ABSTRACT

CHEN, HSIN-YING. T cell response to infection by the Porcine Reproductive and Respiratory Syndrome Virus. (Under the direction of Dr. Scott M. Laster.)

The purpose of the research has been to characterize the response of naïve or virus-specific swine T lymphocytes from different lymphoid compartments to Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). Peripheral blood mononuclear cells (PBMCs), tracheobronchial lymph node (TLN) and lateral retropharyngeal lymph node (LLN) cells were labeled with PKH67 green fluorescence dye to measure cell proliferation and surface phenotypes were examined using anti-CD4, anti-CD8 or anti-CD25 monoclonal antibodies. Stimulation with Concanavalin A was also included as a positive control. Our results show that potential virus-specific T lymphocytes were found in the peripheral blood, although no cell proliferation was found in cultures of lymph node cells. We did find that the percentages of CD8+ T cells in cultures of lymph node cells from the virus-infected pig increased after in vitro stimulation with the Powell virus compared to the lymph node cells cultured in media only, suggesting that CD8+ T lymphocytes may play a role in the virus clearance and immune memory to PRRSV.

T CELL RESPONSE TO INFECTION BY THE PORCINE REPRODUCTIVE AND RESPIRATORY SYNDROME VIRUS

by
HSIN-YING CHEN

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

IMMUNOLOGY

Raleigh, North Carolina

2004

APPROVED BY:

Scott M. Laster, Ph.D Chair of Advisory Committee	Frederick J. Fuller, Ph.D
Susan Tonkonogy, Ph.D	Monte McCaw, D.V.M., Ph.D

BIOGRAPHY

Hsin-Ying Chen was born in February of 1975 in Kaohsiung, Taiwan to Shu-Hsun and Hsi-Hao Chen. She attended both elementary and junior high schools in Kaohsiung, Taiwan. In 1990, she passed the national admission examination and admitted in the Kaohsiung Girls' Senior High School in her home city. After three years, Hsin-Ying completed high school, passed the competitive national admission examination and obtained the admission of the Department of Veterinary Medicine of the College of Agriculture at National Taiwan University (in Taipei, Taiwan). In 1998, she completed her undergraduate education, obtained her Bachelor of Veterinary Medicine and Veterinary licensure after the national qualification examination and was admitted to Pathology Program of the College of Medicine at National Taiwan University (in Taipei, Taiwan) after Oral Qualification Test. After earning the degree of Master of Science in June of 2000, she decided to go to America for further research training. In August of 2001, Hsin-Ying enrolled in the graduate program of the College of Veterinary Medicine at North Carolina State University (in Raleigh, North Carolina, USA) and started to work on Immunology in the spring of 2002. Under the direction of Dr. Scott M. Laster, she completed her thesis and obtained the degree of Master of Science, majoring in Immunology.

ACKNOWLEDGMENTS

First of all, I would like to thank my family beginning my parents, Dr. Hsi-Hao Chen and Mrs. Shu-Hsun Chen, for their support, encouragement and instruction. Also, I would like to thank the support from my sisters, Dr. Tzu-Hsing Chen and Dr. Ching-I Chen, and from my brother in law, Dr. Kai-Yuan Cheng. I would not have been achieving anything without them. I am blessed to have such wonderful family.

Second, I would like to thank the support and instruction from my adviser, Dr. Scott M. Laster and from my graduate advisory committee, Dr. Susan Tonkonogy, Dr. Monte McCaw and Dr. Fredrick J. Fuller. Also, I appreciate the help from Dr. Wayne and Mary Tompkins, Ms. Janet Dow, Ms. Deborah Anderson, Mr. Jinsheng Xu and Angali Josh.

Finally, I would like to thank my labmates, including Dr. Susan Michalowski, Kang Mi Lee, Cari Culver, Rita Maia, Dawn Eads and Shari Sweeney. I thank other graduate students and staff in the Department of Microbiology including Renny Lan, Alan House, Denise Aslett, Ms. Dawn NewKirk, Dr. Jose Bruno-Barcena and Mr. Jimmy Cooper. I thank the prompt help from Ms. Linda Costine, Ms. Jill Statham and Ms. Peggy Olive. I also thank the encouragement and help from my friends including Kuan-Chih Chen, Chih-Wei Ho, Chung-Jung Chou, Hsin-I Chang, Chon-Chien Hong and Bin Jia. It is a great experience to have these good friends.

TABLE OF CONTENTS

Page

List of Tables ……... vi

List of Figures ……..... vii

Literature Review…….. 1

The biology of Porcine Reproductive and Respiratory Syndrome Virus…….1

Characteristics of swine T cells……4

The cell mediated immune response to PRRSV……. 6

The purpose of this research…….. 11

Materials and Methods……... 13

Experimental animals…….13

Virus infection and rechallenge for the animals……..13

Virus stock preparation and quantification…… 14

Isolation of porcine peripheral blood mononuclear cells…….. 15

Collection of lymph node cells…….. 16

Cell labeling with PKH67 green fluorescent dye……...17

In vitro stimulation of immune cells with virus stock……17

Cluster of differentiation (CD) antigens detection and proliferation assay……18

Results…….. 20

Clinical signs in PRRSV-infected pigs……..….20

The response of normal PBMCs to Con A.....21

The response of normal lymph node cells to Con A …… 22

The response of normal PBMCs and lymph node cells to PRRSV……...……24

The response of PBMCs and lymph node cells from PRRSV-infected pig ……. 26

Discussion……..28

Figures and Tables……35

References…… 50

LIST OF TABLES

Table I Summary of responses to Con A by normal swine T cells ……..……38

Table II Summary of responses to PRRSV by T cells from infected Pigs …….……46

LIST OF FIGURES

Fig.1 The phenotypic profile of PBMCs from the normal (control) pig ……..…….35

Fig.2 The response of PBMCs from the normal pig to Con A ……...……. ……36

Fig.3 The phenotypic profiles of PBMCs from the normal pig after Con A treatment ……..…37

Fig.4 The phenotypic profiles of tracheobronchial or lateral retropharyngeal lymph node cells from the normal pig ……..….39

Fig.5 The response of normal lymph node to Con A in culture after 5 days …….40

Fig.6 The phenotypic profiles of normal lymph node cells to Con A ……..41

Fig.7 The forward and side scatter plots of normal PBMCs and lymph node cells following stimulation with PRRSV…….42

Fig.8 Histograms of PKH67 staining of normal PBMCs following incubation with PRRSV ……...43

Fig 9 The percentages of cells in the gated region R3 expressing CD4, CD8 or CD25 in the normal pig ……...44

Fig.10 The forward and side scatter plots of PBMCs and lymph node cells from the virus infected pig following incubation with PRRS…..……..45

Fig.11 PKH67 staining histograms of lymphoid cells from the infected pig following in vitro culture with PRRSV……...……..47

Fig.12 The percentages of the cells in the R1 regions expressing CD4, CD8 or CD25 in the Powell isolate virus infected and re-challenged pig ……48

Fig.13 Comparisons of the percentages of $CD4^+$, $CD8^+$ or $CD25^+$ T cells from the virus-treated cultures of PBMCs and lymph node cells from the control pig and the PRRSV-infected pig…….....49

Literature Review

The biology of Porcine Reproductive and Respiratory Syndrome Virus

Porcine reproductive and respiratory syndrome virus (PRRSV), a member of the *Arteriviridae* family, is an enveloped single stranded RNA virus. The positive-sense and linear genome is 15 kb in length and consists of 8 overlapping open reading frames; ORFs 1a, 1b, and 2-7. The virus particle is spherical with a diameter of 45-70 nm (1, 2). ORFs 1a and b are located at the 5' end of the genome and occupy 75 % of the entire length. They encode nonstructural proteins involved in viral replication. ORFs 2 to 5 encode the viral structural proteins, including N-glycosylated proteins GP2 to GP5, which represent minor constituents of the virus particles. ORFs 6 and 7 encode the viral membrane M and nucleocapsid N proteins, respectively (3, 4, 5).

Porcine reproductive and respiratory syndrome virus was first isolated in Europe in 1990 and the disease was first detected in the United States in 1987 (6, 7, 8, 9). This disease is characterized by late term pregnancy failure in sows and respiratory distress in adult and young pigs. Extensive strain heterogeneity has been described, and it is the major reason that vaccines fail to control this disease. Although two main genotypes, the European (with its prototype the Lelystad virus) and the North American (with its prototype strain VR-2332), have been reported (10, 11). Rapid evolution of this virus

results in quasispecies, defined as a population of closely related and heterogeneous sequences that are variants of one dominant sequence (12, 13). The principal envelope glycoprotein of PRRSV, GP5, carries a hypervariable region within the ectodomain that is responsible for generating diversity in field isolates (12). GP5 has been considered as the primary neutralization epitope and the humoral immune response to this glycoprotein has been explored. Weiland et al. (14) and Gonin et al. (15) demonstrated that GP5 was the structural protein involved in the production of neutralizing antibodies of both the Dutch isolate Intervet-10 and the Quebec reference cytopathogenic strain IAF-Klop, respectively. Plagemann et al. (16) and Ostrowski et al. (17) identified the neutralizing epitope in the GP5 ectodomain of VR-2332 and other strains. Pirzadeh et al. (18) showed the linear neutralizing determinants recognized by their monoclonal antibodies to the GP5 of the Quebec reference cytopathogenic strain IAF-Klop.

The pathological signs, including pneumonia, lymphadenopathy, vasculitis, myocarditis, encephalitis and lung lesions, were demonstrated by Rossow et al. (19) by infecting gnotobiotic pigs with the strain ATCC VR-2332. In their results, the lung lesions were characterized by alveolar septa thickened by macrophages, alveolar proteinaceous, karyorrhectic debris, alveolar syncytial cells and multifocal type II pneumocyte hypertrophy. They also observed lymph node lesions characterized by germinal center hypertrophy and hyperplasia, lymphocyte necrosis, multiple cystic

spaces and polykaryocytes within the cystic spaces; heart lesions (characterized by subendocardial, myocardial and perivascular foci of lymphocytes) were also shown as a late feature of infection. Similar results were described by Reeth (20). Other clinical signs, like transient loss of appetite, slight hyperthermia and respiratory distress, were also observed by other scientists (21). Nielsen et al. (28) intranasally challenged late term pregnant sows with PRRS-VDV (PRRSV 19407B). This strain was isolated in 1997 from the lungs of a stillborn pig which was born to a sow vaccinated with the attenuated live "Ingelvac® PRRS MLN" vaccine based on the pathogenic American PRRSV field isolate, VR-2332. Their clinical results showed congenital infection, fetal death, abortion and preweaning pig mortality.

Viral persistence and immunosuppression are two important characteristics of porcine reproductive and respiratory syndrome. Many scientists have reported these two refractory problems in their experiments and/or field experiences (21, 23, 24, 25). In field cases that develop acute respiratory symptoms of PRRSV infection, the lung is the preferential site for virus replication; PRRSV could be recovered from the infected lung or alveolar macrophages for up to nine weeks post infection (26). Persistent infection by PRRSV was demonstrated when the virus could be isolated from the tonsils, spleen and lymphoid tissues, but not necessarily from the lungs, of the infected pigs (19, 27). Different strategies, experimental preparations of structural proteins of PRRSV, or

commercial vaccines have been developed in order to control this undesirable disease (28, 29, 25, 30, 31, 32). However, strain specificity of the immune response results in the lack of cross protection to different strains of PRRSV (33, 34, 35). Pigs vaccinated with vaccines containing live mutated virus may shed virus causing the infection of normal pigs (35, 36). Previous exposure may prevent development of clinical signs and reduce virus proliferation of the reinfection by the homologous strain of PRRSV (37). Vaccines do not always provide protective immunity to homologous strains of virus (38, 36); the risk of the reversion of a live attenuated vaccine to virulence under field conditions suggests the likelihood that this virus undergoes mutation (39). Yuan et al. (9) compared the genome of one commercial attenuated vaccine to its parental strain (VR-2332), trying to provide the genetic basis for future manipulation of a PRRSV reverse genetics system. Different strains have different ability to infect and replicate in pregnant gilts and cross the placental barrier; strain predominance was observed after infecting naïve or vaccinated pregnant gilts with multiple strains of PRRSV (40).

Characteristics of swine T cells

The porcine immune system is unique and has significant numbers of lymphocyte subpopulations rarely seen in humans and rodents. The normal range of swine lymphocytes in the peripheral blood is 6.0-10.0 x 10^6 cells/mL, varying according to age,

sex and breed (41). There are three distinct populations of T lymphocytes in porcine peripheral blood, including αβ T cells, ϒδ T cells and non-T-non-B lymphocytes. αβ T cells consist of four subpopulations ($CD4^+CD8^-$, $CD4^+CD8^{lo}$, $CD4^-CD8^{lo}$ and $CD4^-CD8^{hi}$) and ϒδ T cells of three ($CD2^-4^-8^-$, $CD2^+4^-8^{lo}$ and $CD2^+4^-8^-$). Non-T-non-B lymphocytes, most of which are $CD2^+3^-4^-8^{lo}$ surface immunoglobulin-negative, have natural killer activity. The proportion of the ϒδ T cells can be up to 50 % of peripheral T lymphocytes. These two types of T cells are not detected in significant numbers in other species except chickens and ruminants. Porcine $CD4^+CD8^+$ T cells are the circulating mature memory /effector lymphocytes because of their demonstrated reactivity to alloantigens, superantigens or viral antigens (42, 43). Lymphocytes of similar phenotypes were also found in the porcine peripheral lymphoid tissues. The ratio of the ϒδ T cells and non-T-non-B lymphocytes are lesser, comparing to those in the peripheral blood, and most of them express CD2 and/or CD8 in the lymphoid tissues. In thymus, small thymocytes are predominantly of the phenotype $CD3^-4^+8^+$; large mature thymocytes express phenotypes similar to those of peripheral T lymphocytes (42, 44). Porcine $CD8^+$ T cells display high heterogeneity. Substantial numbers of circulating CD8αβ, $CD4^+CD8\alpha\alpha^+$ and $CD4^-CD8\alpha\alpha^+$ cells are found in the peripheral blood. Porcine $CD4^-$ $CD8\alpha\alpha^+$ cells, which have been recently defined by their low staining intensity with the CD8α chain specific monoclonal antibody (mAb 76-2-11), are

subdivided into ϒδ TCR^+ (T cell receptor), αβ TCR^+ and natural killer cells. Porcine $CD4^+CD8\alpha\alpha^+$ cells are found to be $CD8^{lo}$ whereas $CD8\alpha\beta^+$ cells are $CD8^{hi}$ by flow cytometric analysis. Different proliferation abilities of porcine $CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ cells were found in pigs experimentally infected with *Brachyspira* (*Serpulina*) *hyodysenteriae* (45). The pigs recovering from this colitic pathogen showed increased circulating $CD4^-CD8\alpha\alpha^+$ cells compared with non-infected pigs. The proliferating $CD8\alpha\alpha^+$ cells in antigen-stimulated cultures of peripheral blood mononuclear cells from *B. hyodysenteriae*-vaccinated pigs consisted of $CD4^-$, $CD4^+$ and ϒδ TCR^+ cells. Of the $CD8\alpha\alpha^+$ cells that had proliferated, the majority of the $CD4^+CD8^+$ were lymphoblasts (large size), while the $CD4^-CD8^+$ cells were predominantly small. *In vitro* depletion of $CD4^+$ cells did not completely abrogate the proliferative response of cells from vaccinated pigs to the *in vitro* stimulation with *B. hyodysenteriae* antigen. Therefore, $CD4^+$ cells might play a role in this proliferation response. However, $CD4^-CD8\alpha\beta^+$ cells from vaccinated or infected pigs did not proliferate after *in vitro* stimulation with this pathogen (43, 45, 46, 47).

