Cell-mediated immune response to porcine reproductive and respiratory syndrome virus

Wasin Charerntantanakul
Iowa State University
Cell-mediated immune response to porcine reproductive
and respiratory syndrome virus

by

Wasin Charerntantanakul

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
DOCTOR OF PHILOSOPHY

Major: Veterinary Microbiology

Program of Study Committee:
James A. Roth, Major Professor
Kenneth B. Platt
Eileen L. Thacker
Patrick G. Halbur
Douglas E. Jones

Iowa State University
Ames, Iowa
2006

Copyright © Wasin Charerntantanakul, 2006. All rights reserved.
Graduate College
Iowa State University

This is to certify that the doctoral dissertation of

Wasin Charerntantanakul

has met the dissertation requirements of Iowa State University

Signature was redacted for privacy.

Major Professor

Signature was redacted for privacy.

For the Major Program
# Table of contents

Chapter 1. General Introduction 1
   Introduction to the Problem 1
   Aims of the Present Studies 2
   Dissertation Organization 3

Chapter 2. Literature Review of Porcine Reproductive and Respiratory Syndrome Virus 4
   Characteristics of the Virus 4
   Characteristics of the Disease 6
   PRRSV Immunity 10
   Immunity and Protection 15
   PRRSV and Immunosuppression 17
   PRRSV Vaccine 19
   Adjuvants for PRRSV Vaccine 28
   Overview of Porcine Myeloid Antigen-Presenting Cells 34
   Summary 37
   References 37

Chapter 3. Immune Responses and Protection by Vaccine and Various Vaccine Adjuvant Candidates to Virulent Porcine Reproductive and Respiratory Syndrome Virus 53
   Abstract 53
   Introduction 55
   Materials and Methods 57
   Results 68
   Discussion 87
   Acknowledgements 94
   References 94

Chapter 4. Effects of Porcine Reproductive and Respiratory Syndrome Virus Virulence and Antigen-Presenting Cells on T Cell Activation and Antiviral Cytokine Production 97
   Abstract 97
   Introduction 98
   Materials and Methods 100
   Results 111
   Discussion 127
   Acknowledgements 130
   References 131
CHAPTER 5. General Conclusions
Discussion 137
Recommendations for Future Research 137
References 139

ACKNOWLEDGEMENTS 142
Chapter 1. General Introduction

Introduction to the Problem

Porcine reproductive and respiratory syndrome virus (PRRSV) causes approximately 560.32 million dollars in losses each year to US swine industry (Neumann et al., 2005). The virus causes reproductive failures in breeding age swine and respiratory diseases in growing pigs. PRRSV is the most common agent involved in the porcine respiratory disease complex; others include *Mycoplasma hyopneumoniae*, *Pasteurella multocida*, *Actinobacillus pleuropneumoniae*, *Hemophilus parasuis*, *Streptococcus suis*, swine influenza virus, porcine circovirus type 2, and pseudorabies virus.

The adaptive immune response to PRRSV is weak and delayed, compared to adaptive immune response to other swine viral pathogens eg. pseudorabies virus and swine influenza virus. Antibody responses to PRRSV are predominately against non-neutralizing epitopes. Neutralizing antibodies to PRRSV have low neutralizing titers and appear late after infection. The antibodies provide complete protection only after homologous virus infection and partial or no protection after heterologous virus infection. Neutralizing antibodies at low titer may enhance PRRSV infection through antibody-dependent enhancement of infection.

The cell-mediated immune (CMI) response to PRRSV is low in magnitude and also appears late after infection. Little is known about the specific T cell subsets required for protection from PRRSV-induced disease by T cells. The current methods utilized for detection of CMI response to PRRSV include lymphocyte blastogenesis, enzyme-linked immunospot (ELISPOT), and reverse transcriptase PCR and ELISA for detection of cytokine expression. Each method provides useful but incomplete data. Mechanisms that PRRSV
utilizes to delay CMI response and methods to improve CMI response to PRRSV have been poorly identified.

**Aims of the Present Studies**

The broad goal of the work presented in this dissertation was to evaluate CMI response to PRRSV. A four-color flow cytometry technique for detection of specific T cell subset response to PRRSV was developed and used in the experiments described in this dissertation. This technique allows simultaneous detection of 5 different porcine T cell subsets and the surface expression of CD25 molecules and intracellular expression of interferon gamma (IFNγ). The first experiment described in this dissertation utilized four-color flow cytometry technique to detect recall response of T cells after exposure to PRRSV antigens. Different vaccination strategies including PRRS modified-live virus (MLV) vaccine and various vaccine adjuvants as well as PRRSV peptide antigens were tested for their efficacy in inducing and enhancing the CMI response. T cell subsets responsible for protection from clinical disease were characterized. The second experiment described in the dissertation aimed at the ability of PRRSV to suppress T cell response in relation to the virus virulence and type of myeloid antigen-presenting cells the virus infects.

**Dissertation Organization**

Chapter 2 of the dissertation is a literature review of prior PRRSV research. Characteristics of the virus, pathogenesis of PRRSV-induced disease, immune response to PRRSV and PRRSV vaccines, and PRRSV immunity and protection are discussed in the review. Chapter 3 is a paper published in *Veterinary Immunology and Immunopathology*
Chapter 4 is a paper submitted to *Viral Immunology*. Chapter 5 is the conclusion of the Ph.D. dissertation and recommendations for future research in PRRSV immunology.
Chapter 2. Literature Review of Porcine Reproductive and Respiratory Syndrome Virus

Characteristics of the Virus

Virus Structure and Genomic Organization

Porcine reproductive and respiratory syndrome virus (PRRSV) is a small, positive-sense, single-strand enveloped RNA virus classified in genus Arterivirus, family Arteriviridae, order Nidovirales (Meulenberg et al., 1993). The viral genome is approximately 15 kb in size and is composed of 9 open reading frames (ORF), designated ORF1a, 1b, 2a, 2b, and 3-7 (Meulenberg et al., 1993). Genomic sequence analysis suggests the existence of at least 2 genotypes of PRRSV- the North American and the European-which have 64-67% and 55-80% nucleotide sequence and amino acid sequence homology, respectively (Meng et al., 1995a; Meng et al., 1995b; Murtaugh et al., 1995; Nelsen et al., 1999).

ORF1a and ORF1b encode viral RNA-dependent RNA polymerase (Allende et al., 1999; Meulenberg et al., 1993). ORF2a and ORF3-5 encode glycosylated proteins, named GP2-GP5, respectively (Dea et al., 2000; Meulenberg and Petersen-den Besten, 1996; Meulenberg et al., 1995). ORF2b encodes unglycosylated 2b protein (Wu et al., 2001). ORF6 encodes an unglycosylated matrix (M) protein, which is connected by a disulfide bond to GP5 (Mardassi et al., 1996; Meulenberg et al., 1995). ORF7 encodes an unglycosylated nucleocapsid protein (Meulenberg et al., 1995).
GP2, GP4, GP5, M, and probably GP3 are associated with the viral envelope (Dea et al., 2000). GP2, GP4, and GP5 contain minor T cell epitopes (Bautista et al., 1999). M protein contains major T cell epitope (Bautista et al., 1999). GP4 also contains a minor neutralizing epitope (Bautista et al., 1999; Kwang et al., 1999; Meulenberg et al., 1997; Weiland et al., 1999). The N-terminal ectodomain of GP5 contains the major neutralizing epitope (Gonin et al., 1999; Kwang et al., 1999; Ostrowski et al., 2002; Plagemann et al., 2002; Weiland et al., 1999; Wissink et al., 2003).

Among PRRSV isolates, GP5 has the highest amino acid sequence variation (81.96-99.72% homology) (Kwang et al., 1999; Murtaugh et al., 1998; Pirzadeh and Dea, 1998; Weiland et al., 1999). M protein, on the other hand, has the least amino acid sequence variation (95.94-100% homology) (Allende et al., 1999; Kapur et al., 1996; Meng et al., 1994; Meng et al., 1995a; Murtaugh et al., 1995).

**PRRSV Replication**

PRRSV infects myelomonocytic cell lineages, including monocytes, macrophages, and dendritic cells (Halbur et al., 1996a; Molitor et al., 1996; Thacker et al., 1998; Thanawongnuwech et al., 2000; Thanawongnuwech et al., 1997a; Voicu et al., 1994). Pulmonary alveolar macrophages (PAM) are the preferred target cells of PRRSV infection (Molitor et al., 1996). The virus enters the target cells by receptor-mediated endocytosis (Delputte and Nauwynck, 2004; Delputte et al., 2002; Duan et al., 1997; Nauwynck et al., 1999; Vanderheijden et al., 2003). The process is mediated by two consecutive steps. First, the M or M-GP5 protein complex binds to heparan sulfate on target cell membrane (Delputte and Nauwynck, 2004; Delputte et al., 2002; Duan et al., 1997; Nauwynck et al., 1999;
Uncoating of PRRSV takes place in the endosome. This process is facilitated by the low pH in the endosome (Kreutz and Ackermann, 1996). The viral RNA is released from the endosome into the cytoplasm where the viral proteins are translated. The location of virus assembly is not known, but is likely to be in smooth endoplasmic reticulum (ER) (Pol et al., 1997a). PRRSV obtains an envelope by budding through the membrane of the smooth ER (Dea et al., 1995; Mardassi et al., 1994; Pol et al., 1997a). The virus is released from the cell by cell lysis (Pol et al., 1997a). The replication cycle is approximately 9-12 hours (Pol et al., 1997a).

Characteristics of the Disease

Effect of PRRSV on Immune Cells

PRRSV suppresses phagocytic ability and inhibits microbial killing as well as production of reactive oxygen species eg. superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$) in infected PAM and pulmonary intravascular macrophages (PIM) (Chiou et al., 2000; Thanawongnuwech et al., 1997a). PRRSV induces both infected cells and bystander uninfected cells, including lymphocytes, to undergo apoptosis (Sirinarumitr et al., 1998; Suarez, 2000; Sur et al., 1996; Sur et al., 1998). In in vivo observations, pigs infected with PRRSV develop transient monocytopenia and lymphopenia and have reduced numbers of PAM (Done and Paton, 1995; Rossow et al., 1994). PRRSV-induced apoptosis of infected cells was shown to be mediated by a product of ORF5 (Suarez et al., 1996). The expression
of ORF5 protein under a T7 promoter in the recombinant vaccinia virus resulted in internucleosomal DNA degradation (apoptosis) in monkey BSC40 cells (Suarez et al., 1996). The precise mechanism of ORF5 protein induced apoptosis in infected and bystander uninfected cells is not known. Apoptosis of monocytes and macrophages, especially those of the respiratory system, results in increased susceptibility of pigs to secondary bacterial infections (Thanawongnuwech et al., 1997a; Thanawongnuwech et al., 1998a).

**Clinical Disease**

Pigs develop cell-free and cell-associated viremia within 24-96 hours after PRRSV infection (Christopher-Hennings et al., 2001; Halbur et al., 1996a; Halbur et al., 1995). The virus can be isolated from serum for at least 5 weeks after infection (Christopher-Hennings et al., 2001; Halbur et al., 1996a; Halbur et al., 1995). The virus spreads systemically to tissues including lung, heart, thymus, spleen, intestine, brain, testes, and ovaries within 48 hours of infection (Halbur et al., 1996a; Halbur et al., 1995; Rossow et al., 1994; Rossow et al., 1996; Sur et al., 1997; Sur et al., 2001; Thanawongnuwech et al., 1997b).

Clinical disease induced by PRRSV varies significantly, depending upon virus genotype, virus virulence, and host factors ie. breed and age (Christopher-Hennings et al., 2001; Halbur et al., 1996a; Halbur et al., 1995; Halbur et al., 1996b; Mengeling et al., 1998a; Opriessnig et al., 2002; Thacker et al., 1998; van der Linden et al., 2003). In general, North American PRRSV induces more severe clinical disease than European PRRSV (Labarque et al., 2002; Plana et al., 1992; Roof et al., 2003; van der Linden et al., 2003; Van Reeth et al., 1996). Neonatal and nursery pigs manifest more severe clinical disease than older pigs (van der Linden et al., 2003). Neonatal and nursery pigs may experience high fevers, anorexia,
dyspnea, tachypnea, chemosis, conjunctivitis, and failure-to-thrive (Collins et al., 1992; Halbur et al., 1995; Halbur et al., 1996b; Rossow et al., 1994; van der Linden et al., 2003). Grow to finish pigs may experience respiratory disease that can vary from subclinical to fatal pneumonia (Collins et al., 1992; Halbur et al., 1995; Halbur et al., 1996b; Rossow et al., 1994; van der Linden et al., 2003). Poor growth performance and mortality may result (Johnson et al., 2004). Breeding pigs may demonstrate late-term abortions, increased numbers of stillborn pigs, mummified fetuses, weak live-born piglets, and delayed return to estrus (Christiansen et al., 1992; Collins et al., 1992; Lager and Halbur, 1996b; Lager et al., 1997a; Lager et al., 1997b; Mengeling et al., 1994; Mengeling et al., 1998b; Rossow et al., 1996). Congenital infection can occur if sows are infected with PRRSV near farrowing (90 days of gestation) (Lager et al., 1997a; Lager et al., 1997b). Piglets born from these sows can have viral RNA in their tissues until at least 210 days of age (Christopher-Hennings et al., 1997).

**Pathological Lesion**

Gross and histopathological lesions caused by PRRSV are pronounced in two different organs- lung and lymphoid tissues (Halbur et al., 1995; Halbur et al., 1996b; Rossow et al., 1994). Pneumonia is primarily seen in the cranial, middle, and accessory lobes and the ventromedial portion of the caudal lobes of the lung and is characterized by failure of the lung to collapse, multifocal firmness, and tan discoloration (Halbur et al., 1995; Halbur et al., 1996b). Microscopic lesions are consistent with interstitial pneumonia, characterized by septal thickening with lymphocytes and macrophages, pneumocyte type 2 hypertrophy and hyperplasia, and accumulation of normal and necrotic macrophages in
alveolar spaces (Halbur et al., 1995; Halbur et al., 1996b; Rossow et al., 1994). The lymphoid tissues especially lymph nodes are enlarged due to polyclonal B-cell proliferation in lymphoid follicles in conjunction with infiltration of macrophages and lymphocytes into the follicles (Halbur et al., 1995; Halbur et al., 1996b; Lemke et al., 2004; Rossow et al., 1994). Lymphocytes infiltrating into the lung and lymphoid follicles are predominately CD4-CD8+ T cells (Tingstedt and Nielsen, 2004). Pyknotic macrophages are frequently observed in the lymphoid follicles (Halbur et al., 1995; Halbur et al., 1996b; Rossow et al., 1994).

Other lesions may include rhinitis, encephalitis and myocarditis (Collins et al., 1992; Halbur et al., 1996a; Halbur et al., 1995; Halbur et al., 1996b; Rossow et al., 1994).

**Persistent Infection**

Pigs exposed to PRRSV demonstrate prolonged viremia (Albina et al., 1998b; Benfield et al., 1997; Foss et al., 2002; Mengeling et al., 1999; van der Linden et al., 2003; Wills et al., 1997). The virus can be isolated from serum and peripheral blood mononuclear cells (PBMC) of infected pigs until at least 5 weeks after infection and from tissues eg. heart, small intestine, and brain for days to months after infection (Christopher-Hennings et al., 1997; Foss et al., 2002; Halbur et al., 1996a; Halbur et al., 1995; Rossow et al., 1994; Wills et al., 1997). The virus can be isolated from the palatine tonsils as long as 157 days after infection (Wills et al., 1997). During PRRSV infection, pigs may shed infectious virus via semen (Christopher-Hennings et al., 1995a; Christopher-Hennings et al., 1997; Christopher-Hennings et al., 1995b; Nielsen et al., 1997b; Swenson et al., 1994), transplacental at late gestation (Lager et al., 1997a; Lager et al., 1997b), urine (Rossow et al., 1994; Wills et al.,
PRRSV Virulence

In general, virulent PRRSV induces (1) more rapid and prolonged viremia and higher virus titers in the serum (Johnson et al., 2004; van der Linden et al., 2003), (2) more rapid and severe pneumonia, lymphadenopathy, encephalitis, and myocarditis and the lesions resolve faster (usually within 2-4 weeks after infection) (Halbur et al., 1996a; Halbur et al., 1995; Halbur et al., 1996b), (3) more tissues infected and more virus antigens in each infected tissue (Halbur et al., 1996a; Haynes et al., 1997), (4) poorer growth performance and higher mortality rate especially in young pigs (Halbur et al., 1996a; Halbur et al., 1995; Halbur et al., 1996b), and (5) more rapid and higher magnitude of antibody response (Johnson et al., 2004; van der Linden et al., 2003) than low virulent PRRSV.

PRRSV Immunity

Innate Immune Response

Pigs do not develop a good innate immune response after PRRSV infection. The level of type I interferon (IFN) was minimal or undetectable in serum and bronchoalveolar lavage (BAL) fluid of PRRSV-infected pigs (Albina et al., 1998a; Van Reeth et al., 1999). PAM and PBMC do not produce IFNα in response to PRRSV infection (Albina et al., 1998a). When PAM are infected with PRRSV and superinfected with transmissible gastroenteritis virus, a virus that strongly induces type I IFN production, no type I IFN can be detected (Albina et al., 1998a; Charley and Lavenant, 1990; Nowacki and Charley, 1993).
Type I IFN production has been reported to be up-regulated after transmissible gastroenteritis virus, swine influenza virus, and porcine respiratory coronavirus (Albina et al., 1998a; Buddaert et al., 1998; Pfeffer et al., 1998; Van Reeth et al., 1999).

The levels of pro-inflammatory cytokines ie. interleukin (IL)-1 and tumor necrosis factor alpha (TNFα) were minimal in BAL fluid after PRRSV infection (Labarque et al., 2003a; Van Reeth et al., 1999). IL-1 and TNFα were detected at high levels in BAL fluid after swine influenza virus infection (Van Reeth et al., 1999). PAM infected with PRRSV do not up-regulate IL-1α and TNFα mRNA expression and TNFα protein production (Chiou et al., 2000; Lopez-Fuertes et al., 2000). In addition, PRRSV-infected PAM do not up-regulate TNFα mRNA expression in response to phorbol 12-myristate 13-acetate (PMA) plus calcium ionophore (ionomycin), a combination of potent TNFα inducers (Lopez-Fuertes et al., 2000).

**Humoral Immune Response**

Pigs develop antibodies to all structural proteins of PRRSV (Meulenberg et al., 1995). The majority of PRRSV-specific antibodies are against the nucleocapsid protein (Murtaugh et al., 1995; Plana-Duran et al., 1997). These antibodies appear to develop faster than antibodies to other structural proteins (Nelson et al., 1994; Yoon et al., 1995; Loemba et al., 1996).

**Total Antibody Response**

PRRSV-specific IgM can be detected in the serum and BAL fluid within 9 days of infection and last 3-4 weeks following infection (Joo et al., 1997; Labarque et al., 2000; Loemba et al., 1996; Park et al., 1995). PRRSV-specific IgA appears in serum and BAL fluid at 2 weeks after infection and decline to undetectable levels at about 6 weeks of
infection (Labarque et al., 2000). IgG1 is the dominant isotype of PRRSV-specific antibody, arising at 9 days after infection and remaining at high titer in serum and BAL fluid (8-12 of log₂ titer) until at least 7-8 weeks after infection (Labarque et al., 2000). IgG2 is the second dominant isotype of PRRSV-specific antibody, appearing in the serum and BAL fluid at 2 weeks after infection and remaining at considerable titers (6-8 of log₂ titer) until at least 7-8 weeks after infection (Labarque et al., 2000). In a study that measuring the duration of antibodies, PRRSV-specific antibodies were detected in the serum by commercial IDEXX ELISA for at least 210 days after infection (Wills et al., 1997). Specific antibodies are developed faster and at higher titers against virulent PRRSV isolates than attenuated ones (Johnson et al., 2004; van der Linden et al., 2003).

Absence of an anamnestic antibody response was reported after a challenge with homologous virus as detected by IDEXX ELISA (Bassaganya-Riera et al., 2004; Foss et al., 2002; Lager et al., 1997a; Lager et al., 1997b). In contrast, an anamnestic antibody response to original virus was detected after heterologous virus challenge (Botner et al., 1999; Nilubol et al., 2004).

**Neutralizing Antibody Response**

Neutralizing antibodies generally appear around 4 weeks after infection and persist at low titers (3-5 of log₂ titer) throughout the infection (at least until 210 days after infection) (Wills et al., 1997; Yoon et al., 1995). The neutralizing antibodies are directed against GP4 and GP5 of the virus (Bautista et al., 1999; Gonin et al., 1999; Kwang et al., 1999; Ostrowski et al., 2002; Plagemann et al., 2002; Weiland et al., 1999; Wissink et al., 2003). Neutralizing antibodies can completely neutralize homologous virus but only partially (or not at all) neutralize heterologous virus (Lopez and Osorio, 2004).
Cell-Mediated Immune (CMI) Response

In Vivo Response

PRRSV causes a transient decrease of CD4$^+$ and CD8$^+$ T cells in peripheral blood 3 days after infection (Nielsen and Botner, 1997a; Rossow et al., 1994; Shimizu et al., 1996). The decrease of CD4$^+$ T cells lasts at least 2 weeks after infection, whereas the decrease of CD8$^+$ T cells lasts 4 weeks after infection (Lamontagne et al., 2003; Shimizu et al., 1996). Following a decrease, the population of CD8$^+$ T cells, primarily CD4$^+$CD8$^+$, increases significantly in peripheral blood and BAL fluid (Lamontagne et al., 2003; Samsom et al., 2000; Shimizu et al., 1996).

