Infection of porcine bone marrow-derived macrophages by porcine respiratory and reproductive syndrome virus impairs phagosomal maturation

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Porcine reproductive and respiratory syndrome virus (PRRSV), a positive-sense, ssRNA virus of the genus Arterivirus, is a devastating disease of swine worldwide. Key early targets of PRRSV infection in pigs include professional phagocytes in the lung, such as alveolar and interstitial macrophages and dendritic cells, the dysfunction of which is believed to be responsible for much of the associated mortality. In order to study the effect of virus infection on phagocyte function, the development of a robust, reproducible model would be advantageous. Given the limitations of current models, we set out to develop a porcine bone marrow-derived macrophage (PBMM\(_W\)) cell model to study phagosomal maturation and function during PRRSV infection. Derivation of PBMM\(_W\)s from marrow using cultured L929 fibroblast supernatant produced a homogeneous population of cells that exhibited macrophage-like morphology and proficiency in Fc-receptor-mediated phagocytosis and phagosomal maturation. PBMM\(_W\)s were permissive to PRRSV infection, resulting in a productive infection that peaked at 24 h. Assessment of the effect of PRRSV infection on the properties of phagosomal maturation in PBMM\(_W\)s revealed a significant decrease in phagosomal proteolysis and lowered production of reactive oxygen species, but no change in PBMM\(_W\) viability, phagocytosis or the ability of phagosomes to acidify. In this study, we present a new model to investigate PRRSV infection of phagocytes, which demonstrates a significant effect on phagosomal maturation with the associated implications on proper macrophage function. This model can also be used to study the effect on the phagosomal microenvironment of infection by other viruses targeting porcine macrophages.

INTRODUCTION

Porcine reproductive and respiratory syndrome is an economically devastating disease of swine, prevalent in most swine-producing countries (Cho & Dec, 2006). Estimates of the economic impact on the pork industry in the USA exceeded US$600 million in 2013 alone (Holtkamp et al., 2013). The causative agent is the porcine reproductive and respiratory syndrome virus (PRRSV), an enveloped positive-sense ssRNA virus of the genus Arterivirus. PRRSV targets cells of the monocyte–macrophage lineage, primarily alveolar and interstitial macrophages and dendritic cells in the lungs, and macrophages found in the spleen, lymph nodes, Peyer’s patches, placenta and endometrium (Beyer et al., 2000; Duan et al., 1997a, b; Halbur et al., 1995; Lawson et al., 1997; Thanawongnuwech et al., 2000; Van Breedam et al., 2010). The targeting of professional phagocytes, in particular alveolar macrophages, by PRRSV leads to significant additional morbidity and mortality in part due to impairment of the ability of the alveolar macrophages to clear infections by invading concurrent and secondary pathogens in the lungs (Drew, 2000; Thanawongnuwech et al., 1997).

Central to many of the functions of macrophages and other professional phagocytes is the phagosome organelle. Upon phagocytosis of particulate material, the phagosome undergoes a well-characterized series of maturation steps: fusion with the early endosomal system, lumenal acidification, fusion with the late endosomal system and lysosomes and the recruitment and activation of numerous degradative and antimicrobial components including hydrolytic...
enzymes (Fairn & Grinstein, 2012; Flannagan et al., 2012). The microbicidal activity of the phagosome is primarily mediated in three ways: (1) by lowering the pH in the nascent phagosome by acquisition of vacuolar ATPases (vATPases) through fusion with endosomes; (2) by production of reactive oxygen species (ROS) and reactive nitrogen intermediates through assembly and activation of the phagocyte NADPH oxidase complex and inducible nitric oxide synthase, respectively; and (3) by the acquisition and activation of antimicrobial peptides and hydrolytic enzymes within the phagosomal lumen through fusion with lysosomes. Phagocyte-tropic viruses often interfere with phagosomal maturation processes following infection (Mercer & Greber, 2013), although very little is known about the effect of PRRSV infection on phagosomal maturation in porcine macrophages. With the development of several quantitative fluorescence-based phagosomal maturation assays (Rohde et al., 2007; Yates et al., 2009; Yates & Russell, 2008), it is now possible to qualitatively and quantitatively assess various stages of phagosomal maturation to identify infection-related defects in phagosomal function.

Here, we assessed the ability of PRRSV infection to impair phagosomal maturation in a newly characterized bone marrow-derived macrophage infection model. We demonstrated a significant defect in production of both the superoxide respiratory burst and the proteolytic capacity of the phagosomes of PRRSV-infected macrophages.

