Porcine reproductive and respiratory syndrome virus-infected alveolar macrophages contain no detectable levels of viral proteins in their plasma membrane and are protected against antibody-dependent, complement-mediated cell lysis

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INTRODUCTION

Porcine reproductive and respiratory syndrome is characterized by reproductive failure in sows and is associated with the respiratory disease complex affecting pigs of all ages (Collins et al., 1992; Keffaber, 1989; Wensvoort et al., 1991). The causative agent, PRRSV, belongs to the family Arteriviridae, order Nidovirales (Cavanagh, 1997). In vivo, the virus infects a subpopulation of macrophages characterized by expression of sialoadhesin (Duan et al., 1997b; Vanderheijden et al., 2003). In vitro, PRRSV can be propagated in porcine alveolar macrophages and cells derived from African green monkey kidney cells, such as MA-104, Marc-145 and CL2126 (Benfield et al., 1992; Duan et al., 1997a; Kim et al., 1993; Wensvoort et al., 1991, 1992). So far, two PRRSV receptors have been identified on primary macrophages: heparan sulphate and sialoadhesin (Delputte et al., 2002, 2005; Duan, 1998; Vanderheijden et al., 2003). Recently, vimentin has been suggested as a putative PRRSV receptor on Marc-145 cells (Kim et al., 2006), but the role of this molecule as a PRRSV receptor on the in vivo target cell, the macrophage, has not been established.

The PRRSV virion is composed of three major proteins, glycoprotein (GP) 5, matrix protein (M) and nucleocapsid protein (N), and three minor proteins, GP2a, E and GP4 (Dea et al., 2000; Meulenberg & Petersen-Den Besten, 1996; Meulenberg et al., 1995; Wu et al., 2005). The structural nature of GP3 remains controversial, as GP3 of the PRRSV isolate Lelystad virus (LV) is incorporated into the virus particle, whereas it has been found in the medium as a secreted protein for some North American strains (Gonin et al., 1998; Mardassi et al., 1998; van Nieuwstadt et al., 1996). As demonstrated previously for Equine arteritis virus (EAV) (de Vries et al., 1995), the prototype virus of the Arteriviridae, PRRSV GP5 and M occur as disulphide-linked heterodimers that are formed in the endoplasmic reticulum (ER) (Mardassi et al., 1996; Meulenberg et al., 1995; Verheije et al., 2002). It has been demonstrated for EAV and PRRSV that N, M and GP5 are essential and sufficient for particle formation, but that the minor proteins are
required for virus infectivity (Wieringa et al., 2003, 2004; Wissink et al., 2005). As the EAV and PRRSV minor envelope proteins are interdependent for their incorporation into virions, it has been suggested that they form heteromultimeric complexes prior to or during virion assembly (Wieringa et al., 2003, 2004; Wissink et al., 2005).

