EFFECTS OF PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS ON PORCINE ALVEOLAR MACROPHAGE SURFACE PROTEIN EXPRESSION

by

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Abstract

Currently, porcine reproductive and respiratory syndrome virus (PRRSV) is the most economically significant disease affecting the swine industry. PRRSV is known for its restricted cell tropism, primarily infecting porcine alveolar macrophages (PAM) via receptor-mediated endocytosis. PRRSV infects only a portion of the PAM population both *in vivo* and *in vitro*, which suggests that not every macrophage is PRRSV-permissive. Three surface proteins that can act as receptors for PRRSV have been identified on PAM, however, little else is known about the regulation of macrophage tropism. Factors determining cellular permissibility or resistance to PRRSV infection remain largely uncharacterized, although a recent study from our laboratory demonstrated that 1) permissiveness to PRRSV infection increased with time in culture, 2) macrophages from infected pigs could be superinfected, and 3) addition of actinomycin D, which inhibits mRNA synthesis, blocked infection. These data suggest that a PRRSV-permissive subpopulation of cells derives from a non-permissive precursor population and depends on new mRNA synthesis. The current studies were designed to examine the effects of PRRSV on both infected and uninfected PAM cells *in vitro*, specifically focusing on the expression of MHC I, MHC II, CD14, CD163 and CD172a surface proteins. The results show upregulation of MHC II, CD14, CD163 and CD172a expression in PRRSV-infected cells and a downregulation on the uninfected cells within the PRRSV-inoculated cultures. The role of apoptosis in the PRRSV-inoculated cultures was investigated, with results showing similar, low levels of apoptosis in control and infected PAM. PAM cytokine responses to PRRSV and LPS were also examined and, although they were uniquely different relative to control PAM, no trends were detected in the responses of PAM infected with PRRSV compared to uninfected and classically stimulated PAM. These data confirm that there are at least two subsets of macrophages within the alveolar population and suggests that the subsets are differentially affected by PRRS virus. We also demonstrated that MHC I

becomes undetectable on PAM as a result of the freezing process, and that PRRSV-permissiveness is greater in the cell population after freezing.

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CHAPTER 1 - Porcine Reproductive and Respiratory Syndrome Virus Literature Review

Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV), one of the most important infectious agents of swine, is endemic in the world's largest pork producing nations and accounts for massive global economic losses each year. PRRSV is very infectious and capable of transmission through many routes, causing reproductive failure in pregnant sows and respiratory disease and mortality in young pigs. Although it is difficult to assess the cost of PRRS to the industry, estimates currently approximate losses of $600 million in the United States each year. One reason control of PRRSV has been difficult is that the mechanisms by which PRRSV evades host immune responses are poorly understood. Therefore, the purpose of this work was to examine the effects of PRRSV on one of its principal target cells, the alveolar macrophage.

PRRSV

History

Outbreaks of a new disease began occurring in swine in the United States and Western Europe at approximately the same time in the late 1980's. The syndrome, which caused reproductive failure in pregnant sows and respiratory disease in young pigs (7, 21), was initially given several different names, including: mystery swine disease, blue-eared pig disease, porcine epidemic abortion and respiratory syndrome (PEARS) and swine infertility and respiratory syndrome (SIRS) (67). Within a few years the disease, now commonly referred to as Porcine Reproductive and Respiratory Syndrome (PRRS), had become endemic in the world's largest swine producing countries of North and South America, Europe, and Asia, and continues to result in substantial annual economic losses in those countries today. Etiology

The etiologic agent, PRRSV, was isolated in 1991 in the U.S. and the Netherlands (7, 21, 108). PRRSV is a enveloped, single-stranded positive-sense RNA virus with an approximately 15 kb genome containing nine open reading frames (23). The envelope surrounds the RNA and N protein capsid and consists of six structural proteins: the two major membrane proteins,

glycoprotein (GP) 5 and matrix protein (M), and minor structural proteins GP2a, GP3, GP4, envelope protein (E) (23, 85). ORFs 1a and 1b code for non-structural viral replicase proteins, ORFs 2a/b, 3, 4, and 5 and 6 encode structural membrane proteins and ORF7 codes for the nucleocapsid (N) proteins (101, 114).

PRRSV belongs to the *Arteriviridae* family, which is grouped together with the *Coronaviridae* and *Roniviridae* families to form the order *Nidovirales*. Other arteriviruses include lactate dehydrogenase-elevating virus (LDV) in mice, simian hemorrhagic fever virus (SHFV) and equine arteritis virus (EAV) (12), all of which are characterized by persistent infections in the presence of an active host immune response and replication in cells of the monocyte lineage (5, 18). The European PRRSV strain, originally referred to as the Lelystad virus, is representative of genotype 1 viruses (106). VR-2233 isolated in North America, is representative of genotype 2 viruses (7, 21). Although type 1 and type 2 (North American) PRRS viruses share similar morphological and biochemical properties, they share only about 67% nucleotide identity at the genomic level and are antigenically distinct.

Cell Tropism

Both *in vivo* and *in vitro* evidence suggests that, while cells of the monocyte/macrophage lineage are the primary targets for PRRSV, only small subpopulations of these cells are actually permissive to PRRSV infection (16, 34). *In vitro*, several cell types have been determined to be PRRSV-permissive and to propagate viral replication. Besides primary porcine alveolar macrophage (PAM) cell cultures, cultivated primary peripheral blood monocytes (PBMC), monocyte-derived dendritic cells (mo-DC), and the African green monkey kidney cell line MA-104 and cells derived therefrom, such as MARC-145 and CL-2621, have been demonstrated to sustain productive viral replication (34, 45, 55, 90, 107).

PRRSV enters cells via receptor-mediated endocytosis, which is thought to be initiated through viral attachment to cellular receptors and/or coreceptors (49, 65). At present, three potential PRRSV receptors have been identified on PAM: heparin sulfate, sialoadhesin and CD163 (11, 28, 29, 104). In contrast, sialoadhesin is not expressed on the cell surface of MARC-145 cells (44, 113), although two different cellular proteins, CD151 and simian vimentin, have been identified as possible PRRSV-receptors (46, 86). Thus, depending on the cell type there might be as many as five different receptors for PRRSV entry. Additional cofactors involved in susceptibility to PRRSV may be involved in viral attachment an entry.

Transmission

Horizontal PRRSV transmission results when susceptible animals, naïve for a specific PRRSV strain, have contact with infected animals. PRRSV has been isolated in porcine blood, saliva, feces, milk and colostrum (106, 112), and can be acquired through the oral-nasal route. Semen from infected boar studs has been demonstrated to contain virus for up to 92 d.p.i. (19), and is also a common direct route for PRRSV transmission (20). In addition, it has recently been demonstrated that pigs can acquire PRRSV through breaks in the skin barrier (41). While normal pig behavior is sufficient to result in cuts and scrapes, standard management practices including vaccination, ear notching/tagging, tooth clipping and tail docking may create additional routes for PRRSV entry (41). Vertical transmission of PRRSV occurs when pregnant sows and gilts acquire infection during mid to late gestation (17).

PRRSV is highly infectious, and in addition to transmission through direct contact, can be spread by many indirect routes. Fomites, such as boots, coveralls, coolers/containers (71), and transport vehicles (24, 26) can carry virus to new locations and cause infections in naïve pigs. Insects, specifically mosquitoes (*Aedes vexans*) and houseflies (*Musca domestica*) found commonly during the summer months in swine facilities, can also to function as mechanical vectors of PRRSV (71, 73). Insects are thought to function strictly in transport of PRRSV, however, and there has been no documentation of insects serving as biological vectors (72, 85). Rodents, raccoons, opossums, skunks, starlings, sparrows, dogs and cats have all been eliminated as potential biological and mechanical vectors (111) although it is yet undetermined if migratory waterfowl facilitate PRRSV spread (78, 117). Finally, it has been proposed that aerosol transmission of PRRSV may occur both between farms and within pig populations, although conclusive evidence is lacking and the issue remains unresolved (71, 78).

PRRSV Infection

Pathology

PRRS is characterized by reproductive and respiratory signs, and the severity is dependent upon several factors, including: pathogenicity of the viral strain, reproductive status of the infected animal, and age at time of infection (77, 96). In the respiratory form of PRRS, young animals exhibit the most severe pathology and respiratory disease, which is often accompanied by secondary infections (95, 96, 111). During acute PRRS, the virus can be

isolated from most organs and tissues. Later, the virus disappears from the serum and organs, except for continued replication in tonsils, lungs and lymph nodes (5, 77, 110). Adult pigs may also be susceptible to PRRSV-infection. In non-pregnant animals PRRS is typically nonfatal and results in transient flu-like symptoms, including mild fever and inappetance (87). When pregnant gilts and sows become infected during mid- to late-gestation, however, clinical signs are primarily reproductive and include late-term abortions, mummified fetuses, premature farrowing, stillbirths and weak neonates exhibiting high mortality and severe respiratory disease (7, 21).

While strain pathogenicity differences can greatly affect the level of viral replication within a host, differences between individual pigs can also affect both the susceptibility and immune response of an animal to PRRSV infection. For example, individual susceptibilities to PRRSV infection have been demonstrated to have a genetic association, although the exact correlation has yet to be determined (1, 2, 86, 105). Also, health status of an animal may affect PRRSV disease intensity, as several studies demonstrate that concurrent infections with organisms such as porcine circovirus type 2 (PCV2) and *Mycoplasma hyopneumoniae* result in intensified disease and pathology (13, 80).

Persistence

The prolonged period of asymptomatic viral shedding and replication is a hallmark of PRRSV infection, and is generally referred to as a period of chronic viral persistence because of its extended duration compared to typical viral respiratory infections (37). Several studies have examined the duration of PRRSV persistence in pig tissues, but the results are variable, likely the result of differences in pathogenicity of the PRRSV strain used and animal susceptibility (34, 50, 77, 112). PRRSV has been recovered from infected swine populations up to 105 days post-inoculation (d.p.i.) (42), from individual animals 225 d.p.i. (4, 110), and from the serum of congenitally infected animals at 210 days post-farrowing (15). Analysis of tissues in PRRSV-infected animals has identified the thymus, tonsil, and male reproductive organs as the locations of PRRSV replication during the persistent stage of infection (5, 20, 79, 112). Although intensive research efforts have been focused on PRRSV since its emergence, the mechanism by which the virus evades host immune responses is unknown (5, 110) and persistent infection remains a principal contributing factor in the world-wide spread of PRRSV.

Cellular and Humoral Immune Responses

Various features of the porcine immune response to PRRSV infection have been characterized as atypical. For instance, a PRRSV-specific humoral response can be detected at approximately 5-7 d.p.i., however it consists primarily of a non-neutralizing antibody response (23, 50, 116) that lacks the capability to induce complement-mediated lysis of infected cells (22). Studies suggest that these antibodies differ from the normal repertoire in that they are polyclonally activated, containing non-mutated germline configurations in their heavy-chain variable regions (10). At approximately 4 weeks d.p.i., low titers of virus neutralizing antibody can be detected, but their role in protective immunity to PRRSV is controversial (50, 53, 54). While data are available that demonstrate a protective role for neutralizing antibodies in PRRSV infections (27, 50, 69), other studies have shown that viremia not only occurs in the presence of these antibodies (112), but that low titers might be associated with enhanced viral infection (a phenomenon termed antibody-dependent enhancement of infection, ADE) (116).

A delayed T-cell response is also seen in PRRSV infections, and results in unusually weak Th1 cytokine secretion and high Th2 responses. Generally, interferon gamma (IFNγ) secreting-cells and proliferative lymphocyte responses occur by 4-8 weeks p.i. (50, 115), but both the T-cell mediated immune response and antibody titers to PRRSV have been reported to decline over time while abundant viral antigen is still present in lymphoid tissues (63, 115). Thus a delay in certain functions of the humoral and cellular arms of the immune response may be involved in the inefficient viral clearance resulting in persistent infections.

Cytokine Production

Intracellular pathogens, such as viruses, are generally processed via major-histocompatibility I (MHC I) pathways which utilize a proteosome to degrade cytosolic proteins into linear peptides, then display them in antigen binding grooves on MHC I proteins. Cytotoxic T-lymphocytes (CTLs) are required for recognition of the foreign antigens displayed on MHC I proteins, and recognition stimulates an immune response appropriate for those antigens. Extracellular pathogens (e.g. bacteria and whole viruses) are broken down and processed via the major-histocompatibility complex MHC II pathway which utilizes specialized antigen-presenting cells to engulf and degrade the pathogens and present the components via the MHC II protein. Since several types of cells are involved in an active immune response, the host system uses

cytokines to specifically activate the appropriate effector cells, which ensures that responses proceed along the correct processing pathway for efficient pathogen elimination (38).

Current data suggest that a Th-2 mediated humoral immune response occurs during PRRSV infection, resulting in inefficient elimination of virus and potentially constituting persistent infections in viremic pigs (64, 107). This hypothesis is supported by studies that show that interleukin-10 (IL-10), a Th2 cytokine that protects cells from apoptosis, is upregulated *in vivo* in infected pigs (80, 91, 92, 98), and *in vitro* in infected cultured macrophages, monocytes and dendritic cells (35, 91). Interestingly, the Th-1 associated cytokine, Interferon-γ (IFNγ), is also upregulated in response to PRRSV infection (32, 57, 62, 109), and although it has been shown to be involved in immune responses against PRRSV, its appearance is generally delayed (6, 84, 102).