RESULTS

L929-conditioned medium drives differentiation of macrophage-like cells from porcine bone marrow precursors

Study of PRRSV infection requires useful cellular models of which there are few. As PRRSV is tropic for cells of the monocyte–macrophage lineage, a phagocytic cell model is essential for the study of phagocyte dysregulation/dysfunction during infection. The two key models being used currently are MARC145 cells (epithelial cells isolated from the African green monkey) and porcine alveolar macrophages (PAMs). Each model has its drawbacks: MARC145 cells are neither from pig origin nor are they similar to cells infected in this animal, and PAMs require alveolar lavage of animals, a process that is time-consuming and inconsistent. PAMs are also primary cells which do not divide, and their functionality is heavily dependent on the age of the animal and the environment in which the animal lives (du Manoir et al., 2002). Lee et al. (2010) developed an easy-to-culture cell line derived from PAM that stably express CD163, and are thus susceptible to PRRSV infection. We set out to add to this model using a primary cell model that was relatively easy to harvest, culture, manipulate and characterize. Our laboratory routinely works with murine bone marrow-derived macrophages, derived using culture supernatant from the L929 fibroblast cell line that secretes murine macrophage colony-stimulating factor (M-CSF) (Englen et al., 1995). We extracted bone marrow from mid-costal ribs from adult pigs and cultured the resultant haematopoietic stem cells in medium containing L929 supernatant. To determine whether using L929 supernatant as a source of mouse M-CSF could drive the differentiation of porcine monocyctic precursors to macrophages, we microscopically observed the cells over a 10 day period (Fig. 1a). Over this period, an increasing number of cells became adherent to the non-treated plastic Petri dish, and between 6 and 10 days, the adherent cell population became more homogeneous as they began to adopt a macrophage-like morphology (Fig. 1b). These cells expressed the leukocyte marker CD45 (Fig. 1c), and had cytoplasmic and nuclear morphologies characteristic of cultured macrophages (Fig. 1d). To determine if these cells were indeed phagocytic, we incubated the adherent cells with IgG-conjugated 3 μm latex beads labelled with Alexa Fluor 488. Intracellular and extracellular beads were distinguished by incubation with the live-cell-impermeant dye Trypan blue, which quenches extracellular fluorescence. Phagocytic index was determined by calculating the ratio of intracellular versus extracellular beads. Phagocytosis of these beads was confirmed microscopically (Fig. 2a; see also Fig. 4b). Phagocytosis of IgG-conjugated and unconjugated latex experimental beads was quantified in the presence and absence of cytochalasin D (CytD), a potent inhibitor of actin polymerization and thus phagocytosis. Conjugation of IgG onto the surface of the beads enhanced phagocytic uptake (Fig. 2a), indicating that these cells express the IgG-specific receptor FcγR, a key marker of phagocytic cells. When IgG-conjugated beads containing the pH-sensitive dye carboxyfluorescein succinimidyl ester (CFSE) were incubated with cells, real-time monitoring indicated that ingested beads were taken up into a cellular compartment that acidifies over time, a process that was inhibited by the specific vATPase inhibitor concanamycin A (CMA) (Fig. 2b, c). Cells incubated with IgG-conjugated beads conjugated to the self-quenched proteolytic substrate DQ-Green BSA (DQ-BSA) showed a characteristic increase in proteolytic activity over time. This was inhibited by CMA (Fig. 2d, e). Taken together, these data suggest that porcine bone marrow stem cells can be differentiated into macrophage-like cells in the presence of L929 supernatant, and may provide a suitable model to study the functional consequences of PRRSV infection in macrophages in an in vitro setting.

Porcine bone marrow-derived macrophages (PBMMΦs) are productively infected by PRRSV

In order to determine whether these BMΦs were susceptible to PRRSV infection, BMΦs were infected at an m.o.i. of 0.01 and the supernatants harvested over time. Virus production was quantified by plaque assay on MARC145 cells (Fig. 3a). As anticipated, virus infection...
was productive, reaching a maximum output of $10^5$ p.f.u. ml$^{-1}$ at 24 h, with a sustained production until approximately 72–96 h. After demonstrating infectability of the PBMM\textsuperscript{W}s, we proceeded to characterize the various phagosomal properties of these cells in order to determine the effect of PRRSV infection on the function of phagosomes in PBMM\textsuperscript{W}s.