The cell mediated immune response to PRRSV

Many scientists have been studying the porcine immune response to infection by the porcine reproductive and respiratory syndrome virus by experimentally infecting pigs of

various ages with different PRRSV strains. Wills et al. (48) intranasally infected 35-day-old pigs with the PRRSV isolate 16244B for 251 days and detected viral RNA in the serum and tonsil biopsy by reverse transcription polymerase chain reaction. They found that most pigs cleared that strain of PRRSV within three to four months, but some pigs remained persistently infected for several months longer. Nielsen et al. (49) intranasally challenged pregnant sows with a sixth porcine pulmonary alveolar macrophage cell culture passage of a Danish PRRSV isolate and determined the leukocyte phenotypes in peripheral blood and bronchoalveolar lavage fluid of the piglets by flow cytometric analysis. Viremia was shown in the piglets up to six weeks after birth; high levels of $CD8^+$ cells were found in the peripheral blood throughout the post-natal experimental period and in the bronchoalveolar lavage fluid up to four weeks after birth (50). Linden et al. (51) intranasally infected young pigs (six to eight weeks old) and old pigs (six months old) with three different PRRSV strains: LV ter Huurne (a European wild-type strain), LV4.2.1 (a cell-line adapted European strain) and SDSU#73 (an American strain). They found that young pigs were susceptible to infection as shown by a higher number of viremic and virus excreting pigs. They also observed different abilities of these three strains to induce viremia and virus shedding. In addition, the kinetics of the antibody response was different after injection with each of the three strains. Halbur et al. (28) intranasally infected five-week-old pigs with four different

PRRSV isolates: VR2385, ISU-984, ISU-22 (these three are highly pneumovirulent strains) and VR2431 (the least virulent isolate of all). They found that these strains caused non-regenerative anemia and increased myeloid:erythroid ratios from three to 21 days after inoculation. Labarque et al. (52) intranasally inoculated four- to five-week-old gnotobiotic pigs and then detected the appearance of anti-PRRSV antibody and characterized the bronchoalveolar lavage cell populations as well as the pattern of virus replication. In their results, total bronchoalveolar cells increased dramatically at 25 days post-infection and remained high until the end of the experiment; the highest number of monocytes/macrophages observed was also found at this time. Differentiated macrophages were reduced between nine and 20 days post-infection; monocytes and non-phagocytes entered the alveolar spaces between the same time interval. Highest virus replication was displayed at seven to nine days post-infection. At nine days post-infection, anti-PRRSV antibodies were detected, but neutralizing antibodies did not appear until 35 days post-infection. The investigations did not determine if the neutralizing antibodies contributed to the clearance of the virus from the lungs.

Cell mediated immunity to the infection of porcine reproductive and respiratory syndrome virus in different compartments of porcine immune system has been demonstrated. Some scientists used live or attenuated whole PRRS virus particles to

infect their experimental animals. Feng et al. (53) demonstrated dramatic loss of circulating lymphocytes, partially explained by the significant drop of peripheral $CD4^+$ T cells in the piglets infected in utero with strain SD23983 of PRRSV (at birth and 7 days of age). Decreased $CD4^+$: $CD8^+$ ratio resulting from the rebound circulating $CD8^+$ T lymphocytes was shown in the 14 day-old infected piglets. Small thymuses, cortical involution and severe cortical depletion of thymocytes were also accompanied immunosuppression in the neonatal piglets. Albina et al. (54) observed increased $CD2^+$, $CD8^+$ and IgM^+ three weeks after intranasal and subsequent intravenous infection with the strain SDRPI5D (an eighth passage of a Spanish isolate recovered from a sick piglet. In the experiment of Samsom et al. (55), the investigators demonstrated $CD2^+CD8^+CD4^-\gamma\delta TCR^-$ cells of both $CD8^+CD6^+$ (cytotoxic T cells) and $CD8^+CD6^-$ (natural killer cells) phenotypes in the broncho-alveolar lavage fluid of the gnotobiotic piglets infected with PRRSV strain ter Huurne. Molitor et al. (56) was the first group to develop and standardize methods allowing the study of the T-cell mediated mechanisms involved in PRRS; they optimized the conditions to detect specific T-cell proliferation responses to *in vitro* stimulation with PRRSV antigens (VR-2332 strain) by using miniature pigs that were homozygous for the major histocompatibility complex carrying the haplotype swine leukocyte antigen $(SLA)^{c/c}$. However, no strong evidence of the *in vitro* proliferating lymphocytes was PRRSV antigen-specific were shown in their study.

$CD4^+$ T cell proliferation was obtained after stimulating the peripheral blood mononuclear cells of pigs that had recovered from infection with European isolate 5710; however, $CD4^+CD8^+$ and $CD4^-CD8^+$ cells were the populations maintained in culture for up to three weeks after stimulating responding cells with the same virus strain, whereas $CD4^+CD8^-$ T cells declined over time in culture (57). Sipos et al. (58) did not note significant changes in CD4 and CD8 markers and cytokine expression (including IL-1α, IL-2, IL-4, IL-6, IL-8, IL-10, tumor necrosis factor-α, interferon-γ and IL-2 receptor) in the piglets (6-week old) vaccinated with a European modified-live PRRSV, strain DV.

Bautista et al. (59) intranasally inoculated five to six week-old pigs with the strain VR-2332 twice with the interval ten to twelve weeks and performed lymphocyte proliferation assays using the structural polypeptides, ORF 2 to 7, as the stimulants for the peripheral blood lymphocytes. The investigators observed significant antigen-specific and dose-dependent proliferative responses to ORF2, 5 and 6 in virus-infected but not in control pigs. Pizadeh et al. (60) intradermally and intramuscularly immunized piglets with a DNA plasmid encoding glycoprotein 5 (GP5) of the Quebec cytopathic strain IAF-Klop three times at two-week intervals; they demonstrated the specific cellular immune response to GP 5 by stimulating the peripheral blood mononuclear cells from the DNA-vaccinated pigs with *E.*

coli-expressing recombinant ORF5-encoded protein. However, these investigators did not identify the phenotypes of the proliferating lymphocytes.

The persistence of porcine reproductive and respiratory syndrome virus in the infected host suggests that the cell-mediated immune does not efficiently cleat the virus. Lamontagne et al. (61) studied the relationship between viral persistence and cytotoxic cells in blood, spleen, mediastinal lymph nodes and tonsil of PRRSV (the LHVA-93-3 isolate) infected pigs. In their results, no change in the proportions of $CD2^{+}CD4^{+}$ cells in the blood or lymphoid organs was observed; the percentage of $CD2^{+}CD8^{high}$, but not $CD2^{+}CD8^{low}$, increased in spleen, blood and mediastinal lymph node from ten to sixty days post-infection, but decreased in tonsils; the $CD2^{+}$MIL-4^{+} cells (natural killer cells) were not significantly modified in blood or lymphoid organs. The investigators concluded that the impaired $CD2^{+}CD8^{high}$ cell response in mediastinal lymph node and tonsils favors viral persistence in these organs, and the same cell population helped viral elimination in blood and spleen.

The purpose of this research

Porcine reproductive and respiratory syndrome virus (PRRSV) causes serious disease in pigs and results in enormous economic losses to the pork industry. The available commercial vaccines do not always provide cross-protection against heterologous strains. Therefore, it is necessary to develop new vaccines that provide

effective and, ideally, cross-protective, immunity to various strains of PRRSV. To generate these vaccines, it is necessary to understand which components of the porcine immune system are important for resistance to this viral pathogen. In this study, we have focused on the T cell compartment of the swine immune system and characterized the phenotypes of T cells that respond to a North Carolina strain of PRRSV, the Powell isolate. Our results suggest that virus-specific T lymphocytes could be found in the peripheral blood and lymph nodes of the pig that recovered from experimental virus infections.

Materials and Methods

Experimental animals

The experimental animal protocol used in this study was approved by the Institutional Animal Care and Use Committee (IACUC) of Laboratory Animal Resources at North Carolina State University. Male Yorkshire pigs, six weeks old, were purchased from a specific pathogen free herd in North Carolina and housed in an isolation facility in the College of Veterinary Medicine at North Carolina State University. A total of six pigs were divided into two groups of three and kept in two isolated animal rooms.

Virus infection and rechallenge for the animals

The viremic serum of titer 10^3 $TCID_{50}$/ml used to infect animals was collected from clinically affected pigs in a North Carolina herd experiencing an acute outbreak of the Powell isolate PRRSV. Mr. Jinsheng Xu confirmed the presence of PRRSV using an indirect immunofluorescence assay with ten-fold serial dilutions of the serum from the infected animal onto porcine pulmonary alveolar macrophages. Each of the three pigs was injected 0.5 ml of this viremic serum intramuscularly at both sides of the neck (2-3 centimeters behind the ears). Thirty-three days after the first infection, these three pigs received the same amount of the serum at the same sites. Rectal temperatures were

taken once daily for the first weeks after the two infections. During the entire course of this study, the three pigs used as control animals did not receive any injections, treatments or administrations.

Virus stock preparation and quantification

The porcine alveolar macrophages used to amplify and quantify the Powell isolate of porcine reproductive and respiratory syndrome virus were kindly provided by Mr. Jinsheng Xu. Viremic serum used to amplify the Powell virus was the one used to infect the experimental pigs of this study. After thawing in the 37 ^{o}C water bath, the porcine alveolar macrophages were washed with 30 ml serum-free RPMI1640 media containing 100 u/ml Penicillin-Streptomycin-Amphotericin (Gibco BRL). Porcine alveolar macrophages (2 x 10^{6}) were plated in a T25 tissue culture flask (Corning) with 4 ml of complete RPMI1640 media [containing 10 % heat-inactivated fetal bovine serum (FBS, Atlanta Biologicals), 100 u/ml Penicillin-Streptomycin-Amphotericin (Gibco BRL) and 5 x 10^{-5} M 2-mercaptoethanol (Sigma)]. When the cells attached to the bottom of the flask, the medium was removed. Viremic serum (0.75 ml) was added into the flask and incubated at the room temperature for 1 hour on the slow-spinning shaker. Then, 7 ml of complete RPMI1640 media was added the flask. The flask was incubated at 37 ^{o}C in a 5 % CO_2 incubator. After 50 hours, the flask was frozen at –80 ^{o}C and thawed at room temperature to completely lyse the cells. The cell suspension was collected and

centrifuged at 3000 rpm at 4 ^{o}C. The supernatant, referred as Powell Virus Stock, was harvested, aliquoted and stored at –80 ^{o}C until use. To determine the titer of this virus stock, 2 x 10^5 porcine alveolar macrophages were plated in each well of a 96-well tissue culture plate (Corning) with 100 μL complete RPMI1640 media per well. Powell Virus Stock was diluted 10-fold in complete RPMI1640 media. 100 μL of diluted Powell Virus Stock was added to each well of 8 replicates. Then the tissue culture plate was incubated at 37 ^{o}C in a 5 % CO_2 incubator. The titer of Powell Virus Stock, expressed as $TCID_{50}$ (tissue culture infective dose 50)/ml, was determined after 48 hours. The $TCID_{50}$ of the Powell Virus Stock is defined as the dilution required to infect 50 % of a given batch of inoculated cell cultures of porcine alveolar macrophages (63). The infection of porcine alveolar macrophages by virus in our experiment was determined by cellular cytopathy observed by microscope.

Isolation of porcine peripheral blood mononuclear cells

Porcine peripheral blood was drawn into Sodium Heparin-coating tubes (VACUTAINER®) from jugular veins using 20.5 G blood collection needles (VACUTAINER®). Porcine peripheral blood mononuclear cells (PBMCs) were separated from heparinized blood by Histopaque-1077 (Sigma). Heparinized blood was diluted 1:1 in Hank's Balanced Salt Solution (HBSS, without Ca^{2+} and Mg^{2+}, Sigma). The blood mixture was overlaid over an equal volume of Histopaque 1077® (Sigma)

and then underwent centrifugation in 650 x g for 30 minutes at 25 ^{o}C. The cells at the interface were harvested, washed 2 times with 10 ml HBSS without Ca^{2+} and Mg^{2+} and then resuspended with complete RPMI1640 media.

Collection of lymph node cells

To euthanize the pigs, 10 ml of Beuthansia Solution (Schering-Plough) was injected into the jugular vein. One of the retropharyngeal lymph nodes, located in the retropharyngeal lymph center, was found level with the temporomandibular joint between the parotid gland laterally and the cleidomastoideus medially, a few centimeters caudomedial to the parotid nodes. The retropharyngeal lymph nodes drain the superficial parts of the head-neck junction and their efferents go to the dorsal superficial cervical nodes. One of the tracheobronchial lymph nodes was dissected from the bifurcation of the trachea, which is the part of the bronchial lymph center. The lymph nodes in the bronchial lymph center drain the lungs, heart and pericardium; their efferents go to the cranial mediastinal nodes or to the thoracic duct (64). The tracheobronchial and lateral retropharyngeal lymph nodes were separately kept in complete RPMI1640 media on ice after dissection. A lymph node was placed in a 10 mm sterile petri dish and a 20 G needle was used to repeatedly poke the lymph node. Complete RPMI1640 (15 ml) was injected into the lymph node by a needle attached syringe. The cell suspension was collected, passed through 150 μm nylon mesh

(Sefar-America) and then centrifuged at 1500 rpm for 5 minutes at room temperature. The cell pellet was resuspended with complete RPMI1640 media and used in following experiments.