In contrast, studies to evaluate the involvement of other cytokines in the immune response to PRRSV have shown mixed results. For example, weak upregulation of tumor-necrosis factor-α (TNFα), an important Th1 cytokine, was documented in some studies (14, 37), while substantial TNFα induction was observed in others (13, 80). Likewise, evidence of poor interferon-α (IFNα) induction compared to other porcine respiratory viruses exists for both *in vivo and in vitro* infections (3, 8, 61, 102), and correlates with reports that IFNα blocks PRRSV replication (55) but contrasts with an *in vitro* study that demonstrates that pre-treatment of monocytes with IFNα results in a strong increase in susceptibility to PRRSV-infection (30). Collectively, however, data suggest that PRRSV manipulates host cells to regulate production of cytokines which are involved in viral replication and transmission.

Apoptosis

Apoptosis is an innate defense mechanism that often functions to eliminate virally infected cells through tightly regulated induction of cell death. Some viruses, however, regulate apoptosis in target cells as a means of facilitating viral propagation. Recently, the predominance of transcripts involved in promoting cell survival has been demonstrated in PRRSV-infected PAM using microarray analysis (37). This study showed an upregulation of anti-apoptotic transcripts and downregulation of pro-apoptotic genes during a 12 hour viral incubation, which corresponds with other studies that describe an initial upregulation of anti-apoptotic genes during the early stages of PRRSV infection and pro-apoptotic genes in later stages (47), and contributes to data suggesting that PRRSV actively manipulates apoptosis regulatory genes in order to

complete its viral replication cycle. While several studies have demonstrated the induction of apoptosis by PRRSV *in vivo* and *in vitro* it is controversial whether the apoptosis is directly or indirectly associated with PRRSV infection (37, 47, 51, 61, 89). Some data that suggest that PRRSV directly induces apoptosis in infected cells, while contrasting information indicates that apoptosis occurs in bystander, non-infected cells.

Objectives of this study

Factors determining cellular permissiveness or resistance to PRRSV infection remain largely uncharacterized. A recent study from our laboratory (36) demonstrated that 1) permissiveness to PRRSV infection increased with time in culture, 2) cultured PAM from infected pigs could be superinfected, and 3) addition of actinomycin D, which inhibits mRNA synthesis, blocked infection. Implications were that a PRRSV-permissive subpopulation of cells arises in culture from a non-permissive precursor population. The current study was designed to examine potential factors involved in susceptibility to PRRSV infection by investigating phenotypic differences between infected and non-infected PAM within a single population *in vitro*.

CHAPTER 2 - Materials and Methods

Cells and Virus

PAM cells were obtained from 4-6 week old PRRSV-seronegative pigs (confirmed via ELISA) using 1L of sterile PBS to lavage lungs from each animal immediately following euthanasia. Lavage fluid was collected by filling each set of lungs with PBS three times, gently massaging to help separate the cells from tissue between PBS aliquots, and pouring the solutions into 50 ml conical tubes. Cells were subsequently washed two times in Dulbecco's modified eagles medium (DMEM) (Gibco), then frozen and stored in liquid nitrogen at 2 x 10^7 cells/vial in Opti-Freeze DMSO Cryopreservation Medium (Fisher). Centrifugation was at 730 g for 5 minutes unless otherwise noted. PAM were incubated in a 37ºC water bath for approximately five minutes after removal from liquid nitrogen to rapidly thaw the cells, then were washed in RPMI-1640 medium (Cellgro) to remove the DMSO-containing freezing medium. PAM were

re-suspended in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% fungizone and 1% penicillin/streptomycin, checked for viability using .04% trypan blue to stain a representative portion of the cells, then seeded on 12-well polystyrene tissue culture plates. Medium was replaced after one hour of incubation at 37°C and 5% CO_2 to remove non-adherent cells, then incubation was resumed. Because PAM have been demonstrated to have increased permissiveness to PRRSV infection after a 24 hour culture period (34, 36), cells were infected after 24 hours of incubation using PRRSV isolate NVSL 97-7895. Cells were removed from the plastic cell culture plates using a plastic scraper after 18 hrs of incubation V- bottom plates (Starstedt) and individually incubated with specific monoclonal antibodies to surface proteins found on macrophage lineage cells, then with secondary, isotype specific antibodies. Negative and secondary isotype controls were included for each experiment. After labeling, cells were fixed in 4% paraformaldehyde for a minimum of 30 minutes, then transferred PBS, pipetted into 5 ml polystyrene round-bottom falcon tubes (BD Biosciences) and analyzed by flow cytometry.

PRRSV isolate NVSL 97-7895 was used in this study at a multiplicity of infection (m.o.i.) of approximately 0.1 TCID50 per cell. Virus stocks were grown on confluent MARC-145 cells in T-75 tissue culture flasks using minimal essential medium (MEM) (Gibco) + 7% FBS + 1% pen/strep + 1% fungizone and incubated at 37°C, in 5% CO2. Virus stocks were prepared by collecting high-titer (106-8 TCID50 per ml) culture supernatants 48-72 hr post inoculation (p.i.) and freezing them in cryovials (Nalgene) at -80°C in until used. Titrations were performed in triplicate on confluent MARC-145 cells using 1:10 serial dilutions, and m.o.i. was calculated as the ratio between the TCID50 value and the number of cells in culture Cytopathic effect (CPE) was identified by the characteristic rounding and detachment of PAM and MARC-145 from the culture plate surface as visualized by microscopic observation. In addition to NVSL 97-7895, several other PRRS viruses were examined for their ability to infect PAM cells, including PR-GFP, VR2332, SD23983, 98-87, 06-625.22, two SD type 2 PRRSV strains (2672 and 2367), and passages 13 and 29 of 97-7895 PRRSV isolates.

Antibody Labeling

PAMs were removed from tissue culture plates using plastic disposable scrapers and washed twice using cold first wash buffer (FWB; PBS + 10% ACD + 2% FBS + 1% phenol red + .4% sodium azide (at 20%)). Surface marker expression was evaluated by individually incubating approximately 1 x 10^6 PAM with recommended concentration of monoclonal antibodies specific for the antigen of interest (see Table 1). After incubation, cells were washed three times in FWB to remove unbound antibody, then incubated with R-Phycoerytherin- (RPE) conjugated secondary antibody (Caltag, Serotec) specific for the isotype of the primary antibody. Cells were washed four times in second wash buffer (SWB; PBS + 10% ACD + 1% phenol red + .4% sodium azide (at 20%)), then fixed in 4% paraformaldehyde. PRRSV-infected cells were detected using the Cytofix/Cytoperm™ Fixation/Permeabilization kit (BD Biosciences, protocol followed as directed) and a standard dilution of 1:100 fluorescein isothiocyanate (FITC)-conjugated SDOW17 antibody specific to the PRRSV nucleocapsid protein. SDOW17 antibody is reactive with a portion of the N-protein that is conserved between type 1 and type 2 PRRSV isolates, and was generously provided by Dr. Eric Nelson of South Dakota State University in Brookings (67). After incubation with SDOW17 antibody, cells were washed four times, then were resuspended in PBS and analyzed using flow cytometry.

Immunofluorescence Assay

Medium was aspirated from PAM cultures 18 hours after viral inoculation. The adherent cells were fixed in 80% acetone for ten minutes, and then were allowed to air dry. PAMs were labeled using FITC-conjugated SDOW17 antibody, and the percentage of infected cells was determined using fluorescence microscopy.

Measurement of Cytokine Production

Bacterial lipopolysaccharide (LPS) (Sigma-Aldrich) was used as a positive control to stimulate the classical activation pathway in PAM. LPS was added to cultured PAM at 1 ug/ml when media was changed after one hour of incubation. Intracellular cytokine proteins made in infected and control PAM were evaluated using the Cytofix/Cytoperm™ Fixation/Permeabilization kit with GolgiPlug™ containing the protein transport inhibitor brefeldin A (BD Biosciences). In this assay, the addition of brefeldin A to cell cultures blocks intracellular transport, resulting in

accumulation of cytokine proteins in the Golgi complex. Cells were treated with GolgiPlug™ 12 hours before collection. PAM were removed from tissue culture plates using plastic disposable scrapers and permeablized. Cytokines were labeled using monoclonal antibodies to TNFα, TGFβ1 and TGFβ2 (Santa Cruz Biotechnology) and FITC-conjugated secondary antibodies (Caltag), then analyzed by flow cytometry. Controls for surface expression of the cytokines were included.

Apoptosis Assay

Three different techniques were used to evaluate apoptosis in infected and control PAM. The first assay utilized FITC-conjugated Annexin V (Serotec) to bind to membrane phospholipid phosphatidylserine (PS) which is exposed on the cell surface during the early stages of apoptosis (according to literature provided by the manufacturer), and was analyzed using flow cytometry. Detection of PS identifies cells entering apoptosis that have not yet undergone nuclear changes such as DNA fragmentation. For the second assay, proteolytic enzymes called caspases were assayed to identify cells between the early and late stages of apoptosis. Caspases are a central component of apoptosis that participate in substrate cleavage, and were detected by binding of carboxyfluorescein labeled fluoromethyl ketone (FMK)-peptide inhibitors (Cell Technology Incorporated) to the activated caspases then analyzed using flow cytometry. The third technique examined cellular DNA for the fragmentation characteristic of late stages in apoptosis, using QIAamp® DNA Mini Kit (Qiagen) to isolate DNA, followed by agarose gel electrophoresis. Actinomycin-D (Ac-D) (EMD Biosciences), which at 500 ng/ml can induce apoptosis, was included as a positive control in each experiment. In all assays, PAM were cultured for 24 hours as described above, infected and incubated an additional 18 hours, then collected by scraping. Ac-D was added to positive control wells after one hour incubation.

Flow Cytometric Analysis

After labeling, cells were stored at 4°C until analyzed. Ten thousand cells from each control and antibody-labeled cell suspension were acquired using a FACSCalibur flow cytometer (BD Biosciences). Parameters used include forward scatter (FSC), side scatter (SSC), fluorescence one (FL1) (green) and fluorescence two (FL2) (orange-red). Data was analyzed using CellQuest

software (BD Biosciences) to calculate percentages of labeled cells and to evaluate fluorescence intensity in the PAM population.

CHAPTER 3 - Results

Selection of virus strain for use in study

Because many PRRSV isolates are available for cell culture and *in vivo* work, this study was initiated by screening different isolates for their capacity to infect macrophages. Ten existing PRRSV-stock viruses were tested. The percentage of PRRSV-infected cells was determined using two different methods: flow cytometry and IFA. The data, presented in Figure 1, showed that isolate 97-7895 (P13) of PRRSV infected the largest percentage of PAM *in vitro*. However, because it is an isolate recently obtained from pigs, because the genome has been completely sequenced, and because it demonstrated a high percentage of infectivity for PAM in culture, we used PRRSV isolate NVSL 97-7895 for the remaining experiments.

Regulation of PAM surface protein expression

Surface Protein Expression in Fresh vs. Frozen PAM Cells

The first experiments were designed to determine whether or not there were differences between freshly isolated and frozen PAMs, in order to confirm whether or not whether freezing had an effect on permissiveness for PRRSV and cell surface marker expression. Surface marker expression was examined by culturing fresh PAM and PAM from the same pig that had been frozen in liquid nitrogen for two months. PAM were cultured for 24 hours then inoculated with PRRSV and re-incubated for an additional 18 hours. Uninfected control cultures were included. Results indicated that fresh PAM cells were less permissive for PRRSV-infection than were frozen PAM, suggesting that the freezing process may result in development of a PRRSV-permissive state in PAM (Figure 2). Surface molecule phenotyping revealed that MHC II, CD14, CD163 and CD172a were not affected by freezing, but that MHC I was no longer detectable. Dot plots illustrate these findings (Figure 3) and a bar graph summarizes the data from PAM taken from three animals (Figure 4).

Differential protein expression in PRRSV-positive and PRRSV-negative cells

In order to gain insight into the phenotypic characteristics of PRRSV-permissive PAM, MHC I, MHC II, CD14, CD163 and CD172a surface proteins on PRRSV-infected and non-infected cells within the same PAM populations were evaluated. There were no detectable differences in the percentage of cells expressing each surface molecule between PRRSV-infected uninfected controls. However, when analyzed using mean fluorescence intensity (MFI), differences were seen between infected and non-infected PAM populations (Figure 5). Mean fluorescence intensity was used to assess the relative number of surface molecules on approximately 90% of the gated population (Figure 6). Mean fluorescence intensities for surface molecules on the PRRSV-infected and non-infected PAM in infected cultures were then compared to control PAM to determine whether or not PRRSV had an effect on surface protein expression in these subpopulations. An overlaid histogram illustrates the upregulation of CD14 in PRRSV-infected cells and downregulation in PRRSV-negative cells from the same culture when compared to control cell surface profiles (Figure 7). Bar graphs representing data from PAM taken from 5 pigs depict the differences between PRRSV-permissive and non-permissive populations, and illustrate the differences in expression levels between the PAM taken from different animals (Figure 8). Raw expression percentages are also shown (Table 1).

Cytokines

In order to characterize the activation pathway utilized by PRRSV infected PAM, we evaluated cytokine expression of PAM within control and LPS or PRRSV-stimulated cultures. Figure 9 depicts profiles of cytokine synthesis in PAM from infected and uninfected cultures. Using tumor necrosis factor α (TNFα) and tumor growth factor β (TGFβ) 1 and 2 to examine classical vs. alternative activation pathways, respectively, we labeled intracellular cytokines with fluorescently tagged antibodies and analyzed them using flow cytometry.

Although cytokine responses to PRRSV and LPS were uniquely different relative to control PAM, no trends were detected in the responses of PAM infected with PRRSV compared to uninfected and classically stimulated PAM. Figure 10 shows the cytokine production in the PAM cultures taken from pigs in this study. Since time courses of cytokine synthesis were performed prior to these experiments, we were confident that the timing of collection was correct. One possible explanation is that the levels of cytokines produced are too low to be

detectable at a significant level using flow cytometry. Because of the electronics systems of flow cytometers, a difference of five percent or greater is recommended before are considered statistically relevant, so that detection of cytokines that are produced in small amounts might be better accomplished using a more sensitive technique (e.g. PCR).

