CD163 expression and ASFV infection, it was shown that the CD163-specific mAb 2A10 inhibits binding of ASFV to macrophages, thereby interfering with virus infection. Interestingly, the same mAb 2A10 had no effect on PRRSV infection of macrophages in this study, suggesting that both viruses interact differently with CD163 during infection. This hypothesis is further sustained by the fact that no CD163 binding was observed for PRRSV contrasting with the results described for ASFV.

For PRRSV, sialoadhesin is established as binding and internalization receptor and CD163 is proposed to be mainly involved in virus uncoating. Surprisingly, some, but not all non-target cell lines expressing only CD163 can be infected in the absence of sialoadhesin; however, less efficient compared with cells expressing both sialoadhesin and CD163. Nevertheless, these results suggest that, depending on the cell line used, PRRSV is able to enter some CD163-expressing cells via a sialoadhesin-independent pathway. The efficiency of this alternative entry pathway seems to be dependent upon the cell line used, since not all cells expressing CD163 support PRRSV infection with the same efficiency, as shown in this study and by Calvert *et al.* (2007). Furthermore, also in its *in vivo* target cell the macrophage, or potentially in monocytes, it cannot be excluded that PRRSV uses, in addition to the sialoadhesin-mediated internalization, a less efficient, alternative entry pathway, because incubation of macrophages with sialoadhesin-specific antibodies at 37 °C never completely blocks PRRSV infection, even if higher antibody concentrations are used than described in this study (data not shown). Although this residual infection could be caused by recycled sialoadhesin that is cleared of the blocking mAb, clearly more research is needed to address this question. Alternatively, the virus could use another receptor or a rather non-specific process like e.g. macropinocytosis in addition to the sialoadhesin-dependent entry pathway to enter CD163-expressing non-target cells or macrophages. In macrophages, macropinosomes are described to rapidly develop classical early endosome characteristics, suggesting they may also acquire CD163 (Jones, 2007). The existence of an alternative entry pathway for PRRSV would not be an exception compared with several other viruses, like e.g. human immunodeficiency virus and influenza, which are also able to use multiple entry pathways with differing efficiencies (Daecke *et al.*, 2005; Sieczkarski & Whittaker, 2002).

In summary, we show that besides sialoadhesin, CD163 is also involved in PRRSV infection of its *in vivo* target cell the macrophage. The role for sialoadhesin as attachment and internalization receptor of PRRSV is confirmed, while for CD163 a role in PRRSV uncoating is proposed. Finally, non-target cells expressing both sialoadhesin and CD163 are clearly more susceptible to PRRSV infection and produce more virus compared with cells expressing only CD163.

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