Cell labeling with PKH67 green fluorescent dye

To label cells with PKH67 green fluorescent dye (Sigma), 2 x 10^7 peripheral blood mononuclear cells or lymph node cells were wash with serum-free RPMI-1640 medium containing 100 u/ml Penicillin-Streptomycin-Amphotericin and resuspended in 1 ml of Diluent C (Sigma). PKH67 fluorescence dye (2 x 10^{-6} M, Sigma) was added to the cell suspension and incubated for 5 minutes. Heat-inactivated fetal bovine serum (2 ml) was added to the cell mixture, incubated for 1 minute and then washed three times with complete RPMI-1640 medium. All steps described above were performed at room temperature (65).

In vitro stimulation of immune cells with virus stock

PKH67-stained cells were plated in 6-well tissue culture plates (Corning; 2x10^6 cells/well) and treated with medium only, Concanavalin A (Con A, 5 μg/ml, Sigma), or Powell Virus Stock of 10^3 or 10^5 $TCID_{50}$/ml as the final concentration. Complete RPMI1640 media (4 ml) was used in each well in the plates. Cells were incubated at 37 ^oC in a 5 % CO_2 incubator for 5 days.

Cluster of differentiation (CD) antigens detection and proliferation assay

At the end of the 5-day incubation, the cells were harvested and equally distributed into 12 x 75 mm polystyrene round-bottom tubes (Falcon) and centrifuged at 1200 rpm for 5 minutes at room temperature. Complete RPMI1640 media (100 μL), R-phycoerythrin-conjugated goat-anti-mouse IgG secondary antibody (Jackson ImmunoReseach, West Grove, Pennsylvania), mouse-anti-porcine CD4 monoclonal antibody (Veterinary Medical Research and Development, VMRD, Pullman, Washington), mouse-anti-porcine CD8 monoclonal antibody (VMRD), or mouse-anti-porcine CD25 monoclonal antibody (VMRD) were added in different tubes. All these monoclonal antibodies and the goat-anti-mouse IgG secondary antibody were 1:100 diluted in complete RPMI1640 media. The cells were incubated with the diluted antibodies at room temperature for 2 hours. After washing 2 times with phosphate buffered saline (PBS, Sigma) containing 5 % Bovine Serum Albumin (BSA, Sigma), 100 μL of 1:100 diluted R-phycoerythrin-conjugated goat-anti-mouse IgG secondary antibody was added to the cells incubated with mouse-anti-porcine CD4, CD8 or CD25 monoclonal antibodies and incubated at room temperature for 1 hour. The cells were then washed 2 times with PBS containing 5 % BSA and resuspended in 100 μL of complete RPMI1640 media plus 10 % heat-inactivated FBS. The PKH67 dye intensity and the expression of CD4, CD8 or CD25 of the cells were detected and analyzed by flow cytometrs (FACSCalibur®, Becton Dickinson) using CellQuest acquisition and

analysis software (version 3.3, Becton Dickinson).

Results

Clinical signs in PRRSV-infected pigs

To investigate the T cell response to PRRSV, an animal model needed to be established. We chose to use the Powell virus isolate, since this strain causes devastating economical loss in the pork industry in North Carolina. The virus-infected pigs in our experiment became lethargic four days after the first infection. Starting from five days after the first infection, bloody diarrhea, serious nasal and eye discharge, depression and blushing skin was observed in all three infected pigs. They also developed fever and consumed more water than the uninfected control pigs. These clinical signs of porcine reproductive and respiratory syndrome began to decline eight days after the first infection and completely disappeared after thirteen days. Thirty-three days after the first infection, these three pigs were re-challenged with 1.0 ml of the same viremic serum (10^3 $TCID_{50}$/ml). Similar clinical signs, although much less severe than described above, were observed in all these three pigs within two days after virus re-challenge. Diarrhea was also observed, but the stool was not bloody. The clinical signs declined completely within six days after virus re-challenge. During the entire course of this study, all three of the control pigs were healthy and did not show any clinical signs of disease. Based on these results, we concluded that those pigs recovered

from infection by the Powell virus isolate were suitable for use as a model for the study of the T lymphocyte response to experimental infection by this virus.

The response of normal PBMCs to Con A

The goal of our study was to characterize the T cell response to infection by PRRSV. Therefore, we had to characterize the phenotypes of both resting and activated T lymphocytes in the normal pigs. Con A, a lectin, was used as a non-specific stimulator for activating lymphoid cells in our study. The effect of Con A on normal swine peripheral blood mononuclear cells was investigated by culturing the PKH67 labeled peripheral blood mononuclear cells from a normal pig with different concentrations of Con A (1 μg/ml, 5 μg/ml and 25 μg/ml) for five days. Cell proliferation in a dose-dependent manner was observed in our results (data not shown), with 25 μg/ml causing the greatest proliferative effect. In our study, we chose to use the concentration 5 μg/ml to avoid activation-induced apoptosis throughout this study, color coding is used to display flow cytometric data. The region R2, labeled as pink, is the region in which resting lymphocytes with small size and low amount of granularity reside. The region R1, labeling with green, represents the area where activated or proliferating lymphocytes exhibiting increased size and granularity. The orange-colored region R3 is the combination of R1 and R2. However, the color of the region R3 cannot be observed because the overlapping of the colors of R1 and R2. Each PKH67 intensity diagram and

dot plot (including CD4, CD8 and CD25 expression) versus PKH67 intensity is shown in orange, because the PKH67 intensity and the CD molecule expression of the R3 regions were used to present the data. As shown in Fig. 1, in PBMCs from the normal pig, the percentage of the resting lymphocytes was 33.3 % (of the total cell events of Fig. 1A), while activated lymphocytes or lymphoblasts was 2.3 % of the total cell events. In this compartment, the percentages of the CD4, CD8 and CD25 expressing cells of total lymphocytes (R3 regions) were 11.3 % (of 5524 cells), 22.9 % (of 7022 cells) and 1.0 % (of 6502 cells), respectively (Fig. 1B, C and D). After treating with Con A (5 μg/ml) for five days in culture, the cell numbers increased dramatically in the R1 region (5938 cells/39.6 % of total cell events, Fig. 2B), which did not happen in the R1 region of the media only control (122 cells/0.8 % of total cell events, Fig. 2A). As shown in Fig. 2D, cells with decreasing amounts of PKH67 intensity appeared in the culture, indicating that cell proliferation had occurred. The percentages of the cells expressing CD4, CD8 or CD25 among the activated lymphocytes increased after Con A treatment (Fig. 3). Reduced levels of PKH67 staining also indicated that these cells were proliferating (Fig. 3). Similar results as described above were obtained from three independent experiments (Table I).

The response of normal lymph node cells to Con A

To characterize and evaluate the ability of the lymph node cells of the normal pig to

respond to a stimulant, we incubated the cells of the tracheobronchial or lateral retropharyngeal lymph nodes from the normal pig with or without Con A for five days. Different profiles of phenotypes, cell composition, and the response to Con A were observed in the tracheobronchial or lateral retropharyngeal lymph node cells of the normal pig. The forward and side scatter plot of normal tracheobronchial lymph node reveled fewer cells in both R1 and R2 regions (the cell numbers/percentages of total cell events: R1 = 173/1.2 %, R2 =1616/10.8 %; Fig. 4A), compared to the normal lateral retropharyngeal lymph node in (R1 = 1010 cells/6.7 %, R2 = 7942 cells/53.0 %, Fig. 4B). The percentages of CD4, CD8 or CD25 expressing cells among the total lymphocyte population of the lateral retropharyngeal lymph node (31.1 %, 18.4 % and 21.3 %, respectively; Fig. 4D, F and H) were also more than that of the tracheobronchial lymph node (27.7 %, 7.4 % and 5.7 %, respectively; Fig. 4C, E and G). After treatment with Con A, the tracheobronchial lymph node cells proliferated (Fig. 5J), as the population in the gated region M2 had declined and the number of a population with lower PKH67 dye labeling increased in the gated region M4 (Fig. 5J), compared to Fig. 5F. In the case of lateral retropharyngeal lymph node, the cells in this compartment were activated after Con A treatment as increased cell percentage was shown in the region R1 in Fig. 5K. Cell proliferation occurred in the media only treated culture of lateral retropharyngeal lymph node (Fig. 5H), since the PKH67 staining profile of Fig.

5H is different from that of before culture (Fig. 5D). However, Con A treatment did not result in further proliferation as demonstrated by the similar profile of cells cultured in media only (Fig. 5H), compared to cells stimulated with Con A (Fig. 5L). The percentages of CD4, CD8 or CD25 expressing cells among total lymphocytes in R3 regions in both tracheobronchial and lateral retropharyngeal lymph nodes increased dramatically after treated with Con A [for tracheobronchial lymph node, the percentages among total lymphocytes (R3 regions): CD4 = 35.7 % of 6252 cells, CD8 = 24.0 % of 8728 cells, CD25 = 21.9 % of 6697 cells; Fig. 6A, C and E, respectively; for that of lateral retropharyngeal lymph node: CD4 = 47.6 % of 5535 cells, CD8 = 33.4 % of 6845 cells, CD25 = 30.1 % of 6357 cells; Fig. 6 B, D and F, respectively].

The response of normal PBMCs and lymph node cells to PRRSV

To demonstrate the potential lymphoid compartments and lymphocyte subpopulations of the naive pig that were capable of responding to the Powell virus, we treated peripheral blood mononuclear cells and lymph node cells from the normal pig with Powell virus1of 10^5 $TCID_{50}$/ml as the final concentration. Compared to the corresponding compartments of the media only control (cell numbers/percentages in the gated regions over total cell events: 365/0.7 %, 755/5.0 % and 584/3.9 %, respectively; Fig. 7A, C and E), the Powell virus isolate of porcine reproductive and respiratory syndrome virus induced slight increase of cell percentages (over total cell events) in the

R1 regions of the peripheral blood (762/2.5 %), tracheobronchial lymph node (962/6.4 %) and lateral retropharyngeal lymph node (1271/8.5 %) from the normal pig (Fig. 7 B, D and F). Cell proliferation did not occur in the culture of peripheral blood mononuclear cells before or after the treatment with the Powell virus since similar profiles of PKH67 fluorescence intensity for the R3 regions were observed for that of the media control (Fig. 8B) and of the cells treated with Powell virus (Fig. 8C), as well as that of before culturing (Fig. 8A). Because different cell population carrying very different PKH67 intensity were observed in the lymph node cultures before and after culturing with media only (Fig. 8D and E; Fig. 8G and H), certain cell populations in both lymph nodes were able to proliferate *in vitro* without any stimulants. However, since similar PKH67 intensity profiles were shown in Fig. 8E and F and Fig. 8H and I, the treatment of Powell virus did not cause further cell proliferation in the cultures of lymph node cells. Compared to the media only control, the percentages of CD4 or CD8, but not CD25, positive cells in the R3 regions of lateral retropharyngeal lymph node increased after stimulated with Powell virus (Fig. 9). But the CD4, CD8 or CD25 positive cells did not increase in the compartments of peripheral blood and tracheobronchial lymph node after stimulation of the Powell virus (Fig. 9). The CD4, CD8 or CD25 positive cells in the R1 regions of all three compartments decreased after treatment of this virus (Fig 9 legend).

The response of PBMCs and lymph node cells from the PRRSV-infected pig to PRRSV

In order to study the Powell virus-specific T lymphocytes and to determine which compartment(s) they reside in, we used the pig that recovered from infections with this virus. We stimulated the peripheral blood mononuclear cells and lymph node cells with or without the same virus of 10^5 $TCID_{50}$/ml for five days. After *in vitro* stimulation of the cells from three different compartments, including peripheral blood, tracheobronchial and lateral retropharyngeal lymph nodes, of the Powell virus infected and re-challenged pig with the same virus, we observed increased cell percentages in the R1 regions, although the increase for tracheobronchial lymph node was minimal (Table II). In vitro simulation with the Powell virus resulted in increased activated lymphocytes in the cultures of all three compartments (Fig. 10B, D and F; Fig. 11E, K and Q). Cell proliferation occurred in the peripheral blood, tracheobronchial and lateral retropharyngeal lymph node after culturing with (Fig. 11F, L and R) or without (Fig. 11D, J and P) Powell virus as demonstrated by different PKH67 staining, compared to those before culturing (Fig. 11B, H and N). The percentages of CD4, CD8 or CD25 expressing activated lymphocytes were clearly increased in the lateral retropharyngeal lymph node (Fig. 12). In the cultures of peripheral blood mononuclear cells and tracheobronchial lymph node cells from this Powell virus-infected pig, the percentages of CD4, CD8 or CD25 expressing activated lymphocytes did not increase after the

co-culture for five days with the same strain of virus (Fig. 12). The percentage of $CD8^+$ cells in the R2 region of the virus-treated culture of tracheobronchial lymph node increased (Fig. 12 legend), suggesting that some effector $CD8^+$ cells maybe resided in this lymph node. In Fig. 13 A and B, the treatment of Powell virus caused increase in the percentages of $CD4^+$ T cells in both R1 and R2 regions of all three compartments from the virus infected pig. Co-culture with Powell virus also resulted in the increased percentages of $CD8^+$ T cells in R1 regions of both lymph nodes and in R2 regions of peripheral blood mononuclear cells and tracheobronchial lymph node cells from the virus infected pig. The percentages of $CD25^+$ T cells increased in the R2 regions of the peripheral blood and tracheobronchial lymph node from the virus infected pig after virus treatment (Fig. 13 B).