Apoptosis

To determine whether or not apoptosis occurred in the infected PAM cultures, we evaluated apoptosis in control, PRRSV- and Ac-D-treated PAM cultures. Since apoptosis is a complex process, and PRRSV infection can result in atypical apoptosis (47), we used three detection techniques to address the issue. Early apoptosis was evaluated using Annexin V. The results indicated that, while all of the macrophages took up propidium iodide (PI), likely through pinocytosis, fewer than 1% of the PAM labeled for Annexin V, consistent with the idea that apoptosis had not occurred in the infected cells (Figure 11). Caspase detection assays were used to evaluate middle apoptosis, and also show no detectable apoptosis in infected control cultures (Figure 12). Finally, a DNA fragmentation assay was utilized to evaluate late apoptosis; no fragmentation was detected, suggesting that no cells were in the late stages of apoptosis (Figure 13). Thus, the PAM in PRRSV inoculated cultures do not appear to have been in apoptosis. However, it remains to be determined whether or not apoptosis has occurred in either the infected cells or the uninfected cells in the inoculated cultures.

Figures and Tables

specificity	clone name	isotype	source
MHC I	2G5	IgG2b	Serotec
MHC II DQ	K274.3G8	IgG1	Serotec
CD14	MIL-2	IgG2b	Serotec
CD163	2A100/11	IgG1	Serotec
CD172a	74-22-15A	IgG2b	VMRD
TNFα	MAb11	IgG1	Santa Cruz Biotechnology
TGFβ1	C-16	IgG	Santa Cruz Biotechnology
TGFβ2	Zg-12	IgG2b	Santa Cruz Biotechnology

Table 1: Monoclonal antibodies used in these studies

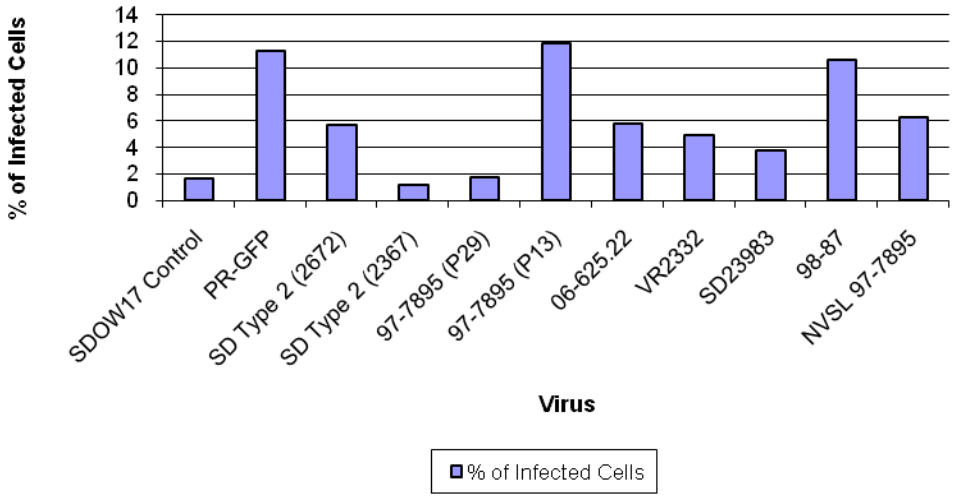


Figure 1: Virus Selection. Virus comparisons were performed by flow cytometric assessment of the percentage of infected cells resulting form inoculation with each isolate. Each bar represents results from a single culture and the virus isolates are named at the bottom of the graph.

	% of Infected Cells
SDOW17 Control	3.3
PR-GFP	13.6
SD Type 2 (2672)	7
SD Type 2 (2367)	3.6
97-7895 (P29)	2.6
97-7895 (P13)	14.3
06-625.22	7.6
VR2332	6.3
SD23983	4
98-87	11.6
NVSL 97-7895	8.3

Table 2: IFA results for virus selection. Virus comparisons were performed by immunofluorescence of PAM detected using a fluorescence microscope. Values are slightly higher than those in Figure 1, likely due to differences in equipment sensitivity.

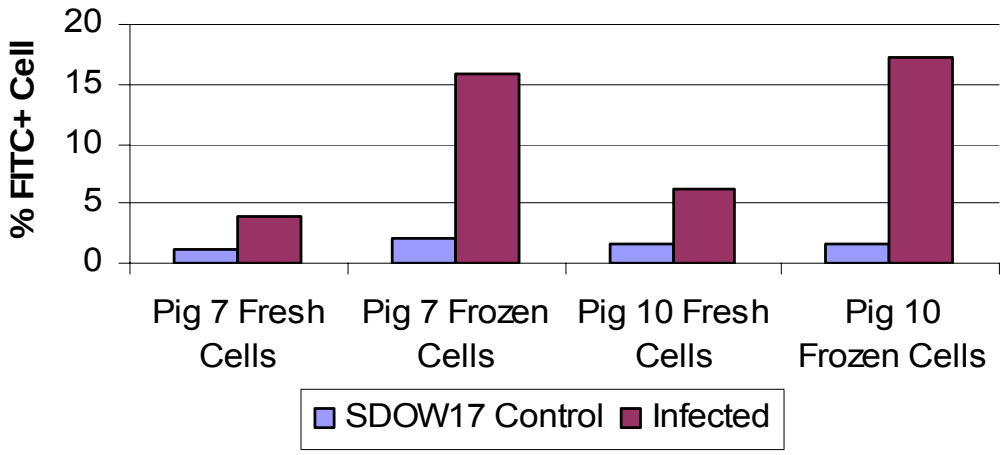
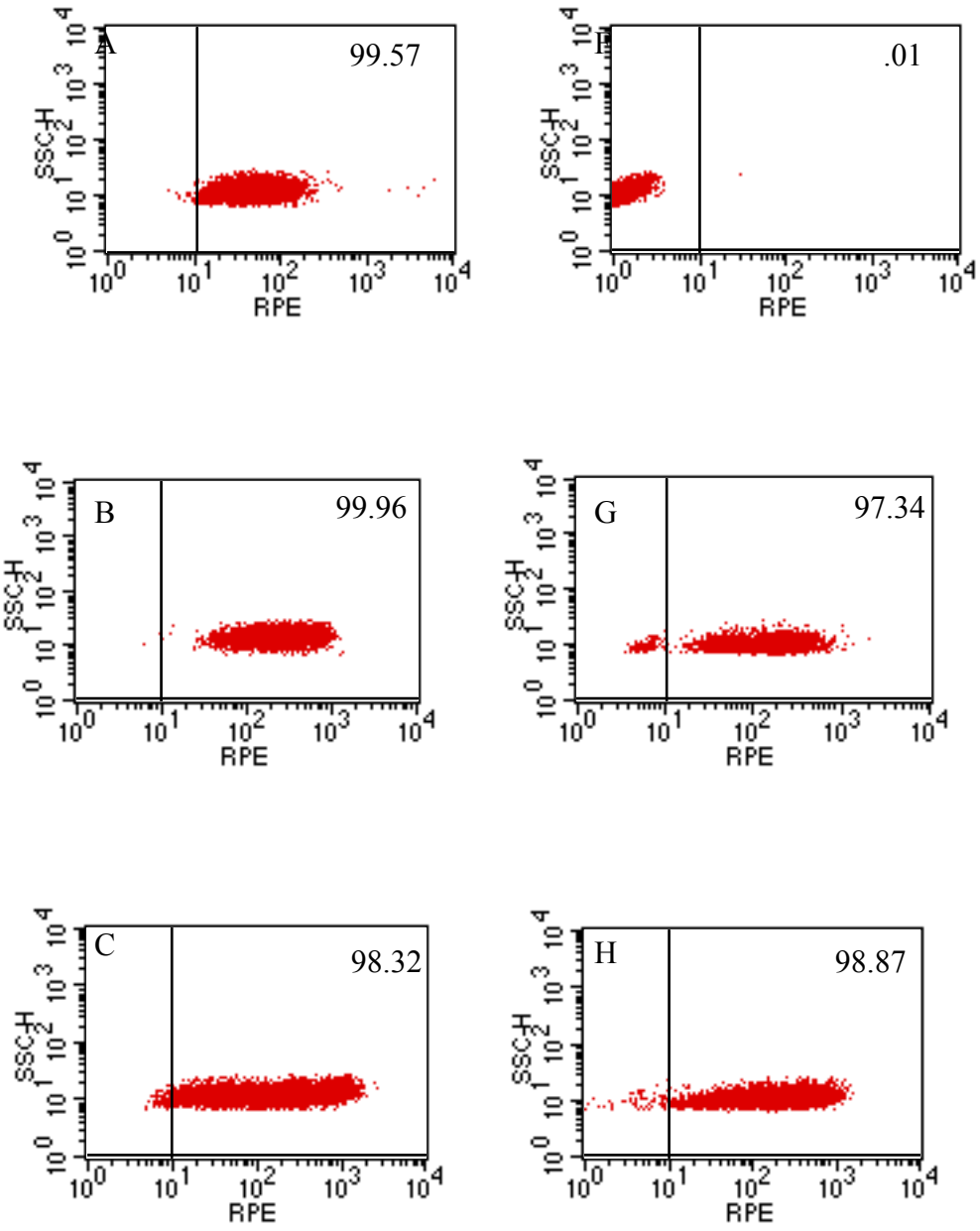


Figure 2: Fresh vs. Frozen PAM Infectability. PAM susceptibility to PRRSV infection after freezing was determined by flow cytometric analysis of the percentage of infected cells resulting from inoculation of either fresh PAM or those that had been frozen. PRRSV permissiveness in PAM from two animals is depicted, and each bar represents results from a single culture.


A
99.57
E
.01
B
99.96
G
97.34
C
98.32
H
98.87
SSC-H
RPE

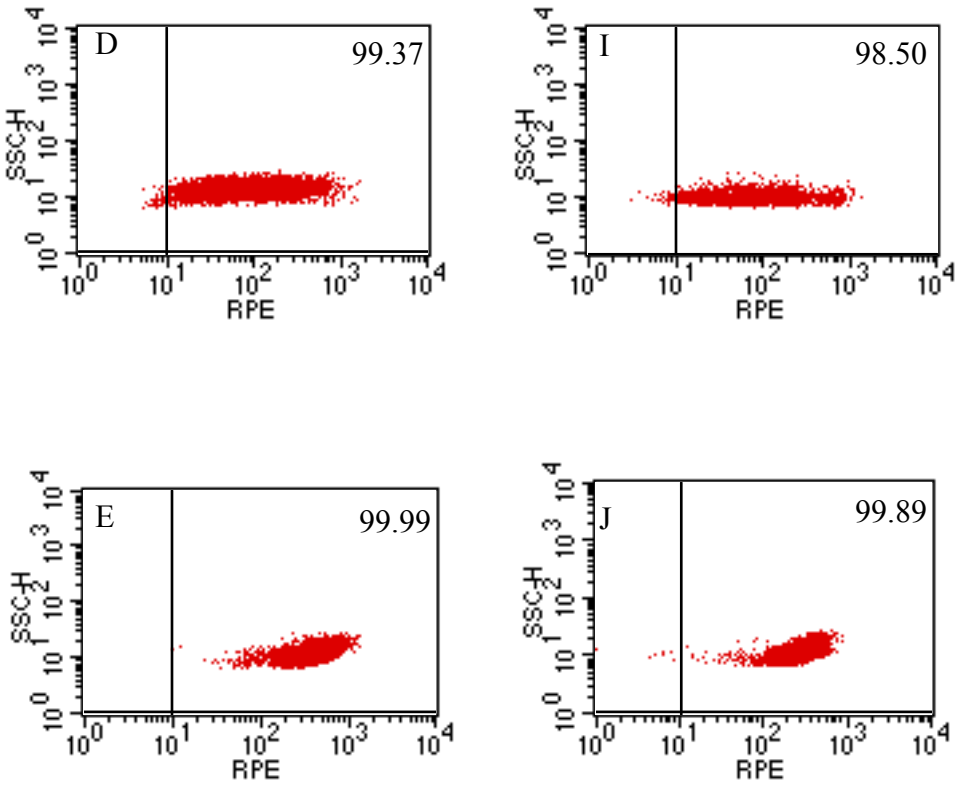


Figure 3: Dot plots illustrating surface protein expression levels in fresh and frozen PAM. Surface antigen comparisons were performed by flow cytometric assessment of the percentage of cells expressing individual surface proteins in fresh and frozen PAM from the same animal (given in upper right of each dot plot). Dot plots represent results from a single culture. A-E = fresh PAM cultures, F-L = frozen PAM cultures. Panels A and F depict MHC I expression levels, panels B and G depict MHC II expression levels, panels C and H depict CD14 levels, panels D and I depict CD163 expression levels, and panels E and J depict CD172a levels.

