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Characterization of age-related susceptibility of macrophages to porcine reproductive and respiratory syndrome virus

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ABSTRACT

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) is the most economically important disease affecting swine production worldwide. The severity and susceptibility of PRRSV infection varies with age. Nursery pigs have been shown to be more susceptible to PRRSV infection and a more severe and prolonged infection is observed as compared to growing or adult pigs. However, antibody responses to PRRSV are observed independent of age. Swine are the only known hosts of PRRSV, infection is restricted to cells of monocytic lineage, and fully differentiated porcine alveolar macrophages are the primary target of natural infection. Pulmonary intravascular macrophages from young pigs have been shown to be more susceptible to infection than those from adult pigs. A better understanding of why young pigs and macrophages from young pigs are more susceptible to PRRSV infection is critical to identify mechanisms of infection that can be explored for enhanced treatment or prevention of disease. This study examined PRRSV susceptibility of porcine alveolar macrophages isolated from the lungs of pigs of different age groups, and the presence of cell surface receptors to determine if differences correlated with infection level. The younger the pigs were, the more susceptible the macrophage were to PRRSV infection, but no differences in cellular receptor expression were observed between pigs of different ages. Resistance to infection is likely related to intracellular innate immune mechanisms rather than receptor-mediated entry.

1. Introduction

Porcine Reproductive and Respiratory Syndrome Virus (PRRSV), a member of the order *Nidovirales*, family *Arteriviridae*, genus *Porartevirus*, is the most economically important disease affecting swine production worldwide. Clinically, PRRSV is observed as a respiratory disease particularly of growing pigs, and a reproductive disease in pregnant sows characterized by late-term abortions, mummified piglets, stillbirths and weak born pigs (Butler et al., 2014; Christianson et al., 1993; Rossow et al., 1995).

Swine are the only known hosts of PRRSV with infection restricted to cells of monocytic lineage, and the primary target of natural infection are fully differentiated porcine alveolar macrophages (PAM) (Duan et al., 1997; Lunney et al., 2016). In the laboratory setting, PRRSV can replicate to varying degrees in the African green monkey kidney cells lines MA-104 and its derivative MARC-145 (Snijder et al., 2013). PRRSV infection involves entry through receptor-mediated endocytosis using a variety of receptors or co-receptors, but the macrophage-specific protein CD163 alone is necessary and sufficient for PRRSV infection (Welch and Calvert, 2010). Other cellular factors identified to play a role in PRRSV infection are heparin sulfate and CD169 (sialoadhesin) which have been shown to be involved in internalization of the virus and vimentin, CD151, and DC-SIGN/CD209 which have been shown to interact with PRRSV (Welch and Calvert, 2010).

The severity and susceptibility of PRRSV varies with age. Nursery pigs have been shown to be more susceptible to PRRSV infection and a more severe and prolonged infection is observed as compared to growing or adult pigs (Cho et al., 2006; Klinge et al., 2009; van der Linden et al., 2003). However, antibody responses to PRRSV are observed independent of age (Klinge et al., 2009).

Pulmonary intravascular macrophages from young (4 week old) pigs have been shown to be more susceptible to infection than those from older (4 months old) animals (Thanawongnuwech et al., 1998). A better understanding of why young pigs and macrophages from young pigs are more susceptible to PRRSV infection will identify mechanisms of infection that can then be explored for enhanced treatment or

