Institut für Tierwissenschaften, Abteilung Tierzucht und Tierhaltung

Global mRNA and miRNA transcriptome profiling of peripheral blood mononuclear cells to investigate the host immunogenetic response to PRRSV vaccination in pigs

Inaugural-Dissertation

zur

Erlangung des Grades

Doktor der Agrarwissenschaften

(Dr. agr.)

der

Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

von

Md. Aminul Islam

aus

Tangail, Bangladesh

Bonn, 2017

Referent:	Prof. Dr. Karl Schellander
Korreferent:	Prof. Dr. Brigitte Petersen
Tag der mündlichen Prüfung:	17. januar 2017

Dedicated to

My beloved parents, my wife and my lovely sons

Global mRNA and miRNA transcriptome profiling of peripheral blood mononuclear cells to investigate the host immunogenetic response to PRRSV vaccination in pigs

This dissertation aims to identify the candidate genes of the functional network of host immune response to porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs; to explore the breed differences on vaccine induced transcriptional response between German Landrace (DL) and Pietrain (Pi) pigs; and to elucidate the post transcriptional regulatory mechanism of vaccine induced gene expression in the peripheral blood mononuclear cells (PBMCs). The Affymetrix gene chip microarray technique was employed for global expression profiling of messenger RNA (mRNA) and microRNA (miRNA) in PBMCs collected in a time series manner following PRRSV vaccination in purebred DL and Pi pigs. Additionally, microarray expression results were validated by qRT-PCR and the PRRSV-specific plasma antibody titre was monitored by ELISA. The PRRSV-specific plasma antibody titre indicated the piglets free from maternal antibody at the time of primary vaccination and rose above the threshold following two weeks of the primary vaccination that subsequently reached a plateau at four weeks post vaccination. The global mRNA profiling of PBMCs from PRRSV vaccinated and age-matched unvaccinated Landrace pigs at immediately before (0 h), and at 6, 24 and 72 h after PRRSV vaccination revealed a distinct host innate immune transcriptional response. A total of 14,231 transcripts were found to be expressed in PBMCs of vaccinated and unvaccinated pigs. Differential expression analysis (FDR < 0.01 and FC > ±1.5) identified 542, 2,263 and 357 differentially expressed genes at 6, 24 and 72 h post vaccination. APP, TRAF6, PIN1, FOS, CDKN1A and TNFAIP3 identified to be potential candidate genes for early stage PRRSV vaccine response in Landrace pigs. In Pietrain pigs, 295 and 116 transcripts were found to be differentially expressed in PBMCs at 1 and 28 days post vaccination, respectively. This study suggested that the innate immune transcriptional network is likely to be regulated by LCK, STAT3, ATP5B, UBB and RSP17; while TGFβ1, IL7R, RAD21, SP1 and GZMB were found to be predictive for the adaptive immune transcriptional response to PRRSV vaccine in PBMCs of Pi pigs. The global microRNA profiles of PBMCs identified 12, 259 and 14 differentially expressed (DE) miRNAs in DL; and 0, 222 and 13 DE miRNAs in Pietrain at 6, 24 and 72 h post vaccination, respectively. There were remarkable differences on expression dynamics of both mRNAs and miRNAs between DL and Pi pigs. Integrated mRNA-miRNA network revealed the inverse correlation between vaccine induced altered mRNAs and miRNAs in PBMCs. Results of this immunogenomics study advances our understanding on the genetic control of PRRS.

Erstellung von globalen mRNA und miRNA Transkriptomprofilen in mononukleäre Zellen des peripheren Blutes zur Untersuchung der Wirts immunogenetischen Reaktion auf eine PRRSV Impfung bei Schweinen

Die vorliegende Arbeit zielt darauf ab, Kandidatengene des funktionellen Netzwerks der wirtsspezifischen Immunantwort auf den PRRS-Virus (PRRSV) Impfstoff bei Schweinen zu identifizieren; um transkriptionale Unterschiede durch den induzierten Impfstoff in den zwei Schweinerassen Deutschen Landrasse (DL) und *Piétrain* (Pi) zu erkunden; und die Aufklärung von Post-transkriptionellen Mechanismen bedingt durch den Impfstoff in den mononukleären Zellen des peripheren Blutes (PBMCs). Zur Erstellung der Transkriptomprofile der Boten-RNA (mRNA) sowie der microRNA (miRNA) in reinrassigen DL und Pi Schweinen zu unterschiedlichen Zeitpunkten nach der PRRSV Impfung wurde die Affymetrix Gen-Chip-Microarray-Technik eingesetzt. Zusätzlich wurden die Microarray Ergebnisse mittels qRT-PCR validiert und die PRRSV-spezifischen Plasma Antikörpertiter durch ELISA bestimmt. Durch den PRRSV-spezifischen Plasma Antikörpertiter zeigte sich, dass die Ferkel frei von mütterlichen Antikörpern zum Zeitpunkt der Erstimpfung waren. Nach der ersten Impfung stieg der Titer in den folgenden zwei Wochen über dem Grenzwert, und erreichte sein Plateau vier Wochen nach der Impfung. Die Betrachtung der globalen mRNA Profile von PBMCs von PRRSV geimpft und ungeimpften DL Schweinen unmittelbar vor 0 und mit 6, 24 und 72 h nach der Impfung ergab eine deutlich angeborene transkriptionelle Wirts Immunreaktion. Insgesamt waren 14.231 Transkripte in PBMCs von geimpften und nicht geimpften Schweine exprimiert. Die Expressionsanalyse (FDR <0,01 und FC> ± 1,5) identifiziert 542, 2263 und 357 differentiell exprimierte Gene 6, 24 und 72 h nach der Impfung. Als potenzielle Kandidatengene für das frühe Stadium der Impfreaktion konnten APP, TRAF6, PIN1, FOS, CDKN1A und TNFAIP3 identifiziert werden. In *Piétrain* Schweinen waren 295 und 116 Transkripte in PBMCs an Tag 1 und 28 nach der Impfung unterschiedlich exprimiert. Diese Ergebnisse zeigen, dass das angeborene Immunnetzwerk wahrscheinlich durch LCK, STAT3, ATP5B, UBB und RSP17 geregelt wird; während sich TGFβ1, IL7R, Rad21, SP1 und GZMB für die adaptive Immunreaktion auf den PRRSV-Impfstoff in PBMCs von Pi-Schweinen als prädiktiv erwiesen. Die microRNA-Profile von PBMCs identifiziert 12, 259 und 14 unterschiedlich exprimiert miRNAs in DL; und 0, 222 und 13 miRNAs in Pi, 6, 24 und 72 h nach der Impfung. Es gab deutliche Unterschiede bei der Expressionsdynamik sowohl bei der mRNAs als auch miRNAs zwischen DL und Pi Schweine. Integrierte mRNA-miRNA-Netzwerke zeigen eine inverse Korrelation zwischen der durch den Impfstoff induzierten veränderten mRNAs und miRNAs Expression in PBMCs. Die Ergebnisse dieser immunogenomischen Studie erweitert unser Verständnis über die genetische Kontrolle von PRRS.

Table of contents

List of abbreviations

ACTB	Actin, beta
APC	Antigen presenting cell
bp	Base pair
Ct	Cycle threshold
cDNA	Complementary deoxyribonucleic acid
cRNA	Complementary ribonucleic acid
CTLs	Cytotoxic T cells
ddH_2O	Double distilled water
DEGs	Differentially expressed genes
DL	Deutsche / German Landrace
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxy ribonuclease
dNTP	Deoxy nucleotide triphosphate
dpi	Days post infection
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
F	Forward
FC	Fold change
FDR	False discovery rate
Fig	Figure
IFN	Interferon
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GO	Gene ontology
GSEA	Gene set enrichment analysis
hpv	Hours post vaccination
IL	Interleukin
LV	Lelystad virus
M	Matrix protein
MAPK	Mitogen activated protein kinase
miRNA	Micro RNA
MHC	Major histocompatibility complex

MLV	Modified live virus vaccine
mRNA	Messenger RNA
NAb	Neutralizing antibody
NFkB	Nuclear factor kappa beta
OD	Optical density
PAMs	Pulmonary alveolar macrophages
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffer saline
PBMCs	Peripheral blood mononuclear cells
Pi	Pietrain
pi	Post infection
PRR	Pattern recognition receptor
PRRS	Porcine reproductive and respiratory syndrome
PRRSV	Porcine reproductive and respiratory syndrome virus
qRT-PCR	Quantitative real time polymerase chain reaction
QTL	Quantitative trait loci
SSC	*Sus scrofa* chromosome
ss-cDNA	Sense strand complimentary DNA
R	Reverse
RBC	Red blood cells
RISC	RNA-induced silencing complex
rpm	Revolutions per minute
RT	Room temperature
TLRs	Toll-like receptors
TGFβ1	Transforming growth factor, beta 1
vs.	Versus

List of figures

List of tables

Chapter 1: General overview

1.1 Introduction

The porcine reproductive and respiratory syndrome (PRRS), an emerging viral infectious disease, results tremendous economic loss in the swine industry worldwide through reproductive failure in breeding sows and respiratory disorders in young and growing pigs. The PRRS has been continued to be of high concern for swine health, production and welfare issues in commercial farms. Despite much emphasis has been placed on development of preventative measures, sustainable PRRS control has not been established yet. However, there are possibilities for genetic control of PRRS through improvement of the host genetics by marker assisted selection breeding. There have been major breakthroughs in understanding the biology and ecology of PRRS virus (PRRSV), but the complexities of virus-host interaction in terms of genetic and epigenetic resistance mechanism are yet to be elucidated (Lunney et al. 2016). There are serious deficits in our knowledge of the key immunological targets for both B- and T-cell-directed protection, and the genetic bases of the immunological events occur at the transcriptome level in peripheral blood following early stage of PRRSV vaccination. To uncover the immunogenetic insights of virus-host interaction, this dissertation project conducted the global expression profiling of mRNA and microRNAs in the peripheral blood mononuclear cells (PBMCs) of PRRSV vaccinated German Landrace and Pietrain pigs. This study aimed to identify the potential candidate genes, biological pathways, and transcriptional networks involved with host immune response to PRRSV vaccination. The aim was seconded with estimating the breed differences on host transcriptional response to PRRSV vaccination as well as to explore the post transcriptional epigenetic regulatory mechanisms for vaccine induced differential gene expression in PBMCs. Herein below, general introduction to the dissertation topic with relevant literatures are presented briefly.

1.1.1 Porcine reproductive and respiratory syndrome (PRRS)

The PRRS is the most economically important infectious diseases of pigs caused by PRRS virus (PRRSV). PRRS causes huge economic loss in commercial farms through increasing morbidity and mortality of infected pigs. PRRS affects swine health by increasing susceptibility to secondary infection and is of major concern as animal welfare issue in consumers and business perspective (Lunney et al. 2011, Xiao et al. 2010). The impact of PRRS is substantial through all the stages of commercial pig production. The weaned and finishing pigs suffer from respiratory disorders due to secondary infection, which are worsened by the immuno-modulatory properties of the PRRSV. The post-weaning pneumonic phase can become chronic, reducing daily gain by 85% and increasing mortality to 10%–25%

(Dee 2014). In breeding herds, the losses are dramatic but, in general, only lasting for a few months until the breeding herd reaches stability. However, the reproductive problems may reoccur if the breeding herd becomes destabilized due to recycling of PRRSV from the finishing herd or excreting gilts. The impact of breeding stock includes reproductive impairment along with respiratory disorders. PRRS can cause up to 25 %–35 % stillbirths and mummies, and more than 10 % abortions in the infected breeding sows (Dee 2014). The PRRS associated cost usually include the producers' costs for vaccination against PRRS virus, treatments, diagnostics performed, or costs associated with the increased levels of biosecurity implemented to minimize the spread of the virus. The PRRS is currently endemic in majority of the pork producing countries through out the world (Shi et al. 2010). The average prevalence of PRRSV infection was estimated in eight European countries as 75 % of sows and in 67 % of nursery or growing pigs having no clinical signs. While the prevalence of PRRS clinical cases were estimated to occur in 10 % of sows and in 14 % of weaned or growing pigs (De Paz et al. 2015). The annual impact of PRRS in Europe in 2013 was estimated to be around 1.5 billion € being the impact on the growing-finishing pigs higher (54 %) than in breeding herds (46 %); (De Paz 2015). The prevalence and impact of PRRS is not uniform across all Europe, however, in most of the countries it costs between 5 € and 10 € per marketed pig or between 100 € and 200 € per inventoried sow annually (De Paz 2015). The estimated annual costs for PRRS in the USA alone were around $664 million (Holtkamp et al. 2013).

Before the etiological agent causing the PRRS disease was known, the syndrome was given various names such as 'pig plague 89', 'swine reproductive and respiratory syndrome', 'swine infertility and respiratory syndrome', 'porcine epidemic abortion and respiratory syndrome', 'blue ear disease', 'porcine reproductive and respiratory syndrome' (Goyal 1993). However, at the first international symposium on SIRS/PRRS held at St. Paul, Minnesota, USA, in 1992, where it was decided to name the syndrome as 'porcine reproductive and respiratory syndrome' and it's causal virus as 'porcine reproductive and respiratory syndrome virus (PRRSV)'. The PRRS was first described in 1987 in North America (Collins 1991), and during the winter of 1990-91 the disease appeared in Germany and in the Netherlands (Wensvoort et al. 1991). The first isolate of the causal agent of PRRS was named Lelystad Virus (LV) after the Dutch city where it was isolated, later referred as EU-strain or Type-1 PRRSV (Wensvoort et al. 1991). Shortly after the isolation of LV, a virus showing

resembling clinical field signs of PRRS was isolated in USA and referred as type-2 or VR-2332 or type-2 PRRSV (Collins et al. 1992).

The clinical manifestations of PRRS are of two major forms: reproductive and respiratory form (Wensvoort et al. 1991). The reproductive impairment caused by PRRSV infection depends on the age of the pig infected and on the pregnancy status and trimester of gestation of the infected sow/gilt (Rossow 1998). Studies found that pigs experimentally infected with nine different isolates of Type 2 PRRSV had major differences in clinical disease, rectal temperatures, and gross and histological lung lesion (Halbur et al. 1995a, Halbur et al. 1995b). Pigs infected with mildly virulent isolates or the LV had transient pyrexia, dyspnea and tachypnea, whereas infection with highly virulent isolates induced labored breathing, pyrexia, lethargy, and anorexia. Furthermore, studies have reported that the impact on reproductive performance may be isolate dependent (Halbur et al. 1995a, Halbur et al. 1995b, Mengeling et al. 1996). The clinical signs in sows are characterized by inappetence, anorexia, and reproductive disorders such as abortion, premature birth, birth of dead or weak piglets, and foetal death with or without mummification. A less frequently observed sign is transient blue discoloration of the ears, abdomen, or vulva (Terpstra et al. 1991). PRRSV infection in weaned pigs is characterized by fever, pneumonia, lethargy, and failure to thrive (Rossow 1998). Gross lesions observed following PRRSV infection vary widely and may be dependent on the virus isolate, genetics of the infected pig, and stress factors (environment and health status of the pig herd). The pulmonary lesions vary from none to diffuse consolidation and are commonly complicated by lesions resulting from concurrent bacterial infections which can localize separate or intermixed (Rossow 1998).

Once the PRRSV infection establishes, it can be divided into at least three distinct stages: acute infection, persistence, and extinction, which are each unique in terms of immunology, virology, and clinical disease (Lunney et al. 2016). The first stage is represented by acute infection, during which the lung serves as a preferential site of infection. The acute post infection phase is characterized by high viremia within 6–12 h post infection and high viral load in tissues which may last up to 28 days post infection despite the presence of circulating antibodies (Halbur et al. 1995a, Halbur et al. 1995b). Followed by the acute post infection stage, a persistent phase of infection continues with lymphoid tissues including tonsil and lymph nodes but not spleen (Allende et al. 2000, Rowland et al. 2003) as the primary site of virus replication. During this stage of persistent infection, virus replication subsides to the

point where virus is no longer detected in blood and lungs and pigs no longer exhibit overt signs of clinical disease. Continuous virus replication in regional lymph nodes accounts for the efficient transmission of virus to naıve pigs via oral-nasal secretions and semen (Christopher-Hennings et al. 2008). Subsequently, virus replication gradually decays until the virus becomes extinct in the host. The eventual disappearance of virus represents the final stage of infection. It is not known exactly when virus disappears, but replication can be maintained for as long as 250 days after infection (Wills et al. 2003). Therefore, PRRSV replication does not establish steady-state equilibrium but gradually declines over time, with the lymphoid organs as the site of the last vestige of virus replication before viral extinction (Allende et al. 2000). However, it should be noted that in the context of the typical commercial pork production setting, during which pigs are maintained for 250 days, PRRSV establishes a "life-long" infection for the majority of pigs if once infected (Chand et al. 2012).

1.1.2 PRRS virus (PRRSV)

The PRRSV is a member of the *Arteriviridae* family, along with along with equine arteritis virus (EAV), lactate dehydrogenase elevating virus (LDV) of mice, and simian hemorrhagic fever virus (SHFV); (Terpstra et al. 1991). Based on the similarities in the genomic orientation and replication mechanism, the *Arteriviridae* family together with the *Coronaviridae*, *Roniviridae*, and *Mesoniviridae* families is placed in the order *Nidovirales* (Cavanagh 1997). The common properties of arterivirus in terms of host-virus interactions include cytopathic replication in macrophages, the capacity to establish a persistent infection, and ability to produce severe disease (Snijder and Spaan 2007).

The PRRSV possesses pleomorphic morphology varying the shape from spherical to oval with a size about 50 to 65 nm and a layered nucleocapsid core of around 40 nm diameter (Spilman et al. 2009); (Fig 1). The nucleocapsid core is surrounded by a lipid membrane, the envelope where the structural proteins are embedded. The major protein components of the lipid envelope are GP5 and M, which together encompass at least half the amount of the viral proteins. The GP5 and M forms a disulfide-linked heterodimer through conserved cysteine residues in both proteins (Verheije et al. 2002). The minor structural proteins GP2, GP3, and GP4 forms a multimeric complex incorporated in the lipid envelope and for at least the Type 1 PRRS viruses E is also a part of this complex (Wissink et al. 2005, Music and Gagnon 2010, Das et al. 2010). The recently discovered ORF5a protein is believed to be the eighth

structural protein of PRRSV, but its orientation in the virion particle and its interaction with the other structural proteins still needs to be clarified (Firth et al. 2011).

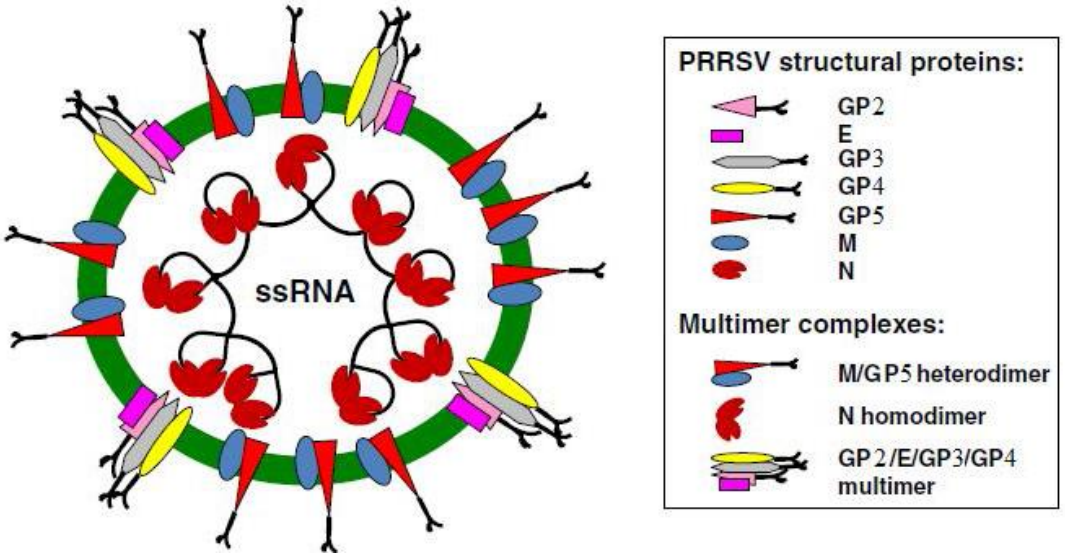

Figure 1. Schematic representation of the PRRSV virion structure. The orientation of the structural proteins of GP2, E, GP3, GP4, GP5, M, and N protein are shown. GP5/M forms a heterodimer and the minor glycoproteins and E forms a multimeric complex. The N protein homodimers are shown surrounding the PRRSV RNA genome (Music and Gagnon 2010 Kvisgaard et al. 2013).

The genome of PRRSV is a single-stranded RNA molecule with a positive-sense orientation. The genome is about 15-15.5 kb long with a 5'-end methylated cap structure and a 3'-end polyadenylated tail (Fig. 2) the untranslated regions (UTR) are present at both termini (Allende et al. 1999, Meulenberg et al. 1993). The genome encodes at least 10 open reading frames (ORFs), including the recently discovered ORF5a (Firth et al. 2011). The ORF1a and ORF1b constitute about 75 % of the genome, and encodes two long non-structural polyproteins, pp1a and pp1ab, with the synthesis of the latter depending on a ribosomal frameshift near the 3'-end of ORF1a (Meulenberg et al. 1993a, Snijder 1998). The ORF2-5 encodes the membrane glycoproteins (GP), GP2-GP5, and ORF6 and ORF7 encodes a non-glycosylated membrane protein (M) and the nucleocapsid (N) protein, respectively. Two small genes, ORF2b and ORF5a, are fully embedded in ORF2. Depending on the genotype partially or fully embedded in ORF5, encodes the non-glycosylated proteins E and ORF5a protein, respectively (Firth et al. 2011).

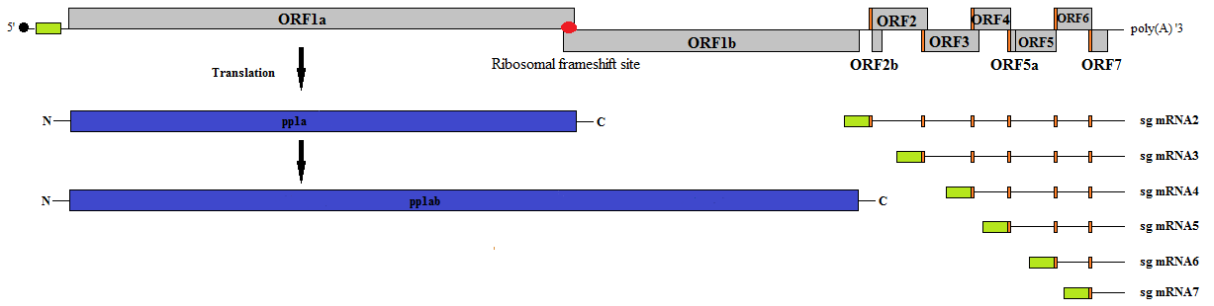

Figure 2. Schematic representation of PRRSV genome orientation. Each ORF encoded by the PRRSV genome is represented as a rectangle marked with the respective name of the gene. The 5' methylated cap structure is shown as a black sphere and the ribosomal frameshift is marked with a red sphere. The black lines at both termini represent the UTRs. The green box at the 5'-UTR represents the common leader sequence and the orange boxes located 5' to the ORF of the structural proteins represent the mRNA bodies. sg mRNA2-7 is shown to the right of the figure and the polyproteins pp1a and pp1ab are shown to the left (Kvisgaard et al. 2013).

Whole genome sequence analysis revealed that there is a marked genetic and antigenic variation between two PRRSV genotypes. The type 1 and type 2 strains are about 70% identical at the nucleotide level; and nucleotide sequence diversity within each genotypic group can be as much as 10% (Rowland et al. 2012). Initially it was believed that Type 2 PRRS viruses were more genetically diverse, while Type 1 PRRS viruses exhibited a lower degree of variations (Meng et al. 1995, Kapur et al. 1996). This perception has been changed following extensive sampling of Type 1 viruses which revealed an even greater diversity among European isolates than North American isolates (Stadejek et al. 2002, Stadejek et al. 2008). The country-specific clusters of PRRSV isolates have also been reported in Great Britain, Italy, and Denmark (Forsberg et al. 2002, Frossard et al. 2013). Based on host responses (e.g. cytokine responses), PRRSV can be divided into three immunotype such as attenuated, suppressive and inflammatory type (Amadori and Razzuoli 2014). Moreover, PRRSV of both genotypes show spontaneous genetic mutation and recombination producing new antigenic strains, which confronts the success of vaccination.

The PRRSV of both genotypes is highly infectious, about 10 or fewer particles are capable of establishing an infection when exposed by the intranasal route. The other routes (oral, vaginal, or eye) require higher doses, usually of the order of 10^3 to particles. The virus is spread by nasal secretions, saliva, feces and urine and field studies suggest it can be airborne up to 3 km. The breeding aged females can be infected with both undiluted and extended semen carrying the virus (Gradil et al. 1996).

1.1.3 Host-PRRSV interaction

The domestic pig (*Sus scrofa domesticus*) is the only natural host for PRRSV infection. Being an obligate intracellular organism, the PRRSV needs to enter into host cells for its survival and replication. In addition to host species specificity, PRRSV has very restricted tissue/cell specificity. PRRSV primarily enters into the alveolar macrophages through receptor-mediated endocytosis (Duan et al. 1998). Initial binding of PRRSV to its host cell occurs through interactions with heparan sulphate glycosaminoglycans (Delputte et al. 2005). After entry into the host cell, PRRSV enters the early endosome where it co-localizes with the scavenger receptor cluster differentiation 163 (CD163); (Van Gorp et al. 2009). The receptor CD163 together with at drop in pH is believed to be involved in the uncoating of the virus and release of its genome into the cytosol (Van Gorp et al. 2008, Van Gorp et al. 2009). The ATPase H+ Transporting V1 Subunit B2 (ATP6V1B2) gene encodes a component of vacuolar ATPase (V-ATPase) that mediates acidification of endosomal organelles (Hinton et al. 2009), facilitates the uncoating of the virus. Viral nucleic acids could be sensed by Toll-like receptors (TLRs) pathway or RIG-I pathway both of which lead to type-I IFN induction by activating IRF3 and IRF7, and to inflammatory cytokines expression by activating the MAPK signaling pathway. The molecular events of interaction between porcine alveolar macrophage and highly pathogenic PRRSV at the transcriptome level are presented in Fig 3, as described by Zhou et al. (2011).

The replication of the PRRSV genome takes place in the cytoplasm of the host cell. The proteins involved in replication are encoded in the two major ORFs, ORF1a and ORF1b. Before the replication of the viral genome can take place, the viral proteins involved in this process have to be synthesized. The ORF1a is translated directly from the genomic RNA to the polyprotein 1a (pp1a) where ORF1b is translated through A-1 ribosomal frameshift just upstream of the ORF1a termination codon resulting in the synthesis of polyprotein 1ab (pp1ab) from ORF1ab (Snijder and Meulenberg 1998). After the protein synthesis, the polyproteins are cleaved into 14 functional nonstructural proteins (nsps). The key enzymes for the RNA replication are the RNA-dependent RNA polymerase (RdRp) and the RNA helicase both encoded in ORF1b (nsp9 and nsp10); (van Dinten et al. 1996). The structural proteins are not translated from the genomic RNA, as the 3' proximal third of the genome is not accessible for ribosomes involved in genome translation, but instead the structural proteins are translated from a nested set of sub-genomic mRNA's (sg mRNA2-7); (Pasternak et al. 2006); (Fig. 2).

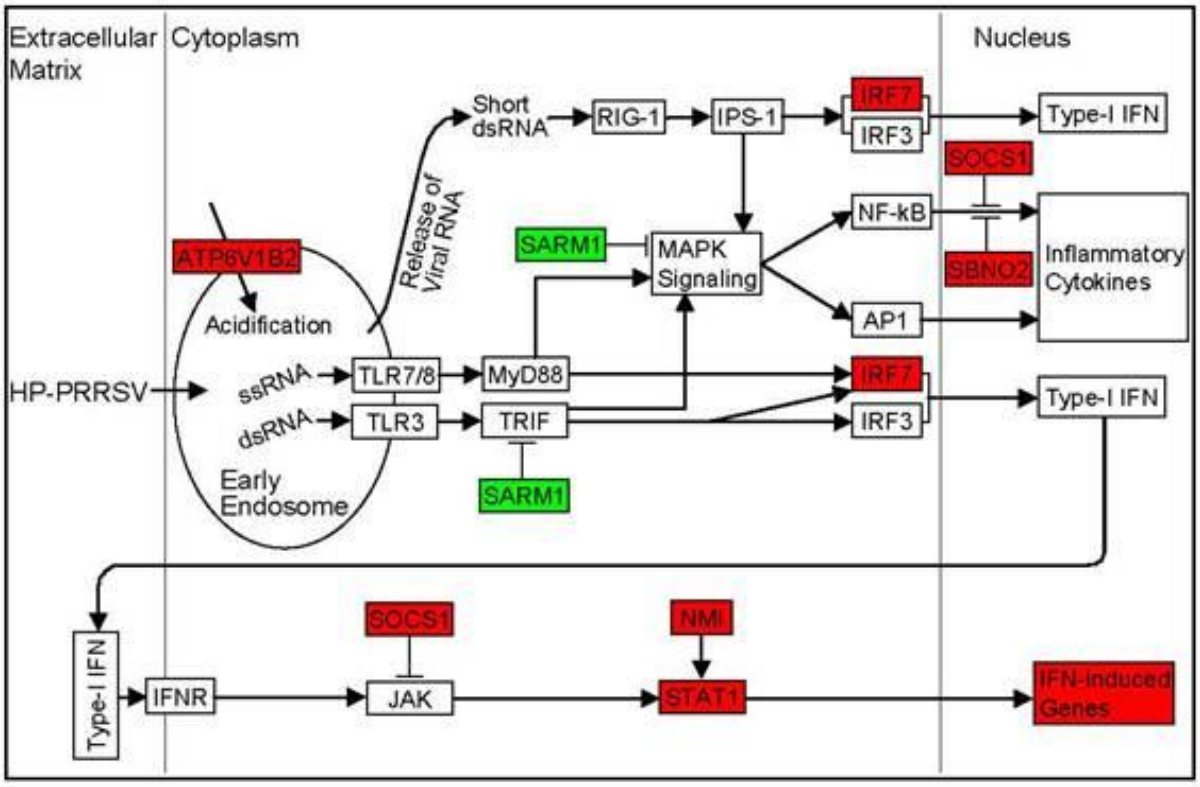

Figure 3. Transcriptome alterations induced by PRRSV infection in the pulmonary alveolar macrophages of pig. Red background in the gene box indicates upregulation of the gene expression, green indicates downregulation, and white indicates no change of the gene expression in pulmonary alveolar macrophage after in vivo infection with highly pathogenic PRRSV (Zhou et al. 2011).

1.1.4 Vaccination for PRRS control

Generally, the control strategy for infectious disease is directed into two dimensions: either to eliminate the causal agent or to improve the host defense. The vaccination is aimed to train the host immune system for preventing subsequent infection of highly pathogenic PRRSV. The goal of control strategies in production herd is to minimize economic losses through reducing clinical manifestation and mortality; while the control strategies in breeding herds aim to produce PRRS-negative piglets at weaning, minimize the PRRSV shedding and consequent horizontal transmission in growing pigs (Corzo et al. 2010). Overall, the PRRS control strategies include proper gilt acclimatization, partial depopulation, temporal herd closure, management practices to minimize PRRSV horizontal transmission, and routine vaccination to the pigs.

Vaccination has been considered to be the primary and most economic method to achieve immunity and protecting herds from losses associated with infections by highly virulent strains of PRRSV (Corzo et al. 2010). The immunization method consists of inoculating antigens derived either from killed adjuvant or live attenuated virus in pigs with an attempt to

build herd immunity against the subsequent virus infection. At least 20 PRRS vaccines are commercially available, worldwide including both attenuated live vaccines (e.g. Porcilis® PRRS and Ingelvac® PRRS MLV) and inactivated vaccines (e.g. Progressis® and PRRomiSe®) are commercially available for immunization of pigs against PRRSV (Murtaugh and Genzow 2011). The attenuated live virus vaccines and/or killed virus vaccines and resident live virus can be used as immunogens (Fano et al. 2005). In general, inactivated virus vaccines are not effective as it does not elicit protective immunity in piglets when used as sole immunization method (Zuckermann et al. 2007). A recent study to evaluate the effect of killed autogenous vaccines on PRRSV infection dynamics concluded that "the experimental heterologous inactivated vaccines and the commercial inactivated vaccine had no or only a limited influence on viremia" (Geldhof et al. 2012). However, the modified live virus (MLV) vaccines are effective in protecting the pig from challenge with a genetically similar or "homologous" virus, but provide little protection against heterologous (genetically diverse) PRRSV isolates (Murtaugh and Genzow 2011).

1.1.5 Host immune response to PRRSV

The immune system of pig is composed of three components such as passive immunity, innate immunity and adaptive immunity. All three components of the immune system take part in developing immune response to PRRSV infection or vaccination.

Innate immunity is the inherent property of immune system which provides the initial protection against the invading pathogen or toxins through inflammatory response. Once the virus antigen come in contact to the host immune cells, pattern recognition receptors (PRRs), such as Toll-like receptors (TLR) and RIG-I-like receptors (RLR), recognize pathogen-associated molecular patterns (PAMPs); (Luo et al. 2008). Following PAMP binding, TLRs and RLRs communicate through specific adaptor proteins that activate transcription factors interferon (IFN) regulatory factor 3 (IRF3), IRF7, and NF-κB, which in turn induces Type 1 IFNs and proinflammatory cytokines. Interferons attach to IFN receptors located on neighboring cells, which induces the JAK-STAT pathway, and signals IFN stimulated genes (Sun et al. 2012). These genes encode the proteins that ultimately block virus transcription, translation, or replication. One characteristic feature of PRRSV infection that probably contributes to the retarded development of a specific cell-mediated immune response is the apparent lack of an adequate IFN−α response to the viral infection. Pigs infected with PRRSV fail to generate any significant inflammatory cytokine expression in the lungs, including the type I interferons (IFN-α/β), interleukin-1 (IL-1), and TNF-α (Thanawongnuwech et al. 2001).

The expression of type I interferon is important for the activation of innate immune response (Kimman et al. 2009). The downregulation of INF-α can be a crucial step in PRRSV pathogenesis as INF-α has been shown to inhibit PRRSV replication (Albina et al. 1998), hence the weak initial innate immune response may lead to longer survival of the virus in the infected animal (Kimman et al. 2009).

The bridging between innate and adaptive immunity in viral infections occurs through the interaction of dendritic cells with type I interferon and the dendritic-cell mediated polarization of T-cell function (Loving et al. 2015). The production of IFN−α by plasmacytoid dendritic cells (pDCs) has an autocrine effect that promotes their functional and phenotypic activation events necessary for their optimal expression of co-stimulatory molecules and subsequent ability to cause naïve T cells to differentiate into IFN−γ secreting cells (Levy et al. 2003). There are two alternative routes (IL-12- or type I IFN-dependent) that can lead to an adaptive Th 1 cell-mediated immune response with potent antiviral effects (Loving et al. 2015). According to a scenario involving the presence of less than a requisite amount of IFN−α, IL-12 could provide the necessary impetus for the development of an anti-viral IFN−γ response. In this regard, IL-12 mRNA has been detected in porcine macrophages infected with PRRSV (Thanawongnuwech et al. 2001), and transiently in the lungs of PRRSV-infected pigs (Chung and Chae 2003). However, this pathogen is also apparently a poor stimulator of IL-12 production, since a negligible quantity of IL-12 mRNA or protein was produced by porcine PBMCs exposed in vitro to PRRSV (Royaee et al. 2004, Calzada-Nova et al. 2011).

The pig's adaptive immune response against PRRSV is characterized by being delayed and defective mainly because of suboptimal induction of innate immune response, specially the induction of type 1 IFNs (Beura et al. 2010). Following a natural infection, it takes at least 3 months to reach immunity at peak levels and it does not appear to be solid enough to prevent reinfection, especially if the reinfection is caused by antigenically heterologous PRRSV strains (Zuckermann et al. 2007). Following vaccination, the earliest and strongest antibody response is directed against the N protein which is measureable 5-9 days post infection (PI); (Kimman et al. 2009). Antibodies against the two non-structural proteins nsp1 and nsp2 are evident at 14 days PI, and reach peak levels at 28-35 days PI (Oleksiewicz et al. 2001, De Lima et al. 2006, Johnson et al. 2007). All these early produced antibodies are non-neutralizing whereas the neutralizing antibodies first appear 4 weeks PI or even later (Lopez and Osorio 2004). The neutralizing antibody response against the GP5 neutralizing epitope is

weak and delayed, and some animals fail to make a detectable antibody response against GP5 (Chand et al. 2012).

In addition to innate and adaptive immunity, the passive immunity also provides short term protection for the piglets against PRRSV infection. The passive immunity is acquired by the newborns from mother through intra-colostrum transmission of immunoglobulins. If sows are immunized through previous PRRSV infection or vaccination, piglets are likely to be immunized at least for certain periods after birth. The passively acquired host immunity provides a short-term protection to PRRS disease in young piglets. The maternally derived antibody (MDA), however, can adversely affect on vaccination success by interfering with vaccine induced antibody response, and can increase disease severity through antibody dependent enhancement process (Yoon et al. 1996). Therefore, relatively lower level of MDA is appreciated at the time of primary vaccination with modified live virus vaccine against PRRS.

1.1.6 Breed differences on host response to PRRSV

Breed has been considered one of the most potential host determinants influencing the susceptibility to PRRSV infection in pigs (Lunney and Chen 2010). The breed differences on the host resistance or susceptibility to PRRSV infection among swine breeds has been reported in several studies (Halbur et al. 1998, Christopher-Hennings et al. 2001, Petry et al. 2005, Vincent et al. 2005, Reiner et al. 2010, Ait-Ali et al. 2011, Xing et al. 2014). The difference on relative resistance to PRRSV infection has been observed between Chinese Meishan and European pig breeds (Halbur et al. 1998). The variation in host innate immunity to European type PRRSV infection has been reported between Landrace and Pietrain pigs through global gene expression profiling of in vitro PRRSV infected pulmonary alveolar macrophage (Ait-Ali et al. 2011). Halbur et al (1998) infected Duroc, Hampshire, and Meishan pigs with PRRS virus (VR-2385) at 22 to 38 days of age and compared the cytopathic lesions 10 days post infection. Hampshire pigs had significantly more severe lung lesions than Duroc or Meishan pigs. The Meishan pigs had significantly less PRRS virus detected in the lungs, but significantly more heart and brain lesions. The Duroc pigs had significantly lower serum antibody titers against PRRS virus. The investigators concluded that the differences observed could, in part, be influenced by breed genetics (Halbur et al. 1998). The non-lean pigs show a reduced susceptibility to PRRSV (Petry et al. 2005), as also shown by the comparative evaluation of PRRSV infection of a local German breed and of commercial Pietrain pigs (Reiner et al. 2010). The breed comparisons on degree of

susceptibility to PRRSV has been summarized from higher order to lower as Hampshire > Large White > Duroc > Landrace (reviewed by Lewis et al. 2007). One of the recent studies of our group revealed the differences between Duroc and Pietrain pigs in terms of transcriptome profiles of lung dendritic cells after in vitro PRRSV infection (Pröll et al. 2016, unpublished data). In the current study, we therefore tested whether any differences on PRRSV vaccine induced transcriptional responses between German Landrace (DL) and Pietrain (Pi) pigs.

Landrace and Pietrain are two leading breeds for commercial pig production worldwide. The breeding of the German Landrace pig was commenced in the northwestern parts of Germany and particularly in the Lower Saxony State in about the year 1900 with local pigs being used as the seed stock. Between 1948 and 1958, importations of Danish and Dutch Landrace enhanced the improvement of the DL breed in Germany. The DL is one of the leading pig breeds in Germany, with white body coat and heavy drooping ears similar to that of the Landrace strains in other countries of the world. The DL pig breed has been chosen for meatiness and are not as great in length and size as a few of the Landrace strains of other countries. Selection attempts have been particularly directed toward outstanding fertility, intense milking and good mothering traits. There has been special concentration, after weaning, to effectiveness in feed change and to elevated cut-out values. The DL tends to be slightly shorter and smaller than rather extreme size seen in the Landrace of some other nations. The DL pigs have small lungs compared to their body size and they do not sweat a lot and serve as reservoir for many infectious pathogens. On the other hand, the Pietrain breed originated from Belgium in the 1950's and later exported to other countries. The breed is of medium size with erected ears and white body coat with black spots (Brings, 1983). Around the black spots there are characteristic rings of light pigmentation that carries white hair. The breed is commonly referred to as being of piebald markings and well known for having extremely high lean to the fat ratios.

1.1.7 Genetic control for PRRS

Genetic control through improving the host resistance has been considered another promising strategy to combat the PRRSV infection. To implement the genetic control, it is necessary to identify genomic regions and DNA markers useful for selecting pigs with improved PRRS resistance while retaining desired production traits. Several research groups have probed for genes and genetic variants and identified quantitative trait locus (QTL) involved in swine health, immune response, and disease resistance traits (reviewed by Lunney and Chen 2010).

With the full length swine genome sequence completed and immunome annotated (Groenen et al. 2012, Dawson et al. 2013, Tuggle et al. 2016), studies have accelerated. Genome-wide association study revealed a major QTL on *Sus scrofa* chromosome 4 (SSC4) associated with host resistance to in-vivo PRRSV infection (Boddicker et al. 2012). The association of this region on SSC4 with PRRS resistance was further validated by the presence of single nucleotide polymorphism (SNP) marker, WUR10000125 (WUR) in the same region (Boddicker et al. 2014a, Boddicker et al. 2014b). Gene expression study in PRRSV infected pulmonary alveolar macrophages over 24 h post infection period suggested that myxovirus resistance 1 (MX1) and ubiquitin specific protease (USP) genes may play important role in clinical disease during PRRSV infection (Zhang et al. 1999). It has been reported that the overexpression of the porcine USP18 resulted a limited replication of PRRSV (Ait-Ali et al. 2009) and a subsequent study suggested that the SNP G-1533A polymorphism in the promoter region of porcine USP18 gene is a potential DNA marker for the resistance to PRRSV (Li et al. 2014). Recently, a single nucleotide polymorphism (SNP) WUR10000125 (WUR) at the interferon-inducible guanylate-binding protein 1 gene (GBP1) has been found to be associated with European PRRS resistance and growth performance in pig (Abella et al. 2016).

The advent of PRRS research suggests that the robust and sustainable PRRS control could be achieved by improving the host genetics through selective breeding for PRRS resistance (Lunney et al. 2011, Rowland et al. 2012, Lunney et al. 2016). However, research on genetic resistance to PRRS is multifactorial as reviewed by Lunney et al. (2016); it is aimed at identifying and understanding the host allelic variation associated with virus replication, which is dependent on the isolate, its virulence, tissue tropism, persistence, and route of infection, as well as on the host immune response and the speed and regulation of innate and adaptive antiviral immunity. The genetic variations in PRRS resistance/susceptibility are polygenic and are likely influenced by the pig's health status and its microbiome, concomitant infections, and nutritional plane. Taken together, it is imperative to understand well the immunological events resulted from host-vaccine interactions at the transcriptional level for stepping forward to genetic control of PRRS through improving the host resistance.

1.1.8 Alteration of mRNA transcriptome profile in response to PRRSV

Transcriptome refers to the complete set of RNA transcripts produced by the genome at a time point. The transcriptome is dynamic and changes under different circumstances due to

different patterns of gene expression. The global mRNA transcriptome profiling enables to generate a comprehensive, genome-wide picture of what genes are active in PBMCs in response to PRRSV vaccination in pigs. The transcriptome data provide a good place to start for exploring potential candidate genes responsible for the host immunity, and thereby susceptibility or resistance to PRRSV in pigs. The availability of high-throughput omics technology including microarray, facilitates the detection of transcriptome alterations to understand molecular genetics behind the complex interaction of vaccine-host immune cells in pigs (Tuggle et al. 2007, Tuggle et al. 2010, Schroyen and Tuggle, 2015). The microarray-based transcriptome profiles have been investigated to characterize the host immune response to PRRSV by several authors (Genini et al. 2008, Ait-Ali et al. 2011, Zhou et al. 2011, Wysocki et al. 2012). Badaoui et al (2013) recently illustrated how the information of multiple PRRS studies could be used simultaneously to gain insight on host response to PRRSV challenges. They have collected all publicly available microarray data covering multiple porcine immunology studies and including many different breeds, tissues, pathogens, and array platforms. The data of 779 general immune response arrays were assembled, and separate meta-analyses for differential expression were performed using these 779 arrays as well as a subset of 279 arrays specifically from PRRS experiments (Badaoui et al. 2013). However, transcriptome data on peripheral blood in response to in vivo PRRSV vaccination in pig is rare.

1.1.9 Alteration of microRNA transcriptome profile in response to PRRSV

Expression dynamics of global microRNAs in host cells in response to PRRSV has recently been a subject of intensive research. MicroRNAs are endogenous, small non-protein-coding single stranded RNAs and are known to be involved in post transcriptional epigenetic regulation of gene expression (Bartel 2004). Several attempts have been made to determine the effects of PRRSV infection on the expression changes of miRNAs of the host cells. Analyses of miRNA profiles of PRRSV infected alveolar macrophages have identified a total of forty cellular miRNAs whose expression was significantly altered within the first 48 hours of infection (Julie et al. 2013). These findings suggest that miRNAs are likely important mediators of PRRSV replication and host defense to infection. Li et al (2015) identified microRNAs related to PRRSV replication and host immune responses using eight lung microRNA transcriptomes from pigs infected with a highly pathogenic PRRSV. They also identified the microRNAs that could bind to the PRRSV genome and candidate editing sites on microRNA sequences (Li et al. 2015). The functional role of some candidate microRNAs

on replication of PRRSV and host immune pathways upon PRRSV infection has also been studied by several authors. Accumulated reports indicated that miR-181 and miR-23a inhibited PRRSV replication through binding to PRRSV genome (Guo et al. 2013, Zhang et al. 2014), while miR-181, miR-125b and miR-506 suppressed PRRSV replication through regulating host antiviral pathways (Gao et al. 2013, Wang et al. 2013, Wu et al. 2014). To this end, it would be interesting to investigate the consequences of PRRSV vaccination on expression dynamics of global miRNAs and thereby exploring the integrated miRNA-miRNA network of vaccine induced immune response. Most of the previous PRRSV transcriptome studies were based on lung tissue, particularly the alveolar macrophage, may be since lungs provide primary site of viral replication during natural infection. However, expression profiles of global microRNAs as well as mRNAs in peripheral blood cells following PRRSV vaccination in pigs has not yet been systematically analyzed.

1.1.10 PBMCs transcriptome model for evaluating the PRRSV vaccine immunity

The present dissertation work implemented the peripheral blood mononuclear cells (PBMCs)-transcriptome model to characterize the host-PRRSV vaccine interaction in terms of immune responses. The PBMCs are subset of white blood cells that include lymphocytes (T cells, B cells and NK cells), monocytes and dendritic cells. The proportion of lymphocytes are typically in the range of 70–90% of PBMCs, monocytes range from 10–30% of PBMCs, while dendritic cells are rare, being only 1–2% of PBMCs. The PBMCs are readily accessible from anticoagulated whole blood samples through density gradient centrifugation. In addition, the PBMCs model has several advantages over respiratory tissue/cells including quick and convenient sampling; minimum stress to the study animals, and possibility of time course investigation through repeated sampling from the same individual.

Transcriptome profiling of PBMCs throughout the course of immune responses has been widely used to identify the extent and kinetics of differential gene expression (Ojha and Kostrzynska 2008, Gao et al. 2010, Huang et al. 2011, Wilkinson et al. 2012, Adler et al. 2013a). The global transcriptome studies have shown that porcine PBMCs can display gene expression patterns which are characteristic for certain pathogenic infection, for example, classical swine fever (Li et al. 2010) and tetanus toxoid (Adler et al. 2013b). Moreover, the PBMCs transcriptome represents not only the primary immune function of leukocytes, but also displays transcriptomic shifts of other tissues and organs due to physiological and environmental alterations (Liew et al. 2006, Kohane and Valtchinov 2012). Therefore,

PBMCs transcriptome model was used for evaluation of host transcriptional response to PRRSV vaccination in pigs.

1.2 Aim and objectives of the dissertation

The overall intention of the dissertation was to elucidate the immunogenetic insights of host-virus interaction for better understanding the genetic control of PRRS. To make the dissertation aim achievable, the following specific objectives were settled:

1. Investigation of the global mRNA profiles of PBMCs to:
   - characterize the innate immune transcriptional response to PRRSV vaccination in German Landrace and Pietrain pigs
   - identify potential candidate genes and functional network of PRRSV vaccine altered transcriptomes associated with innate and adaptive immunity to PRRSV vaccine in Pietrain pigs
   - estimate the breed difference on vaccine induced mRNA alteration between German Landrace and Pietrain pigs
2. Investigation of the global microRNA profiles of PBMCs to:
   - characterize microRNAome expression dynamics in PBMCs associated with innate immune response to PRRSV vaccination
   - explore breed variation on PRRSV vaccine induced microRNAome alteration between German Landrace and Pietrain pigs
3. Integrated analysis of miRNA-mRNA expression profiles of the same PBMCs to understand the cellular regulatory network of gene expression associated with host immune response to PRRSV vaccine.

1.3 Materials and methods

To achieve the dissertation goal, several materials and methods were used. The particular materials and methods are described elaborately in the respective chapters of this dissertation. The importance of key methods and their technical procedure are briefly pointed out here.

1.3.1 Experimental setup and ethics statements

Two in vivo vaccination studies were designed to investigate the host immunogenetic response to PRRSV vaccine in pigs of two breeds. The first experiment was conducted on purebred German Landrace pigs followed by the second one on purebred Pietrain pigs. Piglets those were clinically healthy, and had no history of respiratory diseases and birth defects were included in the experiments. Animals of both experiments were housed in the pig research station at Frankenforst, University of Bonn, Germany. The *in-vivo* experiments were performed according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2003). The research proposal has been approved by the Veterinary and Food Inspection Office, Siegburg, Germany (ref. 39600305-547/15). The blood sampling protocol was also approved by the State Agency for Nature, Environment and Consumer Protection, North Rhine-Westphalia, Germany (permission nr. 84-02.05.04.14.027).

1.3.2 Vaccination and blood sampling

For the first experiment, 12 female piglets from two sows farrowed at the same day were allocated into two separate pens having at least three from each sow in either vaccinated (n=6) or unvaccinated (n=6) group (Fig 4A). The piglets of vaccinated group were treated with intramuscular vaccination with primary dose at day 28 and booster dose at day 56 of age using the modified live PRRSV vaccine of European strain (Porcilis® PRRS, MSD Animal Health, Germany) according to the routine farm vaccination program. The piglets of control group were kept unvaccinated as health control and managed with the same husbandry protocol. About 8 ml whole blood samples with 1.5 mL anticoagulant (0.5 M EDTA) were collected longitudinally from all pigs immediately before (0 h) and several time points after 1st and 2nd vaccination (Fig 4A). For the second experiment, only one group of six Pietrain piglets was vaccinated and the blood sampling was performed immediately before and several time points after vaccination (Fig 4B).