**PRRSV infection of PBMM\textsuperscript{W}s impairs the ability of phagosomes to hydrolyse protein and to generate ROS following phagocytosis**

Since less than 5% of alveolar macrophages show PRRSV antigen during the acute phase of infection in vivo (Duan et al., 1997b), and our in vitro infection shows a peak of

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**Fig. 1.** Porcine bone marrow cells isolated from mid-costal (rib) bones can be differentiated *in vitro* by culturing in the presence of L929 cell supernatant containing M-CSF. (a) Cells were cultured for 10 days post-isolation, following seeding onto plastic bacteriological Petri dishes. Cells took on macrophage-like morphology by day 10, forming large cells with extensive cytoplasm and dendritic projections at the cell surface. Bar, 10 \( \mu \text{m} \). (b) Ten-day-old PBMM\textsuperscript{W}s were prepared for SEM. Cells were imaged at \( \times 250 \) (left), \( \times 2000 \) (centre) or \( \times 16,000 \) magnification (right panel). Bars, 200 \( \mu \text{m} \) (left); 20 \( \mu \text{m} \) (middle); 2 \( \mu \text{m} \) (right). (c) CD45 expression of 10-day-old PBMM\textsuperscript{W} cultures as determined by flow cytometry using FITC-conjugated anti-CD45 antibodies. (d) Ten-day-old PBMM\textsuperscript{W} cultures were fixed and stained using aqueous Diff-Quik modified Romanowsky stain to examine cytosolic and nuclear morphologies. Bar, 10 \( \mu \text{m} \).
Following PRRSV infection, PBMMΦs were incubated at 37 °C with proteolysis reporter experimental beads consisting of 3 μm silica beads conjugated to self-quenched DQ-BSA, calibration fluor Alexa Fluor 594 and IgG. Relative proteolytic activity was calculated using Student’s t-test. *P<0.05.

e) To quantify proteolytic activity, the slopes of the linear portions of the real-time traces were calculated and expressed relative to uninhibited samples. Error bars represent SEM. P values were calculated using Student’s t-test. *P<0.05.

As there is an inverse relationship between the production of ROS and proteolysis in classical activation of macrophages, and due to the importance of ROS as an antimicrobial agent within the phagosome, we investigated how PRRSV infection affected the production of ROS following phagocytosis in PBMMΦs. Production of ROS in response to phagocytosis of serum-opsonized zymosan was quantified after 1 h using the fluorescent extracellular H2O2 reporter Amplex UltraRed. A significant decrease in production of ROS was observed in infected cells when compared to uninfected (Fig. 3g), suggesting an additional defect in phagosomal function, and that the defect in bulk proteolytic activity was not due to oxidative inactivation of phagolysosomal cysteine proteases, as observed in classically activated (‘M1’) macrophages. To control for lower observed proteolysis and respiratory

**Fig. 2.** PBMMΦs differentiated in vitro exhibit characteristic phagosomal properties. (a) To assay phagocytosis, cells were incubated with experimental beads consisting of fluorescently labelled latex beads covalently coupled to BSA only, or to both BSA and human IgG, for 30 min at 37 °C. In order to distinguish between ingested and extracellular experimental beads, Trypan blue (0.01 %, v/v, in PBS) was added to the medium to quench the fluorescence of extracellular beads. Phagocytic index was calculated and is expressed as described in the text. Cells pre-treated with CytD (5 μM) were negative controls for the experiment. Data represent the mean percentage of beads phagocytosed from three independent experiments. Error bars represent SEM. *P<0.05. (b) In order to assay phagosomal acidification, profiles were generated in untreated and CMA (100 nM)-treated PBMMΦs. Cells were incubated with IgG-coupled 3 μm silica beads labelled with the pH-sensitive fluor CFSE (λem. 485 nm, λex. 520 nm) for 90 min. Acidification was measured in real-time by reading the fluorescence change in CFSE, and regressed to a standard curve in order to determine pH. (b) Representative real-time trace of pH measurement. (c) Mean data from three independent experiments of calculated phagosomal pH at 45 min. Error bars represent SEM. P values were determined using Student’s t-test. *P<0.05. (d, e) To assay phagosomal proteolysis, PBMMΦs were incubated at 37 °C with proteolysis reporter experimental beads consisting of 3 μm silica beads conjugated to self-quenched DO-BSA, calibration fluor Alexa Fluor 594 and IgG. Proteolytic efficiencies of phagosomes were assessed in real-time. (d) Representative real-time trace of proteolytic activity. (e) To quantify proteolytic activity, the slopes of the linear portions of the real-time traces were calculated and expressed relative to uninhibited samples. Error bars represent SEM. P values were calculated using Student’s t-test. *P<0.05.
Fig. 3. PRRSV-infected PBMMWs exhibit defects in phagosomal proteolysis and generation of ROS. (a) To determine if infection of PBMMWs by PRRSV results in a productive infection, differentiated PBMMWs in 24-well plates were infected by PRRSV at an m.o.i. of 0.01 and incubated for the indicated time at 37 °C. Supernatants were harvested at the indicated time point and titrated by plaque assay on MARC-145 cells. PBMMWs were infected in triplicate for each time point and supernatants were titrated in triplicate. Error bars represent SEM. (b–f) To measure phagosomal proteolysis, cells infected with PRRSV at an m.o.i. of 0.1 for 24 h, and uninfected control cells (UI), were incubated at 37 °C with proteolytic reporter experimental beads consisting of IgG-conjugated 3 μm silica beads, also conjugated to DQ-BSA and calibration fluor Alexa