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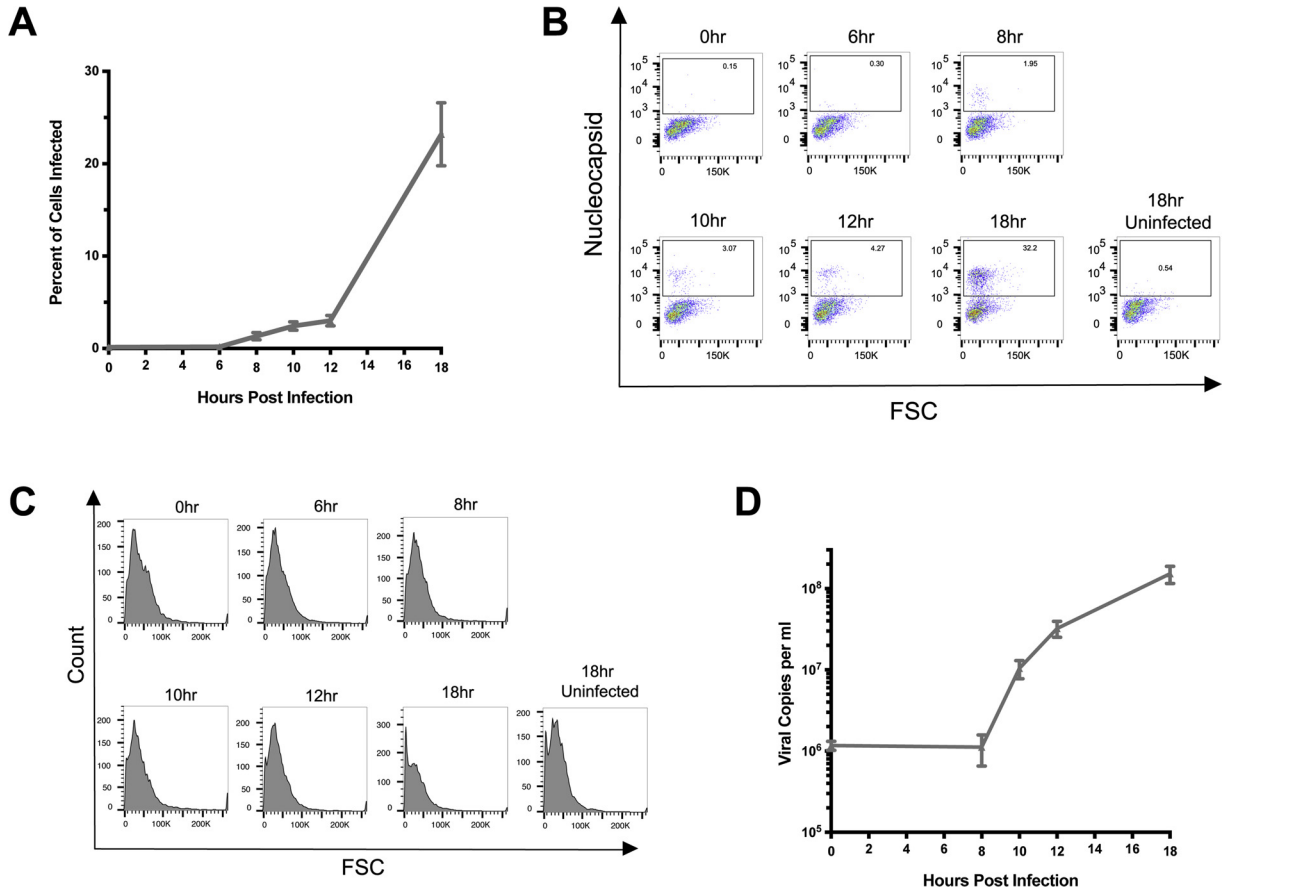

Fig. 1. PRRSV infection over time in PAMs from 8 to 10 week old pigs. PAMs isolated from 8 to 10 week old pigs (n = 3) were infected in vitro with PRRSV MN184 at an MOI of 0.3. A) Cells were harvested over an 18 h time course and the percent of PRRSV positive cells was determined using flow cytometry based on the total number of single cells. Representative flow cytometry plots over the time course are shown indicating B) the percent of infected cells and C) the change in cell morphology (FSC). D) Cell supernatants were harvested over an 18 h time course and viral copy numbers were determined using RT-qPCR relative to a standard curve with a cut-off value for PRRSV positive samples at 1×10^5 viral copies/ml with SEM error bars shown. Mock infected PAMs were negative for PRRSV across the time course.

prevention of disease.