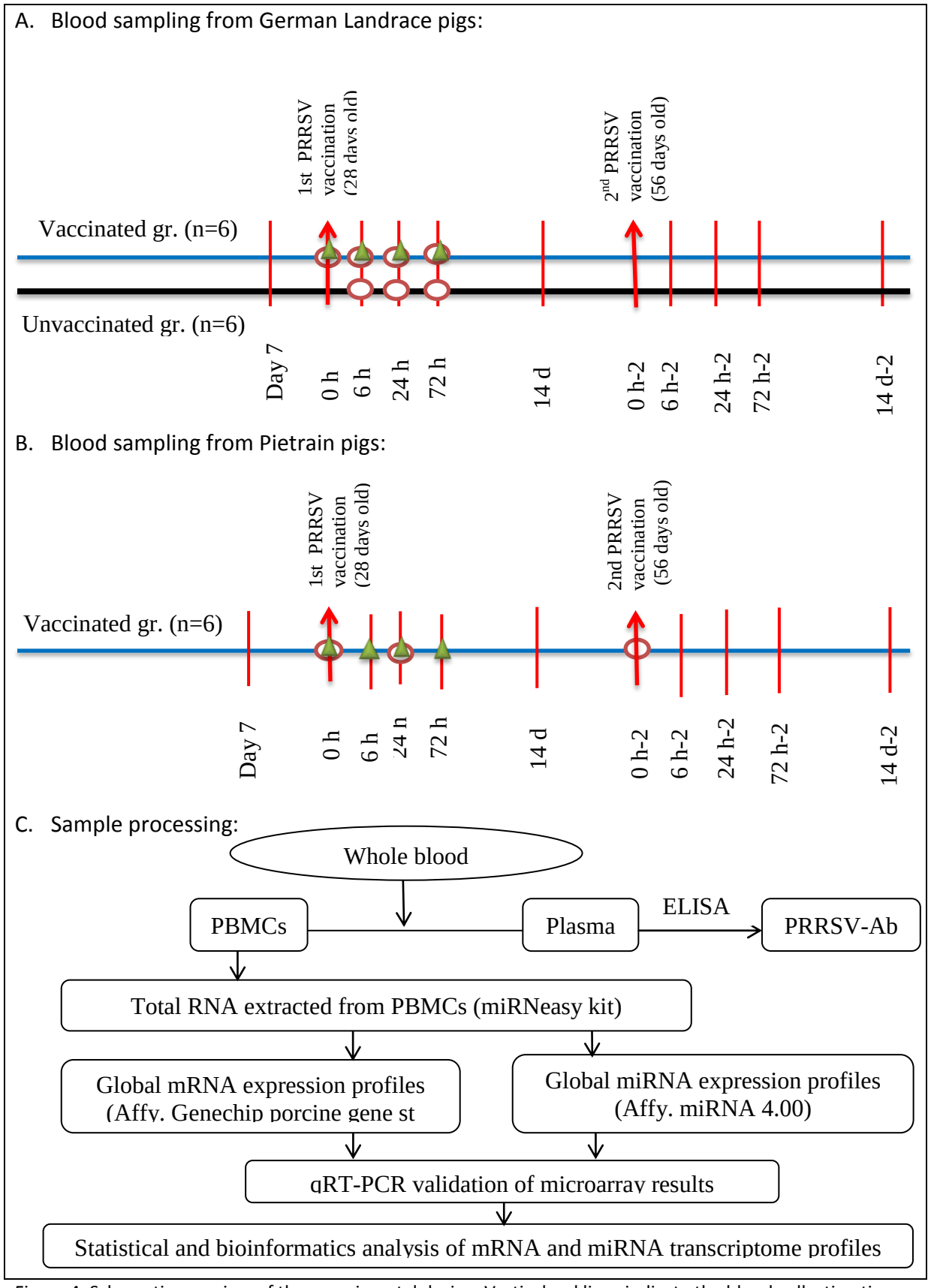

Figure 4. Schematic overview of the experimental design. Vertical red lines indicate the blood collection time points, vertical red arrow indicated the vaccination time points, red hollow circle indicated the time points used for mRNA expression profiling and green triangle indicate the time points used for miRNA expression profiling

1.3.3 Isolation of PBMCs and plasma

Isolation of peripheral blood mononuclear cells (PBMCs) was performed by density gradient centrifugation with Histopaque®-1077 (Sigma-Aldrich, Munich, Germany). In brief, whole blood were diluted 1:1 with phosphate buffered saline (PBS, pH=7.4); and carefully layered over 8 mL of Histopaque®-1077 solution kept in a 50 ml conical tube and centrifuged at 1500 rpm for 30 min at room temperature. After centrifugation, PBMC preparation was carefully aspirate and treated with RBC lysis buffer (Invitrogen, Darmstadt, Germany) to make free of erythrocytes. Finally PBMCs were washed twice with PBS and harvested as pellet. The PBMC was stored at -80 °C until RNA isolation.

1.3.4 Measurement of plasma antibody level

To monitor the PRRSV-specific antibody titre, the plasma samples were screened by ELISA (PRRSV-AK screening, Synlab Vet GmbH, Augsburg, Germany) according to manufacturer's protocol. The optical density (OD) of each well was measured at 650 nm using the Bio-Rad 680 microplate reader. The presence or absence of PRRSV antibody was determined by calculating the sample to positive (S/P) ratio. The S/P ratio was calculated according to the following equation: S/P ratio (%) = 100 × [(OD of test sample – Mean OD of negative controls) / (Mean OD of positive controls – Mean OD of negative controls)]. The samples were considered to be positive for PRRSV antibody if the S/P ratio was more than 0.4 as described by Kittawornrat et al (2012).

1.3.5 Extraction and quality control of total RNA

Total RNA was extracted from PBMCs by using the miRNeasy mini kit (P/N 217004, Qiagen, Co.) according to the manufacturer's instruction along with on-column DNase treatment (P/N 79254, Qiagen, Co). RNA concentration and purity were measured by NanoDrop® spectrophotometry (ND-8000; NanoDrop Technologies). Extracted RNA was determined to be of high purity, as indicated by the absorbance ratio (A260:A280) being very close to 2.00 (1.80 – 2.20). RNA integrity was checked by visualization on 2 % agarose gel containing ethidium bromide followed by micro capillary electrophoresis on an Agilent 2100 Bioanalyzer with RNA 6000 Nanochip Kit (Agilent Technologies, Waghäusel - Wiesental, Germany).

1.3.6 Microarray-based global mRNA expression profiling of PBMCs

The microarray technology enables a snapshot of the entire cellular transcriptome (either messenger RNA or microRNA) on a single microarray chip, furnishing investigation with a global perspective of the complex interactions among thousands of genes simultaneously (Schena 1996). In fact, the RNA profile obtained from microarrays is a static representation of the biological state of the sample and yields the highest information and throughput of any classification assay (Ebert and Golub 2004). In the present study, the Affymetrix oligonucleotide microarray platform has been employed for global expression profiling of both mRNA and miRNA in the same PBMCs samples to determine the consequences of PRRSV vaccination on cellular regulatory networks of gene expression.

For mRNA expression profiling, the GeneChip® WT PLUS Reagent kit (Affymetrix, Santa Clara, CA, USA) was used to synthesize the microarray target probes. The cDNA-based microarray target preparation protocol based on WT PLUS kit was considered for the following reasons: 1). This kit enables priming of entire length of each transcripts in the sample including both polyadenylated (poly-A) and non-poly-A mRNA while many traditional 3´ based expression arrays do only for poly-A containing end. 2). This kit enables synthesis of biotinylated sense strand DNA target which produce a DNA-DNA duplex upon hybridization instead of biotinylated antisense RNA (cRNA) resulting RNA-DNA duplex on hybridization using standard protocol for 3´ based expression arrays (Eklund et al. 2006). Therefore this protocol provides more specific, unbiased and maximum coverage of the transcriptomes expressed in the sample. A set of poly-A RNA controls was used as exogenous positive controls to monitor the whole process of target preparation.

The first-strand cDNA was synthesized from 100 ng of total RNA. Then it was converted to the second-strand cDNA by DNA polymerase in the presence of RNase H (Fig 5). The double-strand cDNA was subjected to *in-vitro* transcription for the synthesis of the antisense RNA (cRNA). After bead purification, the cRNA was converted into the sense-strand cDNA (ss-cDNA). The purified ss-cDNA was fragmented followed by labeling with biotin. Then the biotinylated microarray target probes were hybridized using the hybridization, washing and staining steps, the GeneChip® Hybridization, Wash and Stain Kit (Affymetrix, Inc.). About 130 µL of the fragmented and labeled ss-cDNA preparation in a cocktail was loaded on the whole transcript microarrays (GeneChip® Porcine Gene 1.0 ST Array of 81/4 format) and hybridized for 16 h at 45 °C and 60 rpm. The hybridization and scanning was performed in

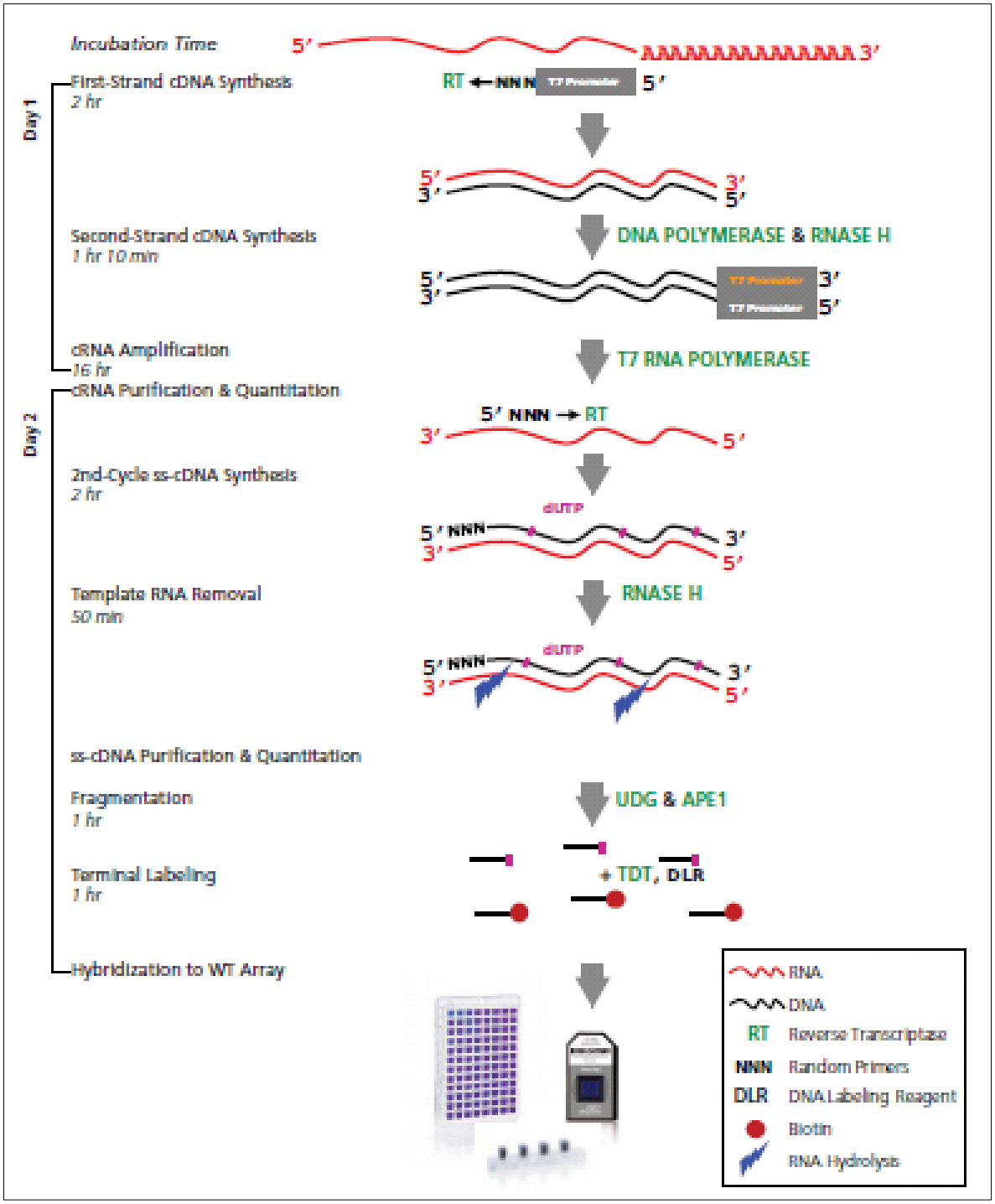

Figure 5. Workflow for the preparation and labeling of microarray target probes for mRNA expression. It was based on the Gene chip WT PLUS kit (Affymetrix Santa Clara, USA)

the Affymetrix array processing unit at Life and Brain center, Institute of Human Genetics, University of Bonn, Germany. The array images were then processed with the Affymetrix GeneChip Command Console™ (AGCC) software to align spots, to integrate ID data files and to export reports of spot intensity data.

1.3.7 Microarray-based global microRNA expression profiling of PBMCs

For global microRNA expression profiling, the FlashTag™ Biotin HSR RNA Labelling kit (P/N 901910; Affymetrix, Santa Clara, CA, USA) was used for synthesizing the labelled microarray target probes (Fig 6). The ELOSA assay was performed to evaluate the labelling efficiency of the kit. The biotinylated total RNA was then hybridized for 16 h using the Affymetrix GeneChip miRNA Array v.4.0 (Affymetrix, Santa Clara, CA, U.S.). Then hybridized array chips were washed and stained using the Affymetrix GeneChip Hybridization Wash and Stain Kit and were then scanned with the Affymetrix GeneChip Scanner 3000 7G (Affymetrix, Santa Clara, CA, U.S.).

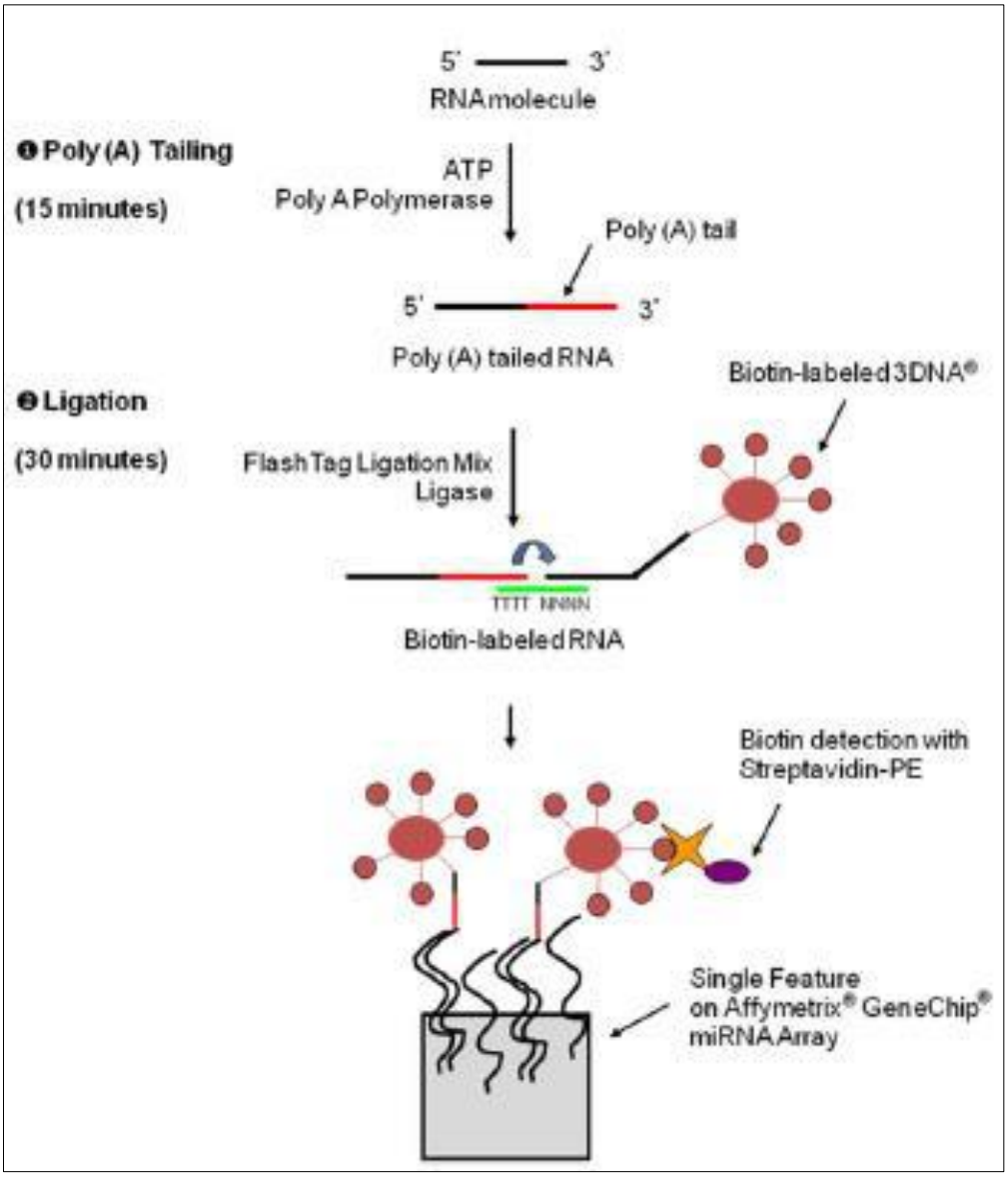

Figure 6. Workflow for the preparation and labeling microarray target probes for miRNAs expression. It was based on the FlashTag™ Biotin HSR RNA Labelling kit (P/N 901910; Affymetrix, Santa Clara, CA, USA)

1.3.8 The qRT-PCR validation of microarray expression results

The quantitative real-time PCR (qRT-PCR) has been considered one of the sensitive methods for technical validation of expression changes observed in multiple gene sets in microarray (Chuaqui et al. 2002). Therefore, the current microarray results for both mRNA and miRNAs expression were validated by qRT-PCR through measuring selected mRNA and miRNAs in the same RNA samples as used for microarray hybridization.

For validation of mRNA expression, the total RNA was transcribed into cDNA using the First Strand cDNA Synthesis Kit (P/N K1612, Thermo Scientific, Co.). The qRT-PCR reaction was set up taking 1.0 µl of cDNA template, 8.0 µl of deionized RNase free water, 0.5 µl of upstream and downstream primers, and 10 µl iTaq™ Universal SYBR® Green Supermix (Bio-Rad laboratories GmbH, Germany) in a total reaction volume of 20 µl and were amplified by the StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Darmstadt, Germany). The thermal cycling conditions were 95 °C for 3 min, 95 °C for 15 sec, 6 °C for 45 sec (40 cycles); 95 °C for 15 sec, 62 °C for 1 min, 95 °C for 15 sec. All reactions were run in duplicate and the average value was used as expression value. Gene-specific expression was measured as relative to the geometric mean of the expression of two housekeeping genes (GAPDH and ACTB). The comparative cycle threshold (ΔCt) [$\Delta Ct = Ct_{target} - Ct_{housekeeping\ genes}$] values were calculated as the difference between target gene and reference genes and expression was calculated as $2^{(-\Delta Ct)}$ (Pfaffl 2001). The correlation between microarray and qRT-PCR results was analyzed by Spearman's Rho test. The significance level was set as $p < 0.05$.

For validation of microRNA expression, gene specific primers were designed based on an open source primer designing software Primer3web version 4.0 (Rozen and Skaletsky 2000). The cDNA was synthesized from 80 ng of miRNA-enriched total RNA, using a miRCURY LAN Universal cDNA synthesis kit (Exiqon) according to the manufacturer's instructions. The resulting cDNA was diluted into 40 times and used for qPCR analysis of candidate miRNAs by using ExiLENT SYBR Green Master Mix (Exiqon). Thermal cycling conditions were preheated at 95 °C for 10 min, followed by 40 cycles of amplification at 95 °C for 10 sec and 60 °C for 1 min. The specificity of miRNA amplification was evaluated by melting curve analysis. The geometric mean of the expression of U6 small noncoding small nuclear RNA (snRNA) and 5S ribosomal RNA was used to normalize the expression values of candidate

miRNAs. The qPCR data were analyzed using the comparative cycle threshold (Ct) method (Livak and Schmittgen 2001).

1.3.9 Statistical analyses of microarray data

The normalization and statistical analyses of both mRNA and miRNA expression data were performed using packages of Bioconductor-platform implemented in R-project software (v3.1.2); (Gentleman et al. 2004).

1.3.9.1 Normalization, background correction, filtering and summarization

The RMA (Robust Multi-array Average) based quantile normalization of both mRNA and miRNA array data was performed using 'oligo' package (Carvalho and Irizarry 2010). For the quality control, some diagnostic plots of the raw intensity data were checked before and after the normalization. Followed by the normalization, different internal control probes of the chip were filtered out. In addition, the interquartile range (IQR) based filtering (variance cutoff value 0.25) was applied to reduce the heterogeneity of expression across the samples. Probe to gene transcript annotation was performed with recent Affymetrix annotation file for assigned array (Liu et al. 2003).

1.3.9.2 Differential gene expression analysis

The principal aim of analyzing the global gene expression data is to identify genes whose patterns of expression differ according to phenotype or experimental condition. The statistical approach to discover these quantitative changes in gene expression levels between experimental groups are known as differential expression analysis. The differential gene expression analysis was performed using the linear analysis of microarray technique from the '*limma*' package (Smyth 2005) with empirical Bayes adjustment to the variance, followed by Benjamini and Hochberg (BH) correction for multiple testing (Benjamini and Hochberg 1995). The thresholds of false discovery rate (FDR) and $\log_2$ fold-change were fixed based on dataset and contrast pairs for determining the number of differentially expressed genes. The number of differentially expressed genes in each contrast pair and their interaction were exported in intersecting Venn diagram.

1.3.9.3 Alternative splicing analysis

The microarray chip we used for mRNA expression can provide the exon level information, we took this advantage and determined the alternative splicing events using the '*limma*' package (Smyth 2005). For this purpose, we used the probe level RMA normalized data for

downstream analysis instead of transcript level data as used for differential expression analysis.

1.3.10 Bioinformatics analyses of microarray data

Followed by statistical analyses, the list of interested transcriptomes (differentially expressed mRNA and miRNAs) were subjected to in-silico functional analyses using several open source online tools for synthesizing the meaningful biological interpretations.

1.3.10.1 Gene set enrichment analysis

The gene set enrichment analysis (GSEA) is a computational method that determines whether an a priori defined set of genes shows statistically significant, concordant difference between two biological states (e.g. phenotypes). The current study generated global mRNA profiles of PBMCs from vaccinated German Landrace pigs along with age-matched unvaccinated pigs (Chapter 2, Fig 9). The GSEA algorithm was employed for characterization of phenotypic groups (i.e. vaccinated and unvaccinated) through genome-wide comparison of PBMC transcriptome profiles. The GSEA first ranks all genes expressed based on the correlation (positive or negative) of their expression values with one of two phenotypes tested, then seeks the significance of over-representation of pre-defined gene sets (pathways) with the ranked gene list (Subramanian et al. 2005). By this way, GSEA focused on identifying the pathways not the individual genes differentially expressed between two contrast phenotypic groups. The GSEA used here took the advantage of its ability to utilize biological information about well-characterized biological pathways from literature to guide analysis (Cantu et al. 2013). It also used maximum information on dataset by taking into account the effect of co-expressed genes and genes having low expression differences between contrast groups (Abatangelo et al. 2009). For this analysis, the normalized and filtered expression values of all expressed genes of PBMCs were used to differentiate the transcriptome response between vaccinated and unvaccinated phenotype. The details of application are available in chapter 2.

1.3.10.2 Gene ontology and pathway analyses

For comprehensive biological interpretation, the significantly over-represented gene ontology terms and biological pathways were explored with the InnateDB pathway analysis tool (Breuer et al. 2013). The InnateDB is a publicly available database of the genes, proteins, experimentally-verified interactions and signaling pathways involved in the innate immune response of humans, mice and bovines to microbial infection. For this analysis, the identifiers

of DEGs from microarray data were converted to their human ensembl orthologues using the BioDBnet tool (http://biodbnet.abcc.ncifcrf.gov/). The list of ensembl gene identifiers was then uploaded in InnateDB web and performed the over-representation analysis with implementation of the hypergeometric algorithm and the Benjamini-Hochberg (BH) multiple test correction method. The gene ontology (GO) and pathways were considered significantly over-represented if they had a FDR<0.05.

1.3.10.3 Transcription factor binding site analysis

Transcription factors (TFs) are potential regulators of gene expression. In mammalian genome, genes are usually in a default 'off' state and TFs serve mainly to turn gene expression 'on'. Transcription factors bind to a DNA promoter sequence near to the transcription start site and facilitate the formation of transcription initiation complex. Therefore, it is important to know if there is any transcription factor binding sites (TFBSs) in the genes of interest. For this purpose, the significantly over-represented TFBSs in the differentially expressed genes were explored with the InnateDB tool (Breuer et al. 2013). The TFBSs were considered significantly over-represented with the cutoff for FDR < 0.05.

1.3.10.4 Cell-type enrichment analysis

The cellular sub-populations of PBMCs are likely having individual roles on development of vaccine mediated immunity. Therefore, the ability to distinguish the effects of variation in cellular demographics from the global gene expression would improve our understanding on which subtypes of PBMCs contribute in vaccine induced differential gene expression. For this purpose, the differentially expressed genes were analyzed using the CTen web-portal. The CTen (cell type enrichment) is an online bioinformatics tool for identifying enriched cell types in heterogeneous microarray data (Shoemaker et al. 2012). This tool implements a highly expressed, cell specific (HECS) gene database comprises of 10,058 genes of human and mouse origin. For this analysis, human orthologus symbol of differentially expressed genes were uploaded and compared with human HECS database. The significance of enrichment was determined using the one-tailed Fisher exact test and P values were adjusted with Benjamini-Hochberg (BH) method across all cell types. The enrichment score estimated as -log10 of the BH-adjusted P value and created the color-coated output figures indicating this enrichment score.

1.3.10.5 Sub-network enrichment analysis

Gene expression is a well coordinated system where expression measurement of particular gene is not fully independent in an in vivo setting. Like other quantitative traits, immune response is regulated by multiple genes which interact with each other through an interconnecting network. The potential regulatory genes of the network promote or inhibit the expression of other connecting genes to maintain the biological functions (Macneil and Walhout 2011). Therefore, network based approaches are believed to be more sensible over the pathway/gene based linear modeling to find the regulatory molecules for transcriptome alterations. To visualize the PRRSV vaccine induced transcriptional network as well as to identify the regulatory genes, the sub-network analysis was performed using NetworkAnlayst online tool (Xia et al. 2014). This tool uses the InnateDB (downloaded June 20, 2014) protein-protein interaction (PPI) datasets comprised of 14,755 proteins and 145,955 experimentally validated interactions for human. NetworkAnlayst implements the R package 'igraph' for network analysis and 'Gephi Toolkit' for finalizing the network layout. Human orthologous ensembl gene identifiers of the DEGs were uploaded into the NetworkAnlayst to construct the interacting network. A default network was assembled based on the Walktrap algorithm taking only direct interaction of seed genes flowed by adjustment of the network size for high-performance visualization. Two topological measures such as degree and betweenness centrality were taken into account for detecting highly interconnected hubs of the network. Finally, weighted network based module detection was performed to cluster the genes of similar biological functions. The p value of a given network module was calculated using a Wilcoxon rank-sum test of the "internal" and "external" degrees. The functional enrichment of modules was performed with REACTOME.db pathway database incorporated in this tool.

1.3.10.6 Differential expression analysis for miRNAs

To identify the differentially expressed miRNAs, the normalized probes were analyzed using the linear analysis of microarray technique from the 'limma' package (Smyth 2005) with empirical Bayes adjustment to the variance, followed by Benjamini and Hochberg (BH) correction for multiple testing (Benjamini and Hochberg 1995). The thresholds of false discovery rate (FDR) and $\log_2$ fold-change were fixed based on dataset and contrast pairs for determining the number of differentially expressed microRNAs. The number of differentially expressed miRNAs in each contrast pair and their interaction were exported in intersecting Venn diagram.

1.3.10.7 In-silico prediction for target genes of differentially expressed miRNAs

Investigating the alteration of miRNA profiles is crucial to understanding genetics of immunological processes. However, microRNAs are not directly involved in phenotypic changes but regulate the gene expression at post-transcriptional level. MicroRNAs work through recognition of complementary sequence target elements followed by either inhibiting messenger RNA (mRNA) translation or inducing mRNA degradation (Bartel 2004). Therefore, the predicted mRNA targets of interested microRNA are required for biological interpretation. For this purpose, the in-silico miRNA target prediction studies were performed using the both TargetScan v.7.1 (Bartel 2009) and miRDB v5.0 (Nathan and Wang 2015) were used to predict the target gene candidates based on complementarity of the miRNA seed sequence (position 2-8 of the miRNA 5′-end) and target binding site on the 5′ UTR, 3′ UTR and protein coding region of the porcine mRNA sequences (Sus scrofa 10.2); (Lewis et al. 2005). The miRDB server utilizes the miRNAs source from miRBase v21 and implements the MirTarget prediction algorithm. The combined list of predicted mRNA targets obtained from both tools was processed further.

1.3.10.8 Integrated mRNA-miRNA network analysis

The miRNA-mRNA interactome networks were constructed for the PRRSV vaccine response in PBMCs as previously described with minor modifications (Coll et al. 2015). First, we refined the list of predicted targets scanned for potential target genes of DE miRNAs. For accomplishing this, we used the differentially expressed genes (DEGs) list obtained from our previous microarray-based mRNA expression data to integrate with the differentially expressed miRNAs. The overlapped results from predicted mRNA targets and DEGs in PBMCs were extracted as true differentially expressed target genes (TDETGs) of the DE miRNAs. In a second phase of the integration procedure we identified those miRNA-target pairs showing negative correlation between miRNA and mRNAs. To accomplish this, Pearson correlation of the expressions of all possible combinations of deregulated mRNAs vs deregulated miRNAs were computed. Multiple testing correction was performed in order to reduce the number of false positive correlations and a final cut-off was set to FDR (false discovery rate) < 0.05. Finally, the miRNA-mRNA pairs with significant negative correlation (FDR < 0.05 and Pearson coefficient < 0) were used for functional co-regulatory network of miRNA-mRNA. The miRNA-mRNA network was visualized using the Cytoscape v3.2.1 (Cline et al. 2007).

1.4 Results

Detailed results are presented with figures and tables in different chapters of this thesis. Only the major findings are highlighted here.

1.4.1 The PRRSV-specific antibody response

In order to exclude the maternally derived antibody (MDA) of PRRSV as well as to evaluate the vaccine induced antibody response, plasma samples from all pigs at day 7, 28, 42, 56 and 70 of age were screened by ELISA. The plasma antibody level confirmed that experimental pigs were negative for MDA of PRRSV considering the sample to positive (S/P) ratio of 0.4 (40%) as threshold. On the other hand, there was an increasing trend of plasma antibody titre in pigs following vaccination. The antibody titre got above the threshold after two weeks, and subsequently reached a plateau after four weeks of the primary vaccination.

1.4.2 Expression dynamics of PBMCs transcriptome after PRRSV vaccination

To investigate the transcriptional responses to PRRSV vaccine over the first three days of vaccination in German Landrace pigs, we performed the global mRNA expression profiling of PBMCs from vaccinated and age-matched unvaccinated pigs at right before (0 h), and at 6, 24 and 72 h after PRRSV vaccination. A total of 14,231 transcripts were found to be expressed in PBMCs of vaccinated and unvaccinated pigs. The genome-wide comparison of PBMCs transcriptome profiles between vaccinated and unvaccinated pigs revealed a distinct host innate immune transcriptional response to PRRSV vaccine. There was a significant temporal variation in transcriptional responses of PRRSV vaccine in PBMCs accounting 542, 2263 and 357 differentially expressed genes (DEGs) at 6, 24 and 72 h post vaccination, respectively compared to the time point before vaccination. Gene ontology analysis revealed the involvement of these DEGs in various biological process including innate immune response, signal transduction, positive regulation of MAP kinase activity, TRIF-dependent toll-like receptor signaling pathway, T cell differentiation and apoptosis. Immune response specific pathways such as cytokine-cytokine receptor interaction, chemokine signaling pathway, signal transduction, JAK-STAT pathway and regulation, TRAF6 mediated induction of NF-kB and MAPK, the NLRP3 inflammasome, endocytosis and interferon signaling were under regulation during the early stage of PRRSV vaccination. Network enrichment analysis revealed *APP, TRAF6, PIN1, FOS, CTNNB1, TNFAIP3, TIP1, CDKN1, SIRT1, ESR1 and HDAC5* as the highly interconnected hubs of the functional network of PRRSV vaccine induced transcriptome changes in PBMCs (detailed results are available in chapter 2).

1.4.3 Transcriptome signatures for innate and adaptive immunity to PRRSV vaccination

To characterize the regulatory genes and networks associated with PRRSV vaccine induced innate and adaptive immunity, we performed the global mRNA expression profiling of PBMCs from vaccinated Pietrain pigs collected at immediately before (D0), at one (D1) and 28 days (D28) post PRRSV vaccination with three biological replications. With FDR <0.05 and log2 fold change ± 1.5 as cutoff criteria, 295 and 116 transcripts were found to be differentially expressed in PBMCs during the stage of innate and adaptive response, respectively. The microarray expression results were technically validated by qRT-PCR. The gene ontology terms such as viral life cycle, regulation of lymphocyte activation, cytokine activity and inflammatory response were enriched during the innate immunity; and cytolysis, T cell mediated cytotoxicity, immunoglobulin production were enriched during adaptive immunity to PRRSV vaccination. Significant enrichment of cytokine-cytokine receptor interaction, signaling by interleukins, signaling by the B cell receptor (BCR), viral mRNA translation, IFN-gamma pathway and AP-1 transcription factor network pathways were indicating the involvement of altered genes in the antiviral defense. Network analysis revealed that four network modules were functionally involved with the transcriptional network of innate immunity, and five modules were linked to adaptive immunity in PBMCs. The innate immune transcriptional network was found to be regulated by LCK, STAT3, ATP5B, UBB and RSP17. The TGFß1, IL7R, RAD21, SP1 and GZMB are likely predictive for the adaptive immune transcriptional response to PRRSV vaccine in PBMCs (detail results are available in chapter 3).

1.4.4 Breed-specific transcriptome signature after PRRSV vaccination

To explore the breed difference in innate immune response to PRRSV vaccination between purebred German Landrace (DL) and Pietrain (Pi) pigs, we analyzed 12 microarray-based transcriptome profiles of PBMCs collected before (0h) and 24h after PRRSV vaccination from DL and Pi breed with three biological replicates. With FDR < 0.01 and log2 fold change ±1.5 as cutoff criteria, 4269 transcripts were found to be differentially expressed in PBMCs among four contrast pairs (*i.e.* DL-24h *vs.* DL-0h, Pi-24h *vs* Pi-0h, DL-0h *vs.* Pi-0h and DL-24h *vs.* Pi-24h) tested. The number of vaccine induced differentially expressed genes (DEGs) was much higher (2459) in Landrace pigs than that of Pietrain pigs (291). After 24 h of PRRSV vaccination, 1046 genes were differentially expressed PMBCs of Landrace pig compared to that of Pietrain (DL-24h *vs.* Pi-24h) which indicated the breed differences in vaccine responsiveness as well. Before vaccination, 3255 genes showed differential

expression between DL and Pi (DL-0h *vs.* Pi-0h) which indicated the genetic variation between two breeds. The top ten biological pathways significantly affected by genes differentially expressed in four contrast pairs tested includes Cytokine signaling in immune system, Pathway in cancer, GPCR signaling, JAK STAT signaling, Interferon signaling, Autoimmune thyroid disease, Natural killer cell mediated cytotoxicity, Hepatitis C, Toll-like receptor signaling pathway and RIG-like receptor signaling pathway. Majority of the pathways are linked to immune response functions. This study revealed that German Landrace pigs showed greater transcriptional responses indicating more immunity developed from PRRSV vaccination compared to that of Pietrain pigs (detailed results are available in chapter 4).

1.4.5 The microRNA expression profiles of PBMCs after PRRSV vaccination

MicroRNAs, small non coding RNAs, posttranscriptional regulator of gene expression, have been emerged as potential tools for evaluating host immune response to infection or vaccination. We showed that peripheral mononuclear cells are able to mount immune response PRRSV vaccination in pigs through global mRNA profiling. We extended our aim to investigate the expression dynamics of global miRNAs in the same PBMCs samples as used for global mRNA profiling. The GeneChip® miRNA 4.0 arrays contain 30,424 total mature miRNA probe sets including 2.578 mature human miRNAs and miRNAs of 202 other organisms. The differential expression analysis (Fold change > ±1.0, FDR < 0.05) identified 12, 259 and 14 differentially expressed (DE) miRNAs in PBMCs of DL; and 0, 222 and 13 DE miRNAs in PBMCs of Pietrain at 6, 24 and 72 h post vaccination, respectively.

1.4.6 The integrated miRNA-mRNA regulated host response to PRRSV vaccination

We extended our aim herein to integrate the mRNA profiles with the miRNA profiles to uncover the miRNA-mRNA regulated host immune response to PRRSV vaccines in PBMCs. This study generated 12 miRNA profiles of PBMCs collected at right before (0 h), and 6, 24 and 72 h post PRRSV vaccination in three German Landrace pigs, from the same sample pool with same parameter, 12 mRNA profiles have been generated and reported in our recent publication. We integrated these two miRNA and mRNA dataset for better understanding of host-vaccine interaction. The miRNA and gene co-regulatory network revealed that miR-181a-5p, miR-4454, miR-6267, miR-23a and miR-125a-5p are the putative regulators of the immune response developed in PBMCs after PRRSV vaccination in pigs.

1.5 References

Abatangelo L, Maglietta R, Distaso A, D'Addabbo A, Creanza TM, Mukherjee S, Ancona N (2009): Comparative study of gene set enrichment methods. BMC Bioinformatics 10, 1471-2105

Adler M, Murani E, Brunner R, Ponsuksili S, Wimmers K (2013a): Transcriptomic response of porcine PBMCs to vaccination with tetanus toxoid as a model antigen. PLoS ONE 8(3), e58306

Adler M, Murani E, Ponsuksili S, Wimmers K (2013b): PBMC transcription profiles of pigs with divergent humoral and immune responses and lean growth performance. Int J Biol Sci. 9(9), 907-916

Ait-Ali T, Wilson AW, Finlayson H, Carré W, Ramaiahgari SC, Westcott DG, Waterfall M, Frossard JP, Baek KH, Drew TW, Bishop SC, Archibald AL (2009): Functional analysis of the porcine USP18 and its role during porcine arterivirus replication. Gene 439(1-2), 35-42

Ait-Ali T, Wilson AD, Carre W, Westcott DG, Frossard JP, Mellencamp MA, Mouzaki D, Matika O, Waddington D, Drew TW, Bishop SC, Archibald AL (2011): Host inhibits replication of European porcine reproductive and respiratory syndrome virus in macrophages by altering differential regulation of type-I interferon transcriptional response. Immunogenetics 63, 437-448

Albina E, Carrat C, Charley B (1998): Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. J Interferon Cytokine Res 18, 485-490

Abella G, Pena RN, Nogareda C, Armengol R, Vidal A, Moradell L, Tarancon V, Novell E, Estany J, Fraile L (2016): A WUR SNP is associated with European Porcine Reproductive and Respiratory Virus Syndrome resistance and growth performance in pigs. Res Vet Sci 104, 117-122

Allende R, Laegreid WW, Kutish GF, Galeota JA, Wills RW, Osorio FA (2000): Porcine reproductive and respiratory syndrome virus: description of persistence in individual pigs upon experimental infection. J Virol 74, 10834-10837

Allende R, Lewis TL, Lu Z, Rock DL, Kutish GF, Ali A, Doster AR, Osorio FA (1999): North American and European porcine reproductive and respiratory syndrome viruses differ in non-structural protein coding regions. J Gen Virol 80, 307-315

Amadori M, Razzuoli E (2014): Immune Control of PRRS: Lessons to be Learned and Possible Ways Forward. Front Vet Sci 1, doi: 10.3389/fvets.2014.00002

Badaoui B, Tuggle CK, Hu Z, Reecy JM, Ait-Ali T, Anselmo A, Botti S (2013): Pig immune response to general stimulus and to porcine reproductive and respiratory syndrome virus infection: a meta-analysis approach. BMC Genomics 14, 1471-2164

Bartel DP (2004): MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281-297

Benjamini Y, Hochberg Y (1995): Controlling the false discovery rate: A typical adn powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 57, 289-300

Beura LK, Sarkar SN, Kwon B, Subramaniam S, Jones C, Pattnaik AK, Osorio FA (2010): Porcine reproductive and respiratory syndrome virus nonstructural protein 1beta modulates host innate immune response by antagonizing IRF3 activation. J Virol 84, 1574-1584

Breuer K, Foroushani AK, Laird MR, Chen C, Sribnaia A, Lo R, Winsor GL, Hancock RE, Brinkman FS, Lynn DJ (2013): InnateDB: systems biology of innate immunity and beyond--recent updates and continuing curation. Nucleic Acids Res 41 (Database), D1228-D1233

Briggs HM (1983): International Pig Breed Encyclopedia. Accessed at 29 September 2016 http://www.ansi.okstate.edu/breeds/swine/pietrain/index.html

Boddicker N, Waide EH, Rowland RR, Lunney JK, Garrick DJ, Reecy JM, Dekkers JC (2012): Evidence for a major QTL associated with host response to porcine reproductive and respiratory syndrome virus challenge. J Anim Sci 90, 1733-1746

Boddicker NJ, Bjorkquist A, Rowland RR, Lunney JK, Reecy JM, Dekkers JC (2014a): Genome-wide association and genomic prediction for host response to porcine reproductive and respiratory syndrome virus infection. Genet Sel Evol 46, 1297-9686

Boddicker NJ, Garrick DJ, Rowland RR, Lunney JK, Reecy JM, Dekkers JC (2014b): Validation and further characterization of a major quantitative trait locus associated with host response to experimental infection with porcine reproductive and respiratory syndrome virus. Anim Genet 45, 48-58

Calzada-Nova G, Schnitzlein WM, Husmann RJ, Zuckermann FA (2011): North American porcine reproductive and respiratory syndrome viruses inhibit type I interferon production by plasmacytoid dendritic cells. J Virol 85, 2703-2713

Cantu E, Lederer DJ, Meyer K, Milewski K, Suzuki Y, Shah RJ, Diamond JM, Meyer NJ, Tobias JW, Baldwin DA, Van Deerlin VM, Olthoff KM, Shaked A, Christie JD

(2013): Gene set enrichment analysis identifies key innate immune pathways in primary graft dysfunction after lung transplantation. Am J Transplant 13, 1898-1904

Carvalho BS, Irizarry RA (2010): A framework for oligonucleotide microarray preprocessing. Bioinformatics 26, 2363-2367

Cavanagh D (1997): Nidovirales: a new order comprising Coronaviridae and Arteriviridae. Arch Virol 142, 629-633

Chand RJ, Trible BR, Rowland RR (2012): Pathogenesis of porcine reproductive and respiratory syndrome virus. Curr Opin Virol 2, 256-263

Christopher-Hennings J, Holler LD, Benfield DA, Nelson EA (2001): Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. J Vet Diagn Invest 13, 133-142

Christopher-Hennings J, Nelson EA, Althouse GC, Lunney J (2008): Comparative antiviral and proviral factors in semen and vaccines for preventing viral dissemination from the male reproductive tract and semen. Anim Health Res Rev 9, 59-69

Chuaqui RF, Bonner RF, Best CJ, Gillespie JW, Flaig MJ, Hewitt SM, Phillips JL, Krizman DB, Tangrea MA, Ahram M, Linehan WM, Knezevic V, Emmert-Buck MR (2002): Post-analysis follow-up and validation of microarray experiments. Nat Genet 32, 509-514

Chung HK, Chae C (2003): Expression of interleukin-10 and interleukin-12 in piglets experimentally infected with porcine reproductive and respiratory syndrome virus (PRRSV). J Comp Pathol 129, 205-212

Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, Christmas R, Avila-Campilo I, Creech M, Gross B, Hanspers K, Isserlin R, Kelley R, Killcovne S, Lotia S, Maere S, Morris J, Ono K, Pavlovic V, Pico AR, Vailva A, Wang PL, Adler A, Conklin BR, Hood L, Kuiper M, Sander C, Schmulevich I, Schwikowsky B, Warner GJ, Ideker T, Bader GD (2007): Integration of biological networks and gene expression data using Cytoscape. Nat Protoc 2, 2366–82

Coll M, El Taghdouini A, Perea L, Mannaerts I, Vila-Casadesus M, Blaya D, Rodrigo-Torres D, Affo S, Morales-Ibanez O, Graupera I, Lozano JJ, Najimi M, Sokal E, Lambrecht J, Gines P, van Grunsven LA, Sancho-Bru P (2015): Integrative miRNA and Gene Expression Profiling Analysis of Human Quiescent Hepatic Stellate Cells. Sci Rep 5:11549, doi:10.1038/srep11549

Collins JE. 1991. Diagnostic note: newly recognized respiratory syndromes in North American swine herds. American Association of Swine Practitioners Newsletter 3, 7-11

Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrison RB, Joo HS, et al. (1992): Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest 4, 117-126

Corzo CA, Mondaca E, Wayne S, Torremorell M, Dee S, Davies P, Morrison RB (2010): Control and elimination of porcine reproductive and respiratory syndrome virus. Virus Res 154, 185-192

Das PB, Dinh PX, Ansari IH, de Lima M, Osorio FA, Pattnaik AK (2010): The minor envelope glycoproteins GP2a and GP4 of porcine reproductive and respiratory syndrome virus interact with the receptor CD163. J Virol 84, 1731-1740

Dawson HD, Loveland JE, Pascal G, Gilbert JG, Uenishi H, Mann KM, Sang Y, Zhang J, Carvalho-Silva D, Hunt T, Hardy M, Hu Z, Zhao SH, Anselmo A, Shinkai H, Chen C, Badaoui B, Berman D, Amid C, Kay M, Lloyd D, Snow C, Morozumi T, Cheng RP, Bystrom M, Kapetanovic R, Schwartz JC, Kataria R, Astley M, Fritz E, Steward C, Thomas M, Wilming L, Toki D, Archibald AL, Bed'Hom B, Beraldi D, Huang TH, Ait-Ali T, Blecha F, Botti S, Freeman TC, Giuffra E, Hume DA, Lunney JK, Murtaugh MP, Reecy JM, Harrow JL, Rogel-Gaillard C, Tuggle CK (2013): Structural and functional annotation of the porcine immunome. BMC Genomics 14, 1471-2164

Dee Scott A (2014): Overview of porcine reproductive and respiratory syndrome. In: the Merck Veterinary manual, Generalized condiction. Online accessed on 10.10.2016 http://www.merckvetmanual.com/mvm/generalized_conditions/porcine_reproductive_and_respiratory_syndrome/overview_of_porcine_reproductive_and_respiratory_syndrome.html

De Lima M, Pattnaik AK, Flores EF, Osorio FA (2006): Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North American strain of porcine reproductive and respiratory syndrome virus. Virology 353, 410-421

De Paz X, Vega D, Duran CO, Angulo J (2015): PRRS prevalence in Europe: Perception of the Pig Producers. ESPHM annual conference on 22-24 April, 2015, Nantes, France

De Paz X (2015): PRRS cost for the European swine industry. Online accessed on 06.10.2016.https://www.pig333.com/what_the_experts_say/prrs-cost-for-the-uropean-swine-industry_10069/

Delputte PL, Costers S, Nauwynck HJ (2005): Analysis of porcine reproductive and respiratory syndrome virus attachment and internalization: distinctive roles for heparan sulphate and sialoadhesin. J Gen Virol 86, 1441-1445

Duan X, Nauwynck HJ, Favoreel HW, Pensaert MB (1998): Identification of a putative receptor for porcine reproductive and respiratory syndrome virus on porcine alveolar macrophages. J Virol 72, 4520-4523

Ebert BL, Golub TR (2004): Genomic approaches to hematologic malignancies. Blood 104, 923-932

Eklund AC, Turner LR, Chen P, Jensen RV, deFeo G, Kopf-Sill AR, Szallasi Z (2006): Replacing cRNA targets with cDNA reduces microarray cross-hybridization. Nat Biotechnol 24(9), 1071-83

Fano E, Olea L, Pijoan C (2005): Eradication of porcine reproductive and respiratory syndrome virus by serum inoculation of naive gilts. Can J Vet Res 69, 71-74

Firth AE, Zevenhoven-Dobbe JC, Wills NM, Go YY, Balasuriya UB, Atkins JF, Snijder EJ, Posthuma CC (2011): Discovery of a small arterivirus gene that overlaps the GP5 coding sequence and is important for virus production. J Gen Virol 92, 1097-1106

Forsberg R, Storgaard T, Nielsen HS, Oleksiewicz MB, Cordioli P, Sala G, Hein J, Botner A (2002): The genetic diversity of European type PRRSV is similar to that of the North American type but is geographically skewed within Europe. Virology 299, 38-47

Frossard JP, Hughes GJ, Westcott DG, Naidu B, Williamson S, Woodger NG, Steinbach F, Drew TW (2013): Porcine reproductive and respiratory syndrome virus: genetic diversity of recent British isolates. Vet Microbiol 162, 507-518

Gao L, Guo XK, Wang L, Zhang Q, Li N, Chen XX, Wang Y, Feng WH (2013): MicroRNA 181 suppresses porcine reproductive and respiratory syndrome virus (PRRSV) infection by targeting PRRSV receptor CD163. J Virol 87, 8808-8812

Gao Y, Flori L, Lecardonnel J, Esquerre D, Hu ZL, Teillaud A, Lemonnier G, Lefevre F, Oswald IP, Rogel-Gaillard C (2010): Transcriptome analysis of porcine PBMCs after in vitro stimulation by LPS or PMA/ionomycin using an expression array targeting the pig immune response. BMC Genomics 11, 1471-2164

Geldhof MF, Vanhee M, Van Breedam W, Van Doorsselaere J, Karniychuk UU, Nauwynck HJ (2012): Comparison of the efficacy of autogenous inactivated Porcine

Reproductive and Respiratory Syndrome Virus (PRRSV) vaccines with that of commercial vaccines against homologous and heterologous challenges. BMC Vet Res 8, 1746-6148

Genini S, Delputte PL, Malinverni R, Cecere M, Stella A, Nauwynck HJ, Giuffra E (2008): Genome-wide transcriptional response of primary alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. J Gen Virol 89, 2550-2564

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004): Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5, 15

Goyal SM (1993): Porcine reproductive and respiratory syndrome. J Vet Diagn Invest 5, 656-664

Gradil C, Dubuc C, Eaglesome MD (1996): Porcine reproductive and respiratory syndrome virus: seminal transmission. Vet Rec 138, 521-522

Groenen MA, Archibald AL, Uenishi H, Tuggle CK, Takeuchi Y, Rothschild MF, Rogel-Gaillard C, Park C, Milan D, Megens HJ, Li S, Larkin DM, Kim H, Frantz LA, Caccamo M, Ahn H, Aken BL, Anselmo A, Anthon C, Auvil L, Badaoui B, Beattie CW, Bendixen C, Berman D, Blecha F, Blomberg J, Bolund L, Bosse M, Botti S, Bujie Z, Bystrom M, Capitanu B, Carvalho-Silva D, Chardon P, Chen C, Cheng R, Choi SH, Chow W, Clark RC, Clee C, Crooijmans RP, Dawson HD, Dehais P, De Sapio F, Dibbits B, Drou N, Du ZQ, Eversole K, Fadista J, Fairley S, Faraut T, Faulkner GJ, Fowler KE, Fredholm M, Fritz E, Gilbert JG, Giuffra E, Gorodkin J, Griffin DK, Harrow JL, Hayward A, Howe K, Hu ZL, Humphray SJ, Hunt T, Hornshoj H, Jeon JT, Jern P, Jones M, Jurka J, Kanamori H, Kapetanovic R, Kim J, Kim JH, Kim KW, Kim TH, Larson G, Lee K, Lee KT, Leggett R, Lewin HA, Li Y, Liu W, Loveland JE, Lu Y, Lunney JK, Ma J, Madsen O, Mann K, Matthews L, McLaren S, Morozumi T, Murtaugh MP, Narayan J, Nguyen DT, Ni P, Oh SJ, Onteru S, Panitz F, Park EW, et al. (2012): Analyses of pig genomes provide insight into porcine demography and evolution. Nature 491, 393-398

Guo XK, Zhang Q, Gao L, Li N, Chen XX, Feng WH (2013): Increasing expression of microRNA 181 inhibits porcine reproductive and respiratory syndrome virus replication and has implications for controlling virus infection. J Virol 87, 1159-1171

Halbur PG, Miller LD, Paul PS, Meng XJ, Huffman EL, Andrews JJ (1995a): Immunohistochemical identification of porcine reproductive and respiratory syndrome virus (PRRSV) antigen in the heart and lymphoid system of three-week-old colostrum-deprived pigs. Vet Pathol 32, 200-204

Halbur PG, Paul PS, Frey ML, Landgraf J, Eernisse K, Meng XJ, Lum MA, Andrews JJ, Rathje JA (1995b): Comparison of the pathogenicity of two US porcine reproductive and respiratory syndrome virus isolates with that of the Lelystad virus. Vet Pathol 32, 648-660

Halbur PG, Rothschild MF, Thacker BJ, Meng XJ, Paul PS, Bruna JD (1998): Differences in susceptibility of Duroc, Hampshire, and Meishan pigs to infection with a high virulence strain (VR2385) of porcine reproductive and respiratory syndrome virus (PRRSV). J Anim Breed Genet 115, 181-189

Hinton A, Bond S, Forgac M (2009): V-ATPase functions in normal and disease processes. Pflugers Arch 457, 589-98

Holtkamp DJ, Kliebenstein JB, Neumann EJ (2013): Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod 21, 72-84

Hu ZL, Dracheva S, Jang W, Maglott D, Bastiaansen J, Rothschild MF, Reecy JM (2005): A QTL resource and comparison tool for pigs: PigQTLDB. Mamm Genome 16, 792-800

Huang TH, Uthe JJ, Bearson SM, Demirkale CY, Nettleton D, Knetter S, Christian C, Ramer-Tait AE, Wannemuehler MJ, Tuggle CK (2011): Distinct peripheral blood RNA responses to Salmonella in pigs differing in Salmonella shedding levels: intersection of IFNG, TLR and miRNA pathways. PLoS ONE 6(12), e28768

Julie AH, Yoo D, Liu HC (2013): Characterization of the microRNAome in porcine reproductive and respiratory syndrome virus infected macrophages. PLoS ONE 8(12), e82054

Johnson CR, Yu W, Murtaugh MP (2007): Cross-reactive antibody responses to nsp1 and nsp2 of Porcine reproductive and respiratory syndrome virus. J Gen Virol 88, 1184-1195

Kapur V, Elam MR, Pawlovich TM, Murtaugh MP (1996): Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J Gen Virol 77, 1271-1276

Kimman TG, Cornelissen LA, Moormann RJ, Rebel JM, Stockhofe-Zurwieden N (2009): Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine 27, 3704-3718

Kittawornrat A, Prickett J, Wang C, Olsen C, Irwin C, Panyasing Y, Ballagi A, Rice A, Main R, Johnson J, Rademacher C, Hoogland M, Rowland R, Zimmerman J (2012): Detection of Porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in oral fluid specimens using a commercial PRRSV serum antibody enzyme-linked immunosorbent assay. J Vet Diagn Invest 24, 262-269

Kohane IS, Valtchinov VI (2012): Quantifying the white blood cell transcriptome as an accessible window to the multiorgan transcriptome. Bioinformatics 28, 538-545

Kvisgaard LK, Larsen LE, Hjulsager CK (2013): Porcine Reproductive and Respiratory Syndrome Virus (PRRSV). Kgs. Lyngby: Technical University of Denmark.