A delayed type hypersensitivity (DTH) response characterized by swelling and redness of the skin appeared 24 hours after intradermal challenge of PRRSV-infected pigs with UV-inactivated homologous virus (Bautista and Molitor, 1997). The DTH response declined 72 hours after the challenge (Bautista and Molitor, 1997).

Lymphocyte Blastogenesis

In vitro studies utilizing lymphocyte blastogenesis assay revealed that antigen-specific lymphocytes appeared in the peripheral blood approximately 4 weeks after infection and persisted 3 months after infection (Bassaganya-Riera et al., 2004; Bautista and Molitor, 1997; Lopez Fuertes et al., 1999). Secondary exposure to the homologous virus induced an anamnestic T-cell proliferative response in which T cells proliferated within 2 weeks after challenge (Bautista and Molitor, 1997). Analysis by flow cytometry revealed that the responsible T-cell subset for lymphocyte blastogenesis was CD4$^+$CD8$^+$ (Lopez Fuertes et al.,
Other subsets of T cells that are responsible for T cell proliferation included CD8\(^+\) T cells which co-express γδ T-cell receptor (TCR) (Lopez Fuertes et al., 1999).

**Cytokine Response**

Primary exposure to PRRSV in vitro and in vivo induces an increase of IL-10 mRNA expression and IL-10 protein production in PBMC, BAL cells, and BAL fluid (Chung and Chae, 2003; Labarque et al., 2003a; Royaee et al., 2004; Suradhat and Thanawongnuwech, 2003; Suradhat et al., 2003). No change or minimal and transient increase of IL-1, IL-2, IL-4, IL-6, IL-8, IL-12, IL-15, IL-18, IFNγ, and TNFα mRNA expression was detected in naïve PBMC incubated with PRRSV in vitro and PBMC collected from PRRSV-infected pigs (Chung and Chae, 2003; Feng et al., 2003; Royaee et al., 2004; Sipos et al., 2003; Suradhat et al., 2003). An elevation of IL-6 in serum has been reported in PRRSV-infected pigs (Asai et al., 1999; Feng et al., 2003).

Piglets infected with PRRSV in utero demonstrated a significant increase of IL-6, IL-10, and IFNγ mRNA expression in their PBMC and BAL cells at the time of farrowing and at 2 weeks of age (Feng et al., 2003; Johnsen et al., 2002). No change in IL-12 mRNA expression was observed in BAL cells at 2, 4, and 6 weeks after farrowing (Johnsen et al., 2002).

In vitro recall response to homologous PRRSV showed an increase of IFNγ mRNA and protein and IL-2 mRNA expression, but not IL-4 mRNA expression, in PBMC (Foss et al., 2002; Lopez Fuertes et al., 1999; Meier et al., 2003; Royaee et al., 2004). IFNγ-producing PBMC determined by ELISPOT assay was detected after 4-8 weeks after PRRSV infection (Foss et al., 2002; Meier et al., 2003; Meier et al., 2004; Royaee et al., 2004). The numbers of IFNγ-producing cells varied from 50-100 cells/10\(^6\) PBMC at 4-10 weeks after
infection and gradually increased to approximately 400 cells/10^6 PBMC after 48 weeks of infection (Meier et al., 2003). The IFN\(\gamma\) secretion was mediated primarily by CD4\(^+\)CD8\(^+\) T cells as determined by a marked decrease of IFN\(\gamma\) secretion after complement-mediated depletion of these cells (Meier et al., 2003).

**Immunity and Protection**

**Innate Immunity**

The cytokines produced by innate immune cells ie. IFN\(\alpha\) and TNF\(\alpha\) have been demonstrated to have an inhibitory effect on PRRSV replication (Albina et al., 1998a; Buddaert et al., 1998; Lopez-Fuertes et al., 2000). Pre-treatment of PAM with recombinant porcine IFN\(\alpha\) or TNF\(\alpha\) significantly reduced PRRSV yield and the numbers of PRRSV-infected cells (Buddaert et al., 1998; Lopez-Fuertes et al., 2000). Pre-inoculation in the lungs of pigs with porcine respiratory coronavirus, a potent IFN\(\alpha\) inducer, followed 2 days later with PRRSV showed a significant reduction of PRRSV titers in the lungs, compared to those infected with PRRSV alone (Buddaert et al., 1998). No evidence of synergistic effect between IFN\(\alpha\) and TNF\(\alpha\) on inhibition of PRRSV replication was reported (Lopez-Fuertes et al., 2000).

**Humoral Immunity**

Humoral immunity has been reported to be a double-edged sword. The antibody may protect pigs from clinical disease or it may enhance PRRSV infection. This ambiguous effect of humoral immunity was demonstrated by Yoon et al (1996), whose study showed that pigs passively transferred with antiserum containing neutralizing antibody to PRRSV
(VR-2402) at high neutralizing titer (1:16) were protected from viremia after homologous virus challenge. Pigs that received neutralizing antibody at lower titer (1:4), on the other hand, were not protected and had increased duration and level of viremia when compared to challenge control pigs. The increased viremia in the presence of low neutralizing antibody titers were suggested to be due to antibody-dependent enhancement of infection (Yoon et al., 1996).

The protection conferred by maternal-derived humoral immunity was elucidated by Osorio et al (2002). This study revealed that sows passively transferred with antiserum containing neutralizing antibody to PRRSV (97-7895) at neutralizing titer ≥1:256 at 87 days of gestation were completely protected from viremia and were negative for virus isolation from tonsils, lung, lung lavage, and lymph nodes after homologous virus challenge 3 days later. These sows passed neutralizing antibodies through colostrum and milk and yielded significantly higher viable piglets than challenge control sows. No PRRSV was isolated from the lung, lymph nodes, tonsils and spleen at 15 and 65 days of age in piglets born from these sows.

Cell-Mediated Immunity

The evidence of PRRSV-specific CMI conferring clinical protection is minimal. In in vitro studies, recombinant porcine IFNγ was shown to inhibit PRRSV replication in monocytes, PAM, and monkey kidney cell line (MARC-145) (Bautista and Molitor, 1999; Rowland et al., 2001). The expression of IFNγ in the recall reaction by PBMC of sows infected with PRRSV significantly correlates with protection from reproductive failure (Lowe et al., 2005). Sows that have higher numbers of IFNγ-producing PBMC yield higher
number of viable piglets at weaning than sows that have lower number of IFNγ-producing cells (Lowe et al., 2005). IFNγ-producing lymphocytes were recruited to the lungs after PRRSV infection (Thanawongnuwech et al., 2003). However their contribution to protection against respiratory disease has not been evident.

**PRRSV and Immunosuppression**

PRRSV has been presumed to possess immunosuppressive properties. This idea comes from at least 3 lines of evidence:

1. PRRSV-infected pigs seem to be more susceptible to co- and secondary infections and have more severe disease than pigs infected with co-infected pathogens alone. In field observations, PRRSV-infected pigs were demonstrated to have increased incidence of infections with *Streptococcus suis*, *Hemophilus parasuis*, *Mycoplasma hyopneumoniae*, *Actinobacillus pleuropneumoniae*, *Salmonella* spp., and swine influenza virus (Done and Paton, 1995; Rossow et al., 1995; Zeman et al., 1993). Experimental dual infection studies revealed that PRRSV exacerbated the severity of the disease caused by *S. suis*. Pigs infected with PRRSV and *S. suis* developed more severe suppurative meningitis and had higher numbers of *S. suis* in the brain, meninges, and blood than pigs infected with *S. suis* alone (Feng et al., 2001; Galina et al., 1994; Thanawongnuwech et al., 2000).

2. The immune response to PRRSV is delayed, compared to that to other pathogens (Thacker, 2001).

3. An IL-10 response dominates the response of other cytokines after PRRSV infection. The production of IL-10 is increased in BAL fluids of PRRSV-infected pigs (Chung and Chae, 2003; Johnsen et al., 2002; Labarque et al., 2003a; Thanawongnuwech and
IL-10 mRNA expression is increased in PBMC of PRRSV-infected pigs and naïve PBMC exposed to PRRSV in vitro (Royaee et al., 2004; Suradhat et al., 2003). IL-10 has been demonstrated in pigs to have an inhibitory effect on IFNγ production by T cells (Waters et al., 1999). It was shown in other species to have suppressive effects on T cell activation, cytokine production eg. IL-1, TNFα, IFNγ, and activation of antigen-presenting cells (APC) (Moore et al., 2001).

In contrast to this evidence, there are at least 2 lines of evidence that do not support the idea of PRRSV and immunosuppression.

(1) Co-infection between PRRSV and *H. parasuis* (Cooper et al., 1995; Solano et al., 1997), *S. suis* (Cooper et al., 1995), *S. choleraesuis* (Cooper et al., 1995), *Pasteurella multocida* (Carvalho et al., 1997; Cooper et al., 1995), *M. hyopneumoniae* (Thacker et al., 1999; Van Alstine et al., 1996), *A. pleuropneumoniae* (Pol et al., 1997b), swine influenza virus (Pol et al., 1997b), and transmissible gastroenteritis virus (Wesley et al., 1998) did not exacerbate the severity of the disease caused by these pathogens.

(2) Lack of evidence that pigs infected with PRRSV had impaired immune response to other pathogens. Pigs inoculated with PRRSV two weeks prior to immunization with killed pseudorabies virus (PRV) vaccine showed no significant difference in antibody response to killed PRV vaccine when compared to control pigs (Albina et al., 1998b). Instead, these PRRSV-infected pigs produced significantly higher antibody titers to PRV 1-2 weeks after PRV challenge, compared to pigs not previously infected. No significant difference of DTH responses to intradermal challenge with PRV glycoproteins was observed between PRRSV-infected and control pigs (Albina et al., 1998b). No significant difference
on the antibody response to circovirus type 1 infection was observed between PRRS MLV-vaccinated and control pigs (Foss et al., 2002).

**PRRSV Vaccines**

1. **MLV Vaccine**

   MLV vaccines used in the United States are derived from North American PRRSV isolate VR-2332 and JA-142 and are licensed under the trade name Ingelvac® PRRS MLV (licensed in 1994) and Ingelvac® PRRS ATP (licensed in 1999), respectively (Boehringer Ingelheim Vetmedica, Inc.). The Restriction Fragment Length Polymorphism (RFLP) pattern of ORF5 of Ingelvac® PRRS MLV and Ingelvac® PRRS ATP is 2-5-2 and 1-4-2, respectively (Roof et al., 2003).

   Ingelvac® PRRS MLV vaccine is recommended for use in sows, gilts, and piglets as an aid in the reduction of disease associated with PRRSV, reproductive and respiratory forms. In sows and gilts, the vaccine is recommended to be administered intramuscularly in a 2-ml single dose at 3-4 weeks prior to breeding and should be repeated prior to each subsequent breeding. In piglets, the vaccine is recommended to be injected intramuscularly in a single 2-ml dose at 3 weeks of age or older.

   Ingelvac® PRRS ATP is recommended for use only in pigs at 3-18 weeks of age as an aid in the reduction of respiratory disease associated with PRRSV. The vaccine is recommended to be injected intramuscularly in a single 2-ml dose at 3 weeks of age or older.

   **Immune Response:**

   Ingelvac® PRRS MLV vaccine induces both antibody and CMI responses. Specific antibody response detected by IDEXX ELISA appears around 2 weeks after vaccination.
Neutralizing antibody response arises around 4 weeks after vaccination (Meier et al., 2004). A CMI response determined by in vitro proliferation of peripheral blood T cells appears approximately 4 weeks after vaccination (Bassaganya-Riera et al., 2004) and IFNγ ELISPOT appears approximately 4-8 weeks after vaccination (Meier et al., 2003; Meier et al., 2004; Royaee et al., 2004).

**Protection:**

**Homologous virus challenge:** Pigs vaccinated with Ingelvac® PRRS MLV and challenged at 4 weeks after vaccination with vaccine parental strain (VR-2332) (99.7% ORF5-6 sequence homology to Ingelvac® PRRS MLV) were completely protected from viremia and demonstrated significant reduction of macroscopic lung lesions (Foss et al., 2002; Roof et al., 2003). Reduced titers or no VR-2332 virus was shed in semen of boars vaccinated and challenged with VR-2332 at 50 days after vaccination (Christopher-Hennings et al., 1997).

Complete protection from viremia after homologous virus challenge was a characteristic of PRRSV infection (Lager et al., 1997a; Lager et al., 1997b; Mengeling et al., 1999; Mengeling et al., 2003a; Nielsen et al., 1997b; van Woensel et al., 1998). No PRRSV can be isolated from PAM, uterine lymph node, and tonsil after homologous virus challenge (Lager et al., 1997a; Lager et al., 1997b). One hundred per cent of fetuses at 111 days of gestation were protected from abortion, viremia, and were negative for virus isolation from PAM, lymph nodes, and tonsils when sows were challenged at 90 days of gestation (Lager et al., 1997a; Lager et al., 1997b; Mengeling et al., 1999). This protection was observed even when a homologous virus challenge was conducted 604 days after primary exposure (Lager et al., 1997a).
**Heterologous virus challenge:** Pigs vaccinated with Ingelvac® PRRS MLV or Ingelvac® PRRS ATP and challenged at 3 to 17 weeks after vaccination with heterologous virus eg. SDSU-73, 17198-6, MN/01/A1, NADC-8, NADC-9, NVSL-14, IA-1-4-2 or with European PRRSV showed reduced titers of viremia of challenged virus, fewer viremic pigs, and significantly reduced macroscopic lung lesions (Meier et al., 2004; Mengeling et al., 1999; Mengeling et al., 2003b; Nielsen et al., 1997b; Nodelijk et al., 2001; Roof et al., 2003). The North American challenge PRRSV have 84.9%-91.7% ORF5-6 nucleotide sequence homology to the vaccine viruses (Roof et al., 2003). The protection from European PRRSV viremia by Ingelvac® PRRS MLV has not been consistent in all studies. Labarque et al (2003) reported that pigs vaccinated with Ingelvac® PRRS MLV and challenged at 6 weeks after vaccination with European PRRSV showed no reduction in number of viremic pigs and titer of challenged virus in serum and BAL fluid.

Gilts vaccinated with Ingelvac® PRRS MLV vaccine and challenged 30 days later (at 90 days of gestation) with heterologous PRRSV (NADC-8) showed shorter duration and reduced titers of viremia (Mengeling et al., 1999). There were fewer positive virus isolations from tonsil swabs and reduced numbers of pre- and postnatal deaths in vaccinated gilts in comparison to non-vaccinated challenge control gilts (Mengeling et al., 1999). Piglets born from MLV-vaccinated gilts were partially protected from virus infection as determined by negative virus isolation from tonsil swab and serum in some piglets (Mengeling et al., 1999). Piglets had higher body weight at 15 days of age than those born from non-vaccinated challenge control gilts (Mengeling et al., 1999). Boars vaccinated with Ingelvac® PRRS MLV vaccine and challenged 35 days later with European PRRSV showed reduction of virus shedding in semen (Nielsen et al., 1997b).
Concerns of PRRSV MLV vaccine:

Virus spreading: Ingelvac® PRRS MLV vaccine induces viremia after vaccination (Foss et al., 2002; Mengeling et al., 2003b; Nielsen et al., 1997b). The vaccine virus can be isolated from BAL fluid and serum at least 3 and 6 weeks after intramuscular injection, respectively (Mengeling et al., 2003b; Thacker et al., 2000). The virus can be shed in semen of boars up to 2 weeks after vaccination (Christopher-Hennings et al., 1997). The vaccine viral antigens and viral RNA can be detected in non-vaccinated boars that were housed in the same facility with boars previously immunized with Ingelvac® PRRS MLV (Botner et al., 1997; Madsen et al., 1998). The vaccine virus spread from vaccinated pigs (3-18 weeks of age) to non-vaccinated sows in a different facility as determined by seroconversion of sows (Sorensen et al., 1998).

Reversion to virulence: Ingelvac® PRRS MLV has been shown to revert to a state of virulence. Pregnant sows infected with vaccine virus which was shed from vaccinated pigs (3-18 weeks of age) demonstrated late-term abortion (Botner et al., 1997; Sorensen et al., 1998). The vaccine-like virus was isolated from dead fetuses and piglets of these sows (Botner et al., 1997). The virus has 99.2-99.5% ORF2-7 nucleotide sequence homology to the Ingelvac® PRRS MLV (Madsen et al., 1998; Storgaard et al., 1999). Intranasal inoculation of vaccine-like virus isolated from dead piglets into seronegative sows at 90 days of gestation caused congenital infection, stillborn pigs, and high pre-weaning mortality (Nielsen et al., 2002). The vaccine-like virus, however, causes mild respiratory disease in growing pigs when compared to VR-2332 (Opriessnig et al., 2002).

Poor immune response after long-term use: Multiple vaccinations with Ingelvac® PRRS MLV vaccine may result in absence of anamnestic IDEXX ELISA antibody and
neutralizing antibody response as well as antigen-specific T cell proliferation (Bassaganya-Riera et al., 2004; Osorio et al., 1998). Booster immunization with an inactivated genetically divergent virus has been reported to be helpful for boosting an antigen-specific T cell response (Bassaganya-Riera et al., 2004). The magnitude of antigen-specific T cell proliferation after booster immunization with inactivated heterologous virus, however, is still significantly lower than that obtained from animals vaccinating with Ingelvac® PRRS MLV for the first time (Bassaganya-Riera et al., 2004).

2. Killed Virus (KV) Vaccine

Commercial KV vaccine used in the United States is derived from North American PRRSV isolate VR-2402 (also known as ISUP) and is licensed under the trade name PRRomiSe™ (Intervet Inc.). The vaccine is recommended for use in female breeding age swine as an aid in the reduction of losses at farrowing due to PRRSV. The vaccine is recommended to be administered intramuscularly twice in a 2-ml dose; the first dose at 5-8 weeks after breeding and the second dose at 2-4 weeks later. Vaccination is recommended to be repeated on subsequent breedings. The KV vaccine is adjuvanted with Spur (Intervet’s Proprietary Technology) to enhance the immune response.

Immune Response:

PRRomiSe™ vaccine did not induce antibody responses detectable by IDEXX ELISA and serum virus neutralization (SVN) test (Bassaganya-Riera et al., 2004). The vaccine induces a CMI response determined by in vitro CD8αβ T cell proliferation which can be detected 27-38 days after the first dose of vaccination (Bassaganya-Riera et al., 2004).
Repeated vaccination of PRRomiSe™ vaccine generates poorer antigen-specific T cell proliferation (Bassaganya-Riera et al., 2004).

**Protection:**

Little data is available regarding PRRomiSe™ vaccine’s efficacy. An experiment conducted in Europe, using European strain of PRRSV as a source of experimental inactivated PRRSV vaccine demonstrated the protection from reproductive failures in sows and gilts previously infected with field European PRRSV. The number of stillborn pigs and mummies were significantly reduced after 3 doses of inactivated PRRSV vaccine (Reynaud et al., 1998). The health status of piglets born from vaccinated and experimentally challenged sows was improved in comparison to those born from non-vaccinated sows (Plana-Duran et al., 1997b).

Experimental killed PRRSV vaccine (VR-2402) appeared to decrease the duration of viral shedding in the semen of boars experimentally infected with homologous PRRSV (VR-2402) (Swenson et al., 1995). PRRomiSe™ vaccine, however, did not protect pigs from viremia and virus infection in tonsils when pigs were infected with heterologous virus (VR-2385) (Nilubol et al., 2004). Pigs demonstrated increased neutralizing antibody titers to VR-2385 after PRRomiSe™ vaccination (Nilubol et al., 2004).

**Concerns of PRRSV KV vaccine:**

Poor CMI response after long-term use: The main attribute of killed PRRSV vaccine is to boost neutralizing antibody response, not CMI response (Bassaganya-Riera et al., 2004). Multiple vaccinations with PRRomiSe™ vaccine resulted in poor antigen-specific T cell proliferation. Sows that were repeatedly vaccinated with PRRomiSe™ and boosted with Ingelvac® PRRS MLV showed some improvements of magnitude of antigen-specific T cell proliferation (Bassaganya-Riera et al., 2004).
proliferation. However, such response was still significantly lower than that observed in naïve sows receiving PRRomiSe™ vaccination for the first time (Bassaganya-Riera et al., 2004).

3. DNA Vaccine

A plasmid DNA expressing PRRSV ORF was studied for its efficacy in inducing immune responses and protecting pigs from clinical disease after PRRSV challenge. The advantage of DNA vaccine over PRRSV MLV is its safety (no virus shedding and reversion to virulence). In addition, DNA vaccines could ideally elicit immune response to various PRRSV isolates in one vaccination. The DNA vector contained immunostimulatory CpG motifs which enhance CMI response in pigs (Kamstrup et al., 2001).