This study examined the PRRSV susceptibility of porcine alveolar macrophages (PAMs) isolated from the lungs of pigs of different ages and hypothesized that differences in expression of surface receptors associated with PRRSV entry and replication would be related to infection level. The younger the pigs were, the more susceptible the PAMs were to PRRSV infection, but no differences in surface marker expression was observed between pigs of different ages. Thus, resistance to infection is likely related to innate intracellular immune mechanisms rather than receptor-mediated entry.

2. Materials and methods

2.1. Virus and cells

Porcine alveolar macrophages (PAMs) were harvested from the lungs of commercial SPF pigs by lung lavage, cryopreserved, and cultured in vitro as previously described (Robinson et al., 2015). Collection of PAMs was approved by and conducted according to protocol number 1402–31319A under the guidelines of the University of Minnesota Institutional Animal Care and Use Committee. PAMs were obtained from a total of 17 nursery (3–16 days old), 6 growing (8–12 weeks old) and 9 adult pigs.

North American (Type 2) PRRSV isolate MN184 (GenBank ID EF442777) was propagated on PAMs as previously described (Robinson et al., 2015). Infectious viral titers on PAMs ($TCID_{50}$) were determined using an indirect florescence antibody assay as previously described (Dvorak et al., 2018). Basically, PAMs were seeded on polyethylinimine-coated 96-well plates at 6×10^4 to 1×10^5 cells per well overnight, infected with ten-fold serial dilutions of virus for 1 h, incubated for 48 h at 37 °C/5% CO_2 and fixed with methanol. Infected cells were visualized by immunofluorescence analysis using a PRRSV anti-nucleocapsid monoclonal antibody SR30 A (RTI, Brookings SD) and an AlexaFluor 568 goat anti-mouse immunoglobulin G (IgG) secondary antibody (Invitrogen, Carlsbad, CA). Nuclei were stained using bisbenzimide (Sigma-Aldrich, St. Louis, MO). Stained monolayers were visualized using an Olympus IX-70 inverted photomicroscope (Olympus America, Inc., Center Valley, PA) provided by the University Imaging Centers at the University of Minnesota. $TCID_{50}$'s were calculated as described (Reed and Muench, 1938).

2.2. PRRSV infection of PAMs

PAMs were thawed from liquid nitrogen, seeded at 2×10^5 cells/tube in clear 5 ml polystyrene snap cap tubes, and incubated overnight at 37 °C/ 5% CO_2. Caps were shut to the first stop to allow airflow while maintaining sterility during the overnight incubation. Cells were then infected at either an MOI of 0.3 or 0.1 with PRRSV isolate MN184, or mock infected, in the absence of fetal bovine serum (FBS) for 1 h at 37 °C/5% CO_2. The inocula was then removed and cells were washed and resuspended in 2 ml of culture media and incubated at 37 °C/5% CO_2 for the desired amount of time (0–48 h). Cells and supernatants were harvested and used for flow cytometry and qPCR for presence of surface markers and detection of virus.