PRRSV is able to persist in pigs for several weeks to several months after initial infection (Duan et al., 1997b; Labarque et al., 2000; Rowland et al., 2003; Wills et al., 1997). The mechanisms by which PRRSV persists are not well known. PRRSV-infected pigs develop a humoral immune response consisting of non-neutralizing antibodies starting from 7 days post-infection, with neutralizing antibodies appearing at 25–35 days post-infection (Delputte et al., 2004; Labarque et al., 2000; Loemba et al., 1996; Yoon et al., 1995). Several reports indicate that virus-neutralizing antibodies are correlated with virus elimination from infected pigs and protection against PRRSV replication and virus-induced disease (Labarque et al., 2000; Osorio et al., 2002). Virus-specific antibodies can help to resolve infection, not only by direct virus neutralization, but also by antibody-mediated cytotoxicity. For many viruses, newly synthesized viral glycoproteins are incorporated in the plasma membrane, rendering infected cells visible to the host humoral immune system. Virus-specific antibodies can then bind to the cell surface, which can result in antibody-dependent, complement-mediated cell lysis (ADCML) or cell lysis by activation of natural killer cells, neutrophils, monocytes or macrophages (antibody-dependent, cell-mediated cytotoxicity) (reviewed by Burton, 2002). The absence of viral proteins in the plasma membrane can make virus-infected cells invisible to the humoral immune system (Favoreel et al., 2003) and can be caused by intracellular retention or internalization from the cell surface. Retention of viral proteins within the cell has been described for coronavirus (Lim & Liu, 2001; Locker et al., 1994; Machamer et al., 1993), adenoviruses (Nilsson et al., 1989; Pääbo et al., 1987), flaviviruses (Cocquerel et al., 1993), togaviruses (Hobman et al., 1997) and bunyaviruses (Andersson et al., 1997). Clearance of viral glycoproteins from the plasma membrane by internalization has been reported for retroviruses and herpesviruses (Alconada et al., 1996; Bu et al., 2004; Favoreel et al., 1999, 2002; Ficinska et al., 2005; Fultz et al., 2001; Heineman & Hall, 2001; Marsh & Pelchen-Matthews, 2000; Olson & Grose, 1997; Van de Walle et al., 2003). In the case of Pseudorabies virus (PRV), this results in protection of virus-infected cells against ADCML (Van de Walle et al., 2003). In this study, we investigated whether PRRSV proteins were incorporated into the plasma membrane of primary macrophages infected in vitro and in vivo and whether infected cells could be lysed by the action of antibody and complement.

**METHODS**

**Cells and viruses.** Alveolar macrophages were obtained and cultivated as described by Wensvoort et al. (1991). Marc-145 cells were maintained as described by Kim et al. (1993). LV, the prototype European PRRSV isolate (Wensvoort et al., 1991), was cultivated in porcine alveolar macrophages (13th passage) or in Marc-145 cells (4th passage). For some experiments, PRRSV was semipurified by ultracentrifugation at 100 000 g for 3 h through a 30% sucrose cushion using an SW41Ti rotor (Beckman Coulter). The Kaplan strain of PRV was cultivated in swine testicle cells (second passage) (Kaplan & Vatter, 1959). To investigate the presence of viral proteins in the plasma membrane of in vivo PRRSV-infected macrophages, two 3-week-old PRRSV-negative pigs were inoculated with 10^6 TCID_{50} LV in 3 ml PBS (1.5 ml in each nostril). The pigs were euthanized at 5 and 6 days post-inoculation (p.i.) and alveolar macrophages were obtained by bronchoalveolar lavage.

**Antibodies**

**Polyclonal antibodies.** Polyclonal antibodies (pAbs) A (immunoperoxidase monolayer assay (IPMA) antibody titre, 10240; serum neutralization assay (SN) antibody titre, 4) were derived from a pig that was subsequently inoculated at an m.o.i. of 1 or 10. The m.o.i. was optimized the conditions to obtain a high number of infected cells during the first infection cycle. Macrophages were cultivated for 24 plasma membrane of PRRSV-infected cells was analysed, we first showed neutralizing activity (SN antibody titre, 24). Sera were heat-inactivated before use.

**mAbs.** The mAbs used were: 126.02 (Meulenberg & Petersen-Den Besten, 1996), 122.29 (Meulenberg et al., 2001), 126.3 (Meulenberg et al., 1995) and p3/27 (Wiczkorek-Krohmner et al., 1996), which recognized LV GP3, GP4, GP5, M and N respectively. PRRSV-specific serum (used in the ADCML assay) was derived from a PRRSV-negative pig. PRRSV-specific serum (used in the ADCML assay) was derived from a PRRSV-negative pig. The PRV-specific pAbs used in this study have been described by Favoreel et al. (1997). All pAbs were purified by using protein A-Sepharose (Amersham Biosciences) and, for some experiments, they were biotinylated by using a protein biotinylation module (Amersham Biosciences). As established by Western immunoblotting, pAbs A and B recognized LV GP3, GP4, GP5, M and N, respectively.