Levy DE, Marie I, Prakash A (2003): Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity. Curr Opin Immunol 15, 52-58

Lewis CR, Ait-Ali T, Clapperton M, Archibald AL, Bishop S (2007): Genetic perspectives on host responses to porcine reproductive and respiratory syndrome (PRRS). Viral Immunol 20, 343-358

Li J, Chen Z, Zhao J, Fang L, Fang R, Xiao J, Chen X, Zhou A, Zhang Y, Ren L, Hu X, Zhao Y, Zhang S, Li N (2015): Difference in microRNA expression and editing profile of lung tissues from different pig breeds related to immune responses to HP-PRRSV. Sci Rep 5, doi: 10.1038/srep09549.

Li J, Yu YJ, Feng L, Cai XB, Tang HB, Sun SK, Zhang HY, Liang JJ, Luo TR (2010): Global transcriptional profiles in peripheral blood mononuclear cell during classical swine fever virus infection. Virus Res 148, 60-70

Li Y, Sun Y, Xing F, Kang L, Wang P, Wang L, Liu H, Li Y, Jiang Y (2014): Identification of a single nucleotide promoter polymorphism regulating the transcription of ubiquitin specific protease 18 gene related to the resistance to porcine reproductive and respiratory syndrome virus infection. Vet Immunol Immunopathol 162, 65-71

Liew CC, Ma J, Tang HC, Zheng R, Dempsey AA (2006): The peripheral blood transcriptome dynamically reflects system wide biology: a potential diagnostic tool. J Lab Clin Med 147, 126-132

Lewis BP, Burge CB, Bartel DP (2005): Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20

Liu G, Loraine AE, Shigeta R, Cline M, Cheng J, Valmeekam V, Sun S, Kulp D, Siani-Rose MA (2003): NetAffx: Affymetrix probesets and annotations. Nucleic Acids Res 31, 82-86

Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408

Lopez OJ, Osorio FA (2004): Role of neutralizing antibodies in PRRSV protective immunity. Vet Immunol Immunopathol 102, 155-163

Loving CL, Osorio FA, Murtaugh MP, Zuckermann FA (2015): Innate and adaptive immunity against Porcine Reproductive and Respiratory Syndrome Virus. Vet Immunol Immunopathol 167, 1-14

Lunney JK, Chen H (2010): Genetic control of host resistance to porcine reproductive and respiratory syndrome virus (PRRSV) infection. Virus Res 154, 161-169

Lunney JK, Fang Y, Ladinig A, Chen N, Li Y, Rowland B, Renukaradhya GJ (2016): Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. Annu Rev Anim Biosci 4, 129-154

Lunney JK, Steibel JP, Reecy JM, Fritz E, Rothschild MF, Kerrigan M, Trible B, Rowland RR (2011): Probing genetic control of swine responses to PRRSV infection: current progress of the PRRS host genetics consortium. BMC Proc 3, 1753-6561

Luo R, Xiao S, Jiang Y, Jin H, Wang D, Liu M, Chen H, Fang L (2008): Porcine reproductive and respiratory syndrome virus (PRRSV) suppresses interferon-beta production by interfering with the RIG-I signaling pathway. Mol Immunol 45, 2839-2846

Macneil LT, Walhout AJ (2011): Gene regulatory networks and the role of robustness and stochasticity in the control of gene expression. Genome Res 21: 645-657.

Meng XJ, Paul PS, Halbur PG, Lum MA (1995): Phylogenetic analyses of the putative M (ORF 6) and N (ORF 7) genes of porcine reproductive and respiratory syndrome virus (PRRSV): implication for the existence of two genotypes of PRRSV in the U.S.A. and Europe. Arch Virol 140, 745-755

Mengeling WL, Vorwald AC, Lager KM, Brockmeier SL (1996): Comparison among strains of porcine reproductive and respiratory syndrome virus for their ability to cause reproductive failure. Am J Vet Res 57, 834-839

Meulenberg JJ, de Meijer EJ, Moormann RJ (1993): Subgenomic RNAs of Lelystad virus contain a conserved leader-body junction sequence. J Gen Virol 74, 1697-1701

Murtaugh MP, Genzow M (2011): Immunological solutions for treatment and prevention of porcine reproductive and respiratory syndrome (PRRS). Vaccine 29, 8192-8204

Music N, Gagnon CA (2010): The role of porcine reproductive and respiratory syndrome (PRRS) virus structural and non-structural proteins in virus pathogenesis. Anim Health Res Rev 11, 135-163

Nathan W, Xiaowei W (2015): miRDB: an online resource for microRNA target prediction and functional annotations. Nucleic Acids Res. 43(D1), D146-152

Ojha S, Kostrzynska M (2008): Examination of animal and zoonotic pathogens using microarrays. Vet Res 39, 25

Oleksiewicz MB, Botner A, Toft P, Normann P, Storgaard T (2001): Epitope mapping porcine reproductive and respiratory syndrome virus by phage display: the nsp2 fragment of the replicase polyprotein contains a cluster of B-cell epitopes. J Virol 75, 3277-3290

Pasternak AO, Spaan WJ, Snijder EJ (2006): Nidovirus transcription: how to make sense...? J Gen Virol 87, 1403-1421

Petry DB, Holl JW, Weber JS, Doster AR, Osorio FA, Johnson RK (2005): Biological responses to porcine respiratory and reproductive syndrome virus in pigs of two genetic populations. J Anim Sci 83, 1494-1502

Pfaffl MW (2001): A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9), e45

Reiner G, Willems H, Pesch S, Ohlinger VF (2010): Variation in resistance to the porcine reproductive and respiratory syndrome virus (PRRSV) in Pietrain and Miniature pigs. J Anim Breed Genet 127, 100-106

Rossow KD (1998): Porcine reproductive and respiratory syndrome. Vet Pathol 35, 1-20

Rowland RR, Lawson S, Rossow K, Benfield DA (2003): Lymphoid tissue tropism of porcine reproductive and respiratory syndrome virus replication during persistent infection of pigs originally exposed to virus in utero. Vet Microbiol 96, 219-235

Rowland RR, Lunney J, Dekkers J (2012): Control of porcine reproductive and respiratory syndrome (PRRS) through genetic improvements in disease resistance and tolerance. Front Genet 3, doi: 10.3389/fgene.2012.00260

Royaee AR, Husmann RJ, Dawson HD, Calzada-Nova G, Schnitzlein WM, Zuckermann FA, Lunney JK (2004): Deciphering the involvement of innate immune factors in the

development of the host response to PRRSV vaccination. Vet Immunol Immunopathol 102, 199-216

Schena M (1996): Genome analysis with gene expression microarrays. Bioessays 18, 427-431

Schroyen M, Tuggle CK (2015): Current transcriptomics in pig immunity research. Mamm Genome 26, 1-20

Shi M, Lam TT, Hon CC, Hui RK, Faaberg KS, Wennblom T, Murtaugh MP, Stadejek T, Leung FC (2010): Molecular epidemiology of PRRSV: a phylogenetic perspective. Virus Res 154, 7-17

Shoemaker JE, Lopes TJ, Ghosh S, Matsuoka Y, Kawaoka Y, Kitano H (2012): CTen: a web-based platform for identifying enriched cell types from heterogeneous microarray data. BMC Genomics 13, 1471-2164

Siegrist C-A (2012): General aspects of vaccinations. In: Vaccines, 6th ed. Elsevier Health Sciences Saunders, USA. pp. 17-36

Smyth GK (2005): Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, editors. Bioinformatics and computational biology solutions using r and bioconductor. Springer USA pp. 397–342

Snijder EJ (1998): The arterivirus replicase. The road from RNA to protein(s), and back again. Adv Exp Med Biol 440, 97-108

Snijder EJ, Meulenberg JJ (1998): The molecular biology of arteriviruses. J Gen Virol 79, 961-979

Snijder EJ, Spaan WJM (2007): Fields Virology Lippincott Williams and Wilkins, Philadelphia, pp. 1337–1355

Spilman MS, Welbon C, Nelson E, Dokland T (2009): Cryo-electron tomography of porcine reproductive and respiratory syndrome virus: organization of the nucleocapsid. J Gen Virol 90, 527-535

Stadejek T, Oleksiewicz MB, Scherbakov AV, Timina AM, Krabbe JS, Chabros K, Potapchuk D (2008): Definition of subtypes in the European genotype of porcine reproductive and respiratory syndrome virus: nucleocapsid characteristics and geographical distribution in Europe. Arch Virol 153, 1479-1488

Stadejek T, Stankevicius A, Storgaard T, Oleksiewicz MB, Belak S, Drew TW, Pejsak Z (2002): Identification of radically different variants of porcine reproductive and respiratory syndrome virus in Eastern Europe: towards a common ancestor for European and American viruses. J Gen Virol 83, 1861-1873

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005): Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102, 15545-15550

Sun Y, Han M, Kim C, Calvert JG, Yoo D (2012): Interplay between interferon-mediated innate immunity and porcine reproductive and respiratory syndrome virus. Viruses 4, 424-446

Terpstra C, Wensvoort G, Pol JM (1991): Experimental reproduction of porcine epidemic abortion and respiratory syndrome (mystery swine disease) by infection with Lelystad virus: Koch's postulates fulfilled. Vet Q 13(3), 131-136

Thanawongnuwech R, Young TF, Thacker BJ, Thacker EL (2001): Differential production of proinflammatory cytokines: in vitro PRRSV and Mycoplasma hyopneumoniae co-infection model. Vet Immunol Immunopathol 79, 115-127

Tuggle CK, Bearson SM, Uthe JJ, Huang TH, Couture OP, Wang YF, Kuhar D, Lunney JK, Honavar V (2010): Methods for transcriptomic analyses of the porcine host immune response: application to Salmonella infection using microarrays. Vet Immunol Immunopathol 138, 280-291

Tuggle CK, Giuffra E, White SN, Clarke L, Zhou H, Ross PJ, Acloque H, Reecy JM, Archibald A, Bellone RR, Boichard M, Chamberlain A, Cheng H, Crooijmans RP, Delany ME, Finno CJ, Groenen MA, Hayes B, Lunney JK, Petersen JL, Plastow GS, Schmidt CJ, Song J, Watson M. (2016): GO-FAANG meeting: a Gathering On Functional Annotation of Animal Genomes. Anim Genet 47(5), 528-33

Tuggle CK, Wang Y, Couture O (2007): Advances in swine transcriptomics. Int J Biol Sci 3, 132-152

Van Dinten LC, Wassenaar AL, Gorbalenya AE, Spaan WJ, Snijder EJ (1996): Processing of the equine arteritis virus replicase ORF1b protein: identification of cleavage products containing the putative viral polymerase and helicase domains. J Virol 70, 6625-6633

Van Gorp H, Van Breedam W, Delputte PL, Nauwynck HJ (2009): The porcine reproductive and respiratory syndrome virus requires trafficking through CD163-positive early endosomes, but not late endosomes, for productive infection. Arch Virol 154, 1939-1943

Van Gorp H, Van Breedam W, Delputte PL, Nauwynck HJ (2008): Sialoadhesin and CD163 join forces during entry of the porcine reproductive and respiratory syndrome virus. J Gen Virol 89, 2943-2953

Verheije MH, Welting TJ, Jansen HT, Rottier PJ, Meulenberg JJ (2002): Chimeric arteriviruses generated by swapping of the M protein ectodomain rule out a role of this domain in viral targeting. Virology 303, 364-373

Vincent AL, Thacker BJ, Halbur PG, Rothschild MF, Thacker EL (2005): In vitro susceptibility of macrophages to porcine reproductive and respiratory syndrome virus varies between genetically diverse lines of pigs. Viral Immunol 18, 506-512

Wang D, Cao L, Xu Z, Fang L, Zhong Y, Chen Q, Luo R, Chen H, Li K, Xiao S (2013): MiR-125b reduces porcine reproductive and respiratory syndrome virus replication by negatively regulating the NF-kappaB pathway. PLoS ONE 8(2), e55838

Wensvoort G, Terpstra C, Pol JM, ter Laak EA, Bloemraad M, de Kluyver EP, Kragten C, van Buiten L, den Besten A, Wagenaar F, et al. (1991): Mystery swine disease in The Netherlands: the isolation of Lelystad virus. Vet Q 13(3), 121-130

Wilkinson JM, Dyck MK, Dixon WT, Foxcroft GR, Dhakal S, Harding JC (2012): Transcriptomic analysis identifies candidate genes and functional networks controlling the response of porcine peripheral blood mononuclear cells to mitogenic stimulation. J Anim Sci 90, 3337-3352

Wills RW, Doster AR, Galeota JA, Sur JH, Osorio FA (2003): Duration of infection and proportion of pigs persistently infected with porcine reproductive and respiratory syndrome virus. J Clin Microbiol 41, 58-62

Wissink EH, Kroese MV, van Wijk HA, Rijsewijk FA, Meulenberg JJ, Rottier PJ (2005): Envelope protein requirements for the assembly of infectious virions of porcine reproductive and respiratory syndrome virus. J Virol 79, 12495-12506

Wu J, Peng X, Zhou A, Qiao M, Wu H, Xiao H, Liu G, Zheng X, Zhang S, Mei S (2014): MiR-506 inhibits PRRSV replication in MARC-145 cells via CD151. Mol Cell Biochem 394, 275-281

Wysocki M, Chen H, Steibel JP, Kuhar D, Petry D, Bates J, Johnson R, Ernst CW, Lunney JK (2012): Identifying putative candidate genes and pathways involved in immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) infection. Anim Genet 43, 328-332

Xia J, Benner MJ, Hancock RE (2014): NetworkAnalyst--integrative approaches for protein-protein interaction network analysis and visual exploration. Nucleic Acids Res 42(Web Server issue), W167-74

Xiao S, Mo D, Wang Q, Jia J, Qin L, Yu X, Niu Y, Zhao X, Liu X, Chen Y (2010): Aberrant host immune response induced by highly virulent PRRSV identified by digital gene expression tag profiling. BMC Genomics 11, 1471-2164

Xing J, Xing F, Zhang C, Zhang Y, Wang N, Li Y, Yang L, Jiang C, Wen C, Jiang Y (2014): Genome-wide gene expression profiles in lung tissues of pig breeds differing in resistance to porcine reproductive and respiratory syndrome virus. PLoS ONE 9(1), e86101

Yoon KJ, Wu LL, Zimmerman JJ, Hill HT, Platt KB (1996): Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. Viral Immunol 9, 51-63

ZDS (2003): Richtlinie Fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit Beim Schwein. Zentralverband der Deutschen Schweineproduktion eV, Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn

Zhang Q, Guo XK, Gao L, Huang C, Li N, Jia X, Liu W, Feng WH (2014): MicroRNA-23 inhibits PRRSV replication by directly targeting PRRSV RNA and possibly by upregulating type I interferons. Virology 451, 182-195

Zhang X, Shin J, Molitor TW, Schook LB, Rutherford MS (1999): Molecular responses of macrophages to porcine reproductive and respiratory syndrome virus infection. Virology 262, 152-162

Zhou P, Zhai S, Zhou X, Lin P, Jiang T, Hu X, Jiang Y, Wu B, Zhang Q, Xu X, Li JP, Liu B (2011): Molecular characterization of transcriptome-wide interactions between highly pathogenic porcine reproductive and respiratory syndrome virus and porcine alveolar macrophages in vivo. Int J Biol Sci 7, 947-959

Zuckermann FA, Garcia EA, Luque ID, Christopher-Hennings J, Doster A, Brito M, Osorio F (2007): Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Vet Microbiol 123, 69-85

Chapter 2: Transcriptome profiles of PBMCs after PRRSV vaccination

Article published in BMC Genomics: 2016, Vol 17(1): 641
[doi:10.1186/s12864-016-2849-1]

Deciphering transcriptome profiles of peripheral blood mononuclear cells in response to PRRSV vaccination in pigs

Md. Aminul Islam[1,2], Christine Große-Brinkhaus[1], Maren Julia Pröll[1], Muhammad Jasim Uddin[2], Sharmin Aqter Rony[1], Dawit Tesfaye[1], Ernst Tholen[1], Michael Hoelker[3], Karl Schellander[1], Christiane Neuhoff[1,*]

[1]Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany
[2]Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
[3]Teaching and Research Station on Frankenfrost, Faculty of Agriculture, University of Bonn

*Corresponding author:
Christiane Neuhoff, PhD
Institute of Animal Science, University of Bonn
Endenicher Allee 15, 53115 Bonn, Germany
Email: cneu@itw.uni-bonn.de
Phone: 0049-228/73-3583
Fax: 0049-00228/73-2284

Article type and status: Research and new

Running title: Transcriptome profiles of PBMCs after PRRSV vaccination

Keywords: pig; PRRSV vaccine; PBMCs; innate immunity; microarray; gene; antibody

2.1 Abstract

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important viral diseases affecting swine industry worldwide. Despite routine farm vaccination, effective control strategies for PRRS remained elusive which underscores the need for in-depth studies to gain insight into the host immune response to vaccines. This study aimed to investigate transcriptional responses to PRRS Virus (PRRSV) vaccine in the peripheral blood mononuclear cells (PBMCs) within 3 days following vaccination in German Landrace pigs. Transcriptome profiling of PBMCs from PRRSV vaccinated and age-matched unvaccinated pigs at right before (0 h), and at 6, 24 and 72 h after PRRSV vaccination was performed using the Affymetrix gene chip porcine gene 1.0 ST array. Comparison of PBMCs transcriptome profiles between vaccinated and unvaccinated pigs revealed a distinct host innate immune transcriptional response to PRRSV vaccine. There was a significant temporal variation in transcriptional responses of PRRSV vaccine in PBMCs accounting 542, 2263 and 357 differentially expressed genes (DEGs) at 6, 24 and 72 h post vaccination, respectively compared to the time point before vaccination (controls). Gene ontology analysis revealed the involvement of these DEGs in various biological process including innate immune response, signal transduction, positive regulation of MAP kinase activity, TRIF-dependent toll-like receptor signaling pathway, T cell differentiation and apoptosis. Immune response specific pathways such as cytokine-cytokine receptor interaction, chemokine signaling pathway, signal transduction, JAK-STAT pathway and regulation, TRAF6 mediated induction of NF-kB and MAPK, the NLRP3 inflammasome, endocytosis and interferon signaling were under regulation during the early stage of PRRSV vaccination. Network enrichment analysis revealed APP, TRAF6, PIN1, FOS, CTNNB1, TNFAIP3, TIP1, CDKN1, SIRT1, ESR1 and HDAC5 as the highly interconnected hubs of the functional network of PRRSV vaccine induced transcriptome changes in PBMCs. This study showed a distinct transcriptional response to PRRSV vaccine in PBMCs. Within first 3 days of vaccine exposure, the highest transcripts abundance was observed at 24 h after vaccination compared to before vaccination. This study suggested that APP, TRAF6, PIN1, FOS, CDKN1A and TNFAIP3 could be considered as potential candidate genes for PRRSV vaccine responsiveness in pigs.

2.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is an emerging viral infectious disease characterized by reproductive failures in breeding sows and respiratory disorders in growing pigs (Lunney et al. 2011). PRRS causes huge economic loss, and is of major concern as animal welfare issue in swine industry worldwide (Lunney et al. , Xiao et al. 2010b). The disease is caused by an enveloped, positive-sense, single-stranded RNA virus called porcine reproductive and respiratory syndrome virus (PRRSV). The PRRSV, a member of arterivirus group under the family arteriviridae, and is divided into two distinct genotypes namely European and North American (Sun et al. 2012). The PRRSV genome is approximately 15 kb containing 10 open reading frames (ORF) encoded with seven structural and 14 nonstructural proteins (Fang and Snijder 2010, Johnson et al. 2011). The virulent PRRSV primarily infects pulmonary alveolar macrophages, and destroy infected cells through cytopathic replication. The host-virus interaction results in a deficient host's innate immune response indicated by a poor induction of type I interferon (IFN α/β), the potent antiviral immune responsive cytokines (Ait-Ali et al. 2011, Albina et al. 1998). Some of the non-structural proteins (Nsp1, Nsp2 and Nsp11) and a structural protein (N protein) of PRRSV are known to be associated with IFN suppression in the infected cells (Sun et al. 2012). The RIG-I/ MDA5 and JAK-STAT pathways are two major signaling pathways for IFN production which are found to be impaired by PRRSV during acute infection (Sun et al. 2012). Overall, the timing and the potency of the host cellular and immunological events that occur following infection are likely potential determinants governing the pathogenesis (Pollock and Neill 2002).

Vaccination with modified live virus has been widely practiced in the commercial swine herd as one of the cost-effective control approaches for PRRS. The live attenuated PRRSV vaccine provides sufficient protection against homologous virus but limited protection against reinfection of genetically variant strains (Martelli et al. 2009). Live viral vaccines can efficiently trigger the activation of the host immune system through evolutionarily conserved pathogen associated molecular patterns (PAMPs) allowing their recognition by pattern recognition receptors (PPRs) of immune cells (Hoebe et al. 2004). Following administration, vaccine antigen produces a 'danger signals' which activate the monocytes and dendritic cells in such a way to secrete proinflammatory cytokines and chemokines (Iwasaki and Medzhitov 2004). These cytokines and chemokines lead the extravasation and attraction of monocytes, granulocytes and natural killer cells, and generate an inflammatory microenvironment, in which monocytes differentiate into macrophages, and immature dendritic cells become mature

(Pashine et al. 2005). Through changing the surface receptors, macrophages and mature dendritic cells migrate towards the draining lymph nodes and induce the activation of T and B lymphocytes. The generation and maintenance of both B and CD8+ T cell responses is supported by growth factors and signals provided by CD4+ T helper lymphocytes 1 and 2 (Th1 and Th2). Th1 and Th2 are controlled by regulatory T cells (Treg) involved in maintaining the immune tolerance (Bacchetta et al. 2005). The peripheral blood mononuclear cells (PBMCs) are the population of immune cells which includes lymphocytes (T cell, B cell and NK cells), monocytes and dendritic cells. Altogether, they play a central role in immune system against virus infection. Therefore, deciphering the PRRSV vaccine induced global transcriptome changes in PBMCs might lead to identify the molecules and signaling pathways associated with host immune response.

The innate immunity against viruses like PRRSV is critical as such virus is continuously changing their antigenic epitopes (Beutler 2004). Innate immunity is the first line defense mechanism of host cells against foreign antigen which typically occurs within hours in a non-specific manner and may persists up to 3-5 days (Beutler 2004). The innate immune system recruits effector cells upon antigen exposure which secret cytokines, chemokines and proteins and subsequently activate the adaptive immune system (Janeway et al. 2001). By that means, the innate immune response acts as precursor to initiate the adaptive immunity against a specific pathogen (Pancer and Cooper 2006). Innate immune traits have been considered as potential selection goals for disease resistance in pig breeding as innate immunity is likely to provide a common protection mechanism against multiple pathogens (Rowland et al. 2012a). Genome-wide association study revealed a major quantitative trait locus (QTL) on chromosome 4 (SSC4) associated with host resistance to in-vivo PRRSV challenge (Boddicker et al. 2012). The association of this region on SSC4 with PRRS resistance was further validated by the presence of single nucleotide polymorphism (SNP) marker, WUR10000125 (WUR) in the same region (Boddicker et al. 2014a, Boddicker et al. 2014b). Candidate genes in this locus on SSC4 include the interferon induced guanylate-binding protein gene family which is functionally linked to the innate immunity (Vestal and Jeyaratnam). Therefore, genes and molecular pathways associated with improved innate immune response to PRRSV vaccine could possibly be implemented in breeding program for PRRS resistant pigs (Rowland et al. 2012). To this end, key molecules regulating the transcriptional network of PRRSV vaccine induced innate immune response in peripheral blood are highly sought.

PBMCs are the primary immune cells in blood (Fairbairn et al. 2011) and have suitably been used for the evaluation of vaccine induced global gene expression changes for several diseases in human and non-human primates (reviewed by Wang et al. 2012). The porcine PBMCs have also been used for microarray analysis of immune response genes following in-vitro lipopolysaccharide stimulation (Gao et al. 2010), in-vivo mycoplasma vaccination (Mach et al. 2013) and tetanus toxoid vaccination (Adler et al. 2013). Transcriptional responses to natural as well as experimental infection of PRRSV have been studied through global gene expression profiling of pulmonary alveolar macrophages in pig (Dwivedi et al. 2012, Badaoui et al. 2014). However, little is known about the global transcriptome alterations in peripheral blood after PRRSV vaccination in pigs. Therefore, the aim of this study was firstly to investigate the transcriptional response to PRRSV vaccine in PBMCs of vaccinated pigs compared to unvaccinated pigs. Secondly to characterize the temporal patterns of global gene expression changes in PBMCs over three days following PRRSV vaccination.

2.3 Materials and methods

2.3.1 Study design and blood sampling

A total of 12 German Landrace female piglets were housed in the pig research farm at Frankenforst, University of Bonn, Germany. Piglets were selected from two sows farrowed at the same day; all piglets were clinically healthy with no history of respiratory diseases and birth defects. After weaning, experimental piglets were divided into two groups: 6 in vaccinated and 6 in unvaccinated group. The piglets of vaccinated group were vaccinated with a modified live PRRSV vaccine of European strain (Porcilis® PRRS, MSD Animal Health, Germany) with primary dose at day 28 and booster dose at day 56 of their age according to the routine farm vaccination program. The unvaccinated group was maintained for health control without vaccine treatment. About 8 mL whole blood samples with 1.5 mL anticoagulant (0.5 M EDTA) were collected at different time points before and after vaccination from pigs of both groups (Fig 9: Additional file 1). The blood samples collected at day 7 of age from all piglets were used for screening the PRRSV-specific maternally derived antibody response. The blood samples collected at four time points (0, 6, 24 and 72 hpv) following primary vaccination from both groups (except 0 h in unvaccinated group) were used for microarray hybridization. Three individual biological replicates from both groups were selected based on their RNA quality for the microarray experiment In addition, the blood

samples from all piglets collected just before, and two weeks post primary vaccination as well as just before, and two weeks post booster vaccination were used for monitoring the vaccine induced antibody response by ELISA.

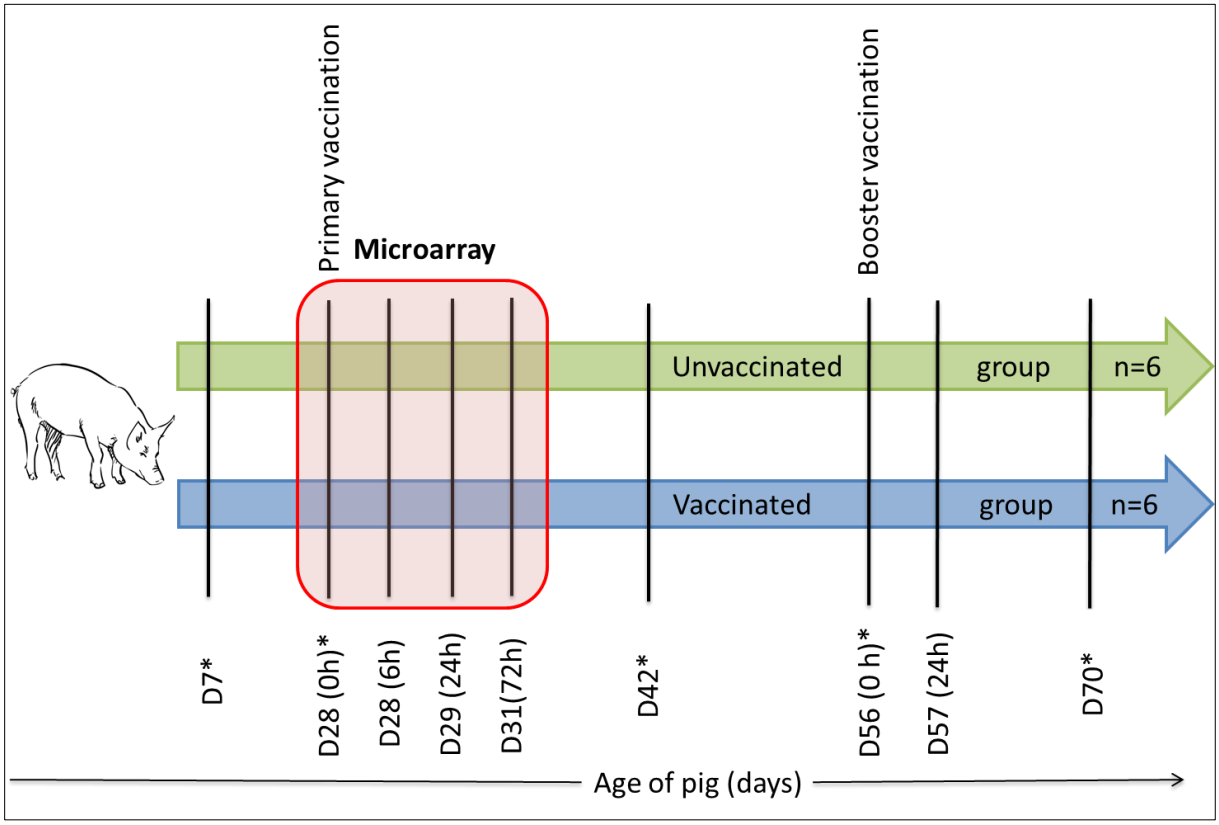

Figure 1(Additional file 1). Experimental design in Landrace pigs. The figure depicts experimental design and blood sampling schedule from PRRSV vaccinated and unvaccinated German Landrace pigs used for this study. Vertical lines indicated the blood sampling time points over the age of pigs (days). Primary and booster vaccination were performed at day 28 and 56 of age, and blood was collected immediately before vaccine injection in those days. Blood samples collected at 0, 6, 24 and 72 h after primary vaccination from both group except 0 h in unvaccinated group used for whole transcriptome microarray study. Three biological replicates from both groups were used for microarray hybridization. The same RNA samples used for microarray were quantified by qRT-PCR for technical validation of microarray data. Blood samples collected from all pigs at day 7, 28, 42, 56 and day 70 of their age were used for ELISA based monitoring of PRRSV specific antibody response.

2.3.2 Isolation of PBMCs and plasma

PBMCs and plasma were separated from the whole blood sample through density gradient centrifugation (1500 rpm for 25 min) with Histopaque®-1077 (Sigma-Aldrich, Munich, Germany) according to the protocol described by Uddin et al. (Uddin et al. 2012). The PBMCs were washed three times (pelleted at 1000 rpm for 5 min) using phosphate-buffered saline with purity of >99% determined by Wright-Giemsa staining.

2.3.3 Measurement of plasma antibody level

To monitor the PRRSV-specific antibody titre, the plasma samples from all study animals collected at day 7, 28, 42, 56 and 70 of their age (Additional file 1) were screened by ELISA (PRRSV-AK screening, Synlab Vet GmbH, Augsburg, Germany) according to manufacturer's protocol. The optical density (OD) of each well was measured at 650 nm using the Bio-Rad 680 microplate reader. The presence or absence of PRRSV antibody was determined by calculating the sample to positive (S/P) ratio. The S/P ratio was calculated according to the following equation: S/P ratio (%) = 100 × [(OD of test sample – Mean OD of negative controls) / (Mean OD of positive controls – Mean OD of negative controls)]. The samples were considered to be positive for PRRSV antibody if the S/P ratio was more than 0.4 as decribed by Kittawornrat et al (2012) .

2.3.4 Isolation and quality control of total RNA

The total RNA was extracted from PBMCs using the miRNeasy mini kit (P/N 217004, Qiagen, Hilden, Germany) according to the manufacturer's protocol along with DNase treatment (P/N 79254, Qiagen, Hilden, Germany). RNA concentration and purity were measured by NanoDrop® spectrophotometry (ND-8000; NanoDrop Technologies). RNA integrity was checked by micro capillary electrophoresis on an Agilent 2100 Bioanalyser with RNA 6000 Nanochip kit (Agilent Technologies, Waghäusel - Wiesental, Germany).

2.3.5 Microarray target preparation and hybridization

To prepare the target probes of 21 microarray, about 100 ng of total RNA samples from each of seven selected time points were processed with the GeneChip® WT PLUS Reagent kit (P/N 902281; Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. In brief, the total RNA was subjected to synthesize the first-strand cDNA containing a T7 promoter sequence at the 5´ end followed by synthesis of the second-strand cDNA by DNA polymerase in the presence of RNase H. This double-strand cDNA was subjected to in-vitro transcription with T7 RNA polymerase for synthesis of the antisense RNA (complementary RNA, cRNA). The cRNA preparation was then purified using purification beads to improve its stability. From 15 µg of purified cRNA, the sense-strand cDNA (ss-cDNA) was synthesized by reverse transcription using random primers. The ss-cDNA contained dUTP at a fixed ratio relative to dTTP and the remaining cRNA was degraded by RNase H. After purification and quantification, 5.5 µg of ss-cDNA in a 31.2 µL volume was fragmented by uracil-DNA glycosylase (UDG) and apyrimidinic endonuclease 1 (APE 1) at the unnatural

dUTP residues and breaks the DNA strand. The fragmented ss-cDNA was then labeled by terminal deoxynucleotidyl transferase (TdT) using the Affymetrix proprietary labeling reagent that is covalently linked to biotin. The hybridization of microarray probes followed by washing and staining was performed with the GeneChip® Hybridization, Wash and Stain kit (P/N 900720, Affymetrix Inc., Santa Clara, CA). For hybridization, about 130 µL of biotinylated ss-cDNA probes was injected into the GeneChip® Porcine Gene 1.0 ST array strip of 81/4 format (P/N 901976, Affymetrix Inc., Santa Clara, CA, USA) and incubated for 16 h in a hybridization oven (GeneChip® Hybridization oven 640; Affymetrix Inc.) at 45 °C with 60 rpm. The hybridized chips were stained and washed in a fluid station (GeneChip® Fluidics Station 450; Affymetrix Inc.) and scanned by Affymetrix GeneChip® scanner 3000 7G. The Affymetrix GeneChip® Command Console™ (AGCC) software was used to evaluate the array images and to export the reports of spot intensity data in .CEL file format.

2.3.6 Microarray data processing

Pre-processing, normalization and statistical analyses of microarray dataset were performed using packages of Bioconductor-platform implemented in R-project software (v3.1.2) (Gentleman et al. 2004). The 'oligo' package was implemented for the RMA (Robust Multi-array Average) based quantile normalization of microarray data at transcript level (Carvalho and Irizarry 2010). For quality control, some diagnostic plots of the raw intensity data were checked before and after the normalization. After excluding two arrays at 72 h post unvaccinated sample which did not pass the quality control, 19 arrays were used for further analysis. After normalization, the main probes (19,218) of the array were extracted. Then interquartile range (IQR) based filtering (variance cutoff value 0.25) was applied which further excluded about 4,978 low expressed probes. Finally the expression dataset comprising 14,231 transcript probes were subjected for downstream analysis. Probe to gene transcript annotation was performed with recent Affymetrix annotation file for assigned array (Liu et al. 2003). Gene annotations were extended by their orthologous human gene symbol as well as ensembl gene identifiers. Until otherwise mentioned, downstream functional analyses of this dataset were performed based on human genome database.

2.3.7 Characterization of phenotypic groups

To characterize the differences of transcriptional responses between pigs of vaccinated and unvaccinated group, the annotated gene expression profiles of PBMCs were subjected to an exploratory functional analysis through gene set enrichment analysis (GSEA) algorithm

implemented in GSEA-P tool (Subramanian et al. 2007). Two pairs of vaccination-time point group (6 hp vaccinated vs. 6 hp unvaccinated and 24 hp vaccinated vs. 24 hp unvaccinated) were considered as input phenotype for this analysis. The normalized expression dataset of 12 arrays containing human orthologous symbols of gene transcripts with their corresponding expression values (Additional file 2) were uploaded into the GSEA-P to generate the list of ranked order gene markers. The 'immunologic signature' catalog of gene set from Molecular Signatures Database (C7: MSigDB v5.0, Broad Institute, Cambridge, MA) was screened against the ranked gene list. The normalized enrichment score (NES) of each gene set was estimated by the number of over-representation of members of gene set towards the top or bottom of the ranked gene list through applying a weighted Kolmogrov-Smirnov statistics (Subramanian et al. 2005). Then the enrichment score p-values were estimated using a phenotype based permutation test procedure. The statistical significance was defined by the cutoff value of false discovery rate (FDR) <0.15 and the NES p <0.05.

2.3.8 Differential gene expression analysis

To explore the temporal variation of transcriptional response to vaccination, differential gene expression analysis was performed using the linear analysis of microarray technique from the 'limma' package (Smyth 2005) with empirical Bayes adjustment to the variance, followed by Benjamini and Hochberg (BH) correction for multiple testing (Benjamini and Hochberg 1995). To check whether there was temporal variation among the pigs of unvaccinated control group, two contrast pairs (i.e. 0h_vac vs. 6h_unvac and 0h_vac vs. 24h_unvac) were tested. Then within the vaccinated group, three pairwise comparisons (6 hpv vs. control; 24 hpv vs. control and 72 hpv vs. control) were taken in to account for the differential expression analysis. Gene transcripts were considered as differentially expressed when passing the thresholds of false discovery rate (FDR) of <0.01 and $\log_2$ fold-change either >1.5 or <-1.5. The number of differentially expressed genes in each contrast pair and their interaction were exported in intersecting Venn diagram.

2.3.9 Gene ontology and pathway analysis

For biological interpretation of the transcriptome dataset, the significantly over-represented gene ontology terms and biological pathways were explored with the InnateDB pathway analysis tool (Breuer et al. 2013). First, the identifiers of DEGs from microarray data were converted to their human ensembl orthologues using the BioDBnet tool (http://biodbnet.abcc.ncifcrf.gov/). The list of ensembl gene identifiers was then uploaded in

InnateDB web and performed the over-representation analysis with implementation of the hypergeometric algorithm and the Benjamini-Hochberg (BH) multiple test correction method. The gene ontology (GO) and pathways were considered significantly over-represented if they had a FDR<0.05.

2.3.10 Sub-network enrichment analysis

To visualize the PRRSV vaccine induced transcriptional network as well as to identify the regulatory genes, the sub-network analysis was performed using NetworkAnlayst online tool (Xia et al. 2014). This tool uses the InnateDB (downloaded June 20, 2014) protein-protein interaction (PPI) datasets comprised of 14,755 proteins and 145,955 experimentally validated interactions for human. NetworkAnlayst implements the R package ‘igraph’ for network analysis and ‘Gephi Toolkit’ for finalizing the network layout. Human orthologous ensembl gene identifiers of the DEGs were uploaded into the NetworkAnlayst to construct the interacting network. First, a default network was assembled based on the Walktrap algorithm taking only direct interaction of seed genes (first-order interactors). The network size was then adjusted for <500 seeds and 200~2000 nodes using the ‘reduce’ panel for high-performance visualization. Two topological measures such as degree (number of connections to other nodes) and betweenness centrality (number of shortest paths going through the node) were taken in to account for detecting highly interconnected hubs of the network. Centrality measures of hub nodes were evaluated serially with degree followed by betweenness. Nodes having higher degree and betweenness values were considered as potentially important network hubs in cellular signal trafficking. Finally, weighted network based module detection was perform to cluster the genes of similar biological functions. The p-value of a given network module was calculated using a Wilcoxon rank-sum test of the "internal" (edges within in a module) and "external" (edges connecting the nodes of other modules) degrees. The p values were calculated based on their connectivity assuming null hypothesis that there is no difference between the number of "internal" and "external" connections to a particular node in the module. Module having more internal than external edges was like to be statistically significant. The functional enrichment of modules was performed with REACTOME.db pathway database incorporated in this tool for comprehensive biological illustration of the network.

2.3.11 Quantitative real-time PCR (qRT-PCR)

For technical validation of microarray results, five selected DEGs (Table 1) known to be involved in immune response function were quantified by qRT-PCR in the same RNA samples as used for microarray expression. Primers were designed based on an open source primer designing software Primer3 (Rozen and Skaletsky 2000). First Strand cDNA Synthesis Kit (P/N K1612, Thermo Scientific, Co.) was used for reverse transcription with oligo (dT) primer. The qRT-PCR reaction was set up taking 1.0 µl of cDNA template, 8.0 µl of deionized RNase free water, 0.5 µM of upstream and downstream primers, and 10 µl iTaq™ Universal SYBR® Green Supermix (Bio-Rad laboratories GmbH, Germany) in a total reaction volume of 20 µl and were amplified by the StepOnePlus™ Real-Time PCR System (Applied Biosystems®, Darmstadt, Germany). The thermal cycling conditions were 95°C for 3 min, 95°C for 15 sec, 6°C for 45 sec (40 cycles); 95°C for 15 sec, 62°C for 1 min, 95°C for 15 sec. All reactions were run in duplicate and the average value was used as expression value. Gene-specific expression was measured as relative to the geometric mean of the expression of two housekeeping genes (GAPDH and ACTB) (Table 1). The delta delta Ct (ΔΔCt) [$\Delta Ct = Ct_{target} - Ct_{housekeeping\ genes}$] values were calculated as the difference between target gene and reference genes and expression was calculated as $2^{(-\Delta\Delta Ct)}$ (Pfaffl 2001). The correlation between microarray and qRT-PCR results was analyzed by Spearman's Rho test. The significance level was set as $p < 0.05$.

2.4 Results

2.4.1 PRRSV-specific antibody responses

In order to exclude the maternally derived antibody (MDA) of PRRSV as well as to evaluate the vaccine induced antibody response, plasma samples from all pigs at day 7, 28, 42, 56 and 70 of age were screened by ELISA. The plasma antibody level confirmed that experimental pigs were negative for MDA of PRRSV considering the sample to positive (s/p) ratio of 0.4 (40%) as threshold (Fig 2). The optical density (OD) values indicated relative higher MDA titre in suckling piglets which felt down and remained stable towards the base line along with increased age of unvaccinated pigs. On the other hand, there was an increasing trend of plasma antibody titre in pigs following vaccination. The antibody titre got above the threshold after two weeks, and subsequently reached a plateau after four weeks of the primary vaccination (Fig 2).

Table 1. The list of primers and their sequences of selected candidate genes used for qRT-PCR validation of microarray data.

GenBank Accession number	Gene name	Primer sequence (5`-3´)
NM_213770.1	IRF3: Interferon regulatory factor 3	F: CCAGTGGTGCCTACACTCCT R: AGAGGTGTCTGGCTCAGGAA
NM_001044580	STAT3 : Signal transducer and activator of transcription 3 (acute-phase response factor)	F: TGCTGGAGGAGAGAATCGT R: GGGAATTTGACCAGCAATC
NM_214087	CD80: Cluster of differentialtion-80	F: TCAGACACCCAGGTACACCA R: GACACATGGCTTCTGCTTGA
NM_001105286.1	TRAF6: Tumor necrosis factor receptor-associated factor	F:GGGAACGATACGCCTTACAA R:CTCTGTCTTAGGGCGTCCAG
NM_213779	CCL4 : Chemokine (C-C motif) ligand 4	F: CTCTCCTCCAGCAAGACCAT R: CAGAGGCTGCTGGTCTCATA
HQ013301	GAPDH : Glyceraldehyde-3-phosphate dehydrogenase*	F: GCTGGTGCTGAGTATGTCGT R: AAGCAGTTGGTGGTACAGG
XM_003124280.3	ACTB: Actin, Beta*	F: AAGGACCTCTACGCCAACAC R: CTGGCTGATCCACATCTGCT

* are the house keeping genes used for normalization

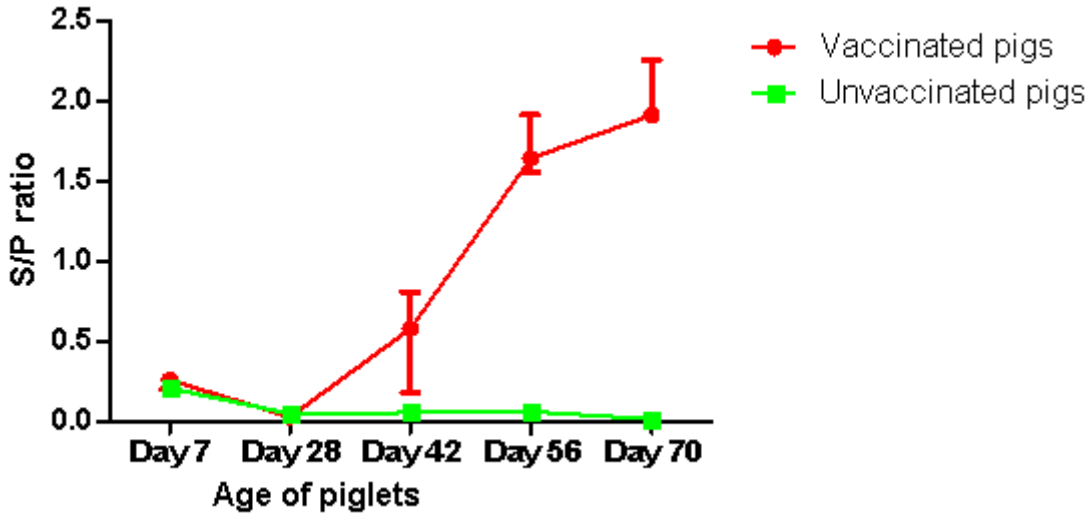

Figure 2. PRRSV-specific antibody responses in Landrace pigs. The figure depicts the reactivity of maternally derived antibody and vaccine derived antibody to PRRSV in plasma detected by PRRSV-AK Enzyme Immunoassay. Values in the Y-axis represent the sample to positive (s/p) ratio, and the s/p values of 0.4 was considered as threshold to classify the individuals either positive or negative. Values in X-axis represents the piglet ages at which blood samples were evaluated. Primary and booster vaccination were performed at day 28 and 56 of age, respectively in pigs of vaccinated group. In vaccinated group, the optical density (OD) values of samples at day 7 and 28 of age represent for maternally derived antibody (MDA), and samples at day 42, 56 and 70 for vaccine induced antibody response. While samples from the control group were used for only monitoring the way of declining the MDA over the age of animals in absence of further PRRSV exposure.

2.4.2 Transcriptome profiles of PBMCs following PRRSV vaccination

To uncover the transcriptional modification underlying the innate immune response to a live attenuated PRRSV vaccine, we performed the global transcriptome profiling of PBMCs from pigs of vaccinated group at before (control) and 6, 24 and 72 h after vaccination; and from unvaccinated group at 6, 24 and 72 h post vaccination time points using the Affymetrix GeneChip Porcine Gene 1.0 ST Array. This array was encoded with 394,580 probe (20-22 probes per gene) representing a total of 19,212 genes. After normalization the current study identified a total of 27,558 probes having higher signal intensity than the background. After filtering, 14,231 transcripts were found to be expressed in PBMCs, 10,217 of which could be annotated and were implemented in the downstream analyses.

2.4.3 Variation of PBMCs transcriptome profiles between vaccinated and unvaccinated pigs

The gene set enrichment analysis (GSEA)-based comparison of genome-wide expression distinguished the vaccine induced transcriptome changes between the vaccinated pigs and the age-matched unvaccinated control pigs. GSEA algorithm revealed that a total of 42 and 36 gene sets (pathways) were significantly upregulated at 6 and 24 hpv in vaccinated group, respectively compared to their unvaccinated counterparts. Of these, the chemokine signaling,

JAK-STAT signaling and cytoskeleton activation are the most significantly enriched gene sets which indicated the potential of vaccine to switch on the transcriptional machinery in PBMCs. The normalized enrichment scores of top 15 up regulated gene sets in vaccinated group ranged from 2.04 to 2.52 (Table 2). The enrichment score of most of the upregulated gene sets in the vaccinated group was increased at 24 hpv from that of 6 hpv indicating the number of core genes of particular gene set increased over the time of immunization.

2.4.4 Differential gene expression in PBMCs after PRRSV vaccination

To get a comprehensive overview of transcriptional modifications associated with innate immune response, we performed the differential gene expression analysis over three time points (6, 24 and 72 hpv) after vaccination compared to the control (before vaccination). The normalized expression values for only main probes of the chip were included for differential analysis and filtered by the thresholds of FDR <0.01 and $\log_2$ fold-change >1.5 or < -1.5. Using this criterion, 2,453 transcripts were found to be differentially expressed in PBMCs after PRRSV vaccination. Among them, 1,087 (44.31%) gene transcripts could be annotated. A complete list of the differentially expressed genes (DEGs) in PBMCs at three time points following PRRSV vaccination is provided in Additional file 2, 3 and 4.

The number DEGs and their direction of expression in three pairwise comparisons are plotted in Fig 3. A total of 542 DEGs including 423 up regulated and 119 down regulated genes were detected at 6 hpv. The highest number (2263) of DEGs was identified at 24 h post vaccination. The number of upregulated genes (2060) was also much higher than the down regulated ones (203) at 24 hpv. A total of 357 genes showed differential expression at 72 hpv in which 188 and 169 were up and down regulated genes, respectively. The fold change (FC) of differential expression ranged from -3.76 to 3.94; from -3.7 to 4.45 and from -4.15 to 3.11 at 6 hpv, 24 hpv and 72 hpv, respectively. A higher proportion of upregulated genes at each comparison indicated that vaccination induces active gene expression processes which may be associated with development of innate immune response.

Table 2. Significantly enriched gene sets obtained from gene set enrichment analysis

Gene sets	6hpv_vacc vs 6hpv_unvacc		24hpv_vacc vs 24hpv_unvacc	
	NES	FDR	NES	FDR
Regulation of actin cytoskeleton	2.51	0.001	2.75	0.001
Chemokine signaling pathway	2.48	0.001	2.56	0.001
JAK-STAT signaling pathway	2.39	0.001	2.55	0.001
Integrin cell surface interactions	2.46	0.001	2.5	0.001
Cell adhesion molecules	2.31	0.001	2.38	0.001
Integrin signaling pathway	2.28	0.001	2.37	0.001
Cell surface interactions at the vascular wall	2.27	0.001	2.33	0.001
Signal transduction by L1	2.24	0.007	2.27	0.007
Cytokine cytokine receptor interaction	2.20	0.008	2.25	0.007
Apoptosis by serum deprivation up	2.17	0.008	2.23	0.007
Immortalized by HPV31 DN	2.16	0.008	2.23	0.008
Signaling by FGFR1 mutants	2.14	0.011	2.21	0.010
TNF signaling up	2.12	0.014	2.19	0.013
ECM receptor interaction	2.10	0.025	2.13	0.021
TRAF trafficking pathway	2.08	0.013	2.12	0.011
Leukocyte transendothelial migration	2.04	0.008	2.14	0.007

NES, Normalized enrichment score; FDR, False discovery rate

The intersecting Venn diagram (Fig 4) revealed that 44, 1733 and 128 genes showing differential expression exclusively at 6, 24 and 72 hpv, respectively. Among the time point specific DEGs, 32, 1404 and 88 were up regulated and 12, 329 and 30 were down regulated at 6, 24 and 72 hpv, respectively. On the other hand, 161 genes showed differential expression constantly over the three days of post vaccination. Differential expression of 480 genes shared between the time points of 6 hpv and 24 hpv; 211 genes between 24 hpv and 72 hpv, and 179 genes shared between 6 hpv and 72 hpv time points.

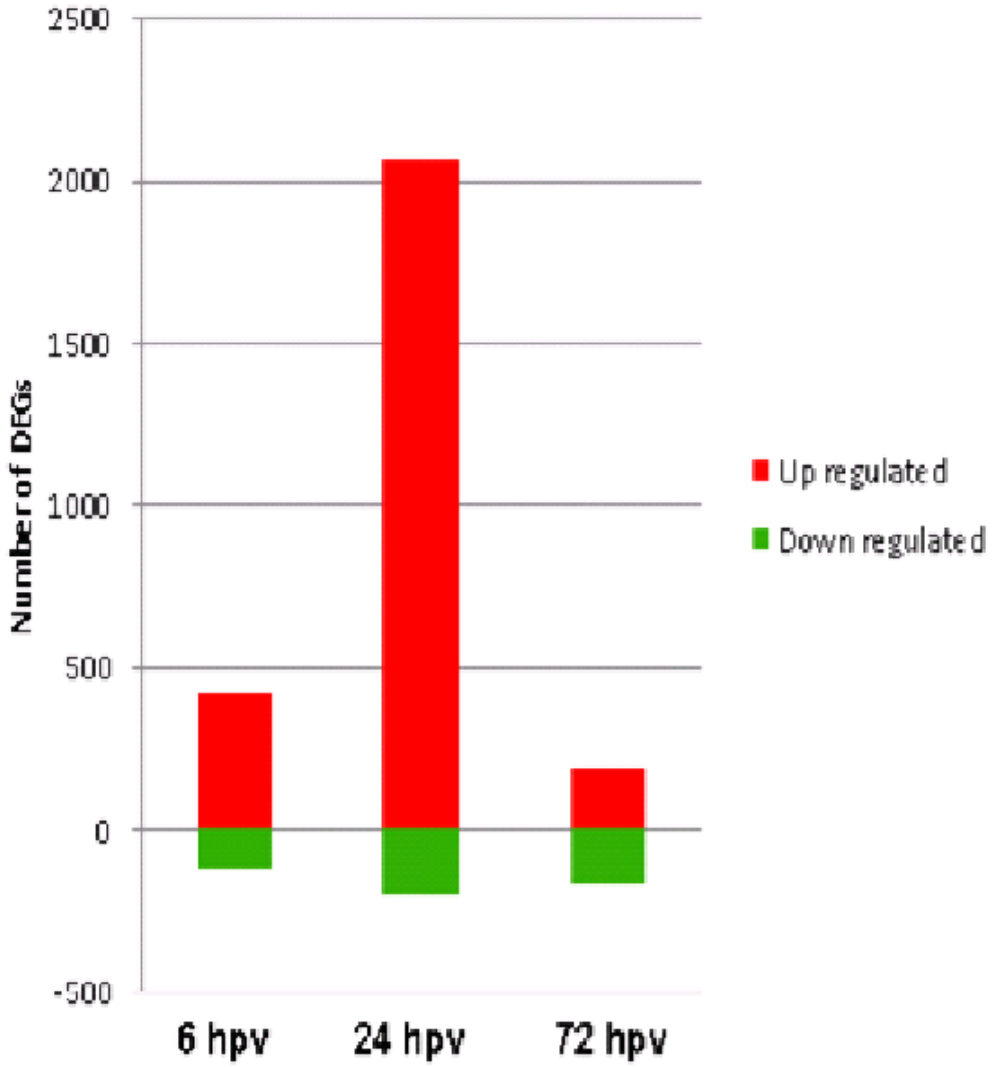

Figure 3 : Number of differentially expressed genes after PRRSV vaccination. The figure depicts the number and direction of DEGs identified at three time points (6, 24 and 72 hpv) of PRRSV vaccination compared to the control (before vaccination).

