IMMUNOPATHOGENESIS AND IMMUNOMODULATION INDUCED BY

PRRSV STRAIN VR2332

Dissertation

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By

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Abstract

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease of swine throughout the world. The disease is a chronic viral respiratory and reproductive disease causing high mortality in nursery piglets and abortion in sows. The PRRS virus (PRRSV), the causative agent, has been continuously evolving over the past two decades. Curbing this disease is the biggest challenge faced by the swine industry, and is complicated by the incomplete knowledge of PRRSV immunology, strain dependent immunomodulation, and vaccine failures under field conditions.

Being an RNA virus, the PRRSV undergoes genetic and antigenic modifications, giving rise to different strains. VR2332 was the first PRRSV strain identified in the US, and has been considered as the North American prototype strain. This strain was attenuated and has been in use in the production of modified live PRRSV vaccine (MLV-PRRS). In recent years, the vaccine strain has been found to be persistent and mutated in swineherds vaccinated with MLV-PRRS. Therefore, we wanted to understand immunopathogenesis and immunomodulation in pigs infected with wildtype VR2332 strain. We chose three different time points for sampling: day 15, day 30 and day 60 post infection, since the virus induces a chronic infection. Viral persistence was observed throughout the study in lungs, serum and lymphoid tissues including tonsils, iliac lymph nodes, and tracheobronchial lymph nodes (TBLN), by virus isolation and viral RNA
detection. The recall antibody response was delayed and virus neutralization titers were low. Immunoregulatory cytokines, interleukin (IL)-10 and transforming growth factor (TGF)-β levels were increased, whereas levels of antiviral cytokine, interferon (IFN)-γ, was reduced in serum of infected pigs. There was a significant modulation of the frequency of immune cells in different tissues. However, increased T-regulatory cell frequency was observed in mononuclear cells isolated from bronchoalveolar lavage (BAL) fluid, blood and TBLN. The cytotoxic function of natural killer (NK) cells was abrogated in blood and lungs of infected pigs.

When the pigs were vaccinated with a commercial MLV-PRRS + adjuvant, M. tuberculosis whole cell lysate(Mtb WCL), intranasally, and then challenged with the parental VR2332 strain; there was a significant reduction in viral titers in the blood of vaccinated pigs up to 60 days post-infection compared to unvaccinated pigs. Given that the virus is a respiratory virus, we observed that the adjuvant and vaccine combination reduced pneumonic lesions in the lungs as evaluated both by gross and histopathologic examination. The MLV-PRRS + Mtb WCL induced increased PRRSV specific antibody responses, reduced IL-10 cytokine levels and reduced number of regulatory T cells in lung mononuclear cells. This suggests that PRRSV induces an immunosuppressive microenvironment in the lungs that leads to impaired function of innate and adaptive immunity.

Innate immunity plays a major role in antimicrobial responses, not only as the first line of defense, but also in inducing adaptive immune responses. Based on our study, we demonstrated that the adaptive immune response against PRRSV VR2332 was
insufficient and that PRRSV induces an immunosuppressive milieu in infected pigs. Recently, immature myeloid cells commonly called “myeloid derived suppressor cells (MDSC)” have been implicated in immune suppression in various cancer conditions and microbial infections in humans, mice and dogs. In pigs, this subset of myeloid cells has not been characterized. Hence, we cultured the porcine peripheral blood mononuclear cells (PBMC) isolated from adult pigs with cytokines GM-CSF and IL-6 in vitro to induce generation of MDSCs. A subset of cells characterized by CD172⁺CD11R3⁺ expression was upregulated. The same subset of cells were also found to produce increased levels of reactive oxygen species (ROS) intracellularly, and IL-10 in the culture supernatants at higher levels than cells cultured without cytokine stimulation at days 4 and 7 cultures. A similar trend was seen in PBMC cultured with UV-inactivated PRRSV VR2332 virus at day 4 of culture, whereby the CD172⁺CD11R3⁺ cells producing ROS and IL-10 were increased. Further analysis of immune markers and more functional characterization needs to be done to confirm that these immunosuppressive cells are indeed MDSC. Overall, there is a possibility of induction of MDSC in PRRSV infection, which could be one of the mechanisms of virus induced immunomodulation seen in infected pigs.

In summary, our experiments show that the PRRSV vaccine strain VR2332 persist in blood, lungs and lymphoid tissues up to 60 days post-infection in pigs. The virus was found to be capable of modulating the immunity by delaying humoral responses, and inducing immunoregulatory cytokines and T-regulatory cells. This was alleviated by vaccinating pigs with MLV-PRRS +Mtb WCL which induced anti-PRRSV
immunity, but still it was not able to completely clear the virus. We further examined the different mechanisms by which the virus might be inducing immunosuppression and hypothesized that MDSC could be a possible link in this virus induced persistence and immunosuppression. We characterized CD172$^+$CD11R3$^+$ cells having certain immunosuppressive properties in pig PBMCs. UV-inactivated virus treatment of PBMC was also capable of inducing the same subset of cells, indicating that this immunosuppressive subset could be one of the important factors in modulating the porcine immune system during PRRSV infection. However, further functional characterization of MDSC is necessary to extrapolate its likely induction in PRRSV infected pigs.
Dedication

To my family, friends and God Almighty
Acknowledgments

I thank Dr. Renukaradhy Gourapura (Aradhya), Dr. Linda Saif, Dr.Ramesh Selvaraj and Dr. Prosper Boyaka for being my committee members. Their motivation, support and guidance helped me greatly in the completion of this degree. I thank Aradhya for his patience and mentorship through this process. His inspirational guidance, constant encouragement and his easily approachable nature instilled in me the confidence to pursue this study; and helped me in the preparation of this document. I thank Linda and Ramesh for helping me to write better and to think differently. I thank Prosper for his motivational talk.

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Fields of Study

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Chapter 1

Literature Review

Porcine Respiratory Reproductive Syndrome

1.1 PRRS, the disease

The mystery swine disease of the early 1990s was identified as porcine reproductive and respiratory syndrome (PRRS) in the U.S and Europe (Benfield et al., 1992; Wensvoort et al., 1991) and since then, it has become prevalent in almost all swine production areas of the world. The disease has also been referred to as Wabash syndrome, mystery pig disease, porcine reproductive and respiratory syndrome, swine plague, porcine epidemic abortion and respiratory syndrome, blue abortion disease, blue ear disease, abortus blau, and Seuchenhafte Spätabort der Schweine. Pigs are the only known host for this disease. As the name indicates the PRRS disease manifests in two forms, namely respiratory and reproductive form. The respiratory form can be seen in all age groups and is characterized clinically by pneumonia, reduced feed intake with chronically recurring illness, debilitation and potentially high mortality. The reproductive disease can range from a lack of conception, early farrowing of congenitally-infected piglets with increased mummies and still born piglets, to abortion storms with significant piglet death loss and even sow death (Mengeling et al., 1998; Meulenberg, 2000; Rowland, 2007).
the US alone, there is a widespread prevalence of the causative agent, porcine reproductive and respiratory syndrome virus (PRRSV). According to the Animal and Plant Health Inspection Service (APHIS) report of January 2009, 49.8% of unvaccinated pigs are seropositive to PRRSV in the US, based on collective data from 94% of pork producers in 17 states.

PRRS is also called as the ‘blue ear disease’ due to the representative symptom of infected pigs and is typically characterized by high morbidity and low mortality. In 2006, the emerging disease lead to an epidemic of atypical PRRS called the ‘swine high fever disease’ in pigs in China. The highly pathogenic PRRS outbreak in China showed variations in clinical disease characterized by high body temperature, neurological symptoms, rubefaction on the skin, respiratory disorder and high mortality and morbidity (Tian et al., 2007; Zhou & Yang, 2012).

1.1.1 Economic importance of PRRSV

PRRS is the most devastating problem plaguing the global swine industry within the last two decades, with economic losses of $664 million (Holtkamp & Kliebenstein, 2011) estimated in the U.S. pork industry. According to UN-FAO, the disease is endemic with only a few countries, namely Australia, New Zealand and Switzerland free of PRRSV. Recent outbreaks of PRRSV in countries on a global level include Sweden (2008), South Africa (2005-2007), Russian Federation, Vietnam and China.
PRRSV negatively impacts swine fertility and growth which leads to the economic losses that the swine industry experiences. Abortions, still births, stunted fetal growth and decreased weight gain in pregnant gilts adversely affects the breeding stock. In nursery and finishing pigs, economic losses are attributed to increased morbidity and mortality rates, decreased appetite, and diminished feed intake, delayed growth rate and increase in unmarketable pigs. In 2005, a study by Neumann et al., (Neumann et al., 2005), indicated the cost of PRRSV to swine farmers due to the following attributes: 1) US $250 million (45%) due to decline in average daily weight gain and feed efficiency in growing pigs; 2) US $243 million (43%) due to mortality in growing pigs; 3) US $63 million (12%) being attributed to reproductive losses. The total estimated losses which amounted to $560 million in 2005, has increased to $664 million in 2011 (Holtkamp & Kliebenstein, 2011). This estimated loss is exclusive of costs related to PRRS vaccination, treatment, diagnostics, and biosecurity; collectively responsible for close to $3 million loss daily to the US pork Industry.

1.1.2 Control of PRRSV

PRRS outbreaks have managed to evade vaccination and management strategies for the past two decades. The main reason for the failure in preventive strategies is the incomplete knowledge of the viral biology, immunopathogenesis, antigenic and genetic variations among strains. The vaccines commonly employed under field conditions include modified live vaccines (MLV) and killed vaccines. The first introduction of MLV
against PRRS in 1994 was hailed as a significant achievement for the swine industry experiencing huge setbacks due to PRRS. Modified live vaccines provide good protection in pigs against homologous infections, but incomplete protection against heterologous infections (Cano et al., 2007). However, limitations of MLV-PRRS are virus shedding, persistence, potential reversion to virulence, incomplete protection against heterologous infections and re-infections, and possibility of spread from vaccinated to naive animals (Ciechanowska et al., 2008; Lager et al., 2003; Mateu & Diaz, 2008; Zuckermann et al., 2007). Killed vaccines are considered ineffective and are used only for vaccinating pregnant sows and breeding boars in which live vaccines are contraindicated (Charerntantanakul, 2009; Zuckermann et al., 2007).

Prevention of PRRSV by proper biosecurity measures needs to be implemented. Several strategies have been shown to be successful in eradicating PRRSV from the infected herd, such as whole herd depopulation/repopulation, test and removal and herd closure (Dee & Joo, 1997; Dee et al., 1997; Dee & Molitor, 1998). This requires purchasing PRRSV negative animals and consistent testing of incoming groups. The disadvantages of these strategies are the increase in production downtime and high cost of implementation and diagnostics (Cho & Dee, 2006). Unfortunately, most of the US swine herds are already infected with PRRSV which impedes the creation of PRRSV negative herds.

Vaccination is the most effective way to control most infectious diseases. Therefore, a more concerted research is needed to formulate new vaccination strategies for the development of attenuated and inactivated marker vaccines. To this end,
knowledge of PRRSV biology and its immunology is critical to manipulate the porcine immune system to induce protection against PRRSV.

1.2 Etiology of PRRS

The PRRS virus is an enveloped, single stranded, positive sense RNA virus belonging to the *Arteriviridae* family, in the order *Nidovirales* (Cavanagh, 1997). The *Arteriviridae* family also includes lactate dehydrogenase-elevating virus (LDV) of mice, equine arteritis virus (EAV) and simian hemorrhagic fever virus (SHFV) which have the common ability to induce prolonged viremia, persistent infection and replicate in macrophages (Plagemann & Moennig, 1992). The Arteriviruses are grouped with coronaviruses, toroviruses and roniviruses in the order *Nidovirales* due to similar genome organization and transcriptional processes (Cavanagh, 1997). Being an RNA virus, PRRSV undergoes major antigenic and genetic variations. Based on its genetic diversity, PRRSV can be divided into two genotypes, Type 1 (European) and Type 2 (American) (Meng *et al.*, 1995) with only 50-60% sequence identity between the two genotypes. Lelystad (Plana *et al.*, 1992) and VR2332 (Benfield *et al.*, 1992; Collins *et al.*, 1992) strains are the prototype wildtype parental strains of Type 1 and Type 2 PRRSV, respectively. Each genotype contains several subtypes and strains, which are genetically highly diverse and therefore display significant differences in their virulence and pathogenicity (Kim *et al.*, 2007). Different PRRSV field isolates within the North
American genotype have genetic diversity ranging from 84 - 100% based on the amino acid homologies of ORFs 2 – 6 relative to prototype VR2332 strain (Kim et al., 2007).

Due to its envelope, the survivability of PRRSV is affected by temperature, pH and exposure to detergents outside its host. Temperature range for the survivability of the PRRSV in a time period of more than 4 months includes temperatures of –70 to –20°C, although viability decreases as the temperature increases, the recovery of virus has been reported for up to 20 min at 56°C, 24 h at 37°C, and 6 days at 21°C (Benfield et al., 1992). The pH range for PRRSV stability is from 6.5 to 7.5; with reduced infectivity observed at pH <6.0 or >7.65 (Bloemraad et al., 1994). Detergents and lipid solvents reduce the virus infectivity as they can disrupt the viral envelope and inactivate replication (Benfield et al., 1992).

1.2.1 Transmission of PRRSV

The direct route of transmission of PRRSV occurs through infected pigs and semen. The virus is excreted from all the body secretions at low levels or intermittently in saliva, nasal secretions, urine, milk, colostrum, feces and in semen of infected pigs (Albina, 1997; Christopher-Hennings et al., 1998; Rossow et al., 1994; Voicu et al., 1994; Wagstrom et al., 2001; Zimmerman, 2003). Reports of vertical transmission during mid to late gestation have also been documented (Christianson et al., 1993). It has also been reported to be transported by fomites and personnel between swine populations (Pitkin et al., 2009), in addition to airborne transport of PRRSV occurring over long
distances (greater than 9 km) (Otake et al., 2010). Therefore, control of PRRSV transmission within and between swine herds is a major challenge to the swine industry.

1.2.2 Persistence of PRRSV

PRRSV infection is persistent in pigs and the virus is present at low levels within the infected animal, eventually decreasing with time. The persistent replication occurs with two periods: the first two weeks of peak replication and then a tail of low replication up to 5-7 weeks of infection (Duan et al., 1997b; Labarque et al., 2000). Upon exposure to infectious body fluids, the resident macrophages in the mucosal surfaces serve as the target cell of replication for the virus (Rossow, 1998). The virus then spreads to systemic organs such as lungs, secondary lymphoid tissues, endometrium, fetal implantation tissues and fetus (Duan et al., 1997b; Karniychuk et al., 2011; Prieto et al., 1997). Prolonged persistence of the PRRSV has been reported up to 154-157 days post infection in individual animals in tonsils and blood (Albina et al., 1994). In intranasally infected boars, infectious PRRSV and PRRSV RNA was detected in the semen for up to 43 and 92 days post- infection, respectively (Christopher-Hennings et al., 1998; Swenson et al., 1994). In another report (Benfield DA, 1997), in utero infected fetuses at 85-90 days became congenitally infected offsprings, with PRRSV RNA detectable in sera even at 210 days post-farrowing.
1.3 Genome and Proteome of PRRSV

Generally, PRRS viral particles are enveloped and have an oval to spherical shape with a diameter of 50-65nm (Spilman et al., 2009), and the positive sense RNA genome encased inside. The viral genome is approximately 15 kb with nine open reading frames (ORFs). The ORF1a and 1b are translated and processed into 14 non-structural proteins that exhibit replicase and polymerase activity, while ORFs 2–7 code for structural proteins (Meulenberg et al., 1995; Snijder & Meulenberg, 1998). ORFs 2-4 encode for GP2a, GP3 and GP4 which are associated with the viral membrane (Fig 1). The major structural proteins include the 25 kDa GP5 protein, 18-19 kDa matrix (M) protein and 15 kDa nucleocapsid protein (N), and are encoded by the ORFs 5, 6 and 7, respectively. A small internal ORF is found within ORF2, which encodes the E (GP2b) protein (Wu et al., 2001).

1.3.1 Non-structural Proteins of PRRSV

ORF1a and ORF1b together span about 75% of the genome. The polyproteins produced from these ORFs are cleaved by viral proteases to generate 14 different NSPs, namely NSP1α, NSP1β, and NSP 2-6, NSP7α, NSP7β, and NSP8-12 (Kroese et al., 2008; Meulenberg, 2000). The NSPs involved in processing of the viral polyproteins, genome replication, and transcription (Meulenberg, 2000) are NSP9 RNA polymerase and NSP10 helicase, the induction of replication-associated membrane rearrangements
by NSP2 and NSP3, and a replicative endoribonuclease (NSP11) (Fang & Snijder, 2010). Various NSPs have been found to modulate innate and adaptive immunity. NSP1α, NSP1β, NSP2, NSP4, and NSP11 antagonize Interferon (IFN) responses and inhibit activation of IFN-β promoter activity (Beura et al., 2011; Chen et al., 2010). The strongest inhibitor of IFN-β was found to be NSP1β, which inhibits TLR3-mediated signaling pathways (Beura et al., 2010) as well as TNF-α promoter activity (Subramaniam et al., 2010). NSP2 is the largest protein encoded by the PRRSV genome. Besides its IFN antagonistic effect, certain regions in NSP2 down regulate Interleukin (IL)-1β and Tumor necrosis factor -α (TNF-α) (Chen et al., 2010). The NSP3–8 region has been identified as possibly containing PRRS virulence factors (Fang & Snijder, 2010).

1.3.2 Structural Proteins of PRRSV

The major envelope proteins include GP5 and M, and the minor envelope proteins are GP2, GP3 and GP4. The E protein is essential for virus infectivity and forms ion channels. GP5 is the most abundant glycoprotein and is a major inducer of neutralizing antibodies. GP5 is highly variable among PRRSV strains (Kapur et al., 1996; Murtaugh et al., 1995). M protein is the most conserved structural protein among the Arteriviruses (Kimman et al., 2009), and is predicted to play an important role in virus assembly and budding. It is involved in heterodimer formation with GP5 through disulfide linkages (Mardassi et al., 1996) and serves as a ligand for CD169, an internalization receptor on
alveolar macrophages (Van Breedam et al., 2010). The N protein is the most abundant protein in PRRSV infected cells (Dea et al., 2000; Snijder & Meulenberg, 1998) constituting about 40% of the total mass of the virion. It is highly immunogenic, leading to development of non-neutralizing and non-protective antibodies (Murtaugh et al., 2002). The N protein, due to the presence of nuclear localization signals (Lee et al., 2006) is postulated to have dual roles - a structural role in the cytoplasm and non-structural role in the nucleus (Yoo et al., 2010). It also induces IL-10, an immunosuppressive cytokine in peripheral blood mononuclear cells (PBMC) of pigs and cultivated porcine alveolar macrophages (PAM) (Yoo et al., 2010). Overall, it has been demonstrated that the three main structural proteins GP5, M and N are essential for particle formation and virus infectivity.

1.4 Viral entry and replication

Although PRRSV is restricted in its cell tropism, it infects differentiating monocytes, differentiated macrophages, immature and mature dendritic cells (Duan et al., 1997a; Flores-Mendoza et al., 2008; Wang et al., 2007). The M-GP5 complex of the virus forms a low affinity attachment to heparin sulfate present on the target cell, the differentiated macrophage (Delputte et al., 2005). Following this attachment, internalization is mediated through binding of macrophage sialoglycoprotein receptor by the sialic acid residues present on GP5 (Delputte & Nauwynck, 2004; Van Breedam et al., 2010). Further, interaction with CD163 receptor, a cellular protein in the scavenger
receptor cysteine rich (SRCR) superfamily, through the GP2 and GP4 proteins leads to virus uncoating and release in the endosomes (Calvert et al., 2007). The E protein has also been found to play a role in the virus uncoating by helping in the disruption of nucleocapsid interactions with envelope proteins (Lee & Yoo, 2006). For the full susceptibility of a target cell to PRRSV infection, the presence of both sialoadhesin and CD163 are required (Van Gorp et al., 2008).