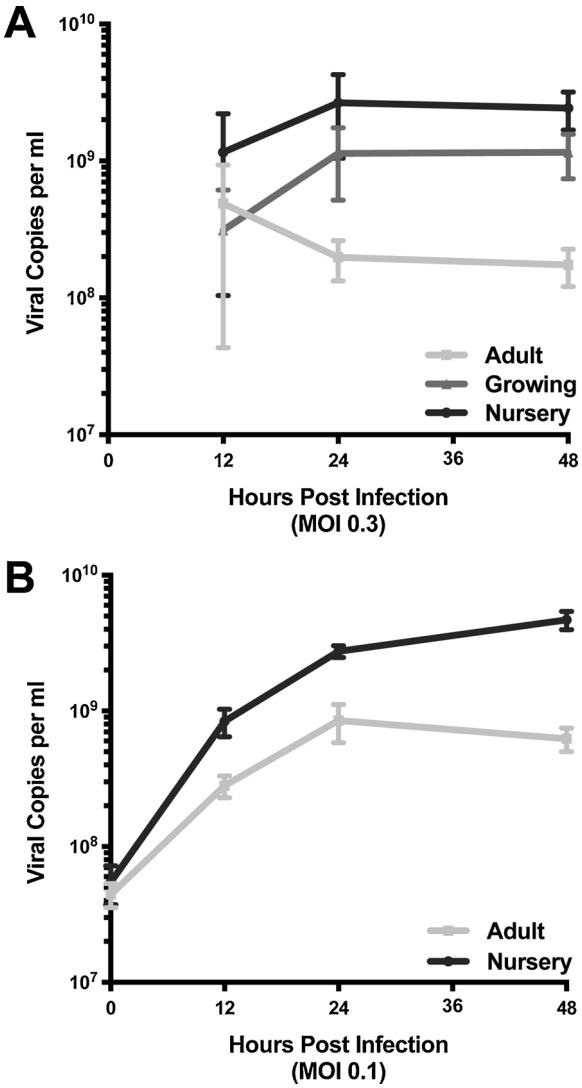

Fig. 2. Age-dependent PRRSV replication in PAMs over a time course. PAMs were isolated from pigs of different ages, infected with PRRSV, and examined for viral replication over a 48 h time course using RT-qPCR. A) Viral replication in PAMs from nursery (n = 2), growing (n = 2), and adult (n = 2) pigs infected at an MOI = 0.3. B) Viral replication in PAMs from nursery (n = 8) and adult (n = 8) pigs infected at an MOI = 0.1. Error bars are shown as SEM.

2.3. Detection of PRRSV-infected cells and cell surface markers by flow cytometry

Samples were stained for the presence of viral infection or for surface markers using flow cytometry as previously described (Robinson et al., 2015). Surface markers were identified using 100 µl of either CD172a (macrophage marker, clone BL1H7), CD163 (ED2, clone 2 A10/11), or CD169 (sialoadhesin, clone 3B11/11) antibodies (AbD Serotec, Raleigh, NC) at 2 µg/ml, 1.2 µg/ml, or 0.5 µg/ml dilution, respectively and 200 µl of a 1:200 dilution of goat anti-mouse APC secondary antibody (Biolegend, San Diego, CA) followed by fixation and permeabilization using a Fixation/Permeabilization Solution Kit (BD Biosciences, San Jose, CA). The SR30-F monoclonal antibody for PRRSV nucleocapsid protein conjugated to FITC (RTI, Brookings, SD) was then added at a 1:100 dilution to identify PRRSV infected cells. Cell counting was performed using a BD FACSCanto flow cytometry system (BD Biosciences, San Jose, CA) and data were analyzed using FlowJo software (FlowJo, Ashland, OR). The flow cytometry data was first gated on single cells to exclude debris and multiple cell clumps followed by gating to include either infected cells (SR30-F$^+$) or the macrophage population (CD172a$^+$). The percent of cell surface markers or infected cells for most experiments was then determined based on the total number of macrophage (CD172a$^+$ cells).

2.4. PRRSV growth in PAMs

PAMs from different ages of pigs were infected as above and supernatants were collected at 0, 6, 8, 10, 12, 18, 24, or 48 h post-infection for isolation of viral RNA and RT-qPCR for evaluation of viral replication over time as previously described amplifying a 198 bp ORF6-7 fragment (Robinson et al., 2018). All samples were run in duplicate and were quantified using a standard curve.

2.5. Statistical analysis

T-test comparisons were performed using GraphPad Prism software (version 7.0c, GraphPad Software, Inc., La Jolla, CA)

3. Results

3.1. Early time course of PRRSV infection in PAMs

PAMs from three 8–10 week old pigs were obtained and infected with PRRSV at an MOI of 0.3 or mock infected as a control. Infected and control cells and supernatants were harvested at 0, 6, 8, 10, 12, and 18 h post-infection. The percent of single cells that were infected with PRRSV was determined by flow cytometry (Fig. 1A and B). Infection increased over the time course up to the final time point at 18 h. By 12 h post infection, an average of 4% of cells were infected, with 22% infected by 18 h post infection. However, by 18 h a change in cell morphology was observed by flow cytometry with a decrease in the forward scatter and a slight increase in the side scatter intensity suggesting a decrease in cell size and increase in granularity of the cells, which may be an early indicator of apoptosis (Fig. 1C). The amount of virus present in cell supernatants over the time course was determined by RT-qPCR showing the amount of viral RNA increasing from 8 h to 18 h post infection (Fig. 1D). All time points from 10 to 18 h showed an observable level of viral infection in cells, but confounding changes to cell size were observed at 18 h. Thus, further analysis of infected cells by flow cytometry was performed at 12 h post-infection.