Discussion

In order to characterize the proliferative ability and phenotype of the circulating lymphocytes of the normal pig, we withdrew the jugular blood stained the purified peripheral blood mononuclear cells with anti-CD4, anti-CD8 or anti-CD25 monoclonal antibodies before and after stimulation with Con A. The percentages of $CD4^+$ and $CD8^+$ T cells were 11.3 % of 6071 cells (the cell events in the gated region R3) and 22.9 % of 7022 cells (the cell events in the gated region R3), respectively (Fig. 1 B and C; which correspond to 4.8 % and 10.8 % of the total cell events, respectively). This result is similar to that of Yang et al., in which the ranges of $CD4^+$ and $CD8^+$ T cells of the total peripheral blood mononuclear cells in the pigs of similar age are 3.4 +/- 2.5 % and 9.9 +/- 5.9 %, respectively (42). The number of $CD25^+$ cells in the normal pig was very low, corresponding to the small cell amounts in the region R1 (Fig. 1A). PKH67, a green fluorescence dye containing long aliphatic carbon tails, labels cells by stably incorporating into the lipid region of the cell membrane. In this study we used this fluorescent dye to monitor cell proliferation, as the PKH67 intensity in the cell membranes of the daughter cells will be half of the PKH67 intensity of the mother cells after one cell division. Con A had clear stimulating effect on the peripheral blood mononuclear cells of the normal pigs: many cells appeared in the region R1 after culturing with Con A for five days (Fig. 2B), compared to the very small number of

cells in the same region of the cells cultured in media only (Fig. 2A). The various cell populations carrying different intensity of PKH67 dye proved that the Con A-treated culture had undergone proliferation (Fig. 2D). The proliferating cells were probably activated $CD4^+$ and $CD8^+$ T lymphocytes, since cells expressing high level of $CD4^+$, $CD8^+$ or $CD25^+$ and carrying diverse PKH67 concentrations were observed in the upper left quadrants in the dot plots of CD molecule expression versus PKH67 intensity (Fig. 3). This result was expected because Con A is a lectin capable of stimulating the proliferation of T lymphocytes. Although similar proliferative responses were observed in three independent experiments, variation of the percentages of the $CD4^+$, $CD8^+$ or $CD25^+$ cells were noted among the three experiments. The possible reasons for this variation could be due to different ages of the pigs since the three experiments were performed at different times. Bautista et al. (59) and Pizadeh et al. (60) also obtained proliferation response of the porcine peripheral blood mononuclear cell from normal pigs to Con A treatment in their experiments, but they did not confirm the phenotype of the proliferating cells. We also characterized the proliferative response and phenotypic profile of the lymph node cells from the normal pig after Con A stimulation. We chose to take the lateral retropharyngeal lymph nodes since this lymph node is the local draining lymph node of the site of virus challenge. The tracheobronchial lymph node was also selected since this lymph node is potentially disease-involved. As shown in Fig.

4 and Fig. 5, the tracheobronchial and the lateral retropharyngeal lymph nodes, although both are secondary peripheral lymphoid organs, have different phenotypic profiles. The normal tracheobronchial lymph node had very few activated lymphocytes and relatively few resting lymphocytes (Fig. 4A), while the normal lateral retropharyngeal lymph node had more activated lymphocytes and resting lymphocytes (Fig. 4B). The percentages of $CD4^+$, $CD8^+$ or $CD25^+$ cells among total lymphocytes (R3) was also higher in this compartment (Fig. 4 D, F and H). Con A treatment resulted in an increase in activated cells in both tracheobronchial and lateral retropharyngeal lymph node (R1 regions; Fig. 5 I and K) and increased percentages of $CD4^+$, $CD8^+$ or $CD25^+$ cells over the cell events in the R3 regions (Fig. 6 A-F). In the case of the tracheobronchial lymph node, co-culture with Con A stimulated cell proliferation as three distinguishable cell populations with different PKH67 staining intensity can be observed in the diagrams and dot plots of CD molecule expression versus PKH67 intensity (Fig. 5 J and Fig. 6 A, C and E), compared to the two cell populations shown before *in vitro* culture (Fig. 4 C, E and G). To our surprise, the proliferating cells were not of $CD4^+$, $CD8^+$ or $CD25^+$ (Fig. 6 A, C and E). However, in the case of lateral retropharyngeal lymph node, Con A treatment did not cause cell division (demonstrated by the similar PKH67 staining profiles of Fig. 5 F and H), although we did observe an increase in activated lymphocytes (Fig. 5 K). The possible explanation could be the lateral retropharyngeal

lymph node cells were pre-activated by other airborne antigens. Therefore the cells in this compartment might not proliferate in response to stimulation with Con A. Another possible reason could be that the lymph node cells require higher concentrations of Con A in order to stimulate the proliferative response.

We also examined the response of normal PBMCs and lymph node cells to the stimulation with PRRSV. Comparing to the R1 regions of the three compartments of the cells cultured with media only (Fig. 7 A, C and E), the treatment with the virus resulted in slight increase of the activated lymphocytes (R1 regions in Fig. 7 B, D and F), but no cell proliferation was obtained since similar PKH67 profiles were observed in the diagrams of the cells in media only control (Fig. 8 B, E and H) and cells treated with Powell virus (Fig. 8 C, F and I). Among total lymphocytes, the percentages of $CD4^+$ and $CD8^+$ T cells increased in the cultures from normal lateral retropharyngeal lymph node cultures after exposure to the Powell virus (Fig. 9), whereas the $CD4^+$ or $CD8^+$ T lymphocytes in the peripheral blood did not survive better when treated with Powell virus in the culture (Fig. 9). The percentages of $CD4^+$, $CD8^+$ or $CD25^+$ cells among total lymphocytes in the tracheobronchial lymph node culture did not change dramatically with or without virus treatment (Fig. 9), so this lymph node may not be critical for virus challenge. The lack of activated cells following exposure to the Powell virus suggests that the normal pig in our study did not have any Powell virus specific lymphocyte. To

investigate virus specific lymphocyte response in pigs that had recovered from infections by the Powell virus, we intramuscularly infected our experimental pigs with the Powell virus of 10^3 $TCID_{50}$/ml twice with the interval of thirty-three days. The peripheral blood mononuclear cells, lateral retropharyngeal lymph node cells and tracheobronchial lymph node cells were collected, labeled with PKH67 dye and stimulated *in vitro* with the Powell virus (10^5 $TCID_{50}$/ml for five days). We found that all lymphocyte cultures from the infected pig displayed considerable proliferative activity, even in the absence of added virus. This spontaneous proliferative activity was not seen with the lymphocytes from the normal pig. It is possible that this activity arises from persistent PRRSV in the pig that was transferred with the lymphocytes in culture. Alternatively, this activity may rise from cytokine or endocrine effects in the recovering pig. We also found higher levels of CD4 and CD8 expression in the activated cells (R1 regions). To demonstrate the phenotypes of the culture of three different lymphoid compartments treated with or without the Powell virus, we incubated the cells with mouse anti- swine CD4, mouse anti-swine CD8 or mouse anti-swine CD25 monoclonal antibodies and then detected expression of these molecules by R-phycoerythrin-conjugated goat-anti-mouse IgG secondary antibody. The percentages of $CD4^+$, $CD8^+$ or $CD25^+$ cells among the total lymphocytes in the peripheral blood of the pig recovered from our experimental infections did not increase after *in vitro*

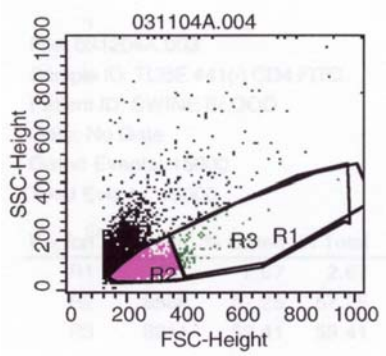
incubation with the same virus (Fig 12). In contrast, the percentages of the activated $CD8^+$ T cells in the lateral retropharyngeal lymph nodes of the same pig did rise obviously after encountering the same virus in culture. In the culture of lateral retropharyngeal lymph node cells with virus treatment, the percentages of activated $CD4^+$ and of activated $CD25^+$ cells also increased (Fig. 12). Our results are different from those of Fuertes et al. (57). Those investigators oro-nasally infected pigs with the European strain 5710 once and allowed them to recover from the infection. Then, they incubated the virus–specific T cell culture (generated by restimulation of peripheral blood mononuclear cells of the recovered pigs weekly with the strain 5710 and recombinant human IL-2) with the same virus for up to three weeks. Their results demonstrated an increase in $CD4^+CD8^+$ and $CD4^-CD8^+$ lymphocytes within the activated cells and the decline of $CD4^+CD8^-$ cells in the culture. The virus-specific T lymphocyte response they observed was not detectable until four weeks after inoculation and remained detectable for more than three months; virus-specific T lymphocytes were generated *in vitro* culture. Our results shown in Table II, Fig. 11 and Fig. 12, obtained using the pigs three weeks after the second experimental infection, show the potential Powell virus specific clone(s) of lymphocytes could be found in peripheral blood. However, the percentages of $CD4^+$, $CD8^+$ and $CD25^+$ T cells in peripheral blood did not increase after treatment with virus compared to the media only

control (Fig. 12). We examined the T cell response of the lymph nodes, the lymphoid compartments serving as the reservoir of various antigens, since the Powell virus infected macrophages and dendritic cells should reside in these two compartments. Different T cell responses to the *in vitro* stimulation with the same virus were also observed in the three lymphoid compartments in the pig infected with the Powell virus (Fig. 12). Since the percentage of activated $CD8^+$ T cells was high in the virus-treated culture of the local draining lymph node at the site of our experimental virus infections (Fig. 12), $CD8^+$ T cells may play an important role in the virus clearance and immune memory for the recalling viral antigen(s); the increase of the percentage of activated $CD4^+$ T cells in this culture might had contributed to the cell mediated immunity and humoral immune response in this pig that recovered from virus infections. Since lymphocyte proliferation was observed in the peripheral blood (Fig. 11 D and F), we conclude the virus specific lymphocyte clone(s) had developed in the Powell virus infected and re-challenged pig in our study. The data in Fig. 13 suggests that those virus-specific lymphocytes developed in the pig that recovered from experimental infections may involve both $CD4^+$ and $CD8^+$ T cells. We did not conduct statistical analysis of our data because of limited sample. For further investigation of this disease, more samples should be collected in order to perform statistical analysis and determine if the data shown in our study is repeatable.

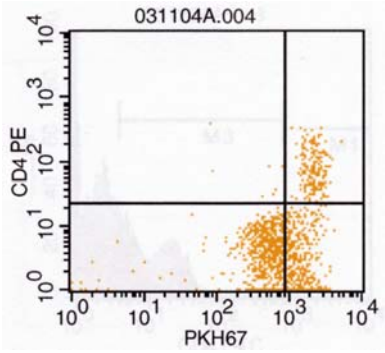
Figures and Tables

Fig. 1

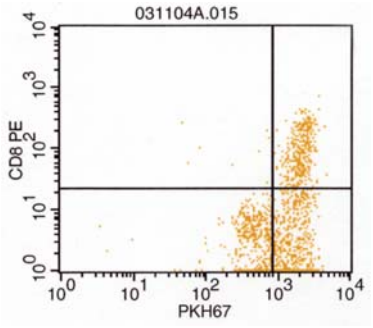
A.



B.



C.



D.

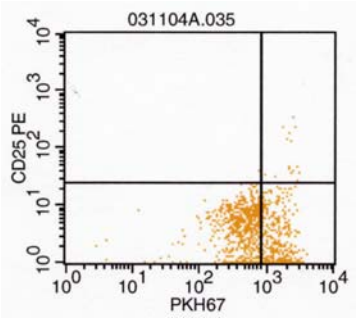
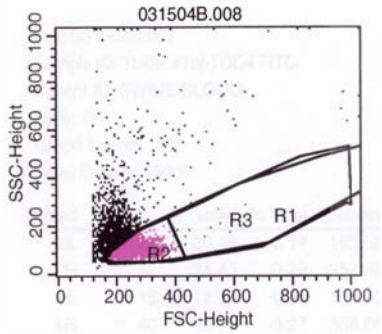


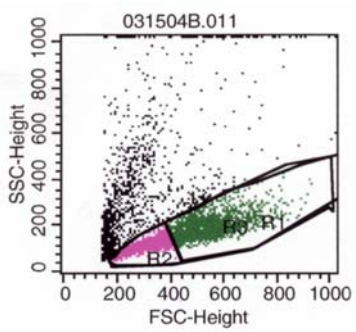
Fig. 1 The phenotypic profile of PBMCs from the normal (control) pig. Fresh PBMCs (without *in vitro* culture) were stained with monoclonal mouse anti-swine CD4, CD8 or CD25 antibodies. A. Forward and side scatter plot. The cell numbers/percentages over total cell events: R1 = 147/2.3 %, R2 = 5524/33.3 % and R3 = 5736/37.8 %. B, C and D. The dot plots of PKH67 intensity versus CD4, CD8 or CD25 expression detected by the R-phycoerythrin-conjugated goat-anti-mouse secondary antibody. The percentages of the cells expressing CD4, CD8 or CD25 in the R3 regions were 11.3 % (of 5524 cells, B), 22.9 % (of 7022 cells, C) and 1.0 % (of 6502 cells, D), respectively. The mean fluorescence intensity (mfi) of PKH 67 dye: B. 1120.0, C. 1489.9, D. 997.1.

Fig. 2

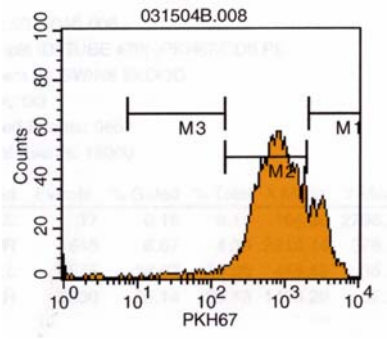
A.



B.



C.



D.

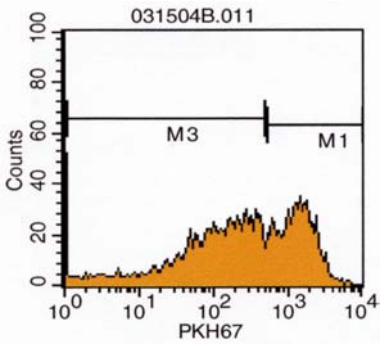
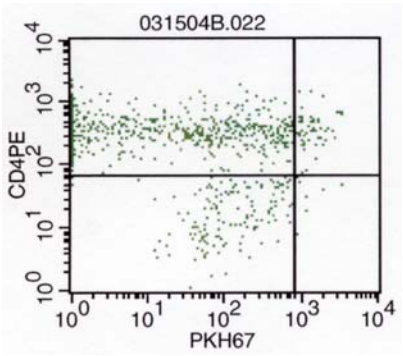


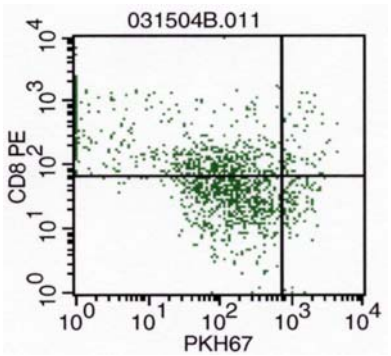
Fig. 2 The response of PBMCs from the normal pig to Con A. A. The normal PBMCs were incubated with Con A of 5 μg/ml for five days at 37°C and 5 % CO_2. The forward and side scatter plot of the cells cultured in media only. The cell numbers/percentages over total cell events: R1 = 122/0.8 %, R2 = 8418/56.1 %, R3 = 8928/59.5 %. B. The forward and side scatter plot of the cells stimulated with Con A. The cell numbers/percentages over total cell events: R1 = 5938/39.6 %, R2 = 4154/27.7 %, R3 = 10158/67.7 %. C. The PKH67 intensity diagram of the gated region R3 from the cells cultured in media only. D. The PKH67 intensity diagram of the gated region R3 from the cells stimulated with Con A. The mean fluorescence intensity (mfi) of PKH 67 dye: C. 1183.3, D. 605.6.