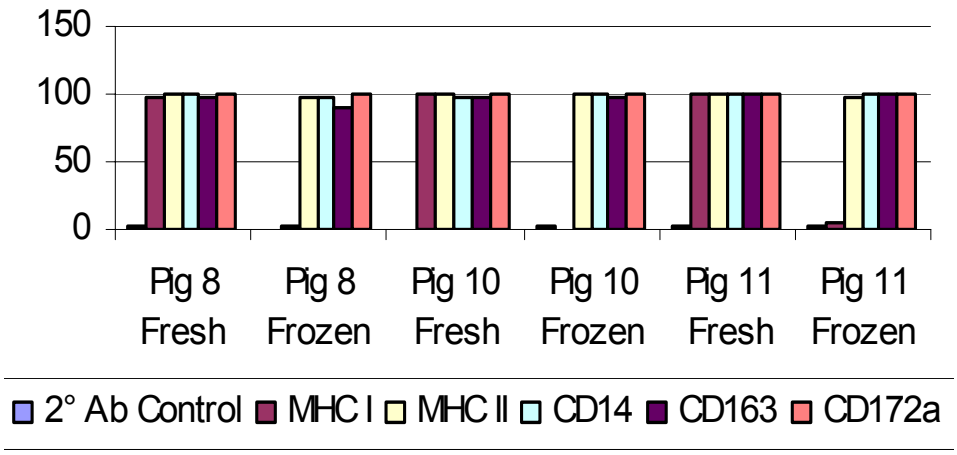


Figure 4: Surface Protein Expression in Fresh and Frozen PAM. Bar graph representation of the data in Figure 3 demonstrates comparable levels of most surface antigens after PAM were frozen. Each bar represents a single surface protein, and three different animals' PAM are shown. Surface molecule name and animal number are listed at the bottom of the graph.

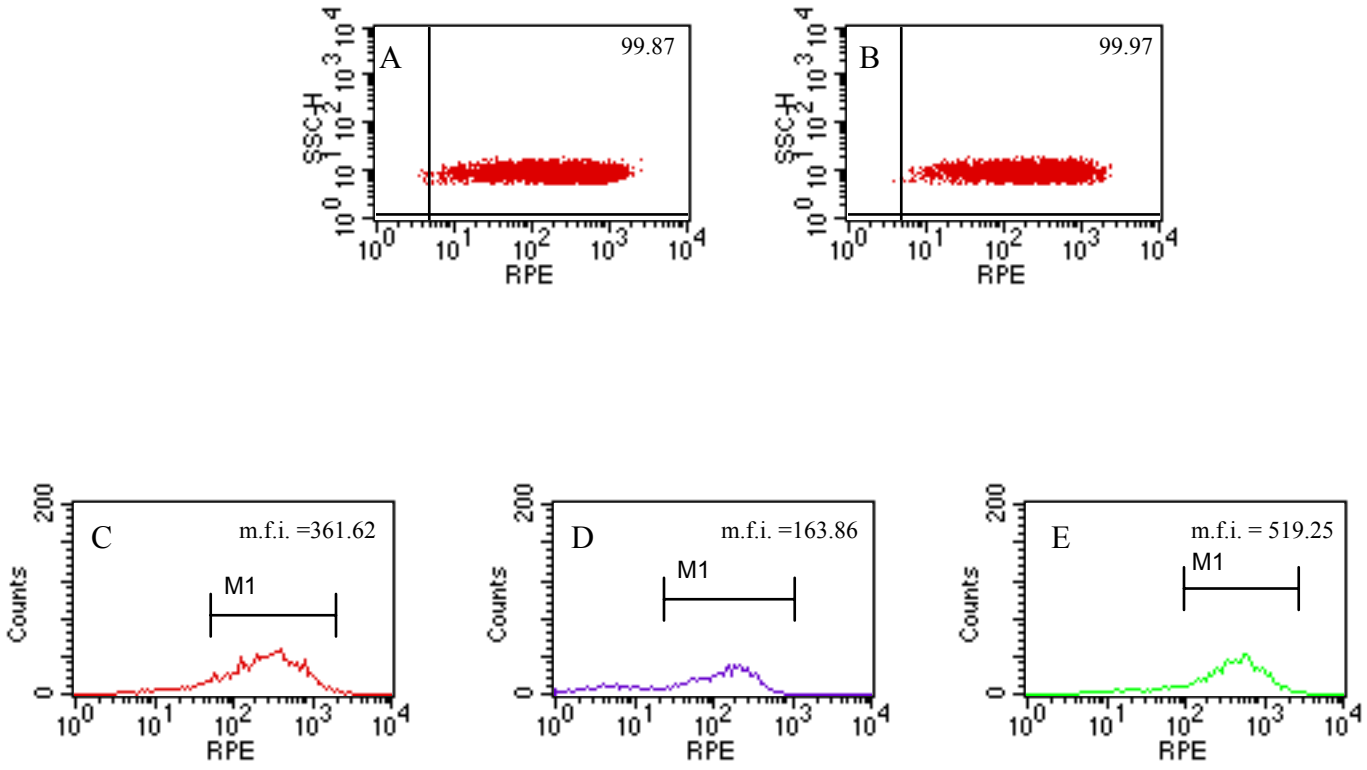
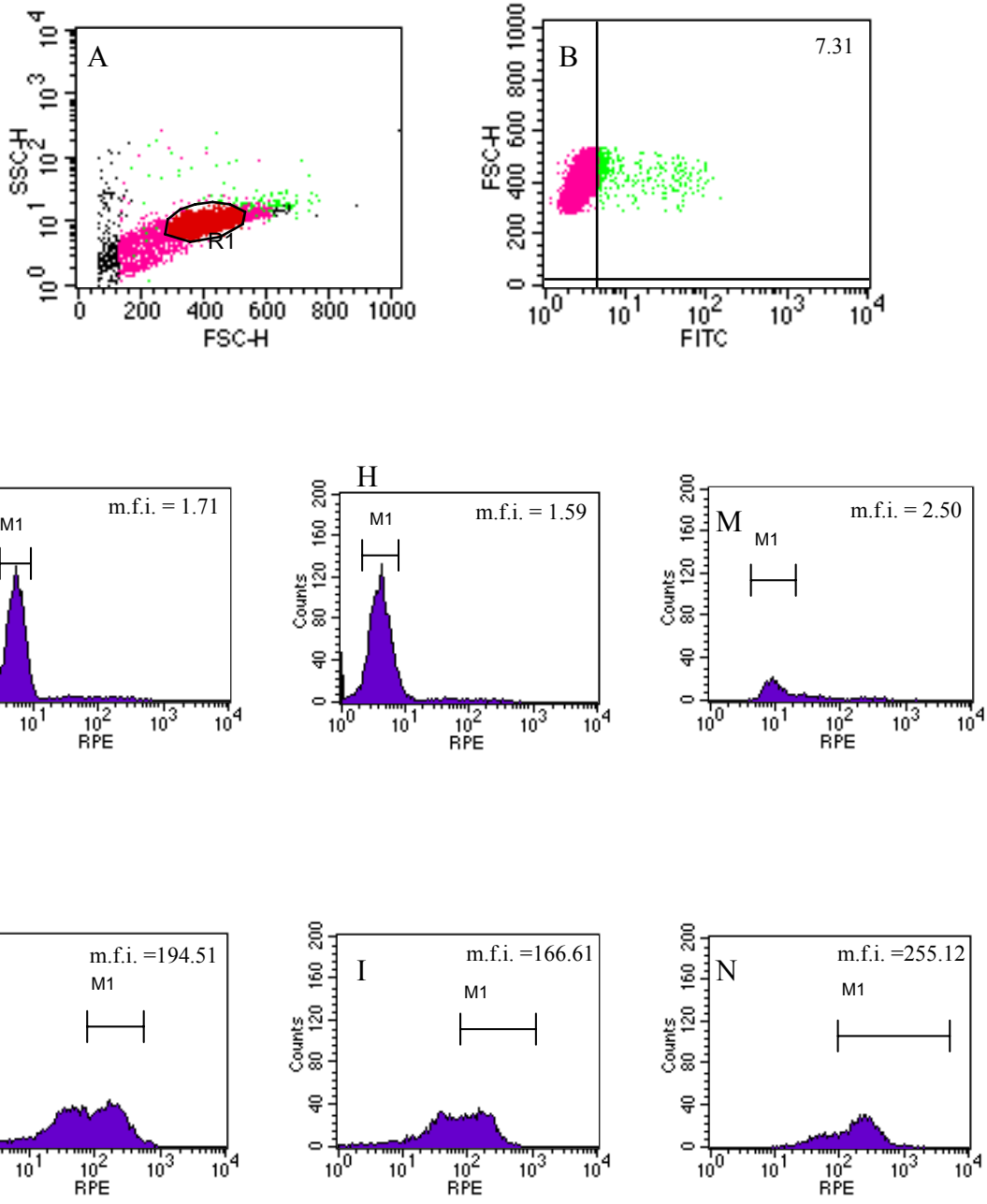


Figure 5: Representative histograms show a difference in CD14 expression levels, while dot plots demonstrated no change. Surface protein expression comparisons were performed by flow cytometric assessment of PRRSV infected and control PAM. A-B depict the percentage of infected cells expressing CD14 using dot plots, and C-E depict the level of CD14 expressed on 90% of the PAM in our population of interest using histograms. Percentage of surface protein expression and MFI are expressed with each figure. Panel A depicts CD14 expression on uninfected control PAM, and panel B depicts CD14 expression on PRRSV infected PAM. Panel C depicts MFI for CD14 expression in uninoculated control cultures, panel D depicts MFI for CD14 expression in PRRSV negative PAM, and panel E depicts MFI for CD14 expression in PRRSV positive PAM.


A
SSC-H
FSC-H
R1
B
7.31
FSC-H
FITC
C
m.f.i. = 1.71
M1
Counts
RPE
H
m.f.i. = 1.59
M1
Counts
RPE
M
m.f.i. = 2.50
M1
Counts
RPE
D
m.f.i. =194.51
M1
Counts
RPE
I
m.f.i. =166.61
M1
Counts
RPE
N
m.f.i. =255.12
M1
Counts
RPE

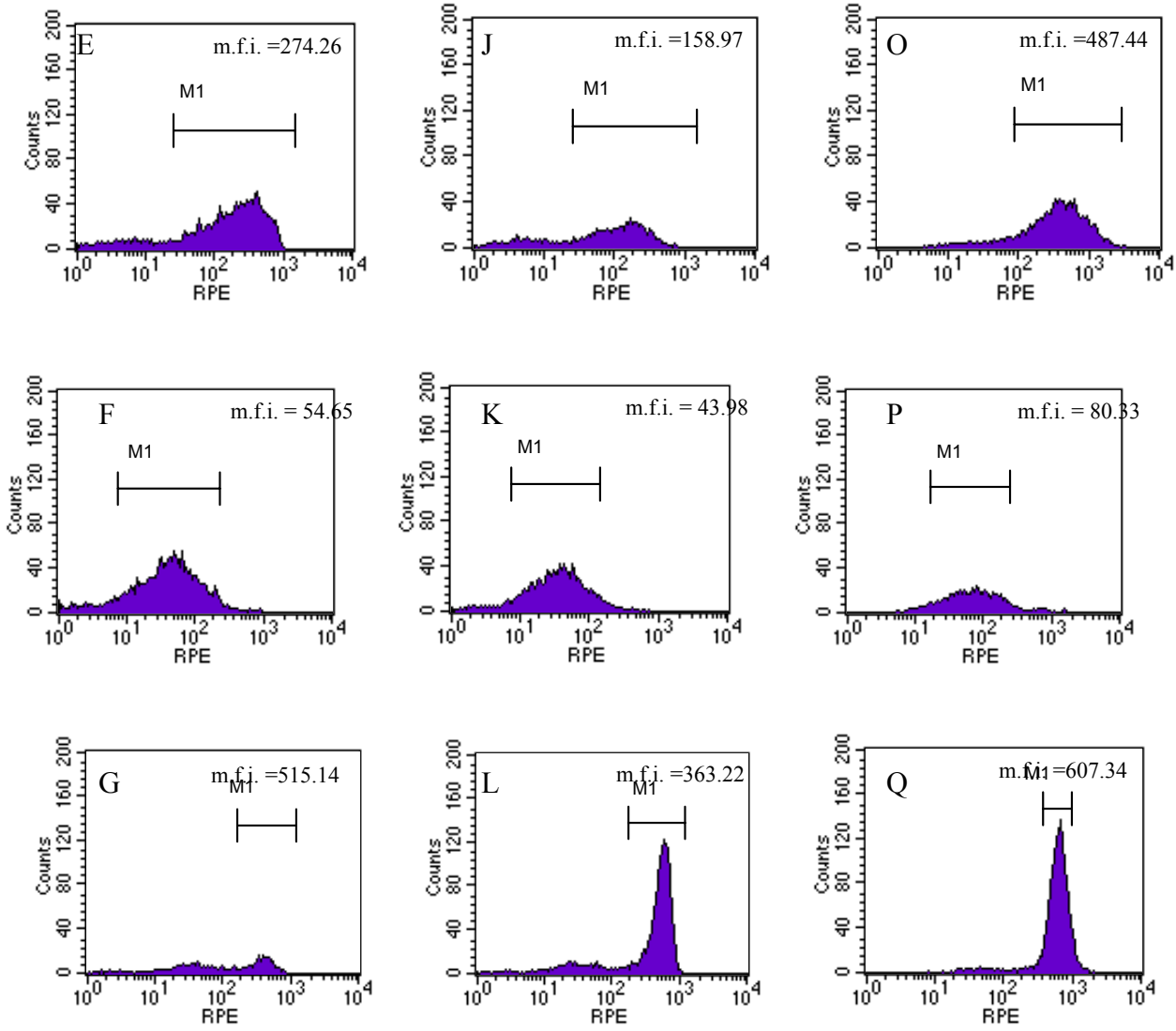


Figure 6: Representative histograms comparing mean fluorescence in PRRSV-positive and negative cells compared to control populations. Surface molecule expression levels on PRRSV infected and uninfected PAM within a single culture are compared to uninoculated control PAM using flow cytometric analysis and histogram representation. Panels A and B depict the cell population of interest, C-G are uninoculated control PAM, H-L depict PRRSV negative PAM within a PRRSV inoculated culture and M-Q depict PRRSV positive PAM. Panels C, H and M depict MHC I expression, panels D, I and N depict MHC II expression, panels E, J and O depict CD14 expression, panels F, K and P depict CD163 expression, and panels G, L and Q depict CD172a expression. The percentage of surface protein expression and MFI are expressed with each figure.