Hierarchical clustering of DEGs in PBMCs following vaccination has also provided a clear image of genes that were regulated in the same or opposite direction in response to vaccination (Fig 5). There was distinction among time points of vaccine exposure in terms of up or down regulation of DEGs as well. A quite remarkable difference was observed at 24 h post vaccination compared to that of control. The hierarchical cluster analysis (HCA) indicates a good cluster of replicate piglets within the group which is suggestive for the homogeneity of the experimental blocks.

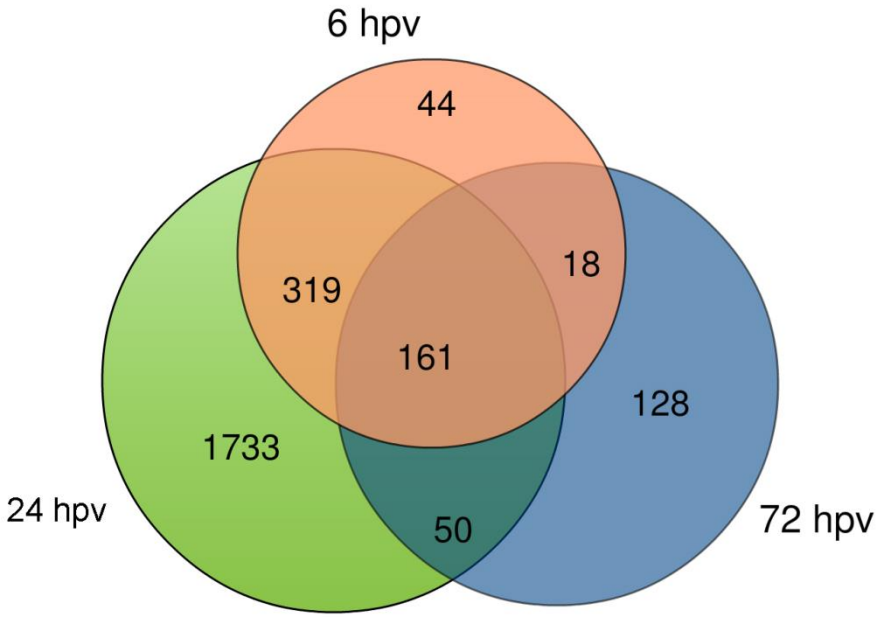

Figure 4. Intersecting Venn diagram showing the abundance of DEGs. The number of genes differentially expressed at three different time points (6, 24 and 72 hpv) of PRRSV vaccination compared to the control (before vaccination). The numbers in overlapping area (s) represent the differential expression of genes shared among the time points.

2.4.5 GO and pathways enriched by PRRSV vaccine induced DEGs

A GO classification of biological processes involved with all differentially expressed genes in PBMCs after PRRSV vaccination is provided in Table 3. The GO categories with a direct relation to immune response function includes innate immune response, signal transduction, viral process, T cell differentiation, chemotaxis, response to light stimulus, cytokine-mediated signaling pathway, complement activation, cell death, cell proliferation and immune system process. Highest representation of genes involved with particular GO terms was observed at 24 hpv compared to 6 hpv and 72 hpv. The pathway analysis paints a similar picture to the GO terms. The statistically significant biological pathways involved with PRRSV induced DEGs are presented in Fig 6. Among the top pathways, cytokine-cytokine receptor interaction, chemokine signaling pathway, signal transduction, JAK-STAT pathway and regulation, TRAF6 mediated induction of NF-kB and MAPK, the NLRP3 inflammasome, endocytosis and interferon signaling were activated.

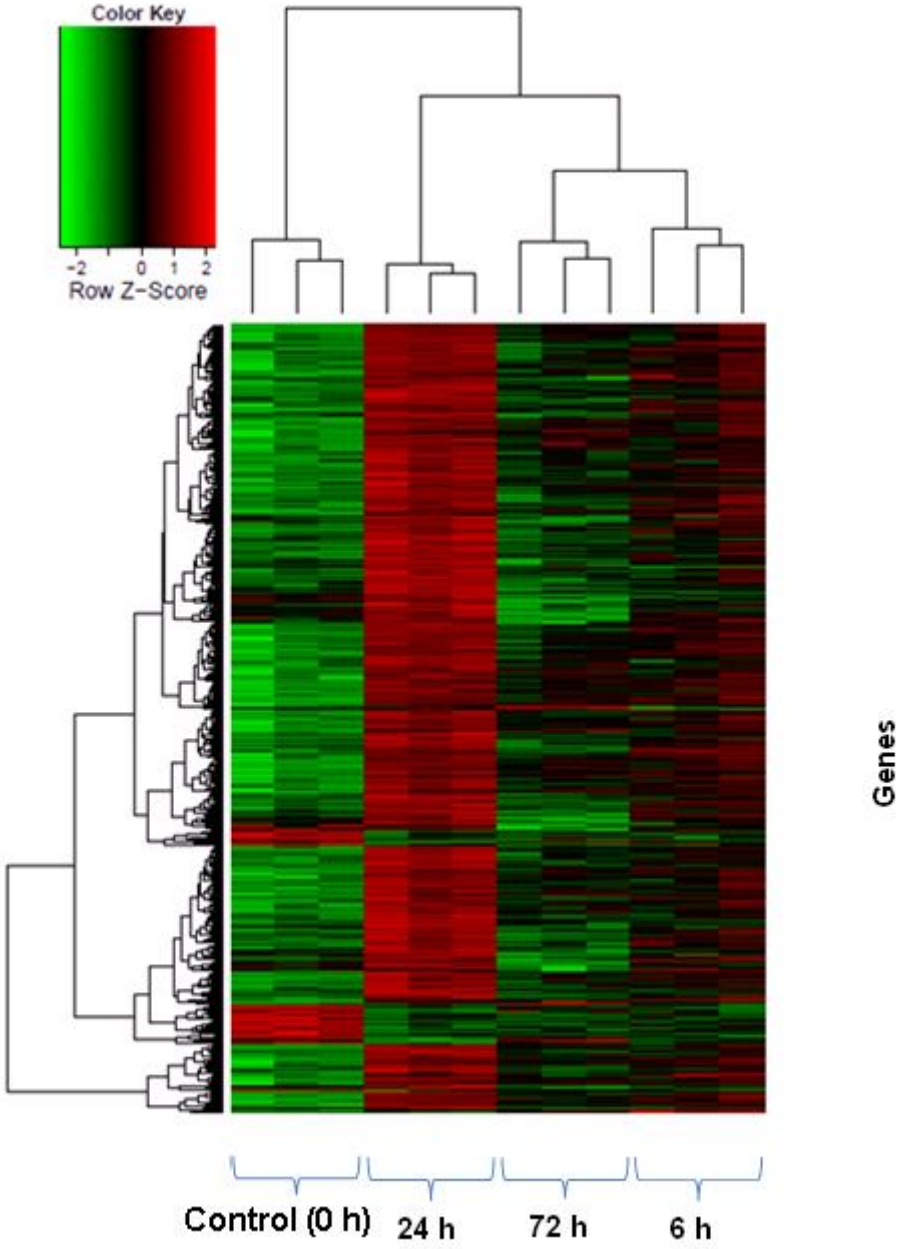

Figure 5. Hierarchical heat map showing differential gene expression over time. The normalized log_2 transformed values as determined by Affymetrix GeneChip® porcine gene 1.0 ST array in PBMCs of German Landrace pigs at 6, 24 and 72 h post PRRSV vaccination. The cutoff value of log_2 fold change as either >1.5 or <-1.5 and FDR <0.05 was considered for statistical significance. Each column represents one array from each of replicate piglets.

Table 3. Gene ontology terms enriched by the DEGs.

Time points	GO ID	GO term	Nr. of genes	Adjusted *p*-value
6 hpv	GO:0045087	Innate immune response	18	0.01
	GO:0042493	Response to drug	9	0.05
	GO:0034097	Response to cytokine	5	0.02
	GO:0016567	Protein ubiquitination	8	0.01
	GO:0043687	Post-translational protein modification	7	0.01
	GO:0044281	Small molecules metabolic process	30	0.01
	GO:0015031	Protein transport	10	0.03
	GO:0006355	Regulation of transcription, DNA-template	28	0.03
	GO:0007186	G-protein coupled receptor signaling pathway	15	0.03
	GO:0044267	Cellular protein metabolic process	14	0.03
24 hpv	GO:0045087	Innate immune response	90	0.01
	GO:0007165	Signal transduction	82	0.01
	GO:0008284	Positive regulation of cell proliferation	45	0.02
	GO:0016032	Viral process	37	0.01
	GO:0051607	Defense response to virus	14	0.02
	GO:0043406	Positive regulation of MAPK kinase activity	9	0.05
	GO:0006874	cellular calcium ion homeostasis	11	0.08
	GO:0007265	Ras protein signal transduction	8	0.02
	GO:0030217	T cell differentiation	5	0.05
	GO:0035666	TRIF-dependent toll-like receptor signaling	8	0.05
72 hpv	GO:0043408	Regulation of MAPK cascade	7	0.04
	GO:0007067	Mitotic nuclear division	6	0.04
	GO:0019221	Cytokine-mediated signaling pathway	5	0.03
	GO:0006935	Chemotaxis	4	0.04
	GO:0007155	Cell adhesion	7	0.05
	GO:0051726	Regulation of cell cycle	9	0.05
	GO:0055085	Transmembrane transport	14	0.03
	GO:0010467	Gene expression	6	0.05
	GO:0006915	Apoptotic process	8	0.04
	GO:0009615	Response to virus	5	0.14

2.4.6 Transcriptional network of PRRSV vaccine induced innate immune response in PBMCs

The network analysis retrieved one giant subnetwork herein called the global network and 12 other smaller networks. The global network comprised of 432 seed genes or nodes and 850 edges or connections. The diameter of each node corresponds to the values of two centrality measures (degree and betweenness) and thereby a larger diameter indicates higher potential of particular node to be the hub of the network (Fig 7). The values of degree and betweenness centrality of all seed genes are presented in Additional file. 6. Based on these two centrality measures, APP (Amyloid beta (A4) precursor protein) was determined to be the top most potential hub gene of the global network having highest values of degree (118) and betweenness centrality (6468). Other potential hubs includes TRAF6 (TNF receptor-associated factor 6), PIN1 (Peptidyl-prolyl cis-trans isomerase NIMA-interacting 1), FOS (FBJ murine osteosarcoma viral oncogene homolog), CTNNB1 (Catenin (cadherin-associated protein) beta 1), CDKN1A (Cyclin-dependent kinase inhibitor 1A (P21, Cip1)-A), TNFAIP3 (Tumor necrosis factor, alpha-induced protein 3), SIRT1 (Sirtuin 1), ESR1 (Estrogen receptor 1) and HDAC5 (Histone deacetylase 5).

The NetworkAnalyst tool has detected five significant (p <0.01) network modules within the global network which are indicated by different colors (Fig 7). Each module was led by one or more of the above mentioned hubs connected to many other genes of similar biological function. Functional enrichment of the modules revealed that PRRSV vaccine induced transcriptional modification involves five major groups of biological functions such as innate immune response; development and differentiation of blood cell; cell death, cell cycle and survival; ubiquitination and glycosylation; and protein metabolism and regulation of gene expression. In particular, the functional involvement of the *APP* led network module (red module) includes membrane trafficking (RAB5C, ARRB1, SEC24C, TBC1D1), chemokine receptor binding (CXCR2, CXCL16), interferon signaling (IFNA8, IFNW1), post-translational protein modification (PIGO, GALNT12, MPI, SEC24C) and asparagine N-linkage glycosylation (MPI, SEC24C).

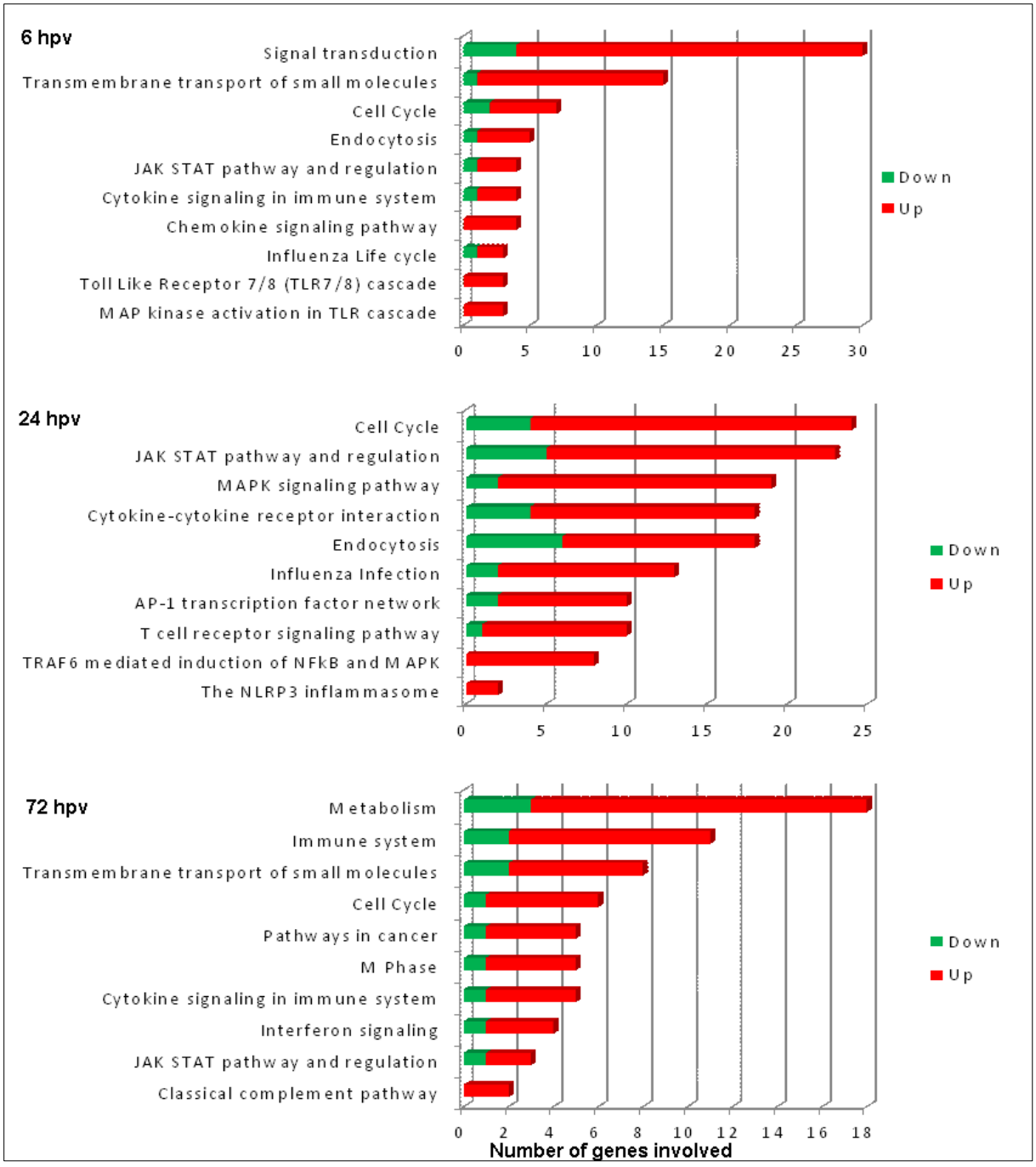

Figure 6. Biological pathways involved with the DEGs following PRRSV vaccination. The figure depicts the top 10 biological pathways regulated by the DEGs in each of three pairwise comparisons. Values in X-axis represents the number of over-expressed genes (red portion of bar) and under-expressed genes (green portion of bar) involved in corresponding pathways. Pathways included here only having the over-representation $p<0.05$ obtained from InnateDB. The upper part of graph (6 hpv) represents the top 10 pathways regulated by DEGs found at 6 h, the middle (24 hpv) for that of 24 h and the bottom one (72 hpv) for that of 72 h of post vaccination time points compared to the control (before vaccination).

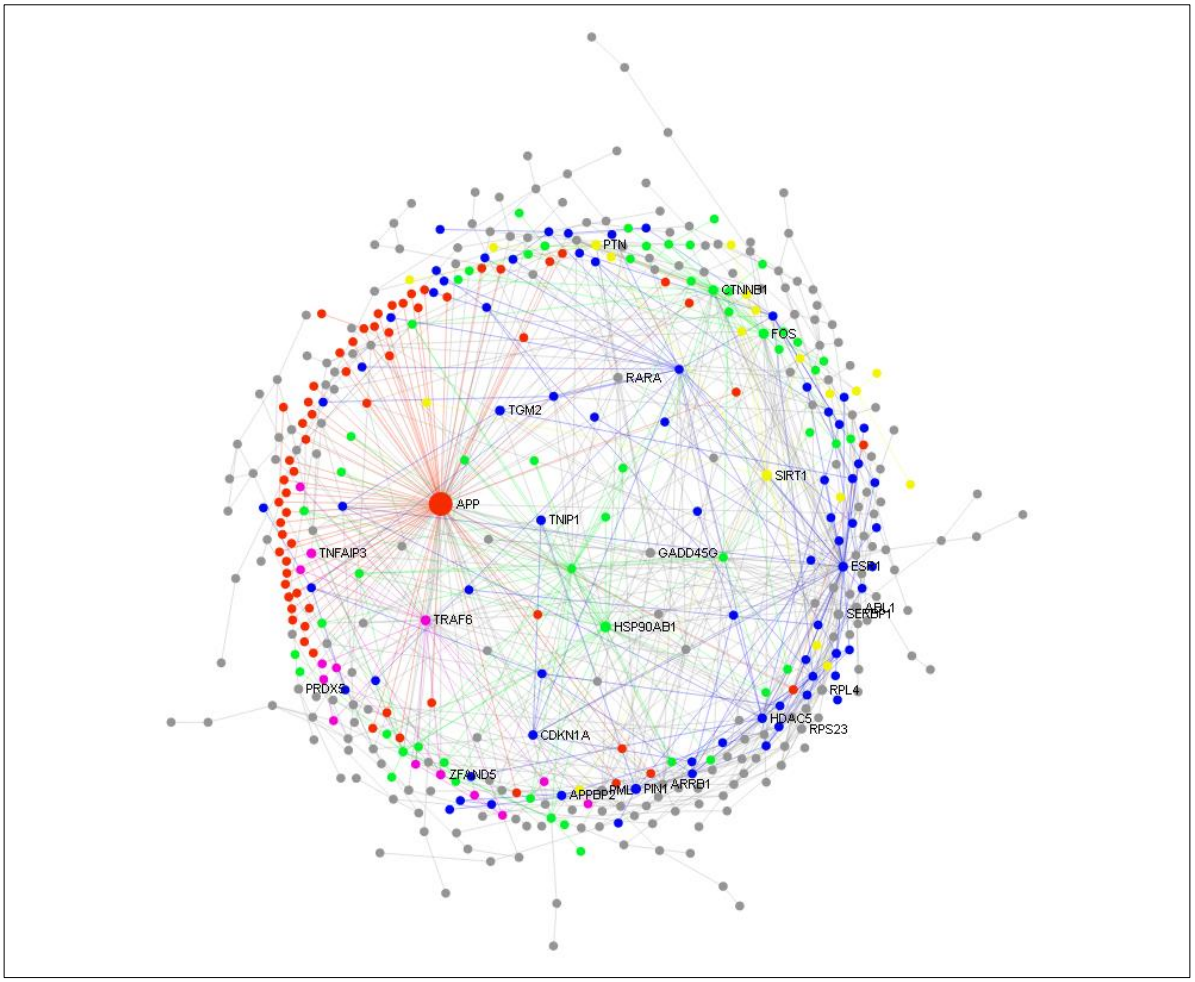

Figure 7. Global transcriptional network of PRRSV vaccine response in PBMCs. The picture depicts the interconnected network of PRRSV vaccine induced differentially expressed genes in PBMCs at 24 hpv. Each circle indicates the node or member genes of the network. The diameter of the circle corresponds to the values of two centrality measures that is degree and betweenness of particular gene. The larger diameter indicates the higher potential of the nodes to be the hub genes of the network. The network modules with corresponding genes are indicated by different colors.

To test whether these hub genes can coordinate the global transcriptional network, we constructed the second network (herein called the core network) taking the top thirteen hubs of global network as seed genes. The higher-order interactions of the core network assembled about 3764 nodes and 5145 connections which reflect the global network. The simplified interconnection among the hub genes is presented in the core network (Fig 8). Among the hub nodes, six (APP, TRAF6, PIN1, FOS, CDKN1A and TNFAIP3) were found to be directly involved with innate immune system and were upregulated in immunized PBMCs. APP, TRAF6, PIN1, FOS and TNFAIP3 have direct connection with six, five, five, four and three

other hubs, respectively. TRAF, APP, CTNNB1, ESR1 and HDAC5 together are responsible signal transduction process. TRAF6 along with FOS participate in toll-like receptor cascades, MAPK signaling, MyD88 dependent & independent cascades and proinflammatory response. TRAF6 and PIN are involved in RIG-1/MDA5 mediated induction of alpha-beta interferon.

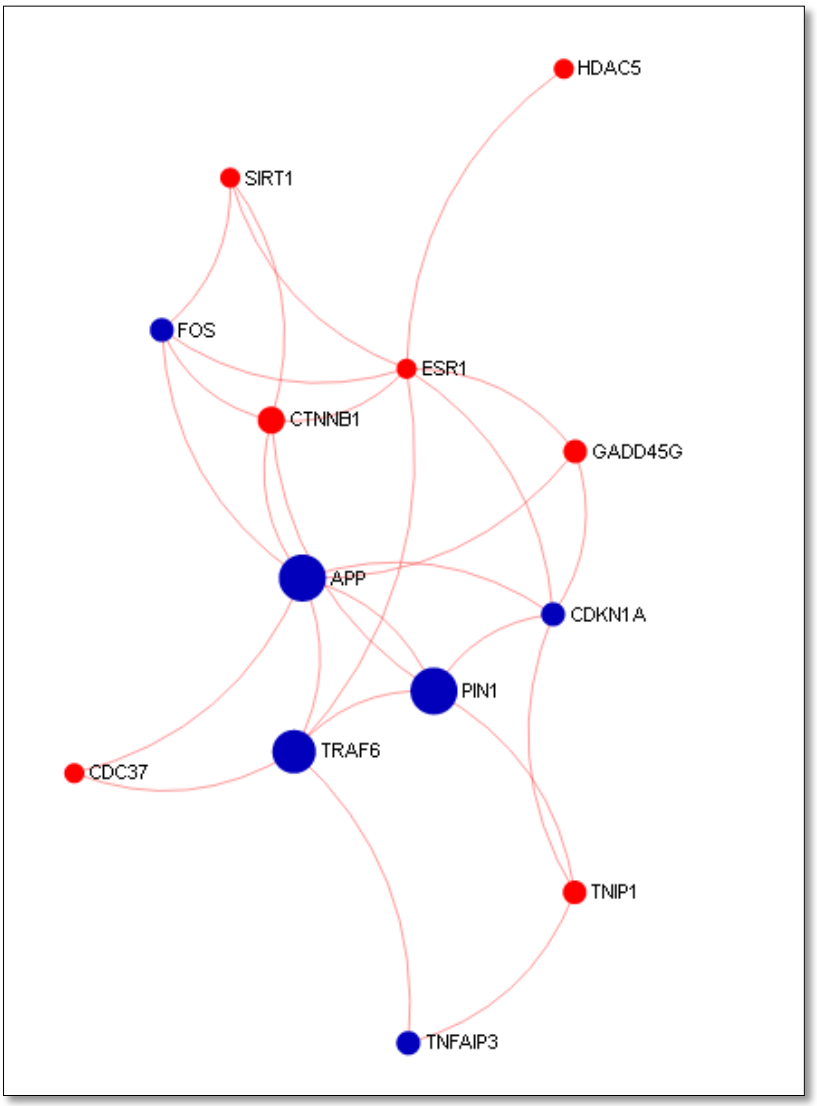

Figure 8. Core transcriptional network of PRRSV vaccine response in PBMCs. The picture depicts the connection among the regulatory genes of the global transcriptional network of PRRSV vaccine response in PBMCs. Only the direct connections among seed genes are presented. The diameter of the circle corresponds to the values of two centrality measures that is degree and betweenness of particular gene. Among the hubs, nodes of blue colors were known to be strongly involved in innate immune response function.

2.4.7 Validation of microarray data

Microarray data was validated through measuring the relative expression level of five differentially expressed genes (STAT3, IRF3, CD80, CCL4 and TRAF6) in PBMCs using qRT-PCR. The expression data (Additional file 7) obtained from microarray and qRT-PCR for the selected genes are plotted in Fig. 9. The qRT-PCR expression values of all five genes confirmed statistically significant ($p<0.01$) differential expression in the same direction as the microarray data with a correlation values of r = 0.949.

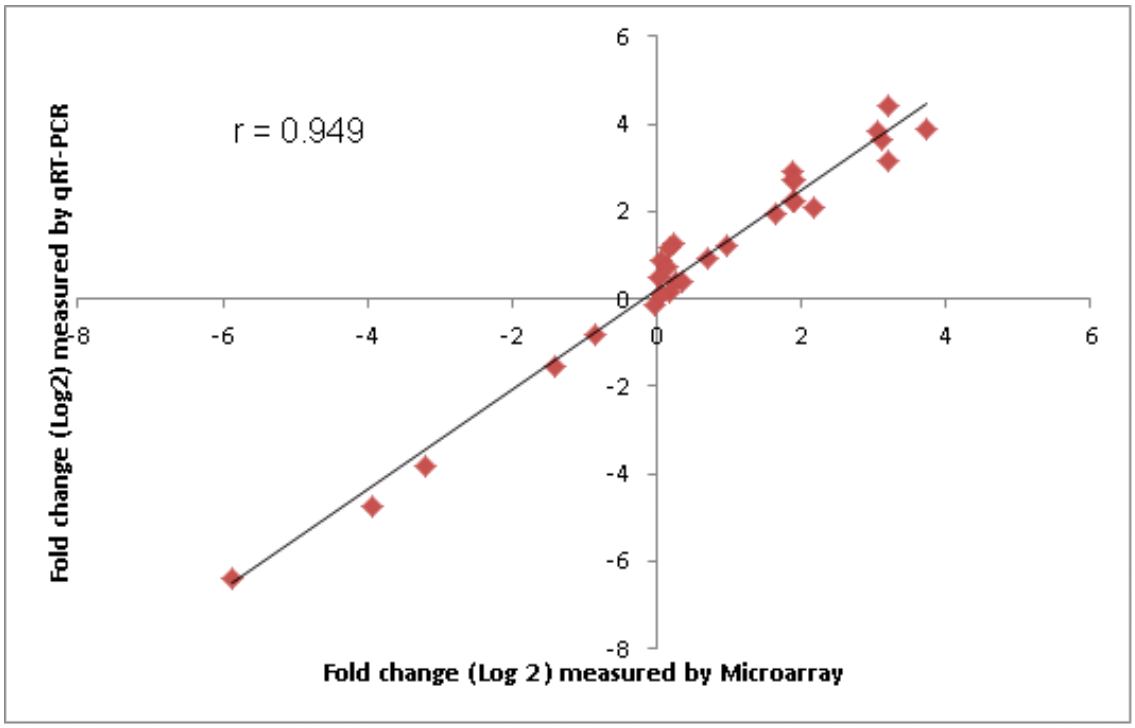

Figure 9. The qRT-PCR validation of the microarray data. The picture depicts the correlation between microarray (X-axis) and qRT-PCR (Y-axis) expression data (Log_2 fold-change) for five selected genes at three different time points (6, 24, 24 hpv) both in vaccinated and unvaccinated pigs. Correlation between microarray and qRT-PCR data was analyzed by Spearman's Rho test. The correlation coefficient was = 0.949, with a statistical significance of $p< 0.01$.

2.5 Discussion

Protective immunity to PRRS virus is a complex and unresolved issue. To date, the live attenuated virus vaccine has been considered to be the most economic method to achieve immunity and protecting herds from losses associated with infections by highly virulent strains of PRRSV (Zuckermann et al. 2007). In this study, the antibody response appeared to start at 2 weeks of primary vaccination and reached a steady state at 4 weeks after primary vaccination in pigs (Fig 2). This reflects the previous reports stating that PRRSV specific antibodies begin to appear in the infected pigs as early as 7-10 days post infection with a low viral titer (Loemba et al. 1996) followed by delayed production of neutralizing antibody (NAb) between 2-4 weeks post infection (Loemba et al. 1996). Besides NAb, components of

innate and cell mediated immune responses have major contribution to the viral clearance in immunized animals. Moreover, the character of the innate immune response to virus is thought to dictate the quality of subsequent adaptive immune response (Miller et al. 2010).

In order to get insight into host's innate immune transcriptional response to modified live virus vaccine, this study has provided for the first time the whole transcriptome profiles of PBMCs from PRRSV (EU strain) vaccinated and unvaccinated German Landrace pigs. The porcine PBMCs have been studied to evaluate the immune response to PRRSV by some authors (Feng et al. 2003, Zhuge et al. 2012), however, these were focused on to the *in-vitro* model with expression profiling of candidate genes. The PBMCs include lymphocytes (T Cells, B cells, and NK cells), monocytes, and dendritic cells in varying frequencies across individuals (Fairbairn et al. 2013). The vaccine induced cellular activation and differentiation may changes the proportion of sub types of PBMCs, which are likely contribute to gene expression changes (Palmer et al. 2006). Thus, the current analyses have limitation in evaluating the cell type specific contribution on vaccine responses. In fact, the reports on, and option for, specific cell subset of PBMCs limited in swine and mostly due to the relative lack of immune-tagged reagents critical for such detail phenotyping (reviewed by Schroyen and Tuggle 2015). However, specific cell type contribution could be partially addressed by bioinformatics approach of gene expression deconvolution. In deed, the unfractionated PBMCs model was used in this microarray study as a rapid and convenient model to evaluate host transcriptional response to PRRSV vaccination.

With the global PBMCs transcriptome profiles, we performed an exploratory functional analysis to characterize the phenotypic groups using gene set enrichment analysis (GSEA) algorithm. The GSEA-based analysis revealed significant enrichment of gene sets (pathways), such as chemokine signaling, JAK-STAT signaling and cytoskeleton activation (Fig 10: Additional file 2) in vaccinated group compared to unvaccinated group indicated the PRRSV vaccine potential to enhance the host's innate immunity. This was consistence with the findings of Badaoui et al (2013) who reported that the host-specific response to PRRSV challenge to be associated with the activation of canonical pathways like TREM1, toll-like receptor and hyper-cytokinemia/hyper-chemokinemia signaling. Moreover, the hybridization

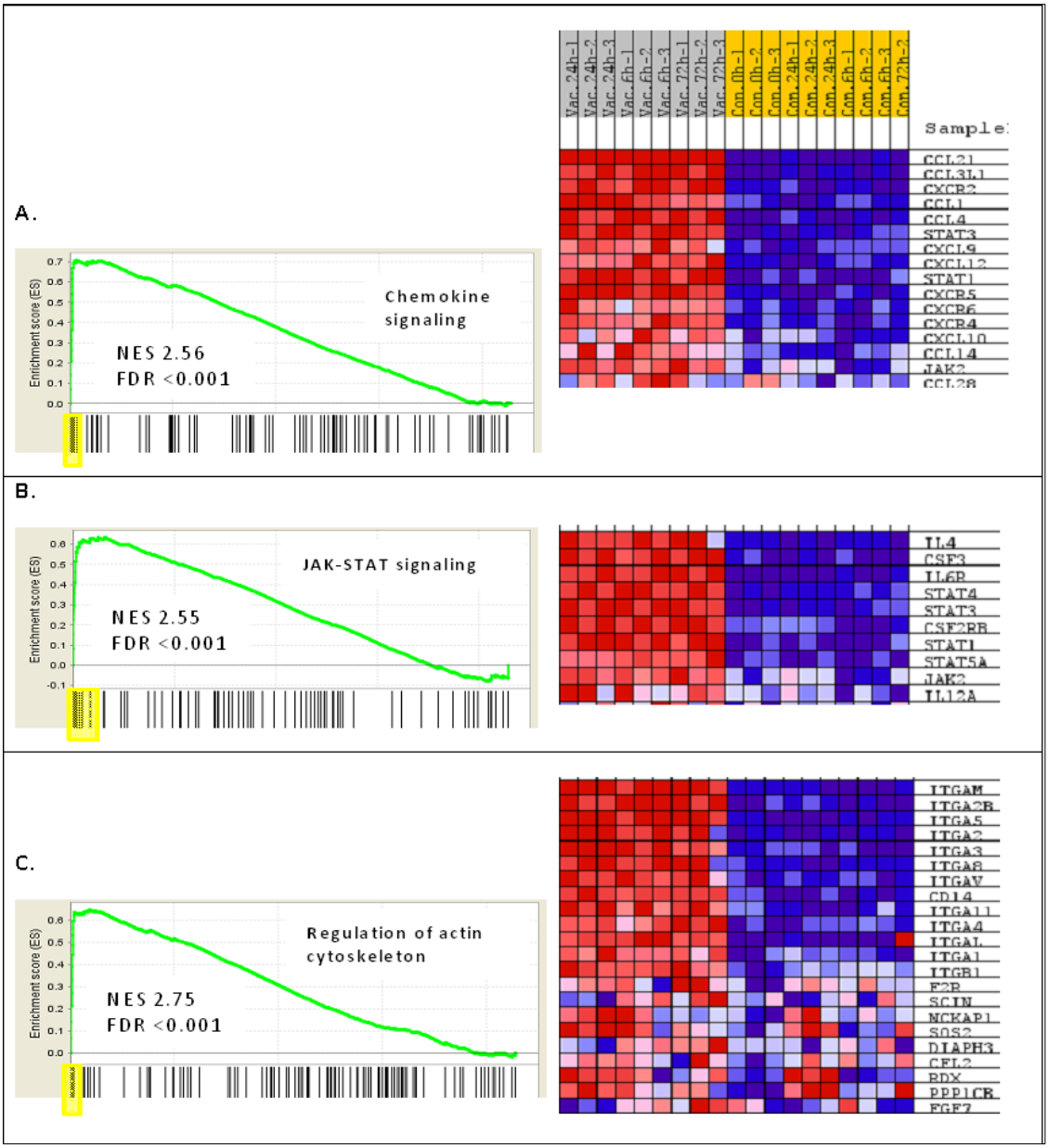

Figure 10 (Additional file 2). Characterization of phenotypic groups by GSEA. The figure depicts the comprehensive results of gene set enrichment analysis of our gene expression dataset against the curated gene set catalogue of "C7: immunological signature (Molecular signature database, v5.0, Cambridge, MA)". Enrichment plots for the 3 gene set (pathways) upregulated both at 6 hpv and 24 hpv in vaccinated cohort compared to their unvaccinated counterparts are shown on the left side with the relative gene positions indicated by the straight lines (line plot) under each graph. Lines clustered to the left represent higher ranked genes in the ranked list. Expression profiles for a subset of genes (shaded in yellow in the line plots) contributing to core enrichment for each pathway are shown to the right as a heatmap. The heatmap compares subject-level gene expression in both vaccinated and control subjects. Gene expression is normalized for each row. Lower levels of expression are represented in shades of blue and higher expression in red.

values of selected cell surface marker genes including CD4, CD14, CD19, CD33 and CD86 (Fig 11: Additional file 8) indicated the changes of the proportion of PBMC-subpopulation following vaccination. These differences justified the application of GSEA to distinguish the transcriptional response between vaccinated and unvaccinated pigs.

Followed by the gene set enrichment analysis, we performed the differential gene expression analyses in a time-series contrast which revealed that transcriptome alteration started at 6 hpv and peaked at 24 hpv followed by a decreased abundance at 72 hpv (Fig 4). The differential expressions of five selective genes were confirmed by qRT-PCR (Fig 9). It was noteworthy that the comparison between 6h and 24 h time points in unvaccinated group with that of 0 h in vaccinated group yielded about ~20 differentially expressed genes (data not shown) which were almost identical in both contrast pairs and did not lead to enrichment of any known immunological pathways. That indicated there was no significant temporal variation of immune response among the pigs having no vaccine exposure. Therefore, before vaccination (0 h) time point were used as control to compare with post vaccination time points. This was also supported by a similar study (Adler et al. 2013), where the pre vaccination sample has also been used as reference to investigate the temporal pattern of transcriptomic response in porcine PBMCs to Tetanus toxoid vaccine. The proportion of up regulated genes was much higher than the down regulated genes at all three time points indicated the potential of PRRSV vaccine to induce gene expression in PBMCs. The differential gene expression analysis of in the present study showed massive changes in the transcript abundance of known immune response genes and of genes that have been implicated in PRRSV infection by several authors (Xiao et al. 2010a, Xiao et al. 2010b, Zhou et al. 2011). Xiao et al (2010b) reported that 4,520 genes were differentially expressed in porcine lungs at 96 h and 168 h after *in-vivo* infection with highly pathogenic PRRSV strain, and those altered genes were functionally linked to host innate immune responses mediated by proinflammatory cytokines and chemokines.

The InnateDB pathway analyses of DEGs revealed that transcriptome modification caused by vaccination are involved with activation of pathway such as toll like receptor 7/8 cascade, endocytosis, cytokine signaling, chemokine signaling, signal transduction, MAPK activation in TLR cascade and JAK/STAT signaling pathway. These pathways are known to be involved in the process of host cell sensing of the viral antigen and subsequent induction of innate immune response. Innate immunity against viral antigen is initiated once after the recognition of viral PAMPs by the specific host cell cytoplasmic PRRs such as TLR3, TLR7, TLR8 The

recognition of viral PAMPs (ss RNA for PRRSV) by the host cell TLR results in a cascade of intracellular signaling through various adapter molecules (e.g. MyD88, MDA5, TRAF6) followed by activation of the MAP kinase family, which in turn switch on transcription factors such as interferon regulatory factors (IRFs) and NF-kB. Among the IRF family

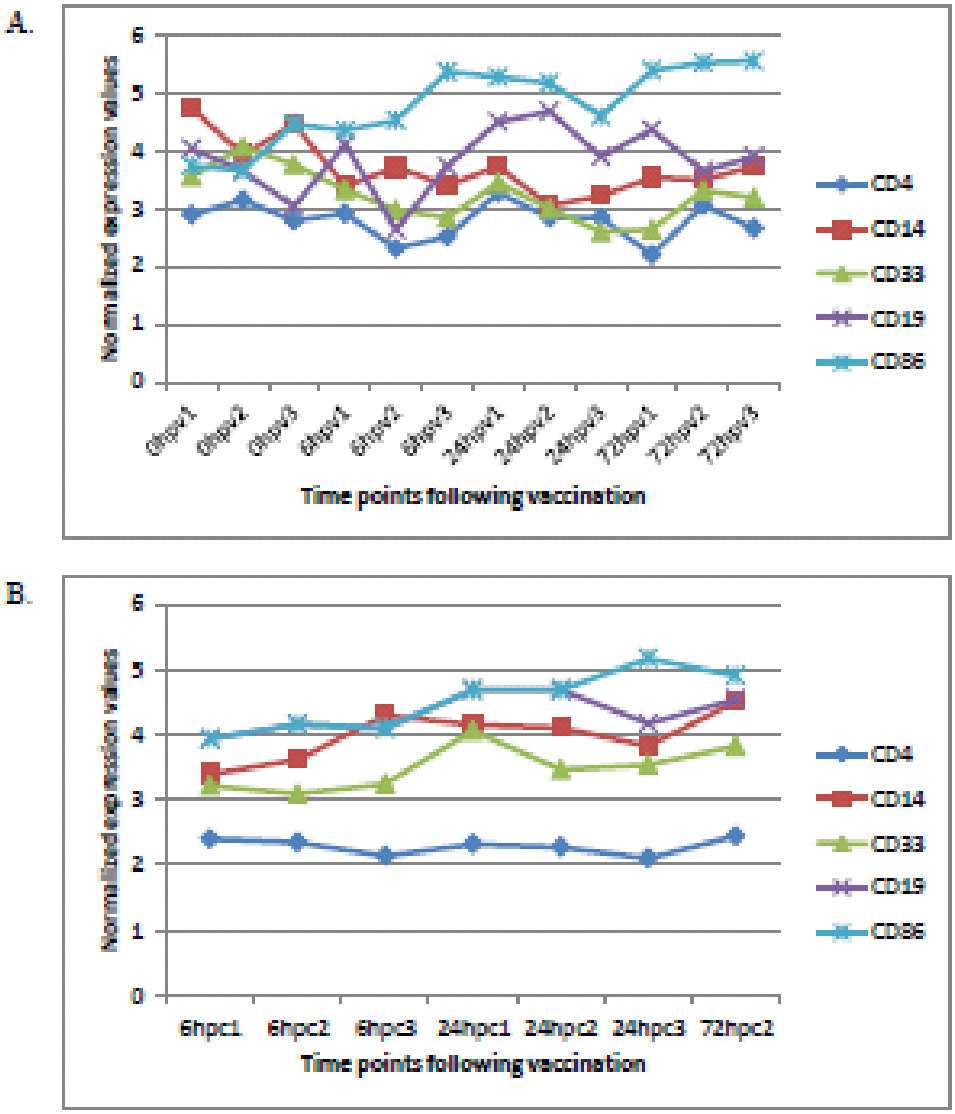

Figure 11 (Additional file 8). Microarray-based expression profiles of selected cell surface markers, (cluster of differentiation (CD)) in PBMCs of vaccinated (A), and unvaccinated pigs (B).

members, IRF2, IRF2BPL, IRF5 and IRF7 were up regulated but IRF3 was down regulated in PBMCs after vaccination. It is known that the members of IRF family such as IRF3, ISGF3, ISG15, IKKα, STAT1/STAT2 are involved in immunosuppressive effects of PRRSV infected cells (Patel et al. 2010, Yoo et al. 2010). The NF-kB induces several downstream signaling leading to the up regulation of proinflammatory cytokines, chemokines and type-I interferon which in turn facilitate the inflammatory process, apoptosis and phagocytosis (Hansen et al. 2011).

Finally we performed the network analysis to extract the regulatory molecules for vaccine responses. the network analysis revealed that genes including APP, TRAF6, PIN1, FOS, CDKN1A, CTNNB1, TNFAIP3 SIRT1, ESR1 and HDAC5 are the most highly interconnected hubs of the functional network of vaccine induced DEGs (Fig 7). The common feature of these master switch genes is that they regulate the induction of several pathways of the innate immune responses including TLR signaling, MAPK kinase cascades, interferon signaling and advanced glycosylation endpoint receptor signaling. This is in line with the recent report on detection of network module containing numerous immune response genes through weighted gene co-expression network analysis of whole blood transcriptome profiles of PRRSV infected pigs (Schroyen et al. 2015). Amyloid beta (A4) precursor protein (APP), the top hub gene, is a protein coding gene which induces the secretion of a number of peptides; two of the peptides were shown to have antibacterial and antifungal activities (Papareddy et al. 2012). The APP has been reported to be over expressed in porcine alveolar macrophages 24 h after in-vitro stimulation with PRRSV (Jiang et al. 2013). The network module led by APP has functional involvement with asparagine N-linked glycosylation of surface glycoprotein 3 (GP3) of PRRSV which regulates the neutralizing antibody response (Ansari et al. 2006). Therefore, the APP led gene network module might contribute to PRRSV vaccine induced transcriptional responses in PBMCs.

The TNF receptor-associated factor 6 (TRAF6) was found to be another prominent hub gene for transcriptional network induced by PRRSV vaccination in PBMCs. The TRAF6 is an adapter molecules required for TLR (TLR7/8) induced signal transduction leading to expression of IFNs (Seth et al. 2006). The peptidyl-prolyl cis-trans isomerase-1 (PIN1) is a nucleus protein which has an essential role in toll-like receptor signaling and type-1 interferon mediated innate immunity. TLR7 and TLR9 activate the isomerase PIN1 which subsequently activates the IRAK1, IRAK2 and IRF7 and induces type I interferons (Tun-Kyi et al. 2011). It appeared that among the top network hubs, APP, TRAF6 and PIN1 are known to be involved in the interferon response, the most potential antiviral innate immunity. Both TRAF6 and PIN1 are located in *Sus scrofa* chromosome 2 (SCC2), where the QTL for interferon-gamma level has already been identified (Uddin et al. 2011). However, no known QTL for immune response capacity found yet on the SSC13 where APP is located. Another hub gene, FOS, is a nuclear phosphoprotein involved in signal transduction, cell proliferation and differentiation, has been reported to regulate the replication of hepatitis-c virus (Kang et al. 2011). The cyclin-dependent kinase inhibitor 1A (CDKN1A) is a protein coding gene known to be

involved in antiviral immune response in human (Zahoor et al. 2014). Both FOS and CDKN1A are located on chromosome 7 (SSC7) where at least two QTLs for PRRS resistance as well as QTL for other innate immune response trait have been reported (Uddin et al. 2011).

Table 4: Additional file 7. Microarray and qRT- PCR expression values obtained for the five selected genes for the validation of microarray results

Genes	Treatment group	Time points	Fold change	
			Microarray	qRT-PCR
STAT3	Unvaccinated control	6h	0.71	0.90
		24h	0.96	1.22
		72h	0.27	0.41
	Vaccinated	6h	3.21	4.41
		24h	3.72	3.90
		72h	3.04	3.82
IRF3	Unvaccinated control	6h	-0.04	-0.12
		24h	-0.84	-0.81
		72h	-1.42	-1.53
	Vaccinated	6h	-3.21	-3.81
		24h	-3.93	-4.73
		72h	-5.88	-6.40
CD80	Unvaccinated control	6h	0.05	0.09
		24h	0.22	1.27
		72h	0.19	0.14
	Vaccinated	6h	1.88	2.9
		24h	2.17	2.10
		72h	1.92	2.21
CCL4	Unvaccinated control	6h	0.19	1.15
		24h	0.34	0.41
		72h	0.05	0.88
	Vaccinated	6h	3.12	3.62
		24h	3.21	3.15
		72h	1.88	2.71
TRAF6	Unvaccinated control	6h	0.04	0.51
		24h	0.14	0.72
		72h	0.06	0.11
	Vaccinated	6h	1.88	2.21
		24h	1.91	2.73
		72h	1.65	1.92

Two house keeping genes (GAPDH and ACTB) were used for normalization of the expression values

Tumor necrosis factor alpha-induced protein 3 (TNFAIP3) is also found as hub of the transcriptional network of PRRSV vaccine response in PBMCs. The TNFAIP3 is a ubiquitin editing enzyme, known to be involved in immune, and inflammatory responses signaled by cytokines, such as TNF-alpha and IL-1 beta, or pathogen sensing via toll-like receptors (TLRs) through terminating NF-kappa-B activity (Feng et al. 2003). TNFAIP3 is located on chromosome1 (SSC1) where at least three QTLs for PRRS susceptibility has been reported (Boddicker et al. 2012, Boddicker et al. 2014a, Serao et al. 2014). There were two close enzymatic products SIR1 and HDAC5 also found as hubs of the transcriptional network. SIRT1 deacetylates a wide range of substrates, including p53, NF-κB, FOXO transcription factors, and PGC-1α, with roles in cellular processes ranging from energy metabolism to cell survival (Cho et al. 2015). However, HDAC5 had strong connection only with ESR1 in the core network. The ESR1 was also found to be in the list of top ten hub genes of the network which was over expressed in vaccinated PBMCs. ESR1 along with PRLR, FSHB, EPOR and RBP4 were reported to have significant association with swine reproductive traits (Onteru et al. 2009). Though reproductive failure is one of the major clinical outcomes of PRRSV infection in breeding sows, ESR1 does not currently have known roles in innate immunity to PRRSV and warrants further investigation. On the whole, the hub genes including APP, TRAF6, PIN1, FOS, CDKN1A and TNFAIP3 (Fig 8), among others, were found to be highly interconnected to maintain the innate immune response function. Therefore, these six hub genes would coordinately be able to control the transcriptional network of PRRSV vaccine induced innate immune responses in PBMCs.

2.6 Conclusions

Herein, we performed a microarray-based transcriptome profiling to investigate genes, pathways and networks that may be involved in innate immune response of PBMCs to PRRSV vaccination in German Landrace pigs. This study identified APP, TRAF6, PIN1, FOS, CDKN1A and TNFAIP3 as potential hub genes which could contribute to the functional network of PRRSV vaccine induced transcriptome changes in PBMCs. Improvement of host genetic resistance has recently been considered as a promising way for sustainable PRRS control. As direct measurement of disease resistance is very difficult, an indirect approach through identification of genomic marker associated with innate immune response to PRRSV vaccine is recommendable. Therefore, it would imply that hub genes of the functional network identified in this transcriptome analysis might be considered for future research to investigate their potential role in PRRS resistance in pigs. However, the genetic diversity

among the pig breeds might contribute to the variation of PRRSV vaccine responsiveness. The correlation between early stage gene expression pattern and the antibody response of PRRSV vaccination could also be tested in larger pig population.

2.7 Declarations

2.7.1 Ethical issues

The experiment was performed according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2003). The experimental design was approved by the veterinary and food inspection office, Siegburg, Germany (Ref. 17-02.04.01-4/15).

2.7.2 Availability of supporting data

The minimum information about microarray experiment (MIAME) standard raw dataset supporting the results of this study has been deposited to the gene expression omnibus database (www.ncbi.nlm.nih.gov/geo/) and is accessible through the number GEO: GSE78254.

[All the spread sheet tables (Additional file 3, 4, 5 and 6) cited in this chapter are available at end the of full text paper published in the BMC Genomics, can be accessed through the link: http://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-016-2849-1]

2.7.3 Competing interests

The author(s) declare that they have no competing interests.

2.7.4 Funding

This work was financially supported by the State of North Rhine Westphalia of Germany with the programme “Umweltverträgliche und Standortgerechte Landwirtschaft (USL)” with the grant nr. 17-02.04.01-4/15.

2.7.5 Authors’ contributions

MAI, CN, KS and MJU conceived and designed the experiments; CN and DT arranged kits and reagents; MAI, MH and MP performed field experiment and blood sampling; MAI and SAR performed the wet lab works: sample processing, microarray and qRT-PCR; MAI and CGB performed dry lab works: statistics and bioinformatics; MAI interpreted the results and

drafted the manuscript; KS, ET, DT, MJU, CGB and CN reviewed and edited the manuscript. All authors have seen and approved the final version of the paper.

2.7.6 Acknowledgements
The authors are thankful to the staffs of the Teaching and Research Station on Frankenfrost, University of Boon for the animal husbandry support.