**Effect of m.o.i. and macrophage cultivation on susceptibility to PRRSV infection.** Before the presence of viral proteins in the plasma membrane of PRRSV-infected cells was analysed, we first optimized the conditions to obtain a high number of infected cells during the first infection cycle. Macrophages were cultivated for 24 or 66 h before inoculation at an m.o.i. of 1 or 10. The m.o.i. was calculated as the ratio between the TCID_{50} value and the number of cells. TCID_{50} values were obtained by performing titrations on macrophages that had been infected for 24 h. The batch of macrophages used for the titration was not the same as the batch used in the experiments. At 12 h p.i., macrophages were fixed with 3% paraformaldehyde (Vel Chemicals), permeabilized with 0.1% saponin (Sigma) and stained with biotinylated PRRSV-specific pAb A and fluorescein isothiocyanate (FITC)-labelled streptavidin (Molecular Probes). PRRSV-infected cells were counted by using fluorescence microscopy.

**Confocal-microscopic analysis of plasma membrane incorporation of viral proteins in PRRSV-infected macrophages and Marc-145 cells.** Before investigating the presence of viral proteins on the cell surface, the presence of intracellular GP3, GP4,
Before flow-cytometric analysis, macrophages were incubated for 1, 3, 6, 9 and 12 h p.i., the cells were washed and kept on ice during the staining. Surface staining was performed either with mAb against GP3, GP4, GP5 or M and FITC-labelled goat anti-mouse IgG or with biotinylated PRRSV-specific pAb (A or B) and FITC-labelled streptavidin. Dead cells were excluded by staining with 0.05 mg ethidium monoazide bromide ml⁻¹ (EMA; Molecular Probes) before fixation with 3% paraformaldehyde. PRRSV-infected cells were identified after permeabilization with 0.1% saponin by staining either with biotinylated PRRSV-specific pAb and Alexa Fluor 350-labelled streptavidin. Background FITC fluorescence was detected by using the FL2 channel and propidium iodide fluorescence was detected by using the FL1 channel. Finally, cells were mounted in glycerin/1,4-diazabicyclo[2.2.2]octane (DABCO) and analysed with a Bio-Rad MRC 1024 confocal laser-scanning system connected to a Nikon Eclipse TE300 inverted microscope.

To investigate whether viral proteins were present in the plasma membrane of in vivo-infected macrophages by confocal microscopy, macrophages were obtained by bronchoalveolar lavage from a PRRSV-infected pig at 5 days p.i. and fixed and stained as described for macrophages infected in vitro.

Flow-cytometric analysis of plasma membrane incorporation of viral proteins in PRRSV-infected macrophages. Macrophages were inoculated with PRRSV (m.o.i. = 10) after 66 h cultivation. At 1, 3, 6, 9 and 12 h p.i., the cells were washed and incubated for 1 h at 4°C with biotinylated PRRSV-specific pAb A, followed by FITC-labelled streptavidin. Background FITC fluorescence was determined by surface staining of PRRSV-infected macrophages with PRRSV-negative biotinylated pAb and FITC-labelled streptavidin. Before flow-cytometric analysis, macrophages were incubated for 5 min with 0.02 mg propidium iodide ml⁻¹ (Molecular Probes) to label and exclude dead cells from the analysis. Analysis was performed with a Becton Dickinson FACScalibur equipped with an argon ion laser. Cells were counted at a speed of 100–200 cells s⁻¹ and a minimum of 10,000 cells were analysed for each sample. FITC fluorescence was detected by using the FL1 channel and propidium iodide fluorescence was detected by using the FL2 channel.

