Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage

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Porcine reproductive and respiratory syndrome virus (PRRSV) emerged in the late 1980s and rapidly became one of the most significant viral pathogens in the swine industry. In vivo, the virus shows a very narrow cell tropism and targets specific subsets of porcine macrophages. The entry of PRRSV into its host cell is the first crucial step in infection and has been the focus of many fundamental studies. This review provides a comprehensive overview of the current knowledge on PRRSV entry into the porcine macrophage, covering virus binding, internalization and genome release, and integrates these findings into a general model of the entry process.

Introduction

Porcine reproductive and respiratory syndrome (PRRS) severely affects swine populations worldwide. The aetiological agent, the PRRS virus (PRRSV), is an RNA virus that is classified within the family Arteriviridae, order Nidovirales (Cavanagh, 1997). PRRSV causes considerable production losses as infection with the virus can result in severe reproductive and respiratory disorders in pigs (Neumann et al., 2005). With the recent outbreaks of highly virulent variants of the virus in China (Li et al., 2007; Tian et al., 2007; Zhou et al., 2008), interest in PRRSV has increased greatly and the demand for safe and effective vaccines for PRRSV control is higher than ever.

The PRRSV virion consists of a nucleocapsid, composed of a positive-strand RNA genome (±15 kb) and the nucleocapsid protein (N), which is surrounded by a lipid bilayer envelope (Benfield et al., 1992; Dea et al., 1995; Mardassi et al., 1994; Meulenberg et al., 1993; Spilman et al., 2009; Wensvoort et al., 1992). The viral envelope contains six structural proteins: the small envelope protein E, the membrane protein M and the N-glycosylated glycoproteins GP2 (or GP2a), GP3, GP4 and GP5. M, N and GP5 are the major structural proteins of PRRSV, while E, GP2, GP3 and GP4 are minor virion components. The M and GP5 proteins occur as disulfide-linked heterodimers in the envelope, while the minor structural proteins E, GP2, GP3 and GP4 appear to associate via non-covalent interactions (Mardassi et al., 1995, 1996; Meulenberg et al., 1993, 1995; Meulenberg & Petersen-den Besten, 1996; van Nieuwstadt et al., 1996; Wissink et al., 2005; Wu et al., 2001). In addition, recent data suggest that interactions exist between major and minor envelope proteins (Das et al., 2010).

Since the discovery of PRRSV, many studies have been performed to gain insight into the biology of this important pathogen. This review reflects on two decades of research on the initial steps of the PRRSV replication cycle, covering virus attachment, internalization and disassembly. A general model of PRRSV entry into the porcine macrophage is proposed and delineates where further research is necessary.

PRRSV cell tropism

Like other members of the family Arteriviridae, PRRSV has a very narrow cell tropism. In vivo, the virus shows a preference for cells of the monocyte/macrophage lineage and infects specific subsets of differentiated macrophages in lungs, lymphoid tissues and placenta. Macrophage precursor cells, i.e. bone marrow cells and peripheral blood monocytes, are largely refractory and also peritoneal macrophages are not susceptible (Duan et al., 1997a, b; Teifke et al., 2001).

Several studies report that porcine dendritic cells (DCs) are susceptible to PRRSV infection. However, these results have to be interpreted with caution. As primary DCs are often hard to obtain, the majority of the studies have been performed with monocyte-derived dendritic cells (MoDCs) or bone marrow-derived dendritic cells (BmDCs), obtained...
by treating peripheral blood mononuclear cells (PBMCs) or bone marrow haematopoietic cells (BmHCs) with both granulocyte-macrophage colony stimulating factor (GM-CSF) and interleukin 4 (IL-4) (Chang et al., 2008; Flores-Mendoza et al., 2008; Park et al., 2008; Silva-Campa et al., 2009; Wang et al., 2007). These cells are clearly susceptible to PRRSV infection, but they are also different from primary DCs. Loving et al. (2007) assessed the susceptibility of both MoDCs and primary lung dendritic cells (L-DCs) to PRRSV infection and showed that L-DCs, in contrast to MoDCs, were not permissive to the virus (Loving et al., 2007). This was suggested to be due to the distinct differentiation pathways of MoDCs and L-DCs. Although MoDCs display many characteristics of primary DCs, they might still retain specific characteristics of monocytes/macrophages that leave them susceptible to PRRSV infection. Clearly, further research is needed to assess the occurrence of PRRSV infection of DCs in vivo.