On virus uncoating, the free nucleocapsid is released into the cytoplasm and primary translation starts with the ORF1a and ORF1b to produce two polyproteins, pp1a and pp1b of approximately 2397 and 3854 amino acids, respectively. These polyproteins are further processed proteolytically into 14 non-structural proteins (Meulenberg et al., 1995; Snijder & Meulenberg, 1998) by auto-cleaving or by Nsp4 which is a serine protease (Meulenberg, 2000). The replication complex consisting of Nsp9 (RNA dependent RNA polymerase), Nsp 11 and Nsp 12 is assembled on an endomembrane scaffold created by Nsp2 and other ORF1 proteins (van der Meer et al., 1998). This replicase complex further produces six genomic mRNAs which leads to production of structural proteins. Assembly of the virion particles occurs in the Golgi complex and/or endoplasmic reticulum. The envelope formation of the virion is determined by GP5-M heterodimers. The virions are then loaded in intracellular vesicles to be transported to cell surface for progeny virus release through the budding process (Wissink et al., 2005).
1.5 PRRSV Immunity

Immunity to PRRSV is complex and in most cases not fully efficient in limiting viral replication. The PRRSV has a specific tropism for differentiated macrophages where it replicates efficiently and persists for a long time, up to 150 days post infection, in pig tonsils and serum (Allende et al., 2000). In addition, the virus infects other cells of the monocyte/macrophage lineage, notably pulmonary intravascular macrophages, subsets of macrophages in lymph nodes and spleen, and intravascular macrophages of the placenta and umbilical cord (Duan et al., 1997a; Lawson et al., 1997). Virus can also infect mature and immature porcine monocyte derived DCs, at least in vitro. Although the infection is less productive in this cell type than in alveolar macrophages, it interferes with many of their functions (Flores-Mendoza et al., 2008; Park et al., 2008). The porcine immune system is unable to mount an efficient anti-PRRSV immune response in the first few weeks post-infection (Mateu & Diaz, 2008; Murtaugh et al., 2002) due to delayed and reduced levels of anti-viral cytokines, neutralizing antibody, and cell-mediated immunity. The immunosuppression caused by the virus makes infected pigs prone to secondary bacterial and viral infections (Done & Paton, 1995). Moreover, different PRRSV field isolates even within the North American genotype have genetic diversity ranging from 84 - 100% (Kim et al., 2007). The different strains have been found to induce differential immunity in the porcine system (Diaz et al., 2006). Immunity generated against the initial infecting genotype of the PRRSV may provide partial to no protection not only to heterologous infections but also to re-infections of the same
genotype (Botner et al., 1997; Kimman et al., 2009; Li et al., 2010; Martelli et al., 2009). This makes understanding the role of viral immunity more complicated and control of the disease difficult.

1.5.1 Innate immune response

PRRSV is known to modulate innate immunity in pigs as early as day-two post-infection (Dwivedi et al., 2012), and continues to do so during first few-weeks of infection (Mateu & Diaz, 2008; Murtaugh et al., 2002) leading to increased susceptibility to secondary microbial infections (Done & Paton, 1995). The most efficient and rapid host response against viruses consists of production of type I IFNs (IFN-α/β), an essential part of the antiviral innate immune system (Thiel & Weber, 2008). The IFN production is triggered by the first contact of cells with the virus, the secreted IFN slows down or even blocks virus multiplication, aiding in establishment of an adaptive immune response (Thiel & Weber, 2008). Coordinated early expression of IL-1β, IL-8 and IFN-γ has been reported in pigs that are able to clear the virus (Lunney et al., 2010). However, PRRSV down regulates the production of inflammatory cytokines such as type 1 interferons (IFN-α, IFN-β), TNF-α, and interleukin-1 (IL-1) (Thanawongnuwech et al., 2001; Van Reeth et al., 1999). The inhibition of this early cytokine production in macrophages and dendritic cells contributes to the weak innate immune response, delayed neutralizing antibodies, slow IFN-γ response and a depressed cytotoxic T cell response (Custers et al., 2009). Increased levels of Th2 cytokines IL-10, IL-4 and TGF-β by the virus have been
proposed to be one of the mechanisms of immune evasion although IL-10 expression varies with different strains of PRRSV (Diaz et al., 2006; Silva-Campa et al., 2009; Thanawongnuwech et al., 2001). The Natural killer cells responsible for innate antiviral cytotoxic activity and IFN-γ production are suppressed in PRRSV infected pigs (Jung et al., 2009; Renukaradhya et al., 2010).

During early stages of infection, PRRSV induces an anti-apoptotic state in infected macrophages and dendritic cells (Costers et al., 2008; Flores-Mendoza et al., 2008), but at later stages it induces apoptosis. The virus has been reported to induce apoptosis of bystander non-infected macrophages/monocytes and infiltrated T cells in in-vivo infection (Karniychuk et al., 2011; Labarque et al., 2003; Sur et al., 1998). This leads to decreased macrophages and dendritic cell populations and dysfunctionality in their ability to secrete inflammatory or immunomodulatory cytokines and antigen presenting ability that are essential for induction of effective adaptive immune responses (Genini et al., 2008; Park et al., 2008). Also PRRSV decreases the capability of alveolar macrophages to phagocytosize and destroy secondary bacterial infections during in-vivo infections (Solano et al., 1998; Thanawongnuwech et al., 1997). Infected macrophages produced significantly reduced levels of superoxide anion and myeloperoxidase-H₂O₂-halide thus limiting their bactericidal function (Thanawongnuwech et al., 1997).

The toll like receptors are transmembrane proteins present on all cell types of immune lineage (B and T cells, dendritic cells, NK cells and monocytes) (Bauer et al., 2008; Takeda & Akira, 2003). TLR3 and TLR7 recognize viral dsRNA and ssRNA, respectively (Alexopoulou et al., 2001; Lund et al., 2004), which initiates a signaling
cascade that results in activation of transcription factors, ultimately leading to production of pro-inflammatory cytokines, chemokines, and anti-viral cytokines such as type 1 IFN (Takeuchi & Akira, 2007). However, there are contradictory reports about the role and expression levels of TLRs in PRRSV infection. Expression of TLRs-3, 4 & 7 increased in TBLN and brain of 8 week old PRRSV experimentally infected pigs (Miguel et al., 2010). On the other hand, increased replication of PRRSV in target cells with down regulated TLR 3 expression was reported (Sang et al., 2008). Calzada-Nova et al., 2010 reported the relation between cytokine production and TLR expression in PRRSV infection, stating that PRRSV failed to induce cytokine secretion from plasmacytoid DCs, suggesting interference with TLR7 and TLR9 signaling (Calzada-Nova et al., 2010).

1.5.2 Adaptive Immunity

In PRRSV infections, the adaptive immunity is delayed, takes about 3 months to reach peak levels and is still not capable of preventing re-infections especially with heterologous strains (Murtaugh et al., 2002; Zuckermann et al., 2007). The memory response against heterologous strains of PRRSV exists but provides partial or incomplete protection upon challenge (Lager et al., 1999). Further, due to the broad antigenic and genetic variation of simultaneously circulating field PRRSV strains, the humoral and cell-mediated immune responses are not efficient to provide protection under field circumstances (Darwich et al., 2010). Also it has been reported that the different strains
of PRRSV induce differential immunity indicating that some strains are capable of inducing strong cellular, in particular IFN-γ production (Diaz et al., 2006).

1.5.3 Humoral Immunity

PRRSV induces abundant humoral immunity that is due to virus mediated polyclonal activation of B cells (Drew, 2000; Lamontagne et al., 2001). Robust antibody responses as early as one week post-infection (Yoon et al., 1995) have been reported, with PRRSV specific antibodies being detected up to at least 604 days post-infection (PI) (Lager et al., 1997). Most antibodies produced during 7-21 days PI are non-neutralizing and are principally targeted to N and NSP2 proteins (Yoon et al., 1995). On the other hand, neutralizing antibodies (VN) appear delayed, around the third or fourth week post infection or later and also in low titers in PRRSV infected pigs. The importance of virus neutralizing (NA) antibodies in providing protection has been debated. In some cases, viremia has been found to resolve even in the absence of detectable levels of NA titers (Diaz et al., 2006). Whereas other reports have shown the passive transfer of neutralizing antibodies prior to infection helped clear the viremia, wherein an NA titer of 1/16 protected pregnant sows against reproductive failure and prevented vertical transmission (Osorio et al., 2002); 1/8 or higher titers protected young piglets from developing viremia; and NA titers of 1/32 provided sterilizing immunity (Lopez et al., 2007). Overall, the neutralizing antibodies are able to eliminate cell free virus (Labarque et al., 2000), but not the virus infected cells, as the viral glycoproteins are not expressed on the
plasma membrane for the antibodies to bind (Costers et al., 2006). This prevents the antibody dependent cell lysis by complement, and therefore phagocytosis is not possible.

Viral neutralizing epitopes (NE) that are capable of inducing neutralizing antibodies have been mapped on M, GP2a, GP3, GP4, and GP5 proteins (Kim & Yoon, 2008; Yang et al., 2000). Of these, neutralizing antibodies to GP5 are most relevant for protection. The major neutralization epitope (NE) of PRRSV is located towards the middle of the GP5 ectodomain (aa 36–52) (Plagemann, 2006). However, decoy epitopes near the NE of GP5 have been reported, which are attributed to be one of the factors responsible for development of non-neutralizing antibodies (Fang et al., 2006; Ostrowski et al., 2002). Decoy epitopes are immunodominant epitopes present on the PRRSV surface glycoproteins which induce an early and strong non-neutralizing antibody response (Osorio et al., 2002; Ostrowski et al., 2002) that helps the virus to evade the host immune system. Another evasion strategy is the glycosylation of proteins in or around the neutralizing epitopes of GP5, which acts as a glycan shield masking the epitope and again leads to production of non-neutralizing antibodies (Faaberg et al., 2006; Mateu et al., 2006; Vu et al., 2011).

1.5.4 Cell-Mediated Immunity

The cell-mediated immunity (CMI) is also delayed when compared to other viral infections. The T-cell responses to PRRSV are induced 2–8 weeks post-infection (Xiao et al., 2004) and are detected against all structural proteins encoded by ORFs 2–7 with the
M protein inducing the highest T cell proliferation (Bautista et al., 1999). Anti PRRSV cell-mediated immunity is characterized by expression of cytokines IFN-γ and IL-2 (Kimman et al., 2009), but it is not efficient in preventing the infection and establishment of persistence by the virus. IFN-γ specific T cell responses have been identified as the indicator of CMI response against PRRSV using IFN-γ ELISPOT assay as the reference assay. Similar to neutralizing antibodies, PRRSV-specific IFN-γ-secreting cells (ISCs) do not develop systemically until 2–3 weeks post-infection, and after 8-10 weeks post-infection, the IFN-γ T cell responses increase until 48 weeks, and are stable for 690 days post-infection (Meier et al., 2003). However, this response is still suboptimal compared to Pseudorabies virus infected pigs. Pigs vaccinated with MLV-PRRSV intranasally developed virus specific IFN-γ secreting cells were in the range of 50-100 cells per million PBMCs and they persisted until 10 weeks post-challenge (Dwivedi et al., 2011a), whereas pigs vaccinated against Aujeszky’s disease virus had 200 - 300 IFN-γ secreting cells per million PBMCs (Meier et al., 2003). Most of the IFN-γ secreting cells were comprised of CD4+ CD8+ T cells with a few CD4−/CD8α+ cytotoxic T cells (Meier et al., 2003). There was an increased number of CD8+ T cells in blood or tissues in the first few weeks PI (Shimizu et al., 1996), but these CD8+ T cells were found to be functionally impaired with respect to their cytotoxic activity (Costers et al., 2009). The reasons proposed for the impairment includes defect in recognition of infected alveolar macrophages, blocked cytotoxicity towards macrophages and detection of CD8 cells which are not virus specific (Kimman et al., 2009).
During PRRSV persistence, T cells were non-responsive to mitogens such as Concavalin A and Phytohemagglutinin (PHA) transiently in blood and lymphoid organs (Lamontagne et al., 2003). A possible reason for this non-specific T cell suppression could be the induction of Th3 regulatory cells in response to PRRSV infected dendritic cells (Silva-Campa et al., 2009). T-regulatory (Tregs) cells suppress the activation of the immune system, secrete IL-10 and TGF-β and have been linked to persistent infections. PRRSV infection has been found to increase the viral specific inducible Tregs in PBMC both in vitro and in vivo (Wongyanin et al., 2010), and also T-helper 3 regulatory cells which produce TGF-β. However, the IL-10 expression varies with different strains of PRRSV (Diaz et al., 2006). Overall, more research is needed to understand the cell-mediated immune response against PRRSV, as this will pave the way to identify strategies to induce protective immune responses.

1.6 Mucosal PRRSV vaccination in pigs and its implications

Studies have demonstrated that intranasal delivery of live vaccines against bovine herpes virus-1, influenza, and parainfluenza-3 has provided protective antiviral immunity (Guillonneau et al., 2009; Karron et al., 1995; Van der Poel et al., 1995). Since PRRSV gains entry through respiratory and reproductive mucosal surfaces and also causes disease primarily at mucosal sites, a protective mucosal vaccine to PRRSV may help to prevent PRRSV infection. A commonly used vaccine is the live attenuated PRRS-Modified live vaccine (MLV-PRRS) (Ingelvac® Boehringer Ingelheim). But the vaccine
itself is capable of delaying the virus specific cell-mediated immune response characterized by virus induced immunosuppression (Diaz et al., 2005; Dwivedi et al., 2011a; Dwivedi et al., 2011b; Johnsen et al., 2002; Renukaradhya et al., 2010; Royaee et al., 2004; Suradhat et al., 2003). To improve the efficacy of the live vaccine, adjuvants have been used. Four injections of IL-12 alone, or cholera toxin alone within 1 week of intramuscular administration of MLV-PRRS to pigs, increased immune responses potentiating the vaccine (Foss et al., 2002). But the MLV-PRRS was not helpful in clearing the viremia when pigs were given a homologous PRRSV challenge.

Complete Freund’s adjuvant (CFA) is known to elicit robust immune responses that are mediated by its important component, heat-killed mycobacteria. However, use of CFA is precluded in humans and food animals, because it induces severe granulomatous inflammatory reactions at the injection site (Chedid & Audibert, 1977). The harmful side effect of CFA is mediated by the cell wall components of mycobacteria, such as mycolic acids, peptidoglycan, wax D etc., (Bekierkunst, 1968; Ungar & Muggleton, 1961). In order to exploit the novel adjuvant properties of killed mycobacteria, a water soluble whole cell lysate of Mtb (Mtb WCL) and its individual components, free from any toxic cell wall core contents have been tested. They were demonstrated to possess excellent adjuvant properties with absence any detectable toxicity (Bansal et al., 2010; Choudhary et al., 2003; Harmala et al., 2002; Narayana et al., 2007; Saito et al., 1976; Singh et al., 2005; Srivastava et al., 1994; Werner et al., 1975). In a study by our group, pigs vaccinated intranasally using MLV-PRRS along with Mtb WCL (Kim et al., 2007), showed protection when challenged with heterologous PRRSV MN184. We detected a
significant increase in body weight gain, reduced lung pathology, enhanced PRRSV neutralizing antibody titers and reduced viremia at early time points post-challenge (Dwivedi et al., 2011a). Increased frequency of T helper (CD3⁺CD4⁺CD8⁻) cells, T helper/memory (CD3⁺CD4⁺CD8⁺) cells, γδ T (CD3⁻γδTCR⁺) cells, dendritic (CD172⁺ CD11c⁺SLAII⁺) cells and a reduced frequency of T regulatory (CD4⁺CD25⁺FoxP3⁺) cells were detected at both mucosal and systemic sites in pigs vaccinated with MLV-PRRS along with Mtb WCL. Further, reduced secretion of immunosuppressive cytokines (IL-10 and TGF-β) and upregulation of the Th1 cytokine IFN-γ in the blood and the lungs were detected (Dwivedi et al., 2011a).

PRRSV specific lymphocyte responses in pigs vaccinated with MLV-PRRS + M. tb WCL and MN184 or VR2332 challenged were generated against internal viral protein of PRRSV, indicated by significantly increased M protein specific IFN-γ and IL-12 cytokine response in restimulated lung MNC and PBMCs. This result has provided evidence that PRRSV internal viral protein specific epitopes targeted response is augmented by intranasal vaccination of MLV-PRRS with M. tb WCL, and that could be responsible for increased cross-protective immune responses (Dwivedi et al., 2011a).

This vaccine + adjuvant combination was capable of inducing NA titers of more than 1/32 that were detected from day 7 until day 35 post-immunization (Dwivedi et al., 2011b). In contrast, in pigs inoculated with MLV-PRRS alone (without any adjuvant), NA titers of less than 1/16 were detected at multiple time points post-immunization (Dwivedi et al., 2011b). In heterologous PRRSV MN184 challenged, pigs immunized with MLV-PRRS plus M. tb WCL, NA titers of more than 1/16 persisted until PID 60
and were associated with significantly reduced viral load in challenged pigs (Dwivedi et al., 2011a). Moreover, the enhanced NA titers in pigs receiving MLV-PRRS plus M. tb WCL was associated with augmented Th1 and suppressed immunosuppressive responses. Also these vaccinated pigs showed increased IFN-γ secreting cells per million PBMCs (Dwivedi et al., 2011a). These results strongly indicate that a potent adjuvant given with MLV-PRRS vaccination can induce enhanced innate and adaptive immune response which could reduce PRRSV replication and dampen the severity of clinical PRRS.

Besides this, the mononuclear cells isolated from lungs and blood of MLV-PRRS + M. tb WCL vaccinated pigs had reduced levels of reactive oxygen species (ROS) than by either unvaccinated VR2332 and MN184 challenged pigs. The ROS produced by immune cells is essential for antimicrobial activity (Acker, 2005; Chvanov et al., 2005; Martin & Edwards, 1993; Quinn & Gauss, 2004), but excess production of ROS causes apoptosis/necrosis of infected and bystander cells (Clutton, 1997; Halliwell & Gutteridge, 2006; Vaughan, 1997). It is one of the important mediators of diffused PRRSV mediated pathology in the lungs and lymph nodes of infected pigs (Sirinarumitr et al., 1998). Analysis of BAL cells and PBMCs in pigs intranasally vaccinated (MLV-PRRS plus M. tb WCL) and challenged with either PRRSV VR2332 or MN184 strains revealed that they had higher levels of ROS production compared to mock control pigs; but the ROS levels in MLV-PRRS plus M. tb WCL vaccinated pigs was significantly lower than in unvaccinated challenged animals (Binjawadagi et al., 2011). This result has suggested that M. tb WCL adjuvant induced the optimal, but not excessive ROS production which
helped to boost immunity to PRRSV without causing lung pathology in virus challenged pigs.

1.7 Mechanisms of viral persistence and immune evasion

Upon pathogen entry, the immune system of the host triggers various arms of immunity such as innate immunity, cytokine responses, antibody responses, T cell responses directed towards resisting and overcoming the infection. In order to overcome the host’s immune response, the virus develops evasion mechanisms. PRRSV has also evolved many immune evasive mechanisms to trick the porcine immune system and some of these mechanisms are still not completely understood. The virus complicates the ability of the host to respond to infection which impedes the control and prevention of PRRS.

1.7.1 Genetic and antigenic diversity

PRRSV achieves genetic drift through point mutations and genetic shift through recombination. It has the highest rate of nucleotide substitution for any RNA virus to date, which is \(4.7 \text{ to } 9.8 \times 10^{-2}/\text{site/year}\) (Hanada et al., 2005; Jenkins et al., 2002). The type 2 viruses can be divided into nine distinct groups or lineages based on the nucleotide sequence analysis of approximately 8500 ORF5 sequences (Shi et al., 2010a). This further illustrates the changing nature of the virus since its emergence two decades ago. The genetic change impacts the resulting peptide sequence variability and
hypervariability within the structural and non-structural proteins which are linked to immune selection of T and B cell epitopes (Chand et al., 2012).

Random mutations are consistently introduced into the viral genome because of the low fidelity of the PRRSV RNA-dependent-RNA polymerase. This leads to the emergence of antigenic heterogeneity of immunodominant epitopes recognized by both antibodies and T cells (Huang & Meng, 2010; Meng, 2000). Especially, mutations occurring in the regions of neutralizing antibody epitopes and envelope proteins are most important. In HIV infection, mutations in the neutralizing antibody targeted epitopes inhibits the binding of the neutralizing antibodies, allowing the virus to escape neutralization (Mascola & Montefiori, 2003). Similarly in PRRSV, mutations in NSP1 and NSP2 may alter the level of virus replication within the cell contributing to viral persistence (Chand et al., 2012). Also PRRSV mutations are so rapid that multiple variants co-exist in the same infected pigs (Chang et al., 2002). Most PRRSV strains exist as quasispecies within the same genotype under field conditions (Goldberg et al., 2003). However their heterogeneity is observed antigenically but is not distinct enough clinically to be regarded as a geographical sub population (Kapur et al., 1996; Meng et al., 1995). Thus the rapidly evolving virus changes both antigenically and genetically which is the major cause of ‘vaccine failures’ under field conditions.
1.7.2 Antibody mediated enhancement

The antibody mediated enhancement of PRRSV infection is highly debated with many researchers rejecting it. Studies of murine models have shown PRRSV infection to be enhanced by certain enhancing antibodies (Cancel-Tirado et al., 2004; Kimman et al., 2009). These virus specific antibodies were either of maternal origin or from previous vaccination and they facilitated the entry of virus into target cells. The antibody mediated enhancement epitopes were reported to be present on N and GP5 proteins of PRRSV (Cancel-Tirado et al., 2004).