**Immune Response:**

Administration of the DNA vaccine induced both antibody and CMI responses (Barfoed et al., 2004a; Kwang et al., 1999; Pirzadeh and Dea, 1998). Pigs receiving intramuscular and intradermal injections of plasmid DNA (100 μg) encoding PRRSV ORF5 developed neutralizing antibodies and in vitro T cell proliferation (Pirzadeh and Dea, 1998). Pigs injected intramuscularly with 400 μg of DNA encoding PRRSV ORF4-7 developed neutralizing antibody and antibody detectable by IDEXX ELISA and Western blot assay, T-cell proliferation, and IFNy production (Kwang et al., 1999). Pigs vaccinated with 10.8 μg of gold particle-coated DNA encoding ORF1-7 developed an antibody response to only ORF7 products (Barfoed et al., 2004a).

**Protection:**
Pigs immunized with DNA vaccine may be protected from viremia and respiratory lesions after homologous virus challenge (Barfoed et al., 2004a; Pirzadeh and Dea, 1998; Rompato et al., 2006). Pigs vaccinated by gene gun containing 60 μg plasmid DNA encoding ORF7 of PRRSV (MI 1257) (2 times, 3-week interval) and intranasally challenged at 4 weeks after the last vaccination with homologous virus (MI 1257) showed reduced virus load in the serum, compared to unvaccinated control pigs (Rompato et al., 2006). Pigs vaccinated intramuscularly and intradermally with DNA encoding ORF5 of PRRSV (Quebec IAF-Klop) (3 times at 2-week intervals) and intratracheally challenged at 2 weeks after the last vaccination with homologous virus showed reduced macroscopic and microscopic lung lesions (Pirzadeh and Dea, 1998). The challenge virus was isolated only from lung and mediastinal lymph nodes with reduced virus titers, while in unvaccinated control pigs, the virus was isolated from lung, spleen, kidney, liver, mediastinal and mesenteric lymph nodes (Pirzadeh and Dea, 1998). Intradermal vaccination with gold particle-coated plasmid DNA encoding ORF1-7 of European PRRSV (DK-111/92) (6 times, 3-week interval) and intranasally challenged at 3 weeks after the last vaccination with homologous virus showed no protection from viremia in term of duration (Barfoed et al., 2004a).

Concerns of PRRSV DNA vaccine:

Difficulty in administration: The administration of DNA vaccine has limited practical application. Studies by Barfoed et al (2004b) reported that pigs vaccinated with plasmid DNA encoding ORF7 of European PRRSV (DK-111/92) 4 times at 3-week intervals developed higher antibody response detected by whole PRRSV (DK-111/92)-coated ELISA when they were vaccinated by gene gun than by conventional intramuscular and intradermal injection. The sites of vaccination by gene gun were the dorsal side of the ear, the inguinal
area, the dorsal thorax, and the ventral side of the tongue (Barfoed et al., 2004b). The intramuscular and intradermal routes of plasmid DNA administration, which are more practical, require 100 times more DNA per vaccination than vaccination by gene gun in order to obtain the same level of antibody response (Barfoed et al., 2004b).

4. **Recombinant PRRSV Protein Vaccine**

Recombinant PRRSV protein was studied for its potential use as a subunit vaccine.

**Immune Response:**

Administration of recombinant PRRSV protein in PRRSV-seronegative pigs induces both antibody and CMI responses (Pirzadeh and Dea, 1998; Plana Duran et al., 1997a). Intramuscular and intradermal injections with 300 μg ORF5 proteins joined at the N-terminus with glutathione S-transferase (GST-ORF5) 3 times at 2-week intervals induced a neutralizing antibody response (titer <1:8) and lymphocyte blastogenesis (Pirzadeh and Dea, 1998). Intramuscular injection with Sf9 cells infected with recombinant baculovirus expressing ORF7 of European PRRSV in the form of water/oil/water emulsion in pregnant sows at 30 and 51 days of gestation induced an antibody response as detected by an immunoperoxidase monolayer assay (IPMA) (Plana Duran et al., 1997a). Anamnestic antibody responses were detected after the second vaccination.

**Protection:**

No clear protection from respiratory lesions conferred by recombinant PRRSV protein vaccine was observed after homologous virus challenge. Pigs vaccinated intramuscularly and intradermally with 300 μg GST-ORF5 of PRRSV (Quebec IAF-Klop) 3 times at 2-week intervals and intratracheally challenged 2 weeks after the last vaccination
with homologous virus (Quebec IAF-Klop) showed no protection from macroscopic and microscopic lung lesions when compared to unvaccinated challenge control pigs (Pirzadeh and Dea, 1998). The challenge virus was isolated from lung, spleen, kidney, and mediastinal lymph nodes with no or slight reduction of virus load when compared to unvaccinated challenge control pigs (Pirzadeh and Dea, 1998).

Some protection from reproductive failure was observed after vaccination with cells expressing recombinant PRRSV proteins. Sows vaccinated intramuscularly with Sf9 cells infected with recombinant baculovirus expressing ORF3, ORF5, and/or ORF7 of European PRRSV (Olot/91) in the form of water/oil/water emulsion at 30 and 51 days of gestation and intranasally challenged at 70-90 days of gestation with homologous virus (Olot/91) showed some protection from reproductive failure. The percentage of piglets born alive after virus challenge was highest in ORF3 sows (68.4%), ORF5 sows (50%), and ORF7 sows (16.6%), respectively. The percentage of piglets born alive in ORF7 sows, the only group that showed an antibody response after vaccination, was not different from that of unvaccinated challenge control sows (12.9%) (Plana Duran et al., 1997a).

**Adjuvants for PRRSV Vaccine**

Immune responses elicited by PRRSV vaccines are relatively weak and delayed, compared to those induced by other virus vaccines eg. pseudorabies and swine influenza (Thacker, 2001). Various reagents have been tested for their potential as adjuvants for PRRSV vaccine. These immune mediators included cytokines (IL-1/IL-6 combination, IL-2, IL-4, IL-12, IFNα), synthetic double-stranded RNA (poly ICLC), and cholera toxin.
Combination of IL-1 and IL-6

IL-1 has been reported in mice to be effective in eliciting in vivo expansion and follicular migration of antigen-stimulated T cells (Pape et al., 1997), causing IL-12-induced IFNγ production by natural killer cells (Hunter et al., 1995), and enhancing antibody production (Reed et al., 1989). Adjuvant properties of IL-1 were demonstrated in swine in conjunction with a Streptococcus suis vaccine (Blecha et al., 1995). Pigs administered recombinant IL-1 at 10 μg/kg body weight had higher antibody responses to S. suis, less severe clinical disease and macroscopic lesions after challenge, and better growth performance during infection.

IL-6 has been reported to promote the terminal differentiation of activated B cells into antibody-secreting cells in mice (Diehl and Rincon, 2002; Kammuller, 1995). This cytokine has an inhibitory effect on T helper 1 (Th1) differentiation (Diehl and Rincon, 2002). In pigs, the adjuvant properties of IL-6 have been tested with swine influenza virus vaccine (Larsen and Olsen, 2002). Administration of an IL-6 expressing plasmid during vaccination for swine influenza virus did not enhance vaccine efficacy for inducing antibody responses and protection after swine influenza virus challenge (Larsen and Olsen, 2002).

In PRRSV, a combination of recombinant porcine IL-1 and IL-6 was studied as an adjuvant for PRRSV MLV vaccine. Pigs vaccinated with Ingelvac® PRRS MLV which received Pichia pastoris-expressed recombinant porcine IL-1 and IL-6 at the time of vaccination and at 2, 4, and 7 days after vaccination (20 μg of each cytokine at each time point) showed no improvement in kinetics and magnitude of antibody response detected by IDEXX ELISA and CMI response detected by IFNγ ELISPOT when compared to those of animals vaccinated with Ingelvac® PRRS MLV alone (Foss et al., 2002).
**IL-2**

IL-2 has been identified as one of the major cytokines responsible for the regulation of CMI responses (Arai et al., 1990; Gaffen and Liu, 2004). The cytokine has been extensively studied as a vaccine adjuvant in mice, rhesus macaque, cattle, and pigs (Chow et al., 1998; Derosa and Sordillo, 1997; Kim et al., 1999; Kim et al., 2001; Mbawuike et al., 1990; Wong et al., 2002). It has been shown that when multiple injections of IL-2 are administered after the immunizing antigen, protection against challenge is enhanced (Derosa and Sordillo, 1997; Mbawuike et al., 1990). The co-administration of a plasmid containing the swine IL-2 gene along with a foot-and-mouth DNA vaccine was able to significantly increase the stimulation index of T cells, when compared to those of animals vaccinated with the DNA vaccine alone (Wong et al., 2002).

Pigs immunized with PRRSV-ORF7 DNA vaccine and DNA encoding porcine IL-2 at day -1, 0 (vaccination day), and 1 (60 μg each) and received booster immunizations with both DNA constructs 21 days later demonstrated a significant increase in T cell proliferation when compared to T cells of pigs immunized with plasmid DNA encoding ORF7 alone (Rompato et al., 2006).

**IL-4**

IL-4 has been reported to be effective in promoting proliferation of resting B cells (Arai et al., 1990), production of antigen-specific antibody (Kim et al., 1999; Kim et al., 2000), and differentiation of Th0 into Th2 cells (Paul, 1991). Adjuvant properties of IL-4 were demonstrated in rhesus macaque in conjunction with a DNA vaccine encoding simian
immunodeficiency virus Gag/Pol proteins. Rhesus macaques administered DNA expressing IL-4 had increased levels of antigen-specific antibodies, when compared to those of animals receiving DNA vaccine alone (Kim et al., 1999).

In PRRSV, plasmid DNA encoding porcine IL-4 did not have adjuvant properties when administered with DNA vaccine expressing PRRSV ORF7 (Rompato et al., 2006). In addition, T cell proliferation was shown to be decreased after homologous PRRSV challenge when compared to T cells of pigs immunized with PRRSV DNA vaccine alone (Rompato et al., 2006).

**IL-12**

IL-12 has been reported to induce IFNγ expression in T cells and natural killer cells in humans, mice, and pigs (Cho et al., 1996; Domeika et al., 2002; Perussia et al., 1992). In pigs, recombinant human IL-12 was studied as a vaccine adjuvant for inactivated PRV vaccine (Zuckermann et al., 1998). Pigs vaccinated with inactivated PRV and recombinant human IL-12 at 2 hours before and 2 hours after vaccination (2 μg each) demonstrated a significant increase in number of IFNγ-secreting PBMC as determined by ELISPOT, compared to pigs that received inactivated PRV vaccine alone (Zuckermann et al., 1998). The amino acid sequences of human and porcine IL-12 have 85% homology (Foss and Murtaugh, 1997).

In PRRSV, recombinant porcine IL-12 has been reported to increase the number of IFNγ-producing PBMC after Ingelvac® PRRS MLV vaccination. Pigs vaccinated with Ingelvac® PRRS MLV and Pichia pastoris-expressed recombinant single-chain (p70) porcine IL-12 at the time of vaccination and at 2, 4, and 7 days after vaccination (20 μg each) had
significantly increased numbers of IFNγ-secreting PBMC as determined by ELISPOT, compared to pigs vaccinated with Ingelvac® PRRS MLV alone (Foss et al., 2002). Recombinant porcine IL-12 given with Ingelvac® PRRS MLV did not increase antibody response as determined by IDEXX ELISA (Foss et al., 2002).

\textit{IFNα}

IFNα has been reported to promote the expression of MHC class I and class II as well as co-stimulatory molecules in monocytes, dendritic cells, and B cells of mice, and thereby enhance the activity in antigen presentation and T cell activation (Honda et al., 2003; Kadowaki et al., 2000; Montoya et al., 2002; Pogue et al., 2004). The cytokine has been demonstrated to enhance IFNγ expression in CD4^+ T cells of humans and mice (Biron, 2001; Brinkmann et al., 1993; Cousens et al., 1999).

IFNα has been studied as a vaccine adjuvant for PRRSV vaccines as IFNα production is reduced after PRRSV infection and vaccination (Albina et al., 1998a; Buddaert et al., 1998; Royaee et al., 2004; Van Reeth et al., 1999). It has been thought that the exogenous addition of this cytokine might compensate for this deficiency and thus may stimulate CMI after PRRSV MLV vaccination.

Pigs vaccinated with Ingelvac® PRRS MLV and a plasmid expressing porcine IFNα at the time of vaccination (200 μg) demonstrated a significant increase in numbers of IFNγ-secreting PBMC when compared to pigs vaccinated with Ingelvac® PRRS MLV alone (Meier et al., 2004). The increased response was detected between 2 and 6 weeks after vaccination. No differences in antibody responses determined by IDEXX ELISA were observed between those two groups (Meier et al., 2004).
**Poly ICLC**

Poly ICLC (polyinosinic:polycytidylic complexed with poly-L-lysine and carboxymethylcellulose) is a synthetic double-stranded RNA reported to effectively induce type I IFN production in humans, mice, and pigs (Finkelman et al., 1991; Loewen and Derbyshire, 1988a; Loewen and Derbyshire, 1988b; Weingartl and Derbyshire, 1990). In pigs, poly ICLC has been studied for its adjuvant activity with PRRSV MLV vaccine (Meier et al., 2004). Pigs vaccinated with Ingelvac® PRRS MLV and poly ICLC at the time of vaccination (0.25 mg/kg body weight) had a significant increase in numbers of IFNγ-secreting PBMC when compared to pigs vaccinated with Ingelvac® PRRS MLV alone (Meier et al., 2004). The significantly increased response was detected between 1 and 3 week after vaccination. No differences in antibody responses as measured by IDEXX ELISA were observed between those two groups (Meier et al., 2004).

**Cholera toxin (CT)**

CT has been reported to promote production of proinflammatory cytokines (IL-1, IL-6, IL-12), expression of co-stimulatory molecules on PBMC and macrophages of humans, mice, and pigs, antigen presentation and T cell proliferation, and isotype switching of murine B cells (Bromander et al., 1991; Cong et al., 1997; Foss and Murtaugh, 1999b; Foss and Murtaugh, 1999c; Foss et al., 1999a; Krakauer, 1996; Lycke and Strober, 1989).

The use of CT as a vaccine adjuvant for PRRSV vaccine was reported by Foss et al (2002). Pigs vaccinated with Ingelvac® PRRS MLV and CT at the time of vaccination and at 2, 4, and 7 days after vaccination (20 µg each) showed improved kinetics and magnitude of
the anti-GP5 antibody response. The response was detected at 21 days after vaccination whereas the maximum response of anti-GP5 antibody to MLV alone was at 28 days (Foss et al., 2002). CT did not improve the CMI response as determined by IFNγ ELISPOT when compared to the response to the MLV vaccine alone (Foss et al., 2002).

Overview of Porcine Myeloid Antigen-Presenting Cells (APC)

Monocytes

Porcine monocytes constitute 10-15% of peripheral blood leukocytes and about 10-40% of PBMC (Chamorro et al., 2005). Monocytes are derived from myeloid progenitors in the bone marrow. They and other myeloid cells express swine workshop cluster 3 (SWC3⁺), a molecule that is specific for cells of myeloid lineage of pigs (Chamorro et al., 2005; McCullough et al., 1999; McCullough et al., 1997). Monocytes express high levels of SWC1, MHC class I, MHC class II and CD14 (Basta et al., 1999). The expression of SWC1, MHC class II, and CD14 is down-regulated after monocytes differentiate to macrophages (Basta et al., 1999). No expression of the co-stimulatory molecule CD80 is reported on monocytes of pigs (Basta et al., 1999).

Monocytes are a potent producer of IL-1 either spontaneously or after activation by bacterial lipopolysaccharide (LPS) (Basta et al., 2000; Basta et al., 1999). The cells respond to bacteria, virus, and plasmid DNA antigens by expression of IL-6, IL-10, and IL-12 (Johansson et al., 2003). Monocytes also are strong inducers of T cell proliferation in recall responses even in the absence of co-stimulatory molecules (Basta et al., 1999). This is probably due to the high IL-1 producing-characteristics of the cells (Basta et al., 1999).
**Macrophages**

Macrophages are derived from peripheral blood monocytes upon migration into tissues. The cells can also be derived in vitro from blood monocytes by continuous plate adherence (Basta et al., 1999; McCullough et al., 1999; McCullough et al., 1997). Monocyte-derived macrophages (MDM) express a specific molecule, SWC9⁺, which is not present in monocytes (McCullough et al., 1999; McCullough et al., 1997). The expression of this molecule increases during the differentiation process of MDM. MDM express high levels of MHC class I, CD80, and β1/β2 integrins and low levels of MHC class II and CD14 (Basta et al., 1999). Phagocytosis and endocytosis and several enzyme activities (e.g., acid phosphatase and glucuronidase) are increased whereas some (e.g., nucleotidase, peroxidase, myeloperoxidase, and esterase) are decreased when compared to monocytes (Basta et al., 1999).

MDM are not good IL-1 producers. The level of IL-1 mRNA and protein expression is low, compared to that of monocytes (Basta et al., 1999). MDM have poorer efficacy in inducing T cell proliferation in a recall response in comparison to monocytes (Basta et al., 1999).

Like MDM, PAM express the SWC1⁻SWC9⁺ phenotype (Basta et al., 1999). PAM express MHC class I, MHC class II, CD80 and β1/β2 integrins to the same level as MDM (Basta et al., 2000; Basta et al., 1999). However, PAM are incapable of inducing T cell proliferation in recall responses (Basta et al., 2000). This is probably due to the poor IL-1 production and other unidentified suppressive effects in PAM (Basta et al., 2000).

**Dendritic cells**
Porcine dendritic cells are derived from myeloid progenitors in the bone marrow. The cells have a characteristic phenotype of enlarged cell diameter, pronounced protusions and microvillous projections of plasma membrane, and abundant multivacuolar and multilamellar vesicles (Johansson et al., 2003; Paillot et al., 2001). The dendritic cells can be derived in vitro from bone marrow progenitor cells and peripheral blood monocytes by continuous culture in complete media supplemented with recombinant porcine granulocyte-macrophage colony stimulating factor (GM-CSF) and recombinant porcine IL-4 and/or recombinant human TNFα (Johansson et al., 2003; West et al., 1999). The resulting monocyte-derived dendritic cells (MDD) are in an immature state (Johansson et al., 2003). The immature MDD have a strong ability to take up foreign antigens (Johansson et al., 2003). The antigen uptake capacity decreases as the cells maturate. The maturation of MDD can be induced by LPS, TNFα, and other necrotic factors (Paillot et al., 2001). MDD express IL-6, IL-10, IL-12, and IFNα in response to bacteria, virus, and plasmid DNA (Johansson et al., 2003). MDD have the ability to induce T cell proliferation in a mixed leukocyte reaction (Johansson et al., 2003; West et al., 1999). This ability is seen more potently in mature MDD than immature MDD (Paillot et al., 2001).

MDD express MHC class I, MHC class II, CD80, CD86, β2-integrins, CD1, CD36, CD68 (lysosome-associated membrane glycoprotein), and p55 fascin (actin-bundling protein) (Johansson et al., 2003; Paillot et al., 2001). Expression of p55 fascin is found only in MDD but not in MDM (West et al., 1999). The expression of MHC II, CD80, and CD86 of MDD are up-regulated upon their differentiation from monocytes (Johansson et al., 2003). The expression of CD14 is down-regulated during differentiation from monocytes (Paillot et al., 2001).
Summary

The humoral and cell-mediated immune responses to PRRSV are weak. The antibody response to PRRSV is predominantly against non-neutralizing epitopes. Antibodies against neutralizing epitopes appear later than non-neutralizing antibodies and have low neutralizing titers throughout the course of infection. The CMI response appears at the same time or later than neutralizing antibodies and has a lower magnitude, compared to the CMI response to other swine viral pathogens.

Protection of pigs by antibodies is restricted by antigenic variation of PRRSV neutralizing epitopes. The antibodies provide complete protection only from homologous virus challenge and partial or no protection from heterologous virus challenge. Low titers of neutralizing antibody enhance PRRSV infection through an antibody-dependent enhancement of infection of macrophages.

Little is known about T cell subsets specific for PRRSV after infection or vaccination and the protection conferred by T cells. Mechanisms that PRRSV utilizes to impair CMI responses and methods to improve CMI response to PRRSV are not well understood.