2.8 References

Adler M, Murani E, Brunner R, Ponsuksili S, Wimmers K (2013): Transcriptomic response of porcine PBMCs to vaccination with tetanus toxoid as a model antigen. PLoS ONE 8(3), e58306

Ait-Ali T, Wilson AD, Carre W, Westcott DG, Frossard JP, Mellencamp MA, Mouzaki D, Matika O, Waddington D, Drew TW, Bishop SC, Archibald AL (2011): Host inhibits replication of European porcine reproductive and respiratory syndrome virus in macrophages by altering differential regulation of type-I interferon transcriptional response. Immunogenetics 63, 437-448

Albina E, Carrat C, Charley B (1998): Interferon-alpha response to swine arterivirus (PoAV), the porcine reproductive and respiratory syndrome virus. J Interferon Cytokine Res 18, 485-490

Ansari IH, Kwon B, Osorio FA, Pattnaik AK (2006): Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J Virol 80, 3994-4004

Bacchetta R, Gregori S, Roncarolo MG (2005): CD4+ regulatory T cells: mechanisms of induction and effector function. Autoimmun Rev 4, 491-496

Badaoui B, Rutigliano T, Anselmo A, Vanhee M, Nauwynck H, Giuffra E, Botti S (2014): RNA-sequence analysis of primary alveolar macrophages after in vitro infection with porcine reproductive and respiratory syndrome virus strains of differing virulence. PLoS ONE 9(3), e91918

Badaoui B, Tuggle CK, Hu Z, Reecy JM, Ait-Ali T, Anselmo A, Botti S (2013): Pig immune response to general stimulus and to porcine reproductive and respiratory syndrome virus infection: a meta-analysis approach. BMC Genomics 14, 1471-2164

Benjamini Y, Hochberg Y (1995): Controlling the false discovery rate: A typical adn powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 57, 289 - 300

Beutler B (2004): Innate immunity: an overview. Mol Immunol 40, 845-859

Boddicker N, Waide EH, Rowland RR, Lunney JK, Garrick DJ, Reecy JM, Dekkers JC (2012): Evidence for a major QTL associated with host response to porcine reproductive and respiratory syndrome virus challenge. J Anim Sci 90, 1733-1746

Boddicker NJ, Bjorkquist A, Rowland RR, Lunney JK, Reecy JM, Dekkers JC (2014a): Genome-wide association and genomic prediction for host response to porcine reproductive and respiratory syndrome virus infection. Genet Sel Evol 46, 1297-9686

Boddicker NJ, Garrick DJ, Rowland RR, Lunney JK, Reecy JM, Dekkers JC (2014b): Validation and further characterization of a major quantitative trait locus associated with host response to experimental infection with porcine reproductive and respiratory syndrome virus. Anim Genet 45, 48-58

Breuer K, Foroushani AK, Laird MR, Chen C, Sribnaia A, Lo R, Winsor GL, Hancock RE, Brinkman FS, Lynn DJ (2013): InnateDB: systems biology of innate immunity and beyond--recent updates and continuing curation. Nucleic Acids Res 41 (Database), D1228-D1233

Carvalho BS, Irizarry RA (2010): A framework for oligonucleotide microarray preprocessing. Bioinformatics 26, 2363-2367

Cho SH, Chen JA, Sayed F, Ward ME, Gao F, Nguyen TA, Krabbe G, Sohn PD, Lo I, Minami S, Devidze N, Zhou Y, Coppola G, Gan L (2015): SIRT1 deficiency in microglia contributes to cognitive decline in aging and neurodegeneration via epigenetic regulation of IL-1beta. J Neurosci 35, 807-818

Dwivedi V, Manickam C, Binjawadagi B, Linhares D, Murtaugh MP, Renukaradhya GJ (2012): Evaluation of immune responses to porcine reproductive and respiratory syndrome virus in pigs during early stage of infection under farm conditions. Virol J 9, 9-45

Fairbairn L, Kapetanovic R, Beraldi D, Sester DP, Tuggle CK, Archibald AL, Hume DA (2013): Comparative analysis of monocyte subsets in the pig. J Immunol 190, 6389-6396

Fairbairn L, Kapetanovic R, Sester DP, Hume DA (2011): The mononuclear phagocyte system of the pig as a model for understanding human innate immunity and disease. J Leukoc Biol 89, 855-871

Fang Y, Snijder EJ (2010): The PRRSV replicase: exploring the multifunctionality of an intriguing set of nonstructural proteins. Virus Res 154, 61-76

Feng WH, Tompkins MB, Xu JS, Zhang HX, McCaw MB (2003): Analysis of constitutive cytokine expression by pigs infected in-utero with porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 94, 35-45

Gao Y, Flori L, Lecardonnel J, Esquerre D, Hu ZL, Teillaud A, Lemonnier G, Lefevre F, Oswald IP, Rogel-Gaillard C (2010): Transcriptome analysis of porcine PBMCs after in vitro stimulation by LPS or PMA/ionomycin using an expression array targeting the pig immune response. BMC Genomics 11, 1471-2164

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004): Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5(10), R80

Hansen JD, Vojtech LN, Laing KJ (2011): Sensing disease and danger: a survey of vertebrate PRRs and their origins. Dev Comp Immunol 35, 886-897

Hoebe K, Janssen E, Beutler B (2004): The interface between innate and adaptive immunity. Nat Immunol 5, 971-974

Iwasaki A, Medzhitov R (2004): Toll-like receptor control of the adaptive immune responses. Nat Immunol 5, 987-995

Janeway CAJr, Travers P, Walport M, Shlomchik MJ (2001): Immunobiology: The Immune System in Health and Disease. 5th edition. Garland Science New York, pp

Jiang Z, Zhou X, Michal JJ, Wu XL, Zhang L, Zhang M, Ding B, Liu B, Manoranjan VS, Neill JD, Harhay GP, Kehrli ME, Jr., Miller LC (2013): Reactomes of porcine alveolar macrophages infected with porcine reproductive and respiratory syndrome virus. PLoS ONE 8(3), e59229

Johnson CR, Griggs TF, Gnanandarajah J, Murtaugh MP (2011): Novel structural protein in porcine reproductive and respiratory syndrome virus encoded by an alternative ORF5 present in all arteriviruses. J Gen Virol 92, 1107-1116

Kang SM, Lim S, Won SJ, Shin YJ, Lim YS, Ahn BY, Hwang SB (2011): c-Fos regulates hepatitis C virus propagation. FEBS Lett 585, 3236-3244

Kittawornrat A, Prickett J, Wang C, Olsen C, Irwin C, Panyasing Y, Ballagi A, Rice A, Main R, Johnson J, Rademacher C, Hoogland M, Rowland R, Zimmerman J (2012): Detection of Porcine reproductive and respiratory syndrome virus (PRRSV) antibodies in oral fluid specimens using a commercial PRRSV serum antibody enzyme-linked immunosorbent assay. J Vet Diagn Invest 24, 262-269

Koyama S, Ishii KJ, Coban C, Akira S (2008): Innate immune response to viral infection. Cytokine 43, 336-341

Liu G, Loraine AE, Shigeta R, Cline M, Cheng J, Valmeekam V, Sun S, Kulp D, Siani-Rose MA (2003): NetAffx: Affymetrix probesets and annotations. Nucleic Acids Res 31, 82-86

Loemba HD, Mounir S, Mardassi H, Archambault D, Dea S (1996): Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Arch Virol 141, 751-761

Lunney JK, Benfield DA, Rowland RR (2010): Porcine reproductive and respiratory syndrome virus: an update on an emerging and re-emerging viral disease of swine. Virus Res 154(1-2), 1-6

Lunney JK, Steibel JP, Reecy JM, Fritz E, Rothschild MF, Kerrigan M, Trible B, Rowland RR (2011): Probing genetic control of swine responses to PRRSV infection: current progress of the PRRS host genetics consortium. BMC Proc 3, 1753-6561

Mach N, Gao Y, Lemonnier G, Lecardonnel J, Oswald IP, Estelle J, Rogel-Gaillard C (2013): The peripheral blood transcriptome reflects variations in immunity traits in swine: towards the identification of biomarkers. BMC Genomics 14, 1471-2164

Martelli P, Gozio S, Ferrari L, Rosina S, De Angelis E, Quintavalla C, Bottarelli E, Borghetti P (2009): Efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: Clinical protection and cell-mediated immunity. Vaccine 27, 3788-3799

Miller LC, Neill JD, Harhay GP, Lager KM, Laegreid WW, Kehrli ME, Jr. (2010): In-depth global analysis of transcript abundance levels in porcine alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. Adv Virol 864181, doi: 10.1155/2010/864181

Onteru SK, Ross JW, Rothschild MF (2009): The role of gene discovery, QTL analyses and gene expression in reproductive traits in the pig. Soc Reprod Fertil Suppl 66, 87-102

Palmer C, Diehn M, Alizadeh AA, Brown PO (2006): Cell-type specific gene expression profiles of leukocytes in human peripheral blood. BMC Genomics 7, 115

Pancer Z, Cooper MD (2006): The evolution of adaptive immunity. Annu Rev Immunol 24, 497-518

Papareddy P, Morgelin M, Walse B, Schmidtchen A, Malmsten M (2012): Antimicrobial activity of peptides derived from human ss-amyloid precursor protein. J Pept Sci 18, 183-191

Pashine A, Valiante NM, Ulmer JB (2005): Targeting the innate immune response with improved vaccine adjuvants. Nat Med 11, S63-68

Patel D, Nan Y, Shen M, Ritthipichai K, Zhu X, Zhang YJ (2010): Porcine reproductive and respiratory syndrome virus inhibits type I interferon signaling by blocking STAT1/STAT2 nuclear translocation. J Virol 84, 11045-11055

Pfaffl MW (2001): A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9), e45

Pollock JM, Neill SD (2002): Mycobacterium bovis infection and tuberculosis in cattle. Vet J 163, 115-127

Rowland RR, Lunney J, Dekkers J (2012): Control of porcine reproductive and respiratory syndrome (PRRS) through genetic improvements in disease resistance and tolerance. Front Genet 3, doi: 10.3389/fgene.2012.00260

Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386

Schroyen M, Steibel JP, Koltes JE, Choi I, Raney NE, Eisley C, Fritz-Waters E, Reecy JM, Dekkers JC, Rowland RR, Lunney JK, Ernst CW, Tuggle CK (2015): Whole blood microarray analysis of pigs showing extreme phenotypes after a porcine reproductive and respiratory syndrome virus infection. BMC Genomics 16, 015-1741

Schroyen M, Tuggle CK (2015): Current transcriptomics in pig immunity research. Mamm Genome 26, 1-20

Serao NV, Matika O, Kemp RA, Harding JC, Bishop SC, Plastow GS, Dekkers JC (2014): Genetic analysis of reproductive traits and antibody response in a PRRS outbreak herd. J Anim Sci 92, 2905-2921

Seth RB, Sun L, Chen ZJ (2006): Antiviral innate immunity pathways. Cell Res 16, 141-147

Smyth GK (2005 pp.397-442): Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, editors. Bioinformatics and computational biology solutions using r and bioconductor. Springer pp. 397–342

Subramanian A, Kuehn H, Gould J, Tamayo P, Mesirov JP (2007): GSEA-P: a desktop application for Gene Set Enrichment Analysis. Bioinformatics 23, 3251-3253

Subramanian A, Tamayo P, Mootha VK, Mukherjee S, Ebert BL, Gillette MA, Paulovich A, Pomeroy SL, Golub TR, Lander ES, Mesirov JP (2005): Gene set enrichment analysis:

a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 102, 15545-15550

Sun Y, Han M, Kim C, Calvert JG, Yoo D (2012): Interplay between interferon-mediated innate immunity and porcine reproductive and respiratory syndrome virus. Viruses 4, 424-446

Tun-Kyi A, Finn G, Greenwood A, Nowak M, Lee TH, Asara JM, Tsokos GC, Fitzgerald K, Israel E, Li X, Exley M, Nicholson LK, Lu KP (2011): Essential role for the prolyl isomerase Pin1 in Toll-like receptor signaling and type I interferon-mediated immunity. Nat Immunol 12, 733-741

Uddin MJ, Cinar MU, Grosse-Brinkhaus C, Tesfaye D, Tholen E, Juengst H, Looft C, Wimmers K, Phatsara C, Schellander K (2011): Mapping quantitative trait loci for innate immune response in the pig. Int J Immunogenet 38, 121-131

Uddin MJ, Nuro-Gyina PK, Islam MA, Tesfaye D, Tholen E, Looft C, Schellander K, Cinar MU (2012): Expression dynamics of Toll-like receptors mRNA and cytokines in porcine peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. Vet Immunol Immunopathol 147, 211-222

Vestal DJ, Jeyaratnam JA (2011): The Guanylate-Binding Proteins: Emerging Insights into the Biochemical Properties and Functions of This Family of Large Interferon-Induced Guanosine Triphosphatase. J Interferon Cytokine Res 31(1), 89-97

Wang IM, Bett AJ, Cristescu R, Loboda A, ter Meulen J (2012): Transcriptional profiling of vaccine-induced immune responses in humans and non-human primates. Microb Biotechnol 5, 177-187

Xia J, Benner MJ, Hancock RE (2014): NetworkAnalyst--integrative approaches for protein-protein interaction network analysis and visual exploration. Nucleic Acids Res 42(Web Server issue), W167-74

Xiao S, Jia J, Mo D, Wang Q, Qin L, He Z, Zhao X, Huang Y, Li A, Yu J, Niu Y, Liu X, Chen Y (2010a): Understanding PRRSV infection in porcine lung based on genome-wide transcriptome response identified by deep sequencing. PLoS ONE 5(6), e11377

Xiao S, Mo D, Wang Q, Jia J, Qin L, Yu X, Niu Y, Zhao X, Liu X, Chen Y (2010b): Aberrant host immune response induced by highly virulent PRRSV identified by digital gene expression tag profiling. BMC Genomics 11, 1471-2164

Yoo D, Song C, Sun Y, Du Y, Kim O, Liu HC (2010): Modulation of host cell responses and evasion strategies for porcine reproductive and respiratory syndrome virus. Virus Res 154, 48-60

Zahoor MA, Philip S, Zhi H, Giam CZ (2014): NF-kappaB inhibition facilitates the establishment of cell lines that chronically produce human T-lymphotropic virus type 1 viral particles. J Virol 88, 3496-3504

ZDS (2003): Richtlinie Fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit Beim Schwein. Zentralverband der Deutschen Schweineproduktion eV, Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn.

Zhou P, Zhai S, Zhou X, Lin P, Jiang T, Hu X, Jiang Y, Wu B, Zhang Q, Xu X, Li JP, Liu B (2011): Molecular characterization of transcriptome-wide interactions between highly pathogenic porcine reproductive and respiratory syndrome virus and porcine alveolar macrophages in vivo. Int J Biol Sci 7, 947-959

Zhuge ZY, Zhu YH, Liu PQ, Yan XD, Yue Y, Weng XG, Zhang R, Wang JF (2012): Effects of Astragalus polysaccharide on immune responses of porcine PBMC stimulated with PRRSV or CSFV. PLoS ONE 7(1), e29320

Zuckermann FA, Garcia EA, Luque ID, Christopher-Hennings J, Doster A, Brito M, Osorio F (2007): Assessment of the efficacy of commercial porcine reproductive and respiratory syndrome virus (PRRSV) vaccines based on measurement of serologic response, frequency of gamma-IFN-producing cells and virological parameters of protection upon challenge. Vet Microbiol 123, 69-85

Chapter 3: Innate and adaptive immunity to PRRSV vaccine

(Manuscript submitted to PLoS ONE: under review)

PBMC transcriptome profiles identifies potential candidate genes and functional networks controlling the innate and the adaptive immune response to PRRSV vaccine in Pietrain pig

Md. Aminul Islam[1,2], Christine Große-Brinkhaus[1], Maren Julia Pröll[1], Muhammad Jasim Uddin[2,3], Sharmin Aqter Rony[1], Dawit Tesfaye[1], Ernst Tholen[1], Michael Hoelker[1,4], Karl Schellander[1,4], Christiane Neuhoff[1,*]

[1]Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany
[2]Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
[3]School of Veterinary Science, The University of Queensland, Gatton Campus, QLD 4343, Australia
[4]Teaching and Research Station on Frankenfrost, Faculty of Agriculture, University of Bonn, Konigswinter, Germany

*Corresponding author:
Christiane Neuhoff, PhD
Institute of Animal Science, University of Bonn
Endenicher Allee 15, 53115 Bonn, Germany
Email: cneu@itw.uni-bonn.de
Phone: 0049-228/73-3583
Fax: 0049-00228/73-2284

Article type and status: Research and new

Running title: Innate and adaptive immunity to PRRSV vaccine

Keywords: pig; PRRSV vaccine; PBMCs; innate immunity; adaptive immunity; microarray; gene; antibody

3.1 Abstract

The porcine reproductive and respiratory syndrome (PRRS) is a devastating viral disease affecting swine production, health and welfare throughout the world. A synergistic action of the innate and the adaptive immune system of the host is essential for mounting a durable protective immunity through vaccination. Therefore, the current study aimed to investigate the transcriptome profiles of peripheral blood mononuclear cells (PBMCs) to characterize the innate and the adaptive immune response to PRRS Virus (PRRSV) vaccination in Pietrain pigs. The Affymetrix gene chip porcine gene 1.0 ST array were used for the transcriptome profiling of PBMCs collected at immediately before (D0), at one (D1) and 28 days (D28) post PRRSV vaccination with three biological replications. With FDR <0.05 and log2 fold change ±1.5 as cutoff criteria, 295 and 116 transcripts were found to be differentially expressed in PBMCs during the stage of innate and adaptive response, respectively. The microarray expression results were technically validated by qRT-PCR. The gene ontology terms such as viral life cycle, regulation of lymphocyte activation, cytokine activity and inflammatory response were enriched during the innate immunity; cytolysis, T cell mediated cytotoxicity, immunoglobulin production were enriched during adaptive immunity to PRRSV vaccination. Significant enrichment of cytokine-cytokine receptor interaction, signaling by interleukins, signaling by the B cell receptor (BCR), viral mRNA translation, IFN-gamma pathway and AP-1 transcription factor network pathways were indicating the involvement of altered genes in the antiviral defense. Network analysis revealed that four network modules were functionally involved with the transcriptional network of innate immunity, and five modules were linked to adaptive immunity in PBMCs. The innate immune transcriptional network was found to be regulated by LCK, STAT3, ATP5B, UBB and RSP17. While TGFß1, IL7R, RAD21, SP1 and GZMB are likely predictive for the adaptive immune transcriptional response to PRRSV vaccine in PBMCs. Results of the current immunogenomics study advances our understanding of PRRS in term of host-vaccine interaction, and thereby contributes to design a rationale for disease control strategy.

3.2 Introduction

Porcine reproductive and respiratory syndrome virus (PRRSV) is the causative agent of an economically important swine disease, which is clinically characterized by reproductive failure in pregnant sows and respiratory disorder in young pigs (Collins et al. 1992). The PRRSV is a positive-sense, single-stranded RNA virus having two distinct genotypes namely European and North American. In swine, the common symptoms of PRRSV infection has

been characterized by prolonged viremia, a deficient induction of innate immunity along with weak and delayed development of neutralizing antibodies (Lopez and Osorio 2004, Meier et al. 2003) which are the major hurdle for control of porcine reproductive and respiratory syndrome (PRRS). Therefore, elucidating the main genomic factors involved in developing protective immune response to PRRSV vaccination is of utmost importance.

The modified live virus (MLV) based vaccination has commonly been practiced as one of the primary and economic tools for swine herd immunization against PRRS (Kimman et al. 2009). The MLV-PRRS vaccination can provide protection at least against reinfection with homologous PRRSV isolates and reduces the clinical outbreaks (Martelli et al. 2009). However, the molecular pathways and functional networks involved during the acquisition of immunity to PRRSV vaccination have not yet been entirely elucidated. It is conceivable that the mode of host response to vaccine antigen may differ in some extend from that of virulent infectious virus. The PRRSV infection has a predilection for the cells of mononuclear phagocyte lineages, like pulmonary alveolar macrophage and blood monocytes (Van Breedam et al. 2010). The virulent PRRSV infection causes depletion of immune cells through cytopathic replication preferably within the alveolar macrophage. While the attenuated virus strain used as vaccine is likely unable to cause cytopathic effects, it is able to sensitize the blood macrophage in the same way as virulent virus and induces immune response afterwards (Weesendorp et al. 2013, Zhuge et al. 2012). Moreover, the quality of immunity derived from natural PRRSV infection seemed not ideal for the implementation in the vaccine development programs (Kapur et al. 1996) that provoked the molecular characterization of host-vaccine interaction.

The host immune response to vaccination is comprised of a complex interplay between components of the innate and the adaptive immune system (Loving et al. 2015). Innate immunity is the initial body defense against invading pathogen, typically occurs within hours to few days of exposure through recognition of conserved epitopes followed by triggering a proinflammatory response (Beutler 2004). While the adaptive immunity represents the neutralizing antibody response usually developed at 2-4 weeks following antigenic stimulation in a pathogen-specific manner through generating the immunological memory (Pancer and Cooper 2006). Antibodies are essential vaccine induced immune effectors produced by B lymphocytes, and are capable of binding specifically to a pathogen or antigen. Other potential effectors are cytotoxic CD8+ T lymphocytes (CTLs) that may limit the spread

of infectious agents by recognizing and killing infected cells or secreting specific antiviral cytokines. The development and maintenance of both B and CD8+ T cell responses are supported by growth factors and signals produced by CD4+ T helper cells, which are commonly subdivided into T helper 1 (Th1) and T helper 2 (Th2). The live attenuated viral vaccine elicits the recruitment of antigen specific CD4+ Th cells in the circulation which lead to induce both higher affinity antibody and immune memory, known as T dependent antibody responses (Lockhart 2003). The balanced host immunocompetence with cell-mediated (Th1) and humoral (Th2) immune responses is a proposed selection goal for general disease resistance (Wilkie and Mallard 1999). Thus, identification of transcriptome signatures for the innate and the adaptive response to PRRSV vaccination might contribute to design a rationale husbandry and breeding scheme for sustainable PRRS control.

The host immune response to PRRSV has been studied through global transcriptome profiling mainly of lung tissue (Wysocki et al. 2012, Xiao et al. 2010a, Xiao et al. 2010b) and pulmonary alveolar macrophage (Ait-Ali et al. 2011, Genini et al. 2008, Zhou et al. 2011) with either in-vitro or in-vivo PRRSV infection, whereas reports on blood-based transcriptional response to vaccination are sparse. Considering the sampling convenience, time entailed, and animal welfare issues, the peripheral blood samples are much preferred to respiratory tissues/cells for evaluating the host immune responses to PRRSV vaccination. Moreover, unlike the sampling of pulmonary alveolar macrophage, repeated blood sampling is possible from the same individual during the course of immune responses, which is especially useful in controlling the baseline variation (Schroyen and Tuggle 2015). Furthermore, blood based genomic biomarkers can significantly advance the herd health management for PRRS by, for example, allowing the rapid and early prediction of host immunocompetence developed from vaccination (Rowland et al. 2012). Among the fractions of whole blood, the white blood cells transcriptome profile assume to reflect the transcriptomes of other porcine immune cells, likely what has been demonstrated in case of human (Kohane and Valtchinov 2012).

Peripheral blood mononuclear cells (PBMCs), a subset of while blood cells have been proved to be a suitable model for characterizing the host immune response to vaccines in human (reviewed by Wang et al. 2012). The porcine PBMCs have also been studied by some authors for the evaluation of immune response to PRRSV in pigs (Aasted et al. 2002, Martelli et al. 2007, Zhuge et al. 2012). However, those studies were mostly focused on expression profiling

of selected candidate genes using the in-vitro model. The current study employed an in-vivo PBMCs model to characterize the innate as well as the adaptive immune response to PRRSV vaccine through a global transcriptome approach. In a recent study, we observed that the highest transcriptional response of PRRSV vaccine during first three days occurred at 24 h after vaccination; we also observed a plateaued plasma antibody response to PRRSV vaccine at 28 days after primary vaccination (Islam et al. 2016). Therefore, we extended our aim herein to investigate the PBMCs transcriptome profiles at day 1, and day 28 post PRRSV vaccination to characterize functional networks associated with the innate and the adaptive immune response to PRRSV in Pietrain pig, respectively.

3.3 Materials and methods

3.3.1 Ethics statements

The research proposal was approved by the Veterinary and Food Inspection Office, Siegburg, Germany (ref. 39600305-547/15). The whole in-vivo experiment was conducted according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2003). The blood sampling protocol was approved by the State Agency for Nature, Environment and Consumer Protection, North Rhine-Westphalia, Germany (permission nr. 84-02.05.04.14.027).

3.3.2 Vaccination and blood sampling

Littermate piglets of two Pietrain sows were housed in the teaching and research station at Frankenforst, University of Bonn, Germany. Six clinically healthy female piglets from two sows, free from history respiratory diseases were included in this study. Piglets were immunized with the commercially available modified live PRRSV vaccine of European strain (Porcilis® PRRS, MSD Animal Health, Germany) through intramuscular injection of primary dose at day 28, and booster dose at day 56 of their age. About 7 mL anti-coagulated venous blood samples were collected from all pigs repeatedly at day 7, 28, 29, 42, 56 and day 70 of their age. All the blood samples were screened by ELISA for monitoring the PRRSV-specific antibody responses. However, for microarray study, the blood samples collected at just before (D0), and one day (D1) and 28 days (D28) relative to the primary vaccination were used with three biological replications.

3.3.3 Isolation of PBMCs and plasma

The PBMCs were isolated from the whole blood by the density gradient centrifugation with Ficoll-Paque (Histopaque®-1077; Sigma-Aldrich, Munich, Germany) according to the protocol described by Uddin et al. (2012). In brief, whole blood were diluted at the ratio of 1:1 with phosphate buffered saline (PBS) and carefully layered over 8 mL of Histopaque solution previously kept in a 50 mL conical tube. Then the tubes were centrifuged at 1250 ×g for 30 min at room temperature. After centrifugation, plasma was aspirated from the upper most layers and kept at -20 °C until used. PBMCs preparation was carefully aspirated and treated with RBC lysis buffer (Invitrogen, Darmstadt, Germany) to eliminate erythrocytes. Finally, PBMCs were washed twice with PBS and harvested as pellet.

3.3.4 Monitoring of plasma antibody response

To monitor the PRRSV-specific antibody titre, the plasma samples from all animals collected at day 7, 28, 42, 56 and 70 of age were screened by ELISA (PRRSV-AK screening, Synlab Vet GmbH, Augsburg, Germany) according to manufacturer's protocol. The optical density (OD) of each well was measured at 650 nm using the Bio-Rad 680 microplate reader. The presence or absence of PRRSV antibody was determined by calculating the sample to positive (S/P) ratio. The S/P ratio was calculated according to the equation described in our previous study (Islam et al. 2016). The samples were considered to be positive for PRRSV antibody if the S/P ratio was more than 0.4.

3.3.5 RNA extraction and microarray hybridization

Total RNA was extracted from PBMCs using the miRNeasy mini kit (P/N 217004, Qiagen, Hilden, Germany) according to the manufacturer's protocol along with on column DNase treatment (P/N 79254, Qiagen, Hilden, Germany). The RNA integrity was checked by micro capillary electrophoresis on an Agilent 2100 Bioanalyser with RNA 6000 Nanochip kit (Agilent Technologies, Waghausel-Wiesental, Germany). The total RNA from three individual piglets collected at D0, D1 and D28 time points were used for preparing the target probes for nine microarrays. About 100 ng of total RNA were processed to synthesize the biotin-labeled sense strand cDNA (ss-cDNA) probes using the GeneChip WT PLUS Reagent kit (P/N 902281; Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. About 130 µL of biotinylated ss-cDNA probes were injected into the GeneChip Porcine Gene 1.0 ST array of 81/4 format (P/N 901976, Affymetrix) and incubated for 16 hours in a hybridization oven (GeneChip Hybridization oven 640; Affymetrix) at 45 °C with

60 rpm for hybridization. The hybridized chips were stained and washed in a fluidics station (GeneChip Fluidics Station 450; Affymetrix) and scanned by Affymetrix GeneChip scanner 3000 7G. The image data were evaluated using Affymetrix Genechip command consol (AGCC) software and the intensity data were exported into .CEL file format. The MIAME (minimum information about microarray experiment) complaint raw data have been submitted into the gene expression omnibus (GEO) database with the accession code GSE84516.

3.3.6 Microarray data processing and statistical analysis

The normalization and background correction of microarray dataset were performed using the `oligo' Bioconductor package (Gentleman et al. 2004) implemented in R project software (v3.1.2). The RMA (Robust Multi-array Average) based quantile normalization (log2) of microarray data was performed at the transcript level (Carvalho and Irizarry 2010). Probe to gene transcript annotation was performed with recent Affymetrix annotation file (Liu et al. 2003). Gene annotations were extended by their orthologous human gene symbol as well as ensembl identifiers using the BioDBnet.org tool (http://biodbnet.abcc.ncifcrf.gov/). To explore the transcriptional modifications in response to vaccination, differential gene expression analysis was performed using the linear analysis of microarray technique from the `limma' package (Smyth 2005) with empirical Bayes adjustment to the variance, followed by Benjamini and Hochberg (BH) correction for multiple testing (Benjamini and Hochberg 1995). Two pairwise contrasts such as D1 vs. D0 and D28 vs. D0 were considered for differential gene expression associated with the innate and the adaptive immune response, respectively. The false discovery rate (FDR) of <0.05 and log2 fold-change either >1.5 or <-1.5 were considered as threshold for differential expression of genes.

3.3.7 Functional analysis of differentially expressed genes

For biological interpretation of the transcriptome dataset, the significantly over-represented gene ontology (GO) terms, biological pathways, and transcription factor binding sites (TFBS) were explored with the InnateDB pathway analysis tool (Breuer et al. 2013). InnateDB platform implements the hypergeometric algorithm with the Benjamini-Hochberg (BH) multiple test correction method for overrepresentation analysis. For this analysis, the list of ensembl gene identifiers was uploaded in InnateDB web and performed the over-representation analysis. GO, pathways and TFBS were considered significantly over-represented with an FDR <0.05.

3.3.8 Cell-type enrichment analysis of differentially expressed genes

To get an overview on which subtypes of PBMCs contribute in vaccine induced differential gene expression, DEGs were analyzed using the CTen web-portal. The CTen (cell type enrichment) is an online bioinformatics tool for identifying enriched cell types in heterogeneous microarray data (Shoemaker et al. 2012). This tool implements a highly expressed, cell specific (HECS) gene database comprises of 10,058 genes of human and mouse origin. For this analysis, human orthologs symbol of differentially expressed genes were uploaded and compared with human HECS database. The significance of enrichment was determined using the one-tailed Fisher exact test and P values were adjusted with Benjamini-Hochberg (BH) method across all cell types. The enrichment score estimated as -log10 of the BH-adjusted P value and created the color-coated output figures indicating this enrichment score.

3.3.9 Network analysis of differentially expressed genes

The ensembl orthologous identifiers of the differentially expressed genes were uploaded into the NetworkAnalayst tool (Xia et al. 2014) to construct the weighted network based on Walktrap algorithm by taking the first order interacts (direct interaction of seed genes). For high-performance visualization, the network size was adjusted for maximum of 500 nodes and 1200 edges using the `trim' function of the tool. Two topological measures such as degree (number of connections it has to other nodes) and betweeness centrality (number of shortest paths going through the node) were taken into account for detecting highly interconnected hub of the network. Nodes having higher degree and betweenness values are the potential network hubs regulating cellular signal trafficking. The weighted network based module detection was performed to stratify the interconnected genes of similar biological function. For statistical significance, p value of a given network module was calculated using a Wilcoxon rank-sum test of the "internal" (edges within in a module) and "external" (edges connecting the nodes of other modules) degrees. Modules having more internal than external edges were like to be significant. Finally, the in-situ functional enrichment of the modules was performed based on REACTOME.db pathway database.

3.3.10 qRT-PCR validation

The RNA samples as prepared for microarray analysis were also used for qPCR validation. Four selected differentially expressed genes known to be involved in immune response were quantified by qRT-PCR (Table 1). Primers were designed based on an open source primer

designing software Primer3 (Rozen and Skaletsky 2000). The reverse transcription was performed using First Strand cDNA Synthesis Kit (P/N K1612, Thermo Scientific, Co.). The qRT-PCR reaction was set up taking 1.0 µl of cDNA template, 8.0 µl of deionized RNase free water, 0.5 µl of upstream and downstream primers, and 10 µl iTaq. Universal SYBR® Green Supermix (Bio-Rad laboratories GmbH, Germany) in a total volume of 20 µl. All reactions were amplified in duplicate by the StepOnePlus. Real-Time PCR System (Applied Biosystems®, Darmstadt, Germany) with thermal cycling conditions of 95 °C for 3 min, 95 °C for 15 sec, 60 °C for 45 sec (40 cycles); 95 °C for 15 sec, 62 °C for 1 min, 95 °C for 15 sec. The delta delta Ct (ΔΔCt) [$\Delta Ct = Ct_{target} - Ct_{housekeeping\ genes}$] values were calculated as the difference between target gene and reference genes and expression was calculated as $2^{(-\Delta\Delta Ct)}$ (Pfaffl 2001). The correlations between qRT-PCR and microarray results were estimated with Pearson correlation test.

Table 1. Sequences of the primers used for qRT-PCR validation of microarray results.

Accession	Symbol	Sequence (5´ - 3´)	Ann. Temp. (°C)	Size (bp)
BNM 213948:1	IFNG	F : AGCTCCCAGAAACTGAACGA R : AGGGTTCAAAGCATGAATGG	60	225
NM 214015	TGFβ1	F : ACTACTACGCCAAGGAGGTCA R : TCTGCCCGAGAGAGCAATAC	60	157
NM 213997:1	IL8	F : TAGGACCAGAGCCAGGAAGA R : CAGTGGGGTCCACTCTCAAT	60	174
NM 214041:1	IL10	F : GTGGAGGAGGTGAAGAGTGCC R : GAGGTACAGCAGGGTTTCCCA	60	266
HQ013301	GAPDH*	F : GCTGGTGCTGAGTATGTCGT R : AAGCAGTTGGTGGTACAGG	56	124
XM 003124280: 3	ACTB*	F : AAGGACCTCTACGCCAACAC R : CTGGCTGATCCACATCTGCT	57	110

* reference gene; Ann. Temp.: Annealing temperature; bp: base pair; F: Forward; R: Reverse.

3.4 Results

3.4.1 Antibody response to PRRSV vaccine

The PRRSV-specific antibody titre in the blood plasma at day 7, 28, 42, 56 and day 70 of age was measured by ELISA to evaluate the antibody response derived from maternal origin and/ or from vaccination. It revealed that piglets had a negligible (P <0.01) level of maternally derived antibody at the time of primary vaccination considering an optical density (OD) value of 0.4 as threshold (Fig 1). The variation of antibody titres among the piglets at before vaccine priming was also significantly (P <0.05) low. The vaccine-specific antibody response found to be appeared (P = 0.0516) at 14 days (day 42 of age) post priming followed by a sharp increased titre at 28 days (day 56 of age) post priming. A significantly (P <0.05) high level of antibody response was observed over the period of four to six weeks of primary vaccination.

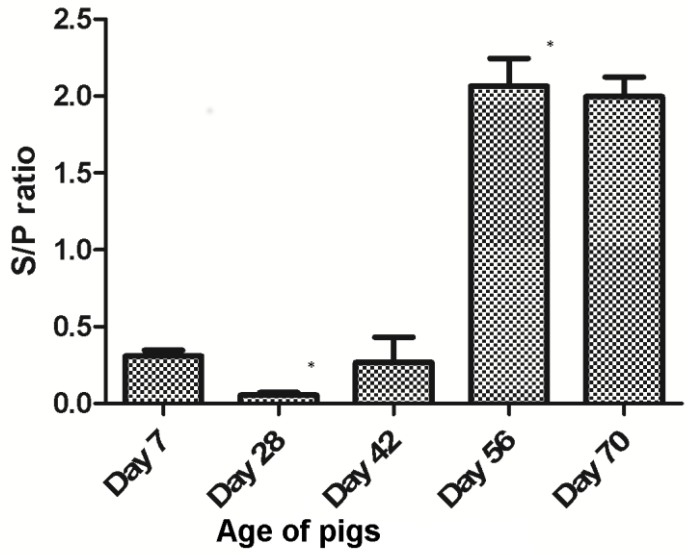

Figure 1. PRRSV specific antibody response in Pietrain pigs. The figure illustrates the reactivity of maternally derived antibody and vaccine derived antibody to PRRSV in plasma detected by ELISA. The optical density (OD) values in the Y-axis represents sample to positive (S/P) ratio, and a S/P value of 0.4 was considered as threshold for positivity of antibody response. Values in X-axis represents the piglet ages at which blood samples were evaluated. Blood sampling at day 28 and day 56 were performed right before the primary and the booster vaccination, respectively indicated by asterisk.

3.4.2 Transcriptome profiling of PBMCs following PRRSV vaccination

In order to investigate the host transcriptional response to PRRSV vaccine, we employed the Affymetrix GeneChip Porcine Gene 1.0 ST Array for the whole transcriptome profiling of PBMCs collected immediately before (D0), and at one (D1) and 28 days (D28) post

vaccination in Pietrain pigs with three biological replicates. First, the normalized gene probe sets were filtered to eliminate those with very low expression summary values and low variability across the samples. After filtering, the normalized expression data yielded a total of 14,212 gene transcripts to be expressed in PBMCs following vaccination. While the array chip used in this study were embedded with probe sets of 19,218 known genes in total.

3.4.3 PBMCs transcriptome alteration associated with innate and adaptive immunity to PRRSV vaccine

Transcripts were considered to be differentially expressed having the log fold change of >1.5 or <1.5 and false discovery rate (FDR) of <0.05. Imposing this cutoff, a total of 295 transcripts were found to be differentially expressed in PBMCs at day one post vaccination compared to control. The expression level of 65 genes including STAT3, LCK, UBB, VAV1, RSP17, SLC2A2, PTGES2 and MESP1 were upregulated and 230 genes including TGFβ1, RTF1, BIN2, TPST2, SNRK and PRKCQ were downregulated (Table 2). The range of log fold change of differentially expressed genes was between -4.461 and 3.46. The extend of fold change of most significantly altered genes (FDR sorted top ten up- and –down regulated) associated with innate immunity are presented in volcanoplot (Fig 2) and a complete list of differentially expressed genes is provided in S1 Table.

Table 2. Number of differentially expressed genes in PBMCs following PRRSV vaccination

Types	Number of genes	
	Innate immunity (D1 *vs.* D0)	Adaptive immunity (D28 *vs.* D0)
Up regulated	65	37
Down regulated	230	79
Total	295	116

At day 28 post vaccination, a total of 116 genes were identified as differentially expressed, with 37 being upregulated and 79 being down regulated under the same threshold as above (Table 2). The volcano plot (Fig 3) demonstrating the FDR sorted top ten altered genes indicated that CXCR2,IFNG, SMAD3, VNN1, F2R and GZMB genes were most significantly upregulated and IL10, MYL9, TPM2, GSTA4, CLU and TGFβ1 were down regulated in PBMCs following PRRSV vaccination, among the list of differentially expressed genes (DEGs). The range of log fold change of DEGs was between -2.51 and 4.50. A complete list

of the differentially expressed genes in PBMCs after 28 days of PRRSV vaccination is provided in S2 Table.

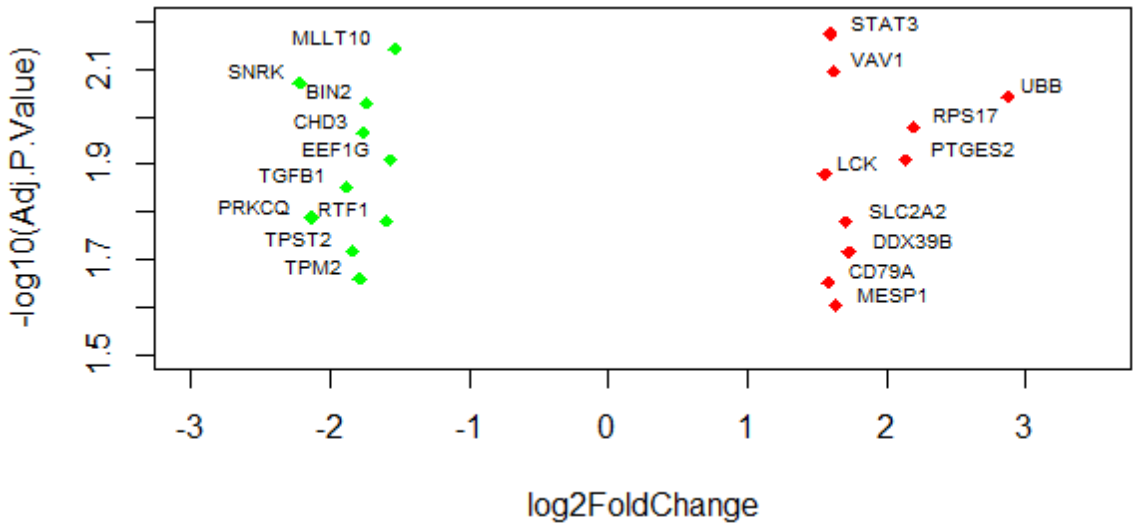

Figure 2. Volcano plot showing the most significantly altered genes at day one post vaccination. The picture demonstrates the range of fold changes of significantly altered transcripts in connection to innate immune response in PBMCs.

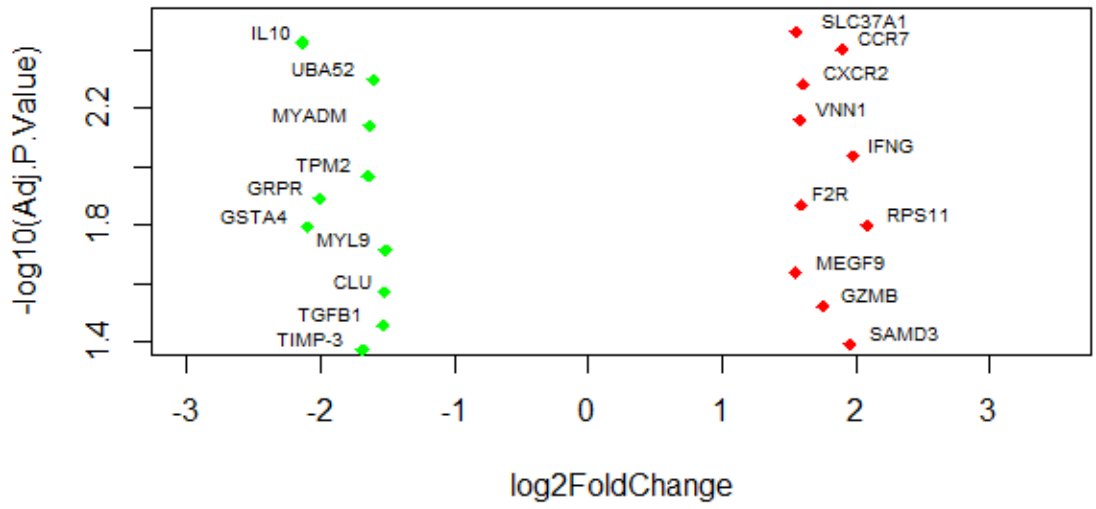

Figure 3. Volcano plot showing the most significantly altered genes at day 28 post vaccination. The picture demonstrates the range of fold changes of significantly altered transcripts in connection to adaptive immune response in PBMCs.

The hierchialcal heatmap demonstrated the visual summary of the dynamic changes in the transcriptional response to PRRSV vaccine at two time points reflecting a gradual upregulation of differentially expressed transcripts with two major clusters (Fig 4). Samples clustering revealed two superior clusters, one for pre vaccinated and another for post vaccinated samples. The replicates of each sampling time points are clustered together indicated a low individual variation on vaccine induced gene expression.

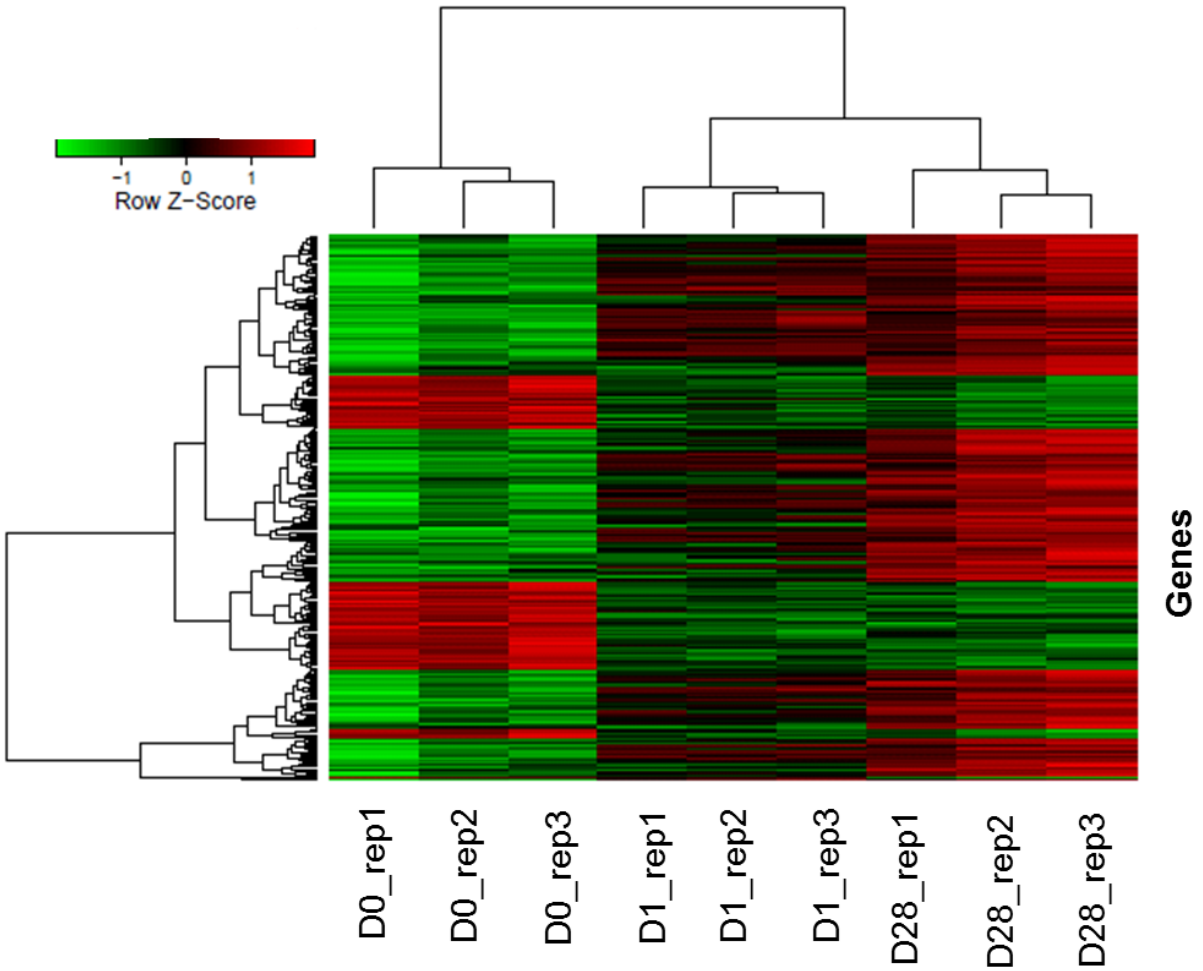

Figure 4. Hierarchical heat map showing the expression dynamics of DEGs. Normalized log2 transformed values as determined by Affymetrix GeneChip® porcine gene 1.0 ST array in PBMCs of Pietrain pigs collected at D0, D1 and D28 of PRRSV vaccination. Each column represents one pig, three replicates at each time point, each horizontal line refers to one gene. The cutoff value of log fold change as >1.5 or <-1.5 and false discovery rate <0.01 was considered.

3.4.4 GO terms and pathways enriched by differentially expressed genes (DEGs)

The Gene Ontology analysis revealed that vaccine induced differentially expressed genes are involved in the process of active cellular process (Table 3), including T cell response (eg, GO:0050852, GO:0051249,GO:00190058), cellular protein metabolism (eg, GO:0044267, GO:0030162, GO:0001948, GO:0005840), gene expression (eg, GO:0010467, GO:0006412, GO:0050852) and regulation of apoptosis (eg, GO:0043065) during the early stage of vaccine exposure. On the other hand, DEGs observed at 28 days post vaccination are involved with enrichment of GO terms including B cell proliferation (eg, GO:0042100) inflammatory response (eg, GO: 0006954), MHC class II biosynthesis (eg, GO:0045348), gene expression (eg, GO: 0010628), antigen processing and presentation (eg, GO:0019882). Overall, there were significantly altered transcripts participating in cellular activation and differentiation, protein metabolism, and gene expression (Table 3).

Pathways enrichment analysis revealed the involvement of several immune response pathways with PRRSV vaccine induced gene expression in PBMCs including signaling by B cell receptor, CD28 dependent VAV1 signaling, signaling by interleukins, influenza infection and TGFβ signaling pathways at one day after vaccination (Fig 5A). Signaling by NOTCH2, peptide-ligand binding receptor, Granzyme mediated apoptosis pathway, AP-1 transcription factor network and TGFβ signaling pathways were significantly enriched at 28 day post vaccination (Fig 5B).

3.4.5 Transcription factor binding sites of DEGs

We explored the involvement of transcription factors in the differential gene expression in vaccinated PBMCs using the InnateDB database. The transcription factor binding site (TFBS) analysis revealed that 120-kDa CRA-binding protein, E4F10, NF1, Tel-2a, HEB and NRF-2 genes have the transcription factor binding sites which are likely contributing to PBMCs transcriptome alteration at early stage of PRRSV vaccination (Fig 6A). The TFBS analysis also revealed that ONECUT1, SMAD1 and MYC have the transcription factor binding sites regulating transcriptional machinery for inducing adaptive immune response in PBMCs (Fig 6B). The PRRSV vaccine induced differentially expressed genes that are predicted to be regulated by the transcription factors are presented in Table 4.

Table 3. Significantly enriched gene ontology (GO) terms involved with DEGs

Contrasts	ID	Description	Catego -ry	Genes[a]	Adj. P
D1 vs. D0	GO:0019058	Viral life cycle	BP	6	0.0067
	GO:0006412	Translation	BP	7	0.0154
	GO:0051249	Regulation of lymphocyte activation	BP	6	0.0259
	GO:0010467	Gene expression	BP	19	0.0269
	GO:0070062	Extracellular vesicular exosome	CC	8	0.0273
	GO:0006200	ATP catabolic process	BP	11	0.0299
	GO:0050852	T cell receptor signaling pathway	BP	6	0.0301
	GO:0030162	Regulation of proteolysis	BP	13	0.0310
	GO:0005840	Ribosome	CC	17	0.0323
	GO:0022857	Transmembrane transporter activity	MF	12	0.0327
	GO:0045747	Positive regulation of Notch signaling pathway	BP	9	0.0336
	GO:0043065	Positive regulation of apoptotic process	BP	6	0.0339
	GO:0044267	Cellular protein metabolic process	BP	16	0.0343
	GO:0001948	Glycoprotein binding	MF	15	0.0343
D28 vs. D0	GO:0005515	Protein binding	MF	19	0.0332
	GO:0005886	Plasma membrane	CC	7	0.0317
	GO:0005576	Extracellular region	CC	8	0.0242
	GO:0006954	Inflammatory response	BP	6	0.0043
	GO:0008284	Positive regulation of cell proliferation	BP	7	0.0143
	GO:0010628	Positive regulation of gene expression	BP	14	0.0198
	GO:0006915	Apoptotic process	BP	6	0.0341
	GO:0042100	B cell proliferation	BP	5	0.0053
	GO:0009615	Response to virus	BP	5	0.0191
	GO:0005125	Cytokine activity	MF	6	0.0224
	GO:0043123	Positive regulation of NFkB signaling	BP	6	0.0240
	GO:0007166	Cell surface receptor signaling pathway	BP	9	0.0299
	GO:0045348	Positive regulation of MHC class II biosynthesis	BP	5	0.0068
	GO:0009611	Response to wounding	BP	6	0.0256
	GO:0019882	Antigen processing and presentation	BP	6	0.0261

a: Number of genes involved in corresponding GO terms, one gene may appear in multiple terms, BP: Biological process, CC: Cellular component, MF: Molecular function, Adj.P: P values adjusted for multiple test correction method

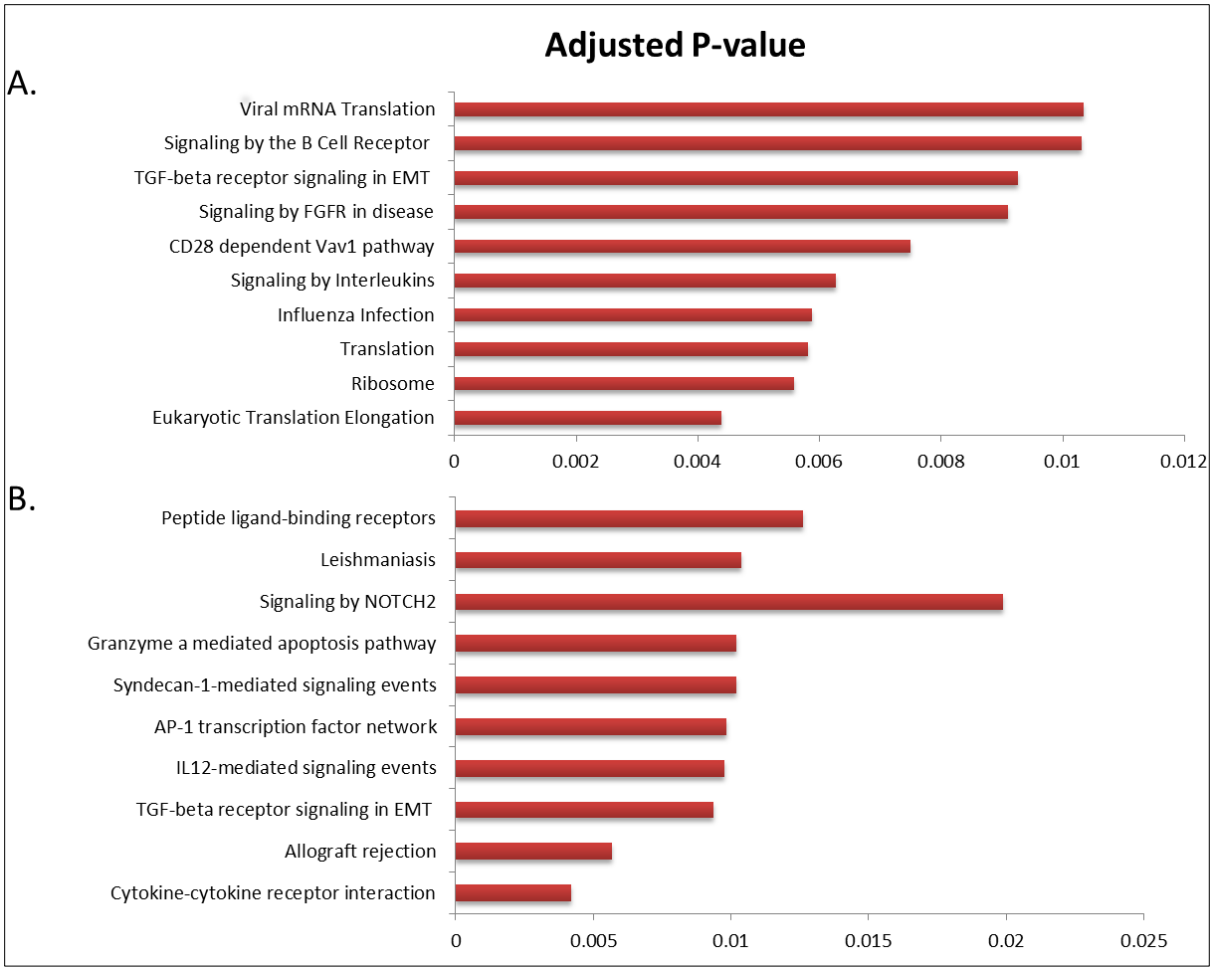

Figure 5. Significantly enriched pathways by DEGs. Significantly enriched pathways in PBMCs at 1 (A), and 28 (B) days post PRRSV vaccination in pig

3.4.6 Cell-type specific pattern of gene expression

To predict the specific cell-type contribution on vaccine induced differential genes expression in PBMCs, we tested the list of DEGs using an enrichment algorithm implemented in CTen web-portal. It revealed that differential expression of transcriptomes at early vaccine exposure was found to be significantly contributed by innate immune cell types including CD56+ NK cells, BDCA4+ dendritic cells, CD4+ T cells and CD8+ T cells (Fig 7A). While differentially expressed genes at 28 days post vaccination were of multiple cell-type origin including CD14+ monocytes, BDCA4+ dendritic cells, thymus, CD8+ T cells, CD4+ T cells, lymphnodes and whole blood (Fig 7B). Some cell types were mutually contributing to differential gene expression in both time points. NK cells were the enriched cell type associated with innate immunity but not with adaptive immunity in PBMCs. On the other hand, thymus, CD14+ and whole blood sample cell types were enriched in connection to adaptive immune response, but not during the stage of innate immunity to PRRSV vaccination in pig.