3.2. PRRSV infection of PAMs from nursery, growing, and adult pigs

PAMs were harvested from pigs of different ages, nursery (3–16 days old), growing (8–12 week old), and adult pigs. Nursery and Adult PAMs were infected with PRRSV at an MOI of 0.3 or 0.1 and PAMs from growing pigs were infected at an MOI of 0.3 and growth was examined over a time course of infection. RT-qPCR was performed to examine viral growth over time (Fig. 2A and B). At an MOI of 0.3, virus replicated from 12 to 24 h in PAMs from nursery and growing pigs and maintained a steady level of virus out to 48 h post-infection, while no increase in virus was observed in PAMs from adult pigs suggesting all susceptible cells were already infected by the 12 h time point (Fig. 2A). At an MOI of 0.1, viral replication was observed in both nursery and adult pigs (Fig. 2B). In PAMs from adult pigs, viral replication increased until 24 h post-infection, but then decreased slightly by 48 h, whereas PAMs from nursery pigs continued to replicate out to 48 h (Fig. 2B). A significant age-dependent difference in viral levels at each time point (12, 24, and 48 h post-infection) was observed ($p < 0.05$) and by 48 h post-infection, viral levels in PAMs from nursery pigs were 7.5 fold higher than that of adult pigs.

Susceptibility of PAMs from pigs of different ages was examined by flow cytometry at 12 h post-infection in order to compare the percent of infected macrophages (Fig. 3). Although the 12 h time point was chosen through examination of PAMs from growing pigs, the time course of growth in the different ages of PAM preparations gave parallel results,