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viability were not.
phagocytosis, phagosomal acidification and cellular
(Fig. 4e). These data indicate that although phagosomal
infected and uninfected PBMM
and uninfected cells using the live cell-impermeant vital
infected cells, cellular viability was enumerated in infected
cell death as a cause of the decrease in proteolysis in
with the pH-sensitive dye CFSE (Fig. 4c, d). To exclude
infected cells, as measured using IgG-conjugated beads labelled
indistinguishable from those observed in uninfected
and the final pH of phagosomes of infected cells were
CytD (Fig. 4a, b). Additionally, the rate of acidification
strated by the ablation of phagocytosis by treatment with
phagosomes over 90 min in uninfected and PRRSV-infected PBMM
from one representative experiment. (e) Mean relative
proteolytic substrate degradation within individual phagosomes over four independent experiments. Error bars represent SEM. P values were calculated using one-way ANOVA. *P<0.05. (d–f) Relative proteolytic efficiencies of individual phagosomes in uninfected and PRRSV-infected PBMM
as measured by high-content microscopy. (d) Relative proteolytic substrate degradation within individual phagosomes over 90 min in uninfected and PRRSV-infected PBMM from one representative experiment. (e) Mean relative proteolytic substrate degradation within individual phagosomes over four independent experiments. Error bars represent SEM. P values were calculated by comparing uninfected and infected samples at each time point using Student’s t-test. *P<0.05. (f) Representative microscopic images of uninfected and PRRSV-infected PBMM
at the 60 min time point presented in (d) and (e). (g) In order to measure the phagosomal production of ROS, cells were incubated with serum-opsonized zymosan at 37 °C for 1 h, followed by 30 min incubation at room temperature with Amplex UltraRed and HRP. Data are presented relative to uninfected samples over three independent experiments. Error bars represent SEM. P values were generated using Student’s t-test. *P<0.05.

burst due to a defect in particle phagocytosis, cells were
assayed using a quantitative bead uptake assay. IgG-con-
jugated beads labelled with the pH- and ROS-resistant
Alexa Fluor 488 were incubated with infected and unin-
fected cells to allow phagocytosis. Intracellular and extra-
cellular beads were distinguished by incubation with
Trypan blue. There was no significant difference in pha-
gocytosis between infected and uninfected cells, a process
which was dependent on actin polymerization, as demon-
strated by the ablation of phagocytosis by treatment with
CytD (Fig. 4a, b). Additionally, the rate of acidification and the final pH of phagosomes of infected cells were
indistinguishable from those observed in uninfected
cells, as measured using IgG-conjugated beads labelled
with the pH-sensitive dye CFSE (Fig. 4c, d). To exclude
cell death as a cause of the decrease in proteolysis in
infected cells, cellular viability was enumerated in infected
and uninfected cells using the live cell-impermeant vital
dye Trypan blue. No difference in cell viability between
infected and uninfected PBMM
was observed
(Fig. 4e). These data indicate that although phagosomal
proteolysis was compromised by PRRSV infection, phagocytosis, phagosomal acidification and cellular
viability were not.