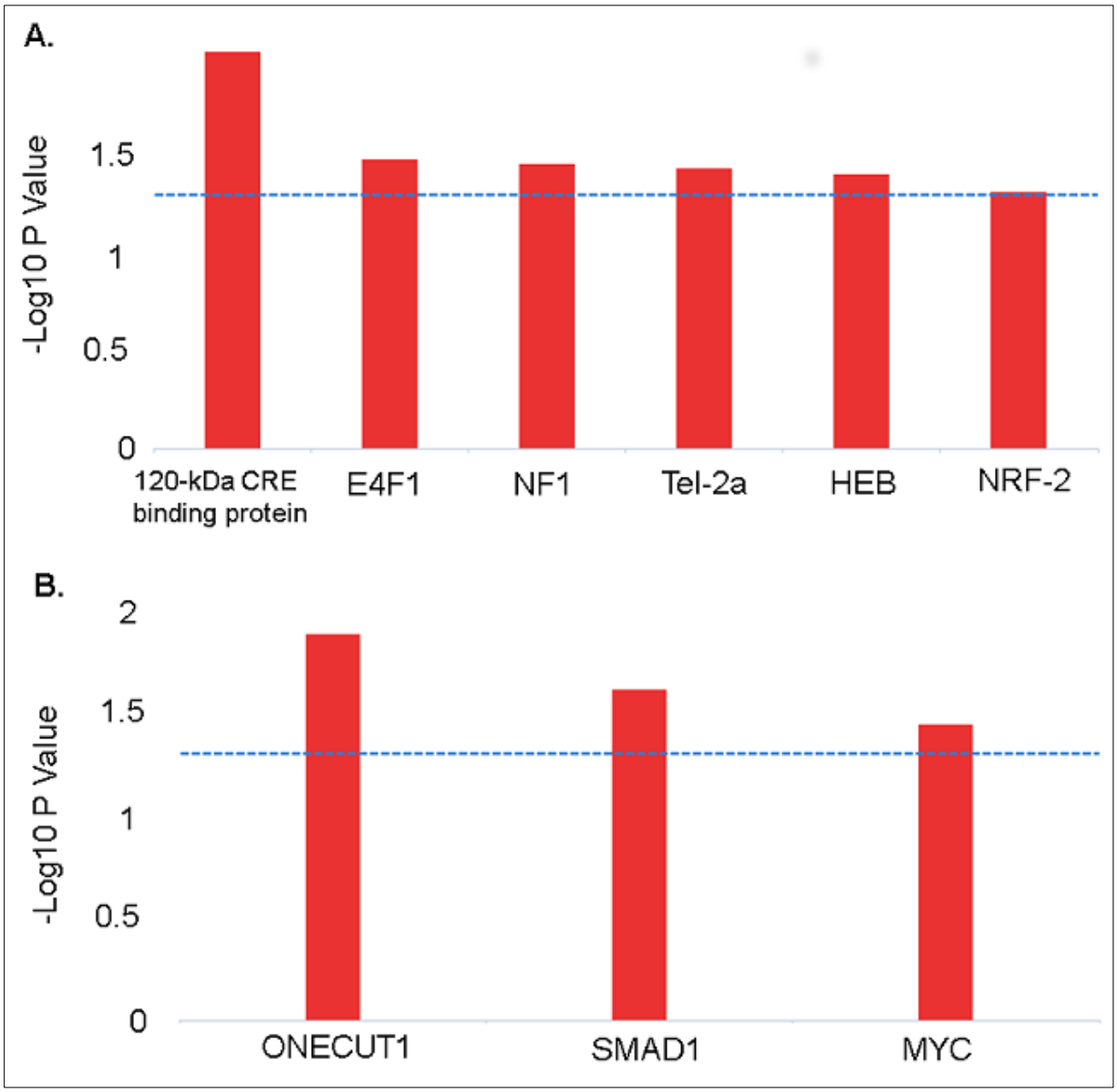

Figure 6. Transcription factors binding sites of DEGs. The figure depicts the TFBS of the genes showing differential expression in PBMCs at one day after vaccination (A), and 28 days after vaccination (B). Blue dotted lines indicate the threshold (-log10 P value of 1.3) for statistical significance.

Table 4. The known target genes bound by transcription factors identified in PBMCs after PRRSV vaccination in pigs

Transcription factors	Potential target genes	P-value
120-kDa CRE-binding protein	DDX39B, RAP2A, RPS11, SERPINC1, STAT3 and TRPC4AP	0.00928
E4F1	DUSP1, RAP2A, RPS11, STAT3 and UBL5	0.03250
NF-1	DAPL1, DDX39B, EN2, LCK, RAP2A, SERPINC1, STAT3, TRPC4AP, UBB and UQCRH	0.03421
Tel-2a	LCK, RPS11, TRPC4AP and UBL5	0.03611
HEB	DAPL1 and TRPC4AP	0.03872
NRF-2	LCK, RPS13, TRPC4AP and UBL5	0.04773
ONECUT1	ANGPT2, DGKA, F2R, GZMB and TGFB1	0.01281
SMAD1	GZMB, IL7R, RSAD2, SMAD3, SCL37A1 and VNN1	0.02389
MYC	ANGPT2, IFNG, PLAC8 and TGFB1	0.03546

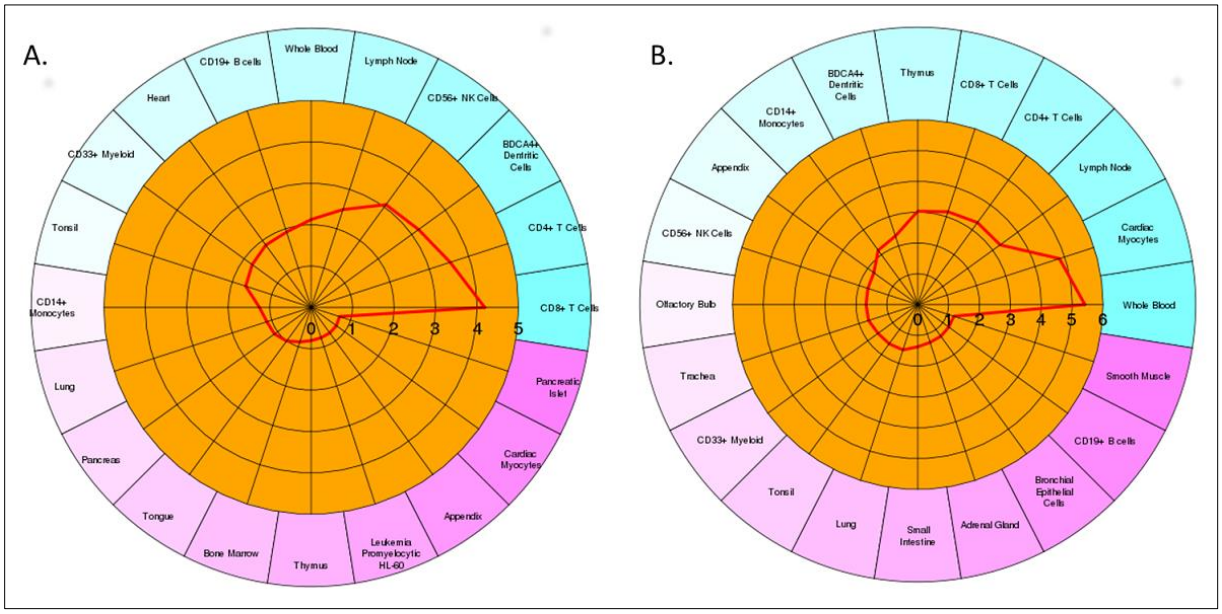

Figure 7. Circular plot showing the cell-type enrichment of DEGs. The figure depicts the cell-type specific enrichment of differentially expressed genes in PBMCs at one day after vaccination (A), and 28 days after vaccination (B). Red bold lines intersecting the cell types indicate the enrichment score (-log 10 adj. P-value) intersecting the cell type. The enrichment score cutoff of 2 or more was considered for statistical significance.

3.4.7 Functional network of innate immune transcripts

The simplified network of PRRSV vaccine induced innate immune transcripts in PBMCs is presented in (Fig 8). The network topology analysis showed that UBD, LCK, STAT3, ATP5B, RPS11, RPS13, RPS17 and EEF1G are the highly interconnected hubs of the network. The majority of the core genes of the network were overexpressed in PBMCs following PRRSV vaccination indicated the upregulation of their underlying function. The network module analysis revealed that differentially expressed genes were clustered in four modules (IM0, IM1, IM2 and IM3) indicated by four different colors (Fig 8).

The purple module (IM0) contains the genes (LCK, SKAP1, MyD88, MAPK14, VAV1, JAK2, SRC, CD79A, PTPRC, AMBP, DOCK8, PTK2B, SMAD3, CSNK2B, UBE3A, KHDRBS1) and is functionally linked to various innate immune response functions such as signaling by interleukins, cytokine signaling in immune system, CD28 co-stimulation, antigen activates B cell receptor leading to generation of second messengers, signaling by SCF-KIT, Fc gamma receptor (FCGR) dependent phagocytosis, interleukin-1 signaling, integrin cell surface interactions, innate immune system, signal transduction, TRAF6 mediated induction

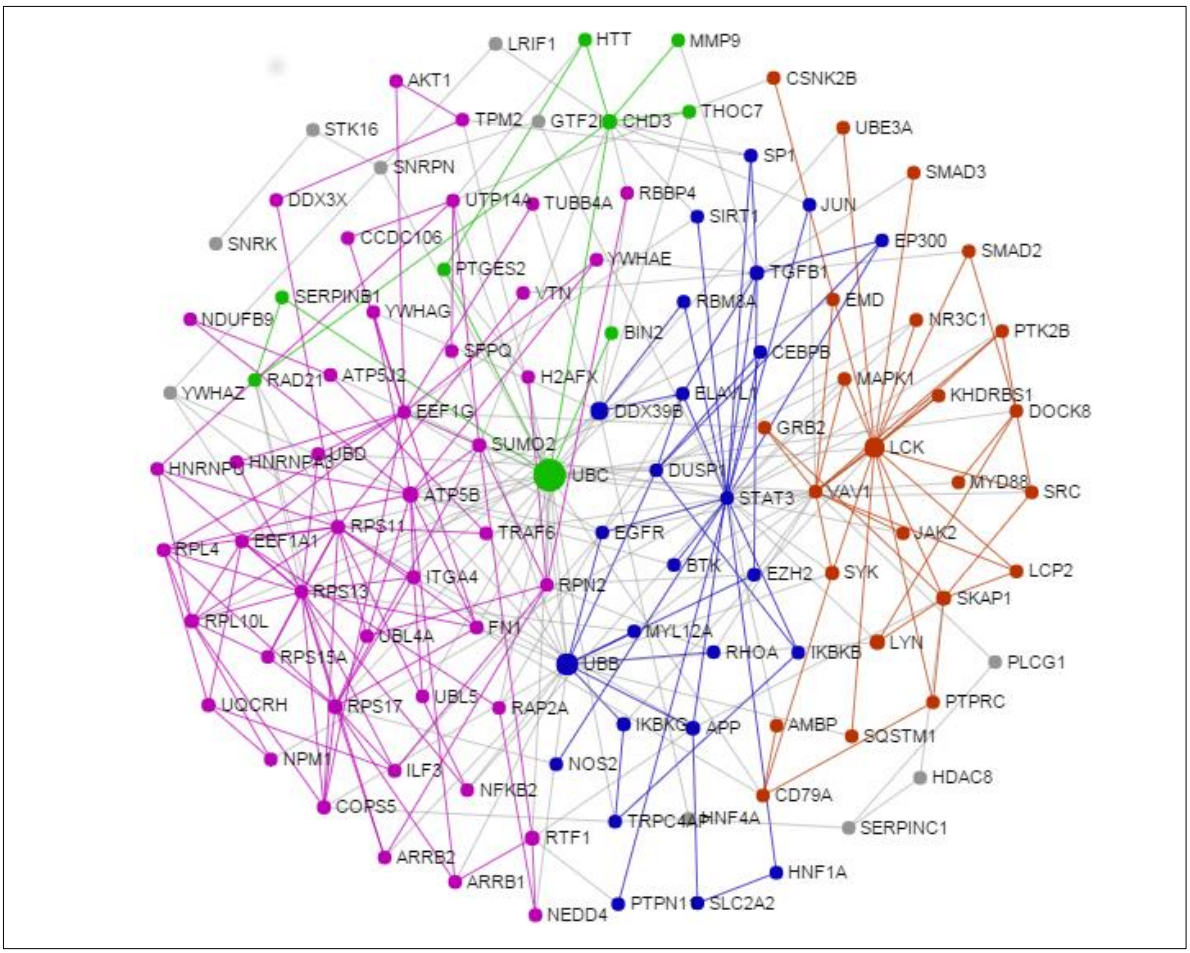

Figure 8. Network of PRRSV vaccine induced innate immune transcriptomes in PBMCs. The figure demonstrates the interconnected network of PRRSV vaccine induced differentially expressed genes in PBMCs at one day after PRRSV vaccination compared to before vaccination in Pietrain pigs. Each circle indicates the node or member genes of the network. The diameter of the circle corresponds to the values of two centrality measures (degree and betweenness). The larger diameter indicates the higher potential of the nodes to be the hub genes of the network. The network modules with corresponding genes are indicated by different colors (purple: IM0, blue: IM1, pink: IM2 and green: IM3).

of NFkB and MAP kinases upon TLR7/8 or 9 activation. The blue module (IM1) containing genes such as STAT3, TGFβ1, APP, DUSP1, ELAVL1DDX39B, MYL12A, EP300, IKBKB, IKBKG, RBM8A, SP1, EGFR, TRPC4A and CEBPB, involved in biological functions like MyD88: Mal cascade initiated on plasma membrane, toll-like receptors cascades, signaling by

interleukins, NFkB activation by phosphorylation and activation of IKKs complex, cytokine signaling in immune system, signaling by the B cell receptor (BCR) and activation of NFkB in B cells. The pink module (IM2) containing genes such ATP5B, RSP11, RSP13, RSP17, UTP14A, EEF1G, EEF1A1, SUMO2, ITGA4, TRAF6, FN1, RPN2, RPL4, RPL10L, COPS5, HNRNPU, HNRNPA3, UBL4A, UBD, UBL5, RTF1, ILF3 and NKKB2, was functionally involved in the process of translation, metabolism of proteins, intrinsic pathway for apoptosis, viral mRNA translation, membrane trafficking, cell cycle and apoptosis. The green module (IM3) containing genes like UBC, CHD3, HTT; RAD21 and PTGES2, was engaged in biological function like activation of matrix metalloproteinases, meiosis, chromosome maintenance and extracellular matrix organization.

3.4.8 Functional network of adaptive immune transcripts

The sub-network enrichment analysis of PRRSV vaccine induced adaptive immune transcripts in PBMCs (Fig 9) identified TGFβ1, IL7R, RAD21 and GZMB as highly interconnected genes, and are likely to be the potential hubs of the functional network. The purple module (AM0) containing genes ILR7, TGFβ1, SP1, IL-10, EP300, IFNG, EGR1, STAT3, TPM2, LEF1, IRF1 and are biologically linked to cytokine signaling in immune system, signaling by TGF-beta receptor complex, influenza virus induced apoptosis and signaling to STAT3. The blue module (AM1)containing the gene GZMA, GZMB, HIST2H2BE, XRCC6, XRCC6 and JUN, was linked to biological function of HIV infection, disease, DNA repair, integration of provirus and nucleosome assembly. The pink module (AM2) containing genes such RAD21, RPS11, ESR1, CLU and HNRNPU, was involved with biological process like cohesin loading onto chromatin, M phase and mitotic prometaphase. The green module (AM3) containing genes like UBC, CTSH, DGKA, STEAP4 and AMIGO, was engaged in biological function like glutathione conjugation, MHC class II antigen presentation and adaptive immune system. Yellow module (AM4) containing genes GPRASP1, UBA52, ARRB1, CXCR2 and YWHAG, was functionally involved with NF-kB activating and signal survival, assembly of HIV virion, STING mediated induction of type 1 IFN, signaling by NOTCH and apoptosis.

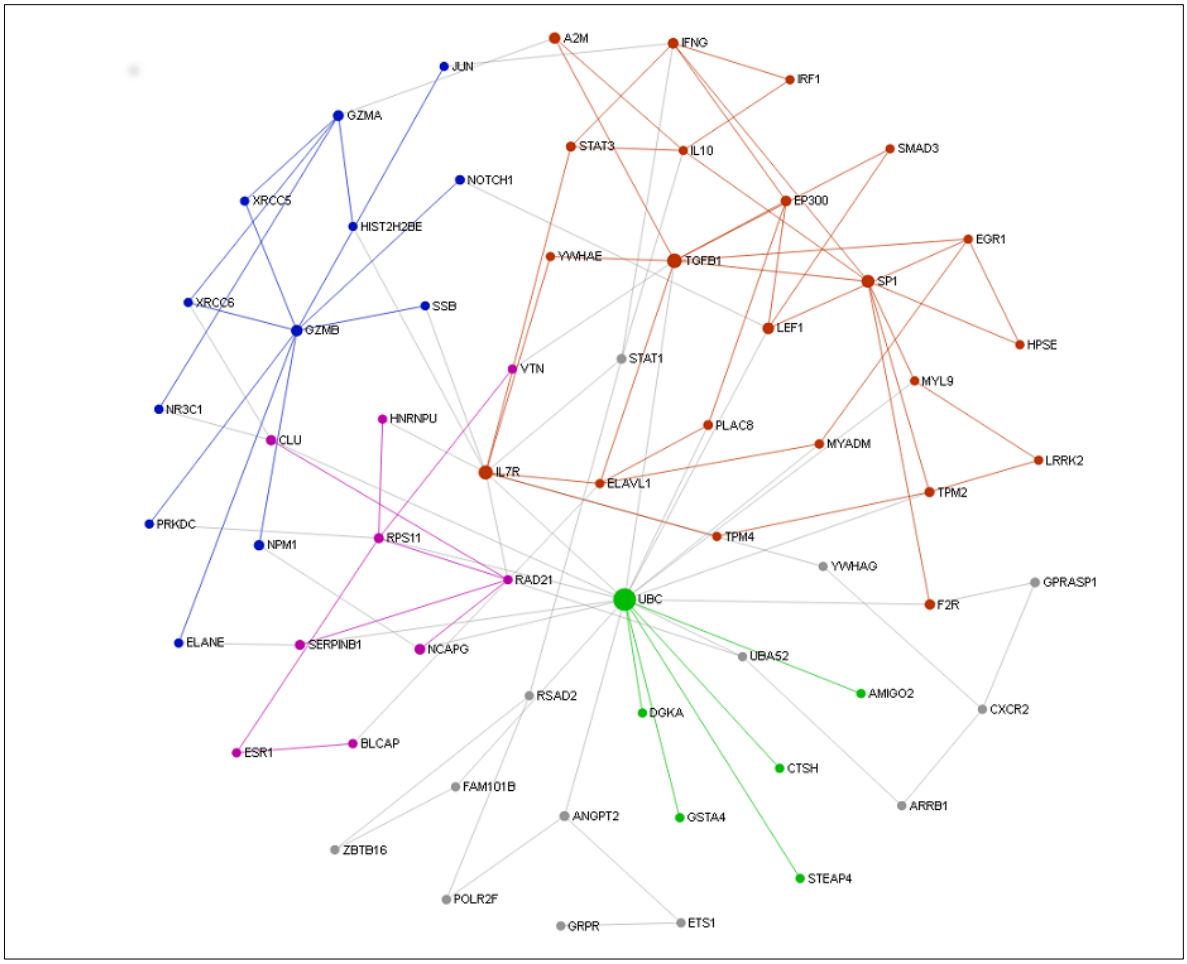

Figure 9. Network of PRRSV vaccine induced adaptive immune transcriptome in PBMCs. The picture depicts the interconnected network of PRRSV vaccine induced differentially expressed genes in PBMCs at 28 days after PRRSV vaccination compared to before vaccination in Pietrain pigs. Each circle indicates the node or member genes of the network. The diameter of the circle corresponds to the values of two centrality measures that is degree and betweenness of particular node. The larger diameter indicates the higher potential of the nodes to be the hub genes of the network. The network modules with corresponding genes are indicated by different colors (purple: AM0, blue: AM1, pink: AM2, green: AM3 and yellow: AM4).

3.4.9 The qRT-PCR validation

To validate the expression level of genes estimated by microarray, four differentially expressed genes such as IFNG, TGFβ1, IL-8 and IL-10 were selected for real time qPCR analysis. The expressions of all selected genes obtained from microarray and the qPCR are presented in Fig 10. The qPCR expression values of all genes were aligned with the microarray data with a high correlation (Pearson correlation coefficient, r = 0.929; *P* = 00154).

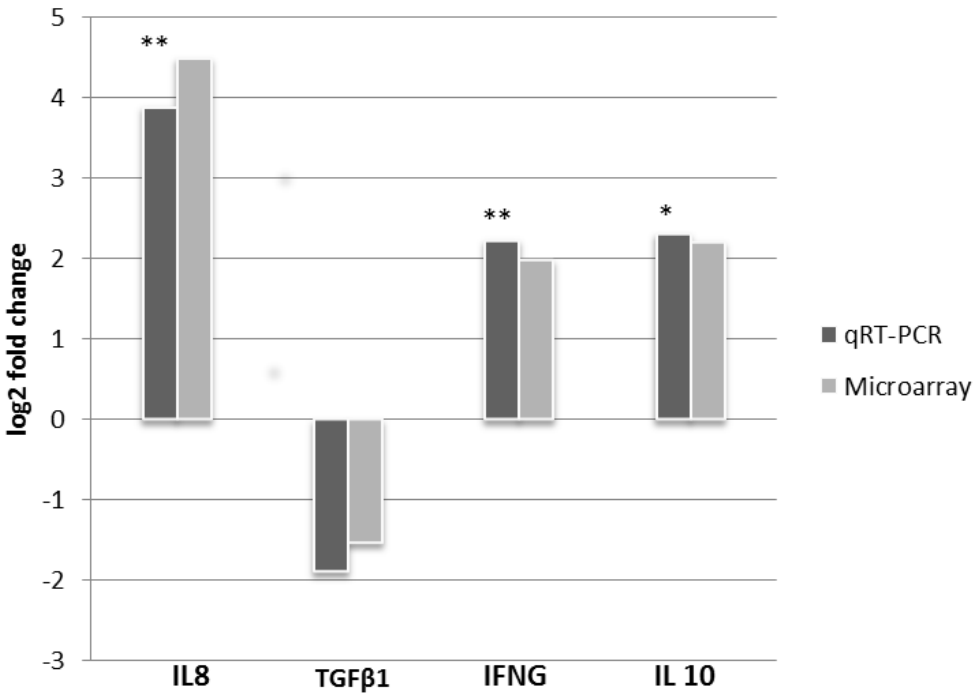

Figure 10. The qRT-PCR validation of the microarray data. The Y-axis of the bars indicates the log2 fold changes of each gene in PBMCs collected at one day after vaccination compared to control determined by qRT-PCR and microarray. Asterisk mark indicate the level of statistical significance (* <0.05; ** <0.01).

3.5 Discussion

Transcriptome profiling of PBMCs is receiving more interest in evaluating host immune response to infectious diseases, since PBMCs play central role in immune system. PBMCs are a heterogeneous population of blood cells that include monocytes, lymphocytes (T cells, B cells and NK cells) and dendritic cells. These blood cells patrol through entire body systems and immediately respond to both internal and external stimuli. Researches have shown that porcine PBMCs can display gene expression patterns which are characteristics for certain pathogenic infection, for instance, classical swine fever (Li et al. 2010a) and tetanus toxoid (Adler et al. 2013). In the current study, whole transcriptome profiling of PBMCs was performed in three individual piglets to characterize the gene expression changes associated with the innate as well as the adaptive immune response to PRRSV vaccine in Pietrain pigs. Though increasing the number of replicates in microarray experiment would leads more robust results, several groups like us have implemented three biological replications in global gene expression studies to characterize the host-PRRSV interaction (Xiao et al. 2010b, Zhou et al. 2011, Genini et al. 2008, Ait-Ali et al. 2011, Badaoui et al. 2013). We compared the global transcriptome profiles of PBMCs collected at day one (D1) and day 28 (D28) post PRRSV vaccination with that of collected before vaccination (D0) from the same pigs. The repeated sampling from the same individual allowed us to reduce the baseline individual

variation (P <0.01) indicated by antibody responses. In a similar design, transcriptome profiles of pre infected (0h) whole blood samples were compared with that of repeatedly collected post infected samples from the same individuals to characterize the immune response to PRRSV (Rowland et al. 2012).

The current study yielded a transcriptome dataset comprised of 411 differentially expressed genes in PBMCs after PRRSV vaccination. The robustness of this dataset was confirmed through measuring the expression levels of four selected differentially expressed genes in the same sample by qRT-PCR (Fig 10). Differential gene expression analysis showed that significant changes in PBMCs transcriptome profiles occurred at day one post PRRSV vaccination in Pietrain pig. The proportion of down regulated genes was higher than the upregulated one for both contrast pairs. The exact mechanism of this global down regulation is yet to be clarified; however, we speculated that this may be attributed by the host genetics. Because, host factors like age (Aasted et al. 2002) and breed (Ait-Ali et al. 2011) in particular, have strong influence on the development of immunity against PRRSV. An aberrant host immune response characterized by the consistent down regulated genes was reported in the PRRSV infected alveolar macrophages of pigs (Genini et al. 2008). In the same line, our recent RNA-seq analysis also revealed the global down regulation of altered transcripts in the PRRSV infected lung dendritic cells obtained from Pietrain pigs (Proll et al. 2016, unpublished). Surprisingly, the gene ontology and pathway analysis revealed a central role in the early vaccine response for genes those are involved in pro-inflammatory responses via cytokine-cytokine receptor signaling pathway, CD28 dependent VAV1 pathway and signaling by interleukins. Over expression of IL8 and CCR7 indicated that PRRSV vaccine is able to induce a proinflammatory response in PBMCs. The development of anti-viral innate immunity launches through sensing the viral protein or nucleic acid by the so-called specific receptor, the pathogen recognition receptors (PRRs), expressed constitutively in the host immune cells (Akira et al. 2006), which in turn induce the proinflammatory response (Thompson et al. 2011). After intramuscular vaccination, vaccine antigen can reach the blood circulation through bypassing the pulmonary alveolar macrophages, where the cytopathic replication takes place. Our results are in line with findings of a recent meta-analysis performed to characterize PRRSV specific immunity from published transcriptome studies (Badaoui et al. 2013). The meta-analysis showed that the differential expression of a cell surface receptor involved in cytokine regulation, TREM1, along with inflammatory responses toll-like receptor genes TLR2, TLR4, cytokines including IL-1b, IL6 and IL18 and chemokine including CCL2 and CCL3 were involved with PRRSV specific host responses (Akira et al.

2006). RNA-seq analyses of transcriptome profiles of PRRSV infected porcine tracheobronchial lymphnodes (Miller et al. 2012) and lung tissue (Xiao et al. 2010a) also revealed that PRRSV induces proinflammatory response.

The interferon response is a well-known innate immune reaction developed upon virus infection or vaccination. We observed an overexpression of IFNG at 28 days post vaccination but not at 1 day post vaccination, which signals a delayed induction of innate anti-viral immunity to PRRSV in PBMCs. This finding is in line with previous studies, where several microarray experiments reported a dampened expression of type I IFN response during PRRSV infection indicating an inadequate stimulation of the innate anti-viral immune response (Xiao et al. 2010a, Ait-Ali et al. 2011, Garcia-Nicolas et al. 2014). Similarly, a gradual development of the interferon-gamma response of swine to PRRS virus infection or vaccination has been reported by Meier et al (2003). In contrast, Genini et al (2008) observed a strong elevation of IFNα at 9 h post infection but a slightly elevated expression of IFNα in alveolar macrophage infected with PRRSV. However, Zhang and colleagues stated that PRRSV does not fail to induce IFNα or IFNβ mRNA expression in monocyte derived dendritic cells, but protein seems to be blocked post-transcriptionally (Zhang et al. 2012) which demands the investigation of potential role of post transcriptional regulators like miRNAs in PRRSV induced IFN responses.

Transcription factors (TFs) are regulators of gene expression. In mammalian genome, genes are usually in a default ‘off’ state and TFs serve mainly to turn gene expression ‘on’ through recognizing specific cis-regulatory DNA sequences at the promoter regions of target genes (Niu et al. 2011). The current analysis revealed the involvement of transcription factors including 120-kDa CRA-binding protein, E4F1, NF1, Tel-2a, HEB and NRF-2 with PRRSV vaccine mediated innate immunity; and ONECTU1, SMAD1 and MYC with adaptive immunity in PBMCs (Fig 6). A total of 27 differentially expressed genes were under regulation of these seven transcription factors identified in PBMCs of PRRSV vaccinated pigs (Table 4), many of altered genes have already been linked to host-PRRSV interaction (Xiao et al. 2010b, Zhou et al. 2011, Genini et al. 2008). Among the TFs identified in this study, MYC has been previously reported to be involved with the swine host response to PRRSV infection (Badaoui et al. 2013). We identified ANGPT2, IFNG, PLAC8 and TGFB1 as potential target genes of MYC transcription factor. The MYC regulates the expression of two immune checkpoint proteins on the tumor cell surface, the innate immune regulator, CD47 (Cluster of

Differentiation 47) and the adaptive immune checkpoint, PD-L1 (programmed death-ligand 1); (Casey et al. 2016), thereby initiates and maintains the tumorigenesis. The involvement of some other transcription factors like interferon regulatory factors (IRF1, IRF3, IRF5 and IRF8), HMGB1, NFkB, EGR1, BCL3, PYCARD MYCN and NFE2L2 in the transcriptional mechanism of immune response to PRRSV in pig has been identified through a meta-analysis (Badaoui et al. 2013). The actions of transcription factors regulate the unique expression of each gene in the different cell types during development process.

The cellular sub-population of PBMCs may have individual roles on development of vaccine immunity. The cell type enrichment analysis revealed that differentially expressed genes specifically expressed in CD4+ T cells, CD8+ T cells, CD14+ and CD33+ monocytes during early stage; and lymphnode, thymus, BDCA4+ dendritic cells, CD4+ T cells and CD8+ T cells in later stage of vaccine immunity (Fig 7). This could indicate that the expression patterns of the genes were not solely due to transcriptional changes but possibly also due to a difference in demographics of PBMCs subsets recruited into the blood. Shimizu et al. observed a remarkable decrease in CD4+ T cells after 3 days PRRSV infection in pigs (Shimizu et al. 1996); this study also reported slight decreases in CD8+ T cells at 3 dpi, followed by substantially increased levels (Shimizu et al. 1996), while at the same time, the ratios of CD4+/CD8+ T cells were significantly lower between day 3 and 28 post-inoculation compared with that of day 0 (Shimizu et al. 1996). However, the proportion of CD4+ and CD8+ T cells were found to be significantly decreased for a few days shortly after PRRSV infection, but returned to pre-infection levels on 8-10 days post infection (Nielsen and Botner 1997). Renukaradhya et al (2010) performed a comprehensive analysis of innate and adaptive immune responses in dual-virus infected pigs and reported that reduced innate NK-cells population along with increased frequencies of CD4+ T cell, CD8+ T cells and myeloid cells resulted from PRRSV infection in pigs. The PRRSV infection is reported to causes an increase in CD14+ expression throughout the early stage of infection, due to a rise in CD14+ monocytes that differentiate to macrophages and migrate to bronchoalveolar spaces (Van Gucht et al. 2004). Silva-Campa et al (2012) observed that PRRSV infection increases the frequency of T cell regulatory cells (Tregs) with the phenotype CD4+, CD8+, CD25+ and Foxp3high. Therefore, this information on cell-type specific contribution to vaccine immunity could be an important add-on for PRRS research.

Innate immune response traits like other quantitative traits are not regulated by straightforward linear pathways but rather by networks of complex molecular interactions (Gardy et al. 2009). Thereby network analyses based on larger immune-specific gene database (Shoemaker et al. 2012) proved to be a more effective strategy for the identification of genes that regulate the immune response to PRRSV vaccine in PBMCs. Among the hub genes of the network, the lymphocyte-specific protein tyrosine kinase (LCK) gene was found to be one of the potential hubs of functional network (Fig 8). LCK encodes p56 (LCK), a non receptor protein-tyrosine kinase of the SRC oncogene family that is involved in transduction of T-cell receptor (TCR)-mediated activation of T-cell. The signal transduction cascades are activated following antigen binding to the TCR, and in concert with engagement of other co-receptors and their associated ligands (such as CD4 and major histocompatibility complex (MHC) class II, CD28, B7, CD8, and MHC I); (Goldman et al. 1998). Functional enrichment of the network module revealed that the innate immune transcripts are clustered in four modules participating in four major groups of biological functions. The ubiquitination was found to be a key cellular processes significantly upregulated with the transcriptome alteration in PBMCs at early after PRRSV vaccination. We observed the over expression of ubiquitin gene family such as UBC, UBB, UBD, UBL5, UBL4A, and UBE3A in PBMCs after vaccination. Moreover, UBC was found to be a potential hub of the functional network of PRRSV vaccine induced innate immune transcriptomes in PBMCs. The ubiquitination is a post-translational modification process that has been implicated in the regulation of a wide variety of cellular process. The genetic and biochemical evidence suggest that protein ubiquitination and deubiquitination are of fundamental importance in the regulation of the innate and adaptive immune system (Sun 2008). The over-expression of porcine ubiquitin specific protease 18 (USP18) is reported to reduce the in-vitro PRRSV replication by altering the cellular distribution of two subunits of NFkB heterodimers (p56 and p50); (Xu et al. 2012) which indicates the role of USP18 as a host restriction factor during innate immune response to PRRSV. In a subsequent study, the SNP G-1533A polymorphism in the promoter region of the porcine USP18 gene has been suggested as a potential DNA marker for the resistance to PRRSV (Li et al. 2014b). Therefore, the ubiquitination process might influence the transcriptional network of PRRSV vaccine induced innate immune response in PBMCs.

The adaptive immunity is specific to the pathogen and the components of the adaptive immune system are also likely contributing to PRRS resistance in pigs. The sub-network analysis of the current microarray study showed the evidence of adaptive B and T cell immunity to PRRSV vaccine in PBMCs. Though adaptive immunity is likely to be

predominated by B cell function, however, T lypmhocytes in parallel also have significant contribution in adaptive immunity (Voskoboinik et al. 2006). The conjugation of viral antigen to a protein carrier (adjuvant) provides foreign peptide antigens that are presented to the immune system and thus recruit antigen-specific CD4+ Th cells which are referred to as T dependent antibody responses (Lockhart 2003). A hallmark of T-dependent responses of live attenuated viral vaccines is to induce both higher-affinity antibodies and immune memory along with generation of CD8+ cytotoxic T cells. Down regulation of immunosuppresive cytokine TGFβ1, and upregulation of interferon IFNG and chemokine CXCR2, VAV1, SMAD3, GYMA, GYM5 and transcription factor STAT1 were found to be among regulators of the transcriptional network of vaccine induced adaptive immunity. This is consistent with the hypothesis that there are possibilities for association of PRRS resistance genes with the cells of adaptive immunity, namely the T and B cells (Glass 2012). Major biological pathways involved were TGF-beta receptor signaling pathways, AP-1 transcription factor network, granzyme mediated apoptosis, NOTCH2 signaling and IL-12 mediated signaling. The perforin-mediated apoptosis is principally regulated by IL-10 secreted from cytotoxic T lymphocytes (CTLs); (Voskoboinik et al. 2006). Type 1 PRRSV strains have been reported to induce IL-10 production in infected dendritic cells (Silva-Campa et al. 2010).

Induction of neutralizing antibody (NAb) response is a potential indicator for the vaccine-based adaptive immunity. However, the specificity as well as the level of NAb titre may vary and are likely attributed to establishment of protective immunity. Previous studies suggested that a higher level of PRRSV-specific NAb titre (1:8 to 1:32) in blood is required to prevent the subsequent infection (Trus et al. 2014). In this study, PRRSV vaccine induced neutralizing antibody titre (S/P ratio) rose around 1:12 at 28 days post vaccination and remain elevated over 42 days post vaccination (Fig 1). This was supported by the findings of Meier et al. 2003 and Yoon et al. 1996, who reported that serum antibodies with PRRSV-neutralizing activity appear only at periods equal or higher than 28 days post infection. The timing of peak response may vary with type of antibodies, for instance the PRRSV-specific IgM could be detected at 7 days post infection (PI), with titre peaking between 14 and 21 day PI and decreasing to undetectable levels around 40 days PI (Loemba et al. 1996). The earliest antibodies detected that are directed against the 15kDa N protein which seems to be unable to provide sufficient protection (Yoon et al. 1996). However, there is a positive correlation between the level of vaccine-induced serum NAb titre and the level of protection against PRRSV infection (Li et al. 2014a). Overall, the anamnestic induction of plasma antibody

response at day 28 post vaccination was suggestive for the development of adaptive immunity to PRRSV vaccine in the studied piglets. Therefore, it would imply that the gene expression changes in PBMCs at 28 days post vaccination may reflect the transcriptional activity associated with adaptive immune response to PRRSV vaccination in Pietrain pigs.

3.6 Conclusions

This study support a model in which PBMCs transcriptome alterations are involved in upregulation of CD28 dependent VAV1 pathway, signaling by interleukins and ubiquitination pathway at the initial 24 hours after vaccination; and upregulation of IL12-mediated signaling events, AP-1 transcription factor network and TGF-beta receptor signaling pathways at 28 days after PRRSV vaccination in pigs. Network analysis sorted out the potential regulatory genes involved with induction of innate immune response and subsequently contributes to the development of adaptive immune response in PBMCs to PRRSV vaccination. Among the vaccine induced genes, LCK, STAT3, ATP5B, UBB and RSP17 were found to be the potential candidates for innate immune responses to PRRSV vaccine in peripheral blood. Further work is required to determine whether polymorphisms linked to genes identified in this study affect the innate immune response trait in pig populations immunized with PRRSV vaccine.

At 28 days post PRRSV vaccination, a plateaued antibody response was observed in plasma, at the same time, significant transcripts abundance was identified by microarray analysis in PBMCs. Among the differentially expressed genes, TGFβ1, IL7R, RAD21, SP1 and GZMB were highly interconnected hub genes of functional network, thereby likely to be the potential candidates to predict the PRRSV vaccine induced adaptive immune response in blood. The degree of association between the antibody response and the transcriptome alteration induced by PRRSV vaccine could further be tested through expression of these adaptive response candidates in the PBMCs of pigs with extreme antibody response phenotype in a larger population.

3.7 Declarations

3.7.1 Acknowledgments

The authors are thankful to the staffs of the Teaching and Research Station on Frankenfrost, University of Bonn for the animal husbandry support.

3.7.2 Funding

This work was financially supported by the State of North Rhine Westphalia of Germany with the programme “Umweltverträgliche und Standortgerechte Landwirtschaft (USL)” with the grant nr. 17-02.04.01-4/15. The funders had no role in study design, data collection and analysis, decision to publish, or presentation of the manuscript.

3.7.3 Author’s contribution

Conceptualization: MAI, MJU, CN, KS; Methodology: MAI, SAR, MP; Software: MAI, CGB; Validation: MAI, SAR; Formal analysis: MAI; Investigation: MAI; Resources: CN, DT, MH; Data curation: MAI; Writing original draft: MAI; Writing review & editing: CN, DT, MJU, CGB, DT, ET, KS; Visualization: MAI; Supervision: CN, KS; Project administration: KS; Funding acquisition: KS.

3.7.4 Competing interests

The author(s) declare that they have no competing interests.

3.7.5 Supporting Information

Table S1. DEGs associated with innate immunity. List of differentially expressed genes in porcine PBMCs associated with innate response to PRRSV vaccine

Table S2. DEGs associated with adaptive immunity. List of differentially expressed genes in porcine PBMCs associated with adaptive response to PRRSV vaccine

3.8 References

Aasted B, Bach P, Nielsen J, Lind P (2002): Cytokine profiles in peripheral blood mononuclear cells and lymph node cells from piglets infected in utero with porcine reproductive and respiratory syndrome virus. Clin Diagn Lab Immunol 9, 1229-1234

Adler M, Murani E, Brunner R, Ponsuksili S, Wimmers K (2013): Transcriptomic response of porcine PBMCs to vaccination with tetanus toxoid as a model antigen. PLoS ONE 8(3), e58306

Ait-Ali T, Wilson AD, Carre W, Westcott DG, Frossard JP, Mellencamp MA, Mouzaki D, Matika O, Waddington D, Drew TW, Bishop SC, Archibald AL (2011): Host inhibits replication of European porcine reproductive and respiratory syndrome virus in macrophages by altering differential regulation of type-I interferon transcriptional response. Immunogenetics 63, 437-448

Akira S, Uematsu S, Takeuchi O (2006): Pathogen recognition and innate immunity. Cell 124, 783-801

Badaoui B, Tuggle CK, Hu Z, Reecy JM, Ait-Ali T, Anselmo A, Botti S (2013): Pig immune response to general stimulus and to porcine reproductive and respiratory syndrome virus infection: a meta-analysis approach. BMC Genomics 14, 1471-2164

Benjamini Y, Hochberg Y (1995): Controlling the false discovery rate: A typical adn powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 57, 289 - 300

Beutler B (2004): Innate immunity: an overview. Mol Immunol 40, 845-859

Breuer K, Foroushani AK, Laird MR, Chen C, Sribnaia A, Lo R, Winsor GL, Hancock RE, Brinkman FS, Lynn DJ (2013): InnateDB: systems biology of innate immunity and beyond--recent updates and continuing curation. Nucleic Acids Res 41 (Database), D1228-D1233

Carvalho BS, Irizarry RA (2010): A framework for oligonucleotide microarray preprocessing. Bioinformatics 26, 2363-2367

Casey SC, Tong L, Li Y, Do R, Walz S, Fitzgerald KN, Gouw AM, Baylot V, Gutgemann I, Eilers M, Felsher DW (2016): MYC regulates the antitumor immune response through CD47 and PD-L1. Science 352(6282), 227-231

Collins JE, Benfield DA, Christianson WT, Harris L, Hennings JC, Shaw DP, Goyal SM, McCullough S, Morrison RB, Joo HS, et al. (1992): Isolation of swine infertility and respiratory syndrome virus (isolate ATCC VR-2332) in North America and experimental reproduction of the disease in gnotobiotic pigs. J Vet Diagn Invest 4, 117-126

Garcia-Nicolas O, Quereda JJ, Gomez-Laguna J, Salguero FJ, Carrasco L, Ramis G, Pallares FJ (2014): Cytokines transcript levels in lung and lymphoid organs during genotype 1 Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) infection. Vet Immunol Immunopathol 160, 26-40

Gardy JL, Lynn DJ, Brinkman FS, Hancock RE (2009): Enabling a systems biology approach to immunology: focus on innate immunity. Trends Immunol 30, 249-262

Genini S, Delputte PL, Malinverni R, Cecere M, Stella A, Nauwynck HJ, Giuffra E (2008): Genome-wide transcriptional response of primary alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. J Gen Virol 89, 2550-2564

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004): Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5, 15

Glass EJ (2012): The molecular pathways underlying host resistance and tolerance to pathogens. Front Genet 3:263, doi: 10.3389/fgene.2012.00263

Goldman FD, Ballas ZK, Schutte BC, Kemp J, Hollenback C, Noraz N, Taylor N (1998): Defective expression of p56lck in an infant with severe combined immunodeficiency. J Clin Invest 102, 421-429

Islam MA, Grosse-Brinkhaus C, Proll MJ, Uddin MJ, Rony SA, Tesfaye D, Tholen E, Holker M, Schellander K, Neuhoff C (2016): Deciphering transcriptome profiles of peripheral blood mononuclear cells in response to PRRSV vaccination in pigs. BMC Genomics 17(1):641, doi: 10.1186/s12864-016-2849-1

Kapur V, Elam MR, Pawlovich TM, Murtaugh MP (1996): Genetic variation in porcine reproductive and respiratory syndrome virus isolates in the midwestern United States. J Gen Virol 77, 1271-1276

Kimman TG, Cornelissen LA, Moormann RJ, Rebel JM, Stockhofe-Zurwieden N (2009): Challenges for porcine reproductive and respiratory syndrome virus (PRRSV) vaccinology. Vaccine 27, 3704-3718

Kohane IS, Valtchinov VI (2012): Quantifying the white blood cell transcriptome as an accessible window to the multiorgan transcriptome. Bioinformatics 28, 538-545

Li J, Yu YJ, Feng L, Cai XB, Tang HB, Sun SK, Zhang HY, Liang JJ, Luo TR (2010): Global transcriptional profiles in peripheral blood mononuclear cell during classical swine fever virus infection. Virus Res 148, 60-70

Li X, Galliher-Beckley A, Pappan L, Trible B, Kerrigan M, Beck A, Hesse R, Blecha F, Nietfeld JC, Rowland RR, Shi J (2014a): Comparison of host immune responses to homologous and heterologous type II porcine reproductive and respiratory syndrome virus (PRRSV) challenge in vaccinated and unvaccinated pigs. Biomed Res Int 416727, doi: 10.1155/2014/416727

Li Y, Sun Y, Xing F, Kang L, Wang P, Wang L, Liu H, Jiang Y (2014b): Identification of a single nucleotide promoter polymorphism regulating the transcription of ubiquitin specific protease 18 gene related to the resistance to porcine reproductive and respiratory syndrome virus infection. Vet Immunol Immunopathol 162, 65-71

Liu G, Loraine AE, Shigeta R, Cline M, Cheng J, Valmeekam V, Sun S, Kulp D, Siani-Rose MA (2003): NetAffx: Affymetrix probesets and annotations. Nucleic Acids Res 31, 82-86

Lockhart S (2003): Conjugate vaccines. Expert Rev Vaccines 2, 633-648

Loemba HD, Mounir S, Mardassi H, Archambault D, Dea S (1996): Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Arch Virol 141, 751-761

Lopez OJ, Osorio FA (2004): Role of neutralizing antibodies in PRRSV protective immunity. Vet Immunol Immunopathol 102, 155-163

Loving CL, Osorio FA, Murtaugh MP, Zuckermann FA (2015): Innate and adaptive immunity against Porcine Reproductive and Respiratory Syndrome Virus. Vet Immunol Immunopathol 167, 1-14

Martelli P, Cordioli P, Alborali LG, Gozio S, De Angelis E, Ferrari L, Lombardi G, Borghetti P (2007): Protection and immune response in pigs intradermally vaccinated against porcine reproductive and respiratory syndrome (PRRS) and subsequently exposed to a heterologous European (Italian cluster) field strain. Vaccine 25, 3400-3408

Martelli P, Gozio S, Ferrari L, Rosina S, De Angelis E, Quintavalla C, Bottarelli E, Borghetti P (2009): Efficacy of a modified live porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs naturally exposed to a heterologous European (Italian cluster) field strain: Clinical protection and cell-mediated immunity. Vaccine 27, 3788-3799

Meier WA, Galeota J, Osorio FA, Husmann RJ, Schnitzlein WM, Zuckermann FA (2003): Gradual development of the interferon-gamma response of swine to porcine reproductive and respiratory syndrome virus infection or vaccination. Virology 309, 18-31

Miller LC, Fleming D, Arbogast A, Bayles DO, Guo B, Lager KM, Henningson JN, Schlink SN, Yang HC, Faaberg KS, Kehrli ME, Jr. (2012): Analysis of the swine tracheobronchial lymph node transcriptomic response to infection with a Chinese highly pathogenic strain of porcine reproductive and respiratory syndrome virus. BMC Vet Res 8, 1746-6148

Nielsen J, Botner A (1997): Hematological and immunological parameters of 4 1/2-month old pigs infected with PRRS virus. Vet Microbiol 55, 289-294

Niu W, Lu ZJ, Zhong M, Sarov M, Murray JI, Brdlik CM, Janette J, Chen C, Alves P, Preston E, Slightham C, Jiang L, Hyman AA, Kim SK, Waterston RH, Gerstein M, Snyder M,

Reinke V (2011): Diverse transcription factor binding features revealed by genome-wide ChIP-seq in *C. elegans*. Genome Res 21(2), 245-254

Pancer Z, Cooper MD (2006): The evolution of adaptive immunity. Annu Rev Immunol 24, 497-518

Pfaffl MW (2001): A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29(9), e45

Renukaradhya GJ, Alekseev K, Jung K, Fang Y, Saif LJ (2010): Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. Viral Immunol 23, 457-466

Rowland RR, Lunney J, Dekkers J (2012): Control of porcine reproductive and respiratory syndrome (PRRS) through genetic improvements in disease resistance and tolerance. Front Genet 3, doi: 10.3389/fgene.2012.00260

Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386

Schroyen M, Tuggle CK (2015): Current transcriptomics in pig immunity research. Mamm Genome 26, 1-20

Shimizu M, Yamada S, Kawashima K, Ohashi S, Shimizu S, Ogawa T (1996): Changes of lymphocyte subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. Vet Immunol Immunopathol 50, 19-27

Shoemaker JE, Lopes TJ, Ghosh S, Matsuoka Y, Kawaoka Y, Kitano H (2012): CTen: a web-based platform for identifying enriched cell types from heterogeneous microarray data. BMC Genomics 13, 1471-2164

Silva-Campa E, Cordoba L, Fraile L, Flores-Mendoza L, Montoya M, Hernandez J (2010): European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells. Virology 396, 264-271

Silva-Campa E, Mata-Haro V, Mateu E, Hernandez J (2012): Porcine reproductive and respiratory syndrome virus induces CD4+CD8+CD25+Foxp3+ regulatory T cells (Tregs). Virology 430, 73-80

Smyth GK (2005 pp.397-442): Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, editors. Bioinformatics and computational biology solutions using r and bioconductor. Springer pp. 397–342

Sun SC (2008): Deubiquitylation and regulation of the immune response. Nat Rev Immunol 8, 501-511

Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA (2011): Pattern recognition receptors and the innate immune response to viral infection. Viruses 3, 920-940

Trus I, Bonckaert C, van der Meulen K, Nauwynck HJ (2014): Efficacy of an attenuated European subtype 1 porcine reproductive and respiratory syndrome virus (PRRSV) vaccine in pigs upon challenge with the East European subtype 3 PRRSV strain Lena. Vaccine 32, 2995-3003

Uddin MJ, Nuro-Gyina PK, Islam MA, Tesfaye D, Tholen E, Looft C, Schellander K, Cinar MU (2012): Expression dynamics of Toll-like receptors mRNA and cytokines in porcine peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. Vet Immunol Immunopathol 147, 211-222

Van Breedam W, Delputte PL, Van Gorp H, Misinzo G, Vanderheijden N, Duan X, Nauwynck HJ (2010): Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. J Gen Virol 91, 1659-1667

Van Gucht S, Labarque G, Van Reeth K (2004): The combination of PRRS virus and bacterial endotoxin as a model for multifactorial respiratory disease in pigs. Vet Immunol Immunopathol 102, 165-178

Voskoboinik I, Smyth MJ, Trapani JA (2006): Perforin-mediated target-cell death and immune homeostasis. Nat Rev Immunol 6, 940-952

Wang IM, Bett AJ, Cristescu R, Loboda A, ter Meulen J (2012): Transcriptional profiling of vaccine-induced immune responses in humans and non-human primates. Microb Biotechnol 5, 177-187

Weesendorp E, Morgan S, Stockhofe-Zurwieden N, Popma-De Graaf DJ, Graham SP, Rebel JM (2013): Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. Vet Microbiol 163(1-2), 1-12

Wilkie B, Mallard B (1999): Selection for high immune response: an alternative approach to animal health maintenance? Vet Immunol Immunopathol 72, 231-235

Wysocki M, Chen H, Steibel JP, Kuhar D, Petry D, Bates J, Johnson R, Ernst CW, Lunney JK (2012): Identifying putative candidate genes and pathways involved in immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) infection. Anim Genet 43, 328-332

Xia J, Benner MJ, Hancock RE (2014): NetworkAnalyst--integrative approaches for protein-protein interaction network analysis and visual exploration. Nucleic Acids Res 42(Web Server issue), W167-74

Xiao S, Jia J, Mo D, Wang Q, Qin L, He Z, Zhao X, Huang Y, Li A, Yu J, Niu Y, Liu X, Chen Y (2010a): Understanding PRRSV infection in porcine lung based on genome-wide transcriptome response identified by deep sequencing. PLoS ONE 5(6), e11377

Xiao S, Mo D, Wang Q, Jia J, Qin L, Yu X, Niu Y, Zhao X, Liu X, Chen Y (2010b): Aberrant host immune response induced by highly virulent PRRSV identified by digital gene expression tag profiling. BMC Genomics 11, 1471-2164

Xu D, Lillico SG, Barnett MW, Whitelaw CB, Archibald AL, Ait-Ali T (2012): USP18 restricts PRRSV growth through alteration of nuclear translocation of NF-kappaB p65 and p50 in MARC-145 cells. Virus Res 169, 264-267

Yoon KJ, Wu LL, Zimmerman JJ, Hill HT, Platt KB (1996): Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. Viral Immunol 9, 51-63

ZDS (2003): Richtlinie Fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit Beim Schwein. Zentralverband der Deutschen Schweineproduktion eV, Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn.