For in vitro study of host cell infection, primary cultures of porcine alveolar macrophages are often used. As these are the main in vivo target cells of PRRSV, findings in these cells provide relevant information on the events occurring in the in vivo situation. In addition, the African green monkey kidney cell line MA-104 and cells derived thereof (MARC-145 and CL2621) sustain PRRSV infection (Kim et al., 1993; Mengeling et al., 1995) and are also often used to explore the PRRSV replication cycle. However, one has to keep in mind that the infection process in these cell lines differs from that in porcine macrophages and that the findings in such cells need to be verified in the porcine target cells to view them in the right perspective.

**PRRSV entry mediators**

Despite its restricted cell tropism, PRRSV is able to replicate in several non-permissive cell lines upon transfection of these cells with the viral genomic RNA. This finding indicates that the cell tropism is determined by the presence or absence of specific entry mediators in the target cell (Kreutz, 1998; Meulenberg et al., 1998). Different cellular factors with a PRRSV entry mediator activity have been identified (Fig. 1). Some of these molecules have been shown to facilitate viral infection upon binding of the virus (Calvert et al., 2007; Delputte et al., 2002; Duan et al., 1998b; Vanderheijden et al., 2001, 2003). However, for other entry mediators the mode of action and possible interaction with the PRRSV virion have not yet been elucidated (Misinzo et al., 2008).

**Heparan sulphate**

Although PRRSV was identified in the beginning of the 1990s (Collins et al., 1992; Wensvoort et al., 1991), it was not until 5 years later that the first entry mediator for the virus was identified. Jusa et al. (1997) found that heparin-like molecules on the surface of MARC-145 cells were involved in the infection of these cells. Incubation of the virus with heparin prior to inoculation of the cells greatly decreased infectivity. Treating the cells prior to inoculation with heparin and heparan sulphate, had a similar effect, indicative of a heparin-like entry mediator on the cell surface (Jusa et al., 1997). Similar experiments on porcine alveolar macrophages suggested the presence of a similar factor on these cells, although the effects of heparin- and heparanase I-treatment were more modest than on MARC-145 cells (Delputte et al., 2002; Vanderheijden et al., 2001). The effect of heparin on infectivity was also strongly dependent on the virus isolate used, arguing that antigenically different PRRSV isolates differ in their capacity to interact with the heparin-like entry mediator on their target cells (Delputte et al., 2002; Vanderheijden et al., 2001).

Additional experiments were undertaken to determine the precise nature of the entry mediator on macrophages and to further characterize its interaction with PRRSV (Delputte et al., 2002). On the mammalian cell surface, heparin-like molecules are present as part of O-glycosylated proteins called proteoglycans. These glycoproteins consist of a core protein carrying one or more covalently attached glycosaminoglycans (GAGs), which are linear polysaccharide chains composed of repeated disaccharide subunits of an amino sugar and a hexuronic acid/galactose residue (for a review on proteoglycans, see reference Prydz & Dalen, 2000). Preincubation of the virus with heparan sulphate GAGs significantly reduced infection of alveolar macrophages. In contrast, infection was not significantly reduced when chondroitin sulphate A or dermatan sulphate GAGs were used (Delputte et al., 2002). In an attempt to identify potential PRRSV ligands for heparan sulphate, a lysate of the Belgian PRRSV isolate 94V360 was incubated with heparin-coated beads (Delputte et al., 2002). The viral M/GP$_3$ complex was found to bind with heparin, suggesting that M/GP$_3$ within the viral envelope may interact with heparan sulphate molecules on the host cell surface.