**DISCUSSION**

A key pathology of PRRSV infection of pigs is the func-
tional impairment of monocytic phagocytes, particularly
those at the interface between the environment and the
animal, such as alveolar macrophages and recruited infil-
trating phagocytes. The inability to properly phagocytose
and kill invading secondary pathogens and present antigen
to T-cells for an adaptive immune response leads to
secondary infections and resulting morbidity (Opiessnig
et al., 2011). An appropriate cell model to directly study
the pathology of PRRSV infection on cells of the monocytic
lineage may assist in the study of PRRSV pathogenesis.
In this study, we present a reproducible, easily isolated
and derived macrophage model for PRRSV infection. While other estimable phagocyte models exist, such as
primary PAMs, some difficulties arise from their isolation
and use. The nature of alveolar macrophage activation is
determined not only by the genetic lineage of the macro-
phages, but by the environment in which they are derived
and survive: they are constantly exposed to inhaled dust,
pollutants and microbes and as such have to maintain a
constant ‘deactivated’ phenotype (du Manoir et al., 2002;
Lambrecht, 2006), and thus can be primed in different
ways depending on the environment in which the
animal was raised. The immortalized PAM model de-
veloped by Lee et al. (2010) is an excellent model with
which to study PRRSV. Culturing PAM _in vitro_ removes
the risk of differential priming of macrophages and gen-
etic and environmental differences in the macrophages.
However, alveolar macrophages have long been known
to be relatively poor at phagocytosis, phagosomal proteol-
ysis, respiratory burst and antigen presentation
(Blumenthal et al., 2001; Hoidal et al., 1981; Holt, 1978;
Weinberg & Unanue, 1981), yet are still known to per-
form, and in fact are required to do, all these functions
in the mammalian lung (Russell et al., 2009). Because
they are rather poor at these functions, it may prove dif-
ficult to characterize the effect of PRRSV infection by
their impairment in PAMs, as low activity levels may
make it difficult to distinguish pathological response
from normal function. PRRSV also targets other cells of
the monocye/macrophage lineage, including pulmonary
interstitial, splenic, placent al, umbilical and endomet rial
macrophages among others (Duan et al., 1997b;
Karniy chuk & Nauwynck, 2013; Lawson et al., 1997;
Thanawongnuwech et al., 2000). It is still not clear how
the infection of maternal and fetal macrophages affects
their function in contributing to the reproductive failure
caused by PRRSV infection, but evidence points to
apoptosis of the macrophages and/or infection of the
fetus causing the failure (Karniy chuk et al., 2011;
Rowland, 2010); however, dysfunction of the
macrophages cannot be ruled out. In view of these points, we set out to develop a more reproducible and robust system that is also easier to isolate and derive, in order to characterize the effect of PRRSV infection on the phagosomal properties of porcine macrophages in an in vitro setting.

We have shown that infection of our PBMMΦs results in a productive infection similar to that observed in alveolar macrophages and bone marrow-derived dendritic cells (Custers et al., 2008; Lee et al., 2010). Since we observed an earlier peak of virus production (24 h versus 36–48 h), we chose a 24 h time point to assay phagosomal function. More rapid infection and virus production may be an additional benefit of using PBMMΦ as functional assays may be performed earlier. Infected macrophages were able to phagocytose beads in our experimental platform, allowing the characterization of phagosomal maturation during PRRSV infection. However, more extensive characterization of phagocytic indices using different particles and ligands in a different experimental platform is needed to determine whether there are subtle differences in the phagocytic efficiency between PRRSV-infected and uninfected macrophages.

Our model demonstrates that PRRSV infection of macrophages induces a change in two key parameters of PRRSV infected

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\caption{Defects in phagosomal proteolysis and generation of ROS in PRRSV-infected PBMMΦ are not due to altered phagocytic uptake or phagosomal acidification or cellular viability. (a, b) To assay phagocytosis, cells were incubated 30 min at 37 °C with 3 μm silica beads covalently coupled to human IgG, BSA and Alexa Fluor 488. (a) To distinguish between phagocytosed and extracellular experimental beads, cells and beads were stained with the vital dye Trypan blue (0.01 %, v/v, in PBS) which quenches fluorescence of extracellular beads. Phagocytic index was calculated as the ratio of the number of phagocytosed experimental beads to the total number of beads per field. CytD (5 μM) was used as negative control for the experiment. Data represent the average percentage of beads phagocytosed from three independent experiments. Error bars represent SEM. P values were calculated using one-way ANOVA. NS, Not significant. (b) Representative microscopic images of uninfected and PRRSV-infected PBMMΦs after 60 min following addition of IgG/BSA-conjugated Alexa Fluor 488-labelled beads, following quenching of extracellular beads with Trypan blue. Bars, 10 μm. (c, d) In order to assay phagosomal acidification, cells were incubated at 37 °C with 3 μm silica beads conjugated to IgG and labelled with the pH-sensitive fluor CFSE. Acidification was measured in real-time by reading the fluorescence change in CFSE, and regressed to a standard curve in order to determine pH. (c) Representative real-time trace of pH measurement. (d) Mean data from three independent experiments of calculated phagosomal pH at 45 min. Error bars represent SEM. (e) In order to assay cell viability, infected and uninfected cells were stained at 24 h p.i. with the vital dye Trypan blue (0.01 %, v/v, in PBS), counted using a haemocytometer and the percentage of dead cells were calculated. Graph represents average from three independent experiments. Error bars represent SEM. P values were calculated using Student’s t-test.}
\end{figure}
phagosomal maturation and macrophage function: the respiratory burst and the hydrolysis of phagocytosed protein. It is unlikely that either of these functions were decreased by infection-induced cell death at 24 h p.i., as evidenced by the observed equivalent cell viability (Fig. 4e), no impairment of phagocytosis (a functional indicator of phagocyte apoptosis; Bodenheimer et al., 1988) (Fig. 4a), and no change in the characteristic acidification of the phagosome (Fig. 4c, d), and in addition, the cells continued to produce high titres of viable virus until 96 h (48 h post-assay, Fig. 3a). In fact, PRRSV replication of macrophages has been shown to depress apoptosis early in infection (Costers et al., 2008). A mechanistic explanation of how PRRSV infection reduces phagosomal proteolysis and phagosome-derived ROS production remains undetermined. Proteolysis within the phagosomal lumen is controlled at several levels, including transcription and translation of lysosomal proteases, trafficking of the proteases to the lysosomes, fusion of the maturing phagosomes with lysosomes, as well as local factors such as phagosomal pH and redox conditions (Balce et al., 2011; Flannagan et al., 2012; Rybicka et al., 2010). Given the long half-life of the lysosomal cathepsins, significant changes to activities of these lysosomal proteases are unlikely to be affected by changes to their expression over 24 h of infection (Nissler et al., 1999; Turk et al., 2002). Additionally, since PRRSV infection did not significantly alter phagosomal acidification and resulted in a reduction of ROS production (which in theory would increase activity of local cysteine cathepsins; Yates, 2013), it is likely that these luminal factors were not responsible for the defect in phagosomal proteolysis observed. Hence, we can speculate that the reduction in phagosomal proteolysis in PRRSV-infected macrophages resulted from delayed maturation of the late phagosome and impaired fusion with lysosomes. Further experimentation using the PBMMΦ model will elucidate a possible mechanism by which PRRSV may impair phagosomal maturation is the focus of a future study.