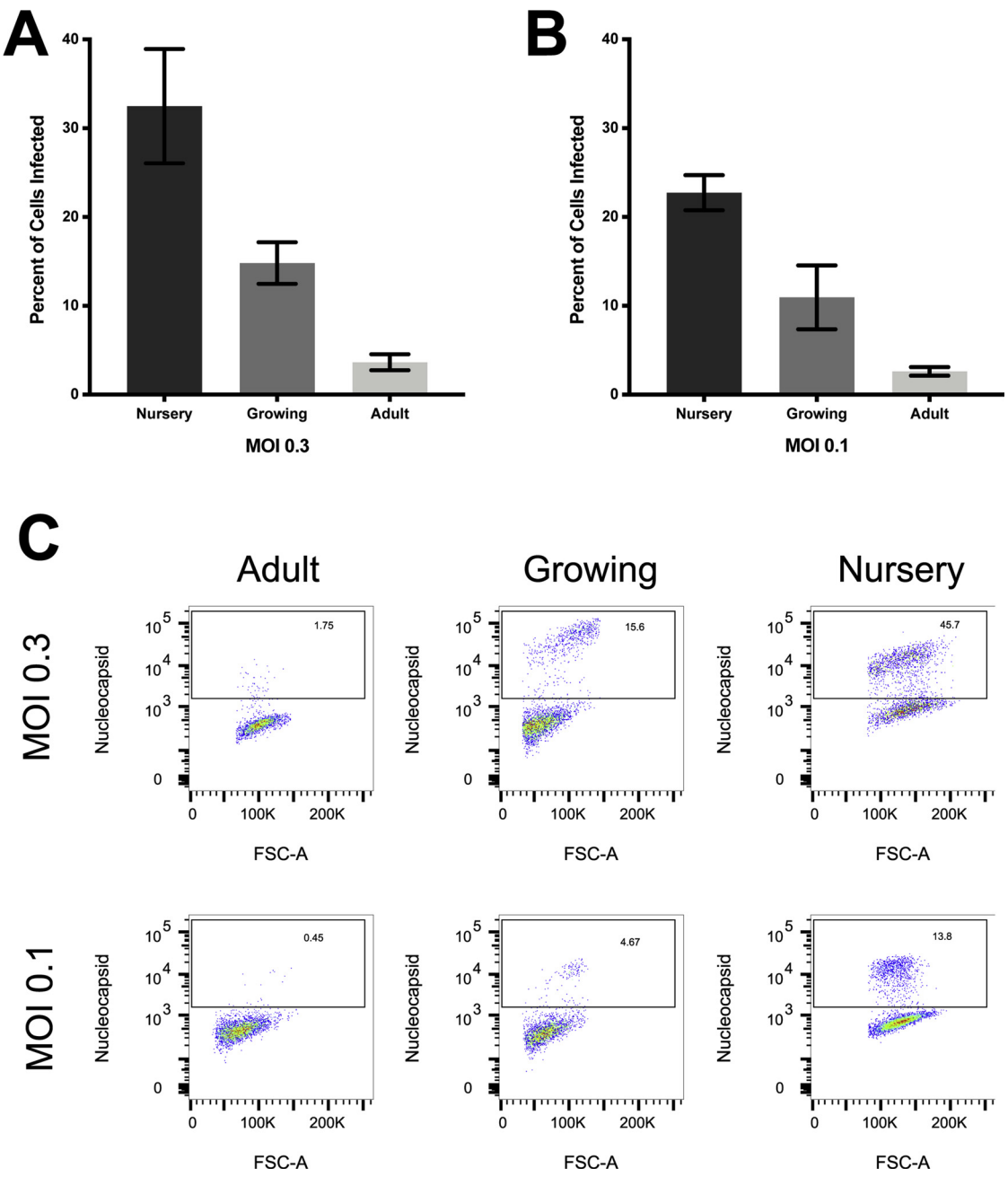

Fig. 3. Age-dependent PRRSV infection. PAMs from animals of different ages were infected with PRRSV and examined for the percent of infected cells at 12 h post-infection by flow cytometry. The average percent of PRRSV positive cells is shown for nursery (n = 9, n = 8), growing (n = 6, n = 3), and adult (n = 9, n = 8) pigs at an A) MOI of 0.3 and B) MOI of 0.1, respectively. Error bars are shown as SEM. C) Representative flow cytometry plots indicating the percent of infected cells in PAMs from animals of different ages.

just at different viral levels. Thus, at 12 h post-infection all ages of PAMs should be at the same stage in the infection cycle. PAMs from younger pigs were more susceptible to infection regardless of infectious dose with 33% and 23% of nursery, 15% and 11% of growing, and only 4% and 3% of adult PAMs being infected at an MOI of 0.3 and 0.1 respectively (Fig. 3A–C). PAMs from nursery pigs were the most susceptible to infection, approximately 2 fold more than growing pigs and 7–8 fold more susceptible than adult pigs and growing pigs were approximately 4 fold more susceptible than adult pigs at either MOI ($p < 0.05$). A higher percent of infected cells was observed following infection at the higher MOI, but similar differences in infection levels between PAMs from different aged pigs was observed.

3.3. Prevalence of cell surface viral receptors

Prevalence of viral receptors on PAMs from pigs of different ages was examined to determine if this contributed to the age-dependent difference in viral infectivity. The number of macrophages present in PAM preparations (as a percent of single cells) differed depending upon the age of the pigs from which they were isolated. Adult pigs had a significantly lower percent of macrophage in their PAM preparations than did nursery pigs ($p < 0.05$, Fig. 4). The percent of CD163 (PRRSV receptor) receptor positive PAMs from nursery, growing, and adult animals showed no age dependent differences, with a range of 90–100% CD163 positive PAMs (Fig. 4). The percent of CD169 (receptor involved in, but not required for PRRSV replication) positive PAMs ranged from 85 to 100% with a significantly higher percent of