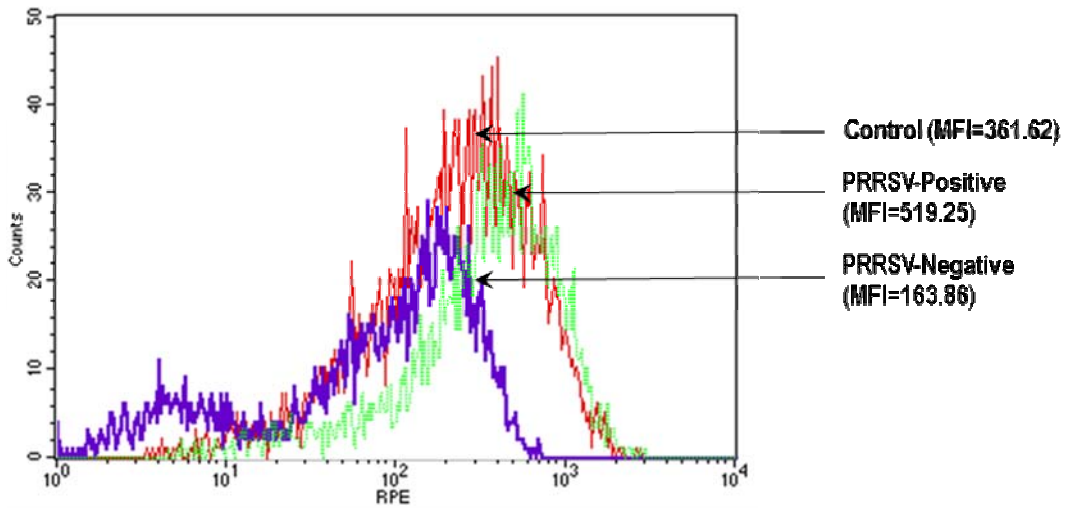
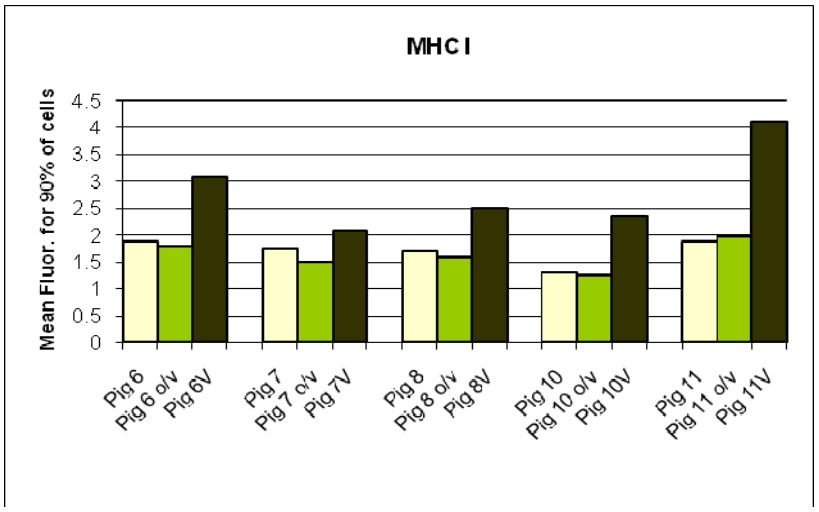
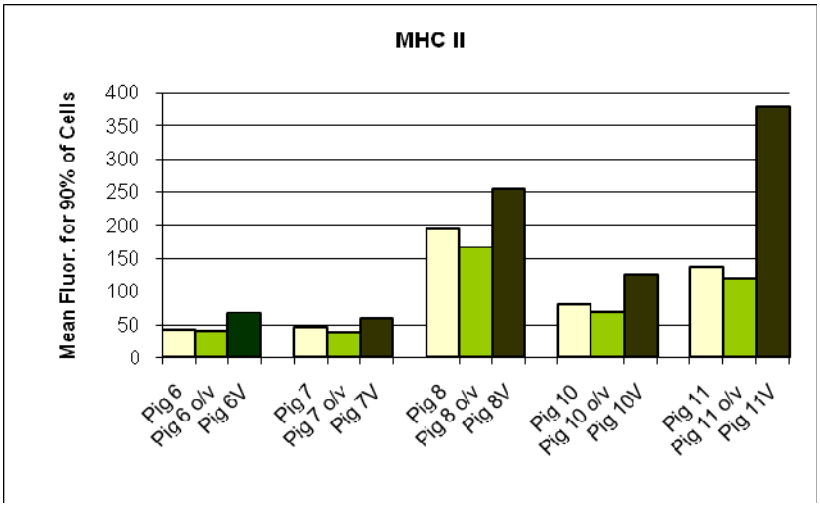


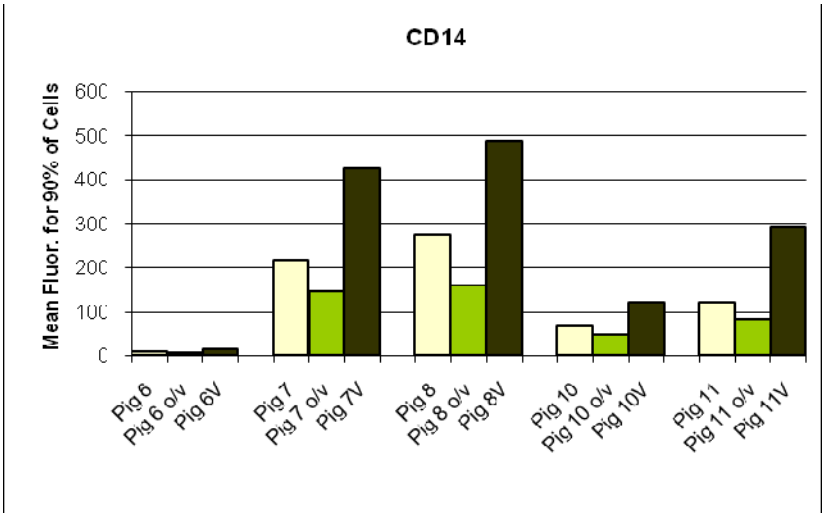
Figure 7: Overlaid CD14 histograms illustrate mean fluorescence intensity differences. A histogram overlay depicts differences in expression levels of CD14 between PRRSV infected and uninfected PAM compared to uninoculated control PAM. Each overlay is labeled with the population it represents and the MFI in the histogram above.

Pig #/treatment	Cell Surface Molecule (MFI)				
	MHC I	**MHC II**	**CD14**	**CD163**	**SWC3a**
P6 PAM[a]	1.87	42.72	7.7	56.18	93.23
P6 PAM Neg[b]	1.8	40.41	6.89	55.85	78.48
P6 PAM Pos[c]	3.08	67.43	14.46	148.29	128.42
P7 PAM	1.74	45.43	216.44	37.97	132.22
P7 PAM Neg	1.51	37.92	145.78	30.37	87.65
P7 PAM Pos	2.09	58.15	424.75	49.96	222.7
P8 PAM	1.71	194.51	274.26	54.65	515.14
P8 PAM Neg	1.59	166.61	158.97	43.98	363.22
P8 PAM Pos	2.5	255.12	487.44	80.33	607.34
P10 PAM[a]	1.29	80.37	65.59	29	163.55
P10 PAM Neg	1.26	70.06	47.1	21.65	137.88
P10 PAM Pos	2.35	125.39	121.42	40.92	225.9
P11 PAM[a]	1.89	137.13	121.05	101.54	291.5
P11 PAM Neg	1.96	118.95	83.37	84.54	202.67
P11 PAM Pos	4.12	379.2	291.37	139.41	315.61

Table 3: Raw data showing differences in expression levels of surface proteins. Data from Figure 6 is presented in table form. a = control PAM, b = PRRSV negative PAM and c = PRRSV positive PAM. Antibody control range was .23-1.89%


MHC I
Mean Fluor. for 90% of cells
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Pig 6
Pig 6 o/v
Pig 6V
Pig 7
Pig 7 o/v
Pig 7V
Pig 8
Pig 8 o/v
Pig 8V
Pig 10
Pig 10 o/v
Pig 10V
Pig 11
Pig 11 o/v
Pig 11V


MHC II
Mean Fluor. for 90% of Cells
400
350
300
250
200
150
100
50
0
Pig 6
Pig 6 o/v
Pig 6V
Pig 7
Pig 7 o/v
Pig 7V
Pig 8
Pig 8 o/v
Pig 8V
Pig 10
Pig 10 o/v
Pig 10V
Pig 11
Pig 11 o/v
Pig 11V


CD14
Mean Fluor. for 90% of Cells
600
500
400
300
200
100
0
Pig 6
Pig 6 o/v
Pig 6V
Pig 7
Pig 7 o/v
Pig 7V
Pig 8
Pig 8 o/v
Pig 8V
Pig 10
Pig 10 o/v
Pig 10V
Pig 11
Pig 11 o/v
Pig 11V

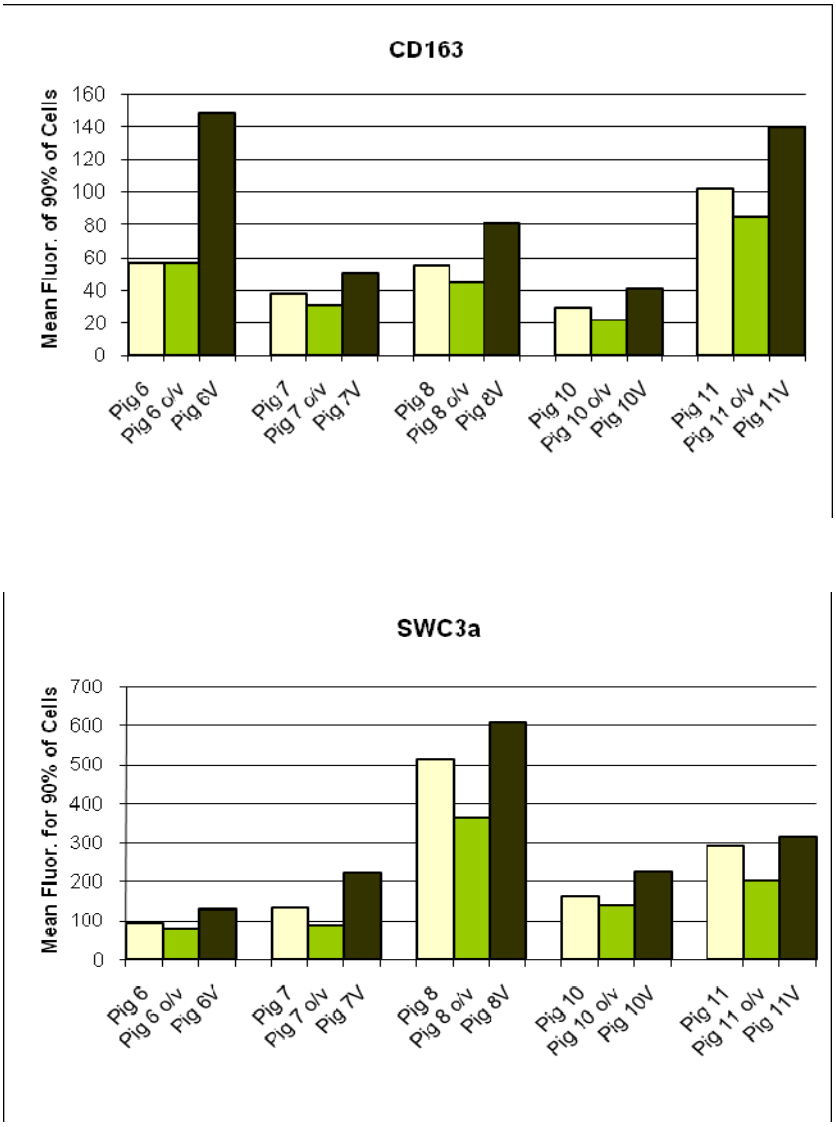
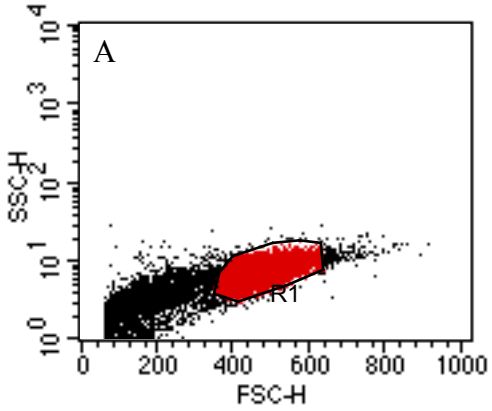
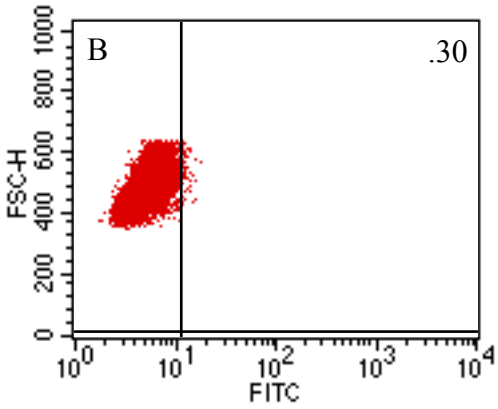
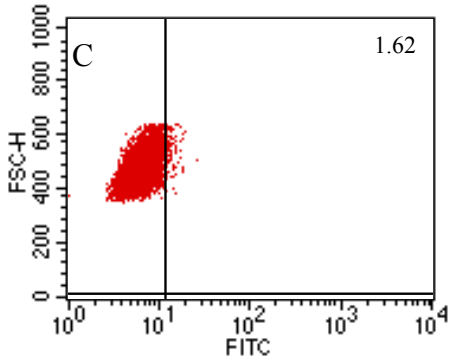
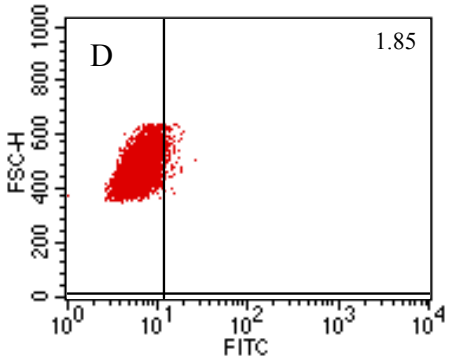


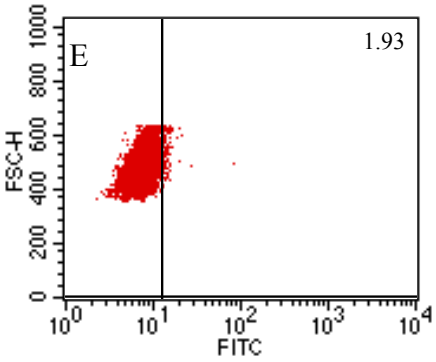
Figure 8: Bar graph representations of surface protein expression levels in five different animals. Surface molecule expression levels were examined for five different animals as described in Figure 6. Data is presented in bar graph form to depict differences between PAM treatment groups as well as expression levels in individual animals. The molecule of interest is labeled above each graph, as are animal ID's. Treatments for each animal include control PAM (labeled as plain animal ID), non-infected PAM within PRRSV-inoculated cultures (labeled as animal ID o/v), and infected PAM from the same culture (labeled as animal ID V).