A reduction in the respiratory burst of the phagosome could have significant effects on the characteristic functions of the macrophage following PRRSV infection. ROS produced by the NADPH oxidase-mediated respiratory burst is a direct antimicrobial effector within the phagosome and has also been shown to affect antigen processing within this compartment (Allan et al., 2014). In combination with the observed reduction in phagosomal proteolysis (likely due to impaired phagosomal fusion with lysosomal compartments), inhibition of ROS production by PRRSV infection is likely to dramatically impede the ability of the macrophages to clear secondary bacterial and fungal infections and may contribute to the morbidity associated with secondary infections in PRRSV-infected animals.

Here we present a robust cell model for the effect of PRRSV infection on pig macrophages. In addition to another cell model to study PRRSV, this paper describes the capability of using infected PBMMΦ to study key phagosomal functions in a reductionist fashion. Each of these functions is critical to the various roles played by phagocytes in the innate and adaptive immune systems, including the clearing of microbes and antigen presentation to T-cells (two steps involved in the significant morbidity and mortality observed in swine herds infected with PRRSV). Our model may also prove useful for the study other important viruses such as porcine circovirus and influenza A, both of which target the alveolar macrophages of swine (Jung et al., 2002; McNeilly et al., 1996). Because little is known about the effects of viral infections in general on phagosomal maturation, the use of bone marrow-derived macrophage models may lead to important discoveries in the pathogenic mechanisms of phagocytic infection. The addition of this model to the pantheon of models to study the pathology of PRRSV will hopefully aid in the fight against this disease that is so devastating to the swine industry.

METHODS

Cell lines and viruses. MARC-145 cells (ATCC CRL-12231, an African green monkey kidney epithelial cell line permissive to PRRSV infection) were cultured at 37 °C, 5% CO₂ using Dulbeco’s modified Eagle’s medium (DMEM; Hyclone) supplemented with 10% FBS (Invitrogen), 2 mM L-glutamine, 100 IU penicillin ml⁻¹ and 100 μg streptomycin ml⁻¹. Cells were passaged twice weekly for maintenance. Cells were seeded to confluence the day prior to infection, functional assays and/or plaque titration. PRRSV isolate NVSL 98-7895 (GenBank accession no. AY545985.1) was a kind gift of Dr John Harding (University of Saskatchewan, SK, Canada). Virus was propagated by growth in MARC-145 cells for 72–96 h. Virus was titrated by plaque assay on confluent monolayers of MARC-145 cells in 6- or 12-well dishes (Greiner Bio-One).