Fig. 3

A.



B.



C.

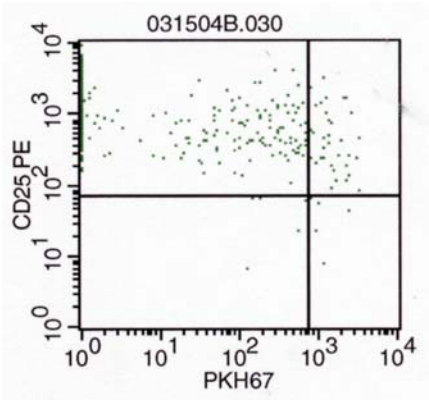


Fig. 3 The phenotypic profiles of PBMCs from the normal pig after Con A treatment. The normal PBMCs were incubated with Con A (5 μg/mL) for 5 days at 37°C and 5 % CO_2. A, B and C The dot plots of PKH67 intensity versus CD4, CD8 or CD25 expression. The data are from R1 regions as shown in Fig. 2. The percentages of the cells expressing different CDs among activated lymphocytes (R1 regions): A. CD4: 84.8 % (of 3891 cells) B. CD8: 50.6 % (of 5938 cells) C. CD25: 99.0 % (of 3125 cells). The percentages of the cells expressing different CDs among total lymphocytes (R3 regions): A. CD4: 49.8 % (of 10941 cells) B. CD8: 43.1 % (of 10158 cells) C. CD25: 74.7 % (of 6671 cells). The mean fluorescence intensity (mfi) of PKH67 dye: A. 749.4, B. 800.2, C 892.4.

Table I

	Day 0 (% in R3)				
CDs	Exp 1	Exp 2	Exp 3	Mean	SE
CD4	11.3	21.0	20.5	17.6	3.86
CD8	22.9	13.4	11.5	15.9	4.32
CD25	1.0	3.9	2.2	2.4	1.03

	After five days culture									
	Media only (% in R1)					Con A (% in R1)				
CDs	Exp 1	Exp 2	Exp 3	Mean	SE	Exp 1	Exp 2	Exp 3	Mean	SE
CD4	20.5	16.8	26.8	21.4	3.58	84.8	85.4	88.5	86.2	1.40
CD8	33.4	35.9	36.1	35.1	1.06	50.6	68.3	66.7	61.9	6.92
CD25	2.6	3.3	2.8	2.9	0.25	99.0	99.8	99.4	99.4	0.28

Table I Summary of responses to Con A by normal swine T cells. The percentages of the cells expressing CD4, CD8 or CD25 with or without Con A (5 μg/mL) before or after 5 days. This table summarizes 3 independent experiments (including the data showing in Fig. 1 and 3).

Fig. 4

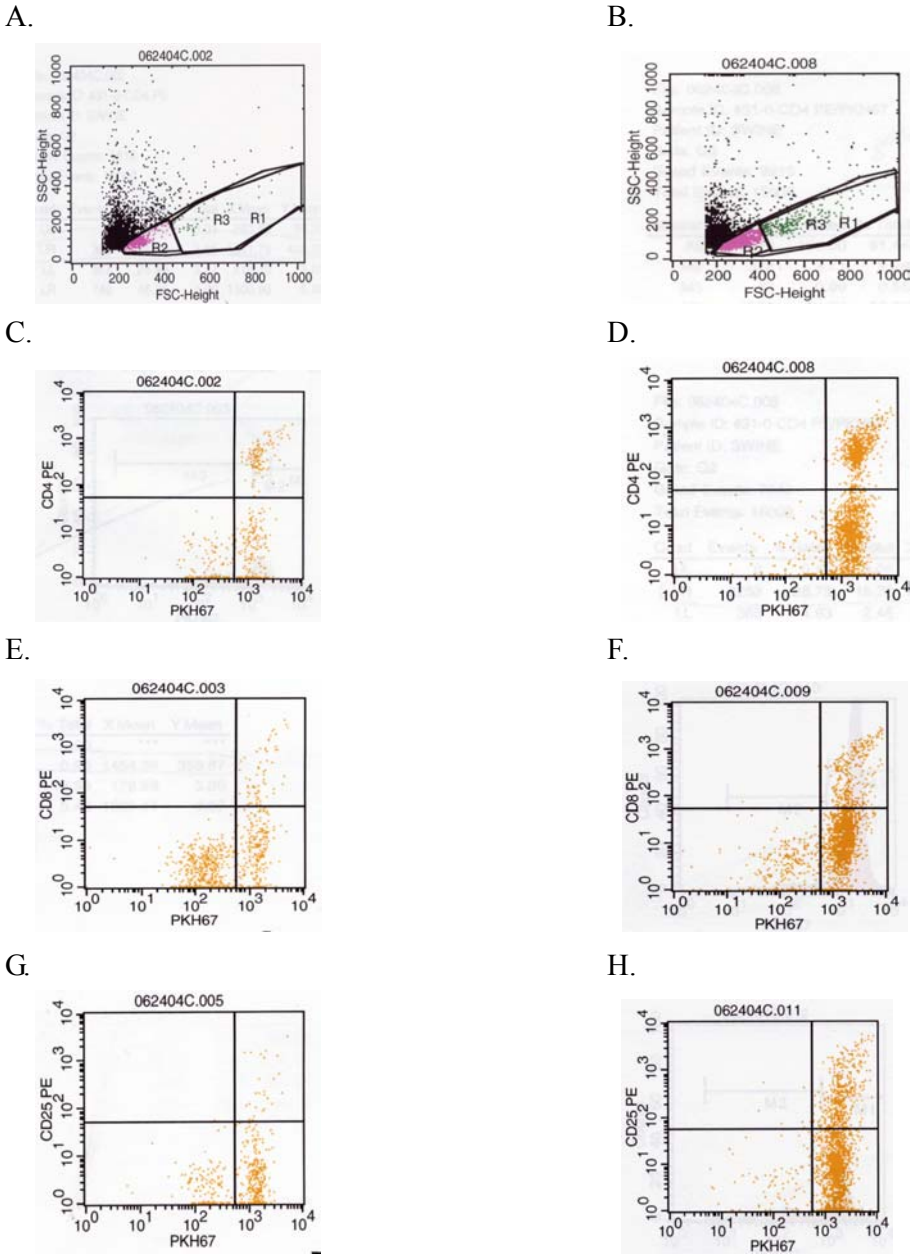


Fig. 4 The phenotypic profiles of tracheobronchial or lateral retropharyngeal lymph node cells from the normal. A, C, E and G Tracheobronchial lymph node. B, D, F and H Lateral retropharyngeal lymph node. The cell numbers/percentages over total cell events: in A. R1 = 173/1.2 %, R2 = 1616/10.8 %, R3 = 1862/12.4 %; in B. R1 = 1010/6.7 %, R2 = 7942/53.0 %, R3 = 9216/61.4 %. The percentages of the cells expressing different CDs in the gated region R3 of the forward and side scatter plots: C. 27.7 % (of 1862 cells) E. 7.4 % (of 3223 cells) G. 5.7 % (of 2285 cells) D. 31.1 % (of 9216 cells) F. 18.4 % (of 7778 cells) H. 21.3 % (of 9249 cells).

Fig. 5

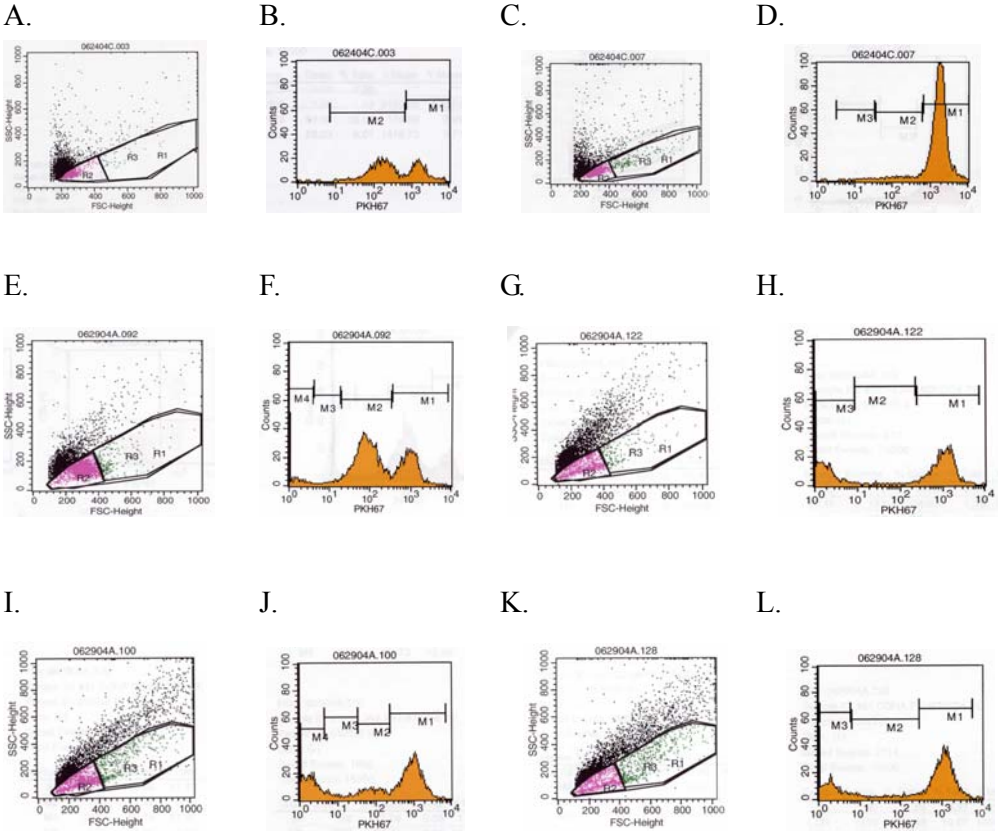


Fig. 5 The response of normal lymph node to Con A in culture after 5 days. Following dissection, lymph node cells were incubated with Con A (5 μg/ml) for five days at 37°C and 5 % CO_2. A, B, E, F, I and J Tracheobronchial lymph node. C, D, G, H, K and L Lateral retropharyngeal lymph node. A, B, C and D The cultures before *in vitro* culture. E, F, G and H Media only control. I, J, K and L Con A treatment. The cell numbers/percentages over total cell events: A. R1 = 168/1.1 %, R2 = 2810/18.7 %; C. R1 = 701/4.7 %, R2 = 7410/49.4 %; E. R1 = 728/4.9 %, R2 = 6299/42.2 %, R3 = 7496/50.0 %; I. R1 = 1682/11.2 %, R2 = 4015/26.8 %, R3 = 6274/41.8 %; G. R1 = 612/4.1 %, R2 = 3550/23.7 %, R3 = 4423/29.5 %; K. R1 = 2114/14.1 %, R2 = 3130/20.9 %, R3 = 5535/36.9 %.

Fig. 6

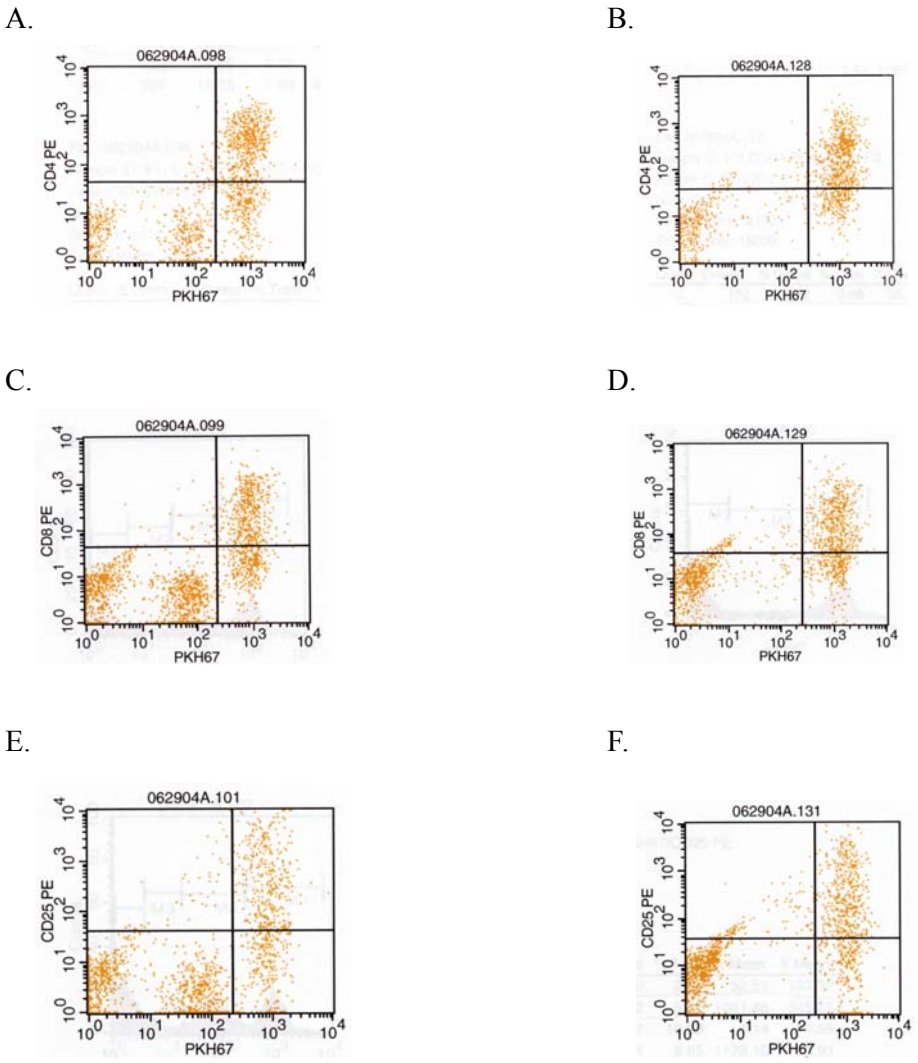


Fig. 6 The phenotypic profiles of normal lymph node cells to Con A. Following dissection, lymph node cells were incubated with Con A (5 μg/ml) for five days at 37^{o}C and 5 % CO_2. A, C and E Tracheobronchial lymph node. B, D and F Lateral retropharyngeal lymph node. The percentages of the cells expressing different CDs in the R3 regions of the forward and side scatter plots: A. 35.7 % (of 6252 cells) C. 24.0 % (of 8728 cells) E. 21.9 % (of 6697 cells) B. 47.6 % (of 5535 cells) D. 33.4 % (of 6845 cells) F. 30.1 % (of 6357 cells). The percentages of the cells expressing different CDs in the R1 regions of the forward and side scatter plots: A. 70.5 % (of 1584 cells) C. 58.9 % (of 1427 cells) E. 64.8 % (of 1351 cells) B. 71.4 % (of 2114 cells) D. 56.0 % (of 2021 cells) F. 61.5 % (of 1443 cells).