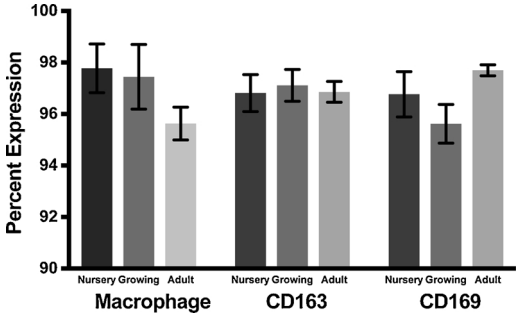

Fig. 4. Presence of PRRSV receptors on PAMs from different ages of pigs. PAMs from nursery (n = 17), growing (n = 4), and adult (n = 9) pigs were stained for macrophage marker CD172a, PRRSV receptor CD163, and CD169/sialoadhesin, and examined by flow cytometry to determine the percent of receptor positive cells. CD172a positive cells (macrophage) were determined as a percent of single cells and CD163 and CD169 are presented as a percent of CD172a positive cells. Error bars are shown as SEM.

CD169 positive adult PAMs as compared to PAMs from growing pigs (Fig. 4). Surface marker expression did not vary between PRRSV-infected cells and mock-infection (data not shown).

4. Discussion

Age-dependent susceptibility to PRRSV infection has been shown previously with younger pigs being more susceptible to infection (Cho et al., 2006; Klinge et al., 2009; van der Linden et al., 2003). Alveolar macrophages are the primary cell target for PRRSV infection (Snijder et al., 2013; Welch and Calvert, 2010). Previously it has been observed that pulmonary intravascular macrophages isolated from younger pigs are more susceptible to PRRSV infection than those from older pigs (Thanawongnuwech et al., 1998). We examined PRRSV infection in alveolar macrophages from pigs of different ages to confirm this observation and determine if an age-dependent difference in the presence of PRRSV receptors was involved in viral susceptibility.

PRRSV infection of PAMs from nursery, growing, and adult pigs confirmed previous observations, that PAMs from younger pigs were more susceptible to infection. The percent of infected cells in PAMs from nursery pigs was significantly higher, 7–8 times, than that of adult PAMs and at 48 h post-infection viral titers were 7.5 times higher ($p < 0.05$). This confirmed previous observational and experimental data that younger pigs are more susceptible and have significantly higher viral titers, predicting a more severe and prolonged infection in vivo in young pigs as compared to adults. Interestingly, we observed that our PAM preparations from adult pigs had significantly lower numbers of macrophage than those from young or nursery pigs ($p < 0.05$). Previously studies observed that bronchoalveolar lavage (BAL) fluids increased in the percent of macrophage from newborn piglets to adults (Islam et al., 2012), but other studies showed preparation purities that were irrespective of age (Islam et al., 2013). Thus, the percentage of macrophage present in a preparation is probably more dependent upon animal to animal variation than upon age.

The PRRSV receptor CD163, has been determined to be necessary and sufficient for viral entry and infection, even though other cellular factors may play a role in infection (Welch and Calvert, 2010). Transfection of CD163 into non-permissive cell-lines, allowed for PRRSV infection and replication (Welch and Calvert, 2010). CD163 is a macrophage-specific membrane protein involved in ligand recognition and is associated with an innate immune response to infection (Welch and Calvert, 2010). A direct correlation between CD163 expression and PRRSV infectivity has been observed previously (Patton et al., 2009). However, in our experiment, age-dependent infection did not correlate with CD163 expression levels, suggesting that CD163 prevalence is not related to age-dependent variation in infection. A significant difference in the percent of infected cells was observed between PAMs from pigs of different ages, but no significant change in the percent of CD163 receptor positive cells was observed. No significant age-dependent difference in CD169 receptor positive cells was observed except between growing and adult pigs with adult pigs having a higher receptor presence. However, growing pigs had a high animal to animal variation in the percent of CD169 positive cells suggesting that this difference may disappear if more animals were examined. Thus, other mechanisms, such as innate intracellular immune mechanisms which may include cellular viral restriction factors rather than receptor-mediated entry, are likely involved in age-dependent susceptibility to PRRSV infection.

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