Heparan sulphate GAGs are found in different animal tissues and are certainly not macrophage specific. The presence of these molecules at the cell surface can explain why many non-permissive cells, incapable of internalizing the virus and not allowing productive infection, can bind PRRSV (Delputte et al., 2005). Therefore, heparan sulphate GAGs on the macrophage surface are assumed to function as PRRSV attachment factors that concentrate virions on the cell surface, hence allowing a more efficient infection.

Although heparan sulphate molecules on the porcine macrophage can play a role in PRRSV infection of these cells, they are not strictly required. While infection of alveolar macrophages could clearly be reduced when virus was preincubated with heparin or heparan sulphate, no complete block of infection could be obtained (Delputte et al., 2002; Vanderheijden et al., 2001). Furthermore, analysis of the binding kinetics of PRRSV to macrophages in the presence of heparin showed that virus binding to the macrophage surface converted from being heparin sensitive.
to being heparin resistant (Delputte et al., 2002, 2005). These data suggest the presence of additional PRRSV attachment factors on the cell surface of the porcine macrophage.

**Sialoadhesin**

In search of other PRRSV entry mediators on macrophages, Duan et al. (1998a, b) generated a set of alveolar macrophage-specific monoclonal antibodies (mAbs). Two of these mAbs, mAb 41D3 and mAb 41D5, were found to block PRRSV infection. Both mAbs recognized a 210 kDa molecule present on the cell membrane of the macrophage and prevented attachment of the virus to the cell surface, indicating that their target antigen was a potential PRRSV attachment factor (Duan et al., 1998a, b). To identify this molecule, the protein was purified from a lysate of alveolar macrophages via immunoprecipitation using mAb 41D3 and subjected to peptide sequencing after tryptic digestion. Analysis revealed a strong homology with mouse sialoadhesin (mSn), indicating that the potential entry mediator was the porcine homologue of this molecule (Vanderheijden et al., 2003). Sialoadhesin is a macrophage-restricted type 1 transmembrane glycoprotein and is the prototype member of a family of sialic acid-binding lectins called siglecS (Munday et al., 1999). Expression of a recombinant porcine sialoadhesin (pSn) in non-permissive mammalian cell lines allowed these cells to efficiently bind and internalize PRRSV virions via clathrin-mediated endocytosis. However, nucleocapsid disassembly and

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**Fig. 1.** Schematic overview of the membrane-associated entry mediators involved in PRRSV infection of the porcine macrophage.
productive infection were not observed in these cells, indicating that other, macrophage-specific factors are required (Vanderheijden et al., 2003). Hence, pSn was postulated to be a PRRSV attachment and internalization receptor. The observation that pSn colocalizes with PRRSV on the cell surface, but also just beneath the plasma membrane, is in line with this (Van Gorp et al., 2009). The fact that deletion of the cytoplasmic tail of pSn completely abrogates PRRSV internalization (H. J. Nauwynck, unpublished data) also suggests a role of pSn in PRRSV internalization.

As preincubation of porcine alveolar macrophages with both heparin and pSn-specific mAb 41D3 results in a full block of attachment, it was concluded that heparan sulphate and pSn are the main factors involved in PRRSV attachment to these cells (Delputte et al., 2005). Analysis of the attachment kinetics of the virus to alveolar macrophages revealed that early attachment occurs predominantly through interaction with heparan sulphate molecules (heparin-sensitive binding) and is followed by a gradual increase in binding to the pSn receptor (mAb 41D3-sensitive binding) (Delputte et al., 2005).