Fig. 7

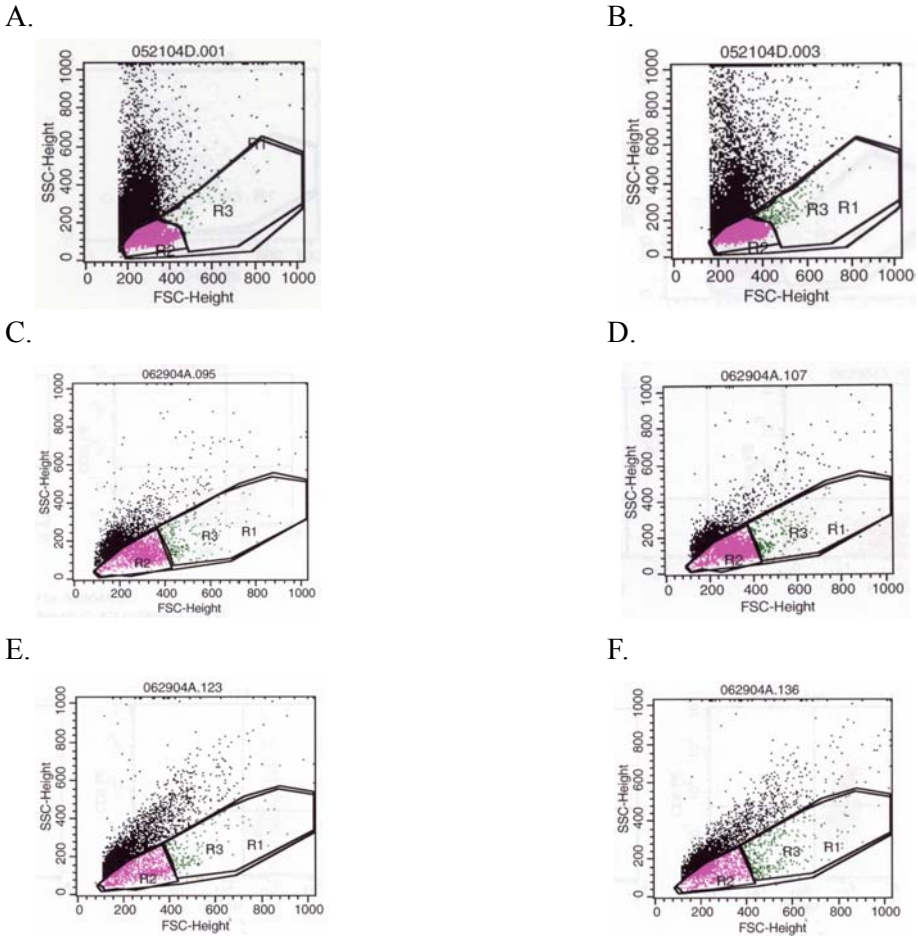


Fig. 7 The forward and side scatter plots of normal PBMCs and lymph node cells following stimulation with PRRSV. Following stimulation with PRRSV, cells were incubated with 10^5 $TCID_{50}$/ml of Powell virus for five days at 37°C and 5 % CO_2. A and B Peripheral blood mononuclear cells. C and D Tracheobronchial lymph node cells. E and F Lateral retropharyngeal lymph node cells. A, C and E Cultures of media-only control. B, D and F Cultures stimulated with Powell strain virus. The cell numbers/percentages over total cell events: A. R1 = 365/0.7 %, R2 = 18048/36.4 %, R3 = 18423/36.4 %; B. R1 = 762/2.5 %, R2 = 11463/38.3 %, R3 = 12254/40.9 %; C. R1 = 755/5.0 %, R2 = 6537/43.6 %, R3 = 7843/52.3 %; D. R1 = 962/6.4 %, R2 = 7925/52.8 %, R3 = 9409/62.7 %; E. R1 = 584/3.9 %, R2 = 3237/21.6 %, R3 = 4105/27.4 %; F. R1 = 1271/8.5 %, R2 = 4507/30.0 %, R3 = 6161/41.1 %.

Fig. 8

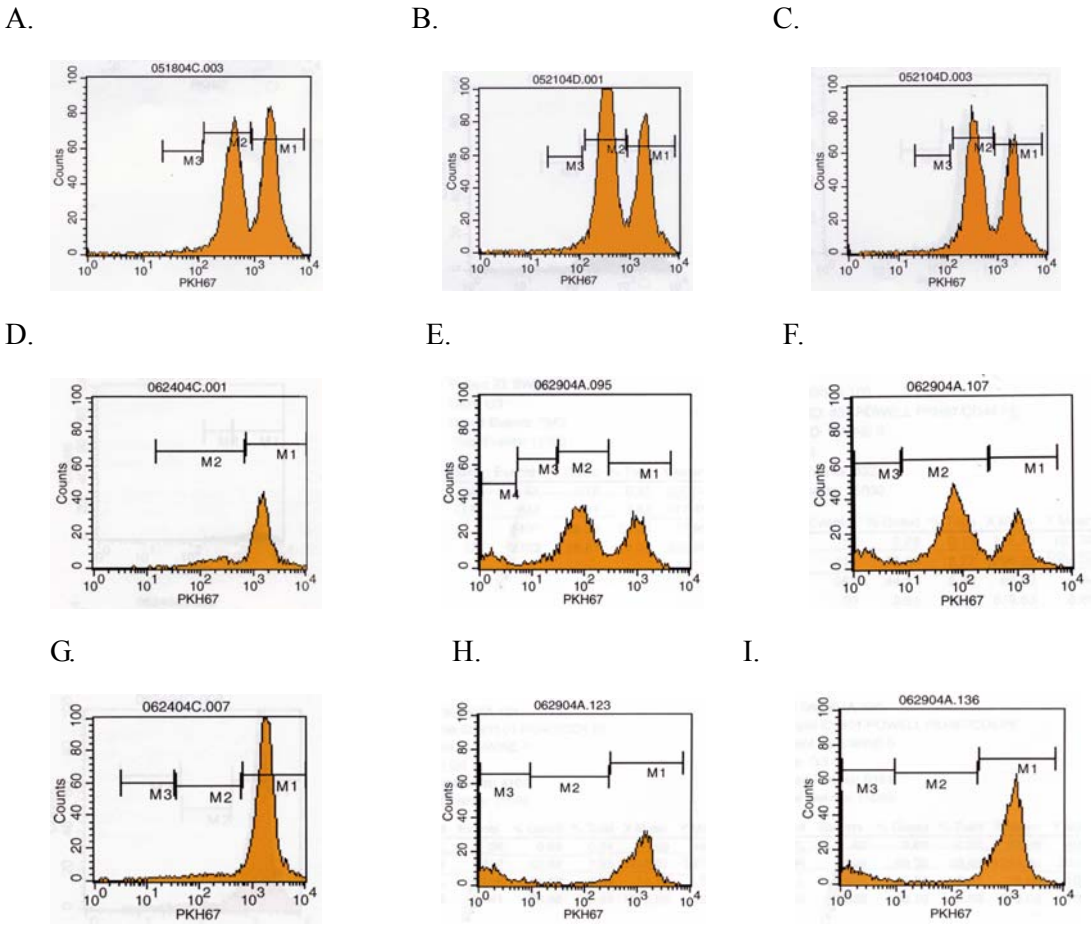


Fig. 8 Histograms of PKH67 staining of normal PBMCs following incubation with PRRSV. Following stimulation with PRRSV, cells were incubated with 10^5 $TCID_{50}$/ml of Powell virus for five days at 37°C and 5 % CO_2. A. Peripheral blood mononuclear cells before *in vitro* culture. B. Peripheral blood mononuclear cells of media-only control. C. Peripheral blood mononuclear cells stimulated with Powell strain virus. D. Tracheobronchial lymph node cells before *in vitro* culture. E. Tracheobronchial lymph node cells of media-only control. F. Tracheobronchial lymph node cells stimulated with Powell strain virus. G. Lateral retropharyngeal lymph node cells before *in vitro* culture. H. Lateral retropharyngeal lymph node cells of media-only control. I. Lateral retropharyngeal lymph node cells stimulated with Powell strain virus.

Fig. 9

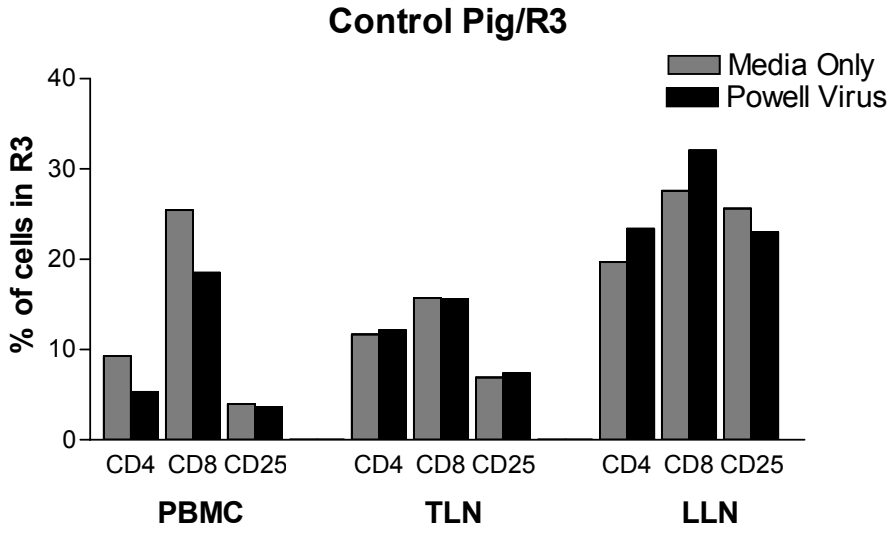


Fig. 9 The percentages of cells in the gated region R3 expressing CD4, CD8 or CD25 in the normal pig. Grey bars: the cultures of media only control. Black bars: the cultures treated with the Powell virus of 10^5 $TCID_{50}$/mL. PBMC: peripheral blood mononuclear cells. TLN: tracheobronchial lymph node. LLN: lateral retropharyngeal lymph node. The percentages of the cells from the PBMC in the R1 regions cultured with media only: $CD4^+$ = 33.4 %, $CD8^+$ = 71.5 %, $CD25^+$ = 37.2 %; and those of cultured with Powell virus: $CD4^+$ = 25.9 %, $CD8^+$ = 63.0 %, $CD25^+$ = 19.3 %. The percentages of the cells from the TLN in the R1 regions cultured with media only: $CD4^+$ = 34.3 %, $CD8^+$ = 44.4 %, $CD25^+$ = 22.8 %; and those of cultured with Powell virus: $CD4^+$ = 31.0 %, $CD8^+$ = 38.5 %, $CD25^+$ = 25.5 %. The percentages of the cells from the LLN in the R1 regions cultured with media only: $CD4^+$ = 28.9 %, $CD8^+$ = 45.0 %, $CD25^+$ = 47.8 %; and those of cultured with Powell virus: $CD4^+$ = 23.0 %, $CD8^+$ = 33.7 %, $CD25^+$ = 37.8 %. The percentages of the cells from the PBMC in the R2 regions cultured with media only: $CD4^+$ = 7.1 %, $CD8^+$ = 20.4 %, $CD25^+$ = 1.9 %; and those of cultured with Powell virus: $CD4^+$ = 3.7 %, $CD8^+$ = 15.8 %, $CD25^+$ = 1.81 %. The percentages of the cells from the TLN in the R2 regions cultured with media only: $CD4^+$ = 8.1 %, $CD8^+$ = 11.4 %, $CD25^+$ = 4.6 %; and those of cultured with Powell virus: $CD4^+$ = 8.7 %, $CD8^+$ = 9.2 %, $CD25^+$ = 4.6 %. The percentages of the cells from the LLN in the R2 regions cultured with media only: $CD4^+$ = 17.2 %, $CD8^+$ = 23.7 %, $CD25^+$ = 19.8 %; and those of cultured with Powell virus: $CD4^+$ = 22.5 %, $CD8^+$ = 31.4 %, $CD25^+$ = 17.4 %.

Fig. 10

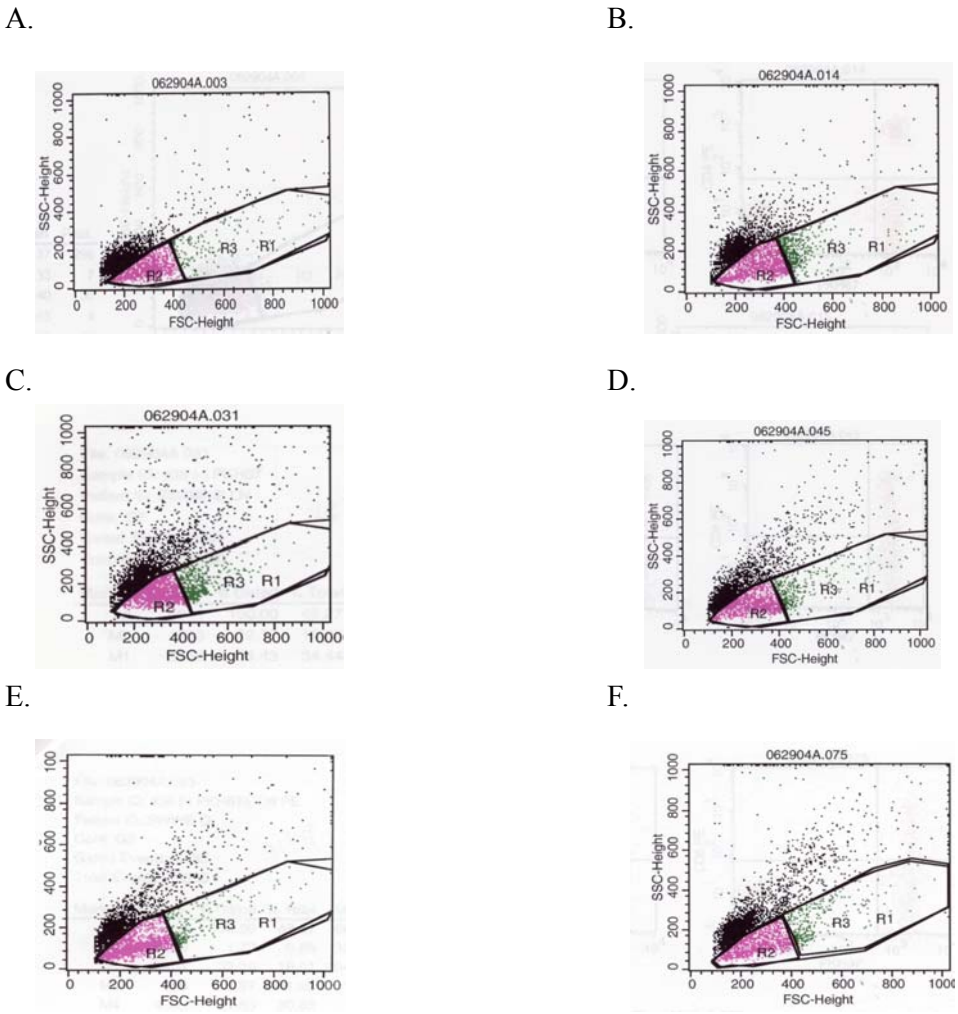


Fig. 10 The forward and side scatter plots of PBMCs and lymph node cells from the virus infected pig following incubation with PRRS. Following stimulation with PRRSV, cells were incubated with 10^5 $TCID_{50}$/ml of Powell virus for five days at 37°C and 5 % CO_2. A and B Peripheral blood mononuclear cells. C and D Tracheobronchial lymph node cells. E and F Lateral retropharyngeal lymph node cells. A, C and E Cultures of media-only control. B, D and F Cultures stimulated with Powell virus. The cell numbers/percentages over total cell events: A. R1 = 708/0.5 %, R2 = 4013/26.8 %, R3 = 5048/33.7 %; B. R1 = 1707/11.4 %, R2 = 5578/37.2 %, R3 = 7743/51.6 %; C. R1 = 1728/11.5 %, R2 = 4805/32.0 %, R3 = 6941/46.3 %; D. R1 = 1403/9.4 %, R2 = 4338/28.9 %, R3 = 6169/41.1 %; E. R1 = 779/5.2 %, R2 = 6380/42.5 %, R3 = 7435/49.6 %; F. R1 = 1180/7.9 %, R2 = 4027/26.8 %, R3 = 5704/38.0 %.