Derivation of PBMMΦs. All animal work was performed in accordance with the Canadian Council of Animal Care guidelines using protocols approved by the University of Calgary Animal Care and Use Committee. Porcine bone marrow was obtained from mid-thoracic costal rib bones removed from humanely euthanized adult pigs. Bones were cleared aseptically of any associated tissue, clipped at their ends and bone marrow flushed out using sterile 100 U heparinized 0.9% (w/v) saline through an 18-gauge needle. Resultant marrow was pelleted and contaminating erythrocytes were removed by osmotic lysis using a 5 min incubation in erythrocyte lysis buffer (0.8%, w/v, NH₄Cl, 0.1 mM EDTA, 10 mM NaHCO₃) followed by restoration of isotonicity with 10x volume PBS (Lonza). Cells were pelleted and resuspended in DMEM containing 20% (v/v) L929 fibroblast culture supernatant, prepared as described previously (Yates et al., 2005). Cells were cultured using untreated bacteriological Petri dishes for approximately 10 days prior to seeding into tissue culture-treated multi-well plates (Greiner Bio-one) for assay. Staining of cells for microscopic evaluation of cytoplasmic and nuclear morphologies was performed using aqueous Diff-Quik modified Romanowsky stain (Baxter) as per the manufacturer’s protocol. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences) for acquisition and the Flow Jo v8.6.2 software (Tree Star) for analysis. Populations were selected using forward scatter/side scatter and a minimum of 5 x 10⁵ events were acquired for each sample. FITC-conjugated mouse anti-swine CD45 mAb and the relevant IgG1 isotype control (AbD Serotec) were used at dilutions recommended by the manufacturer.
PRRSV infection of PBMMΦs. In order to determine whether infection of PBMMΦs with PRRSV was productive, PBMMΦ monolayers in 24-well dishes were infected in triplicate at an m.o.i. of 0.01 with PRRSV diluted in OptiMEM (Invitrogen) for 1 h at 37 °C. Inoculum was removed following incubation and replaced with pre-warmed PBMMΦ culture medium. This time point was considered T=0. Supernatants were harvested and medium replaced with pre-warmed PBMMΦ medium at 0, 12, 24, 48, 72 and 96 h p.i. Virus was titrated for all time points by plaque assay on MARC-145 cells. All titrations were replicated in triplicate for 100-fold dilutions bracketing the expected titre. For all functional assays of infected PBMMΦs, confluent monolayers of macrophages were infected for 24 h at an m.o.i. of 0.1 or not infected (uninfected controls). Cellular viability of infected relative to uninfected PBMMΦs was assayed using 0.01% (v/v) Trypan blue exclusion at 24 h p.i.

Macrophage fluorometric functional assays. For all functional assays, fully differentiated PBMMΦs were seeded to confluency into 24-, 24- or 96-well plates. Confluency was assured prior to assay by microscopic observation.

Phagocytic index. Phagocytic index was determined using an extracellular Trypan blue fluorescence quenching assay. Briefly, carboxylate-modified 3 μm diameter latex or silica experimental beads (Kisker Biotech) were covalently coated with BSA alone or BSA and human IgG (Sigma-Aldrich) and labelled with succinimidyl ester derivatives of Alexa Fluor 488 (Invitrogen) as described previously (Yates & Russell, 2008). These beads were subsequently incubated with confluent monolayers of PBMMΦs for 30 min at 37 °C at a multiplicity of approximately one to three beads per cell, followed by addition of 0.01% (v/v) Trypan blue in PBS to quench extracellular fluorescence. Enumeration of intracellular and extracellular beads was performed using epifluorescence microscopy 30 min after the addition of beads. Cytochalasin D (CytD, 5 μM; Sigma-Aldrich), a potent inhibitor of actin polymerization and thus phagocytosis, was used as a negative control for these experiments. Phagocytic index was calculated as the percentage of beads that were internalized by cells. The percentage of beads internalized by uninfected cells was set as a phagocytic index of 1.0, with values for PRRSV-infected and CytD-treated cells expressed as a ratio of this value. Three fields of view per well were counted for triplicate samples.