Just like mouse and human sialoadhesin, the porcine sialoadhesin shows clear sialic acid-binding activity (Delputte & Nauwynck, 2004; Delputte et al., 2007). Different studies have focussed on the molecular basis of sialic acid binding by mSn. The sialic acid-binding site could be mapped to the N-terminal immunoglobulin-like V-set domain (Nath et al., 1995) and site-directed mutagenesis, nuclear magnetic resonance and crystallography studies have allowed to pinpoint the amino acid residues that are critical for its sialic acid-binding activity. Particularly the R116 residue within the N-terminal domain of mSn appears to be essential, as it forms a salt bridge with the carboxyl group of sialic acid (Crocker et al., 1999; May et al., 1997; Vinson et al., 1996). This is also true for pSn, as changing the R116 residue to an E residue via site-directed mutagenesis results in the loss of sialic acid-binding capacity without significantly altering the overall protein structure (Delputte et al., 2007). The sialic acid-binding activity is also essential for pSn to function as a PRRSV receptor, since cells expressing a recombinant pSn efficiently bind and internalize PRRSV virions, while cells expressing a pSn that lacks sialic acid-binding capacity do not internalize the virus (Delputte et al., 2007). In addition, a recent study showed that the virus is able to bind to a truncated pSn derivative composed of only the N-terminal domain of pSn directly coupled to the transmembrane and tail region (An et al., 2009). Together, these data show that an intact N-terminal V-set domain is both necessary and sufficient for PRRSV binding to pSn.

Not only the sialic acid-binding activity of pSn, but also sialic acids on the virion surface are crucial for PRRSV infection of alveolar macrophages. Removal of sialic acids from the virus strongly reduces the infectivity. Using broad specificity and more linkage-specific sialidases, it was found that α2-3-linked and, to a lesser extent, α2-6-linked sialic acids are essential for infection. Preincubation of the virus with sialic acid-specific lectins gave similar results (Delputte & Nauwynck, 2004). Based on these findings, it was hypothesized that sialic acids on the viral envelope proteins interact with the pSn receptor on the macrophage surface, thereby triggering internalization of the virus. This hypothesis was confirmed by a recent study on the interaction of pSn with the PRRSV envelope (glyco)proteins (Van Breedam et al., 2010). In this study, a soluble pSn receptor was constructed to allow the study of the PRRSV–pSn interaction in a cell-free context. Immunoprecipitation assays on lysates of Lelystad virus (LV), the European prototype PRRSV strain, revealed an interaction between pSn and the PRRSV M/GP₅ complex. This ligand–receptor interaction was clearly dependent on the sialic acid-binding capacity of pSn, as disruption of its sialic acid-binding site (through an R116E mutation) abrogated binding to the M/GP₅ complex. Removal of sialic acids from the virus prior to the immunoprecipitation assay also prevented M/GP₅–pSn interaction.

Upon binding of the virus to the pSn receptor, the virus is internalized via clathrin-mediated endocytosis and enters the endocytic pathway (Nauwynck et al., 1999; Vanderheijden et al., 2003). Productive infection requires release of the viral genome into the cytoplasm of the target cell. A pH drop within the virus-containing endosome is essential for this genome release (Kreutz & Ackermann, 1996; Nauwynck et al., 1999). Via colocalization studies in macrophages, it was found that PRRSV virions generally travel to early endosomes (pH 6.0–6.5), but do not continue to late endosomes (pH 5.0–6.0) and lysosomes (pH 4.6–5.0). These data suggest that the virus releases its genome when present in early endosomes, where it colocalizes with CD163 (Van Gorp et al., 2009), a crucial factor for PRRSV genome release (Van Gorp et al., 2008).

**CD163**

Scavenger receptor CD163 is a type 1 transmembrane glycoprotein mainly expressed on cells of the monocyte/macrophage lineage (Sanchez et al., 1999; Van den Heuvel et al., 1999) and is well known for its haemoglobin-scavenging function. By binding and internalizing haemoglobin–haptoglobin complexes, CD163 protects tissues from free haemoglobin-mediated oxidative damage (Kristiansen et al., 2001; Schaer et al., 2006). Calvert et al. (2007) identified this molecule by screening a cDNA expression library derived from porcine alveolar macrophages for elements capable of conferring a PRRSV-permissive phenotype to otherwise non-permissive cells. Upon expression of porcine CD163, several non-permissive cell types allow productive PRRSV infection, indicating that this molecule can function as a PRRSV entry mediator. Simian CD163 was identified as a key player in PRRSV infection of MARC-145 cells, as its mRNA was clearly present in these cells and infection could be strongly
reduced by CD163-specific antibodies (Calvert et al., 2007). A subsequent study by Van Gorp et al. (2008) pointed out that CD163 also plays an essential role in infection of porcine macrophages. Incubation of macrophages at 37 °C with CD163-specific antibodies strongly reduced PRRSV infection. However, when the cells were incubated with these antibodies at 4 °C, no inhibitory effect was observed. This suggests that CD163 is probably not involved as an attachment factor at the cell surface. Although transient expression of CD163 can render non-permissive cells susceptible to PRRSV infection, only a limited percentage of the CD163-expressing cells is productively infected upon incubation with the virus. Clear and abundant virus internalization as seen in macrophages and pSn-expressing cells is absent in these CD163-expressing cells, suggesting that the low infection efficiency is due to the lack of efficient internalization of the virus into these cells. Indeed, upon expression of both pSn and CD163, cells show efficient virus binding and internalization and many more cells are productively infected. This correlates with what is seen on macrophages and further supports the role of pSn as a PRRSV binding and internalization receptor, while pointing out a role for CD163 in viral uncoating and genome release (Van Gorp et al., 2008).