Table II

Powell strain virus-infected and rechallenged Pig				
After 5 days culture				
Treatment	Media Only		Powell virus	
Gated Region	R1 (%)	R2 (%)	R1 (%)	R2 (%)
PBMC	11.5	88.5	25.8	74.2
TLN	20.8	79.2	24.2	75.8
LLN	16.5	83.5	23.2	76.8

Table II Summary of responses to PRRSV by T cells from infected Pigs. The average percentages in the gated regions R1 and R2 over the cell numbers in R3 regions of the peripheral blood mononuclear cells, tracheobronchial lymph node (TLN) cells and lateral retropharyngeal lymph node (LLN) cells from the Powell virus-infected and re-challenged pig treated with or without the Powell virus (10^5 $TCID_{50}$/mL).

Fig. 11

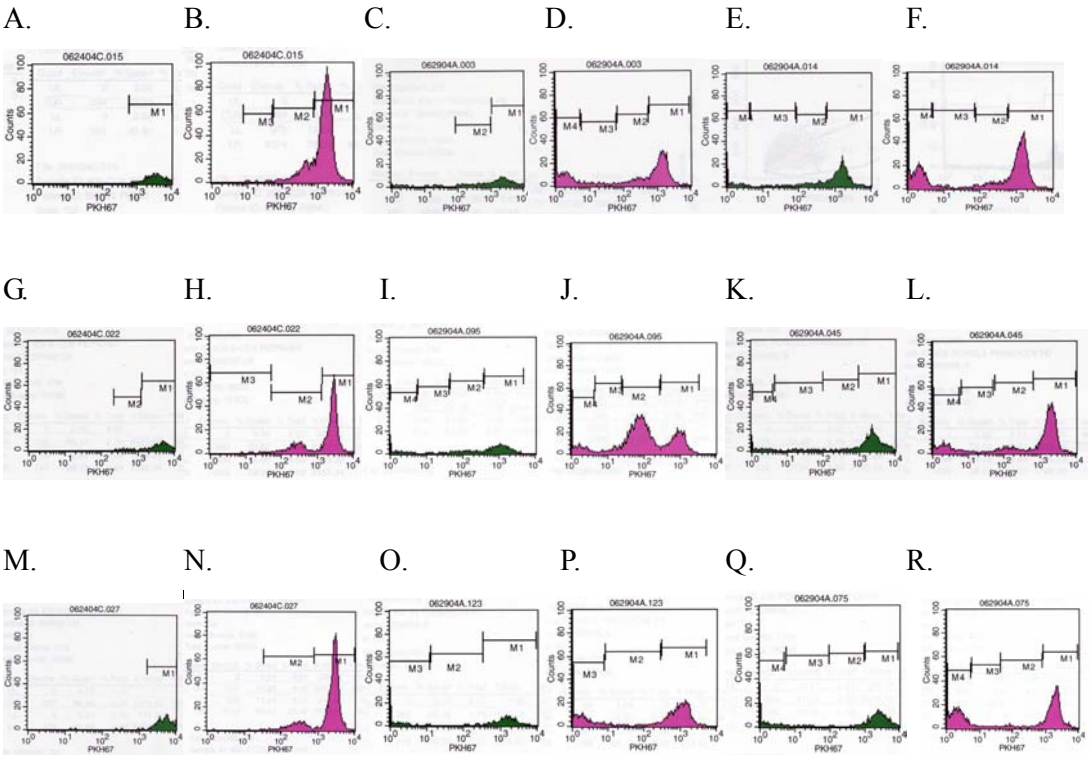


Fig. 11 PKH67 staining histograms of lymphoid cells from the infected pig following in vitro culture with PRRSV. Following stimulation with PRRSV, cells were incubated with 10^5 $TCID_{50}$/ml of Powell virus for five days at 37°C and 5 % CO_2. A. R1 of the peripheral blood mononuclear cells (PBMCs) before *in vitro* culture. B. R2 of the PBMCs before *in vitro* culture. C. R1 of the peripheral blood mononuclear cells (PBMCs) of media-only control. D. R2 of the PBMCs of media-only control. E. R1 of the PBMCs stimulated with Powell virus (10^5 $TCID_{50}$/mL). F. R2 of the PBMCs stimulated with Powell virus. G. R1 of the tracheobronchial lymph node (TLN) cells before *in vitro* culture. H. R2 of the TLN cells before *in vitro* culture. I. R1 of the tracheobronchial lymph node (TLN) cells of media-only control. J. R2 of the TLN cells of media-only control K. R1 of the TLN cells stimulated with Powell virus. L. R2 of the TLN cells stimulated with Powell virus. M. R1 of the lateral retropharyngeal lymph node (LLN) cells before *in vitro* culture. M. R2 of the LLN cells before *in vitro* culture. O. R1 of the lateral retropharyngeal lymph node (LLN) cells of media-only control. P. R2 of the LLN cells of media-only control Q. R1 of the LLN cells stimulated with Powell virus. R. R2 of the LLN cells stimulated with Powell virus.

Fig. 12

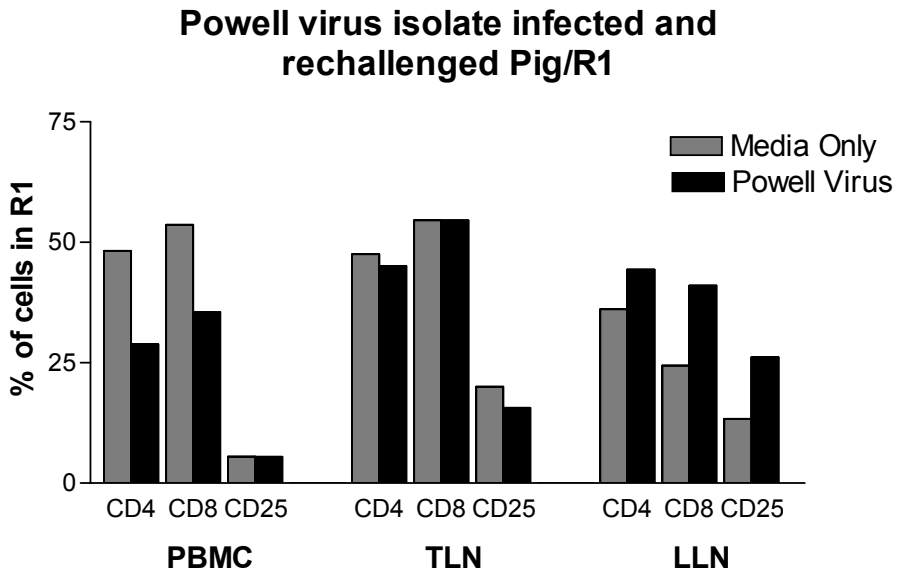
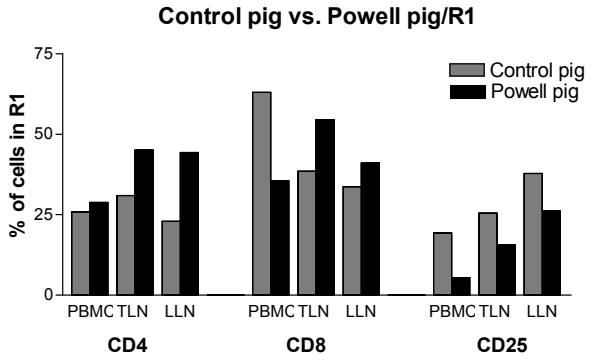


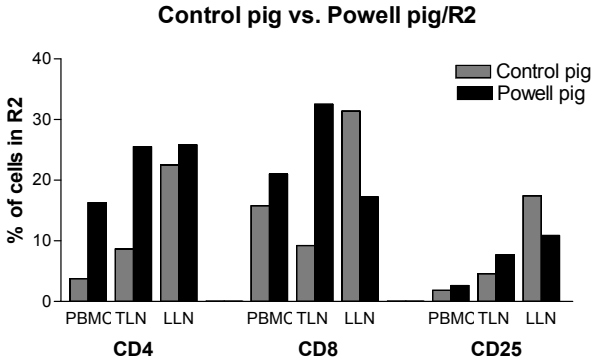
Fig. 12 The percentages of the cells in the R1 regions expressing CD4, CD8 or CD25 in the Powell isolate virus infected and re-challenged pig. Grey bars: the culture of media only control. Black bars: the cultures treated with the Powell virus of 10^5 $TCID_{50}$/mL. PBMC: peripheral blood mononuclear cells. TLN: tracheobronchial lymph node. LLN: lateral retropharyngeal lymph node. The percentages of the cells from the PBMC in the R2 regions cultured with media only: $CD4^+$ = 19.5 %, $CD8^+$ = 23.4 %, $CD25^+$ = 2.5 %; and those of cultured with Powell virus: $CD4^+$ = 16.3 %, $CD8^+$ = 21.0 %, $CD25^+$ = 2.6 %. The percentages of the cells from the TLN in the R2 regions cultured with media only: $CD4^+$ = 25.2 %, $CD8^+$ = 19.2 %, $CD25^+$ = 7.3 %; and those of cultured with Powell virus: $CD4^+$ = 25.5 %, $CD8^+$ = 32.5 %, $CD25^+$ = 7.7 %. The percentages of the cells from the LLN in the R2 regions cultured with media only: $CD4^+$ = 24.6 %, $CD8^+$ = 7.1 %, $CD25^+$ = 2.9 %; and those of cultured with Powell virus: $CD4^+$ = 25.8 %, $CD8^+$ = 17.2 %, $CD25^+$ = 10.9 %. The percentages of the cells from the PBMC in the R3 regions cultured with media only: $CD4^+$ = 23.0 %, $CD8^+$ = 28.5 %, $CD25^+$ = 2.9 %; and those of cultured with Powell virus: $CD4^+$ = 19.6 %, $CD8^+$ = 24.8 %, $CD25^+$ = 3.3 %. The percentages of the cells from the TLN in the R3 regions cultured with media only: $CD4^+$ = 30.8 %, $CD8^+$ = 26.0 %, $CD25^+$ = 10.1 %; and those of cultured with Powell virus: $CD4^+$ = 30.5 %, $CD8^+$ = 37.8 %, $CD25^+$ = 10.2 %. The percentages of the cells from the LLN in the R3 regions cultured with media only: $CD4^+$ = 27.2 %, $CD8^+$ = 9.8 %, $CD25^+$ = 4.5 %; and those of cultured with Powell virus: $CD4^+$ = 31.1 %, $CD8^+$ = 23.3 %, $CD25^+$ = 14.2 %.

Fig. 13

A.



B.



C.

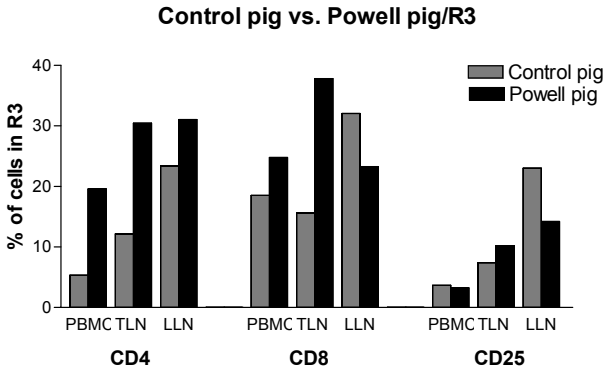


Fig. 13 Comparisons of the percentages of $CD4^+$, $CD8^+$ or $CD25^+$ T cells from the virus-treated cultures of PBMCs and lymph node cells from the control pig and the PRRSV-infected pig. A. The data of the R1 regions. B. The data of the R2 regions. C. The data of the R3 regions, which are the combination of R1 and R2 regions.

References

1. **Fenner, F. J., E. J. Gibbs, F. A. Murphy, R. Rott, M. J. Studdert, D. O. White.** Veterinary Virology, second edition. p 595-601, 1993.

2. **Cavanagh, D.** Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch. Virol. 142(3): 629-33, 1997.

3. **Allende, R., T. L. Lewis, Z. Lu, D. L. Rock, G. F. Kutish, A. Ali, A.R. Doster, and F. A. Osorio**. North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding region. J. Gen. Virol. 80:307-15, 1999.

4. **Meulenberg, J. J., A. Petersen-den Besten, E.P. De Kluyver, R. J. Moormann, W. M. Schaaper and G. Wensvoort**. Characterization of proteins encoded by ORFs 2 to 7 Lelystad virus. Virology. 206: 155-63, 1995.

5. **Nelsen, C. J., M. P. Murtaugh and K. S. Faaberg**. Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73:270-80, 1999.

6. **Keffaber, K. K.** Reproductive failure of unknown etiology. Am. Assoc. Swine Prac. Newsl.: 1-9, 1989.