1.7.3 Decoy epitopes

GP5 is one of the immunogenic proteins which induce neutralizing antibodies. However, immunodominant decoy epitopes adjacent to the neutralizing epitopes evokes robust non-neutralizing antibody responses against PRRSV (Fang et al., 2006; Ostrowski et al., 2002). This masks the early recognition of neutralizing epitopes and skews the antibody response, effectively avoiding neutralization.

1.7.4 Glycan shielding of neutralizing epitopes

The viral envelope glycoproteins shield the neutralizing epitopes thus preventing both neutralizing antibody production and the binding of already present neutralizing...
antibodies to the epitopes. A similar mechanism has been proposed for HIV (Mascola & Montefiori, 2003), Simian Immunodeficiency Virus (SIV) (Reitter et al., 1998) and other viruses including PRRSV. As mentioned earlier, there are four glycoproteins namely GP2, GP3, GP4 and GP5 present in the PRRSV envelope. Glycan moieties present in the GP5 protein of PRRSV have been reported to prevent neutralization of PRRSV by NA (Ansari et al., 2006). Glycosylation of minor envelope proteins GP2, GP3 and GP4, although required for infectious virus production, neither protect against nor enhance neutralizing antibodies (Das et al., 2011).

1.7.5 Subversion of type 1 interferons

The virus’s ability to subdue IFN-α response early in infection, knocks out the porcine innate immune system. Pretreatment of cells with type I IFN was able to inhibit PRRSV replication as shown in many studies (Albina et al., 1998; Buddaert et al., 1998; Overend et al., 2007). Other studies (Lee et al., 2004; Liu et al., 2010) have shown that the type 2 PRRSV viruses vary in their levels of IFN induction. In our previous report (Dwivedi et al., 2012), we found that there was a slight increase of IFN-α at 2 days post-infection in pigs infected intramuscularly with PRRSV strain 1-18-2. However, low levels of IFN-α have been implicated in the stimulation of monocyte/macrophages to express sialoadhesin receptor required for PRRSV internalization (Delputte et al., 2007). It is also interesting to note that multiple non-structural proteins (NSP1, NSP2, NSP4 and
NSP11) function to disrupt the interferon signaling pathways (Beura et al., 2010; Kim et al., 2010; Sun et al., 2012).

1.8 Induction of immune suppressive microenvironment

Protective immunity against tumors or microbes is contributed initially by antigen presenting cells (APC) with subsequent activation of lymphocytes. Immunosuppressive factors can be induced in APC during tumor growth and microbial infection. This immunosuppressive microenvironment might play a major role in PRRSV persistence, as seen in the case of tumors. It is well established that the tumor microenvironment promotes the growth of the tumor and inhibits antitumor immune responses. In the tumor bearing state, elevated serum levels of IL-10 and TGF-β have been reported to block the function of antitumor effector cells (Jarnicki et al., 2006; von Boehmer, 2005).

Generation of Foxp3⁺ CD4⁺ regulatory T cells (Tregs) induced by tumor derived TGF-β further suppresses antitumor immune responses (Chen et al., 2003; Yamaguchi & Sakaguchi, 2006). Also, myeloid derived suppressor cells (MDSC) were generated, leading to dysfunction of antitumor immunity. In viral diseases such as HIV (Qin et al., 2013) where T cell dysfunction and depletion are common in chronically infected patients and similarly following influenza viral infection (De Santo et al., 2008); the simultaneous expansion of the MDSCs leads to dampened T cell function. Similarly, MDSCs have been reported in persistent infection caused by Theiler’s virus in mice (Bowen & Olson, 2009).
In PRRS infected pigs, increased expression of immunosuppressive cytokines namely IL-10, IL-4 and TGF-β following PRRSV infection has been proposed as immune evasive mechanism and cause of secondary infections (Chung & Chae, 2003; Gomez-Laguna et al., 2009; Johnsen et al., 2002; Silva-Campa et al., 2010; Silva-Campa et al., 2009), although differential IL-10 responses are induced by different strains of virus (Diaz et al., 2006). Also, recent papers (Silva-Campa et al., 2010; Silva-Campa et al., 2009; Silva-Campa et al., 2012) have reported the PRRSV induction of T regulatory cells expressing Foxp3 marker. Cytotoxic T cell dysfunction has also been reported in PRRSV infected pigs (Costers et al., 2009). This microenvironment is similar to the immunosuppressive microenvironment of tumors and other microbial infections. This led us to propose that there is a possibility of induction of MDSCs in PRRSV infected pigs. The population of MDSCs has been characterized in mouse, humans and recently in dogs, but not in pigs.

1.8.1 Myeloid derived suppressor cells in mice, human and dogs

The MDSCs are a subset of innate cells comprising of a heterogeneous population of bone marrow derived myeloid progenitors and immature myeloid cells. These cells are absent in healthy hosts but migrate out from the bone marrow and accumulate in blood and peripheral lymphoid organs during pathological conditions such as tumors, inflammatory diseases and autoimmune disorders. They have been characterized in different tumor conditions over the last two decades. In mice, two main MDSCs subtypes
have been reported: (i) Granulocytic MDSCs (G-MDSCs) expressing CD11b\(^+\) Gr-1\(^{\text{hi}}\) Ly-6G\(^+\) Ly-6C\(^{\text{lo}}\) CD49d\(^-\), and (ii) Monocytic MDSCs (M-MDSCs) expressing CD11b\(^+\) Gr-1\(^{\text{hi}}\) Ly-6G\(^-\) Ly-6C\(^{\text{hi}}\) CD49d\(^+\) (Gabrilovich et al., 2012).

In humans, there are no uniform markers for MDSCs but they commonly express myeloid markers CD11b and CD33 but lack maturation marker HLA-DR, with M-MDSCs being predominantly CD14\(^+\) and G-MDSCs being CD15\(^+\) (Gabrilovich & Nagaraj, 2009; Greten et al., 2011). Although, G-MDSCs represent the major subset of the MDSCs circulating in the tumor bearing mice and humans (Gabrilovich et al., 2012), M-MDSCs are proposed to be more immunosuppressive on a per cell basis (Dolcetti et al., 2010; Youn et al., 2008). MDSCs though not present in healthy hosts, can be induced in healthy donor blood in vitro by culturing in the presence of cytokines IL-6 and GM-CSF and also other combinations of GM-CSF with IL-1\(\beta\), PGE\(_2\), TNF-\(\alpha\) or VEGF (Lechner et al., 2010). The immunophenotype of these in vitro characterized MDSCs was CD33\(^+\) CD14\(^+\) CD56\(^+\) from healthy human blood samples and were functionally immunosuppressive, similar to the MDSCs characterized from cancer patients.

Recently, MDSCs were characterized in dogs with naturally occurring tumors. The cells exhibiting a phenotype of CD11b\(^+\) CD14\(^-\) MHCII\(^-\) and with the ability to suppress T cell responses was found to accumulate at higher levels in the blood of canine patients with advanced cancer (Goulart et al., 2012). In another study involving canine cancer patients, MDSCs with immunophenotype of CD11b\(^0\) CADO48A\(^{\text{lo}}\) were characterized (Sherger et al., 2012). Till date, there are no reports on the presence or characterization of MDSCs in
porcine species. Hence, one of my major objectives was to characterize MDSCs from healthy porcine PBMCs by culturing with cytokines GM-CSF and IL-6 in vitro.

1.8.2 MDSCs in viral diseases

MDSCs have been extensively studied in tumors since the last two decades but their role in the pathogenesis of viral diseases still needs to be understood completely. There are very few reports available that incriminates MDSCs in viral infections. Influenza A virus (IAV) infection in mice and human patients led to expansion of MDSCs subset which suppressed virus specific immune responses, with MDSCs function being modulated by invariant Natural Killer T (iNKT) cells (De Santo et al., 2008). Also in another study with IAV, there was increased accumulation of MDSCs in the lungs of mice lacking TLR 7, skewing the immune response towards Th2 biased humoral response (Jeisy-Scott et al., 2011). Hepatitis C viral core protein treated CD33+ mononuclear cells were found to display a CD14+ CD11b+ HLA-DR- phenotype capable of suppressing T cell function by means of increased reactive oxygen species production in vitro; the same phenotype of cells were seen in PBMCs of chronically infected patients (Tacke et al., 2012). Accumulation of MDSCs was observed in a murine model of chronic hepatitis B virus (Chen et al., 2011). In HIV-1 patients, elevated levels MDSCs was observed and was associated with disease progression (Vollbrecht et al., 2012). A recent report showed that culture of PBMCs with HIV-1 or HIV-1 derived Tat protein enhanced MDSCs generation in vitro, and MDSCs from healthy donors were directly infected by HIV-1 playing a role
in HIV-1 replication and transmission (Qin et al., 2013), indicating the immunotherapeutic potential of targeting MDSCs.

1.9 Immunosuppressive mechanisms of MDSCs:

MDSCs apply different mechanisms to suppress the immune system. The two subsets namely M-MDSCs and G-MDSCs can inhibit effector T cell responses through different modes of action but are not exclusive (Gabrilovich & Nagaraj, 2009; Gabrilovich et al., 2012), and often include more than a single mechanism.

1.9.1 Arginase I and iNOS production

The cell-mediated immunity is manipulated by the metabolism of L-arginine (L-Arg). L-Arg is an essential amino acid that is metabolized by enzymes nitric oxide synthase (NOS) and arginase (ARG), which are regulated by T helper 1 (Th1) and Th2 cytokines, respectively (Bronte & Zanovello, 2005). Arginine limitation causes decreased expression of iNOS (inducible NO synthase) (El-Gayar et al., 2003) and the ζ-chain of the T-cell receptor (Taheri et al., 2001). NO synthesized by iNOS pathway and has been shown to be a major effector pathway of inflammatory macrophages (Munder et al., 1999). Macrophages effect their function mostly through the nitric oxide pathway which is activated by Th1 cytokine IFN-γ, while the arginase activity is activated by Th2 cytokines, IL-4 and IL-10.
Role of arginase:

T cells uptake L-Arg from the microenvironment outside the cell. Under low L-Arg conditions, the activated T cells retain some functional ability but do not proliferate, and are arrested in the G0-G1 phase of the cell cycle (Rodriguez et al., 2007; Rodriguez et al., 2002). Increased L-Arg production is one of the mechanisms by which the MDSCs deplete the L-Arg concentration in the microenvironment leading to T cell hyporesponsiveness and reduced specific cell-mediated immunity (Bronte & Zanovello, 2005). Absence of arginase has been reported in mice to improve the survival when infected with Mycobacteria and T. gondii (El Kasmi et al., 2008). This arginase mediated immune dysregulation has been reported in tumors, infections caused by Leishmania major (Munder et al., 2009), Mycobacteria, T. gondii (El Kasmi et al., 2008) and in human pregnancy (Kropf et al., 2007).

Role of NO’ in immunity:

NO’ has many diverse functions. It plays an important role in host immune defense by exerting non-specific antimicrobial action. It has been reported to have cytotoxic and cytostatic effects against various pathogens (Granger et al., 1988; Nathan & Hibbs, 1991). NO’ is produced by the action of nitric oxide synthetase (NOS) on L-Arg. There are three isoforms of NOS namely by endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). Of these, iNOS produces about 10-1000 times
more NO’ than the other two constitutively expressed isoforms (Moncada & Higgs, 1993; Stuehr & Griffith, 1992). iNOS is usually expressed by cells such as inflammatory phagocytic cells, epithelial cells, neuronal cells and others. In bacterial infections, suppression or lack of NO’ leads to impaired clearance of bacteria (Darrah et al., 2000; MacMicking et al., 1997; Shiloh & Nathan, 2000; Umezawa et al., 1997). In many viral infections, iNOS expression is induced by IFN-γ and IL-12 (Akaike & Maeda, 2000) and is protective, but in some cases, overproduction of NO causes injury to tissue. NO interacts with oxygen radicals to produce radical peroxynitrite (ONOO-) that causes pathological manifestations (Beckman et al., 1990; Beckman & Koppenol, 1996; Reiter et al., 2000). Protein nitration caused by the reactive nitrogen species leads to nitrotyrosine accumulation in the T cell receptor impairing its ability to interact with peptide-MHC complexes in a mouse model (Nagaraj et al., 2007). Exposure to peroxynitrites resulted in blockage of antigen presentation and subsequent recognition by cytotoxic T cells (Lu et al., 2011).

**Significance of MDSCs**

Under normal conditions, ARG1 and iNOS regulate each other and therefore are not expressed by the same cell at the same time. ARG1 mediated consumption of L-Arg prevents the expression of iNOS (Lee et al., 2003; Rodriguez & Ochoa, 2008). For instance, M1 macrophages which lead to Th1 response express iNOS and M2 macrophages which lead to Th2 response express ARG1. The hallmark of MDSCs is its
ability to co-express both ARG-1 and iNOS thus exerting a suppressive effect (Poschke & Kiessling, 2012).

1.9.2 Production of Reactive Oxygen Species (ROS)

Predominantly G-MDSCs produce ROS while the M-MDSCs generate NO’ and ARG-1 (Dolcetti et al., 2010; Youn et al., 2008). Other than MDSCs, granulocytes and macrophages also produce ROS which impairs T cell function by inducing T cell receptor ζ-chain. Production of ROS is mediated through the family of NADPH oxidases (NOX) whose activation leads to the release of ROS into the extracellular space. The oxidase enzyme is a multi-protein complex comprised of two membrane proteins gp91 and p22, and four cytosolic proteins p47phox, p67phox, p40phox and a small G protein Rac (Groemping & Rittinger, 2005). These components have been shown to be upregulated in MDSCs, especially p47phox and gp91 (Corzo et al., 2009) in mice models. Upregulation of p47phox was found to be STAT3 dependent in murine MDSCs (Corzo et al., 2009). In the absence of NOX2 activity, the MDSCs failed to induce T cell hyporesponsiveness and differentiate into mature DCs (Corzo et al., 2009). Also abrogation of suppressive activity of murine MDSCs was observed in gp91 knockout mice (Nagaraj et al., 2007). Human PBMCs treated with Hepatitis C viral core protein also up regulated p47phox both at protein and mRNA level, while the addition of ROS inhibiting enzyme catalase restored T cell function (Tacke et al., 2012). ROS reacts with nitric oxide to form peroxynitrites.
1.9.3 Immunosuppressive cytokine production

Immunosuppressive cytokine production is one of the most important suppressive mechanisms of human MDSCs. In many cancer conditions, expression of cytokines IL-10 and TGF-β by MDSCs have been reported (Loercher et al., 1999; Valenti et al., 2006; Vuk-Pavlovic et al., 2010). TGF-β was observed in cytokine induced MDSCs generated in vitro (Lechner et al., 2010; Lechner et al., 2011). A study by Sinha et al (Sinha et al., 2007), showed the cross talk between MDSCs and macrophages through cytokines. The MDSCs secrete increased IL-10 and at the same time down regulate IL-12 secretion by macrophages. This polarizes the macrophages toward M2 phenotype thus skewing CD4 and CD8 T cell responses. IL-10 also interferes with DC maturation (Vicari et al., 2002), which might suggest that MDSCs can potentially block DC function indirectly through immunosuppressive cytokines.

1.9.4 MDSCs and Tregs

MDSCs possess the ability to promote the development of T regulatory cells expressing FoxP3 marker in tumor conditions. In vivo studies of mice have shown the TGF-β dependent (Huang et al., 2006) and independent (Serafini et al., 2008) pathways of de novo development of Tregs by MDSCs. CD14⁺HLADR⁻ cells were able to convert conventional T cells into FoxP3⁺ and IL-10 producing T cells of suppressive nature, whereas CD14⁺HLA-DR⁺ monocytes promoted Th17 cells in human PBMCs ex vivo.
(Hoechst et al., 2011). In a murine model of lymphoma, MDSC induced Tregs expansion was shown to be arginase dependent (Serafini et al., 2008), and in a colon cancer model, IFN-γ and IL-10 were required but not iNOS (Huang et al., 2006). In mice bearing ID8 tumors, high level of CD80 expression on murine MDSCs were correlated with Treg expansion and immune suppression (Yang et al., 2006). There is not much information available on viral diseases with respect to Tregs induction by MDSCs other than in one report. In chronic HIV patients, MDSCs levels correlated with increased frequency of Tregs ex vivo (Vollbrecht et al., 2012).

1.9.5 Suppression of NK cells and NKT cells

Natural Killer (NK) cells and NKT cells are critical players in innate immunity and against viral infections. NK cells are involved in the first defense and regulation of adaptive immunity due to their action on APCs. Human MDSCs interaction with NK cells through NK cell activating receptor NKp30 led to inhibition of NK cell proliferation, cytokine production and cytotoxicity (Hoechst et al., 2009) in vitro. In vaccinia virus infected mice, G-MDSCs suppressed NK cell activation and function through ROS, at early stages of infection (Fortin et al., 2012). In a murine study, absence of iNKT cells resulted in expansion of MDSCs during Influenza A virus infection; adoptive transfer of the iNKT cells led to restoration of immunocompetence and ability to clear the virus (De Santo et al., 2008). In contrast, Nausch et al., (Nausch et al., 2008)
have reported the activation, rather than suppression of NK cells in a mouse model of lymphoma.

1.9.6 Impairment of T cell homing

Naïve T lymphocytes home to lymphoid tissues where they interact with antigen and get activated for which L-selectin (CD62L) is the homing marker. L-selectin directs the naïve T lymphocytes to home to sites of inflammation where they undergo antigen activation, form adhesion and transmigration. In a study by Hanson et al. (Hanson et al., 2009), levels of MDSCs and L-selectin were found to be inversely correlated both under in vivo and in vitro conditions in mouse mammary carcinoma. They further observed the expression of ADAM17, a protease that cleaves L-selectin on the plasma membrane of the MDSCs. Thus, the MDSCs were able to down regulate L-selectin by proteolytic cleavage, impairing T cell trafficking and priming by activated APCs in the lymph nodes and tumor environment.

1.9.7 Cysteine Sequestration

T lymphocytes are unable to generate the amino acid cysteine and cannot import the cysteine, due to lack of the transporter molecule. Cysteine is an essential amino acid for the T cells, especially for their activation, proliferation and differentiation. They are dependent on an exogenous source of cysteine exported by the APC when they are in
close proximity during antigen presentation. Murine MDSCs import cysteine but do not export cysteine thus limiting its availability to T cells and also to macrophages and DCs (Ostrand-Rosenberg, 2010). Low cysteine conditions make cells more prone to oxidative stress (Sakakura et al., 2007). Alleviation of MDSCs mediated immune suppression and arrested tumor growth were observed in mice fed with N-acetyl cysteine, a form of cysteine which cannot be oxidized in the extracellular milieu (Ostrand-Rosenberg, 2010). However, mRNA levels of the amino acid transporters in MDSCs from melanoma patients and healthy controls did not alter significantly; this mechanism of cysteine sequestering in human MDSCs has not been proven (Poschke & Kiessling, 2012).
Chapter 2

Immunomodulatory responses induced by PRRSV strain VR2332 at mucosal tissues of infected pigs

2.1 Abstract

Porcine reproductive and respiratory syndrome (PRRS) is a chronic viral disease of pigs caused by PRRS virus (PRRSV). The PRRSV VR2332 is the prototype North American parental strain commonly used in the preparation of vaccines. The goal of this study was to understand missing information on VR2332 induced immune modulation at the lungs and lymphoid tissues, the sites of PRRSV replication. Pigs were infected intranasally and samples collected at post-infection day (PID) 15, 30 and 60. Microscopically, lungs had moderate interstitial pneumonia, and the virus was detected in all the tested tissues namely lungs, tracheobronchial lymph nodes, iliac lymph nodes, tonsils and serum. Peak antibody response and the cytokine IFN-γ secretion were detected at PID 30, with increased TGF-β until PID 60 in serum. Populations of CD8⁺, CD4⁺, and CD4⁺CD8⁺T cells, Natural killer (NK) cells, and γδ T cells in the lungs and lymphoid tissues were significantly modulated favoring PRRSV persistence. The NK cell-mediated cytotoxicity was significantly reduced in infected pigs. In addition, increased population of immunosuppressive T-regulatory cells (Tregs) and associated
cytokines were also observed in VR2332 strain infected pigs. This study shows the differential mucosal responses elicited by the wildtype PRRSV strain on intranasal inoculation in pigs.

2.2 Introduction

PRRS continues to economically plague the swine industry since the last two decades, causing an estimated annual economic loss of $664 million to the U.S. pork industry (Holtkamp & Kliebenstein, 2011). PRRS was reported in the US and Europe in the early 1990s (Collins et al., 1992; Wensvoort et al., 1991), and is now prevalent in all the swine producing countries. The disease is characterized by both respiratory and reproductive dysregulation (Christopher-Hennings et al., 1995), with losses from reproductive failure in sows, through stillbirths, mummifications, weak born piglets, and high pre-weaning mortality. PRRSV belongs to the family Arteriviridae and it possesses a positive sense RNA genome. The virus is known for frequent mutations resulting in constant emergence of genetic variants. Based on its genetic diversity, the virus was divided into two genotypes, Type1 (European) and Type 2 (North American). Lelystad and VR2332 are the prototype wildtype parental strains of Type 1 and Type 2 PRRSV, respectively. PRRSV infects pigs of all ages; macrophages and dendritic cells are its primary targets. This virus is known to dampen the immune system as early as day-two post-infection (Dwivedi et al., 2012) and continues to do so for several weeks post-
infection (Mateu & Diaz, 2008). Immunosuppression induced by the PRRSV leads to increased susceptibility to secondary microbial infections (Done & Paton, 1995).