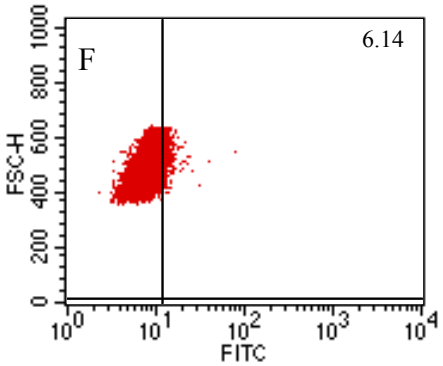

A
SSC-H
FSC-H
R1

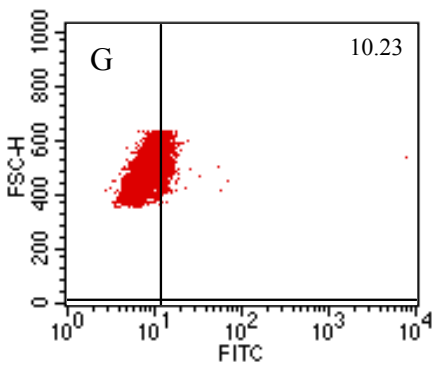

B
.30
FSC-H
FITC

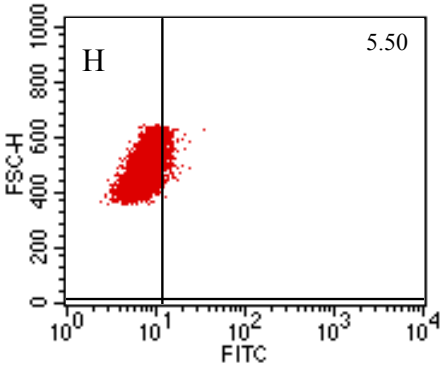

C
1.62
FSC-H
FITC

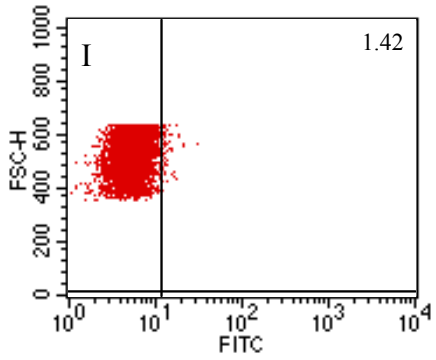

D
1.85
FSC-H
FITC

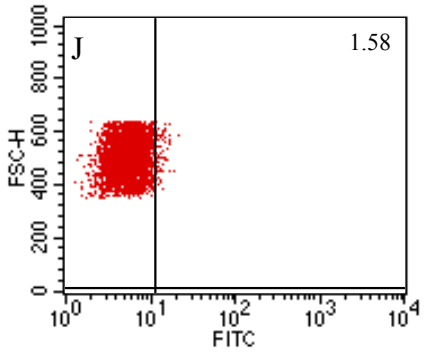

E
1.93
FSC-H
FITC

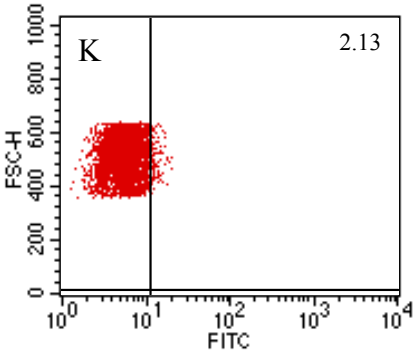

F
6.14
FSC-H
FITC

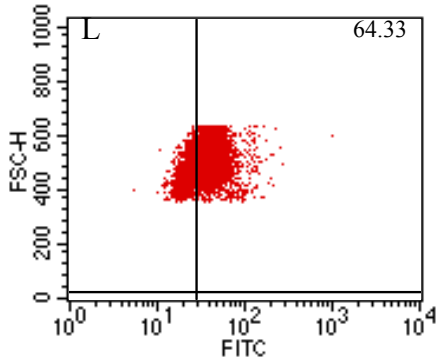

G
10.23
FSC-H
FITC

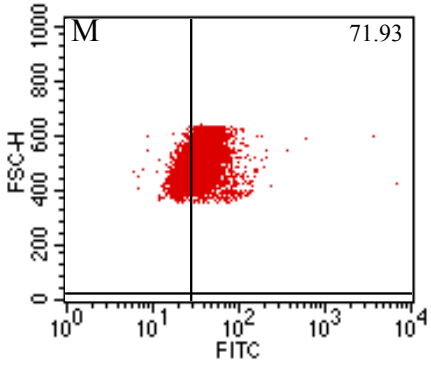

H
5.50
FSC-H
FITC

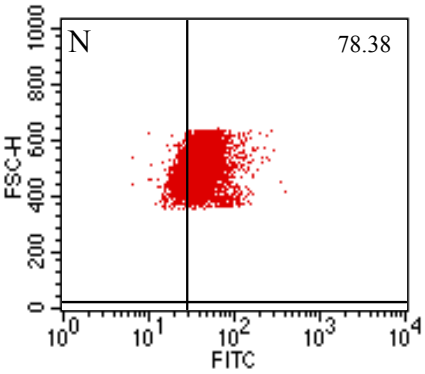

I
1.42
FSC-H
FITC

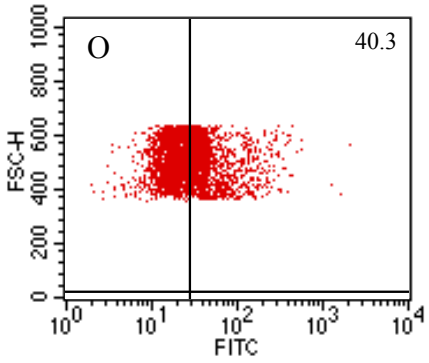

J
1.58
FSC-H
FITC

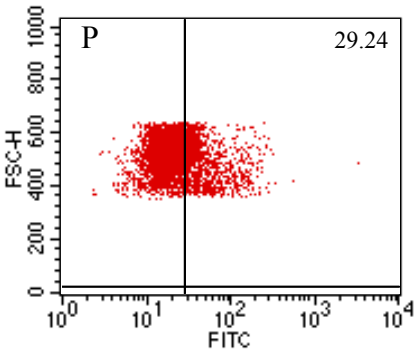

K
2.13
FSC-H
FITC

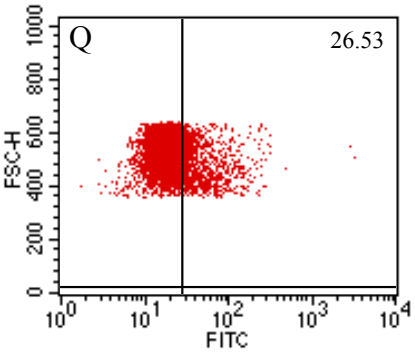

L
64.33
FSC-H
FITC


M
71.93
FSC-H
FITC


N
78.38
FSC-H
FITC


O
40.3
FSC-H
FITC


P
29.24
FSC-H
FITC


Q
26.53
FSC-H
FITC

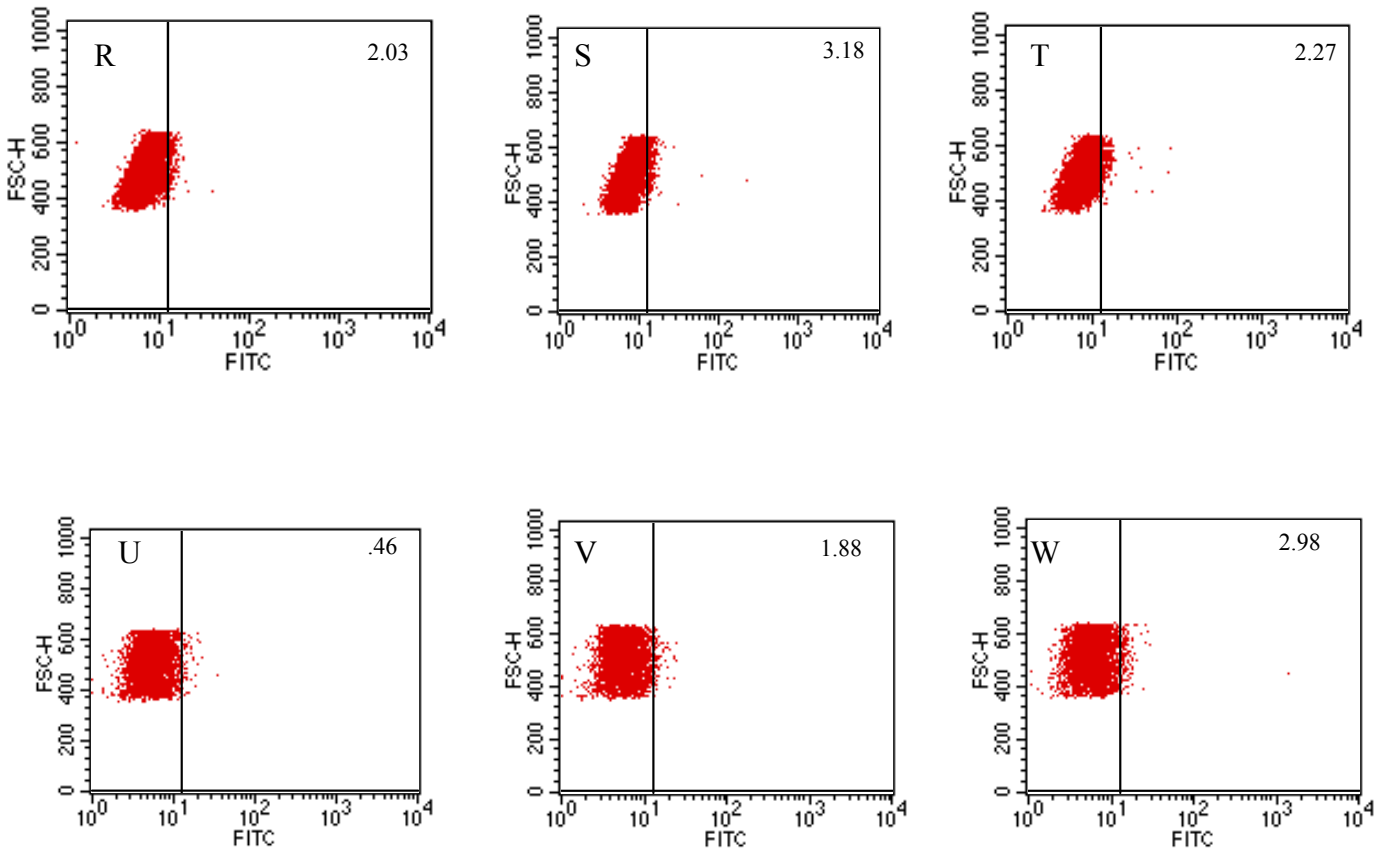
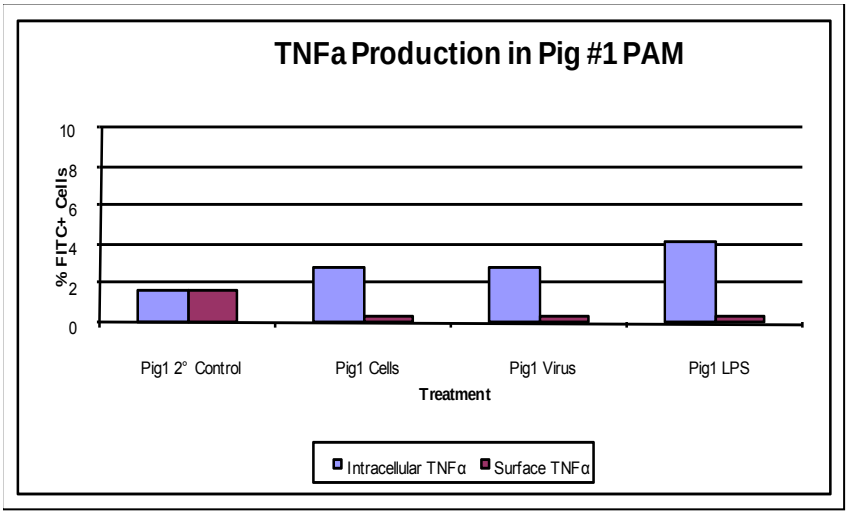
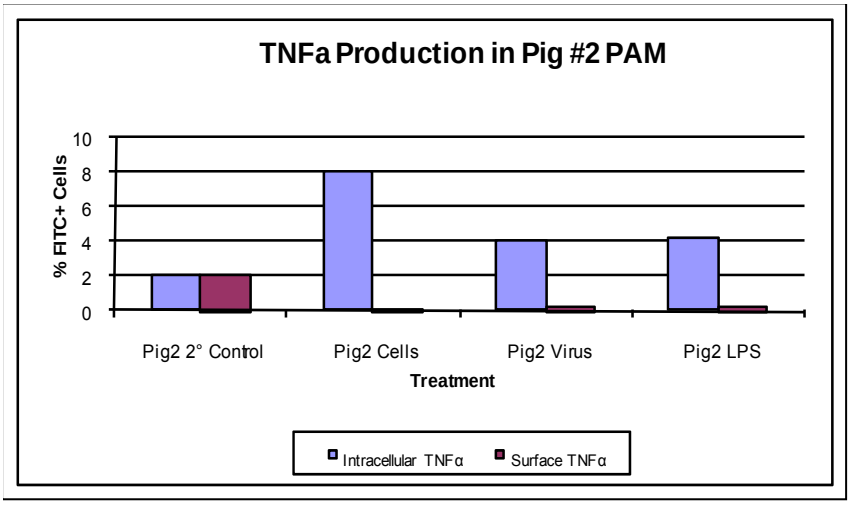
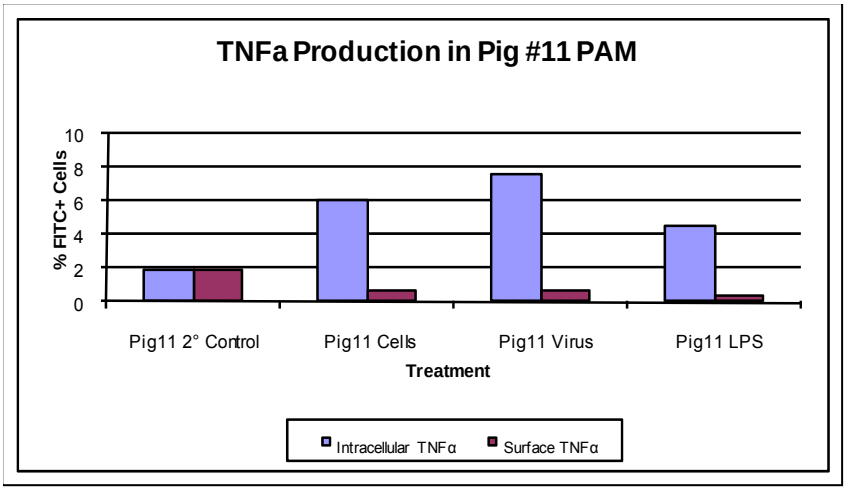
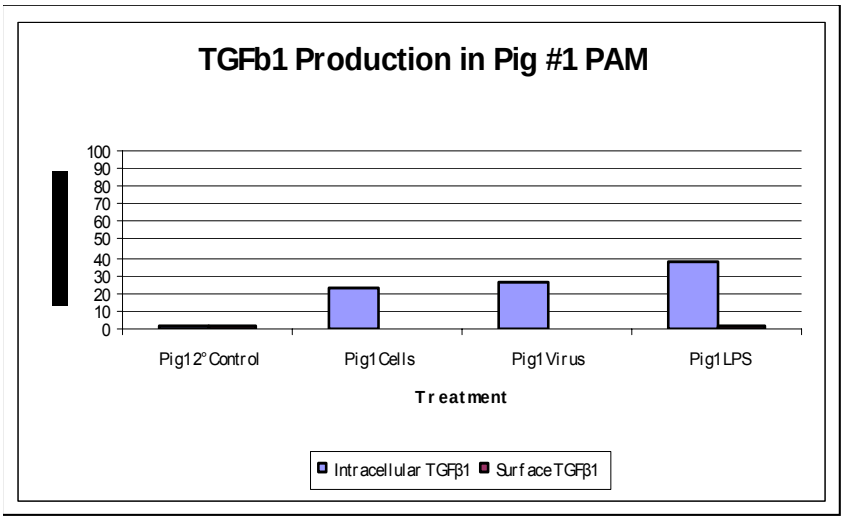