References


Botner, A., Strandbygaard, B., Sorensen, K.J., Have, P., Madsen, K.G., Madsen, E.S.,
Alexandersen, S., 1997, Appearance of acute PRRS-like symptoms in sow herds after
vaccination with a modified live PRRS vaccine. Vet Rec 141, 497-499.
Brinkmann, V., Geiger, T., Alkan, S., Heusser, C.H., 1993, Interferon alpha increases the
frequency of interferon gamma-producing human CD4+ T cells. J Exp Med 178,
1655-1663.
Bromander, A., Holmgren, J., Lycke, N., 1991, Cholera toxin stimulates IL-1 production and
Buddaert, W., Van Reeth, K., Pensaert, M., 1998, In vivo and in vitro interferon (IFN)
studies with the porcine reproductive and respiratory syndrome virus (PRRSV). Adv
Carvalho, L.F., Segales, J., Pijoan, C., 1997, Effect of porcine reproductive and respiratory
syndrome virus on subsequent Pasteurella multocida challenge in pigs. Vet Microbiol
55, 241-246.
Phenotypic and functional heterogeneity of porcine blood monocytes and its relation
with maturation. Immunology 114, 63-71.
Charley, B., Lavenant, L., 1990, Characterization of blood mononuclear cells producing IFN
alpha following induction by coronavirus-infected cells (porcine transmissible
Chiou, M.T., Jeng, C.R., Chueh, L.L., Cheng, C.H., Pang, V.F., 2000, Effects of porcine
reproductive and respiratory syndrome virus (isolate tw91) on porcine alveolar
natural killer cell activity by recombinant human and murine IL-12. Cell Immunol
172, 29-34.
Chow, Y.H., Chiang, B.L., Lee, Y.L., Chi, W.K., Lin, W.C., Chen, Y.T., Tao, M.H., 1998,
Development of Th1 and Th2 populations and the nature of immune responses to
hepatitis B virus DNA vaccines can be modulated by codelivery of various cytokine
Christianson, W.T., Collins, J.E., Benfield, D.A., Harris, L., Gorcerya, D.E., Chladek, D.W.,
Morrison, R.B., Joo, H.S., 1992, Experimental reproduction of swine infertility and
duration of porcine reproductive and respiratory syndrome virus in semen, serum,
peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and
Christopher-Hennings, J., Nelson, E.A., Hines, R.J., Nelson, J.K., Swenson, S.L.,
Zimmerman, J.J., Chase, C.L., Yaeger, M.J., Benfield, D.A., 1995a, Persistence of
porcine reproductive and respiratory syndrome virus in serum and semen of adult
modified-live virus vaccine against porcine reproductive and respiratory syndrome in


Feng, W., Laster, S.M., Tompkins, M., Brown, T., Xu, J.S., Altier, C., Gomez, W., Benfield, D., McCaw, M.B., 2001, In utero infection by porcine reproductive and respiratory syndrome virus is sufficient to increase susceptibility of piglets to challenge by Streptococcus suis type II. J Virol 75, 4889-4895.


porcine reproductive and respiratory syndrome virus infection or vaccination.
Virology 309, 18-31.


vaccine (ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J Virol 76, 11837-11844.


Pogue, S.L., Preston, B.T., Stalder, J., Bebbington, C.R., Cardarelli, P.M., 2004, The receptor for type I IFNs is highly expressed on peripheral blood B cells and monocytes and mediates a distinct profile of differentiation and activation of these cells. J Interferon Cytokine Res 24, 131-139.


Chapter 3. Immune Responses and Protection by Vaccine and Various Vaccine Adjuvant Candidates to Virulent Porcine Reproductive and Respiratory Syndrome Virus

A paper published in *Veterinary Immunology and Immunopathology* *

Wasin Charerntantanakul, Ratree Platt, Wesley Johnson, Michael Roof, Eric Vaughn, James A. Roth

Abstract

Various vaccine adjuvant candidates were assessed with the modified-live porcine reproductive and respiratory syndrome virus (MLV PRRSV) (Ingelvac® PRRS MLV) vaccine. Their influence on humoral-mediated immune (HMI) and cell-mediated immune (CMI) responses as well as protection from virulent PRRSV challenge (MN-184) was evaluated. Ninety seronegative pigs were randomly divided into 9 groups of 10 pigs. One group received MLV vaccine alone. Five groups received MLV vaccine with either bacterial endotoxin-derived adjuvant (ET), mixed open reading frame 5 (ORF5) peptides derived from various PRRSV isolates, porcine interferon alpha (IFNα), polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (poly-ICLC), or porcine interleukin-12 (IL-12). One group did not receive MLV vaccine but was immunized with ORF5 peptides conjugated with cholera toxin (ORF5

peptide/CT). Two groups served as challenged and unchallenged non-vaccinated controls. Four-color flow cytometry was utilized to simultaneously identify 3 major porcine T-cell surface markers (CD4, CD8, and γδ TCR) and detect activation marker CD25 (α chain of IL-2 receptor) or intracellular IFNγ. The MLV PRRSV vaccine alone successfully primed CD4⁺CD8⁺γδ⁻ T cells as demonstrated by a significant increase in %IFNγ⁺ cells when live PRRSV was used as a recall antigen. Booster immunizations of mixed ORF5 peptides and co-administration of IL-12 with MLV PRRSV vaccine significantly enhanced IFNγ expression by some T cell subsets (CD4⁺CD8⁺γδ⁻ and CD4⁺CD8⁺γδ⁺ for mixed ORF5 peptides and CD4⁺CD8⁺γδ⁻ and CD4⁺CD8⁺γδ⁺ for IL-12). All groups receiving MLV-vaccine with or without adjuvants had reduced lung lesions after challenge. The group immunized with only ORF5 peptide/CT did not have significant T cell recall responses and was not protected from challenge. Expression of IFNγ by several T cell subsets correlated with reduced lung lesions and viremia, whereas expression of CD25 did not. Expression of surface CD25 did not correlate with IFNγ production. PRRSV ELISA s/p ratio prior to challenge also correlated with reduced lung lesions and viremia. In conclusion, booster immunizations of the mixed ORF5 peptides and co-administration of IL-12 effectively enhanced the CMI response to MLV vaccine. However, neither adjuvant significantly contributed to reducing clinical effects when compared to MLV alone.

**Keywords:** Vaccine adjuvant; Porcine reproductive and respiratory syndrome virus; Humoral-mediated immune response; Cell-mediated immune response
**Abbreviations:** PRRSV, porcine reproductive and respiratory syndrome virus; CMI, cell-mediated immunity; HMI, humoral-mediated immunity; MLV, modified-live virus; ET, bacterial endotoxin-derived adjuvant; ORF5, open reading frame 5; Poly-ICLC, polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose; ORF5 peptide/CT, ORF5 peptides conjugated with cholera toxin; MFI, geometric mean fluorescent intensities; CD25EI, CD25 expression index; EMEM, Eagle’s minimum essential medium

1. Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is a linear positive-sense, single-stranded, enveloped RNA virus of the family Arteriviridae (Meulenberg et al., 1993). The viral genome is approximately 15 kb in size and consists of 9 open reading frames (ORFs), designated ORF1a, 1b, 2a, 2b, and 3 to 7 (Conzelmann et al., 1993; Meulenberg et al., 1993). ORF5 encodes a major envelope glycoprotein containing a neutralizing epitope(s) (Pirzadeh and Dea., 1997; Kwang et al., 1999; Ostrowski et al., 2002; Plagemann et al., 2002).

PRRSV has been recognized as the causative agent of reproductive failure in adult pigs and respiratory disease in young pigs (Christianson et al., 1992; Collins et al., 1992; Halbur et al., 1996). A modified-live virus (MLV) PRRSV vaccine has been used to control PRRSV infection. The vaccine induces both humoral-mediated immune (HMI) and cell-mediated immune (CMI) responses (Foss et al., 2002; Meier et al., 2003). MLV-vaccinated pigs showed reduced lung lesions (Mengeling et al., 2003) and were protected from viremia after homologous virus challenge (Foss et al., 2002). Antibody responses to MLV vaccine detected by ELISA test appear within 14 days post vaccination, while neutralizing antibodies
arise at 4 weeks post inoculation (Yoon et al., 1995; Foss et al., 2002). Vaccine-induced CMI, determined by enzyme-linked immunospot (ELISPOT) assay for IFNγ production, appears at low level at 14 days post vaccination and develops slowly (Foss et al., 2002; Meier et al., 2003). Vaccine provides incomplete protection to heterologous PRRSV infections (Labarque et al., 2003).

Efforts to use immunomodulating agents to induce robust HMI and CMI responses to vaccine antigens have been attempted for some swine diseases, eg. cholera toxin (CT) for *Ascarid suum* antigens (Tsuji et al., 2001) and IL-12 for inactivated pseudorabies virus vaccine (review in Zuckermann et al., 1999). A recent study by Foss et al. (2002) showed the contributions of CT and IL-12 to the MLV PRRSV vaccine in accelerating neutralizing antibody production and enhancing IFNγ production by PBMCs, respectively.

Various vaccine adjuvants were evaluated in this study for their efficacy to induce more potent HMI and CMI responses to the MLV PRRSV vaccine and to protect pigs from virulent PRRSV challenge. The adjuvants included bacterial endotoxin-derived adjuvant (ET), mixed ORF5 peptides derived from various PRRSV isolates, porcine IFNa, polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose (poly-ICLC), and porcine IL-12.

The responsive T-cell subsets to the recall vaccine antigens were identified by a four-color flow cytometry technique, which allows simultaneous detection of 3 T-cell surface markers (CD4, CD8, and γδ TCR) and surface CD25 (the alpha chain of IL-2R) or intracellular IFNγ. We also correlated the responsive T-cell subsets, and the ELISA s/p ratio, with lung lesions and viremia after virulent virus challenge.
2. Materials and methods

2.1 Experimental design

Ninety 3-week-old PRRSV-seronegative pigs were randomly divided into 9 groups of 10 pigs. Each group was housed in a separate pen at the Veterinary Resources Inc. facilities (Cambridge, IA). Groups receiving MLV vaccine were in separate rooms from groups not receiving MLV vaccine. The non-challenged group was isolated from the challenged groups. Groups 1 to 6 were vaccinated i.m. with 2 ml of Ingelvac® PRRS MLV (Boehringer Ingelheim Vetmedica, Inc. (BIVI), St. Joseph, MO). The vaccination day was referred to as day 0 of the experiment. In addition to Ingelvac® PRRS MLV (group 1), groups 2 to 6 also received different vaccine adjuvants i.m. at different sites of injection than MLV vaccine. Group 2 received a 2 mL dose of ET (BIVI, St. Joseph, MO) on day 0, group 3 received 1 ml dose of mixed ORF5 ectodomain peptides derived from 5 PRRSV isolates (Ingelvac® PRRS MLV, SDSU-73, 17198-6, JA142wt, and MN/01/A1), on day 14 and 28, group 4 received 1 x 10⁵ units of porcine IFNα (PBL Biomedical Laboratories, Piscataway, NJ) on day -1, 0, and 1, and a 400 μg dose of IFNα DNA vaccine on day 0 (Zuckermann F., University of Illinois, Urbana, IL), group 5 received a 0.4 mg dose of poly-ICLC (Ribopharm, Inc. Bethesda, MD) on day 1, and group 6 received a 4.0 μg dose of porcine IL-12 (R&D Systems, Minneapolis, MN) on day 1. Group 7 was immunized i.m. with a 60 μg dose of mixed ORF5 ectodomain peptides conjugated with CT (Sigma, St.Louis, MO) on day 0, 14, and 28. The ORF5 peptides were derived from the same PRRSV isolates as was group 3. Group 8 served as mock negative control, received 2 ml i.m. of Eagle's minimum essential medium (EMEM) (JRH Bioscience, Lenexa, KS). Group 9 served as a strict control without any treatment. All groups except group 9 were challenged intranasally with 10⁵.0 tissue culture infectious dose
of PRRSV isolate MN-184 on day 55. The experimental design is summarized in Table 1. All pigs were necropsied on day 69.

2.2 Viruses

PRRSV isolate MN-184, SDSU-73, and vaccine strain (Ingelvac® PRRS MLV, serial no. JA-787-645, BIVI, St. Joseph, MO) were cultured in confluent MARC-145 cells grown in tissue culture media (DMEM++; DMEM (Life Technologies, Gaithersburg, MD) with 10% heat-inactivated FBS (Atlanta biologicals, Norcross, GA) and 1% tissue culture penicillin (10,000 IU/ml)/streptomycin (10,000 μg/ml) (P/S; Mediatech, Inc., Herndon, VA)). The cultures were incubated in a humidified 5% CO₂ atmosphere at 37°C for 3 days or until 60-80% of cytopathic effect (CPE) were observed. Infected cultures were frozen and thawed, and centrifuged at 1,200 rpm and 4°C for 10 minutes. The supernatant was collected, aliquotted into small volumes, and stored at -80°C. Virus titration was performed by indirect immunofluorescent assay (IFA), using PRRSV-specific mouse mAbs (clone 15E) (Platt KB., Iowa State University, Ames, IA), and FITC-conjugated anti-mouse IgG antibodies (Sigma, St.Louis, MO). Mock antigens were prepared in the same fashion as virus antigens except the viruses were absent.
Table 1. Summary of experimental treatments.

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Antigen</th>
<th>Adjuvants</th>
<th>Timing of adjuvants</th>
<th>Challenge</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>10</td>
<td>MLV</td>
<td>-</td>
<td>-</td>
<td>Day 55</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>MLV</td>
<td>ET</td>
<td>Day 0</td>
<td>Day 55</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>MLV</td>
<td>ORF5 peptides</td>
<td>Day 14,28</td>
<td>Day 55</td>
</tr>
<tr>
<td>4</td>
<td>10</td>
<td>MLV</td>
<td>IFNα</td>
<td>Day -1,0,1</td>
<td>Day 55</td>
</tr>
<tr>
<td>5</td>
<td>10</td>
<td>MLV</td>
<td>Poly-ICLC</td>
<td>Day 1</td>
<td>Day 55</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>MLV</td>
<td>IL-12</td>
<td>Day 1</td>
<td>Day 55</td>
</tr>
<tr>
<td>7</td>
<td>10</td>
<td>ORF5 peptide/CT</td>
<td>-</td>
<td>Day 0,14,28</td>
<td>Day 55</td>
</tr>
<tr>
<td>8</td>
<td>10</td>
<td>EMEM</td>
<td>-</td>
<td>-</td>
<td>Day 55</td>
</tr>
<tr>
<td>9</td>
<td>10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
2.3 Adjuvants and antigen

ORF5 ectodomain peptides were derived from 5 PRRSV isolates, including Ingelvac® PRRS MLV (VLNASNDSSSHLQLIYNLK), SDSU-73 (VLVNANSSSS-HFQSIYNLK), 17198-6 (VLVDANSNSSSHFQILYNLK), JA142wt (ALVNANSSSHLQLIYNLK), and MN/01/A1 (ALVNADNSSSHLQLIYNLK). To couple CT with the peptides, CT were reduced with 50 mM of 1,4-Dithio-DL-threitol (DTT) solution at 37°C for 1 hour and were run over PD-10 desalting column (Amersham, Piscataway, NJ) to remove DTT. Peptides were mixed with sulfo-SMCC (Sulfosuccinimidyl 4-[N-maleimidomethyl] cyclohexane-1-carboxylate) dissolved in DMSO and were coupled with CT at 4°C overnight. The protein concentrations were determined by bicinchoninic acid (BCA) protein assay.

2.4 Virology

2.4.1 Virus isolation

Virus isolations were performed with 100 μl of sera collected on days 55, 56, 59, 62, and 69 in duplicate wells on 48 well plates containing confluent CL 2621 cells. The cells were grown in EMEM supplemented with 2% heat-inactivated FBS, 50 μg/mL Gentamicin (Sigma, St. Louis, MO), and 2.5 μg/mL Fungizone (Invitrogen, UK). The plates were incubated at 37°C with 4.5% CO₂ for 8 days. At the end of incubation, each well was examined for CPE and the percent positive in each group was calculated.

2.4.2 Viremia detection by real-time RT-PCR
Real-time RT-PCR was only performed on sera collected on day 69. The extraction of viral RNA was performed using the QIAamp Viral RNA Mini-Kit® (Qiagen, Valencia, CA) as described in the kit instructions. The real-time RT-PCR assay kit for PRRSV detection was purchased from Tetracore Inc. (Gaithersburg, MD) and used according to kit instructions.

2.5 Serology

Sera from all pigs were collected on day 0 and every week after vaccination until virus challenge (day 55). After challenge, sera were collected on day 56, 59, 62, and 69. Antibodies specific to PRRSV were measured by 2 serological assays; ELISA (HerdChek®, IDEXX Laboratories, Westbrook, ME) and serum virus neutralization (SVN) test. Results of ELISA were presented according to the manufacturer’s protocol as the sample to positive (s/p) ratio, where s/p ratio equal to and greater than 0.4 was considered positive. SVN test was performed on sera collected on day 0 and every other week. The test was performed as described by Yoon et al. (1995). PRRSV isolates MN-184 and SDSU-73 were used as viral antigens. SVN titer equal to or greater than 4 was considered positive (Christopher-Hennings et al., 2001).

2.6 Cell-mediated immunity

2.6.1 Isolation of PBMC

Whole blood of all animals was collected on day 0, 28, 42, 55, and 69 days post vaccination and transferred into a BD Vacutainer™ CPT™ with sodium citrate (Pharmingen, San Diego, CA), and centrifuged at 2,500xg at room temperature for 30 minutes. Two ml of
PBMC at the interface were collected into a 14 ml polypropylene centrifuge tube (BD, Franklin Lakes, NJ) and contaminating RBCs were lysed with 6 ml of buffered water for 90 seconds. The isotonicity was restored with 3 ml of 3x PBS. PBMC were centrifuged at 1,250 rpm at 4°C for 15 minutes and washed once with PBS. After final wash, PBMC were resuspended in 3 ml RPMI++ (RPMI-1640 with 25mM HEPES and L-glutamine (Life Technologies, Gaithersburg, MD), 10% heat-inactivated FBS, and 1% P/S). Cells were counted, using automatic cell counter (Beckman coulter, Inc., Fullerton, CA), and adjusted to 5 x 10^6 cells/ml in RPMI++.

2.6.2 PBMC culture and stimulation

PBMC from each pig were cultured in 2 identical sets in 96-well tissue culture flat-bottomed plates (BD Labware, Franklin Lakes, NJ). One set was for CD25 staining and the other was for intracellular IFNγ staining. In each set, 10^6 PBMC in 200 μl RPMI++ was added into 4 wells. Each well received 100 μl of either 10^2 TCID₅₀/ml of vaccine virus, RPMI++ (unstimulated control), mock antigen (mock control), or ConA (positive control) at final concentration of 1 μg/ml. Plates were incubated in a humidified 5% CO₂ atmosphere at 37°C for 5 days. Wells to be stained for intracellular IFNγ received 50 μl of GolgiStop solution (Pharmingen, San Diego, CA) diluted in RPMI++ at a final concentration recommended by the manufacturer at 12 hours before staining. PMA (Sigma, St.Louis, MO) and ionomycin (Sigma, St.Louis, MO) diluted in RPMI++ at final concentration of 7 ng/ml and 430 ng/ml, respectively, were added to positive control wells at the same time.

2.6.3 Antibodies
Primary mAbs included mouse anti-porcine CD4α mAbs (clone 74-12-4, isotype IgG2b), mouse anti-porcine CD8α mAbs (clone 76-2-11, isotype IgG2a), FITC-conjugated mouse anti-δ chain of porcine γδ TCR (clone PGBL22A, isotype IgG1) (VMRD, Pullman, WA), and R-phycoerythrin (R-PE)-conjugated mouse anti-porcine CD25 mAbs (clone PGBL25A, isotype IgG1) (VMRD, Pullman, WA). The former two mAbs were produced in our laboratory by hybridoma cells purchased from American Type Culture Collection (ATCC) (Manassas, VA). The latter two mAbs were custom-conjugated with FITC and R-PE, respectively (Chromaprobe, Maryland Heights, MO). Secondary antibodies comprised Alexa Fluor®647-conjugated goat anti-mouse IgG2a (γ2a) antibodies (Molecular Probes, Eugene, OR) and Tri-color®-conjugated goat anti-mouse IgG2b (γ) antibodies (Caltag Laboratories, Burlingame, CA). R-PE-conjugated mouse anti-porcine IFNγ mAbs (clone P2G10, isotype IgG1) (BD Pharmingen, San Diego, CA) were used for intracellular IFNγ staining.

2.6.4 Fluorescent antibody labeling

Fresh cell staining: Prior to PBMC culture, 1x10⁶ cells of freshly isolated PBMC from every pig were transferred to 96-well round-bottomed tissue culture plates (BD Labware, Franklin Lakes, NJ) and stained for CD4, CD8, γδ-TCR, and CD25 as described below.

Cultured cell staining: At the conclusion of the culture period, the culture plates were chilled on ice for 15 minutes. PBMC were mixed gently with a micropipette and transferred correspondingly to 96-well round-bottomed plates, centrifuged at 1,200 rpm and 4°C for 1 minute, and washed twice with cold PBS++ (PBS with 0.5% FBS, and 0.1% sodium azide
(Sigma, St.Louis, MO)). For surface CD25 staining, 50 µl of a mixture of four primary mAbs at their previously titrated optimum dilutions was added to all wells, and incubated in the dark at 4°C for 30 minutes. PBMC were then centrifuged at 1,200 rpm and 4°C for 1 minute. The supernatants were discarded and PBMC were washed three times with PBS⁺⁺, and incubated with 50 µl of a mixture of two secondary antibodies at their previously titrated optimum dilutions in the dark at 4°C for 30 minutes. PBMC were washed twice with PBS⁺⁺ and once with PBS then fixed with 150 µl of 1% ultrapure formaldehyde (Polyscience, Warrington, PA) in PBS.

For intracellular IFNγ staining, PBMC were stained in the same fashion as that for CD25 staining except the primary mAb mixture contained no anti-CD25 mAbs. After washing off secondary antibody conjugates, PBMC were stained for intracellular IFNγ. BD Cytofix/Cytoperm kit (Pharmingen, San Diego, CA) was used according to the manufacturer’s protocol. Briefly, PBMC were fixed with 100 µl BD Cytofix/Cytoperm solution for 20 minutes at 4°C, centrifuged at 1,200 rpm and 4°C for 1 minute, washed twice with BD Perm/Wash solution, incubated 30 minutes with 50 µl R-PE-conjugated mouse anti-porcine IFNγ mAbs diluted in BD Perm/Wash at previously titrated optimum dilution, washed three times with BD Perm/Wash, and fixed with 1% ultrapure formaldehyde in PBS.