Innate natural killer (NK) cells provide the first line of innate defense against viral infections (Biron et al., 1999). Viral clearance is mediated by NK cells, virus neutralizing antibodies (VN), and antiviral cytokines followed by cell-mediated immune responses. However, viruses evade the host immunity by multiple mechanisms. PRRSV does it by induction of dampened interferon-α (IFN-α) (Albina et al., 1998), delayed and weak interferon-γ (IFN-γ) secretion and suppression of NK cell-mediated cytotoxicity (Renukaradhya et al., 2010). In addition, PRRSV elicits delayed and weak VN responses (Diaz et al., 2005), and mediates a reduction in the total T lymphocyte population in the lungs (Dwivedi et al., 2011a). Foxp3+ T-regulatory (Tregs) cells suppress the activation of adaptive arm of the immune system, and also secrete the immune suppressive cytokines, interleukin-10 (IL-10) and transforming growth factor-β (TGF-β). Their active role has been linked to several persistent viral infections including PRRS (Peng et al., 2008; Silva-Campa et al., 2012; Vahlenkamp et al., 2005). Increased induction of IL-10 and TGF-β by PRRSV is partially responsible for immune evasion, but induction of IL-10 secretion varies with the strain of PRRSV (Diaz et al., 2006).

PRRSV causes a short viremic phase followed by viral persistence for several months in the lungs, tonsils, and lymphoid tissues (Allende et al., 2000). Although, the immune response in the bronchoalveolar lavage (BAL) fluid and blood of PRRSV infected pigs is extensively studied, similar information for the lung parenchyma and lymphoid tissues is limited. Therefore, the comprehensive anti-PRRSV immunity in the
lungs and lymphoid tissues of wildtype parenteral vaccine strain VR2332 infected pigs was elucidated in this study.

2.3 Materials and Methods

Cells and virus

A stable *Mycoplasma*-free MARC-145 cells (African Green monkey kidney cell line) which supports the growth of PRRSV (Christopher-Hennings *et al.*, 2001) was used to prepare PRRSV stocks and for immunological assays. Cells were maintained in Dulbecco’s minimum essential medium (DMEM, Lonza) with 10% fetal bovine serum (Atlanta Biologicals) at 37°C with 5% CO$_2$. For virus infection, DMEM supplemented with 2% horse serum was used.

Pigs and Inoculations

Conventional Large White-Duroc crossbred weaned specific-pathogen-free pigs were weaned at 16 to 20 days of age and transported to animal facilities at FAHRP, OARDC, The Ohio State University, Wooster, OH. The swine herd was confirmed negative for antibodies in serum to PRRSV, PRCV, TGEV, and PCV2. Blood samples collected on arrival were confirmed for absence of PRRSV antibodies. Pigs were allowed to acclimate for an additional week before initiation of the experiment. Animals were
maintained in our large animal BSL2 facility under the supervision of a veterinarian, and pigs received food and water *ad libitum*. PRRSV VR2332 strain (1×10⁶ TCID₅₀/ml, 2 ml/pig) was administered by the intranasal route. Twelve pigs were used in two groups: group 1 (n=3), mock pigs inoculated with DMEM; group 2 (n=9), infected with VR2332 strain. Three pigs each from group 2 were euthanized on post-infection days (PID) 15, 30, and 60. In another independent study, seven pigs were used in two groups: group 1 (n=3), mock pigs inoculated with MARC-145 mock-infected culture supernatant; group 2 (n=4), infected with VR2332 strain. Two pigs each from group 2 were euthanized at PID 15 and 30. Mock inoculated pigs (total n=6) from the two experiments were euthanized separately. All the pigs were maintained and samples collected as per the protocol approved by The Ohio State University Institutional Animal Care and Use Committee.

**Collection of blood and lung samples for analysis**

For evaluation of viremia and for titration of PRRSV specific serum neutralizing antibodies, 3 to 5 ml of blood samples were collected at PID 0, 4, 7, 14, 21, 28, 31, 35, 42, 49, 56 and 60. Serum was separated and aliquots were kept at −20°C. Pigs were monitored daily for the respiratory disease, and rectal temperature and body weight were recorded twice weekly. We and others found lung homogenates more suitable than BAL fluid for the cytokine and virus evaluation in the lungs (Barbe *et al.*, 2010; Renukaradhya *et al.*, 2010). Approximately 2 - 5 gm of lung tissue was collected in serum-free DMEM from individual pigs during necropsy, minced into tiny pieces, and then blended in a
Stomacher 400 laboratory blender (Seward) for 5 min. Clarified supernatants were aliquoted and kept at -20°C until use in assays.

**Isolation of PBMC, lung MNC, BAL, TBLN, ILN and Tonsils cells**

For the isolation of PBMC, blood was collected in acid citrate dextrose solution from euthanized pigs and processed as described (VanCott *et al.*, 1993). Lung-mono-nuclear cells (lung MNC or LMNC) from individual pigs were isolated as per the described procedure (Dwivedi *et al.*, 2011a) with a few modifications. Briefly, after euthanasia the pulmonary vasculature was flushed with excess sterile PBS to remove the peripheral blood, and the lungs were removed. Airways were lavaged using sterile ice cold PBS containing EDTA (0.03%) to collect BAL cells. The lung tissue was collected in ice cold PBS, washed in sterile PBS, minced, and suspended in PBS containing DNase (Sigma) (40 μg/ml) and Type II collagenase (Invitrogen) (1.5 mg/ml), and incubated on an orbital shaker at 37°C for 2 hr. Released MNCs were collected and fractionated using 43 and 70% Percoll density gradient centrifugation and the interface rich in lung MNCs was harvested. Red blood cells were lysed by hypotonic shock using water, MNCs were counted and the viability was tested by the trypan blue dye exclusion method. Samples of tracheobronchial lymph nodes (TBLN), iliac lymph nodes (ILN) and tonsils were collected in DMEM, and MNCs were isolated as described previously (Dwivedi *et al.*, 2011a).
Microscopic lung pathology

Lung tissue samples taken at different PIDs and from mock pigs were fixed in 10% neutral buffered formalin and processed into paraffin blocks. Four micron sections were cut and stained using hematoxylin and eosin. Lung sections were examined by an unbiased certified veterinary pathologist (Dr. Tracey Papenfuss, OSU, Columbus, OH) and scored in a biased manner for the severity of the interstitial pneumonia: 0 = no microscopic lesions; 1 = mild interstitial pneumonia; 2 = moderate multifocal interstitial pneumonia; 3 = moderate diffuse interstitial pneumonia; 4 = severe interstitial pneumonia.

Virus titration and Virus neutralizing test (VNT)

PRRSV titer and virus neutralizing antibody titer in serum was analyzed by indirect immunofluorescence assay (IFA) as previously described (Christopher-Hennings et al., 2001). Briefly, for virus titration confluent monolayers of MARC-145 cells in 96-well microtiter plates were treated with 10-fold dilution of serum for 24 hr. For VNT, serum was heat inactivated for complement inactivation and then two-fold diluted and incubated with an equal volume of PRRSV (VR2332) containing 500 TCID$_{50}$ per well for 2 hr at 37°C and then 100 μl of the suspension was transferred into a 96-well microtiter plate containing a confluent monolayer of MARC-145 cells and incubated for 24 hr at 37°C in a CO$_2$ incubator. Cytopathic effects in both the plates for virus titration and VNT were
examined following addition of mAb binding to anti-PRRSV N protein (SDOW-17) and Alexa-488 conjugated anti-mouse IgG(H+L) secondary antibody, mounted with glycerol-PBS in 6:4 ratio and then observed using a fluorescent microscope. The PRRS viral titers and PRRSV specific VNT titers were determined based on count of fluorescent plaques and the dilution of the sample.

**Real time PCR detection of PRRSV**

Mononuclear cells from TBLN, tonsils, and ILN were used in the preparation of RNA by the Trizol method. Cells from pigs in each group were pooled and RNA extracted from 10 million cells was reverse transcribed into complementary DNA (cDNA) using a Quantitected Reverse Transcription kit from Qiagen. The cDNA was then used in real time PCR reactions using primers against PRRSV ORF6 in PerfeCta SYBR Green Fast Mix with forward primer (GATAACCACGCATTGTCGTC) and reverse primer (TGCCGTTGTTATTGTCGATA). Standard curves were generated using serial dilutions of known copies of PCR product. Negative and non template controls were also employed in the assay.

**PRRSV specific isotype antibody analysis in the lungs and TBLN**

PRRSV specific IgA and IgG antibodies secreted by restimulated B cells present in the lungs, PBMC, and TBLN were analyzed as described previously (Mulupuri *et al.*, 2003).
Briefly, ELISA plates were coated with pre-titrated semi-purified UV-inactivated PRRSV (VR2332) antigens (10µg/ml) in carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were then washed in sterile PBS (without Tween), and then treated with sterile blocking buffer (1% BSA in PBS) for 2 hr at RT. Isolated lung-MNCs (5x10^5 and 10-fold less 5x10^4 cells in duplicate wells) of individual pigs were plated in enriched RPMI-1640 [10%FBS, gentamicin (100 µg/ml), ampicillin (20 µg/ml), 20 mM HEPES, 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 nM 2-ME] and incubated at 37°C in a CO₂ incubator for 24 hr overnight. Plates were washed with PBS-Tween-20 and the bound PRRSV isotype specific antibody was detected using anti-pig IgA and anti-pig IgG secondary antibodies conjugated with Horesradish peroxidase enzyme (KPL). Finally, plates were developed using a chromogen 2, 2’-Azinodi (3-Ethyl Benzthiazoline Sulfonic Acid (ABTS) and read at 405nm. To eliminate the background activity, we also included control plate with no coated antigen, blocked and treated exactly as above with samples side-by-side. The OD values obtained from control plate were subtracted from the experimental plate to obtain the corrected OD values.

**ELISPOT assay to determine frequency of PRRSV specific IFN-γ secreting cells**

The frequencies of IFN-γ secreting cells in LMNC, PBMC, and TBLN-MNC were determined as described previously (Dwivedi et al., 2011a). As a control, all the MNCs restimulated in the absence of killed PRRSV antigen were included in every
experiment, and the respective background from each pig value was subtracted from the antigen-stimulated cells IFN-γ secreting cells frequency. MNCs restimulated with phytohaemagglutinin (PHA) at 10µg/ml were used as positive controls.

**Analysis of cytokine responses**

Purified and biotin labeled cytokine specific anti-porcine antibodies, IL-6, IL-12, and IL-10 (R&D Systems, Minneapolis, MN), IFN-γ (BD Pharmingen, San Diego, CA), TGF-β (Invitrogen, Camarillo, CA) were purchased from commercial sources and used to perform the sandwich ELISA. Pig sera collected at different PIDs were analyzed for Th1 (IFN-γ, IL-12), pro-inflammatory (IL-6), and immunosuppressive (IL-10 and TGF-β) cytokines by sandwich ELISA (Renukaradhya et al., 2010).

**Flow-cytometric study of different immune cell populations**

Flow cytometric analysis was performed to determine the phenotype and the frequency of different immune cells in a multicolor immunoassay as described previously (Dwivedi et al., 2011a). Briefly, single cell suspensions (PBMC, LMNC, BAL, TBLN and ILN) were suspended in fluorescence-activated cell sorting (FACS) buffer (0.1% BSA, 0.035% sodium bicarbonate and 0.02% Sodium azide in HBSS) and plated (1x10^6 cells) in U-bottom 96-well plates and treated with 2% pig serum to block the Fc receptors. Cells were treated with fluorochrome tagged, purified or biotin labeled pig
specific mAbs, CD3ε (clone PPT3, IgG1, Southern Biotech), CD172 (clone 74-22-15, IgG1, Southern Biotech.), CD4α (clone 74-12-5, IgG2b, Southern Biotech ), CD8α (clone 76-2-11 IgG2a, Southern Biotech), CD25 (clone K231.3B2, IgG1, AbD serotec), TcR1N4 (δ chain specific) (clone PGBL22A, IgG1, VMRD (Davis et al., 2001)), anti- mouse Foxp3 (clone FJK-16s, IgG2a, eBioscience, (Kaser et al., 2008a)) or their respective isotype control mAb. Cells were washed and treated with streptavidin-conjugated fluorochrome or respective anti-species isotype specific secondary antibody conjugated with fluorochrome. Finally, cells meant for determining Tregs population were fixed and washed in FACS buffer. For intracellular Foxp3 staining, the cells were surface stained for CD4 and CD25, and then permeabilized with permeabilization buffer overnight (85.9% deionized water, 11% PBS with no Ca or Mg, 3% formaldehyde solution and 0.1% saponin) and stained with fluorochrome-conjugated pig Foxp3 cross-reactive anti-rat Foxp3 mAb. Immunostained cells were acquired using a FACS AriaII (BD Biosciences) flow cytometer. The analysis was done to determine different immune cell populations based on the phenotypes (Piriou-Guzylack & Salmon, 2008): NK cells (CD3εCD4εCD8α); T-helper cells (CD3εCD4εCD8α); CD8α T cells (CD3εCD4εCD8α); T-helper/memory cells (CD3εCD4εCD8α); γδ T cells (CD8αεTcR1N4ε); T-regulatory cells (CD4εCD25εFoxp3ε); CD172ε (myeloid cells), analyzed using FlowJo software (Tree Star, Inc. OR, USA). Frequencies of individual immune cells were analyzed from a total 50,000 to 100,000 events.

Analysis and gating strategy: Different T lymphocyte subsets were determined by first gating for CD3ε positive cells (total T cells). Subsequently, the CD3ε population was
analyzed for CD4 and CD8 markers which provided us with three different T cell subsets: CD4⁺CD8⁻; CD4⁺CD8⁺; and CD4⁻CD8⁺ which correspond to T-helper, memory/Th, and cytotoxic T cells, respectively. The respective subset was back calculated with reference to CD3⁺ population. For NK cells, from the first gating performed on CD3ε marker, we chose CD3ε negative cells and further analyzed for CD4 and CD8α markers. For Tregs, initial gating was with CD4 and then positive cells were gated for CD25 and Foxp3 markers. For gamma delta T cells, double gating was performed for TCR1N4 and CD8α marker.

**NK cell-cytotoxic assay**

A colorimetric NK cell assay to determine the generic pig NK cell-mediated cytotoxicity in VR2332-infected pigs was conducted as described previously by us (Renukaradhy et al., 2010). Briefly, PBMC and LMNC were used as the source of NK cells (effectors) and K-562 human myeloblastoid leukemia cells as targets. Different ratios of effectors and targets were incubated for 24 hr at 37°C in a CO₂ incubator and the amount of released lactate dehydrogenase (LDH) was measured by a LDH substrate. The percent of NK cell-specific killing was calculated after subtracting the spontaneously released LDH due to nonspecific lysis of targets.
Statistical analysis

All the data were expressed as the mean +/- SEM of 3 to 9 pigs. Pooled data from two independent experiments at PID 15 and 30 are shown, therefore wherever animal tissues were used for analyses the sample number varies between 3 and 6. Statistical analyses were performed using one way analysis of variance (ANOVA) followed by post-hoc Tukey’s test using GraphPad InStat (software version 5.0 for windows) to establish differences between different PIDs or between mock and different PID. Statistical significance was assessed as $P<0.05$.

2.4 Results

Microscopic lung pathology

As expected, being a mild strain, no clinical PRRS symptoms in VR2332 infected pigs were observed. Mild gross lung lesions were observed at PID 30. Hematoxylin and Eosin stained lung sections of the infected pigs were scored microscopically based on severity of lesions and the percentage of area affected. At PID 15, the lung lesions were characteristic of moderate diffuse interstitial pneumonia, whereas at PID 30 moderate focal to diffuse interstitial pneumonia was observed (Fig. 2.1a). Infiltration of mononuclear cells and pneumonic lesions were seen with increased severity score and large infected area at both PID 15 and 30 which subsided by PID 60 (Fig. 2.11b).
Viral load in serum and the lungs of PRRSV infected pigs

PRRSV load in serum was high at PID 15 and gradually decreased by PID 60, but interestingly, a reverse trend was seen in the lungs (Fig. 2.2a). Real time PCR amplification for PRRSV ORF6 also identified the virus in mononuclear cells of TBLN (tracheobronchial lymph nodes), tonsils, and ILN (Iliac lymph nodes). All three lymphoid tissues showed the presence of PRRSV at PID 15 and the viral load was increased by several-fold at PID 30 (Fig. 2.2b). Comparable levels of PRRSV load were detected in all three lymphoid tissues at PID 60. Relatively, tonsils showed greater viral load compared to other two lymphoid tissues at PID 30 (Fig. 2.2b).

Antibody response against PRRSV infection

PRRS virus neutralizing (VN) antibodies were not detected until PID 21 in the serum, and from PID 28 low levels of VN titers (≤8) were maintained until PID 60 (Fig. 2.3a). *Ex vivo* secretion of PRRSV specific antibody by lung MNC (LMNC), peripheral blood MNC (PBMC), and TBLN-MNC of VR2332 infected pigs in cells restimulated with killed VR2332 antigens was detected at PID 30. The MNCs of lungs and TBLN secreted significantly higher amounts of PRRSV specific IgA and IgG antibodies at PID 30 than at the other two PIDs (Fig. 2.3b and 2.3c), however, only an IgG response was detected in PBMC. Antibody secreting cells present in the TBLN-MNC secreted
significantly higher amounts of both isotype specific PRRSV antibodies than LMNC and PBMC (Fig. 2.3b and 2.3c).

Modulated IFN-γ and immunosuppressive cytokines were observed in PRRSV infected pigs

Th1 cytokines, IFN-γ and IL-12, play an important role in antiviral immunity. After VR2332 infection, IFN-γ secretion was undetectable in the serum until PID 28, followed by a significant increase at PID 35 and 42, and subsequently reduced to basal levels (Fig. 2.4a). Secretion of IL-12 was increased gradually from PID 7 and it was significantly higher at PIDs 7, 15, 21, 42, 49 and 57 (Fig. 2.4a). The frequency of IFN-γ secreting cells was analyzed in the lungs, blood, and TBLN, and their frequency was low at PID 15 and increased by 3-fold at PID 30, but completely disappeared in both the mucosal tissues and blood by PID 60 (Fig. 2.4b). The proinflammatory cytokine, IL-6 that helps in initiation of adaptive immunity, was low until PID 35, and a slight increase was detected only from PID 42, with a significant increase at PID 57 (Fig. 2.4c). Immunosuppressive cytokines are IL-10 and TGF-β. In VR2332 infected pigs, TGF-β remained high until PID 49, with significantly increased levels at PIDs 15 and 21 (Fig. 2.4d). Surprisingly, the secretion of cytokine IL-10 remained low until PID 57 but significantly at PID 60 (Fig. 2.4d). The PID 0 levels of the cytokines were similar to mock animals and were used as mock controls.
Modulation in the frequency of immune cells in the lungs and blood

Mononuclear cells in the lungs (LMNC and BAL cells) and blood were immunostained to analyze different immune cell phenotypes. A significant reduction in the total T lymphocyte (CD3\(^+\)) in LMNC and total myeloid cell population (CD172\(^+\)) in BAL (but not in PBMC) was observed at PID 60 (Fig. 2.5a and 2.5g). Frequency of CD8\(^+\) T cells in LMNC were significantly increased and decreased at PID 15 and PID 60, respectively (Fig. 2.5b). T-helper/memory cells (CD4\(^+\)CD8\(^+\)) were significantly increased in PBMC at PID 15 and 30 (Fig. 2.5c), while T helper cell (CD3\(^+\)CD4\(^+\)) population remained unaltered in all the three tissues (Fig. 2.5d). A significant increase in the frequency of Tregs was observed in BAL at PID 15, and which was reduced by PID 60. While they increased to significant levels in PBMC at PID 15 and 60 (Fig. 2.5e). There was a significant decrease in the population of \(\gamma\delta\) T cells in the lungs at both PIDs 30 and 60, and a similar decrease (but not significant) was seen in BAL at PID 60 (Fig. 2.5f).