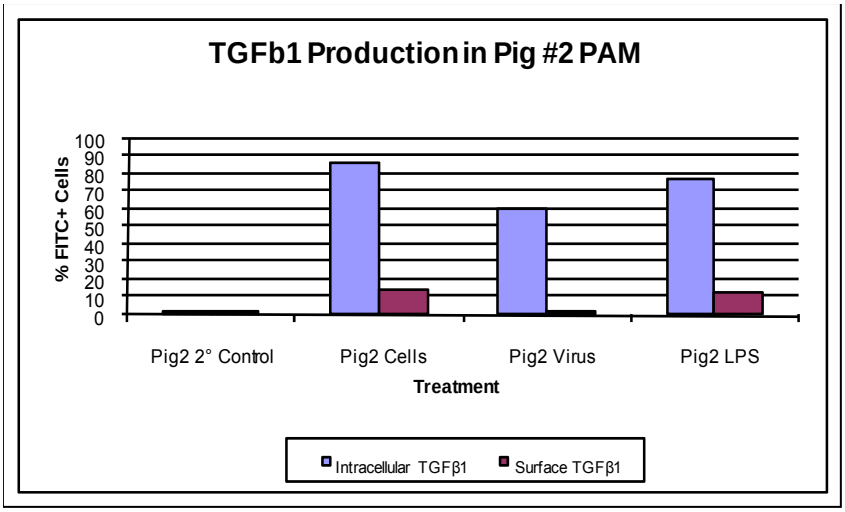
Figure 9: Representative dot plots illustrating cytokine production in infected and control PAM. Cytokine production was assessed using flow cytometry to identify their presence and relative concentrations in PRRSV infected, uninfected, and LPS control PAM. Each dot plot represents an individual culture. Panels A and B depict the cell population of interest, panels C, D and E represent IgG1, IgG, and IgG2b secondary antibody controls, respectably. Panels F, G and H depict TNFα within the cells, and panels F, J and K depict TNFα on the surface of PAM. Panels L, M and N depict TGFβ1 within the cells, and panels O, P and Q depict TGFβ1 on the surface of PAM cells. Panels R, S and T depict TGFβ2 within the cells, and panels U, V and W depict TGFβ2 on the surface of PAM. Percentage of PAM expressing each and treatment descriptions are provided with each dot plot.

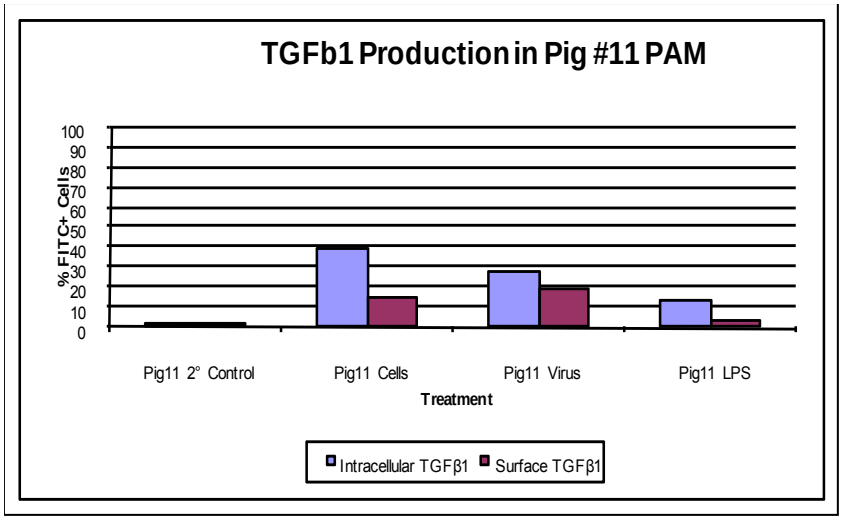

TNFa Production in Pig #1 PAM
% FITC+ Cells
10
8
6
4
2
0
Pig1 2° Control
Pig1 Cells
Pig1 Virus
Pig1 LPS
Treatment
Intracellular TNFα
Surface TNFα


TNFa Production in Pig #2 PAM
% FITC+ Cells
10
8
6
4
2
0
Pig2 2° Control
Pig2 Cells
Pig2 Virus
Pig2 LPS
Treatment
Intracellular TNFα
Surface TNFα


TNFa Production in Pig #11 PAM
% FITC+ Cells
10
8
6
4
2
0
Pig11 2° Control
Pig11 Cells
Pig11 Virus
Pig11 LPS
Treatment
Intracellular TNFα
Surface TNFα


TGFb1 Production in Pig #1 PAM
100
90
80
70
60
50
40
30
20
10
0
Pig1 2° Control
Pig1 Cells
Pig1 Virus
Pig1 LPS
Treatment
Intracellular TGFβ1
Surface TGFβ1


TGFb1 Production in Pig #2 PAM
% FITC+ Cells
100
90
80
70
60
50
40
30
20
10
0
Pig2 2° Control
Pig2 Cells
Pig2 Virus
Pig2 LPS
Treatment
Intracellular TGFβ1
Surface TGFβ1


TGFb1 Production in Pig #11 PAM
% FITC+ Cells
100
90
80
70
60
50
40
30
20
10
0
Pig11 2° Control
Pig11 Cells
Pig11 Virus
Pig11 LPS
Treatment
Intracellular TGFβ1
Surface TGFβ1

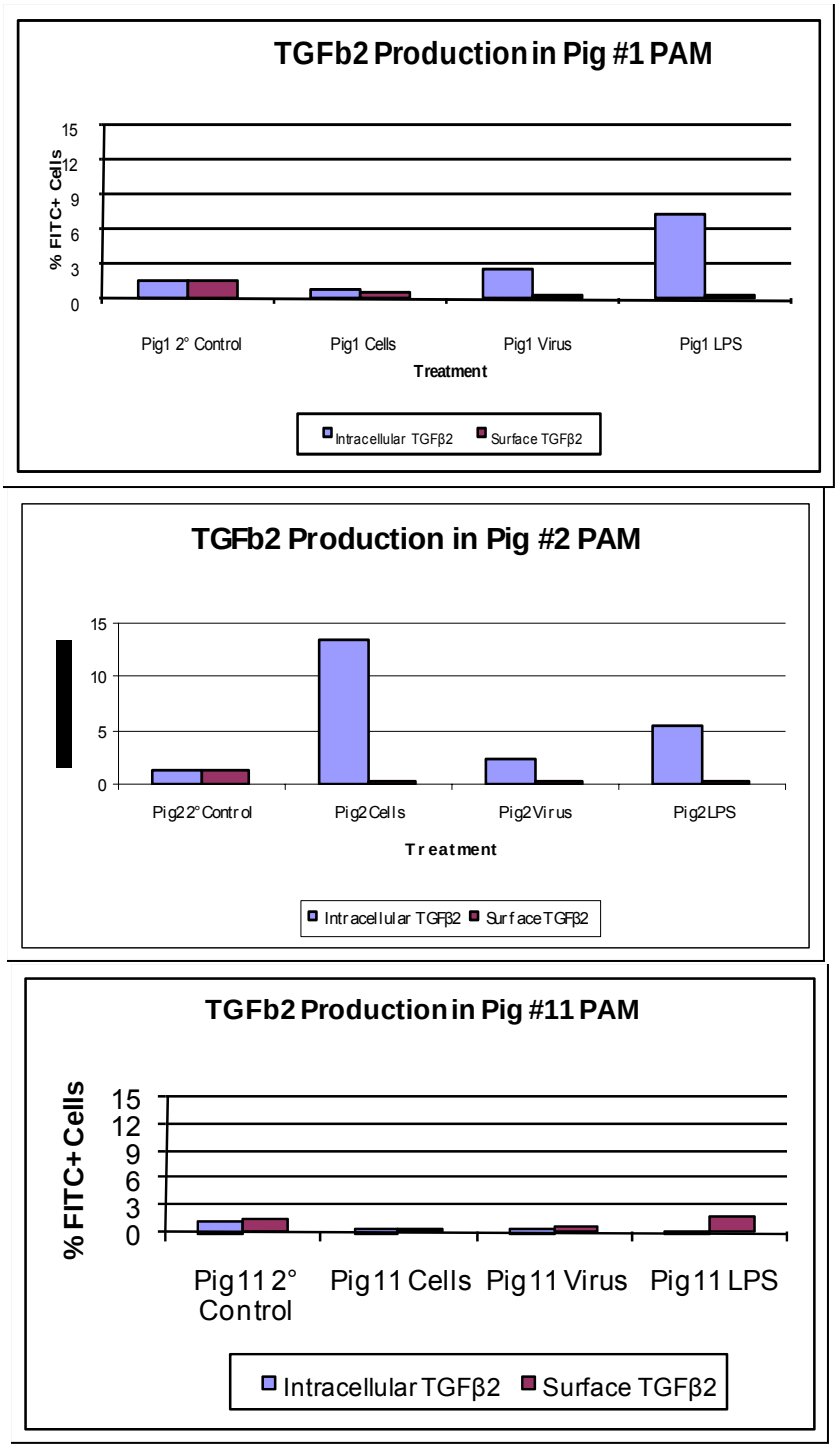


Figure 10: Representative bar graphs showing no trend in expression. Intracellular cytokine levels were examined for three different animals as described in Figure 9. Data is presented in bar graph form to depict differences between PAM treatment groups as well as expression levels in individual animals. The cytokine of interest is labeled above each graph, as are animal ID's. Each bar represents a single culture and treatment descriptions are provided at the bottom of each graph.