Single-color control, secondary antibody control, unstained cells, isotype controls, and unlabeled antibody blocking controls for IFNγ staining, using unlabeled mouse anti-porcine IFNγ mAbs (clone P2G10, isotype IgGl) (BD Pharmingen, San Diego, CA), followed by R-PE-conjugated mouse anti-porcine IFNγ mAbs were performed. The fixed cell suspensions from both stainings were transferred to 5 ml polystyrene round-bottom tubes (BD, Franklin Lakes, NJ) and kept at 4°C until flow cytometric analysis.
2.6.5 Flow cytometry

The flow cytometric analysis was performed by the flow cytometry facility at Iowa State University. Four-color analysis was performed on an Epics Altra cytometer (Beckman Coulter, Fullerton, CA) equipped with both krypton (488 nm) and helium-neon laser (633 nm). The FITC, R-PE, and Tri-Color® dyes were excited by the same 488 nm krypton laser. Alexa Fluor®647 dye was excited by a spatially separated 633 nm helium-neon laser. A gated amplifier was used to differentiate Tri-Color® and Alexa Fluor®647 signals, which were detected by the same photomultiplier tube.

Analysis of flow cytometry data was performed, using FlowJo analysis software (Tree Star, Inc. San Carlos, CA). The analysis targeted five major porcine T-cell subsets; CD4⁺CD8⁺γδ⁺, CD4⁺CD8⁺γδ⁻, CD4⁺CD8⁺γδ⁺, CD4⁺CD8⁺γδ⁺, and CD4⁺CD8⁺γδ⁺, and a non T-cell subpopulation (CD4⁺CD8⁺γδ⁻). To analyze an individual data set, a dot plot of peak forward scatter versus linear forward scatter was plotted to gate out doublet events. A dot plot of linear side scatter versus linear forward scatter of single cells was applied to select live cell populations. Quadrant markers were set on CD4 versus CD8 T cells, generating 4 defined lymphocyte subsets containing CD4⁺CD8⁺, CD4⁺CD8⁻, CD4⁺CD8⁺, and CD4⁺CD8⁻. Histogram based on γδ fluorochrome (FITC) was applied to each subset. A histogram gate separated γδ⁺ and γδ⁻ populations and further identified 4 original T-cell subsets into 8 subsets. Two minor subsets, CD4⁺CD8⁺γδ⁺ and CD4⁺CD8⁺γδ⁺, each represented less than 0.5% of the whole live cell population (Table 2), were not further analyzed. The remaining 6 subsets were gated for their IFNγ⁺ or CD25⁺ percentage on a histogram based on IFNγ or CD25 fluorochrome (R-PE), respectively. The percentage of positive cells of each subset
and their geometric mean fluorescent intensities (MFIs) of the R-PE fluorochrome were collected from all samples.

2.6.6 Expression index (EI) of CD25 and percentage of IFNγ+ cells

The EI of CD25 was calculated as previously described by Sandbulte and Roth (2002). Briefly, products from the multiplication of %CD25+ and MFI of each subset were obtained. The EI derived from dividing the “product” of antigen-stimulated cells by the “product” of unstimulated cells of the same subset of the same animal. Results from IFNγ staining were presented as percentage of positive cells (%IFNγ+) of the same subset.

2.7 Immunity index calculation

An immunity index was calculated to determine if it would be a better predictive indicator for clinical protection from virulent virus challenge. It represents both HMI and CMI collectively. The HMI was represented by the s/p ratio obtained from ELISA test on day 55 (prior to virus challenge). The CMI was represented by the %IFNγ+ calculated for all PBMCs and for 5 individual T-cell subsets and a non T-cell subset averaged from day 42 and 55, which were time points when increased IFNγ responses were observed. The ELISA s/p ratios were multiplied by 10 to weigh their values to similar levels as the %IFNγ+ cells. The immunity index was calculated for each T cell subset for each animal using the following formula:

Immunity index = (s/p ratio x 10) + (%IFNγ+ cells by individual T cell subset)

2.8 Necropsy and lung lesion scoring
Necropsy was performed on day 69. Pigs were euthanized by electric shock and lung lesions were determined as described by Halbur et al (1996). The total score of apical, cardiac, and diaphragmatic lobes on each side were 10%, 10%, and 25%, respectively and the intermediate lobe was 10%.

2.9 Statistical analysis

The CD25EI and %IFNγ+ data for each PBMC subset from day 42 and 55 post vaccination were tested for statistical difference by two-way analysis of variance (ANOVA), followed by Dunnett's test using mean of group 1 and group 9 as control groups. Means of CD25EI and %IFNγ+ shown in the figures were averaged from data of day 42 and 55 post vaccination. Lung lesion scores of every group were tested for statistical difference by one-way ANOVA, followed by Dunnett's test using mean of group 1 and group 8 as control groups. The percentage of virus-positive pigs, number of PRRSV copies, and immunity index were tested for statistical significance in the same way as lung lesion scores except mean of group 1 and group 9 were used as controls. The percentage of virus-positive pigs of each group was averaged from data collected on day 59, 62, and 69. The negative and positive results were defined to be 0 and 1, respectively. Pigs that were positive for virus isolation for 3, 2, and 1 time points were defined to be 100%, 66.7%, and 33.3% positive, respectively. The number of PRRSV copies was transformed to log_{10} prior to analysis and value of 0 was defined to be 1 prior to log_{10} transformation. The SVN titers were transformed to log_{2} to calculate the log mean titer of each group. Anti-log transformation was applied to obtain the final mean titer of each group. The CD25EI and %IFNγ+ data averaged from day 42 and 55 post vaccination were used for correlation analyses with lung
lesion scores and number of PRRSV copies. P<0.05 was set as statistically significant level throughout this study.

3. Results

3.1 Sensitivity of four-color flow cytometry

Six major subsets of PBMC, consisting of 3 subsets of αβ T cells (CD4⁺CD8⁻γδ⁻, CD4⁺CD8⁺γδ⁻, CD4⁺CD8⁺γδ⁺), 2 subsets of γδ T cells (CD4⁺CD8⁻γδ⁺, CD4⁺CD8⁻γδ⁻), and 1 subset that did not express any of the 3 surface markers, denoted CD4⁺CD8⁻γδ⁻, were detected by four-color flow cytometry. The percentage of each PBMC subset was calculated from non-vaccinated pigs (n=20) on day 42 and 55 of the experiment. The CD4⁺CD8⁻γδ⁻ and CD4⁺CD8⁺γδ⁻ subsets made up less than 0.5% of the PBMCs, so no further analysis was performed on those subsets. The percentage of other subsets was comparable to previously reported values for the same age group (Stabel et al., 2000). The percentage of CD4⁺CD8⁻γδ⁻ subset decreased after 5 days of incubation with or without antigen. This may have resulted from the adherence of monocytes/macrophages (CD4⁺CD8⁻γδ⁻) to the culture plate. The percentage of the remaining γδ⁻ subsets did not perceptibly change after culture whereas the CD4⁺CD8⁺γδ⁺ and CD4⁺CD8⁻γδ⁺ had a marked increase (Table 2).
Table 2. PBMC were collected on day 42 and 55 post vaccination from non-vaccinated groups (group 8 and 9), stained immediately or after 5-day culture with culture media or vaccine virus with mAbs directed to surface T-cell subset markers, followed by flow cytometric analysis. Shown are mean percent ± SEM of PBMC subsets before and after 5-day culture of total live cells (n=20).

<table>
<thead>
<tr>
<th></th>
<th>Day 0</th>
<th>Day 5 (media)</th>
<th>Day 5 (Ag-stimulated)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>αβ T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD8⁻γδ⁺</td>
<td>10.1 ± 1.0</td>
<td>9.9 ± 0.9</td>
<td>8.4 ± 0.8</td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁻</td>
<td>4.5 ± 0.3</td>
<td>5.7 ± 0.5</td>
<td>6.1 ± 0.7</td>
</tr>
<tr>
<td>CD4⁻CD8⁺γδ⁻</td>
<td>15.2 ± 0.9</td>
<td>15.0 ± 0.7</td>
<td>14.7 ± 1.2</td>
</tr>
<tr>
<td><strong>γδ T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD8⁻γδ⁺⁺</td>
<td>0.2 ± 0.0</td>
<td>0.4 ± 0.0</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁺⁺</td>
<td>2.2 ± 0.2</td>
<td>8.8 ± 0.8</td>
<td>8.8 ± 0.6</td>
</tr>
<tr>
<td>CD4⁺CD8⁻γδ⁺⁺</td>
<td>16.5 ± 1.0</td>
<td>26.1 ± 1.6</td>
<td>29.8 ± 1.9</td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁺⁺</td>
<td>0.2 ± 0.0</td>
<td>0.3 ± 0.0</td>
<td>0.3 ± 0.0</td>
</tr>
<tr>
<td><strong>Non-T cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD8⁻γδ⁻</td>
<td>51.3 ± 1.2</td>
<td>33.9 ± 2.3</td>
<td>31.5 ± 1.8</td>
</tr>
</tbody>
</table>
3.2 Antibody responses

All pigs were seronegative to PRRSV by ELISA test on the vaccination day (day 0) and remained seronegative until day 14 post vaccination when some MLV-vaccinated pigs became seropositive (Fig. 1). By day 28, all MLV-vaccinated pigs (group 1 to 6) were seropositive and remained seropositive throughout the study with no significant difference among mean s/p ratio (p>0.05). All ORF5 peptide/CT pigs (group 7) remained seronegative until two weeks after the virus challenge (day 55). Challenge control pigs (group 8) became seropositive on day 69, while strict negative control pigs (group 9) remained seronegative throughout the study. No significant difference among mean s/p ratios from virus-challenged groups (groups 1 to 8) was found on day 69 (p>0.05).

The SVN test was performed in parallel with the ELISA test, using sera collected on day 0 and every other week until day 69. The neutralizing activities of the sera were tested with 2 field strain viruses, MN-184 and SDSU-73. Prior to virus challenge, there were no detectable neutralizing antibodies to either strain of virus in any group (data not shown). After challenge with strain MN-184 on day 55, neutralizing antibodies to the homologous strain were detected on day 69 in all MLV-vaccinated groups and one pig each from ORF5 peptide/CT group and challenge control group. The antibody titers ranged from 4 to 32 and the percentage of pigs showing neutralizing antibodies ranged from 40% in MLV-vaccinated pigs with IFNa (group 4) to 100% in MLV vaccine alone (groups 1), MLV-vaccinated pigs with ET (group 2), and MLV-vaccinated pigs with mixed ORF5 peptides (group 3). Cross-neutralizing antibodies to heterologous strain SDSU-73 were detected only in MLV-vaccinated groups (group 1 to 6) on day 69 with
Figure 1. The antibody response of all treatment groups tested over time by IDEXX ELISA. The positive responses were determined at s/p ratio of 0.4 or higher.
the antibody titers ranging from 4 to 32. The percentage of pigs showing cross-neutralizing antibodies ranged from 20% in group 4 to 100% in groups 1 and 2. Strict negative control pigs (group 9) did not show neutralizing antibodies to either strain of virus throughout the study.

3.3 IFNγ responses

The expression of IFNγ was determined in PBMC cultured with live vaccine strain of PRRSV for 5 days and measured by four-color flow cytometry. In all live cells, the background IFNγ expression in unstimulated and mock control cells was less than 5% throughout the study. Low levels of IFNγ responses (<1% IFNγ⁺ cells after background subtraction) were detected in all groups on day 28 post vaccination and no significant differences among the 9 groups were observed (data not shown). Increased IFNγ expression was seen on day 42 and 55 post vaccination. MLV-vaccinated groups had significantly higher %IFNγ⁺ cells than ORF5 peptide/CT and control groups (Fig. 2). Pigs vaccinated with MLV vaccine and IL-12 had the highest %IFNγ⁺ cells, followed by pigs vaccinated with MLV vaccine and poly-ICLC or mixed ORF5 peptides, respectively. ORF5 peptide/CT-administered and control pigs had low %IFNγ⁺ cells. The MFIs of IFNγ of MLV-vaccinated groups were also significantly higher than that of ORF5 peptide/CT and control groups (Fig. 2).

The %IFNγ⁺ cells of each T-cell subset were compared to the same subset of the strict control group (group 9) (Fig. 3). CD4⁺CD8⁺γδ⁻ subset was the only T-cell subset of group 1 (MLV vaccine alone) that showed significantly higher %IFNγ⁺ cells than that of group 9. T-cell subsets in pigs vaccinated with MLV vaccine and mixed ORF5 peptides
Figure 2. IFNγ response of all live cells. PBMCs were incubated with culture media (unstimulated control), supernatant from uninfected MARC-145 cells (mock control), ConA plus PMA and ionomycin (positive control), and vaccine strain of PRRSV (antigen-stimulated). Shown are mean ± SEM of %IFNγ+ cells and MFI of IFNγ+ cells averaged from day 42 and 55 post vaccination. The letters a and b indicate groups that were significantly different from group 9 and group 1, respectively (p<0.05). See Table 1 for treatment group designation.
Figure 3. IFNγ response by T-cell subsets. PBMCs were stained for 3 major porcine T-cell markers (CD4, CD8, and γδ TCR) and intracellular IFNγ after in vitro restimulation with vaccine virus. Shown are mean ± SEM of %IFNγ+ cells averaged from day 42 and 55 post vaccination. The letters a, b, and c indicate groups that were significantly different from group 9, group 1, and both group 9 and 1, respectively (p<0.05). See Table 1 for treatment group designation.
and all but the CD4⁺CD8⁻γδ⁺ T-cell subset for the MLV vaccine and IL-12-treated pigs had significantly higher %IFNγ⁺ cells than that of control group. Pigs vaccinated with MLV vaccine and ET had significantly higher %IFNγ⁺ cells than group 9 in CD4⁺CD8⁻γδ⁻ and CD4⁺CD8⁺γδ⁺ subsets, while pigs vaccinated with MLV vaccine and IFNα had significantly higher %IFNγ⁺ cells than group 9 only in CD4⁺CD8⁻γδ⁻ subset. The non-T cells (CD4⁺CD8⁻ γδ⁻) had higher %IFNγ⁺ cells compared to group 9 for all MLV-vaccinated groups except MLV vaccine with IFNα group.

To test further for the effect of vaccine adjuvants in contributing to an increase in IFNγ response, the %IFNγ⁺ cells of all groups were compared to group 1 (MLV vaccine alone). Only MLV vaccine plus mixed ORF5 peptides or IL-12 showed significantly higher %IFNγ⁺ cells than MLV vaccine alone. The increased responses were seen in CD4⁺CD8⁺γδ⁺ and CD4⁺CD8⁻γδ⁺ T-cell subsets of MLV vaccine plus mixed ORF5 peptides (group 3), and CD4⁺CD8⁺γδ⁻ and CD4⁺CD8⁺γδ⁺ T-cell subsets of MLV vaccine plus IL-12 (group 6).

3.4 CD25 responses

Fresh PBMCs had very low %CD25⁺ cells (<1%) for all treatment groups throughout the study (data not shown). PBMCs cultured for 5 days with live PRRSV had higher %CD25⁺ cells than fresh PBMCs, but the high %CD25⁺ cells were also seen in unstimulated cells and mock control. The high background of %CD25⁺ cells was seen consistently in every time point. To represent the actual antigen-specific CD25 responses, we employed the CD25EI as described by Sandbulte and Roth (2002). CD25EI of all groups were close to 1 at every time point, suggesting that no antigen-specific CD25 response occurred (data not shown).
CD25EI of T-cell subsets are illustrated in Fig. 4. The CD25EI of every group in each subset was close to 1, and thus there was no significant difference of mean CD25EI among 9 groups in most T-cell subsets. CD4⁺CD8⁺γδ⁺ and non-T cells (CD4⁺CD8⁻γδ⁻) were the only subsets that showed mean CD25EI difference. The significant difference was seen in MLV-vaccinated group receiving IFNa, where mean CD25EI was higher than that of both group 1 (MLV vaccine alone) and group 9 (strict negative control). CD25EI of CD4⁻CD8⁺γδ⁺ T-cell subset of MLV-vaccinated group receiving IL-12 was significantly higher than corresponding value in group 9. The low CD25 response in T cells stimulated with recall antigens raised the question of whether T cells expressing IFNγ co-expressed surface CD25. To test this, a group-independent correlation analysis between %IFNγ⁺ and CD25EI of every subset from individual pigs (n=88) was performed. The results consistently showed no significant correlation between IFNγ expression and CD25EI in any subset of T cells except CD4⁺CD8⁺γδ⁻ subset (with p=0.05) (Table 3).

3.5 Lung lesions and viremia

After virus challenge, the challenge control pigs (group 8) developed severe lung lesions with a mean 35% lung damage (Fig. 5a). The ORF5 peptide/CT pigs (group 7) developed a comparable percent of lung damage as challenge control pigs, while MLV-vaccinated pigs (groups 1 to 6) had reduced mean % lung lesions. However, the only significant reduction of lung lesions was seen in MLV-vaccinated pigs receiving ET or
Figure 4. CD25 response by T-cell subsets. PBMCs were stained for 3 major porcine T-cell markers (CD4, CD8, and γδ TCR) and surface CD25 after in vitro restimulation with vaccine virus. Shown are mean ± SEM of CD25EI averaged from day 42 and 55 post vaccination. The letters a, b, and c indicate groups that were significantly different from group 9, group 1, and both group 9 and 1, respectively (p<0.05). See Table 1 for treatment group designation.
Table 3. Correlation coefficients obtained from group-independent correlation analysis between %IFNγ+ cells collected from day 42 and 55 post vaccination and CD25EI obtained from the same days (n=88), lung lesion scores (n=76), or viremia (n=76). The same analysis was done between CD25EI and lung lesion scores (n=76), or viremia (n=76).

<table>
<thead>
<tr>
<th>%IFNγ+</th>
<th>CD25EI</th>
<th>Lung lesion scores</th>
<th>Viremia</th>
</tr>
</thead>
<tbody>
<tr>
<td>All live cells</td>
<td>-0.12</td>
<td>-0.34**</td>
<td>-0.54**</td>
</tr>
<tr>
<td>αβ T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+CD8-γδ+</td>
<td>0.23</td>
<td>-0.16</td>
<td>-0.28*</td>
</tr>
<tr>
<td>CD4+CD8-γδ-</td>
<td>0.25*</td>
<td>-0.22*</td>
<td>-0.25*</td>
</tr>
<tr>
<td>CD4+CD8+γδ-</td>
<td>-0.10</td>
<td>-0.45**</td>
<td>-0.46**</td>
</tr>
<tr>
<td>γδ T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+CD8-γδ+</td>
<td>-0.01</td>
<td>-0.25*</td>
<td>-0.27*</td>
</tr>
<tr>
<td>CD4+CD8-γδ+</td>
<td>-0.16</td>
<td>-0.07</td>
<td>-0.13</td>
</tr>
<tr>
<td>Non-T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4+CD8+γδ-</td>
<td>-0.05</td>
<td>-0.44**</td>
<td>-0.46**</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CD25EI</th>
<th>All live cells</th>
<th>αβ T cells</th>
<th>γδ T cells</th>
<th>Non-T cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CD4+CD8-γδ+</td>
<td>CD4+CD8-γδ-</td>
<td>CD4+CD8+γδ-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD4+CD8-γδ+</td>
<td>CD4+CD8-γδ+</td>
<td>CD4+CD8-γδ+</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.001</td>
</tr>
</tbody>
</table>

* p<0.05, ** p<0.01
IL-12 when compared to the challenge control pigs. The reduced lung lesions of both ET and IL-12 groups were not significantly different from that of MLV vaccine alone (group 1) (p>0.05).

In addition to lung lesions, the presence and level of virus in serum were monitored and quantified. Viremia was detected in every group at 4 days after challenge and persisted for 7 days in MLV-vaccinated groups, and 14 days in ORF5 peptide/CT and challenge control groups (data not shown). MLV-vaccinated pigs had significantly reduced percentage of viremia (Fig. 5b) and number of virus copies (except MLV vaccine with mixed ORF5 peptides) (Fig. 5c) than ORF5 peptide/CT and challenge control pigs. MLV-vaccinated pigs receiving poly-ICLC had the lowest percentage of viremia, while MLV vaccine alone or with IL-12 pigs had the lowest number of virus copies. However, none of these were significantly different (P >0.05) from MLV vaccine alone.

3.6 Correlation of immune responses with lung lesions and viremia

A group-independent correlation analysis between %IFNγ+ or CD25EI and lung lesion scores or virus copies was performed (Table 3). Robust inverse correlations between %IFNγ+ cells and lung lesion scores or number of virus copies were detected. Further analysis by subsets between %IFNγ+ and the two parameters revealed that the correlations between %IFNγ+ and lung lesion scores were robust in CD4+CD8γδ- and CD4+CD8γδ+ subsets, and to a lesser extent, CD4+CD8γδ+ and CD4+CD8γδ+ subsets. Inverse correlation between %IFNγ+ cells and the number of virus copies was seen in all subsets except CD4+CD8γδ+. The strongest correlations were also found in CD4+CD8γδ- and CD4+CD8γδ- subsets, and to a lesser extent, CD4+CD8γδ-, CD4+CD8γδ+, and CD4+CD8γδ+, respectively.
The expression of CD25 did not correlate with the reduction of lung lesion scores or viremia (Table 3). Analysis by subsets also revealed that T cells expressing CD25 did not significantly correlate with either parameter.

A similar correlation analysis between ELISA s/p ratio on day 55 and lung lesion scores or number of virus copies (n=76) was performed. A strong inverse correlation between s/p ratio and the two parameters was found (p<0.01) (Table 4).