Modulation in the frequency of immune cells in the lymphoid tissues

Frequencies of immune cells in three mucosal lymphoid tissues, tonsils, ILN, and TBLN were also analyzed. Tonsils constitute the major organ of PRRSV persistence. ILN are the draining lymph nodes of the reproductive tract and similarly TBLN for the lungs. All three lymphoid tissues had a reduced trend in total T cells at the later two time points post-infection, with a significant reduction in the tonsils at PID 60 and in ILN at PID 30.
CD8⁺ T cells frequency did not change significantly in the three lymphoid tissues (Fig. 2.6b). Both tonsils and ILN showed significantly increased CD4⁺CD8⁺ T cell population at PID 15, which was later, reduced (Fig. 2.6c). A significant increase in T-helper cell population was seen at PID 15 in TBLN and ILN, which was reduced significantly by PID 60 in both tissues (Fig. 2.6d). The frequency of Tregs showed an increased trend in all three lymphoid tissues, with a significant increase in TBLN at PID 60 (Fig. 2.6e). The population of γδ T cells was significantly increased at PID 30 in tonsils and ILN, while their frequency dropped significantly by PID 60 (Fig. 2.6f). The myeloid cell population in the tonsils and ILN was increased significantly at PID 60 (Fig. 2.6g).

**Modulation in the frequency and cytotoxic function of NK cells**

There was a decreasing trend for the populations of NK cell-rich fraction (CD3⁻ CD4⁻CD8α⁺) at all three PIDs in BAL and LMNC, with a significant decrease in BAL at PID 15 compared to mock pigs, whereas in PBMC they were significantly increased at PID 15 and 60 (Fig. 2.5h). NK cells frequency was significantly decreased at all three tested PIDs in tonsils (Fig. 2.6h). Modulation in the frequency of NK cells in VR2332 infected pigs was associated with suppression of its cytotoxicity (Fig. 2.7). At PID 15, a 50% reduction in the NK cytotoxicity and further suppression at later PIDs was observed in both blood (Fig. 2.7a) and in lungs of infected pigs (Fig. 2.7b).
2.5 Discussion

The PRRSV antigenic variations are capable of differentially modulating the immune response depending on the infecting strain (Diaz et al., 2006). Strain VR2332 was isolated from infected pig tissue samples during the first recorded PRRSV epidemic in the US (Collins et al., 1992). Since late 1990s the strain VR2332 has been in use in the preparation of modified live PRRSV vaccine in the US. Persistence of this vaccine strain in lymphoid tissues could be responsible for shedding of vaccine derived mutated viruses in the field (Shi et al., 2010b). The VR2332 strain induces delayed and weak VN titers (<8) in infected pigs. Generally, a PRRSV VN titer of greater than 8 is protective against the infection while a titer of 32 provides sterilizing immunity (Lopez et al., 2007). PRRSV specific antibody responses in serum were detected from PID 14 to 120 in pigs infected with a field virulent isolate (Batista et al., 2004). Following inoculation of MLV-PRRS serum antibodies were detected at PID 15 and 30 (Bassaganya-Riera et al., 2004). But knowledge about the ex vivo antibody secretion by restimulated mucosal immune cells of VR2332 infected pigs is limited. Our study suggests that infected pig lung MNC and TBLN-MNC secreted peak antibody responses only at PID 30.

Increased levels of IFN-γ in serum of VR2332 infected pigs were detected at PID 28 and they stayed high only for a short time. In the PBMC, a similar trend in IFN-γ secreting cell population was detected following VR2332 infection (Xiao et al., 2004). In addition, we identified a similar delayed and weak IFN-γ secreting cell population in the lungs and TBLN, but that population disappeared by PID 60. Delayed secretion of IFN-γ
has been postulated to be one of the immune modulatory mechanism of PRRSV, and has also been observed in pigs inoculated with MLV-PRRS (Meier et al., 2003). IFN-γ is produced predominantly by NK cells, and CD4 and CD8 expressing lymphocyte subsets (Biron et al., 1999). Co-incidentally, the population of NK cells, CD4⁺, CD8⁺, and memory T cells was reduced by PID 60 in VR2332 infected pigs at both systemic and mucosal sites.

Irrespective of increase in the NK cell population in PBMC at PID 15 and 60, their cytotoxicity was not increased, confirming the functional modulation of NK cells in VR2332 infected pigs, as also observed with other PRRSV strains (Renukaradhya et al., 2010). γδ T cells are a lymphocyte subset unusually high in their frequency in pigs (15 – 30%) which were initially characterized as null CD2⁻ cells (Binns, 1994). They are involved in innate and adaptive immune responses against viral infections. In FMD virus infected pigs, an increase in γδ T cells population was associated with upregulation of several important cytokines (Takamatsu et al., 2006). In contrast, γδ T cell population in VR2332 infected pigs was significantly decreased in the lungs, tonsils, and ILN.

CD8⁺ T cell population in VR2332 infected pigs were increased transiently in the lungs, but their frequency was decreased rapidly later, comparable results in Type 1 PRRSV strain infected pigs was observed in the BAL fluid (Samsom et al., 2000). Porcine immune system has a unique increased frequency of circulating population of CD4⁺CD8⁺ T cells representing memory, cytotoxic, and T-helper cell properties (Denyer et al., 2006; Zuckermann & Husmann, 1996). The CD4⁺CD8⁺ and CD4⁺ T cell subsets in VR2332 infected pigs increased significantly at two weeks post-infection in the lungs,
blood, and lymphoid tissues, but rapidly decreased thereafter. Overall, our results suggested that both innate (γδ T cells and NK cells) and adaptive immune cell subsets were modulated in mucosal tissues in VR2332 infected pigs, where the virus persists for long-time.

Foxp3$^{+}$ Tregs have been implicated in mediating immunosuppression (Didierlaurent et al., 2007). Cytokines, IL-10 and TGF-β, are immunosuppressive in nature, produced by Tregs and are upregulated in PRRSV infected pigs (Silva-Campa et al., 2012). In our previous study, we demonstrated that a virulent PRRSV strain MN184 significantly upregulated the production of IL-10 and TGF-β (Dwivedi et al., 2011a) (Dwivedi et al., 2011a). There are reports that claim different PRRSV strains induce secretion of varying amounts of IL-10 (Diaz et al., 2006; Dwivedi et al., 2012; Silva-Campa et al., 2009). In this study, production of IL-10 was upregulated only at PID 60 in infected pigs. But there was a significantly increased production of TGF-β in the serum observed from early time points post-infection.

Recently, several studies have tried to establish Tregs connection in PRRSV infection (Silva-Campa et al., 2009; Wongyanin et al., 2010). Both the wildtype parenteral strain VR2332 and MLV-PRRS upregulate Tregs in PBMCs as early as 10 days post-infection that were decreased by day 14 post-infection (Leroith et al., 2011). But we found a significantly upregulated population of Tregs in PBMC and TBLN only at PID 60. In contrast, in virulent MN184 strain infected pigs the Tregs frequency was upregulated from day 15 post-infection (Dwivedi et al., 2011a). Thus, production of
cytokines IL-10 and TGF-β could be partially contributed by Tregs in VR2332 infected pigs.

In conclusion, although wildtype parenteral strain VR2332 is avirulent, still it dampens the most essential immune components at the site of its replication, the lung parenchyma and lymphoid tissues, resulting in weak and delayed anti-PRRSV immunity. Thus, our study emphasizes the need for a better PRRSV vaccine strain that has the ability to elicit better anti-PRRSV immune responses to combat the menace of constantly increasing PRRSV outbreaks.

2.6 Acknowledgments

We thank Dr. Juliette Hanson, Mathew Weeman, Drs. Mahesh Khatri, and Hadi Yassine for their help in animal studies, Dr. Eric Nelson provided the PRRSV strain VR2332 and control sera.
Fig. 2.1. Microscopic lung pathology in PRRSV VR2332 infected pigs. Pigs infected with PRRSV VR2332 strain were euthanized on PIDs 15, 30 and 60. (a) A representative lung H&E picture of mock and PRRSV infected pigs at indicated PID. (b) Microscopic lesions were graded based on percentage of the lung area affected (filled bars) and the lung lesion scores (open bars) based on the severity of inflammatory pathology. Each bar represents the average value from 3 pigs ± SEM.
Fig. 2.2 Persistence PRRSV load in serum, lungs and lymphoid tissues of VR2332 infected pigs. Pigs infected with VR2332 virus were euthanized at three indicated PIDs. (a) Each bar represents the average viral load in serum (n=5 at PID 15 and 30, and n=3 at PID 60) and lung homogenates (n=5 at PID 15, and n=3 at PID 30 and 60) at indicated PID. Asterisk indicates statistically significant difference (P<0.05) between different PIDs. (b) PRRSV load in the MNCs isolated from TBLN, tonsils, and ILN at all three indicated PIDs (n=3 pigs/group) is shown.
Fig. 2.3. Delayed PRRSV specific antibody response in VR2332 infected pigs. (a) Serum samples collected on the indicated PID were analyzed for anti-PRRSV specific neutralizing antibody titers by a standard immunofluorescence assay. Serum samples from five pigs were analyzed at each indicated time point from PID 0 to 28 and three samples from PID 35 to 56. (b and c) LMNC, PBMC, and TBLN-MNC isolated from PRRSV infected pigs at indicated PIDs were restimulated with killed PRRSV antigens coated plate, and the isotype specific secreted antibody was analyzed by ELISA. Each data point in the graph represents the average OD from 3 to 5 pigs ± SEM. Asterisk indicates statistically significant difference (P<0.05) between different PIDs.
Fig. 2.4. PRRSV infected pigs secreted variable amounts of cytokines. Pigs were infected with PRRSV and euthanized at PIDs 15, 30 and 60. Serum samples collected at weekly intervals was analyzed for: (a) IFN-γ and IL-12; (c) IL-6; and (d) IL-10 and TGF-β by ELISA. Each data point represents the average cytokine levels from 9, 6, and 3 pigs at PID 15, 30, and 60, respectively, ± SEM. Asterisk indicates statistically significant difference (P<0.05) between average cytokine levels from pooled samples at respective PID with average at PID 0. (b) LMNC, TBLN-MNC, and PBMC were restimulated in the absence or presence of killed PRRSV antigens and the frequency of IFN-γ secreting cells were analyzed by ELISPOT assay. Data represented at PID 0 were similar to mock controls. Each bar represents the average number of IFN-γ secreting cell spots after subtracting from the background from 3 pigs ± SEM. Asterisk indicates statistically significant difference (P<0.05) between averages at each PID.
Fig. 2.5. Frequency of immune cells in PRRSV infected pig lung and blood are modulated. Pigs were mock infected (n=6) or infected with PRRSV strain VR2332 and euthanized at PIDs 15 (n=5), 30 (n=5), and 60 (n=3). LMNC, PBMC, and BAL cells were immunostained to determine the frequency of immune cells from all the indicated number of pigs used under each group: (a) Total T cells; (b) CD8+ T cells; (c) T-helper cells; (d) Memory cells; (e) T-regulatory cells; (f) γδ T cells; (g) Myeloid cells; and (h) NK cells, and then subjected to flow cytometry. Each bar represents the average percent of immune cells from 3 to 6 pigs ± SEM. Asterisk indicates statistically significant difference (P<0.05) between averages at each PID and mock.
Fig. 2.6. Frequency of immune cells in PRRSV infected pig lymphoid tissues were modulated. Pigs were mock infected (n=6) or infected with PRRSV strain VR2332 and euthanized at PIDs 15 (n=5), 30 (n=5), and 60 (n=3). Mononuclear cells from tonsils, TBLN, and ILN were immunostained to determine the frequency of different immune cells from all the indicated number of pigs used under each group: (a) Total T cells (b) CD8^+ T cells; (c) T-helper cells; (d) Memory cells; (e) T-regulatory cells; (f) γδ T cells; (g) Myeloid cells; and (h) NK cells, and then subjected to flow cytometry. Each bar represents the average percent of immune cells from 3 to 6 pigs ± SEM. Asterisk indicates statistically significant difference (P<0.05) between averages at each PID.
Fig. 2.7. Dampened NK cell-mediated cytotoxicity in VR2332 infected pigs’ blood and the lungs. Percent NK cell-specific cytotoxicity was measured using: (a) PBMC and (b) LMNC as source of NK cells (effectors) against K-562 target cells. Effectors and targets at indicated E:T ratios were co-cultured and the supernatant harvested after 24 hr was analyzed at OD490 nm for released LDH from the lysed targets using LDH substrate. Each line corresponds to average NK cell-specific lysis from three pigs ± SEM at four indicated E:T ratios. Statistical analysis was performed at every E:T ratio by comparing percent NK lysis between mock vs. individual PID and also between PIDs: ‘a’ denote statistically significant difference between mock vs. PID 15; ‘b’ denote between mock vs. PID 30; ‘c’ denote between mock vs. PID 60; ‘d’ denote between PID 15 vs. 60.
Chapter 3

PRRSV infection creates an immunosuppressive microenvironment in the lungs of pigs; Adjuvanted vaccine potentiates the anti-PRRSV immunity

3.1 Abstract

Porcine reproductive and respiratory syndrome (PRRS) is an economically important disease of pigs worldwide. Currently used PRRSV vaccines provide incomplete protection. Recently, we identified *Mycobacterium tuberculosis* whole cell lysate (*Mtb* WCL) as a potent mucosal adjuvant to modified live PRRSV vaccine (MLV-PRRS). In this study, pigs were unvaccinated or vaccinated with MLV-PRRS plus *Mtb* WCL, intranasally, challenged with the homologous PRRSV (strain VR2332), and subsequently euthanized at three time points post-challenge to evaluate lung immune responses. Examination of lungs revealed reduced lung lesions on gross pathology, and reduced disruption of the lung architecture and less interstitial pneumonia microscopically in vaccinated, compared to unvaccinated VR2332 challenged pigs. Along with this, reduced immunosuppressive IL-10 cytokines and increased IgA response was observed in lung mononuclear cells restimulated with VR2332 antigens. The lung homogenate showed decreased secretion of nitric oxide was detected in vaccinated virus challenged pigs at PID 15. In summary, the adjuvant effects of *Mtb*
WCL to MLV-PRRS resulted in enhanced anti-PRRSV immune microenvironment in the lungs which was however not sufficient to clear the viremia.

3.2 Introduction

Porcine Reproductive and Respiratory syndrome (PRRS) is the most devastating problem plaguing the global swine industry within the last two decades. The annual economic losses due to PRRS is estimated to be $664 million in the US (Holtkamp & Kliebenstein, 2011). PRRS is characterized by both respiratory and reproductive abnormalities (Christopher-Hennings et al., 1995; Collins et al., 1992; Wensvoort et al., 1991), with major losses from reproductive failure associated with stillbirths, mummifications, weak born piglets, and high preweaning mortality (Mengeling et al., 1998; Meulenberg, 2000; Rowland, 2007). The respiratory form leads to pneumonia, reduced feed intake with chronically recurring illness, and debilitation with potentially high mortality. The causative agent, PRRS virus (PRRSV), is an Arterivirus with a positive sense RNA genome. The PRRSV is prone to constant genetic and antigenic variations. Based on its genetic diversity, PRRSV can be divided into two genotypes, Type1 (European) and Type 2 (North American). The strains Lelystad and VR2332 are the reference prototype strains of Type 1 and Type 2 PRRSV, respectively. PRRSV replicates in alveolar macrophages and persists for a long time, up to 150 days in the lymphoid tissues (Allende et al., 2000). The porcine immune system is unable to mount an effective PRRSV immune response in the first few weeks post-infection (Mateu &
Diaz, 2008; Murtaugh et al., 2002); attributed to dampened production of anti-viral cytokines, virus neutralizing antibodies, and cell-mediated immune responses. In addition, immunosuppression caused by the virus makes infected pigs prone to secondary bacterial and viral infections (Done & Paton, 1995).

To control PRRS outbreaks, commonly employed vaccines include modified live PRRSV (MLV-PRRS) and killed virus vaccines. Killed vaccine does not provide adequate protection in terms of reducing viremia or virus shedding compared to MLV-PRRS (Nielsen et al., 1997). MLV-PRRS provides satisfactory protection against homologous infections, but protection against heterologous viruses and reinfections is incomplete. There are reports of results obtained using field isolates of PRRSV and MLV-PRRS administered by either parenteral or intranasal route showing suppression of innate NK cell cytotoxic function and IFN-α production (Albina et al., 1998; Dwivedi et al., 2011b; Renukaradhya et al., 2010), weak and delayed cell-mediated and virus neutralizing antibody responses (Lopez & Osorio, 2004; Yoon et al., 1995), associated with prolonged immunosuppression (renukaradhya. To improve the efficacy of MLV-PRRS in pigs we explored the benefits of inducing anti-PRRSV mucosal immunity by intranasal co-administration of MLV-PRRS with a suitable mucosal adjuvant (Renukaradhya et al., 2012).

Intranasal immunization of certain vaccines has shown great promise in eliciting protective immunity against various respiratory viral infections such as bovine herpes virus-1, influenza, and parainfluenza-3 (Guillonneau et al., 2009; Van der Poel et al., 1995). Mucosal tissues and associated lymphoid organs contain approximately 80% of
total immune cells in the body, and stimulation of the mucosal immune system leads to
generation of both systemic and mucosal immune responses resulting in effective
prevention of entry and establishment of pathogens (Etchart et al., 1996; Karron et al.,
1995). Recently, we have demonstrated that intranasal co-administration of MLV-PRRS
and adjuvant *Mycobacterium tuberculosis* whole cell lysate (*Mtb* WCL) overcome the
vaccine induced immunosuppression with concomitant increase in host innate and
adaptive immune responses, associated with better protection against a virulent
heterologous PRRSV MN184 challenge (Binjawadagi et al., 2011; Dwivedi et al., 2011a;
Dwivedi et al., 2011b; Renukaradhya et al., 2012). In our earlier study, we have also
shown that protective anti-PRRSV immune response elicited in pigs to MLV-PRRS was
mediated by the adjuvant effects of *Mtb* WCL, as pigs vaccinated with only MLV-PRRS
failed to elicit adequate immune responses (Dwivedi et al., 2011a). In continuation of the
prior studies, this paper deals with aspects of lung pathology associated with secretion of
cytokines, production of an important innate molecule ‘nitric oxide’, proliferation of
PRRSV specific CD8⁺ lymphocytes and modulated frequencies of immune cells in the
lungs of pigs vaccinated (MLV-PRRS + *Mtb* WCL) and challenged with PRRSV.
3.3 Materials and Methods

Cells, PRRSV, and adjuvant

A stable Mycoplasma-free MARC 145 cells (African Green monkey kidney cell line) which supports the growth of PRRSV (Christopher-Hennings et al., 2001), was used to prepare PRRSV stocks and for immunological assays. Cells were maintained in Dulbecco’s minimum essential medium (DMEM, Lonza) with 10% fetal bovine serum (Atlanta Biologicals) at 37°C with 5% CO₂. For virus infection, DMEM supplemented with 2% horse serum was used. PRRSV modified live vaccine (MLV-PRRS) (Ingelvac® Boehringer Ingelheim) was a kind gift from Dr. Mike Roof (Bio-R&D, Boehringer Ingelheim Vetmedica Inc). PRRSV strain VR2332 (low pathogenic vaccine strain) used as homologous virus challenge in the study were provided by Eric Nelson (South Dakota State University). For some experiments, M.tb whole cell lysate (Mtb WCL) was provided by Drs. Dobos and Belisle under NIH/NIAID funded contract HHSN266200400091c "TB Vaccine Testing and Research Materials" (Colorado State University).

Pigs and Inoculations

Conventional Large White-Duroc crossbred specific-pathogen-free piglets weaned at 3 to 4 weeks of age were obtained from farms regularly screened and free from
PRRSV, porcine respiratory coronavirus, transmissible gastroenteritis virus, and porcine circovirus type 2 were used in the study. Before the beginning of our study, pigs were tested to confirm absence of PRRSV antibodies by ELISA. Throughout the duration of the study pigs received food and water ad libitum. All inoculations including PRRSV challenge was performed by intranasal delivery. Animals were maintained, samples collected, and pigs were euthanized in our large animal BSL2 facility as per the protocol approved by the Institutional Animal Care and Use Committee (IACUC) and Institutional Biosafety Committee (IBC), The Ohio State University, Ohio. Forty pigs were allocated to one of three groups: group 1, mock pigs (n=4) inoculated with vehicle (normal saline) and DMEM; group 2 was unvaccinated (n=9) and group 3 was vaccinated [MLV-PRRS (Boehringer Ingelheim Vetmedica, Inc.) + Mtb WCL] (n=9). Groups 2 and 3 were challenged with a homologous parenteral PRRSV strain VR2332 (1x10^6 TCID50/ml, 2ml per pig) on day 21 post-immunization. Pigs from each group were euthanized at three time points at 15, 30, and 60 day post-challenge (DPC) (n = 3 per group). Mock inoculated pigs (n=4) were euthanized for negative control.