ADCM assay. Macrophages were inoculated with PRRSV (m.o.i. = 10) after 66 h cultivation in suspension. At 9 h p.i., the cells were incubated for 1 h at 4°C with various concentrations of pAb (purified PRRSV-negative pAb, purified PRRSV-specific pAb A, purified PRRSV-specific pAb B or 25% inactivated serum derived from a PRRSV-infected pig at 0, 7, 10, 14, 21 and 141 days p.i.). Macrophages were washed twice and incubated for 1 h at 37°C with various concentrations of porcine complement (non-inactivated serum of a PRRSV-negative pig). After incubation with complement, macrophages were stained with EMA to label dead cells, fixed with 3% paraformaldehyde, permeabilized with 0.1% saponin and stained with N-specific mAb P3/27 and FITC-labelled goat anti-mouse IgG to identify PRRSV-infected macrophages. Dead, infected cells were counted by using fluorescence microscopy. To confirm the activity of the porcine complement, an ADCML assay was performed on PRRSV-infected macrophages, as described previously (Van de Walle et al., 2003). Therefore, alveolar macrophages were inoculated with PRV Kaplan (m.o.i. = 10). At 12 h p.i., the cells were incubated with 50 μg genistein ml⁻¹ (Sigma) for 45 min at 37°C. Genistein inhibits tyrosine kinase activity and, as a consequence, also inhibits antibody-induced internalization of viral proteins from the cell surface (Van de Walle et al., 2003). Cells were incubated with 1 mg purified PRV-specific pAb ml⁻¹, washed twice and incubated with 5% porcine complement. Thereafter, cells were stained with EMA to label dead cells, fixed with 3% paraformaldehyde, permeabilized with 0.1% saponin and stained with FITC-labelled PRV-specific pAb.

To investigate the effect of antibody and complement on in vivo-infected macrophages, the ADCML assay was performed on fresh macrophages obtained by bronchoalveolar lavage of PRRSV-infected pigs at 5 and 6 days p.i. Statistical analyses of data were based on the non-parametric Kruskal–Wallis test (rejection level 0.05), using SPSS version 12.0.

Internalization of viral proteins from the surface of PRRSV-infected macrophages. After 66 h cultivation, macrophages were inoculated with PRRSV at a m.o.i. of 10. The viral inoculum was removed by washing at 1 h p.i. At 9 h p.i., macrophages were incubated for 1 h at 37°C in the presence of 100 μg biotinylated PRRSV-specific pAb A or B ml⁻¹, fixed with 3% paraformaldehyde and permeabilized with 0.1% saponin. Antibodies were visualized by using FITC-labelled streptavidin. PRRSV-infected cells were identified with mAb P3/27 and Texas red-labelled goat anti-mouse IgG (Molecular Probes). To assess the specificity of this internalization assay, mock-inoculated macrophages were used or PRRSV-inoculated macrophages were incubated with biotinylated pAb derived from a PRRSV-negative pig. To confirm that internalized antibodies could be visualized by using the technique described, PRV Kaplan-inoculated macrophages (9 h p.i.) were incubated with 100 μg biotinylated PRV-specific pAb ml⁻¹ and visualized by using FITC-labelled streptavidin.

RESULTS

Effect of macrophage cultivation on susceptibility to PRRSV infection

In this study, as many infected macrophages as possible were needed in a first replication cycle to facilitate flow-cytometric analysis of PRRSV-infected macrophages. Duan et al. (1997a) showed that the susceptibility of macrophages to PRRSV infection increases by cultivating the cells for 24 h. Therefore, macrophages were cultivated for 24 h, incubated with PRRSV at increasing m.o.i.s and fixed at 12 h p.i. The percentages of infected macrophages were 11.9, 13.3, 14.1, 26.4, 44.6, 46.2 and 45.9% at m.o.i.s of 1, 3, 5, 12.5, 25, 50 and 100, respectively. The maximum level of infection was reached at m.o.i.s of 25, 50 and 100, suggesting that the susceptibility of macrophages was restricted at 24 h p.i. We also investigated whether cultivation of macrophages for 66 h instead of 24 h before infection increased the number of infected cells (Fig. 1). Using an m.o.i. of 1, a 24 h cultivation time resulted in 0.9±0.3, 2.6±1.0, 11.8±1.4 and 40.9±6.6% infected cells at 6, 9, 12 and 24 h p.i., whereas 66 h of cultivation resulted in 3.6±1.2, 8.6±0.6, 30.8±4.1 and 95.1±1.1% infected cells, indicating that ageing makes macrophages more susceptible to PRRSV. Using an m.o.i. of 10, after 66 h macrophage cultivation, >90% infection was obtained at 12 h p.i. These conditions were used throughout the study, except where indicated otherwise.
Viral proteins cannot be detected on the surface of macrophages infected with PRRSV in vitro and in vivo by using confocal microscopy