3.7 Immunity index and protection

Both CMI (represented by %IFNγ⁺) and HMI (represented by ELISA s/p ratio) were significantly correlated with the reduction of lung lesion scores and number of virus copies. However, there were pigs with very low s/p ratios and pigs with very low %IFNγ⁺ that were protected from challenge based on lung scores or viremia (Fig. 7). This raised the possibility that the pigs with low s/p ratios may have been protected by a high %IFNγ⁺ response and that pigs with a low %IFNγ⁺ response may have been protected by a high antibody response. To test this possibility, we calculated an immunity index which approximately equally weighted the HMI and CMI responses. To collectively summarize the protection provided by CMI and HMI, we combined the two aspects of immune response and presented the result as immunity index. The immunity index of all MLV-vaccinated groups was significantly higher than that of ORF5 peptide/CT and control groups in every T-cell subset (Fig. 6). A significant inverse correlation between immunity index and lung lesion scores or number of virus copies was
Table 4. Correlation coefficients obtained from group-independent correlation analysis between ELISA s/p ratio from day 55 post vaccination or immunity index and lung lesion scores or viremia (n=76).

<table>
<thead>
<tr>
<th></th>
<th>Lung lesion scores</th>
<th>Viremia</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ELISA s/p ratio</strong></td>
<td>-0.50**</td>
<td>-0.42**</td>
</tr>
</tbody>
</table>

**Immunity index**

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All live cells</strong></td>
<td>-0.51**</td>
<td>-0.53**</td>
</tr>
<tr>
<td>αβ T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁺</td>
<td>-0.47**</td>
<td>-0.45**</td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁻</td>
<td>-0.50**</td>
<td>-0.46**</td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁺</td>
<td>-0.51**</td>
<td>-0.54**</td>
</tr>
<tr>
<td>γδ T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁺</td>
<td>-0.50**</td>
<td>-0.46**</td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁺</td>
<td>-0.43**</td>
<td>-0.42**</td>
</tr>
<tr>
<td>Non-T cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD4⁺CD8⁺γδ⁻</td>
<td>-0.52**</td>
<td>-0.54**</td>
</tr>
</tbody>
</table>

** p<0.01
Figure 6. Mean ± SEM of immunity index by subsets. Immunity index was derived from the summation of HMI (represented by ELISA s/p ratio on day 55 multiplied by 10) and CMI responses (represented by %IFNγ+ T cells by subsets averaged from day 42 and 55). The letters a and b indicate groups that were significantly different from group 9 and group 1, respectively (p<0.05). See Table 1 for treatment group designation.
observed (Table 4). The immunity index did not correlate any more strongly with protection than either the s/p ratio or the %IFNγ+ response (Tables 3 and 4). There were individual pigs with a low immunity index (ie. no measurable antibody or IFNγ production) that were still protected from challenge based on lung lesion score (Fig. 7). However, all pigs with an immunity index below 15 were viremic 14 days after challenge (Fig. 7).

3.8 Correlation of lung lesion scores with level of viremia

A positive correlation existed between lung lesion scores and levels of viremia 14 days after challenge (Fig. 8). However, many individual pigs had high levels of viremia and essentially no lung lesions while other pigs had high lung lesion scores with no detectable viremia 14 days after challenge (Fig. 8).

4. Discussion

Several vaccine adjuvants for the MLV PRRSV vaccine and a vaccine antigen ORF5 peptide/CT were studied for their potential to increase HMI and CMI responses as well as to protect pigs from virulent PRRSV challenge. CT has been reported to induce neutralizing (anti-GP5) antibodies after MLV PRRSV vaccination (Foss et al., 2002). Its adjuvant effect was further tested in this study with ORF5 peptide antigens.

After vaccination, antibody responses detected by IDEXX ELISA were seen in all MLV-vaccinated pigs by day 28 post vaccination but were not detected in ORF5 peptide/CT pigs until 2 weeks after challenge (day 69). Previous studies reported that
Figure 8. Scatter plot and group-independent correlation analysis between lung lesion scores and log_{10} viremia at day 14 after challenge. Correlation coefficient ($r$) represents a positive correlation between lung lesion scores and log_{10} viremia ($p<0.01$).
IDEXX ELISA preferentially captures antibodies specific to the nucleocapsid protein of the virus which is encoded by ORF7 (Yoon et al., 1995; Kwang et al., 1999; Foss et al., 2002). Therefore, the pigs administered ORF5 peptide/CT may have developed anti-GP5 antibodies but the antibodies were not detected by IDEXX ELISA. The positive ELISA responses of ORF5 peptide/CT group post challenge were likely induced by the challenge virus rather than the ORF5 peptides themselves.

Pigs from all groups failed to develop neutralizing antibodies prior to virus challenge. After challenge, however, only pigs vaccinated with MLV (with or without other adjuvants) developed neutralizing antibodies, whereas ORF5 peptide/CT pigs did not. This suggests that MLV vaccination contributed to rapid induction of neutralizing antibody to the challenge virus. The percentage of pigs showing neutralizing antibodies to MN-184 and SDSU-73 were highest in pigs vaccinated with MLV vaccine alone or combined with ET, suggesting that vaccine adjuvants did not contribute to enhancing the production of neutralizing antibodies. The lack of neutralizing antibody production in ORF5 peptide/CT group correlated with the high lung lesion scores and high viremia observed in pigs in this group. The magnitude of both clinical parameters were similar to those for the challenge control pigs. This suggests that administration with ORF5 peptide/CT did not induce protective immunity against challenge virus. The absence of neutralizing antibody production may be due to the lack of post translational modification of chemically-synthesized peptides. Correct glycosylation and post-translational modification of the GP5 may be required for successful induction of neutralizing antibodies (Pirzadeh and Dea, 1998). The activity of CT after chemical conjugation with ORF5 peptides was not determined. A loss of CT activity could contribute to poor neutralizing antibody response to ORF5 peptides. Another possible
explanation may be that the ORF5 peptides used in this study were derived from virus isolates that were heterologous to the challenge virus (MN-184), therefore, antibodies induced by those peptides may inefficiently protect against the heterologous virus challenge.

Antibodies detected by IDEXX ELISA were previously reported to not have neutralizing activity (Yoon et al., 1995; Foss et al., 2002). Nonetheless, the ELISA s/p ratio prior to virus challenge showed a strong inverse correlation with lung lesions and level of viremia. Since neutralizing antibodies were not detected prior to virus challenge, antibodies to PRRSV detected by IDEXX ELISA may play a protective role other than virus neutralization. This may be through complement activation, opsonization for phagocytosis, or antibody-dependent cell-mediated cytotoxicity. Neutralizing antibody responses in MLV-vaccinated pigs which developed after virus challenge may also have contributed to protection.

CMI responses were evaluated by up regulation of surface expression of CD25 and by intracellular expression of IFNγ by T cells in response to PRRSV recall antigen. Significant up regulation of surface expression of CD25 was rarely seen in T-cell subsets after stimulation with recall antigens *in vitro*. CD4⁺CD8⁺γδ⁺ T cells and non-T cells (CD4⁻CD8⁺γδ⁻) of the MLV plus IFNα group were the only subsets that showed a significant difference from the non-vaccinated control group (Fig. 4). In addition, up regulation of CD25 did not significantly correlate with either lung lesion score or level of viremia (Table 3). While the up regulation of CD25 expression was minor, the intracellular expression of IFNγ was significantly (p<0.05) increased in response to recall antigen in all T-cell subsets for at least some of the vaccinated groups as compared to the non-vaccinated group (Fig. 3). In addition, increased expression of IFNγ was significantly (p<0.05) inversely correlated
with lung lesion score and level of viremia. This finding indicates a lack of correlation between CD25 and IFNγ expression in pigs in response to PRRSV recall antigen. CD25 expression is an early event of T-cell proliferation and is used to detect activated porcine T cells (Saalmuller et al., 1994). CD25 should have been expressed prior to the expression of IFNγ. Similar results were observed in a pseudorabies virus model in which IFNγ production did not correlate with lymphocyte proliferation (Zuckermann et al., 1998). The low level of CD25 up regulation in response to PRRSV recall antigen may contribute to a weak CMI response to PRRSV.

Increased IFNγ responses were detected on day 42 and 55 post vaccination in all MLV-vaccinated groups for at least some lymphocyte subsets. This is later after vaccination than a previous study by Foss et al (2002), using an ELISPOT assay which detected IFNγ production as early as 14 days post MLV PRRSV vaccination. The %IFNγ+ cells detected in the present study were higher than that observed by ELISPOT assay at 42 days post vaccination. The different results may be explained by differences in the assays used, the recall antigens used, and/or the duration of incubation periods. In this study, live vaccine viruses were used at low titers to stimulate antigen-specific T cells and the incubation period was five days, while in the ELISPOT assay, heat-inactivated viruses were used at high titers and the incubation period was much shorter (usually less than 24 hours). During the longer incubation period, cytokines produced by antigen-specific T cells may induce proliferation of antigen-specific T cells and IFNγ secretion in bystander cells (as detected in CD4+CD8-γδ- subset). IFNγ secreted by proliferating T cell and bystander cell populations may contribute to higher %IFNγ+ cells.
A significant IFNγ response to recall vaccine antigens was detected in the CD4+CD8+γδ+ T cell subset of pigs which were vaccinated with MLV vaccine alone, suggesting that MLV vaccine had primed this subset (Fig. 3). CD4+CD8+γδ+ T cells are classified as cytotoxic T cells in pigs (Pauly et al., 1995; Yang and Parkhouse, 1997). IFNγ expression by these cells significantly correlates with the reduction of lung lesions and level of viremia (Table 3).

Porcine IL-12 (group 6) and mixed ORF5 peptides (group 3) when used as adjuvants with the MLV vaccine significantly increased the IFNγ response to recall antigen as compared to MLV vaccine alone (Fig. 3). Booster with mixed ORF5 peptides significantly increased IFNγ response in CD4+CD8+γδ+ and CD4+CD8+γδ+ subsets, whereas co-administration of MLV vaccine with IL-12 significantly increased IFNγ response in CD4+CD8+γδ+ and CD4+CD8+γδ+ subsets. CD4+CD8+γδ+ and CD4+CD8+γδ+ subsets are classified as non MHC-restricted, with natural killer activity in the CD4+CD8+γδ+ subset (Pauly et al., 1996; Yang and Parkhouse., 1996). The CD4+CD8+γδ− cells are characterized as memory Th cells with MHC class II restriction (Summerfield et al., 1996; Zuckermann and Husmann, 1996; Saalmuller et al., 2002). IFNγ expressed by these subsets correlates significantly with clinical protection. The mechanism of enhancement of the IFNγ recall response by these subsets by mixed ORF5 peptides and porcine IL-12 is not clearly understood. The ORF5 peptides have previously been shown to enhance HMI rather than CMI (Pirzadeh and Dea, 1997). IL-12, on the other hand, was shown to induce IFNγ expression when used as an adjuvant for an inactivated pseudorabies virus vaccine (review in Zuckermann et al., 1999) or the MLV PRRSV vaccine (Foss et al., 2002). However, the responsive T-cell subsets were not determined in those studies. The dosage of IL-12 used in this study was 4 μg (single
dose on day 1 post vaccination), while a previous study by Foss et al (2002) administered a total of 60 μg on day 2, 4, and 7 after vaccination. The results reported here indicate that a lower dose of IL-12 was sufficiently potent to induce an increased IFNγ response to MLV PRRSV vaccine. Although mixed ORF5 peptides and porcine IL-12 when used as adjuvants significantly enhanced IFNγ responses to MLV vaccine, there was no statistically significant corresponding improvement in either lung lesion scores or level of viremia (Fig. 5).

ELISA s/p ratio, IFNγ production by CD4⁺CD8⁺γδ⁻ T cells and IFNγ production by non-T cells (Tables 3 and 4) strongly correlated with protection from viremia and development of lung lesion scores. The correlation coefficient was less than -0.40 in each case and was highly significant (P<0.01) (Tables 3 and 4). This implies that both the antibody response and production of IFNγ contribute to protection. In order to determine if the combination of ELISA s/p ratio and IFNγ production by lymphocyte subsets might be a better predictor of resistance to challenge we combined these two parameters into an immunity index which approximately equally weighted the ELISA s/p ratio and IFNγ production by each lymphocyte subset. The immunity index for each cell subset for each pig was then analyzed for correlation with viremia and lung lesion scores. The immunity index did not correlate any more strongly with protection from viremia or lung lesion scores than did the ELISA s/p ratio or IFNγ production alone. Several pigs with low immunity index also had low lung lesion scores, however, all pigs with immunity index < 15 had relatively high viremia (Fig. 7).

In conclusion, pigs receiving MLV vaccine with or without vaccine adjuvants had higher ELISA antibody and IFNγ recall responses prior to virus challenge and lower lung lesion scores and levels of viremia after challenge than non-vaccinated pigs. Both ELISA s/p
ratio and IFNγ expression prior to virus challenge significantly correlated with reduced lung lesion scores and levels of viremia. This suggests that both immune parameters can be used to predict clinical protection.

Acknowledgements

This research was partially supported by Boehringer Ingelheim Vetmedica, Inc. The authors thank Dr. Kenneth B. Platt for mAbs (clone 15E) and Thomas Skadow for technical assistance.

References


Chapter 4. Effects of porcine reproductive and respiratory syndrome virus virulence and antigen-presenting cells on T cell activation and antiviral cytokine production

A paper submitted to Viral Immunology
Wasin Charerntantanakul, Ratree Platt, James A. Roth

ABSTRACT

The ability of porcine reproductive and respiratory syndrome virus (PRRSV) to suppress T cell expression of CD25 (alpha chain of interleukin (IL)-2 receptor), interferon gamma (IFNγ), and tumor necrosis factor alpha (TNFα) was determined by flow cytometry in naïve porcine T cells in response to mitogen (concanavalin A) and cytokine inducers (phorbol 12-myristate 13-acetate plus ionomycin (PMA/I)). Four PRRSV isolates of varying clinical virulence and three different types of porcine myeloid antigen-presenting cells (APC) were used. T cells cultured with monocytes infected with virulent PRRSV (VR-2385, SDSU-73, VR-2332), but not with vaccine strain (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica), demonstrated significantly reduced %CD25⁺ and %IFNγ⁺ when compared to T cells incubated with uninoculated monocyte cultures (p<0.05). T cells cultured with monocytes infected with all four PRRSV isolates demonstrated significantly reduced %TNFα⁺ (p<0.05). The significant reduction of %CD25⁺, %IFNγ⁺, and %TNFα⁺ was not detected in T cells cultured with monocyte-derived macrophages (MDM) and
immature monocyte-derived dendritic cells (MDC) infected with any PRRSV isolates. Heat-inactivated PRRSV did not induce significantly reduced T cell responses in any APC cultures. The reduction of T cell response in monocyte cultures was not due to PRRSV-induced T cell death. Gene expression of IL-10 detected by semi-quantitative reverse transcriptase PCR was significantly increased in virulent PRRSV-infected monocyte cultures after PMA/I, but not con A stimulation, compared to IL-10 gene expression from uninoculated monocyte cultures (p<0.05). Increased IL-10 gene expression contributed to significantly reduced %IFNγ+ and %TNFα+, but not %CD25+, as determined by an IL-10 neutralization assay. This study reports that PRRSV has an ability to suppress T cell response. The suppressive ability of PRRSV associates with virus virulence and is mediated by virus-infected monocytes, but not by virus-infected MDM and immature MDC.

INTRODUCTION

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-sense, single-stranded enveloped RNA virus of the family Arteriviridae (36). The virus causes respiratory disease in pigs of all ages and reproductive losses in breeding age swine (36).

Several lines of evidence suggest that PRRSV may possess immunosuppressive properties. Field investigations revealed that pigs infected with PRRSV had increased incidence of secondary infections with Streptococcus suis, Hemophilus parasuis, Mycoplasma hyopneumoniae, Actinobacillus pleuropneumoniae, Salmonella spp., and swine influenza virus, compared to PRRSV-free pigs (15,48,70). Pigs infected with PRRSV and S. suis developed more severe clinical disease and pathological lesion caused by S. suis than
pigs infected with *S. suis* alone (63). PRRSV infects monocytes, macrophages, and dendritic cells which are antigen-presenting cells (APC) of pigs (10,20,22,38,60,62,63,66). Macrophages infected with PRRSV demonstrate reduced phagocytic ability, microbial killing, production of reactive oxygen species ie. superoxide anion (O$_2^-$) and hydrogen peroxide (H$_2$O$_2$), and production of antiviral cytokines ie. interferon alpha (IFNa) and tumor-necrosis factor alpha (TNFa) compared to uninfected macrophages (2,12,30,62). PRRSV-infected macrophages as well as uninfected bystander lymphocytes may be induced to undergo apoptosis (53,57). Humoral and cell-mediated immune (CMI) responses to PRRSV are poor, compared to those to other swine viral pathogens eg. pseudorabies virus and swine influenza virus (61). Specific antibodies to PRRSV detected by IDEXX ELISA appeared around 2 weeks after infection (17,34,69). Neutralizing antibodies to PRRSV appeared around 4 weeks after infection (69). T cell proliferation and IFNy secretion by peripheral blood mononuclear cells (PBMC) in recall response to PRRSV appeared around 3-8 weeks after infection (5,8,11,17,29,33,34,50). PRRSV-primed T cells did not up-regulate CD25 (alpha chain of interleukin (IL)-2 receptor) expression after re-stimulation in vitro with recall PRRSV (11). CD25 expression is reportedly up-regulated in activated porcine T cells after stimulation with either recall antigen or mitogen (23,52,56).

The objective of this study was to determine the ability of PRRSV to suppress T cell response. Expression of CD25, IFNy, and TNFα by naïve T cells were evaluated after T cells were cultured with PRRSV-infected monocyte, monocyte-derived macrophages (MDM), and immature monocyte-derived dendritic cells (MDC) and stimulated with concanavalin A (con A) for CD25 up-regulation and phorbol 12-myristate 13-acetate plus ionomycin (PMA/I) for IFNy and TNFα up-regulation. PRRSV of varying clinical virulence were used. The effect
of PRRSV virulence as well as PRRSV-infected APC on T cells has not been determined. The effects of PRRSV virulence have been demonstrated in the disease severity and humoral immune response (19-21,26,65). Virulent PRRSV induces more severe respiratory disease and reproductive failure, poorer growth performance, and higher mortality rate than low virulent virus (19-21,26,65). Antibody response to virulent PRRSV appears more rapidly and has higher ELISA s/p ratio than antibody response to low virulent PRRSV (26,65).

This study reported that PRRSV has the ability to suppress T cell response which is determined by significantly reduced CD25, IFNγ, and TNFα expression in response to con A and PMA/I. The suppressive effect associates with virus virulence and is mediated by monocytes, but not by MDM and immature MDC.

**MATERIALS AND METHODS**

*Animals and viruses.* Three 10-week old PRRSV-seronegative pigs were used. The pigs were housed at the laboratory animal facility, College of Veterinary Medicine, Iowa State University (ISU). The animal experiment was approved by the ISU animal care and use committee.

Four PRRSV isolates with known clinical virulence were used (42,47). The high virulent strains, American Type Culture Collection (ATCC) PRRSV isolate VR-2385 (Dr. Patrick Halbur, ISU) and isolate SDSU-73 (Dr. Michael Roof, Boehringer Ingelheim Vetmedica, Inc. (BIVI), Ames, IA), were recovered from bronchoalveolar lavage (BAL) fluid of experimentally infected pigs. The moderate virulent ATCC PRRSV isolate VR-2332 was from stock virus at passage seven in MARC-145 cells (Dr. Kenneth Platt, ISU). The PRRS
MLV was from commercial Ingelvac® PRRS MLV vaccine (BIVI, St. Joseph, MO). All viruses were subsequently propagated in MARC-145 cells grown in DMEM† (DMEM (Life Technologies, Gaithersburg, MD), 10% heat-inactivated fetal bovine serum (FBS; Atlanta biologicals, Norcross, GA), and 1% tissue culture penicillin (10,000 IU/ml)/streptomycin (10,000 μg/ml) (Mediatech, Herndon, VA)) to the titer of 10⁶ tissue culture infectious dose 50% (TCID₅₀)/ml. Virus titration was performed by indirect immunofluorescent assay (IFA) using mouse anti-PRRSV monoclonal antibodies (mAbs) (clone 15E; Dr. Kenneth Platt, ISU), followed by fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse IgG antibodies (Sigma, St. Louis, MO). PRRSV isolates VR-2385, SDSU-73, VR-2332, and MLV were used at cell culture passage five, five, nine, and two, respectively. Mock antigens were prepared in the same fashion from uninfected MARC-145 cells. Heat inactivation of virus was performed at 56°C for 8 hours. Complete virus inactivation was confirmed by negative IFA test in inoculated MARC-145 cells.