**Isolation of PBMCs and lung-MNCs**

For the isolation of PBMCs, blood was collected in acid citrate dextrose solution from euthanized pigs and processed as previously described (VanCott *et al.*, 1993). Lung-mononuclear cells (lung-MNCs or LMNC) from individual pigs were isolated as per the procedure described previously (Calder *et al.*, 2004; Kuroki *et al.*, 2003; Loving *et al.*, 2002).
2007) with modifications. Briefly, after euthanasia the pulmonary vasculature was flushed with excess sterile PBS to remove the peripheral blood and lungs were subsequently removed. Subsequently, the lung tissue was collected in ice cold PBS, washed in sterile PBS, minced and suspended in PBS containing DNase (Sigma) (40 μg/ml) and Type II collagenase (Invitrogen) (1.5 mg/ml) and incubated on an orbital shaker at 37°C for 2 hr. Released cells were collected and fractionated using 43 and 70% Percoll density gradient centrifugation and the interface rich in lung-MNCs were collected. After RBCs lysis by hypotonic shock using sterile distilled water, cells were counted using a hemocytometer and the viability was tested by trypan blue dye exclusion method. The viability of lung-MNCs harvested by this method was >95%.

**Gross lung lesion analysis**

Necropsies were performed and lungs were examined for gross lesions. Grossly evident pulmonary changes were assigned a score based upon the percent of virus-affected lesions (purple-red colored consolidation) in each lung lobe separately, and a total percentage for the entire lung was calculated as described previously (Jung *et al.*, 2007).
Microscopic lung pathology

Lung tissue samples collected from pigs was fixed in 10% neutral buffered formalin and processed into paraffin blocks, three μm sections were cut and stained with haematoxylin and eosin as described previously (Jung et al., 2009). The lung sections were examined in a blinded fashion by a board certified veterinary pathologist (Dr. Tracey Papenfuss, The Ohio State University, Columbus, OH) to determine the percent lung area affected by virus induced pathology (Gomez-Laguna et al., 2010).

PRRSV specific isotype antibody analysis in lungs

Total PRRSV isotype specific IgA and IgG antibodies secreted by activated B cells in pig lungs was analyzed as described previously (Mulupuri et al., 2008; Zhang et al., 2007). Briefly, ELISA plates were coated with pre-titrated semi-purified UV-inactivated PRRSV (VR2332) antigens (10μg/ml) in carbonate- bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. Plates were washed in sterile PBS (without Tween), and then treated with sterile blocking buffer (1% BSA in PBS) for 2 hr at RT. Isolated lung-MNCs (5x10^5 and 10 fold less 5x10^4 cells in duplicate wells) of individual pigs were plated in enriched RPMI-1640 [10%FBS, gentamicin (100 μg/ml), ampicilin (20μg/ml), 20 mM HEPES, 2 mM l-glutamine, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, and 50 nM 2-ME] and incubated at 37°C in a CO2 incubator for 24 hr overnight. Plates were washed with PBS-Tween-20 and the bound PRRSV isotype specific antibody was
detected using anti-pig IgA and anti-pig IgG secondary antibodies conjugated with HRP (KPL). Finally, plates were developed using a chromogen ABTS and read at 405nm. To eliminate the background activity, we also included control plate with no coated antigen, blocked and treated exactly as above with samples side-by-side. The OD values obtained from control plate were subtracted from the experimental plate to obtain the corrected OD values.

**Virus titration and Virus neutralizing test (VNT)**

PRRSV titer and virus neutralizing antibody titer in serum was analyzed by indirect immunofluorescence assay (IFA) as previously described (Christopher-Hennings *et al.*, 2001). Briefly, for virus titration confluent monolayer of MARC-145 cells in 96-well microtiter plate was treated with 10-fold dilution of serum for 24 hr. For VNT, serum was heat inactivated for complement inactivation and then two-fold diluted and incubated with equal volume of PRRSV (VR2332) 1x10^3 FFU units per well for 2 hr at 37°C and then 100 μl of suspension was transferred into 96-well microtiter plate containing confluent monolayer of MARC-145 cells in a 96-well tissue culture plate and incubated for 24 hr at 37°C in a CO₂ incubator. Cytopathic effects in both the plates meant for virus titration and VNT were examined following addition of anti-PPRSV N’mAb (SDOW-17) and Alexa-488 conjugated anti-mouse IgG(H+L) secondary antibody and observed under a fluorescent microscope after mounted with glycerol-PBS in 6:4 ratio. The PRRS viral titers and PRRSV specific VNT titers were determined.
**PRRSV specific recall/memory immune response**

Five million PBMCs, TBLN-MNCs, BAL cells, and lung-MNCs were subjected to *in vitro* restimulation in a 24-well tissue culture plate in the presence semi-purified UV-inactivated PRRSV (VR2332) antigens (50 μg/ml) (Baker *et al.*, 2007; Johnson *et al.*, 2007) in enriched RPMI-1640 for 48 hr at 37°C. Culture supernatants harvested was analyzed for cytokines by ELISA. Cells cultured just in enriched RPMI were included as control. The amount of cytokines secreted by cultured restimulated mock pig cells were subtracted from the experimental pig immune cells cytokines.

**ELISPOT assay to determine PRRSV specific IFN-γ secreting cells**

The levels of IFN-γ secreting cells in PBMCs and lung-MNCs were determined as described previously (Azevedo *et al.*, 2006; Martelli *et al.*, 2009). Briefly, PBMCs and lung-MNCs were plated (5×10⁵ cells/well) in enriched RPMI-1640 in a 96-well *MultiScreen* plate (Millipore) precoated overnight with 10 μg/ml of mouse anti-pig IFN-γ mAb (BD Bioscience) at 4°C. The cells plated were restimulated with killed PRRSV (VR2332) antigens (50μg/ml) for 24 hr at 37°C in a CO₂ incubator. Plates were washed six times with PBST followed by incubation with biotinylated anti-pig IFNγ detection antibody for 1 hr at RT. Plates were washed and treated with strepavidin-HRP conjugate for 1 hr at RT and then developed using an insoluble substrate at RT for 1 hr. Plates were
washed in tap water several times and air dried. The frequency of PRRSV specific IFNγ secreting cells was counted using an AID® ELISpot Reader System. The background values were subtracted from the respective counts of the unstimulated cells and the immune responses were expressed as the number of IFNγ secreting cells per million PBMCs or lung-MNCs. Cells stimulated with PHA and unstimulated were included as positive and negative control, respectively, in every plate.

**Flow-cytometric study of different immune cell populations**

Flow cytometric analysis was performed to determine the phenotype and the frequency of different immune cells in a multicolor immunoassay as described previously (Renukaradhya et al., 2010). Briefly, single cell suspensions (PBMCs, lung-MNCs, BAL, and TBLN) were resuspended in fluorescence-activated cell sorting (FACS) buffer (HBSS containing 0.1% BSA, and 0.02% Sodium Azide) and plated (1x10^6) in U-bottom 96-well plates and then treated with FACS buffer containing 2% pig serum for 10 min at 4°C to block the Fc receptors. Cells were then stained with appropriate mAb, either directly conjugated to specific fluorochrome, biotinylated or purified antibody specific to pig specific immune cell surface markers, such as CD3ε, CD172 (Southern Biotech.), CD4α, CD8α/β, CD11c (BD Biosciences), CD25, MHC class II (Serotec), TcR1N4 (VMRD), FoxP3 (eBioscience) or their respective isotype control mAb and then incubated for 1 hr at 4°C. Cells were then washed with FACS buffer and treated with streptavidin-conjugated fluorochrome or respective anti-species isotype specific
secondary antibody conjugated with fluorochrome. Finally, cells were fixed with 1% paraformaldehyde washed and resuspended in FACS buffer. For intracellular FoxP3 staining, the cells were surface stained for CD4 and CD25 as described above, and then kept for overnight incubation at 4°C in permeabilization buffer and stained with fluorochrome-conjugated pig Foxp3 cross-reactive anti-rat FoxP3 mAb as described previously (Kaser et al., 2008a; b). Immunostained cells were acquired using a FACS AriaII (BD Biosciences) flow cytometer. The analysis was done to determine different immune cell populations based on the cell surface marker phenotypes: natural killer (NK) cells (CD3−CD4−CD8+); T-helper cells (CD3+CD4+CD8−); cytotoxic T lymphocytes (CTLs) (CD3−CD4−CD8+); T-helper/memory cells (CD3+CD4+CD8−); γδ T cells (CD8α+TcR1N4+); T-regulatory cells (CD4−CD25+FOXP3+); CD172+ (myeloid cells); CD4+CD25+ (activated T-helper cells); and dendritic cells rich fraction (CD172−CD11c−SLAII+) using FlowJo software (Tree Star, Inc. OR, USA). Frequencies of individual lymphocyte and myeloid cell subsets were analyzed from a total 50,000 to 100,000 events.

**Carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay**

To determine PRRSV specific proliferation of lymphocytes, lung mononuclear cells (LMNC) and peripheral blood mononuclear cells (PBMC) were isolated as described (Calder et al., 2004; Kuroki et al., 2003; Loving et al., 2007). Cells were stained with an irreversible cytosolic dye, CFSE, as described previously (Ko et al.,
2005). Briefly, five million cells were washed and stained with CFSE at 10 μM concentration in PBS and incubated for 15 min at room temperature. The labeling reaction was stopped using ice-cold RPMI containing 10% FBS, washed twice, and resuspended with the medium and incubated at 37°C for 30 min, and the cell numbers adjusted to 5X10^5 cells per well in a 96-well flat bottom plate. The cells were restimulated with semi-purified PRRSV antigens (20 μg/ml) of VR2332 strain, obtained by ultracentrifugation of clarified PRRSV infected MARC 145 cell culture supernatant. Cells untreated or treated with Concavalin A were used as negative and positive control, respectively. Following three days of incubation, cells were washed and stained with R-phycoerythrine conjugated anti-pig CD8 antibody (clone 76-2-11, BD Biosciences). Finally, cells were fixed and 50,000 to 100,000 events acquired using the FACS AriaII (BD Biosciences) flow cytometer and analyzed for CFSE and PE stained lymphocytes. The cell proliferation data was analyzed by FlowJo software (Tree Star, Inc. OR, USA) to obtain the proliferation index (PI) of each sample. The PI denotes the average number of cell divisions the responding lymphocytes underwent after subtracting the background (initial population of cells at generation ‘0’).

**Griess Assay**

Nitric oxide production in the lungs was determined by measuring nitrite levels, which is formed as nitric oxide breakdown product using a commercial kit (Promega). Lung homogenates were prepared from the lung tissue samples as described previously
(Renukaradhya et al., 2010). Samples were treated with a sulfanilamide solution for 5-10 min in the dark, and subsequently with N-1-naphthylethylenediamine dihydrochloride (NED) solution. The resulting reaction product was measured at an absorbance between 520 and 550nm, and the absorbance was calculated by generating a nitrite standard reference curve.

**Statistical analysis**

All data were expressed as the mean +/- SEM of three pigs. Statistical analyses were performed using two-sample t-test to establish differences between vaccinated and unvaccinated groups. Statistical significance was assessed as \( P < 0.05 \).

### 3.4 Results

**Reduction in lung pathology**

The gross lung examination in unvaccinated VR2332 challenged pigs revealed no appreciable macroscopic lesions until DPC 15 (Fig 3.1A). However, increased lung lesions were recorded at DPC 30 and 60 (\( P = 0.02 \)) in unvaccinated and challenged pigs. Histological examination revealed reduced lung lesion scores (Fig. 3.2 B), which were characterized microscopically by reduced infiltration of mononuclear cells and
interstitial pneumonic changes in vaccinated compared to unvaccinated VR2332
challenged pigs at DPCs 15 and 30 (although differences not significant).

Reduced viral titers in serum

Consistent with the reduced lung lesions, a significantly reduced viral load was
detected in the vaccinated compared to unvaccinated PRRSV challenged pigs at DPC 15
and 30 (Fig. 3.2). Although, the PRRSV load was detected less in vaccinated compared to
unvaccinated virus challenged pigs at DPC 60, the virus was almost cleared from both the
pig groups.

Elevated PRRSV specific humoral immune response in mucosally immunized pigs

We determined anti-PRRSV isotype specific mucosal antibody response secreted by
lung-MNCs restimulated in the presence of killed PRRSV (VR2332) antigens. The anti
PRRSV specific secretory IgA antibody response in mucosally immunized pigs was three
folds higher compared to control challenged pigs (Fig 3.3 A). Further, to confirm whether
increased amounts of anti-PRRSV specific antibodies detected have the ability to
neutralize PRRSV, the levels of the PRRSV neutralizing antibody titers during the course
of the experiment was analyzed by an indirect immunofluorescence assay. Our results
showed an increase in the PRRSV specific neutralization antibody titers in immunized
VR2332 challenged pigs at DPCs 7 to 35 (Figure 3.3 B) albeit not at significant levels.
Reduced secretion of immunosuppressive cytokines in response to PRRSV

The recall/memory cytokine response of LMNCs stimulated with VR2332 antigens was determined in the culture supernatants. PRRSV is a strong inducer of immunosuppressive cytokines (IL-10 and TGF-β). A potent mucosal vaccine should suppress IL-10 and TGF-β and concomitantly should induce secretion of pro-inflammatory and Th1 cytokines. We have demonstrated that inoculation of *Mtb* WCL along with PRRSV-MLV suppresses the vaccine virus induced IL-10 production at DPC 15 and 30 (Fig 3.4 C). This effect was detected both in the presence and absence of restimulation with killed VR2332 antigens, suggesting the *Mtb* WCL mediated response in vaccinated pigs. Interestingly, TGF-β levels did not differ between the groups at DPC 15 and 30, but at DPC 60 the vaccinated animals showed higher levels of TGF-β (Fig. 3.4 D). At DPC 30, there was no difference observed in the frequency of IFN-γ-secreting cells in the lung MNC and PBMCs among the challenged pig groups (Fig. 3.5). However, a significantly increased secretion of IFN-γ by lung MNC was observed in vaccinated compared to unvaccinated pigs challenged with PRRSV (Fig. 3.4B). There was no difference between groups in IL-12 secretion (Fig 3.4 B). The reason for the increased IFN-γ and TGF-β in vaccinated pigs at the later time point (PID 60) is not known.
Increased NO’ production in unvaccinated challenged pigs

Nitric oxide is an important immune molecule involved in both innate and adaptive immunity. It is an important molecule against antiviral immunity, but its overproduction can lead to inflammation. To determine the production of NO’ in the pig lungs, lung homogenates were prepared and used in the analyses. At DPC 15, there was a significant decrease in NO’ levels in vaccinated pigs compared to unvaccinated pigs. The PRRSV infected pig lungs showed higher level of NO’; however, at DPCs 30 and 60 there was no difference between the groups (Fig 3.6).

Frequencies of lymphoid and myeloid immune cells in vaccinated pigs challenged with PRRSV correlate with the cytokine response

It is important to delineate the phenotype and frequency of various immune cells which were directly and/or indirectly responsible for secretion of enhanced Th1 and reduced immunosuppressive cytokines in vaccinated, PRRSV challenged pigs. To associate the adjuvant effects of Mtb WCL, a comparative statistical analysis of the frequency of immune cells of pigs belonging to unvaccinated or vaccinated and PRRSV challenged pigs was performed. At DPC 15, a significant increase in the frequency of dendritic cells, and a marked increase in the frequencies of CD8^+ and CD4^+CD8^+ T lymphocytes, and γδ T cells was detected in the lungs of vaccinated compared unvaccinated virus challenged pigs (Table 1A). These findings were associated with a
significant decrease in the frequency of Tregs in the lungs (Table 1A). At DPC 30, a 50% reduction in the frequency of Tregs in the lungs of pigs was associated with increase in the frequency of CD4⁺, CD8⁺ T lymphocytes, γδ T cells, and NK cells in the lungs, blood, and TBLN of vaccinated, compared to unvaccinated PRRSV challenged pigs (Table 1A-C). At DPC 60, a significant increase in the frequency of dendritic cells was detected in vaccinated, virus challenged pigs (Table 1C).

Overall, an increase in the frequency of all particular immune cell populations was consistent at all the three tested DPCs in vaccinated compared to unvaccinated PRRSV challenged pigs. Conversely, a reciprocal reduction in the frequency of Tregs was also consistent in all those pigs (Table 1). Although, differences in the frequency of many immune cell types between the vaccinated and unvaccinated pig groups showed more than 2 fold changes, the data was not statistically significant likely due to large variations in immune responses among individual pigs. The likely reasons for this discrepancy could be the outbred nature of pigs, which resulted in large degree of heterogeneity in immune responses among individual pigs.

**Proliferation response of T cells**

PBMC and LMNC of pigs were restimulated with VR2332 antigens, and examined for an increased proliferation of total PRRSV specific lymphocytes in the lungs and blood of vaccinated compared to unvaccinated, virus challenged pigs (Fig. 3.7B iii & iv). Further, increased (but not significantly) proliferation of CD8⁺ lymphocytes in the
LMNC of VR2332 challenged pigs stimulated with VR2332 antigens was detected (Fig. 3.7Ai).

3.5 Discussion

Porcine reproductive and respiratory syndrome virus has evolved into a major swine disease over the last two decades. We have recently demonstrated the benefits of intranasal delivery of MLV-PRRS with a potent mucosal adjuvant, *Mtb* WCL, in stimulating favorable anti-PRRSV immune responses against virulent heterologous (MN184) PRRSV (Dwivedi et al., 2011a; Dwivedi et al., 2011b). As a potent mucosal adjuvant we used mechanically lyzed endotoxin-free WCL of *Mtb* which contains predominantly intracellular bacterial proteins was almost free from toxic cell wall lipid contents (Takayama et al., 1975). Clinically, pigs administered *Mtb* WCL did not show any side effects. In vaccinated (MLV-PRRS plus *Mtb* WCL) pigs, reduced gross lung pathology and significantly reduced viremia were attributed to adjuvant mediated innate and PRRSV specific adaptive immune responses compared to only MLV-PRRS vaccinated pigs, when challenged with PRRSV MN184 strain (Dwivedi et al., 2011a). This study provides additional important information on the lung microenvironment when pigs were vaccinated with MLV-PRRS plus *Mtb* WCL, on challenge with homologous strain VR2332.

Results from a similar study of pigs with the same vaccine but challenged with MN184 strain of virus (Dwivedi et al., 2011a), and data from this study wherein pigs
were challenged with VR2332 strain (Fig 3.1) showed reduced viremia during early time points post-challenge in vaccinated pigs irrespective of challenge. Also histological examination showed that PRRSV challenge in unvaccinated pigs leads to interstitial pneumatic changes. The MLV-PRRS + Mtb WCL vaccine was able to significantly reduce the gross lung pathology at PCD 30 and 60 post VR2332 challenge (Fig 3.2).

The nitric oxide (NO’) helps in clearance of intracellular pathogens by inhibiting viral replication. It plays a major role in lung immunity and virus clearance. A large amount of pulmonary NO’ is produced by the epithelial lung inducible nitric oxide synthase (Lane et al., 2004), which acts on L-arginine to produce NO’. It has been shown to inhibit replication of influenza virus and herpes simplex virus type 1 (Akerstrom et al., 2005; Croen, 1993; Rimmelzwaan et al., 1999). However, over production of NO’ and reactive oxygen species (ROS) can lead to inflammation and tissue injury. In the lungs of pigs unvaccinated and challenged with VR2332 strain increased level of NO’ production was detected at DPC 15 (Fig. 3.6), suggesting the possibility of NO’ mediated lung injury. However there was no difference in NO’ levels at later time points between the vaccinated and unvaccinated groups, which suggests that factors other than NO’ was involved in lung pathology. Our earlier report (Binjawadagi et al., 2011) showed increased ROS in the immune cells isolated from bronchoalveolar lavage and blood of the unvaccinated compared to vaccinated MN184 virus challenged pigs at all the DPCs. This shows that NO’ plays a minor role in inducing lung pathology in PRRSV VR2332 infected pigs.
Generally, PRRSV induces immunosuppression resulting in delayed onset of cell-mediated immune (CMI) response (Diaz et al., 2005; Johnsen et al., 2002; Renukaradhya et al., 2010; Suradhat et al., 2003). Production of immunosuppressive cytokines, IL-10 and TGF-β has been found to be involved in antagonizing the pathogen specific protective CMI responses (Johnsen et al., 2002). PRRSV enhances the sustained secretion of IL-10 in pig lungs (Johnsen et al., 2002; Renukaradhya et al., 2010). In the present study, MLV-PRRS + M.tb WCL inoculated and VR2332 challenged pigs had significantly reduced secretion of IL-10 by lung MNC. Infiltrated Tregs in the lungs of infected animals contribute to increased secretion of IL-10 and TGF-β (Didierlaurent et al., 2007). The roles of Tregs have been implicated in several persistent viral infections in humans (Humphreys et al., 2007; Rehermann, 2007; Rouse et al., 2006; Suvas & Rouse, 2006). There are several reports of increased frequency of Tregs in pigs infected with PRRSV, suggesting their involvement in disease pathogenesis (Silva-Campa et al., 2009; Subramaniam et al., 2010; Wongyanin et al., 2010). Results of our study demonstrated the M.tb WCL mediated reduction in the frequency of Tregs in both pig PBMC and lung MNC. Recently, we have demonstrated an increased level of IL-10 and TGFβ in the lungs of pigs inoculated with MLV-PRRS intranasally, which was positively correlated with an increased frequency of Tregs. In contrast, in the lungs of pigs inoculated with MLV-PRRS + Mtb WCL, an opposite trend in the response with respect to secretion of IL-10 and TGFβ, and frequency of Tregs was detected (Dwivedi et al., 2011b). PRRSV-specific neutralizing antibodies play an important role in the viral clearance (Batista et al., 2004; Bautista & Molitor, 1999; Lopez & Osorio, 2004; Lowe et al., 2005).
Unfortunately, like in PRRSV infected pigs, MLV-PRRS administered pigs also have reduced VN antibody response associated with a dampened virus specific CMI response (Charerntantanakul et al., 2006; Foss et al., 2002). In pigs vaccinated with MLV-PRRS + Mtb WCL, an increase in VN antibody titers associated with reduced viremia was observed. For complete viral clearance, apart from effective humoral response, an effective CMI response orchestrated by a Th1 cytokine IFN-γ, which is generally produced by NK cells, γδ T cells, CD4+ T cells, CD8+ T cells, and CD4CD8 double positive T lymphocytes is critical (Costers et al., 2009). In our study, Mtb WCL mediated secretion of IFN-γ but only at PID 60 in viral antigen restimulated LMNCs. Similarly, the increased frequency of NK cells, γδ T cells, CD4+ T cells, activated Th cells, CD8+ T cells, in both the lungs and blood of vaccinated pigs were not significant. Also, we found the vaccinated pigs were not able to show increased CD8+ T cell proliferation response than unvaccinated pigs, which was again not significant. This shows that the adjuvanted vaccine was able to alleviate the PRRSV induced immunosuppression in VR2332 infected pigs but was not able to completely potentiate the vaccine.