As the absence of viral proteins in the plasma membrane of infected cells can protect cells against antibody-mediated immune recognition, confocal microscopy was used to investigate whether viral proteins could be detected on the surface of PRRSV-infected macrophages. Newly synthesized GP3, GP4, GP5, M and N were detected intracellularly in infected macrophages from 6 h p.i. As no structural proteins were observed before 6 h p.i., incorporation of viral proteins into the plasma membrane was only examined starting from 6 h p.i. To investigate whether PRRSV-infected macrophages incorporated viral proteins into their plasma membrane, infected macrophages were stained at 6, 9 and 12 h p.i. as follows: (i) dead cells were stained with EMA to allow exclusion of these cells from the analysis; (ii) viral proteins on the surface of infected cells were stained with mAb against GP3, GP4, GP5 or M, or with PRRSV-specific pAb before permeabilization; (iii) infected cells were identified after permeabilization by using PRRSV-specific pAb or N-specific mAb. At each time point, viral proteins were not detected on the surface of living PRRSV-infected macrophages. Fig. 2 shows representative images of surface-stained PRRSV-infected macrophages at 9 h p.i. In dead, permeable cells, identified by positive EMA staining, surface staining with PRRSV-specific pAb resulted in cytoplasmic fluorescence.

To determine whether incorporation of PRRSV proteins into the plasma membrane was dependent on cell type, Marc-145 cells were inoculated, fixed and stained as described in Methods. Although PRRSV GP3, GP4, GP5, M and N proteins were detected intracellularly at 9 and 12 h p.i., they were not detected on the cell surface. To determine whether these in vitro findings were relevant for the in vivo situation, we fixed and stained PRRSV-infected macrophages at 9 h p.i. in vitro and of macrophages derived by bronchoalveolar lavage from a PRRSV-infected pig at 5 days p.i. Dead cells were stained with EMA (red), viral proteins on the cell surface were stained by using PRRSV-specific pAb (green) and PRRSV-infected cells were identified by using N-specific mAb (blue). Bar, 8 μm.
macrophages derived by bronchoalveolar lavage from the lungs of a PRRSV-infected pig at 5 days p.i. as described in Methods. Viral proteins were detected in the cytoplasm of infected cells, but not on the surface (Fig. 2).

**Viral proteins cannot be detected on the surface of PRRSV-infected macrophages by using flow cytometry**

PRRSV-infected macrophages were surface-stained with PRRSV-specific pAb at 1, 3, 6, 9 and 12 h p.i. and analysed by flow cytometry. Fig. 3(a) shows the median fluorescence intensities (MFI) of surface-stained, infected macrophages at the different time points. Compared with the MFI values of surface staining with PRRSV-negative pAb, a small, non-significant increase in fluorescence intensity was observed for surface staining with PRRSV-specific pAb. This shift could be observed before 6 h p.i., when newly synthesized viral proteins were not yet detected. This was probably due to virus or viral proteins that were bound but not internalized by the macrophages, as surface staining of mock-infected macrophages with PRRSV-specific pAb did not result in such a shift. Fig. 3(b) shows histograms of the results at 9 h p.i. Histograms produced from the results at 1, 3, 6 and 12 h p.i. were comparable. Permeabilization of infected macrophages before staining with PRRSV-specific pAb resulted in a strong fluorescence intensity shift for the whole population, indicating that most cells were infected.

**ADCML assay on macrophages infected with PRRSV in vitro and in vivo**

In PRRSV-infected cells, viral proteins were not detected on the cell surface by using confocal microscopy or flow cytometry, indicating that viral proteins were not incorporated into the plasma membrane of infected cells or that they were not recognized by PRRSV-specific porcine antibodies. To determine whether PRRSV-infected cells were protected against ADCML, an ADCML assay was performed at 9 h p.i. At this time, high levels of viral proteins were detected in infected macrophages, but cell death as a consequence of viral infection was still very low. PRRSV-infected macrophages were incubated with different concentrations of swine pAbs and different concentrations of porcine complement. The results are shown in Tables 1 and 2.