Assessment of phagosomal pH and proteolytic activity. Phagosome pH was measured as described previously (Allan et al., 2014; Balce et al., 2011; Rybicka et al., 2010; Yates et al., 2005; Yates & Russell, 2008). Briefly, BSA/IgG-coated 3 μm silica beads, labelled with the pH-sensitive dye CFSE were incubated with PBMMΦ monolayers, at a multiplicity of one to three beads per cell. Intra-phagosomal pH was calculated by recording the ratio of the fluorescent emission at 520 nm following excitation at 488 and 450 nm using a FLUOstar Optima plate fluorescence reader (BMG Labtech) or Envision multilabel plate reader (Perkin-Elmer), followed by polynomial regression to a standard curve. Real-time proteolytic activity of phagosomes in PBMMΦs was measured using fluorescence dequenching of 3 μm silica beads labelled with IgG and heavily derivatized DQ-BSA substrate (Invitrogen) as described previously (Balce et al., 2011; Rybicka et al., 2010, 2012; Yates et al., 2005, 2007; Yates & Russell, 2008). Briefly, 3 μm silica beads were covalently coupled to IgG and the heavily derivatized and fluoroscently quenched substrate DQ-Green BSA (Invitrogen), and subsequently labelled with the calibration fluor Alexa Fluor 594 succinimidyl ester (AF594). These beads were incubated with PBMMΦ monolayers, at a multiplicity of one to three beads per cell. Proteolysis of the BSA substrate causes fluorescence dequenching of the BODIPY-FL label resulting in increased fluorescence at 488 nm excitation/520 nm emission. The substrate fluorescence was measured over time using a plate reader and normalized to a redox- and pH-insensitive calibration fluorescence (AF594). Fluorescent ratios were plotted and slopes calculated for the linear portion of the curve, generally 45–90 min post-inoculation. Concanaamycin A (CMA, 100 nM; Sigma-Aldrich), a potent vATPase inhibitor that prevents acidification of phagosomes and subsequent activation of proteases, was used as a negative control for all phagosomal maturation experiments. In all cases, cells were inspected microscopically following assay to ensure the complete phagocytosis and expected multiplicity of experimental beads. Single phagosome analysis was performed using an InCell Analyser 2000 High-Content Imaging system (GE Life Sciences) equipped with a Nikon ×40 objective (numerical aperture=0.6). At 0, 30, 60 and 90 min post-particle ingestion, images in the bright-field, FITC (measuring DQ-BSA fluorescence) and Texas red (measuring AF594 calibration fluorescence) channels were taken at 0.15, 0.05 and 0.03 s exposure times, respectively. Raw unmodified images were exported as TIFFs and analysed using IN Cell Developer Toolbox software program (GE Life Sciences). Individual targets (in this case fluoroscent particle-containing phagosomes) were identified by setting the segmentation type to ‘nuclear’ and changing the minimum target area to match the size of the experimental particles (approximately 3.0 μm). Sensitivity of target detection was adjusted so that all particle-containing phagosomes would be included in the analysis. Using the Texas red target as a reference, both DQ-BSA and AF594 density levels were measured. Raw data were exported into Microsoft Excel, and relative fluorescence units (RFU) (DQ-BSA fluorescence/AF594 fluorescence) for each particle was calculated. Any particles which were not internalized (less than 5%) were manually excluded from the analysis. Mean RFU values (four independent experiments) from uninfected and infected experimental groups for each time point were calculated.

Assessment of respiratory burst. Production of hydrogen peroxide following the phagocytosis of serum-opsonized zymosan by PBMMΦs was measured using Amplex UltraRed (Invitrogen) as described previously (Balce et al., 2014). Briefly, confluent monolayers of PBMMΦs in 96-well plates were incubated with serum-opsonized zymosan (0.5 mg ml⁻¹; Sigma-Aldrich) for 1 h at 37 °C. Following incubation, 1 U HRP (0.1 U ml⁻¹; Sigma-Aldrich) and 1 mM Amplex UltraRed reagent were added to each well and incubated in the dark for 30 min at room temperature. Absolute fluorescence intensity values were measured using a plate reader at 550 nm excitation and 615 nm emission wavelengths. Negative control samples consisting of PBMMΦs that were not incubated with serum-opsonized zymosan, which will not produce a respiratory burst, were used to determine background values. The relative ROS production of each sample was determined by dividing the background-adjusted fluorescence intensity values by those of uninfected samples in the same experiment.

Optical imaging and scanning electron microscopy. Bright-field optical and fluorescent imaging were performed using an Olympus IX70 epifluorescence microscope using Q-Capture Pro 6.0 Image and Analysis software package (QIImaging). Confocal images were acquired from a Leica TCS SP5 scanning confocal microscope using Leica Application Suite software (version X). For sample preparation for scanning electron microscopy (SEM), PBMMΦs were initially fixed for 3 h at room temperature in 0.1 M sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde (v/v). Following fixation, cells were washed in ice-cold cacodylate buffer to remove fixative and progressively dehydrated in increasing concentrations of ethanol (25, 40, 60, 80 and 100%). Following dehydration, cells were resuspended and washed in increasing concentrations of hexamethyldisilazane in ethanol (25, 50, 75 and 100%). Small amounts of the resuspended cells were mounted on gold-coated cover slips for imaging using a Phillips XL30 SEM at the University of Calgary Microscopy and Imaging Facility.
Statistical analysis. Numerical values have been represented as means ± SEM. Data were analysed using Graphpad Prism software (version 5; Graphpad Software). To compare statistical significance of experimental outcomes between two experimental groups, P values were calculated using either Student’s t-test or ANOVA, where appropriate. Statistical significance was considered to be established when the observed P values were <0.05.

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