In conclusion, from this study, mucosal immunization of pigs using MLV-PRRS + Mtb WCL dampened the virus-mediated immunosuppressive mechanisms with a concomitant boost in both innate and adaptive immune responses at both mucosal and systemic sites; which was indicated by reduced gross lung pathology, mucosal IgA response, and reduced immunosuppressive cytokine production and reduced Tregs (Dwivedi et al., 2011a) in the lungs. But the adjuvanted vaccine was insufficient in its ability to boost the functional ability of T cells in terms of effector cytokine and antigen specific proliferative
response against VR2332 challenge. Unlike this study, our earlier report with the same adjuvanted vaccine showed the potentiation of PRRSV specific immune response in pigs challenged with a different strain (MN184) virus (Dwivedi et al., 2011a). This difference in vaccine immunity could be due to the induction of differential immunopathogenesis by different stains of the PRRSV. Hence it is imperative to identify a broad vaccine capable of conferring immunity across all strains of the PRRSV.

3.6 Acknowledgments

We thank Ms. Ruth Patterson, Dr. Juliette Hanson, Todd Root, Mathew Weeman, Drs. Mahesh Khatri and Hadi Yassine for their help in animal studies, Dr. Tracey Papaenfuss for histopathological examination and Drs. Eric Nelson and Michael Murtaugh provided PRRSV reagents.
Fig. 3.1 Reduced lung pathology and infiltration of inflammatory cells in vaccinated PRRSV challenged pigs. Pigs were unvaccinated or vaccinated with MLV-PRRS plus Mtb WCL intranasally and challenged with PRRSV strain VR2332, and euthanized at DPC 15, 30, and 60. (A) Gross lung lesion scores of PRRSV challenged pigs at DPC 15, 30 and 60 were scored from all the individual pig lung lobes. Microscopic sections of lung were stained with H&E and scored for inflammatory lesions. Asterisk indicates statistically significant difference (P<0.05) between unvaccinated vs vaccinated pig groups.
Fig. 3.2. Reduced viral titers in serum of vaccinated pigs. Pigs were unvaccinated or vaccinated with MLV-PRRS + *Mtb* WCL intranasally and challenged with PRRSV VR2332 on DPI 21 intranasally and euthanized on days post-challenge (DPC) 15, 30, and 60. Sera collected at the indicated DPC were analyzed for PRRSV titers using standard immunofluorescence assay. Asterisk denotes statistically significant difference (P<0.05) between unvaccinated vs. vaccinated and virus challenged pigs.
Fig. 3.3. Increased virus specific antibody response in vaccinated pigs. Pigs were unvaccinated or vaccinated and then challenged with PRRSV VR2332. (A) Lung-MNCs isolated were cultured in the presence of killed VR2332 antigens precoated ELISA plates and the PRRSV isotype specific antibody secreted was determined by ELISA. (B) Serum samples collected on the indicated Days post infection (DPI) and Days post challenge (DPC) were analyzed for anti-PRRSV specific neutralizing antibody titers by standard immunofluorescence assay. Each bar or data point in the graph represents the average OD or titer from 3 pigs ± SEM. Asterisk indicates statistically significant difference (P<0.05) between unvaccinated vs. vaccinated pig groups analyzed.
Fig.3.4 Intranasally vaccinated and VR2332 challenged pigs secreted less immunosuppressive cytokines. Pigs were unvaccinated or vaccinated and then challenged at DPI-21 with PRRSV strain VR2332. Lung-MNCs were re-stimulated in the absence or presence of killed VR2332 and the culture supernatants were analyzed for cytokines: (A, B, & C) IL-12; and (D & E) IFN-γ by ELISA. Each bar represents the average cytokine levels from 3 pigs ± SEM. Asterisk indicates statistically significant difference (P<0.05) between unvaccinated vs. vaccinated pigs.
Fig. 3.5 Intranasally vaccinated pigs with adjuvant had more of IFNγ secreting cells in PBMCs. Pigs were unvaccinated or vaccinated and then challenged with PRRSV strain VR2332. (A) PBMCs and (B) LMNCs were re-stimulated in the presence of killed VR2332 antigens and the IFNγ secreting cells were analyzed by ELISPOT. Each bar represents the average number of IFNγ cell secreting spots from 3 pigs ± SEM.
Fig. 3.6 Elevated nitric oxide production in unvaccinated pigs. Pigs were unvaccinated or vaccinated (MLV-PRRS + Mtb WCL) intranasally and challenged with PRRSV strain VR2332. Lung homogenates were estimated for nitric oxide production using Griess reagent. Each bar represents the average amount of nitric oxide in mM concentration per gram of lung tissue from three pigs + SEM. Asterisk denotes statistically significant difference (P<0.05) between unvaccinated and vaccinated pigs.
Fig. 3.7 Intranasally vaccinated pigs had enhanced proliferation of CD8$^+$ lymphocytes. (A) A representative overlayed histograms of proliferated PRRSV specific lymphocytes is shown. Pigs were unvaccinated or vaccinated (MLV-PRRS + Mtb WCL) and challenged with PRRSV and euthanized at DPC 15. LMNC were stained with CFSE and restimulated with killed PRRSV VR2332 antigens, subsequently cells were analyzed by flow cytometry to determine total lymphocyte proliferation. Dilution of the dye CFSE indicates higher proliferation of cells. (B) PRRSV specific CD8$^+$ lymphocyte proliferation data (i) LMNC and (ii) PBMC; total T cells proliferation data from (iii) LMNC and (iv) PBMC isolated from pigs unvaccinated or vaccinated and challenged at DPC 30. Each bar represents the average proliferation index of CD8$^+$ lymphocytes or total lymphocytes from three pigs +/- SEM.
Table 3.1. Frequency of immune cells in pigs inoculated intranasally with mock, unvaccinated or vaccinated with MLV-PRRS + Mtb WCL and challenged with PRRSV VR2332.  a Data from four mock control pigs (5 - 6 wks of age); b three each of unvaccinated or vaccinated and virus challenged pigs were euthanized at DPC 15, 30, and 60. Frequency of different immune cell populations present in lung MNC, PBMC, and TBLN MNC were enumerated by flow cytometry: c CD3+ and CD3- cells were gated to enumerate CD4 and CD8α expression; d CD25+ cells were gated to enumerate CD4 and Foxp3 expression and the percent of double positive (CD4+CD25+Foxp3+) cells are shown; e CD25+ cells were gated to enumerate CD4 and Foxp3 expression and the percent of triple positive (CD4+CD25+Foxp3+) cells are shown; f CD172+ cells were gated to enumerate CD11c and SLAII expression and the percent of triple positive (CD172+CD11c+SLAII+) cells are shown. Each number is an average percent of immune cells from three or four pigs +/- SEM. Asterisk indicates a statistically significant difference (P<0.05) between unvaccinated vs. vaccinated pig groups. ND: no difference in the immune cell frequency between unvaccinated and vaccinated pig groups.

<table>
<thead>
<tr>
<th>Immune cells</th>
<th>a Mock</th>
<th>15</th>
<th>30</th>
<th>60</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Lung MNC</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T lymphocytes</td>
<td>29 ± 0.3</td>
<td>ND</td>
<td>22.5 ± 3.8</td>
<td>11.0 ± 2.7</td>
<td>ND</td>
<td>33.8 ± 3.2</td>
<td>14.3 ± 2.9</td>
</tr>
<tr>
<td>Th cells+</td>
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<td>ND</td>
<td>23.8 ± 1.5</td>
<td>18.9 ± 2.5</td>
<td>ND</td>
<td>31.3 ± 6.3</td>
<td>22.1 ± 2.7</td>
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<td>CTLs+</td>
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<td>56.5 ± 4.8</td>
<td>34.3 ± 2.3</td>
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<td>ND</td>
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<td>Th/memory cells+</td>
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<td>26.3 ± 1.1</td>
<td>ND</td>
<td>ND</td>
<td>29.8 ± 2.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
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<td>ND</td>
<td>27.5 ± 5.3</td>
<td>ND</td>
<td>ND</td>
<td>39.9 ± 3.4</td>
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<tr>
<td>γδ T cells</td>
<td>6.9 ± 0.6</td>
<td>17.2 ± 3.6</td>
<td>3.3 ± 0.5</td>
<td>ND</td>
<td>26.7 ± 4.9</td>
<td>5.3 ± 1.4</td>
<td>ND</td>
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<tr>
<td>T-regulatory cells+</td>
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<td>12.8 ± 7.3</td>
<td>38.8 ± 4.6</td>
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Chapter 4
Characterization of Myeloid derived suppressor cells from porcine blood mononuclear cells

4.1 Abstract

Myeloid derived suppressor cells (MDSCs) have been gaining importance in viral diseases due to their ability to suppress the immune system. They have been characterized previously in humans and mice disease conditions. They are a subset of immature myeloid progenitor cells and immature subsets of granulocytes, macrophages and dendritic cells at different stages of differentiation. This makes it difficult to identify the unique phenotypic markers of MDSCs. In pigs, as of now there are no reports of identified markers to delineate MDSCs. A study by Lechner et al, 2010, showed the GM-CSF and IL-6 combination of cytokines to be the most potent in inducing MDSCs from human peripheral blood mononuclear cells (PBMC) in vitro (Ref.). Hence, we have attempted to delineate the MDSCs population from porcine PBMCs by inducing them with the combination of cytokines, GMCSF and IL-6. In vitro stimulation of PBMCs with this cytokine combination resulted in an increased population of cells expressing both CD172 and CD11R3 compared to control PBMC without stimulation at both day 4 and 7 post-culture. These cells also showed increased production of reactive oxygen
species and IL-10 production in the culture supernatant. Our aim was to identify the presence of MDSCs following PRRSV VR2332 treatment; therefore, to begin with we have stimulated the PBMCs with UV-inactivated PRRSV virus in the presence or absence of cytokine combination, GMCSF and IL-6 in vitro. The inactivated virus treated cells also showed a similar trend of increased CD172⁺CD11R3⁺ cells generation, associated with elevated levels of IL-10 and ROS production. These factors indicate the presence of immunosuppressive cells which are likely to be MDSCs.

4.2 Introduction

The myeloid cells including monocytes, macrophages, dendritic cells, and others, are an important component of the immune system. Porcine macrophages are the primary cell target for the PRRSV. How PRRSV circumvents the immune response in lungs of infected pigs by targeting the macrophages is not completely understood. The PRRSV infection has been shown to cause reduced expression of MHC class I, MHC class II, CD14 and CD11b/c; thus, impairing the antigen presentation by these cells (Wang et al., 2007). In mice, there are many reports which identified a subset of immunosuppressive myeloid cells, MDSCs, known to suppress T cell mediated immune responses in pathological situations such as cancer (Gabrilovich & Nagaraj, 2009) and sepsis. The MDSCs block T cell proliferation by nitric oxide (NO’) mediated arginine metabolism in mice injected with BCG and thus help in immune evasion of Mycobacteria (Martino et al., 2010).
Two main MDSCs subtypes have been reported in mice: (i) Granulocytic MDSCs (G-MDSCs) expressing CD11b\(^+\) Gr-1\(^{hi}\) Ly-6G\(^+\) Ly-6C\(^{lo}\) CD49d\(^-\), and (ii) Monocytic MDSCs (M-MDSCs) expressing CD11b\(^+\) Gr-1\(^{hi}\) Ly-6G\(^-\) Ly-6C\(^{hi}\) CD49d\(^+\) (Gabrilovich et al., 2012). In humans, there are no uniform markers, but they commonly express myeloid markers CD11b and CD33, and lack the maturation marker HLA-DR; with M-MDSCs being predominantly CD14\(^+\) and G-MDSCs being CD15\(^+\) (Gabrilovich & Nagaraj, 2009; Greten et al., 2011). The MDSCs mediate immunosuppression through different mechanisms. The commonly observed mechanism includes production of immunosuppressive cytokines IL-10 and/or TGF-\(\beta\) (Loercher et al., 1999; Valenti et al., 2006; Vuk-Pavlovic et al., 2010). Expression of Arginase I (ARG 1), nitric oxide (NO’), or reactive oxygen species (ROS) can impair T cell responses (Bronte & Zanovello, 2005; Lu et al., 2011; Tacke et al., 2012). This leads to reduced cell-mediated immune responses. Various studies (Hoechst et al., 2011; Huang et al., 2006; Serafini et al., 2008) have shown the capacity of MDSCs to induce Tregs.

The phenotype and function of MDSCs in pigs have not been identified, but they could be playing an important role in PRRSV induced immune suppression. Our hypothesis is that PRRSV infected pigs have enhanced frequency of MDSCs which helps the virus to immunomodulate immunity to PRRSV, thus enabling viral persistence in infected pigs. As a first step, we characterized porcine MDSCs from blood of healthy pigs in vitro by cytokine induction using GM-CSF and IL-6. A sub-population of cells of phenotype CD11R3\(^+\) CD172\(^+\) was up regulated in PBMCs upon culture with increased production of the immunosuppressive cytokine IL-10, detected in the culture supernatant.
We also stimulated PBMCs with the UV-inactivated PRRSV VR2332 protein to test whether such conditions could induce accumulation of MDSCs.

4.3 Material and Methods

Isolation of peripheral of blood mononuclear cells (PBMC)

For the isolation of PBMC, blood was collected in acid citrate dextrose solution from euthanized healthy pigs (hogs) around 6 months of age, from the slaughter house and processed as described (VanCott et al., 1993). Briefly, buffy coat separated from the blood was fractionated by ficoll paque density gradient centrifugation. The interface rich in MNC was harvested. RBCs were lysed by hypotonic shock using sterile water, cells were resuspended in enriched RPMI media, counted and the viability was tested by trypan blue dye exclusion method.

In vitro culture of PBMC with cytokines

PBMC (20x10^6 cells) were allowed to attach onto a 6 well plate. After 2 hours, the non-adherent cells were removed and the adherent cells were washed twice with culture media. The adherent cells were cultured in the presence of the following treatments:

1. No treatment with cytokines or viral antigen. (n=11);
2. GM-CSF (Recombinant Porcine GM-CSF, Prospec) at 10ng/ml + IL-6 (Recombinant Porcine IL-6, Thermoscientific) at 10ng/ml (n=11);
3. UV- inactivated PRRSV VR2332 at a concentration of 20µg/ml (n=5);
4. GM-CSF (10ng/ml) + IL-6 (10ng/ml) + UV- inactivated PRRSV VR2332 (20µg/ml) (n=5).

Cells were collected at 4 and 7 days post-culture and subjected to flow cytometry and other assays. Supernatants were collected and subjected to porcine cytokine ELISA.

Flow cytometric analyses of cell phenotype

The *in vitro* cultured PBMC were evaluated for the expression of CD11R3, CD172, CD11c and CD152 (also called CD80/86). CD172 is the porcine myeloid cell marker, also called monocyte/granulocyte marker. CD11R3 has an expression pattern similar to human CD11b marker. It is expressed on granulocytes, monocytes and alveolar macrophages. CD11c recognizes human monocyte/macrophage antigen. CD80/86 is a co-stimulatory molecule and maturation marker of dendritic cells. The dilutions and details of the antibodies are given in the table below.

PBMC after culture were suspended in fluorescence-activated cell sorting (FACS) buffer (0.1% BSA ,0.035% sodium bicarbonate and 0.02% Sodium azide in HBSS) and plated in U-bottom 96-well plates and treated with 2% commercial pig serum (free of PRRSV antibodies) to block the Fc receptors. Cells were treated with fluorochrome tagged, purified or biotin labeled pig specific antibodies for 30 minutes at 4°C. Cells were washed...
twice with FACS buffer and then treated with appropriate secondary antibodies for 15 minutes at 4°C. The cells were fixed in 1% paraformaldehyde and after washing were resuspended in FACS buffer. The immunostained cells were acquired using a Flow cytometer and the data analysed by Flowjo software. Frequencies of individual immune cells were analyzed from a total 50,000 events. After initial gating based on forward scatter and side scatter, cells were double gated for CD172 marker with either CD11R3 or CD11c or CD152 marker each time. Isotype controls were used against each primary antibody as negative controls.

Table 4.1. List of antibodies used.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Isotype</th>
<th>Dilution</th>
<th>Source</th>
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<td>Mouse anti-pig Monocyte/Granulocyte PE</td>
<td>74-22-15</td>
<td>IgG1</td>
<td>1:500</td>
<td>Southern Biotech</td>
</tr>
<tr>
<td>Mouse anti-pigCD11R3 Unlab</td>
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<td>IgG1</td>
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<tr>
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<td>Streptavidin PE-Cy7</td>
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<td></td>
<td>1:500</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>Goat anti-mouse IgG1-APC/Cy7</td>
<td></td>
<td>IgG1</td>
<td>1:500</td>
<td>Southern Biotech</td>
</tr>
</tbody>
</table>
**Analysis of cytokine responses**

Purified and biotin labeled cytokine specific anti-porcine antibodies, IL-10 (R&D Systems, Minneapolis, MN) and TGF-β (Invitrogen, Camarillo, CA) were purchased from commercial sources and used to perform the sandwich ELISA. Culture supernatants collected at different time points were analyzed by sandwich ELISA (Renukaradhya et al., 2010).

**Griess Assay**

Nitric oxide production in the culture supernatants was determined by measuring nitrite levels, which is formed as nitric oxide breakdown product using a commercial kit (Promega). Samples were treated with a sulfanilamide solution for 5-10 min in the dark, and subsequently with N-1-naphtylethylenediamine dihydrochloride (NED) solution. The resulting reaction product was measured at an absorbance between 520 and 550nm, and the absorbance was calculated by generating a nitrite standard reference curve.

**ROS detection**

At Day 4 and 7 post stimulation, cells were cultured in media containing no serum at 37° C in the presence of 2.5μM CM-H2DCFDA (Invitrogen) and with their respective treatments for 45 minutes. The cells were then fixed by 1% paraformaldehyde and
analysed by flow cytometry as described above. The CM-H2DCFDA, a cell permeable, non-fluorescent precursor passively diffuses into cells and is cleaved by intracellular esterases. Subsequent oxidation by ROS yields a fluorescent adduct that is trapped inside cells for long term.

**Arginase Assay**

Arginase activity was assessed in 1X10^5 PBMC by the method described earlier (Corraliza *et al.*, 1994). The cultured cells were lysed with 50 µl of 0.1% triton X-100 containing 1X protease inhibitor cocktail in 25mM Tris-HCL pH 7.5 and incubated at 4°C for 30 minutes. 50 µl of 10mM MnCl2 was added and the enzyme was activated at 55°C for 10 minutes. Arginine hydrolysis was initiated by the addition of 25 µl of 0.5M Arginine pH 9.7 to 25 µl of the lysate and incubated at 37°C for 60 minutes. The reaction was stopped by adding 400 µl of an acid mixture containing H$_2$SO$_4$, H$_3$PO$_4$ and H$_2$O (1:3:7). 25 µl of 4% ISPF was added and incubated at 100°C for 30 minutes. The urea formed was quantified colorimetrically at 540nm. Standards were prepared using 2 fold dilutions of urea concentration starting from 0.1M.

**Statistical analysis**

All data were expressed as the mean +/- SEM of the indicated number of pig PBMCs per group. Statistical analyses were performed using non-parametric Kruskal
Wallis test followed by post-hoc Dunn’s test using GraphPad InStat (software version 5.0 for windows) to establish differences between different treatments. Statistical significance was assessed as $P<0.05$.