The concentration of purified pAb had no effect on cell lysis, as no significant increase in the number of dead, infected cells was observed when increasing concentrations of purified pAb were used in combination with a fixed concentration of complement. A small, but statistically non-significant (P > 0.05), increase in cell death was observed by using 1 mg purified PRRSV-specific pAb ml⁻¹ and 10 % complement. Incubation of cells with 25 % inactivated serum, which had no effect on cell viability, did not result in an increased number of dead, infected cells. The activity of the porcine complement used in our assay was confirmed in an ADCML assay on PRV Kaplan-infected macrophages, as described previously (Van de Walle et al., 2003). The background mortality of PRV-infected cells was 11.7 ± 4.7 %, whereas incubation of PRV-infected cells with 1 mg PRV-specific pAb ml⁻¹ and 5 % complement resulted in 83.1 ± 4.5 % cell death. This corresponded to the results of Van de Walle et al. (2003).

To investigate whether macrophages infected in vivo were sensitive towards elimination by antibody and complement, an ADCML assay was performed on macrophages derived by bronchoalveolar lavage of PRRSV-infected pigs at 5 and 6 days p.i. (Table 3). Incubation of the cells with 1 mg purified PRRSV-specific pAb A or B ml⁻¹ together with 5 or
The effect of inactivated porcine serum and complement on the lysis of PRRSV-infected macrophages is given as the percentage of lysed cells. Data are means ± SD of at least three assays. ND, Not determined.

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<th>Concentration (mg ml⁻¹)</th>
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<td>PRRSV-specific B</td>
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10% complement did not result in a significant increase in cell lysis.

Internalization of viral proteins from the cell surface is not observed in PRRSV-infected macrophages

For some viruses, spontaneous as well as antibody-induced endocytosis of viral proteins on the cell surface has been demonstrated (Favoreel et al., 2002; Ficinska et al., 2005; Fultz et al., 2001; Marsh & Pelchen-Matthews, 2000; Olson & Grose, 1997). As neither confocal microscopy nor flow cytometry revealed viral proteins on the surface of PRRSV-infected macrophages, we investigated whether there was spontaneous or antibody-induced internalization of PRRSV proteins.

PRRSV-infected macrophages were incubated with PRRSV-specific pAb (A or B) that were visualized after fixation and permeabilization (Fig. 4). If internalization of viral proteins occurred, PRRSV-specific antibody would be co-internalized and intracellular fluorescent vesicles would be visualized by using FITC-labelled streptavidin. Compared with PRRSV-infected macrophages incubated with pAb derived from a PRRSV-negative pig or with mock-infected macrophages incubated with PRRSV-specific pAb, no difference in fluorescence pattern or intensity was seen. As a positive control for this internalization assay, PRV Kaplan-infected macrophages were incubated with PRV-specific pAb, as spontaneous as well as antibody-mediated endocytosis of viral proteins from the plasma membrane of infected monocytes has been described for this virus (Favoreel et al., 1999, 2002; Ficinska et al., 2005; Van de Walle et al., 2003). In this study, the PRV-infected macrophages also internalized their viral proteins from the plasma membrane when PRV-specific antibodies were added.