**Antibodies.** Mouse mAbs anti-porcine cell surface antigens were all from VMRD (Pullman, WA). They were anti-major histocompatibility complex (MHC) class II (clone H42A, isotype IgG2a), anti-swine workshop cluster 3a (SWC3a; swine myeloid marker) (clone 74-22-15a, isotype IgG2b), anti-CD3 (clone 8C8, isotype IgG2a), and FITC-conjugated anti-δ chain of γδ T cell receptor (TCR) (clone PGBL22A, isotype IgG1) Anti-CD4 (clone 74-12-4, isotype IgG2b) and anti-CD8α (clone 76-2-11, isotype IgG2a) mAbs were produced in our laboratory by hybridomas from ATCC (Manassas, VA). Secondary antibodies included Alexa Fluor®647-conjugated goat anti-mouse IgG2a (γ2a) (Molecular Probes, Eugene, OR), Tri-color®-conjugated goat anti-mouse IgG2b (γ) (Caltag Laboratories,
Burlingame, CA), and FITC-conjugated goat anti-mouse IgG2b (Southern Biotech, Birmingham, AL).

Mouse mAbs anti-porcine activation marker CD25 (clone PGBL25A, isotype IgG1, VMRD, Pullman, WA) and anti-porcine TNFα (clone 4F4, isotype IgG1, Pierce biotechnology, Rockford, IL) were custom-conjugated with R-phycoerythrin (R-PE) by Chromaprobe Inc. (Maryland Heights, MO). The R-PE-conjugated anti-porcine IFNγ (clone P2G10, isotype IgG1) mAb was from BD Pharmingen (San Diego, CA). Unconjugated mouse anti-swine IL-10 mAb (clone 945A4C437B1, isotype IgG1κ, Biosource, Camarillo, CA) was used for IL-10 neutralization assay. The unconjugated anti-IFNγ and anti-TNFα mAbs of the same clone were used for antibody blocking control in intracellular cytokine staining. All isotype control mAbs were from Southern Biotechnology Associates, Inc. (Birmingham, AL).

**Cell preparation.** Isolation of PBMC. Anti-coagulated blood were collected in 0.1 volume of 2x acid-citrate-dextrose (0.15M sodium citrate, 0.076M citric acid monohydrate, 0.287M dextrose) solution, diluted with equal volume of phosphate buffered saline (PBS) and layered onto lymphocyte separation medium (1.077 gm/ml, Mediatech, Herndon, VA), then centrifuged at 1,200 xg and 25°C for 30 minutes. PBMC were collected, washed once with PBS, and centrifuged at 270 xg and 4°C for 10 minutes. Contaminating red blood cells were lysed with 3 volumes of buffered water (0.15M sodium phosphate dibasic (Na₂HPO₄) and 0.15M potassium phosphate monobasic (KH₂PO₄)) for 90 seconds and restored with 1.5 volumes of 3x PBS. PBMC were resuspended in RPMI++ (RPMI-1640 with 25mM HEPES and L-glutamine (Life Technologies, Gaithersburg, MD), 10% heat-inactivated FBS (Atlanta biologicals, Norcross, GA), and 1% tissue culture penicillin (10,000 IU)/streptomycin
(10,000 µg/ml) (Mediatech, Herndon, VA)) or PBS++ (PBS, 0.25% heat-inactivated FBS, and 0.025% sodium azide (NaN₃) (Sigma, St. Louis, MO)). PBMC resuspended in RPMI++ were for preparation of APC, and PBMC resuspended in PBS++ were for preparation of purified lymphocytes. PBMC were counted by automatic cell counter (Beckman coulter, Inc., Fullerton, CA) and their concentrations were adjusted to 10⁷ cells/ml.

**Preparation of APC.** One hundred µl of PBMC in RPMI++ (10⁷ cells/ml) were placed into each well of a 96-well flat-bottom plate (BD Labware, Franklin Lakes, NJ) and incubated for 4 hours at 37°C in humidified, 5% CO₂ atmosphere. Non-adherent cells were removed and adherent cells were gently washed once with 150 µl pre-warmed (37°C) RPMI++ to obtain monocytes. Adherent monocytes were cultured continuously in the same incubation environment for another 7 days to obtain MDM and immature MDC. Monocytes to be derived to MDM were cultured in 300 µl RPMI++, while monocytes to be immature MDC were cultured in 300 µl RPMI++ supplemented with recombinant human granulocyte-macrophage colony-stimulating factor (rhuGM-CSF) at 20 ng/ml (eBioscience, San Diego, CA) and recombinant porcine IL-4 (rpoIL-4) at 40 ng/ml (Biosource, Camarillo, CA). Half of culture media (150 µl) was removed and replaced with fresh media (with rhuGM-CSF and rpoIL-4 for immature MDC) every other day. Confirmation of monocyte, MDM, and immature MDC phenotype was made by flow cytometry, using a combination of anti-SWC3a and anti-MHC class II mAbs (Fig. 1).

**Preparation of purified lymphocytes.** PBMC in PBS++ at 10⁷ cells/ml were transferred to 50 ml sterile polypropylene centrifuge tubes (Falcon®, BD, Franklin Lakes, NJ) and received 10 µl of anti-SWC3a mAbs (1 mg/ml) per ml of cell suspension. They were then incubated at 4°C for 60 minutes with gentle tumbling motion, centrifuged at 270 xg (4°C, 10 minutes),
and washed twice with cold PBS++. PBMC were then resuspended with Dynabeads® M450 pan mouse IgG (Dynal Biotech, Brown Deer, WI) in PBS++ at the ratio recommended by the manufacturer. Cells were incubated at 4°C for 60 minutes with gentle tumbling motion, then transferred to sterile snap-cap 5 ml polystyrene round-bottom tubes (Falcon®, BD, Bedford, MA), and placed in a magnetic particle concentrator (Dynal A.S., Oslo, Norway) for 3 minutes to deplete antibody-bound monocytes. The resulting cells were transferred to a new snap-cap tube and the monocyte depletion was repeated one more time. At the end of the monocyte depletion processes, the lymphocytes were washed with 10 volumes of PBS++, centrifuged at 270 xg (4°C, 10 minutes), and cell pellets were resuspended in RPMI++ to 10⁷ cells/ml. The purified lymphocytes contained less than 1% monocytes (SWC3+) as determined by flow cytometry (data not shown).

Cultures of cells. The culture plates containing adherent monocyte, MDM, and immature MDC were centrifuged at 270 xg (25°C, 1 minute) and the cells were gently washed once with 150 μl pre-warmed (37°C) RPMI++. Each cell type was then received 100 μl of freshly purified lymphocytes in RPMI++ (10⁷ cells/ml) from the same animal. After addition of lymphocytes, each APC culture then received 100 μl of either live PRRSV (10⁶ TCID₅₀/ml), heat-inactivated PRRSV (10⁶ TCID₅₀/ml), mock antigen (to serve as negative control), or mock antigen (which was later stimulated with con A or PMA/I to serve as positive control). The ratio of lymphocytes added to monocyte, MDM, and immature MDC resembled the ratio of lymphocyte to monocyte in peripheral blood of pigs at the same age, approximately 10:1 (55). The cultures were incubated at 37°C for 2 days in a humidified, 5% CO₂ atmosphere. Infection of PRRSV in monocytes, MDM, and immature MDC was detected by IFA (mouse anti-PRRSV mAbs (clone 15E)) at day 2 of cell culture. At the end of day 2, one plate of
each APC culture received 50 μl of con A (5 μg/ml final, Sigma, St. Louis, MO) in RPMI for all wells except wells containing mock control. Two plates of each APC culture received 50 μl of a combination of PMA (7 ng/ml final, Sigma, St. Louis, MO), ionomycin (430 ng/ml final, Sigma, St. Louis, MO), and GolgiStop® solution (Monensin, Pharmingen, San Diego, CA) at manufacturer recommended dilution in RPMI for all wells except wells containing mock control. The plates that received con A were incubated for 2 more days for CD25 up-regulation, while the plates that received PMA/I were incubated for 12 more hours for IFNγ and TNFα up-regulation. The concentration of con A and PMA/I and the stimulation period used were optimized to obtain significant up-regulation of CD25, IFNγ, and TNFα expression on T cells with minimum T cell death (data not shown).

**Determination of cell viability.** The viability of T cells from each APC culture as well as monocytes, MDM, and immature MDC from the same well was determined at day 2 and after con A (day 4) and PMA/I (day 2.5) stimulation. In all cultures, all cells were mixed gently with a micropipette and transferred correspondingly to 96-well round-bottom plates. Remaining adherent cells were harvested by placing culture plates on ice for 15 minutes, followed by addition of 10 μl of cold PBS plus 5mM EDTA (Sigma, St. Louis, MO) at pH 8.0 and incubated on ice for 10 minutes. All cells were then processed as described in *fluorescent antibody labeling* and stained with a combination of anti-CD3 and anti-SWC3a mAbs at previously titrated optimum dilutions. One hundred μl of propidium iodide (PI; 100 μg/ml final, Molecular Probes, Eugene, OR) was added to the fluorescent-labeled cells prior to flow cytometry.

**Reverse transcriptase (RT)-PCR.** *RNA extraction and reverse transcription.* In all APC cultures, all cells before (day 2) and after con A (day 4) and PMA/I (day 2.5) stimulation
were harvested and transferred to 96-well round-bottom plates (BD Labware, Franklin Lakes, NJ), centrifuged at 270 xg (4°C, 1 minute), washed once with PBS, resuspended with 200 μl of RNAlater (Ambion Inc., Austin, TX), and stored at -20°C until RNA extraction. Total RNA was extracted, using the Nucleospin RNA II kit (BD Clonetech Laboratories Inc., Mountain View, CA) according to manufacturer's instructions. Contaminating DNA was eliminated by Dnase I provided with the kit. Total RNA was eluted in 40 μl of RNase-free water. Of which, 10 μl from each sample was assigned to reverse transcription, using Omniscript RT kit (Qiagen Inc., Valencia, CA), 0.5 μg random hexamers (Promega Corporation, Madison, WI), and 40 U ribonuclease inhibitor (RNaseOUT, Invitrogen Corporation, Carlsbad, CA). The reverse transcription was performed in the total reaction volume of 20 μl and at 37°C for 60 minutes, followed by heat inactivation at 93°C for 5 minutes and rapid cooling to 4°C.

**PCR.** Complementary DNA (cDNA) of samples before stimulation (day 2) was used as a template for multiplex PCR of porcine IL-2, IL-10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), according to the protocols described by Suradhat et al (58) with minor adjustment. cDNA of the samples after con A (day 4) and PMA/I (day 2.5) stimulation was used as a template for traditional PCR of single gene due to unequal expression of the 3 genes after stimulation which was not suitable for multiplex PCR conditions. All primer sequences were from Suradhat et al (58).

Multiplex and traditional PCR were performed in a total reaction volume of 50 μl, consisting of 2 μl cDNA template, 10 mM dNTPs (Invitrogen Corporation, Carlsbad, CA), and 1 unit *Taq* DNA polymerase (Platinum® Taq DNA Polymerase High Fidelity, Invitrogen Corporation, Carlsbad, CA). The volume of primer mix for multiplex PCR was 6 μl (1 μl of
each primer) and for traditional PCR was 2 µl (1 µl of each primer). The final concentration of the primers was 0.6 µM, 0.2 µM, and 0.05 µM for IL-2, IL-10, and GAPDH, respectively. The PCR condition was 'hot start' at 94°C for 2 minutes; denaturation at 94°C for 30s; annealing at 55°C for 45s; extension at 68°C for 45s, and final extension at 72°C for 5 minutes with the total number of 33 cycles for multiplex PCR and 30 cycles for traditional PCR. The number of PCR cycles of multiplex and traditional PCR was optimized by performing different numbers of PCR cycle and selecting the cycle that none of the PCR products reached the plateau phase during PCR amplification (data not shown).

**Quantification of the PCR products.** Gel electrophoresis of PCR products was performed on 2.5% agarose (Gibco BRL, Grand Island, NY) in TBE buffer (Fisher Scientific company, Pittsburgh, PA) with 0.5 µg/ml ethidium bromide. Images of PCR products on agarose gel were visualized by UV illuminator and captured by GeneSnap software (version 4.01.00, Spectronics Corporation, Westburg, NY). Densitometric analysis of the PCR products was performed using GeneTools software (version 3.00.22, Spectronics Corporation, Westburg, NY). The expressions of IL-2 and IL-10 genes before cell stimulation (day 2) were presented as percentage ratio of cytokine/GAPDH expression of the same animal. The gene expressions after con A (day 4) and PMA/I (day 2.5) stimulation were presented as number of fold increase, calculated from the division of percentage ratio of cytokine/GAPDH expression from stimulated cells by corresponding value from mock control cells of the same animal.

**IL-10 neutralization assay.** IL-10 neutralization assay was performed to determine whether significantly increased IL-10 mRNA expression caused significantly reduced CD25, IFNγ, and TNFα expression. The assay was performed only in monocyte culture since it was
the only culture among all three APC cultures that showed significantly increased IL-10 mRNA expression as well as significantly reduced CD25, IFNγ, and TNFα expression. Mouse anti-swine IL-10 mAb (clone 945A4C437B1, Biosource, Camarillo, CA) was used for IL-10 neutralization.

Three plates each containing 48 wells of adherent monocytes were used. They were for CD25, IFNγ, and TNFα staining. In each plate, monocytes received 100 μl of lymphocytes (10^7 cells/ml) which had received either anti-swine IL-10 mAb at 2.5 μl or 25 μl per 1 ml lymphocytes, mouse isotype control mAb (clone 15H6, Southern Biotech, Birmingham, AL) at 25 μl per 1 ml lymphocytes (isotype control), or RPMI++ (no antibody control). Following the addition of purified lymphocytes, each well then received 100 μl of either live PRRSV (10^6 TCID_{50}/ml) or mock antigens. All plates were incubated at 37°C for 2 days in a humidified, 5% CO2 atmosphere. At the end of day 2, the plate that was for CD25 staining received 50 μl of con A (5 μg/ml final) and the plates that were for IFNγ and TNFα staining received 50 μl of PMA/I (7 ng/ml/430 ng/ml final) in RPMI++. Plate receiving con A was incubated for 2 more days for CD25 up-regulation, while plates receiving PMA/I were incubated for 12 more hours for IFNγ and TNFα up-regulation. The final concentrations of the anti-swine IL-10 mAb in the total 250 μl culture were 1 and 10 μg/ml (manufacturer’s recommendations), respectively. The final concentration of the mouse isotype control mAb was 10 μg/ml.

**Fluorescent antibody labeling.** Primary mAb mixture comprised anti-SWC3a and anti-MHC class II mAbs for differentiation of APC; anti-CD3 and anti-SWC3a mAbs for determination of cell viability; anti-CD3 (or mixture of anti-CD4, anti-CD8α, and FITC-conjugated anti-δ chain of γδ TCR mAbs) and R-PE-conjugated anti-CD25 mAbs for surface
CD25 staining; anti-CD3 mAb alone (or mixture of anti-CD4, anti-CD8α, and FITC-conjugated anti-δ chain of γδ TCR mAbs) for intracellular IFNγ and TNFα staining. Secondary antibody mixture consisted of Alexa Fluor®647-conjugated goat anti-mouse IgG2a (γ2a) and Tri-color®-conjugated goat anti-mouse IgG2b (γ). For cell viability determination, FITC-conjugated goat anti-mouse IgG2b was used in place of Tri-color®-conjugated goat anti-mouse IgG2b (γ). All antibodies were used at previously titrated optimum dilutions.

At the conclusion of the culture period, the culture plates were chilled on ice for 15 minutes and the cells were mixed gently and transferred to 96-well round-bottom plates. The plates were centrifuged at 270 x g and 4°C for 1 minute and washed three times with 150 μl of PBS++. The pellets were incubated with 50 μl primary mAb mixture in the dark at 4°C for 30 minutes, followed by three washes with PBS++. Fifty μl of secondary antibody mixture were added and the plates were incubated for 30 minutes at 4°C in the dark, washed twice with PBS++ and once with PBS, then fixed with 150 μl of 1% ultrapure formaldehyde (Polyscience, Warrington, PA) in PBS.

For intracellular IFNγ and TNFα staining, after washing off secondary antibody mixture, cells were fixed with 100 μl of BD Cytofix/Cytoperm solution (Pharmingen, San Diego, CA) for 30 minutes at 4°C in the dark, washed twice with 150 μl of BD Perm/Wash (Pharmingen, San Diego, CA), and received 50 μl of R-PE-conjugated anti-porcine IFNγ or R-PE-conjugated anti-porcine TNFα mAb in BD Perm/Wash solution at previously titrated optimum dilutions. Cells were incubated in the dark for 30 minutes at 4°C, followed by three washes with BD Perm/Wash solution and fixed with 150 μl of 1% ultrapure formaldehyde in PBS.
Single-color controls, secondary antibody controls, unstained cells, isotype controls, and unconjugated antibody blocking controls for IFNγ and TNFα staining were performed. The blocking controls were incubated with unconjugated mouse anti-porcine IFNγ mAb (clone P2G10, isotype IgG1, BD Pharmingen, San Diego, CA), or unconjugated mouse anti-porcine TNFα mAb (clone 4F4, isotype IgG1, Pierce biotechnology, Rockford, IL), followed by their corresponding R-PE-conjugated mAbs. The fixed cell suspensions from all stainings were transferred to 5 ml polystyrene round-bottom tubes (BD, Franklin Lakes, NJ) and stored at 4°C until flow cytometric analysis.

**Flow cytometry.** The flow cytometric analysis was performed by the flow cytometry facility at ISU on an Epics Altra cytometer (Beckman Coulter, Fullerton, CA) equipped with both krypton (488 nm) and helium-neon laser (633 nm). The FITC, R-PE, PI, and Tri-Color® dyes were excited by the same 488 nm krypton laser. Alexa Fluor®647 dye was excited by a spatially separated 633 nm helium-neon laser. A gated amplifier was used to differentiate Tri-Color® and Alexa Fluor®647 signals, which were detected by the same photomultiplier tube.

Flow cytometry data were analyzed, using FlowJo analysis software (version 4.6.2, Tree Star, Inc. San Carlos, CA). Analyses of APC differentiation and cell viability were performed on total cell populations. A histogram based on SWC3a and CD3 was applied to total cell population, followed by a histogram based on MHC class II and PI on SWC3+ population and PI on CD3+ population. The geometric mean fluorescent intensity (MFI) of MHC class II and percentage of PI+ cells of each cell type were collected.

Analyses of CD25, IFNγ, and TNFα expression in T cells were performed on live cell populations after gating out of doublet and dead events from total cell populations according
to forward- and side-scatter distribution. A histogram based on CD3 was applied to live cell population, followed by a histogram of CD25, IFNγ, and TNFα on CD3⁺ population. For T cell subset analysis, a histogram based on γδ TCR was applied to live cell population, followed by a histogram based on CD25, IFNγ, and TNFα on γδ⁺ population. Quadrant markers were set on CD4 versus CD8 T cells on γδ⁻ population, generating 4 defined lymphocyte subsets containing CD4⁺CD8⁻γδ⁻, CD4⁺CD8⁺γδ⁻, CD4⁻CD8⁺γδ⁻, and CD4⁻CD8⁻ γδ⁻. A non-T cell subset (CD4⁻CD8⁻γδ⁻) was not further analyzed. The remaining 3 T cell subsets were gated for their percentage of CD25⁺, IFNγ⁺, or TNFα⁺ on a histogram based on CD25, IFNγ, and TNFα, respectively.

Statistical analysis. All statistical analyses were performed using the JMP5.1 software (SAS Institute Inc., Cary, NC). Mean difference of MFI of MHC class II expression among monocyte, MDM, and immature MDC was tested by one-way analysis of variance, followed by Tukey-Kramer HSD test for multiple mean comparisons. Mean differences of %CD25⁺, %IFNγ⁺, %TNFα⁺, and %PI⁺ of T cells of the same APC culture were tested among treatments by Kruskal-Wallis test, followed by Dunnett's test using mean of positive control as a control group. The same statistical methods (Kruskal-Wallis test and Dunnett's test) were used for analysis of mean differences of %PI⁺, IL-2 mRNA, and IL-10 mRNA of monocyte, MDM, and immature MDC cultures. P<0.05 was set as statistically significant level throughout this study.

RESULTS
Differentiation of porcine APC. Monocytes, MDM, and immature MDC were differentiated from one another by differential MHC class II expression (6,24,32). At the end of cell culture period (day 7), immature MDC had significantly higher MHC class II expression than monocyte and MDM, respectively (p<0.05) (Fig. 1). Monocytes had significantly higher MHC class II expression than MDM (p<0.05) (Fig. 1).

Effect of PRRSV and APC on T cell responses. CD25 response. All three types of porcine myeloid APC were infected with all four PRRSV isolates after 2-day of co-cultivation as determined by IFA. Naïve T cells incubated with monocytes infected with VR-2385, SDSU-73, and VR-2332 demonstrated significantly reduced %CD25$^+$ when compared to those from positive controls (p<0.05) (Fig. 2). No significant reduction of %CD25$^+$ was observed in T cells cultured with monocytes infected with PRRS MLV. T cells from MDM and immature MDC cultures infected with PRRSV of all isolates did not show significantly reduced %CD25$^+$ (Fig. 2). Heat-inactivated PRRSV did not induce significantly reduced %CD25$^+$ in T cells from any APC cultures (data not shown). Analysis by T cell subset revealed that significantly reduced %CD25$^+$ in monocyte cultures occurred in all subsets of αβ T cells (CD4$^+$CD8$^+$γδ$^-$, CD4$^+$CD8$^+$γδ$^+$, CD4$^+$CD8$^+$γδ$^-$) and γδ$^+$ T cells (Fig. 3).