4.4 Results

Increased expression of CD11R3 and CD172

Studies of mice and humans have reported the heterogeneity of the MDSCs phenotype. However, the common marker expressed is CD11b, the porcine homolog of which is CD11R3. Flow cytometric analysis showed that the GM-CSF + IL-6 treatment induced a sub-population of cells which were largely double positive for CD172 and CD11R3 (Fig 4.1). At both day 4 and day 7 of culture, PBMC treated with GM-CSF + IL6 group showed significantly increased levels of CD172$^+$CD11R3$^+$ (Fig 4.2). PBMC treated with inactivated PRRSV VR2332 without cytokine stimulation showed increased CD172$^+$CD11R3$^+$ subset at day 4 (Fig 4.2 A) but not on day 7 (Fig 4.2 A), whereas PBMC treated with VR2332 antigen + GM-CSF + IL-6 showed significantly increased percentage of this subset up to 7 days of culture (Fig 4.2). The cells were also stained for other maturation markers of macrophages and dendritic cells namely CD11c and CD80/86 but did not show differences between groups, due to variations between the individual pigs within the group.
IL-10 levels increased in GMCSF + IL-6 treated cells

At both days 4 and 7 of culture, the culture supernatants of GM-CSF + IL6 group showed significantly elevated levels of IL-10 compared to untreated cells (Fig 4.3). Similarly inactivated VR2332 treated cells irrespective of cytokine stimulation showed elevated levels of IL-10 compared to untreated cells at both days 4 and 7 of culture. However at day 4 of culture, inactivated VR2332 treated groups had significantly increased level of IL-10 in their culture supernatants (Fig 4.3 A). No TGF-β secretion was seen in any of the groups.

Reactive Oxygen Species production elevated in GM-CSF + IL-6 group:

Reactive oxygen species is one of the mechanisms of MDSC mediated immune suppression. PBMC were treated with a dye CM-H$_2$DCFDA along with their respective treatments and then were analysed for the fluorescence of the dye by flow cytometry. Presence of ROS inside the cells reduces this dye into a permanent fluorescent product. Increased fluorescence indicates increased levels of ROS inside the cells. Fig 4.4 A shows the profile of ROS production in untreated cells and cells that were treated with GM-CSF+IL-6 at day 7 of culture. The GM-CSF + IL-6 group showed significantly increased ROS activity at day 4 and 7 of culture than the untreated cells (Fig 4.4 B & C). At day 4 of culture, ROS levels were high in both viral antigen treated groups compared
to untreated group, although their levels were lower than the GM-CSF + IL-6 group (Fig 4.4 B). Interestingly at day 7, both the viral antigen treated groups showed decreased ROS levels that were even lower than the untreated group (Fig 4.4 C).

**Nitric oxide in culture supernatants:**

Culture supernatants were subjected to Griess assay to determine nitric oxide levels. No nitric oxide was produced in the culture supernatants in any of the groups.

**Arginase levels in cultured cells:**

PBMC cultured with different treatments were subjected to arginase assay. No arginase activity was seen in any of the groups.

**4.5 Discussion**

MDSC are a heterogeneous population of immature myeloid cells at different stages of differentiation. They have been characterized in numerous cancer conditions in mice and humans, while in viral diseases their role is still being understood. They are capable of suppressing immune responses by suppressing the innate responses of NK cells and NKT cells, and the adaptive responses of CD4$^+$ and CD8$^+$ T lymphocytes. MDSCs were generated and characterized by Lechner et al., (Lechner et al., 2010) from
normal human PBMCs by inducing them with different combinations of cytokines. They found that the GM-CSF + IL-6 combination generated potent CD33+ MDSCs and did not require T cells. To characterize the same in pigs, we cultured myeloid cells with the GM-CSF + IL-6 combination. We found cells of phenotype of CD11R3+ CD172+ accumulating in the GM-CSF + IL-6 group. The immunosuppressive cytokine, IL-10 was seen at elevated levels in the culture supernatants of the GM-CSF + IL-6 group.

MDSC are known to accumulate in blood, various tissues and lymphoid organs and mediate T cell suppression through various means (Gabrilovich & Nagaraj, 2009; Gabrilovich et al., 2012), such as arginase-1 mediated L-arginine depletion, iNOS and reactive oxygen species production (Lee et al., 2003; Rodriguez & Ochoa, 2008), immunosuppressive cytokine production (Loercher et al., 1999; Valenti et al., 2006; Vuk-Pavlovic et al., 2010), and expansion of Tregs (Loercher et al., 1999; Valenti et al., 2006; Vuk-Pavlovic et al., 2010). Some of these mechanisms have been observed in PRRSV infection but the actual pathogenesis is not yet understood. For example, PRRSV infection induces suppression of innate immunity by increased IL-10 and TGF-β (Chung & Chae, 2003; Gomez-Laguna et al., 2009; Johnsen et al., 2002; Silva-Campa et al., 2010; Silva-Campa et al., 2009), and inhibition of NK cell cytotoxicity (Dwivedi et al., 2011a). Further, suppression of the adaptive immune system (Dwivedi et al., 2011a) results from dampened functional ability of CD8+ T cells (Costers et al., 2009), decreased T cell proliferation and expansion of Tregs (Leroith et al., 2011). Since these aspects are also seen in MDSC mediated immune suppression, we were interested to know if PRRSV is capable of expanding the MDSCs subset, and thus capable of
overcoming the host immune response and establishing persistent infection in pigs. To this end, we cultured PBMCs with inactivated VR2332 virus in the presence or absence of the cytokine combination.

The inactivated VR2332 virus treated cells showed a phenotype similar to the GM-CSF + IL-6 treated group. At Day 4 of culture, inactivated virus treated cells even without cytokine stimulation showed a higher percentage of CD172⁺CD11R3⁺ subset that were able to secrete IL-10 at higher level than the untreated cells. This could indicate that the virus by itself is capable of inducing the accumulation of MDSCs without the need for cytokine supplementation. At day 7, however the frequency of the CD172⁺CD11R3⁺ subset was reduced in the case of cells treated only with inactivated virus, although the IL-10 levels remained high. Treatment with GM-CSF+IL-6 along with inactivated virus increased the immunosuppressive subset significantly at day 7 culture; however there was not much difference in IL-10 levels with or without cytokine stimulation.

As mentioned earlier, MDSCs have been implicated in T cell suppression and the mechanisms include arginase, ROS and nitric oxide. In this study, we found that there was an absence of production of nitric oxide and arginase. On the other hand, we observed significant elevation in ROS levels in cells treated with GM-CSF + IL-6. Studies have shown that the oxidative stress caused by tumor derived macrophages inhibited γ chain expression in T cells and antigen induced proliferation in tumor bearing mice (Otsuji et al., 1996). In another study (Kusmartsev et al., 2004), interaction of MDSC with antigen specific T cells led to significant increase of ROS production and was mediated by integrins namely, CD11b, CD18 and CD29. Peroxynitrites formed from
ROS and NO can lead to nitration of tyrosine residues in TCR and CD8 molecules altering the binding of the TCR/CD8 complex with MHC that is necessary for T cell nitration (Nagaraj et al., 2007). In inactivated virus treated cells there was increased ROS production at day 4 in both the groups of cytokine treated and untreated cells (Fig 4.4 B). However, surprisingly at day 7 of culture, the same treatment showed reduced ROS which was not increased even in the presence of GM-CSF and IL-6 (Fig 4.4 C). Also the ROS level did not correlate with IL-10 levels and CD172⁺CD11R3⁺ subset in the inactivated virus treated groups. However the cell phenotype, IL-10 and ROS levels can be correlated in the GM-CSF + IL-6 group.

Due to the heterogeneity of the MDSCs, a general immunophenotype has been elusive in humans. More studies of characterization of these subsets in pigs is needed to pinpoint the indicative markers. Although, we have identified a subset of immunosuppressive cells of phenotype CD172⁺CD11R3⁺ which is similar to MDSC expression of CD11b⁺ Gr-1hi in mice and CD33⁺CD11b⁺, we still have to characterize them for maturation markers. Also this study is based on blood collected from pigs whose history of disease status, especially PRRSV, is unknown. Hence, they have to be tested for the presence of PRRSV infection. Overall, this study shows that a cytokine combination of GM-CSF and IL-6 is capable of inducing a subset of cells in pigs that are immunosuppressive in nature. Further stimulation with inactivated PRRSV VR2332 virus at day 4 elicited IL-10 and ROS production similar to the GM-CSF+IL-6 group indicating the potential role of these immunosuppressive cells in PRRSV infection.
4.6 Acknowledgments

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Fig 4.1 CD172^+ CD11R3^+ subset increased in GM-CSF + IL-6 treated pig PBMC.
A representative figure showing the immune cells gating strategy used for the analysis of cultured PBMCs for 4 days in the presence or absence of cytokines GM-CSF and IL-6, stained with antibodies against CD11R3 and CD172 and analyzed by flow cytometry.
Fig 4.2 Enhanced expression of CD11R3 in cells.
PBMC stimulated with indicated different treatments in vitro were characterized for markers CD11R3 and CD172 at different time points post-stimulation. Each bar indicates the average percentage of total cells double positive for CD11R3 and CD172 on (A) day 4 and (B) day 7 post stimulation. Asterisk indicates significant difference between the treatment group and no treatment group.
Fig 4.3. Increased IL-10 secretion in culture supernatants.
PBMC were stimulated with different treatments in vitro for 4 and 7 days. The immunosuppressive cytokine IL-10 was detected by ELISA in the culture supernatants collected at (A) Day 4 and (B) Day 7. Each datapoint represents average cytokine levels in pg/ml. Asterisk indicates significant difference between the indicated treatment group and no treatment group.
Fig 4.4 Enhanced production of ROS.
PBMC were stimulated with different treatments in the presence of a dye CM-H$_2$DCFDA. This non-fluorescent dye is reduced inside ROS producing cells to a fluorescent dye. (A) A representative figure showing the profile of fluorescent CM-H$_2$DCFDA in unstained cells, cells treated with no cytokines and cells treated with GM-CSF + IL-6. Each bar represents average percentage of cells expressing CM-H$_2$DCFDA in cells cultured after (B) 4 days and (C) 7 days post in vitro treatment with cytokines and inactivated virus. Asterisk indicates significant difference between the indicated treatment group and no treatment group.
Chapter 5

Summary and suggestions for future work

The swine industry has gone through a lot of economic difficulties due the PRRS disease during the last two decades at a global level. The PRRS continues to be an unabated problem for pork producers with evolving strains leading to high levels of morbidity and mortality of pigs as was observed in China (Li et al., 2007). In US, the first epidemic was caused by the VR2332 strain of PRRSV (Benfield et al., 1992) and this strain has been established as the prototype strain of the North American genotype. The disease by itself causes a severe respiratory infection and abortion storms, and leads to further complications such as secondary bacterial and viral infections or multifactorial disease complexes in infected pigs.

The causative RNA virus undergoes genetic diversity through point mutations and also achieves high levels of antigenic diversity even within genotypes. The immune response induced in infected pigs varies with different strains and genotype. This complexity of the virus confounds the PRRS researchers in their efforts to develop vaccine and control strategies. Both the virus and the modified live vaccine have been found to cause immunosuppression in pigs. Given the enormity of the economic loss caused by this disease, we were interested in understanding the immunopathogenesis of the virus which will provide an insight into how and where the virus persist and what
immunomodulation it induces in innate and adaptive immunity. This knowledge can be used for the formulation of vaccines and to identify the immune correlates. Towards this end, we performed a time-course study of PRRSV infection in pigs. Infection with PRRSV strain VR2332 lead to viral persistence in lungs and lymphoid tissue upto PID 60. In the blood, viral loads were reduced by PID 60, however not cleared completely. Delayed cytokine response of IFN-γ and elevated IL-10 and TGF-β in the serum during the course of the experiment showed that the immunosuppression is established immediately after infection but the animal does not completely recover even by PID 60. The probable reason could be the continued presence of the virus in serum even at lower titers leading to elevated immunosuppressive cytokines. The innate natural killer cells that were increased in PBMC at PID 15 and PID 60 did not translate into elevated functionality (IFN-γ or cytotoxicity) of these cells indicating that cell number do not correlate with function in PRRSV infection. This is similar to the report of dampened cytotoxic function of CD8 T cells even though their frequency was increased in PRRSV infected pigs (Costers et al., 2009).

Similarly, humoral response was delayed, the neutralizing antibodies appearing only by three weeks post infection. This has also been reported by other. But we also found that, on antigenic restimulation, the B cells present in the LMNC, PBMC were not able to secrete Ig A at significant levels thus impairing one of the arms of mucosal immunity. In the case of the immune cell frequencies, the Treg population was increased in PBMC, BAL and TBLN at PID 15 and 60. This correlates with the increased TGF-β and IL-10 seen in the sera of infected pigs. The immunosuppressive cytokines can also be
secreted by other immune cell subsets such as Th2 macrophages, tolerogenic dendritic cells, myeloid derivied suppressor cells and other tolerogenic cell subsets. The increased myeloid cell population observed in tonsil and ILN at PID 60 could possibly be a tolerogenic myeloid population, however we do not know what subset of myeloid cells these are.

The CD4\(^+\), CD8\(^+\) and CD4\(^+\) CD8\(^+\) T cells were found to increase at early timepoints of infection but then declined significantly by PID 60 in almost all the tested tissues. Given the increase in their number, there was no or less IFN-\(\gamma\) secretion seen at early time points of infection, which again shows the impaired functionality of these cell subsets. Also we do not know what percentage of these increased immune cell frequency is PRRSV specific. This leads to further questions such as, how the PRRSV dyregulates the T cells functionally; if PRRSV is directly responsible for the increased cell numbers and whether the decrease in T cell frequency at PID 60 is due to apoptosis or some other mechanism.

The immunopathogenesis study of VR2332 infection in pigs showed that the lungs are important in viral persistence and immune modulation. Most of the PRRSV research is concentrated on identifying the immune correlates of protection in blood and clearance of viremia in vaccinated pigs. But based on our data, we were interested in understanding how the immune response is potentiated by MLV-PRRS both at the lungs and the blood. Lungs are the tissue of target for PRRSV since PRRSV has tropism for alveolar macrophages.
MLV-PRRS is a modified live vaccine that is derived by the attenuation of the VR2332 strain. The vaccine itself is capable of immunosuppressing the porcine immune system. Other limitations include the risk of reversal to virulence, vaccine virus shedding and spread, and incomplete protection against re-infections and heterologous infections (Ciechanowska et al., 2008; Lager et al., 2003; Mateu & Diaz, 2008; Zuckermann et al., 2007). In our earlier study, we identified *M.Tb* WCL as a potent adjuvant to MLV-PRRS when inoculated by intranasal route (Dwivedi et al., 2011b). The adjuvanted vaccine was able to induce increased Th1 cytokines, increased NK cells, γδ T cells and CD4+ and CD8+ T cells, early generation of PRRSV specific neutralizing antibodies and reduced immunosuppressive cytokines compared to vaccine only. Also in challenge study with a different PRRSV strain namely MN184 (Dwivedi et al., 2011a) the adjuvanted vaccine was able to elicit cross protective response. Due to these reasons, the MLV-PRRS + *M.Tb* WCL was used to investigate the PRRSV immune response at both mucosal (lungs) and systemic (blood) sites.

On VR2332 challenge, there was reduction in gross lesions but not in microscopic lesions in lungs of vaccinated pigs at PID 30 and 60. The adjuvanted vaccine was still able to reduce viral titers in serum at PID 15 and 30, but was not able to completely clear the virus even by PID 60. Similar to virus infection, the antibody response was delayed; however the IgA response in restimulated LMNC was significantly higher in vaccinated pigs at PID 30. Neutralizing antibody response is one of the immune correlates of protection in PRRSV infection. Neutralizing antibody response in serum was not at significantly increased titer, however they were higher than 1/8 titer in vaccinated
animals. Others have shown that 1/8 or higher titers of neutralizing antibody protected young piglets from developing viremia in a report of passive transfer of neutralizing antibodies (Lopez et al., 2007).

The PRRSV antigen restimulated LMNCs of vaccinated pigs showed reduced immunosuppressive cytokine IL-10 secretion in culture supernatants at DPC 15 and DPC 30, whereas IFN-γ response was seen only by PID 60 in the culture superantants. The adjuvanted vaccine although able to reduce IL-10 was still not able to increase the anti-viral cytokine IFN-γ. Also IFN-γ secreting cells were not increased in vaccinated pigs. The inadequate IFN-γ and antibody response could be responsible for the non clearance of the virus even though the viral load was reduced. The dampened proliferation of T cells was also not completely rescued in LMNCs and PBMCs of vaccinated and challenged pigs. Similarly the increases in various immune cell subsets in LMNC in vaccinated pigs were not significant compared to unvaccinated pigs after challenge at all timpoints. Interestingly, the Treg population was significantly reduced in LMNCs at DPC 15 which correlated with reduced IL-10 secretion. This indicates that the Tregs contribute majorly to the production of IL-10 levels.

The NO' levels in lung homogenates were also found to be reduced in vaccinated pigs at DPC-15. The overproduction of NO' is capable of lung pathology. At DPC 15, reduced IL-10, reduced Tregs and reduced NO’ could be the factors that help in viral clearance. But the initial clearance did not extend upto DPC 60 as the other immune factors such as antibody response and cell mediated immune response did not kick in. This further suggests that the immune correlates of protection for PRRSV vaccination is a
combination of all the responses and can not be attributed only to neutralizing antibody response. Overall, the vaccine study suggests that the MLV-PRRS + *M. Tb* WCL was able to potentiate the PRRSV immunity to a level of alleviation of PRRSV infection but not completely capable of viral clearance in pigs challenged with strain VR2332. There are various reasons that can be attributed to this partial failure of the vaccine such as the strain mediated immunomodulation and the immunosuppressive nature of MLV-PRRS. This study also shows that a vaccine capable of protecting pigs against one strain of virus need not be able to protect against other strains. Further research towards generation of novel vaccines which are broadly protective is necessary. Given the limitations with attenuated vaccine, new generation inactivated vaccines have to be formulated.

PRRSV has been found to cause immunsuppression through various mechanisms such as dampened T cell response, inhibition of NK cell cytotoxicity, production of NO’ and ROS, induction of Treg cells, immunosuppressive cytokine production. All these mechanisms have been attributed in the myeloid derived suppressor cell (MDSC) mediated immunosuppression seen in tumors, infectious diseases and auto-inflammatory conditions. So we hypothesized that PRRSV mediates the expansion of this myeloid subset that leads to immunosuppression and leads to viral persistence. The MDSCs have been characterized in mice, humans and dogs. The presence of this cell subset is unknown in pigs. So our aim was to characterize MDSC *in vitro* from porcine blood. And further to investigate if there is induction of MDSC when porcine blood mononuclear cells were cultured in the presence of inactivated PRRSV VR2332 viral antigen.
GM-CSF + IL-6 combination was reported to be the most potent combination for the induction of human MDSCs from PBMCs by *in vitro* culture (Lechner *et al.*, 2010). Mononuclear cells isolated from porcine blood were cultured in the presence of the GM-CSF + IL-6 cytokine combination showed increased frequency of cells expressing CD11R3 (porcine CD11b) and CD172 (myeloid cell marker). Further these cells were found to secrete higher levels of IL-10 into the culture supernatants at both day 4 and 7 of culture. These cells also had higher levels of ROS in their cytoplasm. The ROS also leads to dampened T cell function by impairing the T cell receptor. Hence CD172⁺ CD11R3⁺ cells with high IL-10 and ROS production were found to immunosuppressive myeloid cells that were similar to MDSC. To confirm that this cell phenotype is MDSC, more exploration is needed. The MDSC are a heterogeneous population of immature myeloid cells and are immunosuppressive to T cells. So further work will involve investigating the expression of maturation markers namely CD11c, CD80/86 and SLAII on these cells, their ability to inhibit the proliferation of T lymphocytes and induce Tregs.

Porcine blood mononuclear cells were also cultured with UV inactivated PRRSV strain VR2332 virus with or without the cytokine combination. Both at day 4 and day 7, the CD172⁺ CD11R3⁺ were increased in cells cultured with GM-CSF + IL-6 combination. However significant levels of IL-10 were observed only at day 4 in both the groups. The ROS levels of cells cultured in both these groups were higher than mock cells with no treatment at day 4, however interestingly they were reduced by day 7. The reason for this trend is not known.
Since this is the first time the *in vitro* generation and characterization of MDSC has been done in the procine system and given the absence of specific phenotype for these cells, it is not possible to conclude that these cells are MDSC. But these are myeloid cells which exhibit immunosuppressive nature and are likely to be MDSC. After further characterization to pinpoint their phenotype and function, the role of MDSC induction in PRRSV needs to be investigated. If the MDSC are found to play a major role, they will open up new avenues for exploration of vaccine strategies.
Bibliography


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