DISCUSSION

Following virus infection, both cellular and humoral immune mechanisms are induced, mostly resulting in clearance of the virus. However, in PRRSV-infected pigs, virus elimination occurs ineffectively, as prolonged viraemia and persistent infections or re-infections can be observed in infected pigs (Duan et al., 1997b; Labarque et al., 2000; Murtagh et al., 2002; Rowland et al., 2003; Wills et al., 1997). The reason for this ineffective virus elimination is not fully understood. A remarkable delay in the onset of some
arms of the cellular and humoral immune response may explain the ineffective clearance of PRRSV. PRRSV-specific cell-mediated immunity can only be detected from 4 weeks post-infection, with a peak at 7 weeks post-infection (Bautista & Molitor, 1997; López Fuertes et al., 1999; Molitor et al., 1997). PRRSV-specific non-neutralizing antibodies are induced quickly starting from 7 days post-infection, but virus-neutralizing antibodies are only detected starting from 25 days post-infection, and even then only at low titres (Labarque et al., 2000; Loemba et al., 1996; Lopez & Osorio, 2004; Yoon et al., 1995). In general, antibodies can act against free virus or against virus-infected cells, mediating several antiviral activities. Binding of antibody to free virus can result in direct virus neutralization, complement-mediated virolysis and/or phagocytosis, leading to a reduction in viral load (reviewed by Burton, 2002). For PPRSV, the role of virus-neutralizing antibody in protection against PPRSV replication has been studied extensively and a clear correlation has been shown between the appearance of PPRSV-specific neutralizing antibody and a strong reduction in PPRSV or elimination of PPRSV from the body (Delputte et al., 2004; Labarque et al., 2000; Lopez & Osorio, 2004; Osorio et al., 2002). In contrast to what is known about virus-neutralizing antibodies, the effect of antibodies on PPRSV-infected cells has not been studied previously. In this study, the role of PPRSV-specific antibody in lysing PPRSV-infected cells was investigated.

Enveloped mammalian viruses use the biosynthetic transport pathway of the host cell to synthesize and process their envelope proteins. For many viruses, newly synthesized viral proteins are transported to and incorporated into the plasma membrane, rendering infected cells visible to the antibody-dependent immune response (reviewed by Burton, 2002; Favoreel et al., 2003). Until now, whether viral proteins are incorporated into the plasma membrane of

### Table 3. ADCML in PPRSV-infected macrophages derived by bronchoalveolar lavage of PPRSV-infected pigs at 5 and 6 days p.i.

The effect of purified pAb and complement on the lysis of PPRSV-infected macrophages is given as the percentage of lysed cells. Data are means ± SD of two pigs, with three repetitions per pig.

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### Fig. 4. Analysis of viral protein internalization from the cell surface of PRRSV-infected macrophages. Confocal images of PRRSV-infected macrophages incubated with PRRSV-specific pAb (green) or pAb derived from a PRRSV-negative pig (green), and PRV-infected macrophages incubated with PRV-specific pAb (green). Infected cells were identified by intracellular staining with N-specific mAb (for PRRSV) (red) or gD-specific mAb (for PRV) (red). Bar, 8 μm.
PRRSV-infected macrophages has not been investigated. In this study, surface stainings using PRRSV-specific antibodies revealed no viral proteins on the cell surface of macrophages inoculated in vitro and in vivo with PRRSV. This observation was not cell type-dependent, as viral proteins were not detected on the surface of PRRSV-infected Marc-145 cells. The absence of viral proteins from the cell surface is known to make infected cells invisible to virus-specific antibodies, protecting them against antibody-dependent lysis via complement, phagocytosis and destruction by cell-mediated cytotoxicity (Favoreel et al., 1999; Van de Walle et al., 2003; van der Meulen et al., 2003). To investigate whether PRRSV-infected cells were protected against ADCML, an ADCML assay was performed on macrophages infected with PRRSV in vitro. PRRSV-infected macrophages were incubated with purified porcine PRRSV-specific antibody and porcine complement, but only a small, non-significant increase in cell lysis was observed. As the pAbs were purified from sera of PRRSV-inoculated pigs at 141 or 165 days p.i., a total reliance on polyclonal porcine IgG as the specific detection reagent could be suggested. Therefore, ADCML assays were also performed with 25% inactivated serum (derived from an LV-infected pig at 0, 7, 10, 14, 21 and 141 days p.i.) as the source of pAb, but ADCML was not observed, indicating that macrophages infected with PRRSV in vitro are protected against ADCML. An ADCML assay was performed on macrophages infected with PRRSV in vivo and no significant increase in the number of dead cells was observed, indicating that macrophages infected with PRRSV in vivo are also protected against ADCML. The protection of PRRSV-infected macrophages against ADCML is consistent with the fact that viral proteins were not observed in the plasma membrane of macrophages infected with PRRSV in vitro and in vivo, and points towards a limited role for PRRSV-specific antibodies in eliminating PRRSV-infected cells.