The exact mechanism of action of CD163 is not yet known. Recently however, CD163 domains crucial for PRRSV infection were identified. Different mutant forms of CD163 were evaluated for their capacity to confer a PRRSV-permissive phenotype to non-permissive cells. In one approach, mutants of CD163 were obtained by deleting specific domains of the protein. In another approach, chimaeric proteins were obtained by swapping specific domains of CD163 with the corresponding regions of human CD163-L1, a paralogue of human CD163 that does not allow PRRSV infection, or vice versa. The results of this study indicate that the four N-terminal scavenger receptor cysteine-rich (SRCR) domains of CD163 are not involved, while the fifth SRCR domain has a leading role in PRRSV infection. In addition, the presence of the sixth SRCR domain and of the two proline–serine–threonine (PST)-rich interdomains of CD163 has a positive influence on the infection efficiency. The presence of the SRCR domains 7, 8 and 9 seems important for a correct overall structure and presentation of the CD163 domains that are critical for infection. The cytoplasmic tail of CD163, which contains a Yxxφ-motif that directs the constitutive endocytosis of this molecule, is dispensable, arguing against a role for CD163 as an internalization receptor for PRRSV (Van Gorp et al., 2010). Another recent study aimed to identify viral-binding partners for CD163 (Das et al., 2010). The glycoproteins GP2, GP3, GP4 or GP5 were co-expressed with CD163 in BHK-21 cells, after which cells were lysed and co-immunoprecipitation assays were performed with a CD163-specific mAb. Apart from CD163, GP3 and GP4 were found in the bound fraction. Vice versa, CD163 was also found in the bound fraction when GP2- or GP3-specific antibodies were used for precipitation. These results suggest that GP2 and GP3 interact with CD163 and may thus have a role in genome release. Similar results were obtained when a CD163 construct lacking the C-terminal cytoplasmic domain, the transmembrane domain and the ninth SRCR domain was used, indicating that these domains are not involved in interactions with GP2 and GP4 (Das et al., 2010). The above studies provide important information concerning the role of CD163. However, more research is still necessary to unravel the exact functioning of CD163 in PRRSV infection of the porcine macrophage.

**Cellular proteases**

In addition to CD163, cellular proteases have been implicated in the uncoating process (Misinzo et al., 2008). Treatment of macrophages with serum inhibited PRRSV infection of these cells without affecting virus attachment and internalization. The strongest antiviral effect was associated with the z-globulin fraction of serum. As this serum protein fraction displays a high antiproteolytic activity, it was plausible that the antiviral effect would be due to the inhibition of specific cellular proteases. This assumption was supported by the observation that a similar antiviral effect was obtained by treating the cells with a broad-spectrum protease inhibitor cocktail. Treatment of macrophages with broadly active inhibitors of serine or aspartic proteases, but not of cysteine or metalloproteases, inhibited PRRSV infection. Experiments with more specific inhibitors implicated aspartic protease cathepsin E in PRRSV infection of macrophages. The finding that internalized PRRSV partially colocalizes with cathepsin E and that there is a positive correlation between cathepsin E expression and the susceptibility to PRRSV infection corroborates this (Misinzo et al., 2008). Expression of cathepsin E is restricted to specific cell types, where it is found in different non-lysosomal compartments such as endosomes and the ER- and Golgi-complex (Maric et al., 1994; Rawlings & Barrett, 1995; Sastradipura et al., 1998). The enzyme shows optimal activity at low pH (Athauda et al., 1991) and acidic conditions are necessary for the autocatalytic conversion of procathepsin E to the active cathepsin E (Sastradipura et al., 1998). This requirement is reminiscent of the necessity of endosomal acidification for productive PRRSV infection. Apart from the aspartic protease cathepsin E, also serine proteases have been implicated during PRRSV infection (Misinzo et al., 2008). However, their identity and exact function remain to be discovered.