IFNγ and TNFα responses. Naïve T cells from monocyte culture infected with VR-2385, SDSU-73, and VR-2332 demonstrated significantly reduced %IFNγ$^+$ and %TNFα$^+$ when compared to those from positive controls (p<0.05) (Fig. 2). Significant reduction of %TNFα$^+$ but not %IFNγ$^+$ was observed in T cells from monocyte cultures infected with PRRS MLV (p<0.05) (Fig. 2). T cells from MDM and immature MDC cultures infected with PRRSV of all isolates did not show significantly reduced %IFNγ$^+$ and %TNFα$^+$ (Fig. 2).
FIGURE 1. Histogram of geometric mean fluorescent intensity (MFI) of MHC class II expressed in monocyte (left), monocyte-derived macrophage (MDM) (center), and immature monocyte-derived dendritic cell (MDC) (right). Monocytes were obtained by plate adherence of naive PBMC. MDM were derived from monocytes by continuous adherence for 7 days in RPMI\(^{++}\). Immature MDC were derived from monocytes by continuous adherence for 7 days in RPMI\(^{++}\) supplemented with rhuGM-CSF (20 ng/ml) and rpoIL-4 (40 ng/ml). Half of the culture media were removed and replaced with fresh media every other day. Grey-filled histogram represents isotype control. The number represents mean MFI ± standard error of mean (SEM).
Heat-inactivated PRRSV did not induce significantly reduced %IFNγ+ and %TNFα+ in T cells from any APC cultures (data not shown). Analysis by T cell subset in monocyte cultures revealed that significantly reduced %IFNγ+ occurred in CD4+CD8γδ− and CD4−CD8γδ− T cells and significantly reduced %TNFα+ occurred in CD4+CD8γδ−, CD4+CD8γδ−, and CD4−CD8γδ− T cells (Fig. 3).

**Effect of PRRSV on viability of T cells and APC.** No significant difference in %PI+ of T cells was detected in any APC culture between PRRSV-infected groups and controls. The results were consistent in both before and after cell stimulation. The mean %PI+ of T cells before the stimulation, after con A, and after PMA/I stimulation was approximately 30-40%, 60-70%, and 45-55%, respectively, in all treatments of all APC cultures (data not shown).

Significant increase in %PI+ was detected in monocytes, MDM, and immature MDC infected with PRRSV of all isolates (Fig. 4). After 2 days of infection, 40-60% of monocytes, 70-90% of MDM, and 40-60% of immature MDC were dead, whereas only about 20% of corresponding APC in mock control were dead (Fig. 4). The %PI+ were increased in all types of APC after con A and PMA/I stimulation (Fig. 4). MDM always showed the highest %PI+ among 3 types of APC. Heat-inactivated PRRSV did not induce significantly increased %PI+ in any APC culture compared to that in corresponding controls either before or after con A and PMA/I stimulation (data not shown).

**Gene expression of IL-2 and IL-10.** No significant differences in IL-2 and IL-10 mRNA expression were detected before con A and PMA/I stimulation in any APC culture between PRRSV-infected groups and mock controls (Fig. 5). Only MDM cultures infected with SDSU-73 showed significantly increased IL-2 mRNA expression compared to mock control (p<0.05) (Fig. 5).
FIGURE 5. Mean %IL-2 and %IL-10 gene expression of monocyte, MDM, and immature MDC cultures after inoculation with PRRSV for 2 days. RT-PCR was performed on total RNA extracted from each sample in each APC culture. Presented data were after normalization with GAPDH gene expression of the same animal. Error bar indicates SEM. * p<0.05.
Significant increase in IL-2 mRNA expression was detected in monocyte cultures infected with VR-2385, SDSU-73, and VR-2332, and in MDM cultures infected with SDSU-73 and VR-2332 after con A stimulation, compared to corresponding positive controls (p<0.05) (Fig. 6). There was relatively high IL-2 mRNA expression in immature MDC cultures in PRRSV-infected groups and controls compared to that in monocyte and MDM cultures (Fig. 6). No significant difference of IL-10 mRNA expression was detected between PRRSV-infected groups and positive controls in any APC culture after con A stimulation (Fig. 6).

Significant difference of IL-2 mRNA expression was not detected between PRRSV-infected groups and positive controls in any APC cultures after PMA/I stimulation (Fig. 7). The IL-2 mRNA expression was relatively high in immature MDC cultures compared to that in monocyte and MDM cultures in both PRRSV-infected groups and controls (Fig. 7). The IL-10 mRNA expression was significantly increased in monocyte cultures infected with VR-2385, SDSU-73, and VR-2332 (p<0.05) (Fig. 7). No significant increase of IL-10 mRNA expression was observed in MDM and immature MDC cultures infected with any PRRSV isolates (Fig. 7).

**IL-10 neutralization.** Significantly reduced %IFNγ+ and %TNFα+ but not %CD25+ of T cells in monocyte cultures were inhibited in the presence of anti-swine IL-10 mAbs (Fig. 8). Significantly reduced %TNFα+ were completely inhibited in monocyte cultures infected with all PRRSV isolates at low mAb concentration (1 μg/ml). Significantly reduced %IFNγ+ in monocyte culture infected with SDSU-73 and VR-2332 were inhibited at low mAb concentration (1 μg/ml), whereas significantly reduced %IFNγ+ in monocyte culture infected with VR-2385 was inhibited at high mAb concentration (10 μg/ml) (Fig. 8).
FIGURE 6. Mean fold increase of IL-2 and IL-10 gene expression in monocyte, MDM, and immature MDC cultures infected with PRRSV and stimulated with con A. All cultures were incubated with PRRSV for 2 days and con A stimulation for another 2 days prior to RT-PCR analysis. Data were normalized with GAPDH gene expression of the same animal. Error bar indicates SEM. * p<0.05.
FIGURE 7. Mean fold increase of IL-2 and IL-10 gene expression in monocyte, MDM, and immature MDC cultures infected with PRRSV and stimulated with PMA/I. All cultures were incubated with PRRSV for 2 days and received PMA/I stimulation for another 12 hours prior to RT-PCR analysis. Data were normalized with GAPDH gene expression of the same animal. Error bar indicates SEM. * p<0.05.
DISCUSSION

The ability of PRRSV to suppress T cell response was evaluated on CD25, IFNγ, and TNFα expression on naïve T cells in response to con A and PMA/I, respectively. Four PRRSV isolates possessing varying clinical virulence and three different types of porcine myeloid APC were used to compare their effects on T cell response. Significant reductions of %CD25+ and %IFNγ+ were detected only after virulent PRRSV infection, while significant reduction of %TNFα+ was detected after infection with all four PRRSV isolates, suggested the differential effects of PRRSV virulence on different immune parameters. All reductions were detected only in monocyte cultures, indicated the role of monocytes in T cell suppression after PRRSV infection.

Significant up-regulation of CD25 molecules on porcine T cells has been reported in response to con A (56). The expression of CD25 molecules was followed by proliferation of porcine T cells (56). Significantly reduced CD25 expression after virulent PRRSV infection may result in inhibition of clonal expansion of PRRSV-specific T cells, resulting in delayed CMI response. CMI response to PRRSV determined by T cell proliferation in response to recall PRRSV in vitro was detected 4 weeks after infection, which was relatively delayed compared to T cell proliferative response to pseudorabies virus and swine influenza virus which appeared within 1 week after infection (8,29,61).

An inductive effect of PMA/I on IFNγ and TNFα expression by porcine T cells has been reported (1,46). IFNγ and TNFα have been demonstrated to inhibit PRRSV replication (9,30,49). Significantly reduced IFNγ and TNFα expression after virulent PRRSV infection may facilitate PRRSV replication, as demonstrated by significantly higher PRRSV titers in
the serum after virulent PRRSV infection than after low virulent PRRSV infection (26).

Significantly reduced CD25 expression on T cells after virulent PRRSV infection may also inhibit clonal expansion of PRRSV-specific IFN-γ-producing T cells and PRRSV-specific TNFα-producing T cells. T cells producing IFN-γ in response to PRRSV appeared in peripheral blood 3-8 weeks after PRRSV infection (5,11,17,33,34,50). This is considerably delayed compared to 1 week after pseudorabies virus infection (61). The percentage of T cells producing TNFα were significantly reduced when determined directly from peripheral blood of PRRSV-infected pigs (1).

Absence of significantly reduced CD25 and IFN-γ, but not TNFα expression after PRRS MLV infection suggests that the vaccine virus may possess T cell suppressive property of lesser degree than virulent virus. Previous study on CD25 expression in T cells of pigs vaccinated with PRRS MLV showed no CD25 up-regulation in PRRS MLV-primed T cells after re-stimulation in vitro with recall MLV antigens (11). The ability of PRRS MLV to suppress TNFα expression may facilitate virus replication and thereby viremia of PRRS MLV after vaccination (17,35,40).

Effects of monocytes, MDM, and immature MDC on T cells in response to PRRSV have not been determined. Porcine monocytes, macrophages, and dendritic cells have been reported to up-regulate their IL-10 gene expression after PRRSV infection (22,58). Increased IL-10 production has been detected in the lungs of PRRSV-infected pigs during monocyte accumulation (13,16,25,28,64). Porcine monocytes seem to be a more potent IL-10 producer than porcine MDM and immature MDC in response to PRRSV (Fig. 7). The potential of porcine monocytes to express IL-10 in comparison to porcine MDM and immature MDC has been reported in response to bacterial lipopolysaccharide (LPS), *Actinobacillus*
pleuropneumoniae, Sendai virus, and plasmid DNA (6,7,24,43). The ability of porcine monocytes to express IL-10 in response to PRRSV may induce poor T cell response. In pigs, monocytes are a more potent inducer of T cell proliferation than MDM and immature MDC (6). In humans and mice, T cell stimulating activity of monocyte is reduced after a treatment with IL-10 (39,44). IL-10 has been reported to suppress expressions of MHC class II and co-stimulatory molecules (CD80/CD86) on murine monocytes as well as production of pro-inflammatory cytokines ie. IL-1, IL-6, IL-12, and TNFα, which are essential for activation of APC and induction of T helper 1 response (39,41,44,45,59). In pigs, IL-10 suppresses IFNγ production by T cells (68). Increased IL-10 production by monocytes after PRRSV infection may be a strategy of PRRSV to down regulate pig CMI response. IL-10 has chemotactic activity that can attract T cells (44). PRRSV may exploit this activity of IL-10 to attract T cells to the infected lungs and lymphoid tissues where monocytes are accumulated and intense concentrations of IL-10 are being produced.

Significantly increased IL-2 gene expression was detected in monocyte and MDM cultures infected with virulent PRRSV and stimulated with con A (p<0.05) (Fig. 6). T cells from monocyte cultures demonstrated significantly reduced CD25 expression (p<0.05) (Fig. 3). In mice, IL-2 gene expression is directly correlated with CD25 gene expression (31,51,54). Increased IL-2 production enhances CD25 expression in murine T cells (31,51,54). IL-2 binds to constitutively expressed β and γ chains of IL-2 receptor on T cells, which then triggers signaling molecules eg. JAK1, Ras, Raf, MAPK, PI3K, E2F, NFκB and STAT5 to induce transcription of CD25 gene (4,18,37,67). In pigs, the effect of IL-2 on CD25 expression is not known. Some binding motifs for transcription factors used for CD25 transcription in mice were found in the 5'-upstream region of the porcine CD25 gene (27).
In-depth study focusing on signaling pathway for porcine CD25 expression is essential to elucidate the mechanism used by virulent PRRSV to suppress CD25 expression by T cells. In mice, CD25 expression can be reduced by the regulatory effects of suppressors of cytokine signaling 1 (SOCS1) molecules, SOCS3 molecules, Shp-1, and CIS1 (3,14,18). In pigs, SOCS1 gene expression was reportedly increased in T cells in the recall response to PRRSV (50).

In conclusion, this study reported that PRRSV has the ability to suppress T cell activity. The ability was demonstrated by significantly reduced CD25, IFNγ, and TNFα expression of T cells in response to con A and PMA/I. The suppressive activity associates with virus virulence and is mediated by monocytes but not by MDM and immature MDC. The reduced expression IFNγ and TNFα is mediated by increased expression of IL-10.

ACKNOWLEDGEMENTS

This study was supported by the Institute for International Cooperation in Animal Biologics, College of Veterinary Medicine, Iowa State University. The authors wish to thank Dr.Kenneth Platt for mouse anti-PRRSV mAbs (clone 15E) and ATCC VR-2332, Dr.Patrick Halbur for VR-2385, Dr.Michael Roof for SDSU-73, and Dr.Eileen Thacker for UV illuminator and software for PCR quantification. The authors also would like to thank Dr.Shawn Rigby for Epic Altra Flow cytometry analysis at the Flow cytometry facility and Thomas Skadow for excellent laboratory assistance.
REFERENCES


42. Opriessnig, T., P.G. Halbur, K.J. Yoon, R.M. Pogranichny, K.M. Harmon, R. Evans, K.F. Key, F.J. Pallares, P. Thomas and X.J. Meng. 2002. Comparison of molecular and biological characteristics of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine (ingelvac PRRS MLV), the parent strain of the vaccine
(ATCC VR2332), ATCC VR2385, and two recent field isolates of PRRSV. J Virol 76:11837-11844.


Chapter 5. General Conclusions

Discussion

The work presented in this dissertation has generated new knowledge on cell-mediated immune (CMI) response to porcine reproductive and respiratory syndrome virus (PRRSV). Several kinds of vaccine adjuvants have been tested for their adjuvant property for PRRS modified-live virus (MLV) vaccine (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica). The CMI responses were correlated with protection from virulent PRRSV challenge. The ability of PRRSV to suppress T cell responses was investigated.

In the first study, which is described in chapter 3, antigen-specific T-cell subsets responsible for PRRS MLV vaccination were characterized, using a developed 4-color flow cytometry method. Previous methods used to study T cell responses to PRRSV included reverse transcriptase (RT)-PCR for cytokine gene expression, ELISA for cytokine production, enzyme-linked immunospot (ELISPOT) assay for IFNγ production, and incorporation of ³H-thymidine and PKH staining for lymphocyte blastogenesis assay. None of these assays, except PKH staining, provides data of PRRSV-specific T cell subsets and T cell activation, proliferation, or cytokine production at the same time. By the 4-color flow cytometry method, five T-cell subsets including CD4⁺CD8⁻γδ⁻, CD4⁺CD8⁺γδ⁺, CD4⁺CD8⁻γδ⁺, CD4⁺CD8⁺γδ⁺, and CD4⁺CD8⁻γδ⁻ and a non T-cell subset (CD4⁺CD8⁺γδ⁻) were simultaneously characterized for surface expression of CD25 molecules and intracellular expression of IFNγ. The expression of CD25 molecules and of IFNγ was assessed after in vitro exposure to PRRSV. No up-regulation of CD25 expression was detected in any T-cell
subsets after PRRS MLV vaccination. Only CD4⁺CD8⁺γδ⁻ T cells showed significantly increased IFNγ expression after vaccination.

To attempt to improve the CD25 and IFNγ responses of T cells to the PRRS MLV vaccine, five different vaccine adjuvants (bacterial endotoxin-derived adjuvant, mixed open reading frame 5 (ORF5) peptides derived from 5 PRRSV isolates, porcine IFNα, polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose, and porcine interleukin-12 (IL-12)) were used. Administration of mixed ORF5 peptides at 14 and 28 days after PRRS MLV vaccination significantly increased IFNγ production in CD4⁺CD8⁺γδ⁺, CD4⁺CD8⁺γδ⁻, and CD4⁺CD8⁺γδ⁻ T cells. Administration of porcine IL-12 at 1 day after PRRS MLV vaccination significantly increased IFNγ production in CD4⁺CD8⁺γδ⁻, CD4⁺CD8⁺γδ⁻, and CD4⁺CD8⁺γδ⁻ T cells. Significantly increased IFNγ expression in CD4⁺CD8⁺γδ⁻, CD4⁺CD8⁺γδ⁻, and CD4⁺CD8⁺γδ⁻ T cells but not CD4⁺CD8⁺γδ⁺ T cells were correlated significantly with the reduction of lung lesion scores and viremia after virulent PRRSV challenge. Administration of porcine IFNα at -1, 0, and 1 day and porcine IL-12 at 1 day after PRRS MLV vaccination significantly increased CD25 expression in CD4⁺CD8⁺γδ⁻ T cells. However, the increased CD25 expression did not correlate with protection. None of the vaccine adjuvants contributed to the reduction of lung lesion scores and viremia in comparison to PRRS MLV alone.

In the second study, which is described in chapter 4, the ability of PRRSV to suppress T cell expression of CD25, IFNγ, and tumor necrosis factor alpha (TNFα) was determined by flow cytometry in naïve porcine T cells in response to concanavalin A and phorbol 12-myristate 13-acetate plus ionomycin (PMA/I). Four PRRSV isolates of varying clinical virulence and three different types of porcine myeloid antigen-presenting cells (APC) were
used. The effects of PRRSV virulence and PRRSV-infected myeloid APC on T cell response were not known. Naïve T cells cultured with monocytes infected with virulent PRRSV (VR-2385, SDSU-73, VR-2332), but not with PRRS MLV (Ingelvac® PRRS MLV, Boehringer Ingelheim Vetmedica), demonstrated significantly reduced %CD25+ and %IFNγ+ when compared to T cells incubated with uninoculated monocyte cultures. T cells cultured with monocytes infected with all four PRRSV isolates demonstrated significantly reduced %TNFα+. The significant reduction of %CD25+, %IFNγ+, and %TNFα+ was not detected in T cells cultured with monocyte-derived macrophages (MDM) and immature monocyte-derived dendritic cells (MDC) infected with any PRRSV isolates. The reduction of T cell response in monocyte cultures was not due to PRRSV-induced T cell death. Gene expression of IL-10 detected by semi-quantitative RT-PCR was significantly increased in virulent PRRSV-infected monocyte cultures after PMA/I, but not con A stimulation, compared to IL-10 gene expression from uninoculated monocyte cultures. Increased IL-10 gene expression contributed to significantly reduced %IFNγ+ and %TNFα+, but not %CD25+, as determined by an IL-10 neutralization assay.

**Recommendations for Future Research**

The finding that IL-10 contributed to significantly reduced %IFNγ+ and %TNFα+ provides the direction for development of vaccine adjuvants for PRRS MLV vaccine. The vaccine adjuvants for PRRS MLV vaccine should be able to prevent IL-10 production after PRRS MLV vaccination. The prevention of IL-10 production might be achieved by the utilization of RNA interference (RNAi) technology. A recent study on RNAi in pigs revealed that chemically synthesized small interfering RNA (siRNA) targeted to
glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reduced GAPDH transcripts in pulmonary alveolar macrophages (PAM) (Zhang et al., 2005). The use of chemically synthesized porcine IL-10 siRNA as a vaccine adjuvant might be able to reduce IL-10 transcript after PRRS MLV vaccination. The prevention of IL-10 production by porcine IL-10 siRNA may improve CMI responses of pigs to PRRS MLV vaccine.

The finding that virulent PRRSV suppresses CD25 expression on T cells in an IL-10-independent manner suggests that there are yet unknown mechanisms of PRRSV to suppress CMI responses. To elucidate these mechanisms, an in-depth study of signaling pathways of porcine CD25 expression is essential. The knowledge of mechanisms used by PRRSV to suppress CD25 expression might be utilized for development of vaccine adjuvants that can interfere with the pathway stimulated by the virus.

The effect of PRRSV of varying levels of virulence on CMI responses has not been determined in vivo. The knowledge of PRRSV virulence effect on CMI responses in vivo may help explain differential clinical severity among PRRSV strains. CMI response to virulent PRRSV may be delayed, compared to CMI response to low virulent PRRSV. The delayed CMI response may facilitate virulent PRRSV replication and cause more damage to virus-infected cells.

Differential suppressive activity of PRRSV on CD25, IFNγ, and TNFα expression on T cells may be useful for selection of candidate strains for PRRSV vaccine. The candidate PRRSV vaccine strains should have neither clinical virulence nor T cell suppressive activity. The lack of T cell suppressive activity will improve the CMI response of pigs to the vaccine.
References

Acknowledgements

I would like to thank Dr. James Roth, my major professor, for giving me an excellent opportunity to pursue my Ph.D. degree in his laboratory and for his guidance throughout my Ph.D. study. I have learned a great deal from him about analytical thinking, writing, and public speaking. Dr. Roth has offered me interesting projects from which I have learned lots in immunology. I hope the knowledge that I learned from him will be useful for improving quality of life of animal and human throughout my academic career.

I would like to thank Dr. Ratree Platt for her guidance during my Ph.D. study. Without Dr. Platt, I would not have accomplished my Ph.D. degree. I also thank people in Dr. Roth's office—Tom Skadow, Dawne Buhrow, and Naomi Backous for their help and hospitality throughout my graduate study. I appreciate all advice from my POS committee member—Dr. Kenneth Platt, Dr. Eileen Thacker, Dr. Douglas Jones, and Dr. Patrick Halbur.

My parents, Visit and Piyaporn, my siblings, Srisakul and Dr. Roongtham, and my best friend, Pattama, have always supported me through every difficulty during my five years of graduate study. Their love is greatly appreciated. Nawanat Eua-anant, Jumnongjit Phasuk, Dennis Wendell, Pravina Kitikoon, Jose Neto, Andy & Szi Wei Tang, Nakhyung Lee, Yi Mei, and Taran Schilling have made my life so enjoyable during the long hard work of my graduate study. The scholarship for my Ph.D. study was awarded by the Royal Thai Government.