When viral proteins are absent from the surface of infected cells, viral proteins can either (i) be cleared from the plasma membrane, for example by internalization (spontaneous or antibody-mediated), or (ii) be retained inside infected cells. The envelope glycoproteins of Human immunodeficiency virus and Simian immunodeficiency virus are spontaneously internalized and cleared from the plasma membrane, thereby protecting infected cells from the antibody-mediated immune response (Bu et al., 2004; Fultz et al., 2001; Marsh & Pelchen-Matthews, 2000). Spontaneous and antibody-induced endocytosis of viral glycoproteins from the plasma membrane has also been described for alphaherpesviruses, such as PRV, Human herpesvirus 3 (varicella-zoster virus) and Human herpesvirus 1 (herpes simplex virus) (Alconada et al., 1996; Favoreel et al., 1999, 2002; Ficinska et al., 2005; Heineman & Hall, 2001; Olson & Grose, 1997; Van de Walle et al., 2003), and in the case of PRV, this was shown to protect infected monocytes against ADCML (Favoreel et al., 1999, 2002; Ficinska et al., 2005; Van de Walle et al., 2003). However, when PRRSV-infected macrophages were incubated with PRRSV-specific pAb, no internalization of viral proteins from the cell surface was observed, suggesting that the viral proteins are retained within the cells. This is consistent with electron microscopy studies showing that PRRSV particles, like the other arterviruses, assemble within the host cell by budding of nucleocapsids into the lumen of the ER and/or Golgi compartments (Dea et al., 1995; Pol et al., 1997; Snijder & Meulenberg, 1998; Welland et al., 1995). For viruses that bud through intracellular membranes, it has been shown that viral proteins are retained in and accumulate at the budding site, which might be the ER and/or Golgi complex in the case of arterviruses. The suggestion of retention of PRRSV proteins in the ER for intracellular budding is also supported by several findings (Mardassi et al., 1996, 1998; Wissink et al., 2005). Wissink et al. (2005) demonstrated that PRRSV minor proteins are retained in the ER when expressed individually or with one of the other minor proteins and that transport competence through the Golgi complex was only acquired when the minor proteins were expressed together. Intracellular retention of proteins can be established by retention motifs, as described for Hepatitis C virus (Cocquerel et al., 1998; Drummer et al., 2003; Okamoto et al., 2004), Rubella virus (Hobman et al., 1997), Coronaviridae (Lim & Liu, 2001; Locker et al., 1994; Machamer et al., 1993; Vennema et al., 1992), Adenoviridae (Nilsson et al., 1989; Pääbo et al., 1987) and Bunyaviridae (Andersson et al., 1997). Sequences of LV GP2, GP3, GP4, GP5, M and N were screened for the presence of motifs known to be involved in ER or Golgi localization. One putative ER-retention motif was found in GP2: LVXXXL (aa 23–28). In contrast to the North American strain IAF-Klop, where no ER-targeting sequences were detected in GP3 (Mardassi et al., 1998), two putative ER-retention motifs were found in LV GP3: LVXXXL (aa 19–24) and HDEL (aa 87–90), the latter being a motif for ER lumen retention. In LV GP3, one putative Golgi-retention motif was also detected: CXXH (aa 144–147). So far, it is not known whether retention motifs are responsible for the localization of PRRSV proteins in the ER or Golgi, or whether retention is due to other mechanisms, such as interactions with chaperone proteins, as suggested by Mardassi et al. (1998).

In conclusion, this study indicated that viral proteins are not incorporated into the plasma membrane of PRRSV-infected macrophages, which masks the infected cells from PRRSV-specific antibodies and could explain their protection against antibody-dependent cell lysis, both in vitro and in vivo. PRRSV-specific antibodies clearly have the potential to clear free virus from the circulation, but their role in eliminating PRRSV-infected cells is limited, as shown in this study. Cell-mediated immunity may be a necessary component for the elimination of PRRSV-infected cells and its importance will be investigated in the near future.

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