**Other entry mediators**

It can be expected that additional cellular as well as viral molecules are involved in the entry process of PRRSV into the macrophage. Simian vimentin and CD151 were postulated to have a role in PRRSV infection of MARC-145 cells (Kim et al., 2006; Shannukhappa et al., 2007). Additionally, the small envelope protein E of PRRSV has been implicated in infection of MARC-145 cells where it may function as a viroporin that facilitates uncoating of the
virus and release of the viral genome into the cytoplasm (Lee & Yoo, 2006). The role of these molecules in infection of macrophages has however not been established. Further study of these factors and identification of additional molecules involved in virus entry may contribute to the understanding of this complex process.

**PRRSV entry into the porcine macrophage: a model**

Synthesis of the data above suggests a model of the early steps in PRRSV infection of the porcine macrophage (Fig. 2).

Initial contact of the virus with the macrophage occurs via heparan sulphate GAGs on the cell surface (Delputte et al., 2002; Vanderheijden et al., 2001). Subsequently, the virus engages sialoadhesin (pSn) in a more stable interaction (Delputte et al., 2005; Vanderheijden et al., 2003). Interaction of the virus with this receptor involves binding of the viral M/GP5 complex to the N-terminal part of pSn (Van Breedam et al., 2010). The sialic acid-binding domain at the N terminus of pSn and sialic acids on the virion surface are critical for this interaction (Delputte & Nauwynck, 2004; Delputte et al., 2007; Van Breedam et al., 2010). Attachment of the virus to pSn is followed by the uptake of the virus–receptor complex via a process of clathrin-mediated endocytosis (Nauwynck et al., 1999; Vanderheijden et al., 2003). Upon internalization, the viral genome is released into the cytoplasm. This last stage of the entry occurs when the virus is present in the early endosome and is critically dependent on acidification of the endosome and on scavenger receptor CD163 expression (Van Gorp et al., 2008, 2009). The role of CD163 in genome release may require interaction with the GP2 and GP4 glycoproteins (Das et al., 2010) and relies on a functional CD163 SRCR domain 5 (Van Gorp et al., 2010). Also the protease cathepsin E and an as-yet-unidentified trypsin-like serine protease have been implicated in this process (Misinzo et al., 2008). The above model describes the main entry pathway of PRRSV into the porcine macrophage. Nevertheless, it cannot be excluded that PRRSV also uses alternative entry pathways, independent of or partly overlapping the main entry pathway described here. Further research is needed to address this issue.

**Future perspectives**

In comparison with many other viruses, the entry of PRRSV in the porcine macrophage is not a black box anymore. Different studies that have focussed on virus attachment, internalization and genome release have shed light on these early steps of infection to provide the basis for a general model of PRRSV entry in porcine macrophages. However, many questions remain unanswered. The genome release for example is not that well understood. Although some cellular and viral factors have been implicated in this process (Lee & Yoo, 2006; Misinzo et al., 2008; Van Gorp et al., 2008), their precise functions and modes of action remain unknown. Further fundamental research is necessary to obtain a complete picture of the PRRSV entry process.

**Acknowledgements**

This work was supported by the Flemish Institute for the Promotion of Innovation by Science and Technology (I. W. T.-Flanders; SB 61491 and 63491), the Industrial Research Fund (IOP) of Ghent University and the European Union (Seventh Framework Programme; project no. 245141).

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