Zhang H, Guo X, Nelson E, Christopher-Hennings J, Wang X (2012): Porcine reproductive and respiratory syndrome virus activates the transcription of interferon alpha/beta (IFN-alpha/beta) in monocyte-derived dendritic cells (Mo-DC). Vet Microbiol 159, 494-498

Zhou P, Zhai S, Zhou X, Lin P, Jiang T, Hu X, Jiang Y, Wu B, Zhang Q, Xu X, Li JP, Liu B (2011): Molecular characterization of transcriptome-wide interactions between highly pathogenic porcine reproductive and respiratory syndrome virus and porcine alveolar macrophages in vivo. Int J Biol Sci 7, 947-959

Zhuge ZY, Zhu YH, Liu PQ, Yan XD, Yue Y, Weng XG, Zhang R, Wang JF (2012): Effects of Astragalus polysaccharide on immune responses of porcine PBMC stimulated with PRRSV or CSFV. PLoS ONE 7(1), e29320

Chapter 4: Breed specific transcriptome signature for PRRSV vaccine response

(Manuscript is ahead of submission to the Frontiers in Genetics)

Genome-wide gene expression profiles of PBMCs revealed breed-specific transcriptome signatures for PRRSV vaccination in German Landrace and Pietrain pigs

Md. Aminul Islam[1,2], Christiane Neuhoff[1], Sharmin Aqter Rony[1], Christine Große-Brinkhaus[1], Muhammad Jasim Uddin[2,3], Michael Hoelker[1,4], Dawit Tesfaye[1], Ernst Tholen[1], Karl Schellander[1,4], Maren Julia Pröll[1,*]

[1]Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany
[2]Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh
[3]School of Veterinary Science, The University of Queensland, Gatton Campus, QLD 4343, Australia
[4]Teaching and Research Station on Frankenfrost, Faculty of Agriculture, University of Bonn, Konigswinter, Germany

Author's email:
MAI: aisl@itw.uni-bonn.de
CN: cneu@itw.uni-bonn.de
SAR: sron@itw.uni-bonn.de
CGB: cgro@itw.uni-bonn.de
MJU: jasimmedbau@gmail.com
MH: mhoe@itw.uni-bonn.de
DT: dtes@itw.uni-bonn.de
ET: etho@itw.uni-bonn.de
KS: ksch@itw.uni-bonn.de
MJP: mpro@itw.uni-bonn.de

*Correspondence to:
Maren Julia Pröll, PhD
Institute of Animal Science, University of Bonn
Endenicher Allee 15, 53115 Bonn, Germany
Email: mpro@itw.uni-bonn.de
Phone: 0049-228/73-3584
Fax: 0049-00228/73-2284

Article type and status: Meta-analysis and new

Running title: Breed specific transcriptome signature for PRRSV vaccine response

Keywords: PRRSV vaccine, innate immunity, gene, microarray, breed

4.1 Abstract

The porcine reproductive and respiratory syndrome (PRRS) is a devastating viral disease affecting swine industry worldwide. The breed is one of the potential determinants influencing the host immune response to PRRS virus (PRRSV) infection in pigs. Elucidating the role of host genetics in the variation of PRRSV vaccine responsiveness may lead to characterize the host specific immunocompetence, and thereby resistance to PRRS. Therefore, the current study aimed to investigate the breed difference in innate immune response to PRRSV vaccination between purebred German Landrace (DL) and Pietrain (Pi) pigs. We analyzed microarray-based transcriptome profiles of peripheral blood mononuclear cells (PBMCs) collected before (0h) and 24h after PRRSV vaccination from DL and Pi breed with three biological replicates. With FDR <0.05 and log2 fold change 1.5 as cutoff criteria, 4,269 transcripts were found to be differentially expressed in PBMCs in at least any of four contrast pairs (*i.e.* DL-24h *vs.* DL-0h, Pi-24h *vs.* Pi-0h, DL-0h *vs.* Pi-0h and DL-24h *vs.* Pi-24h) tested. The number of vaccine induced differentially expressed genes (DEGs) was much higher (2,459) in DL pigs than that of Pietrain pigs (291). After 24 h of PRRSV vaccination, 1,046 genes were differentially expressed PMBCs of DL pig compared to that of Pietrain (DL-24h *vs.* Pi-24h), indicating the breed differences in vaccine responsiveness. Before vaccination, 3,255 genes showed differential expression between DL and Pi (DL-0h *vs.* Pi-0h) which indicated the genetic variation between two breeds. The top biological pathways significantly affected by genes differentially expressed in PBMCs of both breeds are linked to immune response functions. The network enrichment analysis identified STAT1, MMS19, RPA2, BAD, UCHL5 and APC as potential regulatory genes for the functional network of PRRSV vaccine response specific for DL. While FOXO3, IRF2, ADRBK1, FHL3, PPP2CB, MTOR, EIF3I, RPL8, FLNC, NCOA6, DICER1 were found to be the most potential hubs of the Pietrain-specific transcriptome network. In conclusion, German Landrace pigs differ greatly from Pietrain in terms of PBMCs transcriptome profiles after PRRSV vaccination. The current transcriptome analysis enhances our knowledge on genetic control of the susceptibility to PRRS.

4.2 Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most economically important viral diseases of swine industry worldwide. The PRRS is caused by a positive sense single stranded RNA virus PRRS virus (PRRSV) having two genetically diverse strains namely Type 1 (European) and Type 2 (North American) (Nelsen et al. 1999). The clinical

outcome of PRRSV infection varies widely from mild asymptomatic illness to severe clinical disease depending on virulence of the virus and immune status of the host (Lunney et al. 2011). The PRRSV of either genotype seems to inherently stimulate an imbalanced immune response characterized by an aberrant interferon (IFN) response (Murtaugh et al. 2002). Variability of host immunity is likely responsible for the inconsistency of the clinical outcomes seen upon PRRSV challenge either to naïve or previously immunized pigs (Labarque et al. 2003). Thereby, the severity of PRRSV infection is determined by the ability of the host to overcome the inherent propensity of PRRSV in preventing the timely development of host innate immunity.

The innate immunity, as the first line of host defense mechanism is typically occurs within hours of antigen exposure in a non-specific manner and may persist up to few days (Beutler 2004). Adequate activation of innate immune system is essential for mounting a durable protective immunity (Glass 2012). Genes regulating the innate immune response to pathogenic infection are likely to be the strong candidates for disease resistance trait (Loving et al. 2015). Innate immune related genes, in particular, the members of guanylate-binding protein gene family have been found to be associated with host resistance to PRRSV (Boddicker et al. 2012). The guanylate-binding protein gene family are located on *Sus scrofa* chromosome 4 (SSC4) where the quantitative trait locus (QTL) associated with host resistance to PRSSV infection has been identified (Boddicker et al. 2012). A single nucleotide polymorphism (SNP) WUR10000125 (WUR) at the interferon-inducible guanylate-binding protein 1 gene (GBP1) has recently been found to be associated with European PRRS resistance and growth performance in pig (Abella et al. 2016). Gene expression study in PRRSV infected pulmonary alveolar macrophages over 24 h post infection period suggested that myxovirus resistance 1 (MX1) and ubiquitin specific protease (USP) genes may play important role in clinical disease during PRRSV infection (Zhang et al. 1999). It has also been reported that the overexpression of the porcine USP18 resulted a limited replication of PRRSV (Ait-Ali et al. 2009) through altering the nuclear translocation of NF-κB p65 and p50 (Xu et al. 2012). A subsequent study suggested that the SNP G-1533A polymorphism in the promoter region of porcine USP18 gene is a potential DNA marker for the resistance to PRRSV (Li et al. 2014). Therefore, genes and molecular pathways associated with innate immunity to PRRSV are crucial for genetic improvement of the host through selective breeding.

The breed is one of the potential host determinants affecting the immune response to variety of pathogens or stressors in pig. The existence of variation in the host resistance or susceptibility to PRRSV infection among swine breeds has been reported in several studies (Halbur et al. 1998, Christopher-Hennings et al. 2001, Petry et al. 2005, Vincent et al. 2005, Lewis et al. 2007, Reiner et al. 2010, Ait-Ali et al. 2011, Xing et al. 2014). Difference on relative resistance to PRRSV infection has been observed between Chinese Meishan and European pig breeds (Halbur et al. 1998). Variation in host innate immunity to European type PRRSV infection has been reported between Landrace and Pietrain pigs through global gene expression profiling of in-vitro PRRSV infected pulmonary alveolar macrophages (Ait-Ali et al. 2011). Christopher-Hennings and his colleagues (2001) compared the presence of virus in serum, semen, or peripheral blood mononuclear cells (PBMCs) over time in adult Hampshire (n =3), Yorkshire (n = 3), and Landrace (n = 2) boars inoculated with a PRRSV field isolate (SD-23983). The variation in immune response within such a small population precluded the possibility of detecting statistically significant differences among breeds (Christopher-Hennings et al. 2001). The non-lean type pigs showed a reduced susceptibility to PRRSV (Petry et al. 2005), as also shown by the comparative evaluation of PRRSV infection in German miniature and Pietrain pigs (Reiner et al. 2010). In a recent study, we also observed the differences between Duroc and Pietrain pigs in terms of transcriptome profiles of lung dendritic cells after in vitro PRRSV infection (Pröll et al. 2016, unpublished data). All these above mentioned works have raised the evidence for genetic variation in host transcriptional response to PRRSV among porcine breeds. Therefore, exploring the breed-specific transcriptome signature for PRRSV vaccine response in German Landrace and Pietrain pigs might be an important add-on for the understanding on genetic control of the susceptibility to PRRS.

A handful of studies have conducted to evaluate host transcriptional response to in vitro or in vivo PRRSV infection were based on respiratory tissues/cells, as they provide the primary site of virus replication (Ait-Ali et al. 2011, Xing et al. 2014). In fact, the live attenuated PRRSV vaccine antigens given that administered through intramuscular injection usually bypass the lung tissue and reach faster into the blood circulation and sensitize the blood macrophages to initiate the immune response (Siegrist 2012). Therefore, blood-based investigation of molecular mechanisms of host-vaccine interaction is worthwhile. Moreover, the blood transcriptomics could provide quick insight into the complex biological processes linking between host genotype and vaccine response (Chaussabel 2015). Furthermore, genetic

variation in PRRSV vaccine response at blood transcriptome level among porcine breeds has not yet been entirely explored. Our previous study revealed that temporal variation of innate immune transcriptional responses to PRRSV vaccination in PBMCs with a peak response occurred at 24 hours post vaccination in German Landrace pigs (Islam et al. 2016). Therefore, the time point 24 h post vaccination was selected for the detection of transcriptome signature of innate immune response to PRRSV vaccine. To explore the evidence of genetic variations of PRRSV vaccine induced innate immunity, we compared herein the global gene expression profiles of PBMCs collected immediately before and 24 h post PRRSV vaccination in purebred German Landrace and Pietrain pigs.

4.3 Materials and methods

4.3.1 Ethics statements

The research proposal was approved by the Veterinary and Food Inspection Office, Siegburg, Germany (ref. 39600305-547/15). The whole in-vivo experiment was conducted according to the institutional guidelines and animal husbandry regulations of Germany [28]. The blood sampling protocol was approved by the State Agency for Nature, Environment and Consumer Protection, North Rhine-Westphalia, Germany (permission nr. 84-02.05.04.14.027).

4.3.2 Study animals and vaccination

This study was conducted on purebred German Landrace (DL) and Pietrain (Pi) pigs. Three female piglets from both DL and Pi breed, clinically healthy with no history of respiratory diseases, were housed in the Teaching and Research Station at Frankenfrost, University of Bonn, Germany. All piglets were immunized with the live attenuated PRRSV vaccine of EU strain (Porcillis® PRRS Vaccine, DE) with primary dose at day 28 of age. The anticoagulated venous blood samples were collected at immediately before (0h) and 24 h post vaccination.

4.3.3 Sample preparation and microarray hybridization

The details of sample processing and microarray hybridization are available in our previous publication (Islam et al. 2016). In brief, the PBMCs were isolated from the whole blood through density gradient centrifugation using Histopague®. The total RNA was extracted from PBMCs using the miRNeasy mini kit (P/N 217004, Qiagen, Hilden, Germany) according to the manufacturer's protocol along with on column DNase treatment (P/N 79254, Qiagen, Hilden, Germany). After quality control, about 100 ng of total RNA was processed to synthesize the biotin-labeled sense strand cDNA probes using the GeneChip® WT PLUS

Reagent kit (P/N 902281; Affymetrix Inc., Santa Clara, CA, USA) according to the manufacturer's protocol. The microarray target probes were hybridized into the GeneChip® Porcine Gene 1.0 ST array strip of 81/4 format (P/N 901976, Affymetrix Inc., Santa Clara, CA, USA) followed by staining, washing and scanning using the Affymetrix GeneChip array processing facility at Life and Brain Centre, Uni-bonn, Germany. The microarray expressions of both dataset were technically validated through measuring of qRT-PCR expression of five selected differentially expressed genes in the same RNA sample as used for microarray hybridization. The raw microarray data for DL pigs and Pietrain pigs are available in NCBI-GEO database with the accession code GSE76254 and GSE84516, respectively.

4.3.4 Statistical analysis of microarray data

The raw intensity microarray data was processed for background correction and normalization in R/Bioconductor software (v 3.1.2). The RMA (Robust Multi-array Average) based quantile normalization of microarray data were performed using the 'oligo' package (Carvalho and Irizarry 2010). Then for differential expression analysis, normalized microarray dataset was prepared four contrast pairs: DL-24h *vs.* DL-0h, Pi-24h *vs.* Pi-0h, DL-0h *vs.* Pi-0h and DL-24h *vs.* Pi-24h. Differentially expressed genes were determined using the linear analysis of microarray technique from the '*limma'* package (Smyth 2005) with empirical Bayes adjustment to the variance, followed by Benjamini and Hochberg (BH) correction for multiple testing (Benjamini and Hochberg 1995, Smyth 2005). Threshold criteria for genes to be considered differentially expressed were set as of FDR < 0.05 and $\log_2$ fold-change >1.5 or <-1.5. The hierarchical clustered heat map was generated using the *heatmap.2* function of 'ggplots' package.

4.3.5 Functional annotation of differentially expressed genes

For biological interpretation of the altered PBMC-transcriptomes between the two breeds, the significantly over-represented gene ontology (GO) terms and biological pathways were explored using the InnateDB pathway analysis tool (Breuer et al. 2013). The InnateDB platform implements the hypergeometric algorithm with the Benjamini-Hochberg (BH) multiple test correction method for overrepresentation analysis. First, the differentially expressed genes from microarray data were converted to their human ensembl orthologues using the biological DataBase network (bioDBnet) tool (Mudunuri et al. 2009). Then the list of ensembl gene identifiers was uploaded in InnateDB web and the over-representation analysis performed. The GO and pathways were considered significantly over-represented with an FDR <0.05.

4.3.6 Network analysis for differentially expressed genes

To identify the potential regulatory genes of vaccine mediated immunity in breed specific manner, we performed the network analysis with the DEGs more abundant in vaccinated PBMCs of DL compared to that of Pietrain pigs and vice versa using the NetworkAnalyst online tool (Xia et al. 2014). The human orthologous gene ensambl of the DEGs were imported as seed genes and a default network was constructed based on the Walktrap algorithm taking only direct interaction of seed genes (first-order interactors). The network size was then adjusted for <500 seeds and 200~2000 nodes using the 'reduce' panel for high-performance visualization. Two topological measures such as degree (number of connections to other nodes) and betweenness centrality (number of shortest paths going through the node) were taken in to account for detecting highly interconnected hubs of the network that could regulate the entire network. In addition, weighted network based module detection was perform to cluster the genes of similar biological functions. The p value of a given network module was calculated using a Wilcoxon rank-sum test of the "internal" (edges within in a module) and "external" (edges connecting the nodes of other modules) degrees.

4.4 Results

In order to get a comprehensive insight of vaccine induced transcriptome differences between piglets of German Landrace and Pietrain breed, we conducted a whole transcripts microarray in PBMCs collected immediately before (0h) and at 24 hours after primary PRRSV vaccination. The transcriptome profiling was performed with three biological replications for each sampling time points in both breed groups using Affymetrix GeneChip Porcine Gene 1.0 ST array containing 394,580 probesets representing a total of 19,212 known genes.

4.4.1 Abundance of differentially expressed genes in PBMCs following PRRSV vaccination

Gene transcripts were considered differentially expressed with thresholds set as FDR <0.05 and $\log_2$ fold-change >1.5 or <-1.5. Four contrast pairs such as German Landrace PBMCs between pre and 24h post vaccination (DL-24h *vs.* DL-0h), Pietrain PBMCs between pre and 24h post vaccination (Pi-24h *vs.* Pi-0h), unvaccinated PBMCs between German Landrace and Pietrain pigs (DL-0h *vs*. Pi-0h) and vaccinated PBMCs between German Landrace and Pietrain pigs (DL-24h *vs*. Pi-24h) were taken into consideration for differentially expressed genes (DEGs). Following statistical analysis, 4,269 transcripts were found to be differentially expressed in at least one of the four contrast pairs, while 2,459, 291, 3255 and 1,046 DEGs

were identified in the pairwise comparison of DL-24h *vs.* DL-0h, Pi-24h *vs.* Pi-0h, DL-0h *vs.* Pi-0h and DL-24h *vs.* Pi-24h, respectively (Fig 1A). Notably, 59 genes were differentially expressed in all four contrast pairs irrespective of vaccine responses and breed differences.

In the contrast between vaccinated (24h) and unvaccinated (0h) pigs, 2350 DEGs were more abundant in vaccinated DL pigs and 182 were more abundant in vaccinated Pi pigs (Fig 1A). Among the vaccine induced DEGs, 255 were uniquely expressed in PBMCs of DL and 34 were uniquely expressed in PBMCs Pi pigs while the differential expression of a total of 109 genes shared in vaccinated PBMCs of both breeds. There were 27 vaccine induced DEGs observed in PBMCs of both breeds at 24 h post vaccination, but degree of alteration was more abundant in DL than Pi pigs (Fig 1A). In the contrast between DL and Pi breed accounted 3,255 DEGs in unvaccinated PBMCs, and 1,046 in vaccinated PBMCs, respectively; and 325 DEGs were stable between vaccinated and unvaccinated pairs (Fig 1A). About 721 DEGs were more abundant in vaccinated PBMCs of DL pigs compared to that of Pietrain pigs, which were likely responsible for breed specific host immune response phenotype. Moreover, 2,930 DEGs were more abundant in PBMCs of healthy DL pigs compared to that of Pi pigs regardless of vaccine effect, which indicated the existence of breed variation in phenotypes even other than immune response. Among the breed dependent DEGs, expression of 405 genes were found to be modified by vaccination and alteration of 1,089 genes were caused by factors other than immunization (Fig 1A).

4.4.2 Global expression patterns of DEGs between DL and Pietrain pigs

In PBMCs of DL pigs, a large number of DEGs (2186) were upregulated compared to the down regulated one (273) (Fig 1B). On the other hand, a majority of the altered genes (260) in vaccinated PBMCs of Pietrain pigs were down regulated and only 31 genes were upregulated (Fig 1B). In unvaccinated PBMCs, 2,472 genes were upregulated in DL and 783 were upregulated in Pi pigs. A higher number (933) of upregulated genes were observed in vaccinated PBMCs of DL compared to vaccinated PBMCs of Pietrain pigs (133); (Fig. 1B). The range of log fold changes of the DEGs in four contrasts includes -3.87 to 5.12; -4.71 to 3.63; -5.87 to 6.41 and -3.89 to 6.72 in the contrasts of DL-24h *vs.* DL-0h; Pi-24h *vs.* Pi-0h; DL-0h *vs.* Pi-0h and DL-24h *vs.* Pi-24h, respectively (Fig 2). The hierarchical heatmap (Fig. 3) demonstrated the expression patterns of genes differentially expressed in vaccinated PBMCs of DL pigs compared to that of Pietrain. Replicates were clustered together within the

particular treatment block. The DEGs were clustered in five major group based on the similarities of biological functions.

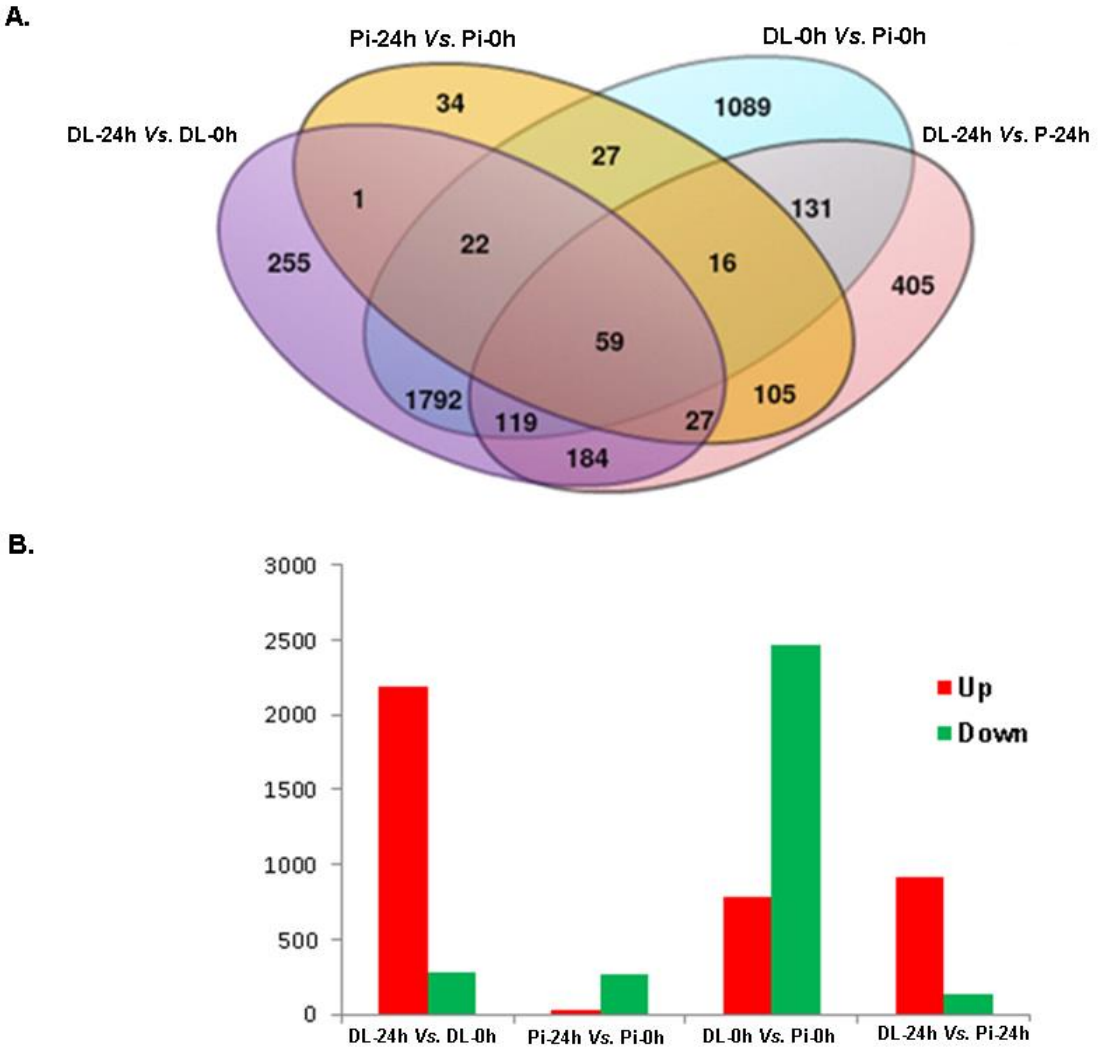

Figure 1. Number of differentially expressed genes after PRRSV vaccination. The intersecting venn diagram demonstrates the number of DEGs identified at four contrast pairs such as German Landrace PBMCs between pre and 24h post vaccination (DL-24h *vs.* DL-0h); Pietrain PBMCs between pre and 24h post vaccination (Pi-24h *vs.* Pi-0h); unvaccinated PBMCs between German Landrace and Pietrain pigs (DL-0h *vs.* Pi-0h), and vaccinated PBMCs between German Landrace and Pietrain pigs (DL-24h *vs.* Pi-24h); (A). The bar graphs depicts the proportion of DEGs showed their expression either upregulated (red bars) or down regulated (green bars) direction at four contrast pairs tested (B).

4.4.3 Expression regulation of shared DEGs between DL and Pietrain pigs

To identify the potential regulatory genes among the shared DEGs between breeds, we performed the network enrichment analysis. The seed genes of the network were ranked based on their degree and betweenness centrality values to detect the most potential hub genes. The

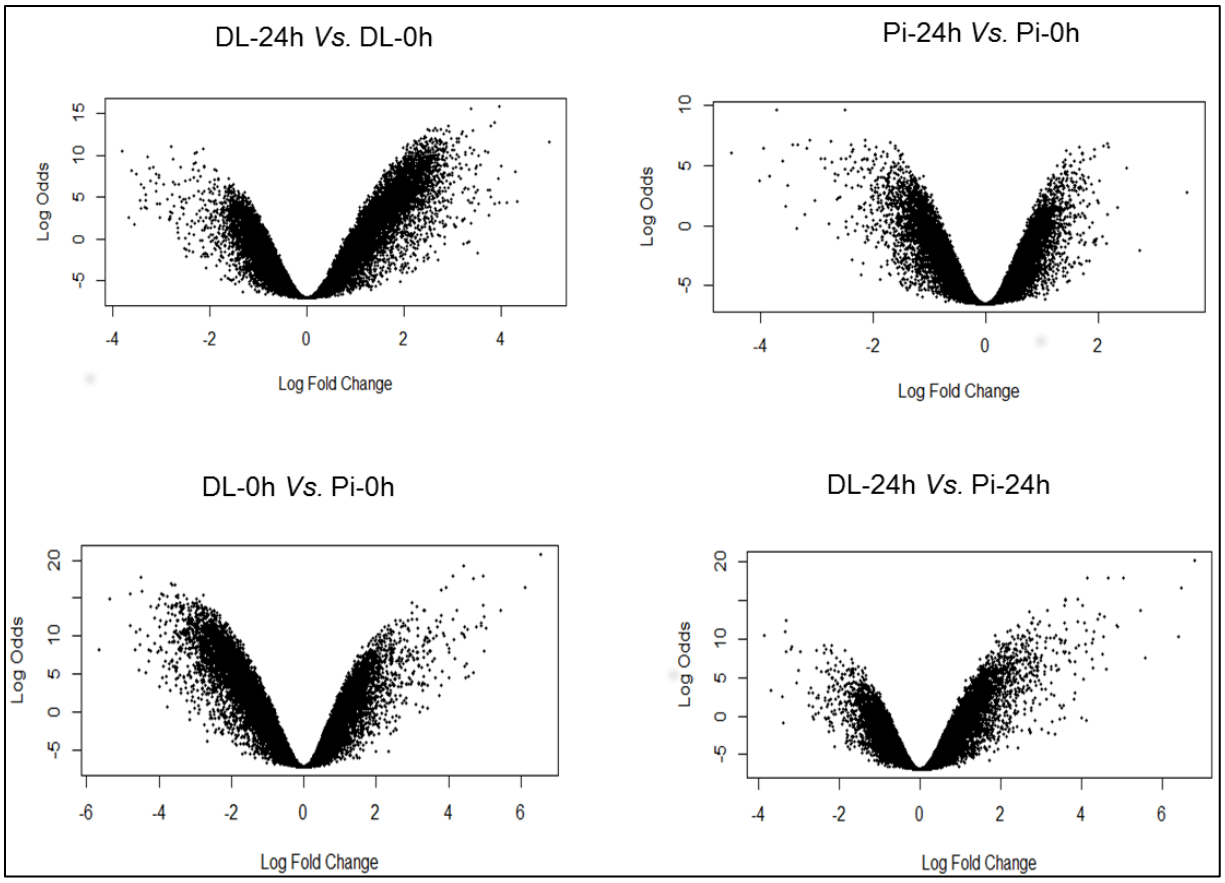

Figure 2. Volcano plots showing the range of fold changes of DEGs observed at four contrast pairs tested for differential gene expression. The picture illustrates the range of fold change for German Landrace PBMCs between pre and 24h post vaccination (DL-24h *vs.* DL-0h); (indicated on top of each figure panels), Pietrain PBMCs between pre and 24h post vaccination (Pi-24h *vs.* Pi-0h); unvaccinated PBMCs between Landrace and Pietrain pigs (DL-0h *vs.* Pi-0h) and vaccinated PBMCs between Landrace and Pietrain pigs (DL-24h *vs.* Pi-24h)

network of shared DEGs in PBMCs of both DL and Pietrain pigs is presented in Fig 4. Based on two centrality measures, the most highly interconnected hubs of the functional network of shared DEGs includes EIF3I (Eukaryotic translation initiation factor 3, subunit I), RRS1 (Ribosome biogenesis regulator homolog (S. cerevisiae)), ARPC1B (Actin related protein 2/3 complex, subunit 1B, 41kDa), BAG3 (BCL2-associated athanogene 3), ATP5J2 (ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F2), CSN2 (Casein beta), ASAP2 (ArfGAP with SH3 domain, ankyrin repeat and PH domain 2), BUD31 (BUD31 homolog (*S. cerevisiae*)), DCTN3 (Dynactin 3 (p22)), NACC1 (Nucleus accumbens associated 1, BEN and BTB (POZ) domain containing) and SLC9A2 (Solute carrier family 9, subfamily A (NHE2, cation proton antiporter 2), member 2). The relative expression values

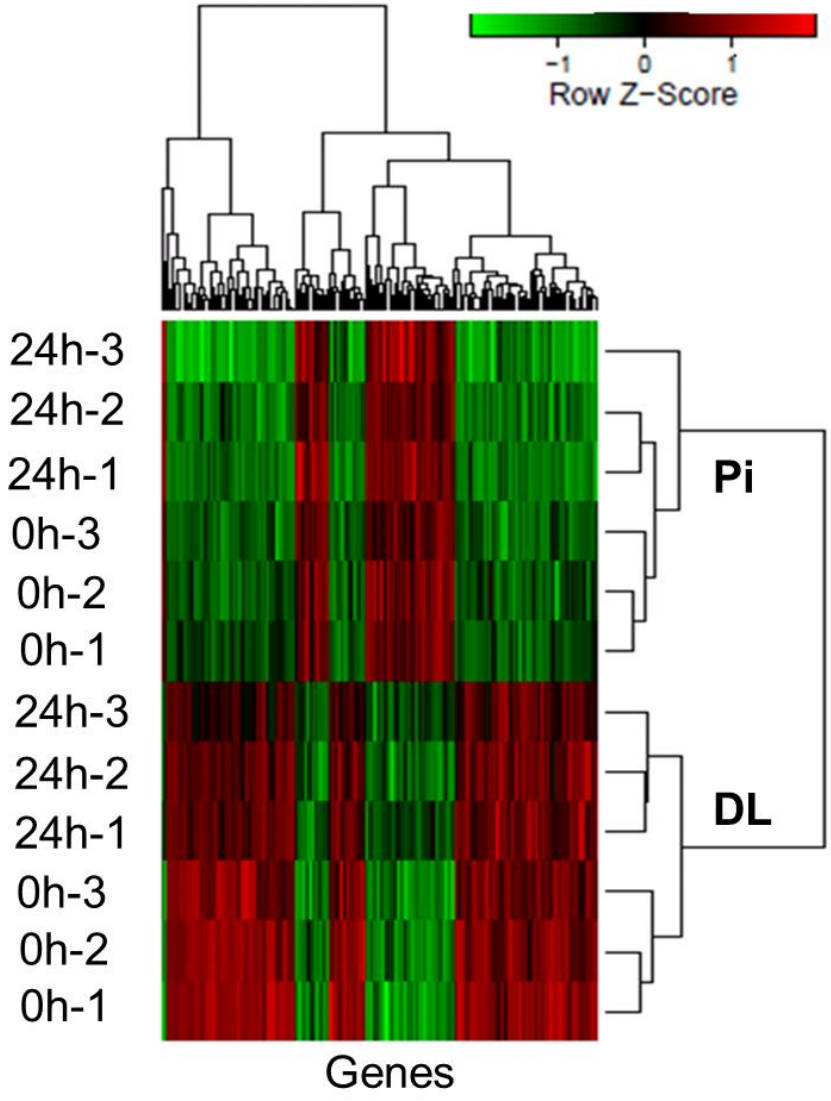

Figure 3. Hierarchical heat map showing differential gene expression over the contrast pairs. The figure only includes the genes differentially expressed between vaccinated PBMCs of German Landrace pigs compared to that of Pietrain pigs. The normalized log_2 transformed values determined by Affymetrix GeneChip® porcine gene 1.0 ST array in PBMCs collected at 0 and 24 h post PRRSV vaccination both in German Landrace and Pietrain pigs. The cutoff value of log_2 fold change as either >1.5 or <-1.5 and FDR <0.05 was considered for statistical significance. Column represents one array from each replicates.

and centrality estimates of the hub genes of shared transcriptome network are presented in the table 1. Surprisingly, all the hub genes except one showed opposite direction of their expression regulation. The relative expression of SLC9A2, ASAP2, BAG3, NACC1, RRS1, DCTN3, BUD31, ARPC1B and ATP5J2 were up regulated after vaccination in DL pigs but down regulated in Pietrain pigs after vaccination. In contrary, EIF3I was under expressed in PBMCs of vaccinated DL pigs but over expressed in that of Pietrain pigs. Only the expression of CSN2 was down regulated in both breeds after vaccination.

Table 1. Network centrality estimates and relative expression values of major hub genes regulating the network of common DEGs between PBMCs of vaccinated DL and Pi pigs

Gene name	Centrality estimates		Relative expression (FC)	
	Degree	Between-ness	DL	Pietrain
Eukaryotic translation initiation factor 3, subunit I (EIF3I)	79	25387	-1.743	1.771
RRS1 ribosome biogenesis regulator homolog (S. cerevisiae); (RRS1)	50	15702	2.005	-1.619
Actin related protein 2/3 complex, subunit 1B, 41kDa (ARPC1B)	42	13702	1.769	-1.764
BCL2-associated athanogene 3 (BAG3)	39	12700	2.594	-1.664
ATP synthase, H+ transporting, mitochondrial Fo complex, subunit F2 (ATP5J2)	25	7719	2.468	-1.931
Casein beta (CSN2)	22	9427	-3.041	-1.534
ArfGAP with SH3 domain, ankyrin repeat and PH domain 2 (ASAP2)	21	6625	2.435	-2.470
BUD31 homolog (S. cerevisiae); (BUD31)	21	6495	2.202	-1.733
Dynactin 3 (p22); (DCTN3)	16	4970	1.780	-1.523
Nucleus accumbens associated 1, BEN and BTB (POZ) domain containing (NACC1)	16	4539	2.216	-1.607
Solute carrier family 9, subfamily A (NHE2, cation proton antiporter 2), member 2 (SLC9A2)	11	3279	2.340	-2.126

FC, Fold Change; DL, German Landrace, Pi, Pietrain

4.4.4 GO and pathways enriched by breed-specific DEGs

For better understanding the biological mechanisms of breed-specific host transcriptional response to PRRSV vaccination, we performed gene ontology (GO) and pathway enrichment analyses for genes showing unique differential expression in vaccinated PBMCs in two breeds. Among the breed dependent DEGs, 913 were more abundant in PBMCs of German Landrace and 133 were more abundant in PBMCs of Pietrain pigs. The top most GO terms enriched in vaccinated PBMCs of DL pigs compared to that of Pietrain pigs include cell surface receptor signaling pathways, small molecules metabolic process, cell death, apoptotic process, positive regulation of cell proliferation, extracellular matrix organization, transport, canonical Wnt signaling pathway, positive regulation of epithelial cell proliferation and response to drug (Fig 5A). The GO for DEGs upregulated in vaccinated PBMCs of Pietrain

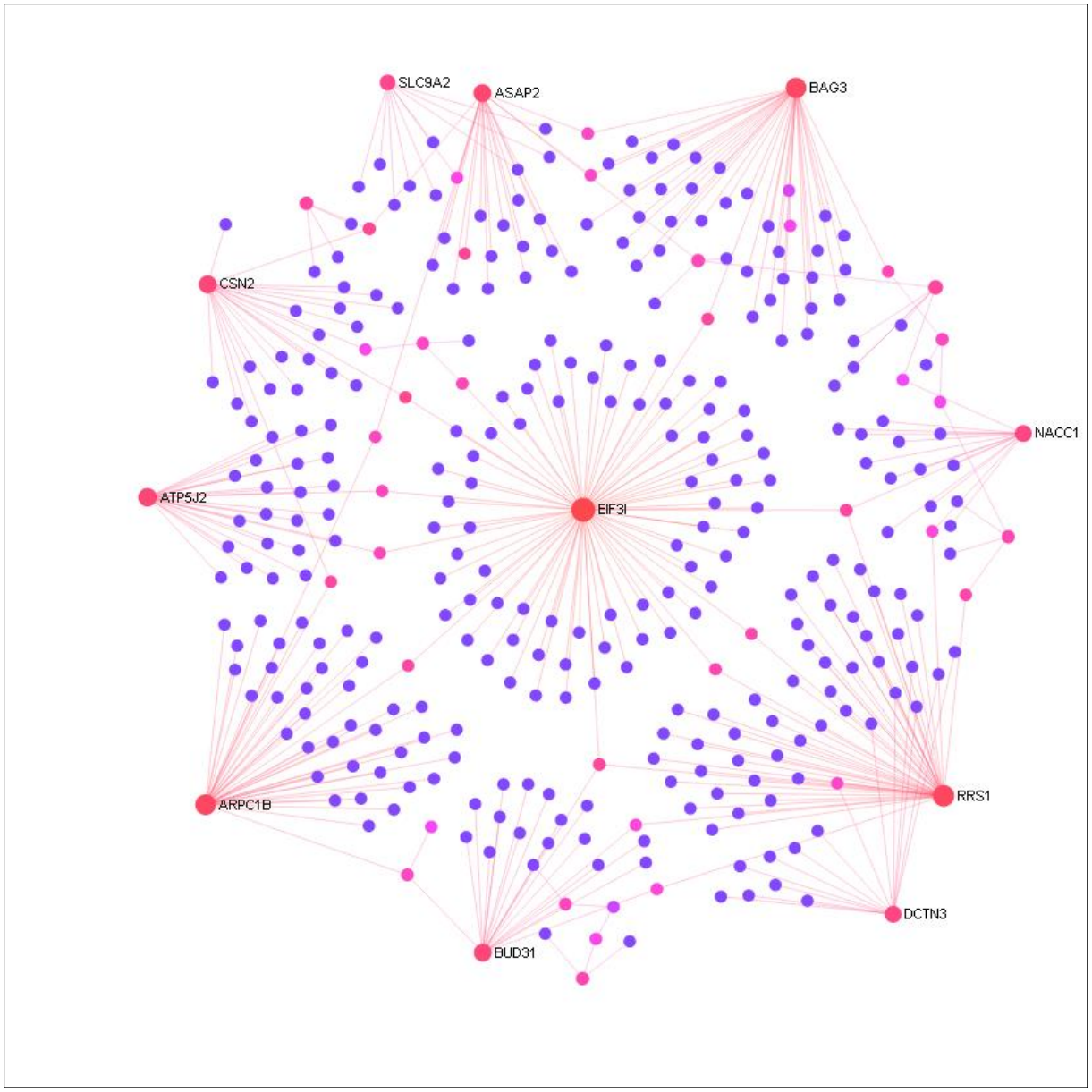

Figure 4. Network of commonly altered genes after PRRSV vaccination both in DL and Pi pigs. The interconnecting network showing the potential hub genes of the functional network of differentially expressed genes commonly observed in PBMCs of both DL and Pi pig at 24h after PRRSV vaccination. Each circle of the network indicates node (seed gene) and the diameter of node accounted for its centrality estimates. Lines between nodes indicate the connectivity.

pigs compared to that of DL includes positive chemotaxis, cell proliferation, inflammatory responses, epidermal growth receptor signaling pathway, positive regulation of endothelial cell proliferation, positive regulation of smooth muscle cell migration, positive regulation of transcription from RNA polymerase II promoter, fibroblast growth factor receptor signaling pathway and innate immune response (Fig 5B). Biological pathways significantly affected by genes differentially expressed in vaccinated PBMCs of Landrace pigs compared to that of Pietrain pigs includes signal transduction, metabolism, extracellular matrix organization, cytokine signaling in immune system, Wnt signaling pathway, apoptosis, Glycolysis/

gluconeogenesis, interferon alpha/beta signaling and TNF receptor signaling pathway (Table 2). The pathways significantly altered by DEGs upregulated in vaccinated PBMCs of Pietrain pigs compared to that of Landrace includes innate immune system, signaling by FGFR in disease, TGF beta receptor, JAK STAT pathway and regulation, chemokine signaling pathway, IL2 signaling events mediated by PI3K, validated targets of C-MYC transcriptional repression, cell-cell communication, glucose metabolism and platelet homeostasis (Table 2).

4.4.5 Breed-specific transcriptome signature for PRRSV vaccine responses

To identify the breed-specific transcriptome signature for PRRSV vaccine mediated immunity in PBMCs, we performed the network analysis for the breed-specific DEGs of DL and Pi pigs using the NetworkAnalyst tool (Xia et al. 2014). The breed-specific transcriptome network labelled with potential hub genes are presented in Fig 6. The degree and betweenness centrality estimates of seed genes are provided in Additional file 5: Table S8 & S9. The hub genes of the DL-specific transcriptome network includes STAT1 (Signal transducer and activator of transcription 1), MMS19 (MMS19 Homolog, cytosolic iron-sulfur assembly component), RPA2 (Replication protein A2), BAD (BCL2 associated agonist of cell death), UCHL5 (Ubiquitin C-terminal hydrolase L5) and APC (Adenomatous polyposis coli). While FOXO3 (Fork head box O3), IRF2 (Interferon regulatory factor 2), ADRBK1 (Adrenergic beta receptor kinase 1), FHL3 (Four and a half LIM domains 3), PPP2CB (Protein phosphatase 2 catalytic subunit beta), MTOR (Mechanistic target of rapamycin), EIF3I (Eukaryotic translation initiation factor 3 subunit), RPL8 (Ribosomal protein L8), DICER1 (Dicer 1, ribonuclease III), FLNC (Filamin C) and NCOA6 (Nuclear receptor coactivator 6) were found to be the most potential hubs of the Pietrain-specific transcriptome network.

4.4.6 Variation of PBMCs transcriptomes between healthy DL and Pietrain pigs

The PBMCs transcriptome profiles of healthy control DL and Pietrain pigs showed massive difference in transcript abundances. The top most GO terms including ribosome, protein metabolism, catabolic process, cellular response to lipid, vasodilatation, phospholipid efflux, and cartilage homeostasis were enriched at unvaccinated PBMCs compared to unvaccinated PBMCs of Pietrain pigs (data not shown). The top most pathways including metal chelating activity, response to acetate, lactose biosynthetic process, tryptophan transport and visual behavior were enriched at unvaccinated PBMCs of Landrace compared to that of Pietrain pigs (data not shown).

Table 2. Biological pathways enriched by breed specific differentially expressed genes in PBMCs following PRRSV vaccination in German Landrace (DL) and Pietrain (Pi) pigs

Breed	Pathway name	p-value	Genes involved*
DL	Signal Transduction	0.001	**ADAM17**, **APC**, **APOE**, **B4GALT1**, **BAD**, **CNGA1**, **CRHR1**, DNAL4, DRD2, DRD3, **FLT4**, FZD3, **GFAP**, GHRHR, GLP1R, GPR68, **GREM2**, LGR6, **OR10H3**, **OR2AE1**, **OR4C46**, **OR4K13**, **OR4N2**, **OR6J1**, **OR7C2**, **OR9K2**, PSME3, **PTPRU**, RDH8, RHOBTB2, **SDC3**, **SDC4**, SFRP1, **SMO**, **STAT1**, **TERT**, **UCHL5**, VIPR2 and **YWHAB**
	Metabolism	0.05	**ACSL6**, **ALDH2**, **APOE**, **ATP5J2**, **B4GALT1**, CA12, CERS3, CYP17A1, DBT, DGUOK, DIO2, **FBP1**, GLP1R, **GPAT2**, HK3, IP6K1, KCNJ11, LRPPRC, LYPLA1, MED27, **MMS19**, MTMR7, **NDUFS2**, **NDUFS3**, **NME2**, PSME3, **SDC3**, **SDC4** and SQLE
	Extracellular matrix organization	0.008	**ADAM17**, BMP1, **SDC3**, **SDC4**, **TGFB2**, TLL1 and TLL2
	Cytokine Signaling in Immune system	0.08	**ADAM17**, HLA-C, **IFNA6**, **MX1**, **STAT1**, TNIP2 and **YWHAB**
	Wnt signaling pathway	0.002	**APC**, FZD3, **SDC3**, **SDC4**, SFRP1 and **YWHAB**
	Apoptosis	0.007	**ADAM17**, **APC**, BAD, PSME3, **YWHAB**
	Glycolysis / Gluconeogenesis	0.02	ALDH2, **ALDH3A1**, **FBP1** and HK3
	Interferon alpha/beta signaling	0.05	HLA-C, **IFNA6** and **MX1**
	Antigen processing and presentation	0.08	HLA-C, **HLA**-DMB and **PSME3**
	TNF receptor signaling pathway	0.09	**ADAM17** and **STAT1**
Pi	Innate Immune System	0.001	**ADRBK1**, **FOXO3**, **IRF2**, **MTOR** and PPP2CB
	Signaling by FGFR in disease	0.001	**ADRBK1**, **FOXO3**, **MTOR** and **PPP2CB**
	TGF_beta_Receptor	0.02	EIF3I, FOXO3 and **MTOR**
	JAK STAT pathway and regulation	0.03	**ADRBK1**, IL1A and **MTOR**
	Chemokine signaling pathway	0.07	**ADRBK1** and **FOXO3**
	IL2 signaling events mediated by PI3K	0.003	**FOXO3** and **MTOR**
	Validated targets of C-MYC transcriptional repression	0.01	**DKK1** and **FOXO3**
	Cell-Cell communication	0.004	**CDH13**, **FLNC** and KIRREL2
	Glucose metabolism	0.01	GYS1 and **PPP2CB**
	Platelet homeostasis	0.02	**P2RX1** and **PPP2CB**

*bold symbols indicate the upregulated genes involved with corresponding pathways

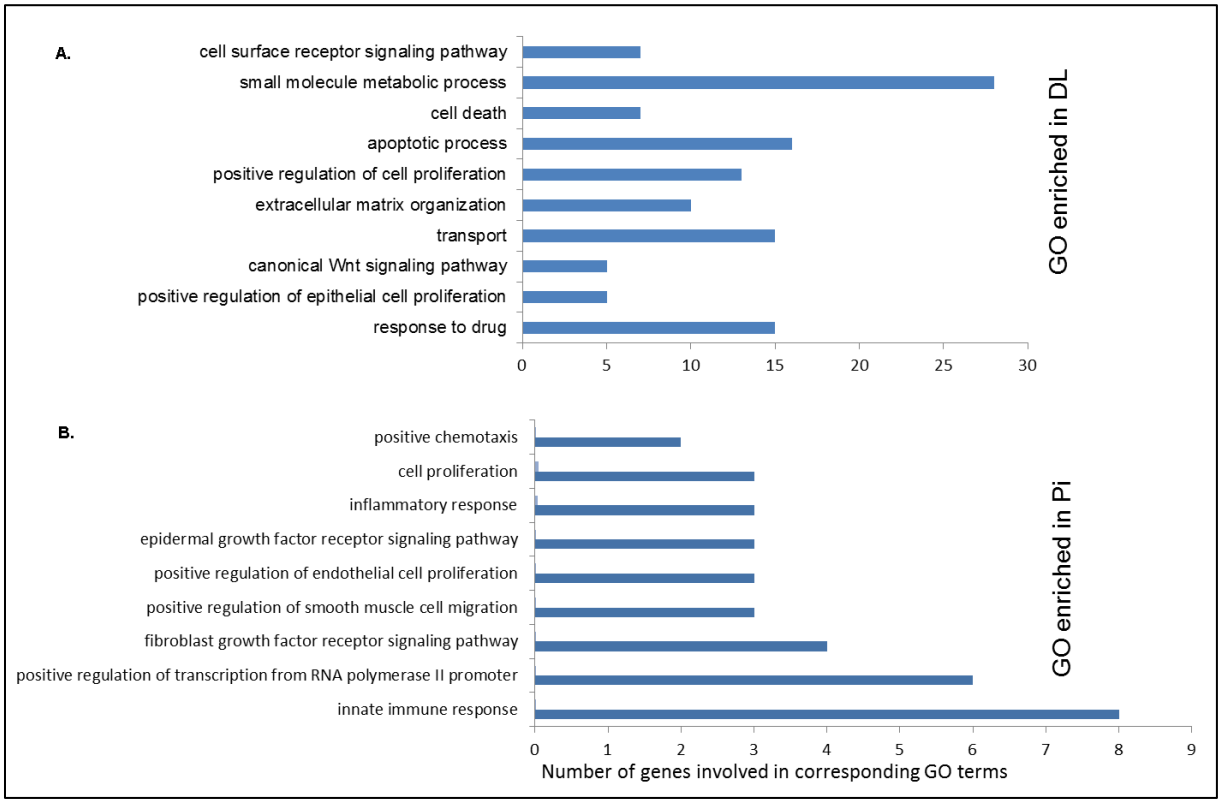

Figure 5. GO terms enriched by breed-specific DEGs. Bar graphs showing the enriched GO's in the vaccinated PBMCs of DL compared to that of Pi (a); and GO's in the vaccinated PBMCs of Pi compared to that of DL (b). The p value of <0.05 was considered for statistically significant enrichment.

4.5 Discussion

Innate host resistance to PRRS is becoming an area of great interest over the recent years because of the possibility for disease-resistant pig breeding. There is a consensus for genetic control of PRRS through improvement of host genetics by selective breeding for PRRS resistance (Lunney and Chen 2010). However, data on innate host resistance to PRRS virus, as measured by replication of virus within the pig is very limited to date. To contribute in this scheme, one promising way to go is the identification of host genotypes associated with improved innate immune response following PRRSV vaccination (Rowland et al. 2012). In spite of having considerably high heritability of disease resistance phenotypes, only a little has been addressed by breeding program as these are difficult to measure (Flori et al. 2011). Hence, an alternative approach of estimating the disease resistance through measuring host immunocompetence developed from vaccination is recommendable (Rowland et al. 2012). Identification of breed specific transcriptome signature associated with host innate immunocompetence following vaccination might increase our understanding not only for PRRS resistance but for other pathogens. To scrutinize the breed-specific transcripts for

vaccine mediated immunity, we compared the global gene expression profiles of PBMCs from DL and Pietrain pigs.