7. **Christianson, W. T., J. E. Collins, D. A. Benfield, L. Harris, D. E. Gorcyca, D. W. Chladek, R. B. Morrison and H. S. Joo.** Experimental reproduction of swine infertility and respiratory syndrome in pregnant sows. Am. J. Vet. Res. 53: 485-488, 1992.

8. **Meulenberg, J. J., M. M. Hulst, E. J. de Meijer, P. L. Moonen, A. den Besten, E. P. de Kluyver, G. Wensvoort and R. J. Moormann.** Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192: 62-72, 1993.

9. **Yuan S., D. Mickelson, M. P. Murtaugh, K. S. Faaberg.** Complete genome comparison of porcine reproductive and respiratory syndrome virus parental and attenuated strains. Virus Res. 74: 99-110, 2001.

10. **Meng, X. J.** Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development. Vet. Microbiol. 74: 309-329, 2000.

11. **Andreyev, V. G., R. D. Wesley, W. L. Mengeling, A. C. Vorwald and K. M. Lager.** Genetic variation and phylogenetic relationships of 22 porcine reproductive and respiratory syndrome virus (PRRSV) field strains based on sequence analysis of open reading frame 5. Arch. Virol. 142: 993-1001, 1997.

12. **Rowland, R. R. R., M. Steffen, T. Ackerman and D. A. Benfield.** The evolution of porcine reproductive and respiratory syndrome virus: quasispecies and emergence of a virus subpopulation during infection of pigs with VR-2332. Virol. 259: 262-266, 1999.

13. **Nelsen, C. J., M. P. Murtaugh and K. S. Faaberg.** Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J. Virol. 73(1): 270-280, 1999.

14. **Weiland, E., M. Wieczorek-Krohmer, D. Kohl, K. K. Conzelmann and F. Weiland.** Monoclonal antibodies to the GP5 of porcine reproductive and respiratory syndrome virus are more effective in virus neutralization than monoclonal antibodies to the GP4. Vet. Microbiol. 66: 171-186, 1999.

15. **Gonin, P., B. Pirzadeh, C. A. Gagnon and S. Dea.** Seroneutralization of porcine reproductive and respiratory syndrome virus correlates with antibody response to the GP5 major envelope glycoprotein. J. Vet. Diagn. Invest. 11: 20-26, 1999.

16. **Plagemann, P. G. W., R. R. R. Rowland and K. S. Faaberg.** The primary neutralization epitope of porcine respiratory and reproductive syndrome virus strain VR-2332 is located in the middle of the GP5 ectodomain. Arch. Virol. 147: 2327-2347, 2002.

17. **Ostrowski, M., J. A. Galeota, A. M. Jar, K. B. Platt, F. A. Osorio and O. J. Lopez.** Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J. Virol. 76(9): 4241-4250, 2002.

18. **Pirzadeh, B. and S. Dea.** Monoclonal antibodies to the ORF5 product of porcine reproductive and respiratory syndrome virus define linear neutralizing determinants. J.

Gen. Virol. 78: 1867-1873, 1997.

19. **Rossow, K. D., J. E. Collins, S. M. Goyal, E. A. Nelson, J. Christopher-Hennings and D. A. Benfield.** Pathogenesis of porcine reproductive and respiratory syndrome virus infection in gnotobiotic pigs. Vet. Pathol. 32: 361-373, 1995.

20. **Reeth, K. V.** Pathogenesis and clinical aspects of a respiratory porcine reproductive and respiratory syndrome virus infection. Vet. Microbio. 55: 223-230, 1997.

21. **Albina, E.** Porcine reproductive and respiratory syndrome: ten years of experience with this undesirable viral infection. Vet. Res. 28(4): 305-352, 1997.

22. **Halbur, P. G., F. J. Pallares, J. A. Rathje, R. Evans, W. A. Hagemoser, P. S. Paul, X. J. Meng.** Effects of different US isolates of porcine reproductive and respiratory syndrome virus (PRRSV) on blood and bone marrow parameters of experimentally infected pigs. Vet. Rec. 151: 344-348, 2002.

23. **Bilodeau, R., D. Archambault, S. A. Vezina, R. Sauvageau, M. Fournier, S. Dea.** Persistence of porcine reproductive and respiratory syndrome virus infection: swine operation. Can. J. Vet. Res. 58(4): 291-298, 1994.

24. **Albina, E. F. Madec, R. Cariolet, J. Torrison.** Immune response and persistence of the porcine reproductive and respiratory syndrome virus in infected pigs and farm units. Vet. Rec. 134: 567-573, 1994.

25. **Nodelijk, G., L. A. M. G. van Leengoed, E. J. Schoevers, A. H. Kroese, M. C. M. De Jong, G. Wensvoort, J. H. M. Verheijden.** Seroprevalence of porcine reproductive and respiratory syndrome virus in Dutch weaning pigs. Vet. Microbiol. 56: 21-32, 1997.

26. **Mengeling, W. L., A. C. Vorwald, K. M. Lager and S. L. Brockmeier.** Diagnosis of porcine reproductive and respiratory syndrome using infected alveolar macrophages collevted from live pigs. Vet. Microbiol. 49: 105-115, 1996a.

27. **Suarez, P.** Ultrastructural pathogenesis of the PRRS virus. Vet. Res. 31: 47-55, 2000.

28. **Nielsen, J., A. Bøtner, V. Bille-Hansen, M. B. Oleksiewicz and T. Storgaard.** Experimental inoculation of late term pregnant sows with a field isolate of porcine

reproductive and respiratory syndrome vaccine-derived virus. Vet. Microbiol. 84: 1-13, 2002.

29. **Dee, S. A., H. Joo**. Strategies to control PRRS: A summary of field and research experiences. Vet. Microbiol. 55: 347-353, 1997.

30. **Plana-Dura′n, J., M. Bastons, A. Urniza, M. Vayreda, X. Vila′ and H. Mane′.** Efficacy of an inactivated vaccine for prevention of reproductive failure induced by porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 55: 361-370, 1997.

31. **Osorio, F. A., F. Zuckermann, R. Wills, W. Meier, S. Christian, J. Galeota and A. Doster.** PRRSV: Comparison of commercial protection against current PRRSV strains of high virulence. Allen D. Leman Swine Conference: 176-182, 1998.

32. **Shibata, I., M. Mori and S. Yazawa.** Experimental reinfection with homologous porcine reproductive and respiratory syndrome virus in SPF pigs. J. Vet. Med. Sci. 62(1): 105-108, 2000.

33. **Mengeling, W. L., K. M. Lager, A. C. Vorwald, K. J. Koehler.** Strain specificity of the immune response of pigs following vaccination with various strains of porcine reproductive and respiratory syndrome virus. Vet. Microbiol. 93: 13-24, 2003.

34. **Lager, K. M., W. L. Mengeling, S. L. Brockmeier.** Homologous challenge of porcine reproductive and respiratory syndrome virus immunity in pregnant swine. Vet. Microbiol. 58: 113-125, 1997.

35. **Verheije, M. H., M. V. Kroese, I. F. A. van der Linden, E. A. de Boer-luijtze, P. A. van Rijn, J. M. A. Pol, J. J. M. Meulenberg and P. J. G. M. Steverink.** Safety and protective efficacy of porcine reproductive and respiratory syndrome recombinant virus vaccines in young pigs. Vaccine 21: 2556-2563, 2003.

36. **Bøtner, A., J. Nielsen, M. B. Oleksiewicz and T. Storgaard.** Heterologous challenge with porcine reproductive and respiratory syndrome (PRRS) vaccine virus: no evidence of reactivation of previous European-type PRRS virus infection. Vet. Microbiol. 68: 187-195, 1999.

37. **Shibata, I., M. Mori and S. Yazawa**. Experimental reinfection with homologous

porcine reproductive and respiratory syndrome virus in SPF pigs. J. Vet. Med. Sci. 62(1): 105-108, 2000.

38. **Sipos, W., C. Duvigneau, P. Pietschmann, K. Höller, R. Hartl, K. Wahl, R. Steinborn, M.Gemeiner, M. Willheim and F. Schmoll.** Parameters of humoral and cellular immunity following vaccination of pigs with a European modified-live strain of porcine reproductive and respiratory syndrome virus (PRRSV). Viral Immunol. 16(3): 335-346, 2003.

39. **Nielsen, H. S., M. B. Oleksiewicz, R. Forsberg, T. Stadejek, A. Bøtner and T. Storgaard.** Reversion of a live porcine reproductive and respiratory syndrome virus vaccine investigated by parallel mutations. J. Gen. Virol. 82:1263-1272, 2001.

40. **Lager, K. M., W. L. Mengeling and R. D. Wesley.** Strain predominance following exposure of vaccinated and naïve pregnant gilts to multiple strains of porcine reproductive and respiratory syndrome virus. Can. J. Vet. Res. 67: 121-127, 2003.

41. http://www.unmc.edu/Eudcation/Animal/guide

42. **Yang, H. and R. M. E. Parkhouse.** Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues. Immunology 89: 76-83, 1996.

43. **Waters, W. R., R. Hontecillas, R. E. Sacco, F. A. Zuckermann, K. R. Harkins, J. Bassaganyariera and M. J. Wannemuehler.** Antigen-specific proliferation of porcine CD8αα cells to an extracellular bacterial pathogen. Immunology 101: 333-341, 2000.

44. **Kirkham, P. A., H. Takamatsu, H. Yang and R. M. E. Parkhouse**. Porcine CD3ε: its characterization, expression and involvement in activation of porcine T lymphocytes. Immunology 87: 616-623, 1996.

45. **Yang, H., R. M. E. Parkhouse**. Differential expression of CD8 epitope amongst porcine CD8-positive functional lymphocyte subsets. Immunology 92: 45-52, 1997.

46. **Zuckermann, F. A., R. J. Husmann.** Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87: 500-512, 1996.

47. **Pescovitz, M. D., A. G. Sakopoulos, J. A. Gaddy, R. J. Husmann, F. A.**

Zukermann. Porcine peripheral blood $CD4^+/CD8^+$ dual expressing T-cells. Vet. Immunol. Immunopathol. 43:53-62, 1994.

48. **Wills, R. W., A. R. Doster, J. A. Galeota, J. H. Sur and F. A. Osorio.** Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. J. Cli. Microbiol. 41(1): 58-62, 2003.

49. **Bøtner, A., J. Nielsen, V. Bille-Hansen.** Isolation of porcine reproductive and respiratory syndrome (PRRS) virus in a Danish swine herd and experimental infection of pregnant gilts with the virus. Vet. Microbiol. 40: 351-360, 1994.

50. **Nielsen J., A. Bøtner, J. E. Tingstedt, B. Aasted, C. K. Johnsen, U. Riber and P. Lind.** In utero infection with porcine reproductive and respiratory syndrome virus modulates leukocyte subpopulations in peripheral blood and bronchoalveolar fluid of surviving piglets. Vet. Immuno. Immunopatho. 93: 135-151, 2003.

51. **Linden, I. F. A., J. J. M. Voermans, E. M. van der Linde-Bril, A. T. J. Bianchi and P. J. G. M. Steverink.** Virological kinetics and immunological responses to a porcine reproductive and respiratory syndrome virus infection of pigs at different ages. Vaccine 21: 1952-1957, 2003.

52. **Labarque G. G., H. J. Nauwynck, K. V. Reeth and M. B. Pensaert.** Effect of cellular changes and onset of humoral immunity on the replication of porcine reproductive and respiratory syndrome virus in the lungs of pigs. J. Gen. Virol. 81: 1327-1334, 2000.

53. **Feng, W. H., M. B. Tompkins, J. S. Xu, T. T. Brown, S. M. Laster, H. X. Zhang and M. B. McCaw.** Thymocyte and peripheral blood T lymphocyte subpopulation changes in piglets following in utero infection with porcine reproductive and respiratory syndrome virus. Virology 302: 363-372, 2002.

54. **Albina, E., L. Piriou, E. Hutet, R. Cariolet and R. L'Hospitalier.** Immune response in pigs infected with porcine reproductive and respiratory syndrome virus (PRRSV). Vet. Immuno. Immunopatho. 61: 49-66, 1998.

55. **Samsom, J. N., T. G. M. de Bruin, J. J. M. Voermans, J. J. M. Meulenberg, J. M. A. Pol and A. T. J. Bianchi.** Changes of leukocyte phenotype and function in the broncho-alveolar lavage fluid of pigs infected with porcine reproductive and

respiratory syndrome virus: a role for $CD8^+$ cells. J. Gen. Virol. 81: 497-505, 2000.

56. **Bautista, E. M. and T. W. Molitor**. Cell-mediated immunity to porcine reproductive and respiratory syndrome virus in swine. Viral Immunol. 10(2): 83-94, 1997.

57. **Fuertes, L. L., N. Domenech, B. Alvarez, A. Ezquerra, J. Dominguez, J. M. Castro and F. Alonso.** Analysis of cellular immune response in pigs recovered from porcine respiratory and reproductive syndrome infection. Virus Res. 64: 33-42, 1999.

58. **Sipos, W., C. Duvigneau, P. Pietschmann, K. Höller, R. Hartl, K. Wahl, R. Steinborn, M.Gemeiner, M. Willheim and F. Schmoll.** Parameters of humoral and cellular immunity following vaccination of pigs with a European modified-live strain of porcine reproductive and respiratory syndrome virus (PRRSV). Viral Immunol. 16(3): 335-346, 2003.

59. **Bautista, E. M., P. Suarez and T. W. Molitor**. T cell response to the structural polypeptides of porcine reproductive and respiratory syndrome virus. Arch. Virol. 144: 117-134, 1999.

60. **Pirzadeh B. and S. Dea.** Immune response in pigs vaccinated with plasmid DNA encoding ORF5 of porcine reproductive and respiratory syndrome virus. J. Gen. Virol. 79: 989-999, 1998.

61. **Lamontagne, L., C. Page, R. Larochelle and R. Magar**. Porcine reproductive and respiratory syndrome virus persistence in blood, spleen, lymph nodes, and tonsils of experimentally infected pigs depends on the level of $CD8^{high}$ T cells. Viral Immunol. 16(3): 395-406, 2003.

62. **Janeway C. A., P. Travers, M. Walport and M. Shlomchik**. Immunobiology. Fifth edition, 2001. p30, 110-111, 178 and 344.

63. **Mahy, B., H. O. Kangro**. Virology Methods Manual. p 35-37, 1996.

64. **Dyce, K. M., W. O. Sack and C. J. G. Wensing**. Textbook of Veterinary Anatomy. Second edition. p769-785, 1996.

65. Waters, W. R., K. R. Harkins and M. J. Wannemuehler. Five-color flow cytometric analysis of swine lymphocytes for detection of proliferation, apoptosis,

viability and phenotype. Cytometry 48: 212-221, 2002.