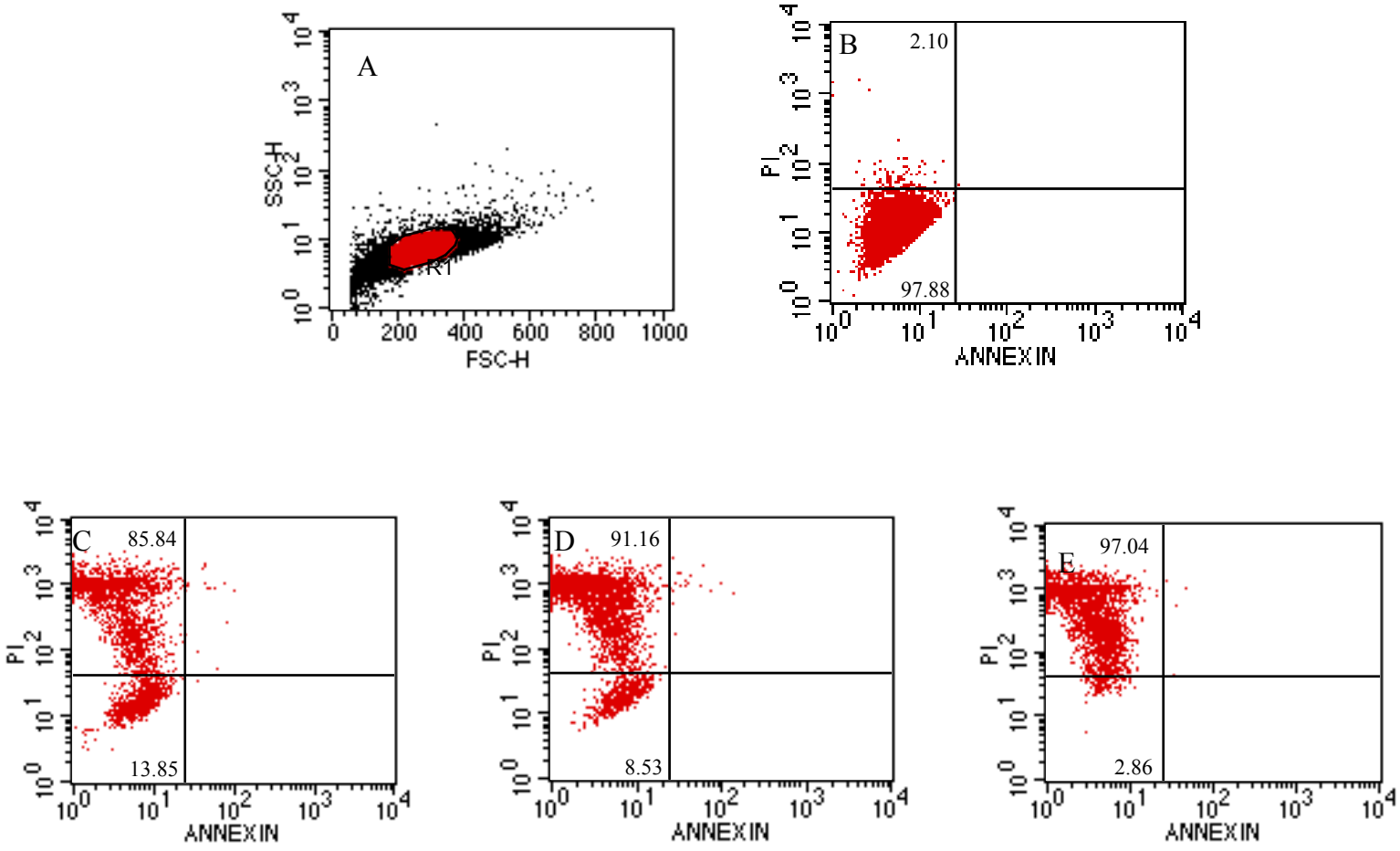


Figure 11: Representative dot plots demonstrating apoptosis levels in PRRSV-inoculated and control cultures using Annexin V. PAM in early apoptosis were examined using flow cytometry. Panels A and B depict the population of interest. Panel C depicts negative control PAM, panel D depicts PRRSV infected PAM, and panel D depicts Ac-D treated control PAM. The percentage of PAM in each quadrant are given with each dot plot.

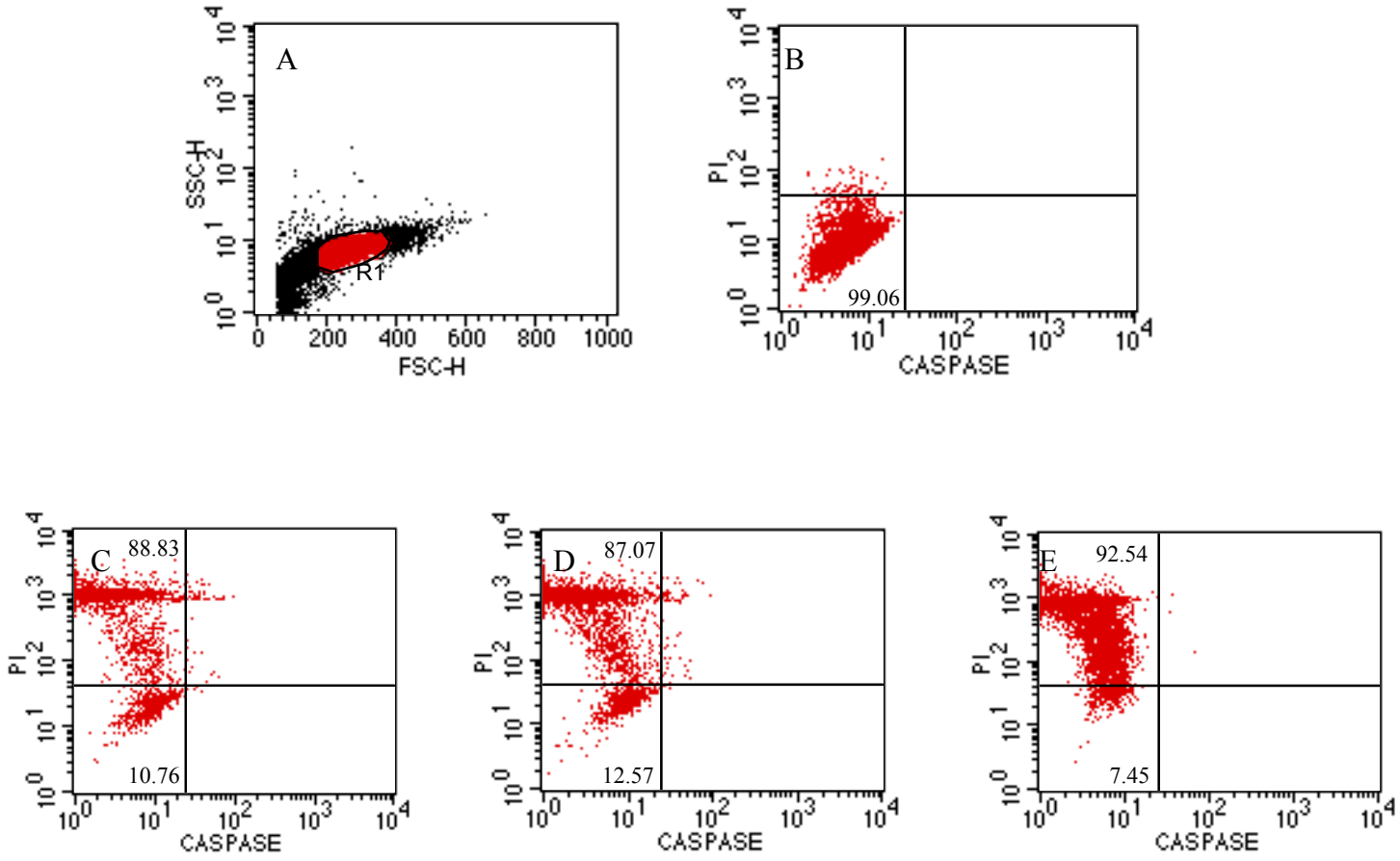


Figure 12: Representative dot plots demonstrating apoptosis levels in PRRSV-inoculated and control cultures by detection of caspases. PAM undergoing apoptosis were examined using flow cytometry. Panels A and B depict the population of interest. Panel C depicts negative control PAM, panel D depicts PRRSV infected PAM, and panel D depicts Ac-D treated control PAM. The percentage of PAM in each quadrant are given with each dot plot.

1 2 3 4 5 6 7 8 9 10

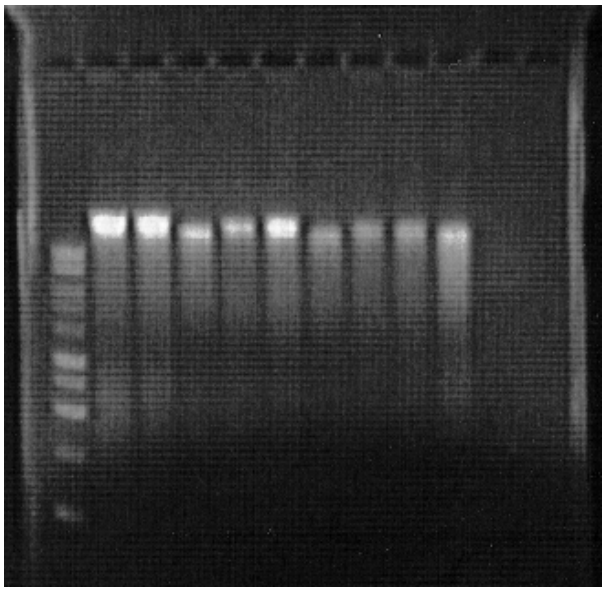


Figure 13: Gel demonstrates the absence of fragmented DNA in infected and control PAM. PAM in late apoptosis were examined by looking for DNA fragmentation. 10 ul of DNA was added to each lane on a 1% agarose gel and ran for approximately 45 minutes at 110v. For each pig, the first lane contains DNA from control PAM, the second lane contains DNA from PRRSV infected PAM and the third lane contains Ac-D control PAM DNA. Lane 1 is the DNA ladder, lanes 2-4 contain DNA from pig #1 PAM, 5-7 contain DNA from pig #2 PAM, and lanes 8-10 contain DNA from pig #9 PAM.

CHAPTER 4 - Discussion

Cells of the monocoyte/macrophage lineage express several surface proteins that are involved in their functions. Among them are MHC I, MHC II, CD14, CD163, and CD172a. The data consistently demonstrated upregulation of MHC II, CD14, CD163 and CD172a expression in PRRSV-infected cells and downregulation on the uninfected cells within the PRRSV-inoculated cultures when compared to control cultures. This observation is consistent with another study in which CD14 upregulation was demonstrated in PRRSV-inoculated cultures (100). Our results suggest that there are at least two subsets of macrophages within the alveolar population, one of which is PRRSV susceptible, and that the subsets have differentially regulated surface molecule expression. Upregulation on infected PAM of molecules involved in macrophage functions suggests that PRRSV manipulates the antigen detection and presentation capabilities of these cells. MHC I was also examined, however the expression was reduced below detectable levels after the PAM had been stored in liquid nitrogen.

Interestingly, we also demonstrated that PRRSV permissiveness appears to have been induced in an additional portion of the cell population by the freezing process. One possible explanation for this observation is that other cells found in lung lavage are lost during freezing/thawing of the cell suspensions, either PRRSV-nonpermissive PAM or another cell type which secretes anti-viral factors. Since PAM are thought to require a specific state of activation or differentiation to be permissive for infection (34), another possible explanation is that freezing results in induction of a PRRSV-susceptible state in a subset of PAM. Finally, the observation that PAM appear to lose MHC I expression after freezing, in conjunction with increasing permissiveness for infection by PRRSV, suggests that a currently unidentified association exists between MHC I expression and permissiveness of PAM to PRRSV. Work supporting this idea has shown MHC I downregulation in PRRSV-infected PAM, PBMC and mo-DC (93, 107).

Because surface protein expression was altered, we sought to determine how the infection might induce such changes. Thus, we evaluated alternative activation and apoptosis as possible explanations for the surface expression changes observed in the infected cultures. Alternative activation of PAM could explain an abnormal host response to PRRSV. Classically activated

macrophages exhibit a Th1-like cytokine profile, secreting TNFα and IL-13 which promote inflammation and apoptosis, while alternatively activated macrophages display a Th2-like phenotype, promoting cell proliferation and angiogenesis, and resolving inflammation. Indeed, the upregulation of CD163 on infected PAM is consistent with the idea of an anti-inflammatory profile for these cells (9). We sought to determine whether PRRSV-infected macrophages were classically or alternatively activated by examining TNFα and TGFβ production in PRRSV-infected, LPS- and non-treated control cells. However, no trend could be detected, suggesting that PRRSV does not alter the activation status of PAM.

Apoptosis induction is also a mechanisms by which PRRSV might control PAM. Since apoptosis occurs in stages, we examined cells for early, middle, and late apoptosis. However, apoptosis was not detectable in infected cultures. Apoptosis has been demonstrated to exhibit late onset (3 d.p.i.) during PRRSV infection of MARC-145 cells (47). This, combined with data showing no apoptosis after 12 hr p.i. in PAM (37) and our 18 hour time course, may account for our observations that PRRSV infected cells do not appear to differ from non-infected controls in the detection of cells in any stage of apoptosis. In addition, our studies compared PRRSV-inoculated and un-inoculated cultures, and it is possible that the apoptotic population is too small for differences to be noticeable without separating the populations for examination. Future experiments are required to investigate the issue further.

CHAPTER 5 - References

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