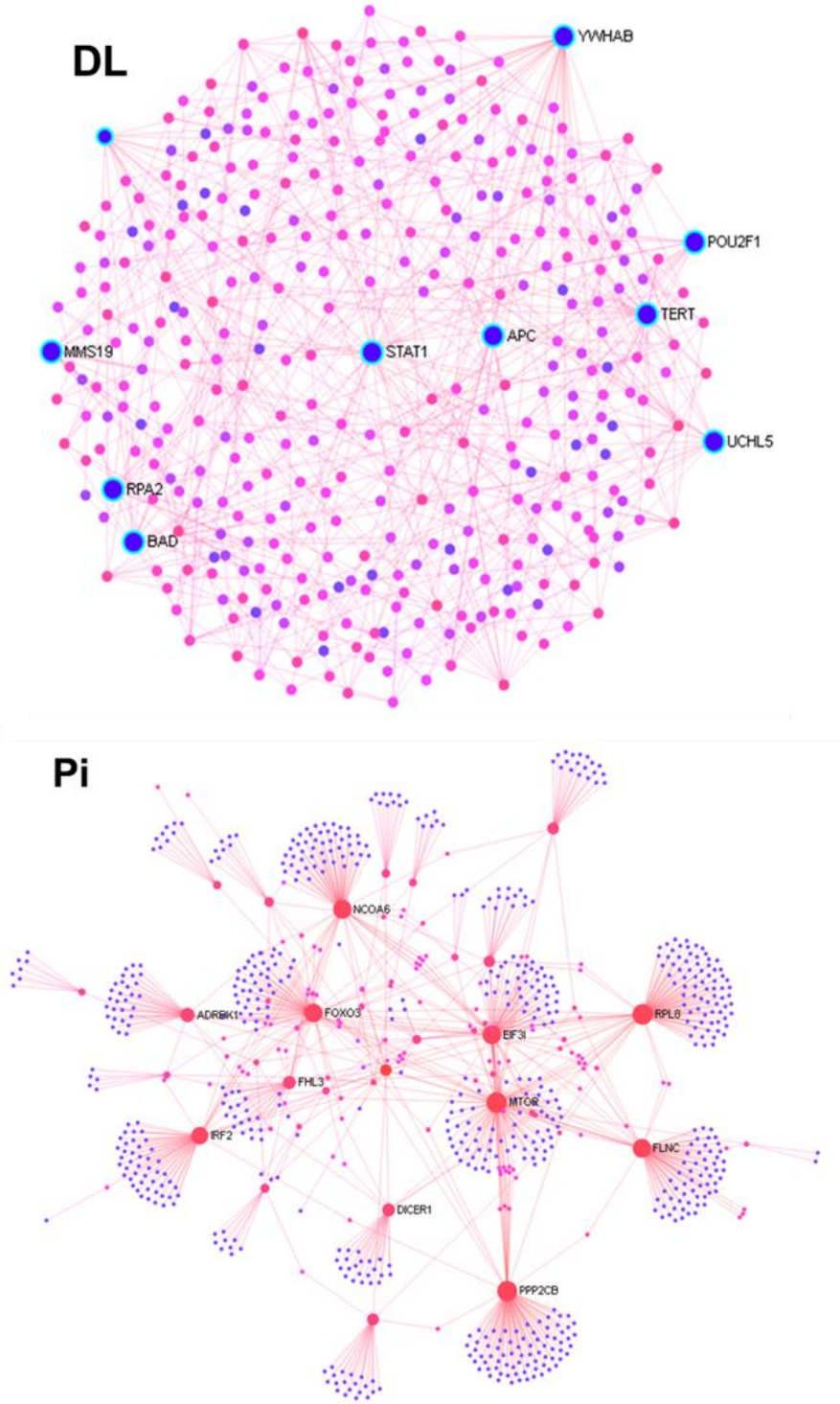

Figure 6. Networks of breed-specific altered transcriptome visualized by NetworkAnalyst. The DL-specific DEGs (upper one) and the Pietrain specific transcriptome network presented in lower part of the figure.

The Landrace pigs used in this study had a massive transcriptional response to PRRSV vaccination, as evidenced by differential expression of more than double the number of genes

as compared with the Pietrain pigs (Fig 1A). The higher number of vaccine induced DEGs in PBMCs of DL compared to that of Pietrain indicated that PRRSV vaccine is able to mount an effective immunity in DL pigs than Pietrain. These findings are closely comparable with a previous report of Ait-Ali et al (2011), who compared the microarray-based gene expression profiling of lung tissue after PRRSV infection in between Landrace and Pietrain pigs. The DL pigs showed a higher number of DEGs within 12h post infection compared to that of Pietrain pigs (Ait-Ali et al. 2011). Another independent study has also reported the difference on host susceptibility between DL and Pietrain in response to porcine circovirus infection (Opriessnig et al. 2009). The variation of susceptibility to PRRSV infection has been reported in some other breed comparisons in pigs as well. For example, macrophage of Large White pigs showed more reactive to in-vitro PRRSV infection than Duroc-Pietrain synthetic line (Vincent et al. 2005). The Hampshire-Duroc cross pigs found to be more susceptible to in-vivo PRRSV infection than NE Index Lines pigs (Petry et al. 2005). Halbur et al (1998) infected Duroc, Hampshire, and Meishan pigs with PRRS virus (VR-2385) at 22 to 38 days of age and compared the cytopathic lesions 10 days post infection. Hampshire pigs had significantly more severe lung lesions than Duroc or Meishan pigs. Meishan pigs had significantly less PRRS virus detected in the lungs, but significantly more heart and brain lesions. Duroc pigs had significantly lower serum antibody titers against PRRS virus (Halbur et al. 1998). Generally, the genetic configuration of each breed can display their specific pattern of coping strategy against stressors which in turn leads the variation of host susceptibility (Lewis et al. 2007). Therefore, we postulate that the variation of transcriptome profiles observed between DL and Pi pigs could, at least in part, be influenced by breed genetics.

A global upregulation of altered transcripts were observed in vaccinated PBMCs of DL pigs indicated that PRRSV vaccine can activate the immune system of German Landrace pigs and may lead to develop better immunocompetence (Fig 1B). On the other hand, PRRSV vaccination resulted in a global down regulation of PBMCs transcriptomes in Pietrain pigs indicating the suppression of immune system functions. This difference may be due to breed-specific host immune response to PRRSV vaccination as indicated by enrichment of different set of biological pathways by breed-specific DEGs in PBMCs (Table 2). The host transcriptional response to PRRSV challenge has been reported to be associated with the activation of well defined canonical pathways like TREM1, toll-like receptor and hyper-cytokinemia/hyper-chemokinemia signaling (Badaoui et al. 2013). There was over expression of member genes (IFN6 and MX1) of interferon alpha/beta pathways in vaccinated PBMCs of

DL pigs compared to that of Pietrain pigs. It was an indication for PRRSV vaccine potential for developing interferon response at least in some extends in PBMCs of DL pigs. The vaccine pulsed immune cells secrete the type I IFN which interacts with a subset of naïve T cells to promote their conversion into virus-specific IFNγ secreting cell, thereby induce the cell mediated interferon response, a strong anti-viral defense (Levy et al. 2003). The early induction of a type I interferon (IFN) response in vitro may be responsible for the reduced susceptibility of Landrace pig macrophages to PRRSV replication (Ait-Ali et al. 2011). Therefore we speculate that, unlike in Pietrain pigs, the early stage development of vaccine mediated immunocompetence in DL pigs might lead to a reduced susceptibility and/or higher tolerance to PRRSV infection.

The immune response traits are likely to be regulated by multiple genes which interact with each other through an interconnecting network (Gardy et al. 2009). Here we performed the network analysis to scrutinize the regulatory genes from the list of vaccine induced DEGs which were common in PBMCs of both breeds (Fig 4), DEGs which were more abundant in DL (Fig 6A) and DEGs which were more abundant in Pietrain pigs (Fig 6B). There are many genes which were differentially expressed in PBMCs after PRRSV vaccination in both breeds but their regulation of expression were mostly in the opposite direction (Table1). The difference in the relative expressions of genes commonly altered in both breeds may be caused by variation of functional regulation individual genes. We therefore checked which genes are more potential regulators of the functional network of shared DEGs. Network analysis detected SLC9A2, NACC1, DCTN3, RRS1, BAG3, BUD31, ATP5J2, ARPC1B, ASAP2, EIF3I and CSN2 as the potential hubs of the functional network of shared DEGs (Fig 4). The predicted hub genes of the network are likely to promote or inhibit the expression of other connecting genes to maintain the biological function (Macneil and Walhout 2011).

Among the hub genes of common network, SLC9A2 is one of the potential hub genes, involved with intracellular pH regulation, and colonic sodium absorption. The SLC9A2 has reported to be predictive for distinguishing between colon adenomatous polyp and carcinoma (Drew et al. 2014). While another hub gene, NACC1 has been reported to be involved with argininemia, an inherited metabolic disease resulted from L-arginine deficiency in human (Wong et al. 2014). The mutations in the SLC7A7 gene cause the lysinuric protein intolerance, an autosomal recessive defect of dibasic amino acid transport, leading to the argininemia (Kamada et al. 2001), which indicates the functional interaction of NACC and

SLC protein family. The RRS1, another hub gene, reported to be involved with disease resistance in plant (Narusaka et al. 2009), and the RRS1-mouse homolog showed altered expression in mouse model for Huntington's disease (Horigome et al. 2011). The DCTN3 is a protein coding gene, involved with diverse array of cellular functions, including cell division and cytokinesis (Karki et al. 1998). The BAG3 is a protein coding gene which is reported to be involved with heat stress and apoptosis pathway. The mutations of BAG3 gene have been implicated as a novel cause of dilated cardiomyopathy in human (Franaszczyk et al. 2014). The ARPC1B is known to be involved with bacterial invasion of epithelial cells and has been reported as prediction marker gene for sensitivity of choroidal malignant melanoma to radiotherapy (Kumagai et al. 2006). The ASAP2, another hub gene, is known to be involved with enrichment of Fc gamma R-mediated phagocytosis pathway (Uchida et al. 2001). The eIF3I is a protein coding gene, and its overexpression involve with the integration of growth signals by mTOR into the mRNA translation process, promoting protein synthesis and tumor growth (Ahlemann et al. 2006). Overall, the hub genes seem to be involved with cellular immune response to disease process but have not been linked well to the PRRSV vaccine mediated immunity in pig before. Therefore, hubs of the shared transcriptome network could be used as candidate genes for expression studies in other porcine breed lines following PRRSV vaccination.

The network analysis also revealed a number of genes which are likely to have control over the PRRSV vaccine induced transcriptome network specific for both DL and Pi pigs (Fig 6A, B). This is comparable with the report of Xing et al (Xing et al. 2014), who identified breed specific gene signatures through comparing the microarray-based global gene expression profiles of lung tissue samples from Dapulian pigs (DPL, a Chinese indigenous breed) and Duroc×Landrace×Yorkshire (DLY) pigs after infection with PRRSV and postulate that USP18 might play important role in the resistance of DPL pigs to PRRSV infection (Xing et al. 2014). Network analysis of breed-specific differentially expressed transcripts indicated that STAT1, MMS19, RPA2, BAD, UCHL5 and APC as potential regulatory genes for the transcriptional activity in the DL pigs after PRRSV vaccination (Fig 6A). Among the hub genes of Landrace-specific network, STAT1 is a highly interconnected one, which is a protein coding gene of the signal transducer and transcription activator (STAT) protein family. The STATs mediate cellular responses to interferons (IFNs), cytokines and other growth factors involved in antiviral innate immunity (Koyama et al. 2008). Network analysis also revealed that FOXO3, IRF2, ADRBK1, FHL3, PPP2CB, MTOR, RPL8, DICER1, FLNC and NCOA6

as potential regulatory genes for PRRSV induced transcriptional activity in Pietrain pigs (Fig 6B). All of these genes were upregulated in vaccinated PBMCs of Pietrain pigs compared to that of DL, suggesting the Pietrain breed specific transcript signature for PRRSV vaccine response in PBMCs. Among the hubs, FOXO3 is the highly interconnected gene, which belongs to the fork head family of transcription factors. FOXO3 functions as a trigger for apoptosis through expression of genes necessary for cell death and participates in post-transcriptional regulation of MYC transcription factor which has been reported to be associated with host response to PRRSV (Badaoui et al. 2013).

Healthy pigs of two breeds were also differed greatly in their transcriptome profiles before vaccination. It suggests that differential gene expression levels may be also caused by the genetic differences between DL and Pi pigs regardless of vaccine stimulation. Several researchers have reported genetic variation in immune traits in healthy pigs (Edfors-Lilja et al. 1994, Flori et al. 2011). Differences in gene expression between phenotypic groups irrespective of infection could be due to the different genetic background of different breeds of pigs (Lunney and Chen 2010). The variation in number and function of neutrophils, monocytes and lymphocyte subsets in blood has been reported between healthy Meishan and Large white pigs (Clapperton et al. 2005). Therefore, even irrespective of external stimulation, the PBMC transcriptome profiles of two porcine breeds are different from each other.

4.6 Conclusions

This study provided with the evidence of host genetic variation in PRRSV vaccine induced gene expression in between German Landrace and Pietrain pigs. A much higher number of gene transcripts were differentially expressed in PMBCs after PRRSV vaccination in Landrace pigs compared to that of Pietrain pigs. The breed-specific differentially expressed genes were biologically linked to innate immune response. The PBMCs transcriptome profiles were also differed between healthy Landrace and Pietrain pigs. Results of this study support the prospects of selective breeding of PRRS resistant pig to establish a sustainable PRRS control regime. The expression patterns of potential hub genes of the shared transcriptome network between Landrace and Pietrain pigs could be tested further in other pig breeds following PRRSV vaccination. The breed-specific gene transcripts identified in this study could be of potential candidate for further functional to explore the polymorphism linked to PRRSV vaccine responses.

Abbreviations

DEGs = Differentially expressed genes; DL = German Landrace; GO = Gene ontology; Pi = Pietrain; PBMCs = Peripheral blood mononuclear cells; PRRS = Porcine reproductive and respiratory syndrome; PRRSV = PRRS virus

4.7 Declarations

4.7.1 Conflict of interest

Authors declare there is no conflict of interest

4.7.2 Author contributions

MAI, CN and KS conceived and designed the experiments; CN and DT arranged kits and reagents; MAI, MH and MP performed field experiment and blood sampling; MAI and SAR performed the microarray experiments, MAI analyzed and interpreted the data; MAI drafted the manuscript. MJU, CN, MP, KS reviewed and edited the manuscripts. All authors have seen and approved the submitted version.

4.7.3 Funding

This work was financially supported by the "Umweltvertragliche und Standortgerechte Landwirtschaft" (USL) project of North Rhine-Westphalia, Germany with the grant number 17-02.04.01-4/15.

4.7.4 Acknowledgements

Authors are thankful to the stuffs of Teaching and Research station at Frankenfrost for the animal husbandry support.

[The additional excel files in support of the results presented in this chapter are available at the internal database of our research group, Institutes of Animal Science, Uni-Bonn; www.citreon.itw.uni-bonn.de]

4.8 References

Abella G, Pena RN, Nogareda C, Armengol R, Vidal A, Moradell L, Tarancon V, Novell E, Estany J, Fraile L (2016): A WUR SNP is associated with European Porcine

Reproductive and Respiratory Virus Syndrome resistance and growth performance in pigs. Res Vet Sci 104, 117-122

Ahlemann M, Zeidler R, Lang S, Mack B, Munz M, Gires O (2006): Carcinoma-associated eIF3i overexpression facilitates mTOR-dependent growth transformation. Mol Carcinog 45, 957-967

Ait-Ali T, Wilson AD, Carre W, Westcott DG, Frossard JP, Mellencamp MA, Mouzaki D, Matika O, Waddington D, Drew TW, Bishop SC, Archibald AL (2011): Host inhibits replication of European porcine reproductive and respiratory syndrome virus in macrophages by altering differential regulation of type-I interferon transcriptional response. Immunogenetics 63, 437-448

Ait-Ali T, Wilson AW, Finlayson H, Carre W, Ramaiahgari SC, Westcott DG, Waterfall M, Frossard JP, Baek KH, Drew TW, Bishop SC, Archibald AL (2009): Functional analysis of the porcine USP18 and its role during porcine arterivirus replication. Gene 439, 35-42

Badaoui B, Tuggle CK, Hu Z, Reecy JM, Ait-Ali T, Anselmo A, Botti S (2013): Pig immune response to general stimulus and to porcine reproductive and respiratory syndrome virus infection: a meta-analysis approach. BMC Genomics 14, 1471-2164

Benjamini Y, Hochberg Y (1995): Controlling the false discovery rate: A typical adn powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 57, 289 - 300

Beutler B (2004): Innate immunity: an overview. Mol Immunol 40, 845-859

Boddicker N, Waide EH, Rowland RR, Lunney JK, Garrick DJ, Reecy JM, Dekkers JC (2012): Evidence for a major QTL associated with host response to porcine reproductive and respiratory syndrome virus challenge. J Anim Sci 90, 1733-1746

Breuer K, Foroushani AK, Laird MR, Chen C, Sribnaia A, Lo R, Winsor GL, Hancock RE, Brinkman FS, Lynn DJ (2013): InnateDB: systems biology of innate immunity and beyond--recent updates and continuing curation. Nucleic Acids Res 41, 24

Carvalho BS, Irizarry RA (2010): A framework for oligonucleotide microarray preprocessing. Bioinformatics 26, 2363-2367

Chaussabel D (2015): Assessment of immune status using blood transcriptomics and potential implications for global health. Semin Immunol 27, 58-66

Christopher-Hennings J, Holler LD, Benfield DA, Nelson EA (2001): Detection and duration of porcine reproductive and respiratory syndrome virus in semen, serum, peripheral

blood mononuclear cells, and tissues from Yorkshire, Hampshire, and Landrace boars. J Vet Diagn Invest 13, 133-142

Clapperton M, Bishop SC, Glass EJ (2005): Innate immune traits differ between Meishan and Large White pigs. Vet Immunol Immunopathol 104, 131-144

Drew JE, Farquharson AJ, Mayer CD, Vase HF, Coates PJ, Steele RJ, Carey FA (2014): Predictive gene signatures: molecular markers distinguishing colon adenomatous polyp and carcinoma. PLoS One 9,

Edfors-Lilja I, Wattrang E, Magnusson U, Fossum C (1994): Genetic variation in parameters reflecting immune competence of swine. Vet Immunol Immunopathol 40, 1-16

Flori L, Gao Y, Laloe D, Lemonnier G, Leplat JJ, Teillaud A, Cossalter AM, Laffitte J, Pinton P, de Vaureix C, Bouffaud M, Mercat MJ, Lefevre F, Oswald IP, Bidanel JP, Rogel-Gaillard C (2011): Immunity traits in pigs: substantial genetic variation and limited covariation. PLoS One 6, 29

Franaszczyk M, Bilinska ZT, Sobieszczanska-Malek M, Michalak E, Sleszycka J, Sioma A, Malek LA, Kaczmarska D, Walczak E, Wlodarski P, Hutnik L, Milanowska B, Dzielinska Z, Religa G, Grzybowski J, Zielinski T, Ploski R (2014): The BAG3 gene variants in Polish patients with dilated cardiomyopathy: four novel mutations and a genotype-phenotype correlation. J Transl Med 12, 1479-5876

Gardy JL, Lynn DJ, Brinkman FS, Hancock RE (2009): Enabling a systems biology approach to immunology: focus on innate immunity. Trends Immunol 30, 249-262

Glass EJ (2012): The molecular pathways underlying host resistance and tolerance to pathogens. Front Genet 3, doi: http://dx.doi.org/10.3389/fgene.2012.00263

Halbur PG, Rothschild MF, Thacker BJ, Meng XJ, Paul PS, Bruna JD (1998): Differences in susceptibility of Duroc, Hampshire, and Meishan pigs to infection with a high virulence strain (VR2385) of porcine reproductive and respiratory syndrome virus (PRRSV). Journal of Animal Breeding and Genetics 115, 181-189

Horigome C, Okada T, Shimazu K, Gasser SM, Mizuta K (2011): Ribosome biogenesis factors bind a nuclear envelope SUN domain protein to cluster yeast telomeres. Embo J 30, 3799-3811

Islam MA, Grosse-Brinkhaus C, Proll MJ, Uddin MJ, Rony SA, Tesfaye D, Tholen E, Holker M, Schellander K, Neuhoff C (2016): Deciphering transcriptome profiles of peripheral blood mononuclear cells in response to PRRSV vaccination in pigs. BMC Genomics 17:641, doi: https://dx.doi.org/10.1186/s12864-016-2849-1

Kamada Y, Nagaretani H, Tamura S, Ohama T, Maruyama T, Hiraoka H, Yamashita S, Yamada A, Kiso S, Inui Y, Ito N, Kayanoki Y, Kawata S, Matsuzawa Y (2001): Vascular endothelial dysfunction resulting from L-arginine deficiency in a patient with lysinuric protein intolerance. J Clin Invest 108, 717-724

Karki S, LaMonte B, Holzbaur EL (1998): Characterization of the p22 subunit of dynactin reveals the localization of cytoplasmic dynein and dynactin to the midbody of dividing cells. J Cell Biol 142, 1023-1034

Koyama S, Ishii KJ, Coban C, Akira S (2008): Innate immune response to viral infection. Cytokine 43, 336-341

Kumagai K, Nimura Y, Mizota A, Miyahara N, Aoki M, Furusawa Y, Takiguchi M, Yamamoto S, Seki N (2006): Arpc1b gene is a candidate prediction marker for choroidal malignant melanomas sensitive to radiotherapy. Invest Ophthalmol Vis Sci 47, 2300-2304

Labarque G, Van Gucht S, Van Reeth K, Nauwynck H, Pensaert M (2003): Respiratory tract protection upon challenge of pigs vaccinated with attenuated porcine reproductive and respiratory syndrome virus vaccines. Vet Microbiol 95, 187-197

Levy DE, Marie I, Prakash A (2003): Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity. Curr Opin Immunol 15, 52-58

Lewis CR, Ait-Ali T, Clapperton M, Archibald AL, Bishop S (2007): Genetic perspectives on host responses to porcine reproductive and respiratory syndrome (PRRS). Viral Immunol 20, 343-358

Li Y, Sun Y, Xing F, Kang L, Wang P, Wang L, Liu H, Jiang Y (2014): Identification of a single nucleotide promoter polymorphism regulating the transcription of ubiquitin specific protease 18 gene related to the resistance to porcine reproductive and respiratory syndrome virus infection. Vet Immunol Immunopathol 162, 65-71

Loving CL, Osorio FA, Murtaugh MP, Zuckermann FA (2015): Innate and adaptive immunity against Porcine Reproductive and Respiratory Syndrome Virus. Vet Immunol Immunopathol 167, 1-14

Lunney JK, Chen H (2010): Genetic control of host resistance to porcine reproductive and respiratory syndrome virus (PRRSV) infection. Virus Res 154, 161-169

Lunney JK, Steibel JP, Reecy JM, Fritz E, Rothschild MF, Kerrigan M, Trible B, Rowland RR (2011): Probing genetic control of swine responses to PRRSV infection: current progress of the PRRS host genetics consortium. BMC Proc 3, 1753-6561

Macneil LT, Walhout AJ (2011): Gene regulatory networks and the role of robustness and stochasticity in the control of gene expression. Genome Res 21, 645-657

Mudunuri U, Che A, Yi M, Stephens RM (2009): bioDBnet: the biological database network. Bioinformatics 25, 555-556

Murtaugh MP, Xiao Z, Zuckermann F (2002): Immunological responses of swine to porcine reproductive and respiratory syndrome virus infection. Viral Immunol 15, 533-547

Narusaka M, Shirasu K, Noutoshi Y, Kubo Y, Shiraishi T, Iwabuchi M, Narusaka Y (2009): RRS1 and RPS4 provide a dual Resistance-gene system against fungal and bacterial pathogens. Plant J 60, 218-226

Nelsen CJ, Murtaugh MP, Faaberg KS (1999): Porcine reproductive and respiratory syndrome virus comparison: divergent evolution on two continents. J Virol 73, 270-280

Opriessnig T, Patterson AR, Madson DM, Pal N, Rothschild M, Kuhar D, Lunney JK, Juhan NM, Meng XJ, Halbur PG (2009): Difference in severity of porcine circovirus type two-induced pathological lesions between Landrace and Pietrain pigs. J Anim Sci 87, 1582-1590

Petry DB, Holl JW, Weber JS, Doster AR, Osorio FA, Johnson RK (2005): Biological responses to porcine respiratory and reproductive syndrome virus in pigs of two genetic populations. J Anim Sci 83, 1494-1502

Reiner G, Willems H, Pesch S, Ohlinger VF (2010): Variation in resistance to the porcine reproductive and respiratory syndrome virus (PRRSV) in Pietrain and Miniature pigs. J Anim Breed Genet 127, 100-106

Rowland RR, Lunney J, Dekkers J (2012): Control of porcine reproductive and respiratory syndrome (PRRS) through genetic improvements in disease resistance and tolerance. Front Genet 3, 260

Siegrist C-A (2012): General aspects of vaccinations. 6th Elsevier Health Sciences Saunders, USA.

Smyth GK (2005 pp.397-442): Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, editors. Bioinformatics and computational biology solutions using r and bioconductor. Springer pp. 397–342

Uchida H, Kondo A, Yoshimura Y, Mazaki Y, Sabe H (2001): PAG3/Papalpha/KIAA0400, a GTPase-activating protein for ADP-ribosylation factor (ARF), regulates ARF6 in Fcgamma receptor-mediated phagocytosis of macrophages. J Exp Med 193, 955-966

Vestal DJ, Jeyaratnam JA The Guanylate-Binding Proteins: Emerging Insights into the Biochemical Properties and Functions of This Family of Large Interferon-Induced

Guanosine Triphosphatase. J Interferon Cytokine Res. 2011 Jan;31(1):89-97. doi:10.1089/jir.2010.0102.,

Vincent AL, Thacker BJ, Halbur PG, Rothschild MF, Thacker EL (2005): In vitro susceptibility of macrophages to porcine reproductive and respiratory syndrome virus varies between genetically diverse lines of pigs. Viral Immunol 18, 506-512

Wong D, Cederbaum S, Crombez EA (2014): Arginase deficiency.

Xia J, Benner MJ, Hancock RE (2014): NetworkAnalyst--integrative approaches for protein-protein interaction network analysis and visual exploration. Nucleic Acids Res 42, 26

Xing J, Xing F, Zhang C, Zhang Y, Wang N, Li Y, Yang L, Jiang C, Wen C, Jiang Y (2014): Genome-wide gene expression profiles in lung tissues of pig breeds differing in resistance to porcine reproductive and respiratory syndrome virus. PLoS One 9(1), e86101

Xu D, Lillico SG, Barnett MW, Whitelaw CB, Archibald AL, Ait-Ali T (2012): USP18 restricts PRRSV growth through alteration of nuclear translocation of NF-kappaB p65 and p50 in MARC-145 cells. Virus Res 169, 264-267

Zhang X, Shin J, Molitor TW, Schook LB, Rutherford MS (1999): Molecular responses of macrophages to porcine reproductive and respiratory syndrome virus infection. Virology 262, 152-162

Chapter 5: Integrated miRNA-mRNA network for PRRSV vaccine responses

(Manuscript under preparation)

Integrated network analysis for miRNAs and mRNAs expressed in PRRSV vaccinated peripheral blood mononuclear cells of pigs

Md. Aminul Islam[1,2], Maren Julia Pröll[1], Sharmin Aqter Rony[1], Christine Große-Brinkhaus[1], Muhammad Jasim Uddin[2,3], Michael Hoelker[1,4], Dawit Tesfaye[1], Ernst Tholen[1], Karl Schellander[1,4], Christiane Neuhoff[1,*]

[1]Institute of Animal Science, University of Bonn, Endenicher Allee 15, 53115 Bonn, Germany

[2]Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Mymensingh-2202, Bangladesh

[3]School of Veterinary Science, The University of Queensland, Gatton Campus, QLD 4343, Australia

[4]Teaching and Research Station on Frankenfrost, Faculty of Agriculture, University of Bonn, Konigswinter, Germany

Author's email:
MAI: aisl@itw.uni-bonn.de
CN: cneu@itw.uni-bonn.de
SAR: sron@itw.uni-bonn.de
CGB: cgro@itw.uni-bonn.de
MJU: jasimmedbau@gmail.com
MH: mhoe@itw.uni-bonn.de
DT: dtes@itw.uni-bonn.de
ET: etho@itw.uni-bonn.de
KS: ksch@itw.uni-bonn.de
MJP: mpro@itw.uni-bonn.de

*Correspondence to:
Christiane Neuhoff, PhD
Institute of Animal Science, University of Bonn
Endenicher Allee 15, 53115 Bonn, Germany
Email: mpro@itw.uni-bonn.de
Phone: 0049-228/73-3583
Fax: 0049-00228/73-2284

Article type and status: Research and new

Running title: Integrated miRNA-mRNA network for PRRSV vaccine response in PBMCs

Keywords: PRRSV vaccine, miRNA-miRNA network, PBMCs, microarray, immunity

5.1 Abstract

The porcine reproductive and respiratory syndrome (PRRS) is the most costly disease of swine industry through out the world. MicroRNAs, small non coding RNAs, posttranscriptional regulator of gene expression, have been emerged as potential tools for evaluating host immune response to infection or vaccination. In a previous study, we showed that peripheral mononuclear cells (PBMCs) are able to alter the global mRNA expression profiles during the course of innate immune response following PRRS virus (PRRSV) vaccination in pigs. We extended our aim herein to integrate the miRNA profiles with the mRNA profiles to uncover the miRNA-mRNA regulated host immune response to PRRSV vaccines in PBMCs. The current study generated miRNA profiles of PBMCs collected at before (0 h), and 6, 24 and 72 h post PRRSV vaccination both in German Landrace (DL) and Pietrain (Pi) pigs with three biological replicates. The global miRNA profiles of PBMCs identified 12, 259 and 14 differentially expressed (DE) miRNAs in DL; and 0, 222 and 13 DE miRNAs in Pietrain at 6, 24 and 72 h post vaccination, respectively. The validated target genes of DE miRNAs are involved with regulation of biological process like response to drug, signal transduction, innate immune response regulation of MAPK kinase activity and apoptosis process. We integrated the miRNA expression dataset obtained from the DL pigs with that of mRNA expression profiles generated from the same sample pool. The miRNA and gene co-regulatory network revealed that miR-6762, miR-23a-5p, miR-181b-5p, miR-4454 and miR-125-5p are the putative regulators of PRRSV vaccine induced gene expression changes in PBMCs.

5.2 Introduction

The porcine reproductive and respiratory syndrome (PRRS) is the most costly swine disease worldwide. The PRRS results reproductive failure in pregnant sows and respiratory distress with high mortality in young pigs (Albina 1997, Neumann et al. 2005). The PRRS associated annual economic losses estimated to be around € 1.5 billion in Europe (De Paz 2015) and around $664 million in USA (Holtkamp et al. 2013). The disease caused by the PRRS virus (PRRSV), which is an enveloped, single-stranded positive-sense RNA virus of the Arteriviridae family (Meulenberg et al. 1993). Like other arteriviruses, PRRSV primarily infect the pulmonary alveolar macrophage (PAMs) of pig following natural infection (Van Breeam et al. 2010) and is reported to modulate the host immune system (Genini et al. 2008). Despite the incomplete success, vaccination remains the cornerstone of PRRS control strategy. Following intramuscular vaccination, the peripheral blood mononuclear cells

(PBMCs), a subset of white blood cells (WBC), come first in contact to vaccine antigen and initiate the immune reaction (Siegrist 2012). Therefore blood-based investigation on the molecular genetics of host immune response to in vivo PRRSV vaccination would be worthwhile.

Vaccines increase the host resistance to disease by priming the immune system for responding to the causal virus. Following administration, the virus vaccine antigens are initially recognized by the innate immune cells through specific molecules of pathogens via a limited number of germline-encoded pattern recognition receptors (PRRs); (Akira et al. 2006). The engagement of PRRs by the invading PAMPs leads to transcriptional changes associated with induction of type I interferon as well as proinflammatory responses (Akira et al. 2006). As the fist line body defense, innate immunity occurs within hours of exposure to the pathogen. MicroRNAs (miRNAs) have recently emerged as key gene-regulators and have been shown to play an important role in innate immune response to infections (O'Connell et al. 2010, O'Neill et al. 2011).

miRNAs are endogenous, non-protein-coding single stranded RNAs ranging from 19 to 24 nucleotides in length (Bartel 2004). Emerging evidence suggest that miRNAs are tightly involved in process of virus-host interaction including virus replication (Guo et al. 2013, Li et al. 2010, Trobaugh et al. 2014) and host antiviral immune responses (Chen et al. 2013, Hussain and Asgari 2010). Moreover, miRNAs are likely to be biomarkers for immunocompetence developed from PRRSV vaccination. Previous analysis of miRNA expression profiles obtained from PRRSV infected alveolar macrophages have identified differential expression of forty cellular miRNAs within the first 48 hours of infection (Julie et al. 2013). This was suggestive that miRNAs are likely important mediators of PRRSV replication and host antiviral defense. Functional studies based on pulmonary alveolar macrophages stimulated with in-vitro PRRSV indicated that miR-181 and miR-23a inhibited PRRSV replication through binding to PRRSV genome (Guo et al. 2013, Zhang et al. 2014), while miR-181, miR-125b and miR-506 suppressed PRRSV replication through regulating host antiviral pathways (Gao et al. 2013, Wang et al. 2013, Wu et al. 2014). However, little is known about the expression dynamics and regulation of microRNAs in PBMCs following PRRSV vaccination in pig. The PBMCs are the primary immune cells of blood, and have been used as a preferred model for evaluating the host transcriptional response to vaccination

in pig (Adler et al. 2013). Therefore, global miRNA expression profiles of PBMCs could provide better insights of mRNA mediated host immune response to PRRSV vaccination in pig.

As with messenger RNA (mRNA) expressions profiles, variability in the miRNA expression profiles is likely to be influenced by host genotype. Breed differences on the PRRSV induced global mRNA expression profiles have been reported by several groups (Reiner et al. 2010, Ait-Ali et al. 2011, Xing et al. 2014), like ours [Proll et al. 2016 (unpublished); Chapter 4 of this thesis]. In addition, the tissue-specific expression and regulation of miRNAome has also been studied in pig (Martini et al. 2014). The miRNA signatures for different porcine tissues such as skeletal muscle (McDaneld et al. 2012), adipose tissue (Li et al. 2012), intestinal tracts (Sharbati et al. 2010), kidney (Timoneda et al. 2013) and brain tissue (Podolska et al. 2011) have been identified. The breed comparison on global miRNA expression profiles obtained from skeletal muscle (Tang et al. 2015), kidney (Timoneda et al. 2013), testis (Li et al. 2016) and placenta (Li et al. 2015a) were led to the identification of breed-specific miRNAs, which could be potentially associated to specific phenotypes. The breed-specific miRNA signatures for host immune response to PRRSV infection in the lungs between Tongcheng pigs and Landrace pigs have recently been reported (Li et al. 2015b). We therefore, hypothesized that variation on PRRSV vaccine induced miRNA profiles between DL and Pi pigs may exist.

The interaction of miRNA-mRNA can result the downregulation of protein expression due to translational repression, mRNA cleavage, or promotion of mRNA decay (Kim 2005). miRNAs have extensive regulatory capacity given that a single miRNA can simultaneously target multiple genes, and multiple miRNAs can co-operatively function while targeting a single gene (Krek et al. 2005, Miranda et al. 2006). However, a clear correlation is known to exist between the expression patterns of miRNAs and their mRNA targets (Farh et al. 2005, Selbach et al. 2008), therefore, miRNA-target relationship analysis has been increasingly used to identify potential interactions between miRNA and mRNA based on paired expression profiles (Ruike et al. 2008, Tian et al. 2008, Gennarino et al. 2009, Siengdee et al. 2013, Jing et al. 2015). With the approach of miRNA and mRNA interaction network, significant molecular insights have been reported on skeletal muscle development (Siengdee et al. 2013) and differential residual feed intake (Jing et al. 2015) in pig. Furthermore, the integrated miRNA-mRNA network for strain-specific (Cong et al. 2014) and breed-specific (Li et al. 2015b) host immune response to PRRSV infection have been studied based on comparative global expression of miRNA and mRNA profiled in the lung tissue. In our previous studies,

we observed a massive gene expression changes in PBMCs at early stage after PRRSV vaccination in DL (Islam et al. 2016a) and Pi (Islam et al. 2016b) pigs. Herein, we conducted the microarray-based global miRNA expression profiling in the same total RNA samples as used for global mRNA profiling, to investigate the breed-specific miRNAome alterations as well as to explore the miRNA-mRNA co-regulatory network for PRRSV vaccine responses in PBMCs.

5.3 Materials and methods

5.3.1 Ethics statements

The research proposal was approved by the Veterinary and Food Inspection Office, Siegburg, Germany (ref. 39600305-547/15). The in-vivo experiment was conducted according to the institutional guidelines and animal husbandry regulations of Germany (ZDS 2003). The blood sampling protocol was approved by the State Agency for Nature, Environment and Consumer Protection, North Rhine-Westphalia, Germany (permission nr. 84-02.05.04.14.027).

5.3.1 Experimental settings and RNA sample preparation

The study pigs were housed in the Teaching and Research Station at Frankenfrost, University of Bonn, Germany. Three female piglets both from DL and Pi pigs were vaccinated with a modified live PRRSV vaccine of European strain (Porcilis® PRRS, MSD Animal Health, Germany) at four weeks of age. The whole blood sampling was performed at before (0), 6, 24 and 72 h post vaccination. The PBMCs were isolated from the whole blood samples by density gradient centrifugation at 1250× g for 25 minutes using Histpaque®-1077 (Sigma-Aldrich Co. WGK, Germany). The PBMCs were then subjected to extract the total RNA enriched with miRNAs using miRNeasy mini kit (Qiagen, Co.) along with on column DNase treatment. The RNA was quantified using ND 8000 NanoDrop® spectrophotometry (Thermo Scientific, Wilmington, USA) followed by quality assessment on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies, Waghäusel - Wiesental, Germany).

5.3.2 Microarray hybridization for microRNA expression profiling

A total of 24 miRNA profiles of PBMCs were generated from PRRSV vaccinated pigs of two breed at four longitudinal time points. About 250 ng of total RNA were used to synthesize microarray probes using a 4DNA array detection FlashTag™ Biotin HSR RNA Labelling kit (Affymetrix, Santa Clara, CA, USA) following the manufacturer's instructions. Initially, poly-A tailing of total RNA was performed followed by labeling with biotin. The ELOSA assay

was performed to evaluate the biotin labeling performance. The hybridization of microarray probes followed by washing and staining was performed using the GeneChip® Hybridization, Wash and Stain kit (Affymetrix Inc., Santa Clara, CA). For the hybridization, a cocktail containing the biotinylated total RNA probes was injected into the GeneChip® miRNA 4.0 array (Affymetrix Inc., Santa Clara, CA, USA) and incubated for 16 h in a hybridization oven (GeneChip® Hybridization oven 640; Affymetrix Inc.) at 45 °C with 60 rpm. The hybridized chips were stained and washed within a fluidics station (GeneChip® Fluidics Station 450; Affymetrix Inc.) and scanned by Affymetrix GeneChip® scanner 3000 7G. The Affymetrix GeneChip® Command Console™ (AGCC) software was used to evaluate the array images and to export the reports of spot intensity data.

5.3.2 Statistical analysis for identification of differentially expressed miRNAs

After quality control the microarray data was normalized using the RMA based approach using the "oligo" package implemented in R/Bioconductor platform (Gentleman et al. 2004). To identify the differentially expressed miRNAs, the normalized probes were analyzed using the linear analysis of microarray technique from the *"*limma" package with empirical Bayes adjustment to the variance (Smyth 2005). To make the analysis more robust and control more strictly for the false discovery rate (FDR), the *p*-values were corrected for multiple testing with Benjamini and Hochberg (BH) method (Benjamini and Hochberg 1995). Threshold criteria for the miRNA to be considered differentially expressed were set as of FDR < 0.05 and $\log_2$ fold-change >1.0 or <-1.0. The number of differentially expressed miRNAs in each contrast pair and their overlapping were exported in an intersecting Venn diagram. The *heatmap.2* function from "ggplots" package was used to generate images.

5.3.3 In-silico target prediction for DE miRNAs

Both TargetScan v.7.1 (Bartel 2009) and miRDB v5.0 (Nathan and Wang 2015) were used to predict the target gene candidates based on complementarity of the miRNA seed sequence (position 2-8 of the miRNA 5′-end) and target binding site on the 5′ UTR, 3′ UTR and protein coding region of the porcine mRNA sequences (Sus scrofa 10.2); (Lewis et al. 2005). The miRDB server utilizes the miRNAs source from miRBase v21 and implements the MirTarget prediction algorithm (Xiaowei 2016). The combined list of predicted mRNA targets obtained from both tools was processed further.

5.3.4 The integrated miRNA-mRNA network analysis

The miRNA-mRNA interactome networks were constructed for PRRSV vaccine response in PBMCs as previously described with minor modifications (Coll et al. 2015). First, we refined the list of predicted targets scanned for potential target genes of DE miRNAs. For accomplishing this, we used the differentially expressed genes (DEGs) list obtained from our previous microarray-based mRNA expression data (GEO accession number GSE76254, (Islam et al. 2016)) to integrate with the differentially expressed miRNAs. The overlapped results from predicted mRNA targets and DEGs in PBMCs were extracted as true differentially expressed target genes (TDETGs) of the DE miRNAs. In a second phase of the integration procedure we identified those miRNA-target pairs showing negative correlation between miRNA and mRNAs. To accomplish this, Pearson correlation of the expressions of all possible combinations of deregulated mRNAs vs deregulated miRNAs were computed. Multiple testing correction was performed in order to reduce the number of false positive correlations and a cut-off was set to FDR < 0.05. Finally, the miRNA-mRNA interaction pairs with significant negative correlation (Pearson coefficient < 0 and FDR < 0.05) were used to construct the regulatory network. The miRNA-mRNA network was visualized using the Cytoscape v3.2.1 (Cline et al. 2007).

5.3.5 GO and pathway analysis

The list of inversely correlated true differentially expressed target genes (TDETGs) obtained from miRNA-mRNA interaction network were subjected to GO and pathway enrichment analysis using the InnateDB pathway analysis tool (Breuer et al. 2013). The hypergeometric test was used to calculate a *p*-value followed by B-H multiple test correction method (Benjamini and Hochberg 1995). An adjusted *p*-value of <0.05 was considered for statistical significance.

5.4 Results

To understand the role of porcine cellular miRNAs in PRRSV vaccine immunity, we performed global expression profiling of miRNAs in the PBMCs obtained from German Landrace and Pietrain pigs vaccinated with modified live attenuated PRRSV of EU strain at before (0 h), and 6, 24 and 72 h post vaccination. The GeneChip® miRNA v. 4.0 arrays used for this study encoded 30,424 total mature miRNA probe sets, including 2,578 mature human miRNAs and miRNAs of 202 other organisms (miRBase v.20).

5.4.1 Temporal expression dynamics of miRNAs after PRRSV vaccination

The time-course distributions of differentially expressed miRNAs in the immunized PBMCs are presented in Fig 1. Imposing the threshold as FDR <0.05 and log fold change more than ± 1.0, a total of 12, 259 and 14 differentially expressed (DE) mRNAs were detected at the 6, 24 and 72 h post vaccination time points, respectively in PBMCs of DL pigs (Fig 1A). While the number of differentially expressed microRNAs identified in PBMCs after PRRSV vaccination was 0, 222 and 13 at 6, 24 and 72 h post vaccination, respectively in Pi pigs (Fig 1B).

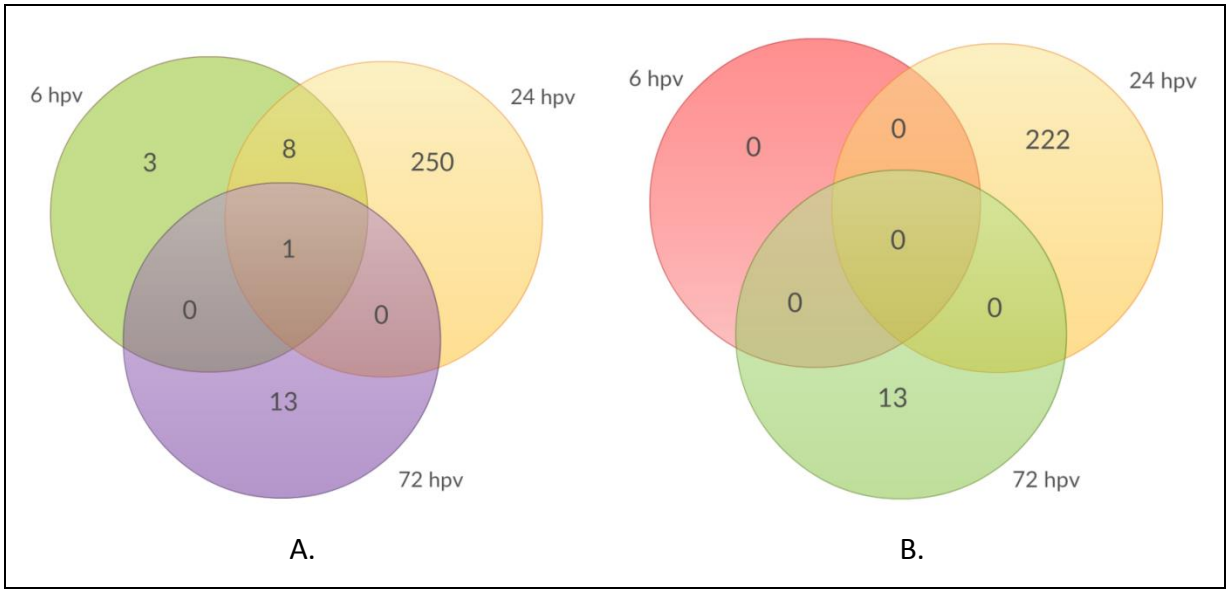

Figure 1. Venn diagram showing the differentially expressed miRNAs in PBMCs of PRRSV vaccinated German Landrace (A) and Pietrain (B) pigs. The picture shows the number of DE miRNAs at three time points (6, 24 and 72 hpv) compared to the control (0 hpv) in PBMCs. The FDR of <0.05 and FC more than ± 1.0 were considered as threshold criteria.

The most significantly deregulated miRNAs which are conserved among species are presented in the heatmap (Fig. 2). The log_2 fold changes of DE miRNAs in PBMCs at three time points compared to the control are presented in Table 1.

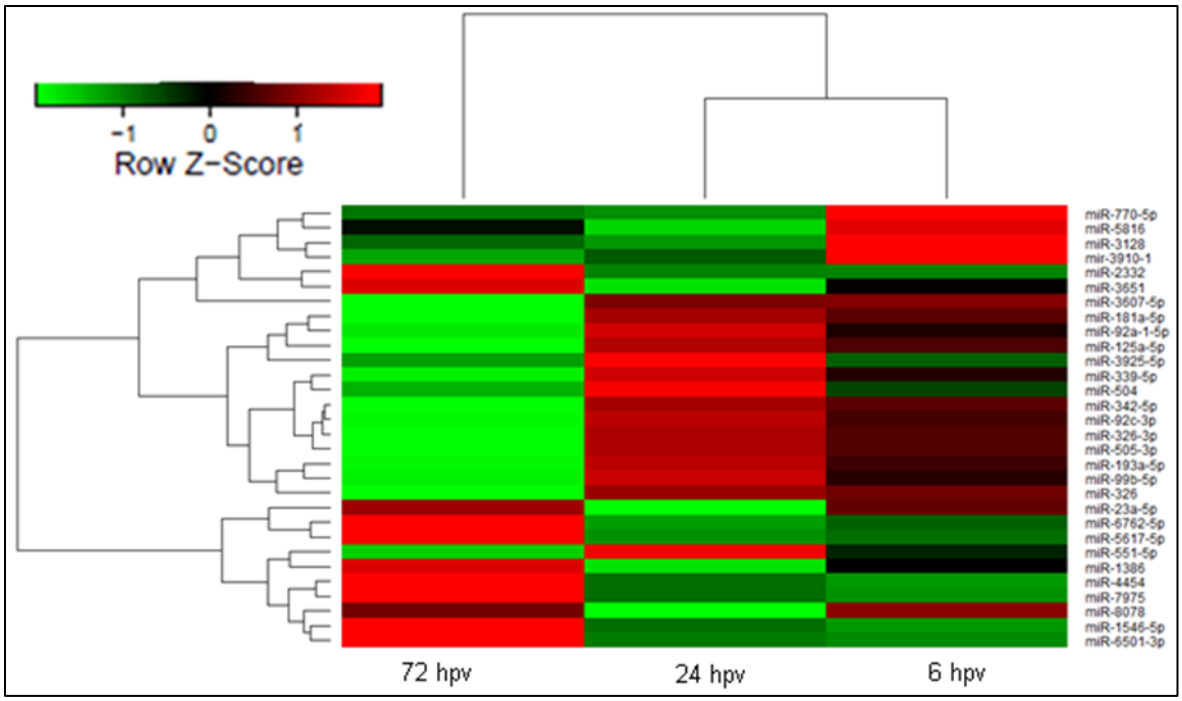

Figure 2. Differential miRNA expression profiles of PBMCs derived from DL pigs after PRRSV vaccination. The log_2 fold change of corresponding De miRNAs at 6, 24 and 72 h post vaccination as compared to the control were used to plot the heatmap.

5.4.2 Breed-specific miRNA expression between DL and Pi pigs

The temporal expression dynamics of PBMCs miRNAs revealed that the alterations miRNAs were more pronounced at 24 h post PRRSV vaccination as compared with control in both breeds. Therefore, the 24 h post vaccination time point was used for breed comparison. Considering a FDR < 0.05 and log_2 fold change > 1.0 to < -1.0 as threshold, a total 512 DE miRNAs showed more pronounced deferential expression in DL pigs and 79 were in Pi pigs. A total of 177 deregulated miRNAs shared in PBMCs both breeds after PRRSV vaccination (Table 2). The hierarchical clustering also revealed that array samples were clustered in two distinct groups as originated from DL and Pi pigs (Fig. 3).

Table 1. Significantly deregulated microRNAs in PBMCs after PRRSV vaccination in DL pigs

miRNA family	DE miRNAs	FC at 6 hpv	FC at 24 hpv	FC at 72 hpv	Adj. *p*-value
miR-99-100-5p	miR-99b-5p	0.323	1.877	-2.414	0.025603
miR-92a-1-5p	miR-92c-3p	0.536	1.0620	-0.901	0.047613
	miR-92a-1-5p	1.267	2.386	-0.366	0.047512
	miR-92a	0.310	0.912	-1.643	0.0452
miR-8078	miR-8078	-0.987	-2.149	-1.038	0.030413
miR-7975	miR-7975	-2.174	-2.033	-0.350	0.041625
miR-770-5p	miR-770-5p	2.952	0.857	0.992	0.047512
miR-6762	miR-6762-5p	-3.071	-3.263	-1.878	0.001859
miR-6501	miR-6501-3p	-1.744	-1.724	-1.176	0.026445
miR-5816	miR-5816	2.294	0.1419	1.117	0.037284
miR-5617/6845-5p	miR-5617-5p	-3.564	-3.714	-2.016	0.013784
miR-551b-5p	miR-551b-5p	-1.817	-1.257	-2.165	0.04174
miR-505	miR-505-3p	0.3188	0.660	-1.001	0.0452
miR-4454	miR-4454	-1.978	-1.778	0.015	0.001859
miR-3925-5p	miR-3925-5p	0.406	2.894	-0.032	0.007915
miR-3651	miR-3651	1.012	-0.156	2.054	0.003625
mir-3607	miR-3607-5p	3.764	3.730	2.139	0.001859
	mir-3607	1.617	1.231	0.611	0.047512
miR-342-5p	miR-342-5p	0.731	1.083	-0.878	0.018467
miR-339-5p	miR-339-5p	0.855	1.523	-0.218	0.0452
miR-326	miR-326	0.282	0.635	-2.946	0.001859
	miR-326-3p	0.650	1.140	-1.146	0.039885
miR-3128	miR-3128	2.266	-0.284	0.018	0.034879
miR-23-5p	miR-23a-5p	-1.939	-5.071	-1.421	0.015001
miR-2332	miR-2332	0.394	0.4054	2.603	0.026445
miR-193-5p	miR-193a-5p	0.919	1.982	-1.712	0.054896
miR-181-5p	miR-181b-5p	0.449	0.585	-1.127	0.0452
miR-181-5p	miR-181a-5p	1.927	2.485	-0.783	0.0452
	miR-181a-2-3p	0.073	1.755	-1.231	0.039851
	miR-181a	1.247	1.766	-0.091	0.026445
miR-1546-5p	miR-1546-5p	-1.298	-1.263	-0.939	0.0452
miR-1386	miR-1386	-2.122	-2.595	-1.670	0.004003
miR-125-5p	miR-125a-5p	2.037	3.120	-1.537	0.001859
	miR-125a-3p	-2.372	-3.452	-1.224	0.025603
	miR-125a	2.544	3.465	-0.763	0.007306

FC, Fold change; hpv, hours post vaccination; Adj. p-value, adjusted p-value after multiple test correction

Table 2. Breed comparison of PRRSV vaccine induced DE miRNAs profiles in PBMCs

Fold change	Number of DE miRNAs		
	More abundant in DL	More abundant in Pi	Shared in both breed
More than ± 1.0 to < 2.0	259	41	76
More than ± 2.0 to < 3.0	158	21	49
More than ± 3.0 to < 4.0	70	10	39
More than ± 4.0	25	7	12
Total	512 (Up: 376, Down: 136)	79 (Up: 51, Down: 28)	177 (Up: 102, Down: 75)

The number generated from two pairwise comparisons (24h.DL vs 0h. DL and 24h.Pi vs 0h.Pi) and their overlapping. The FDR value of <0.05 was considered for statistical significance for all pairwise comparisons.

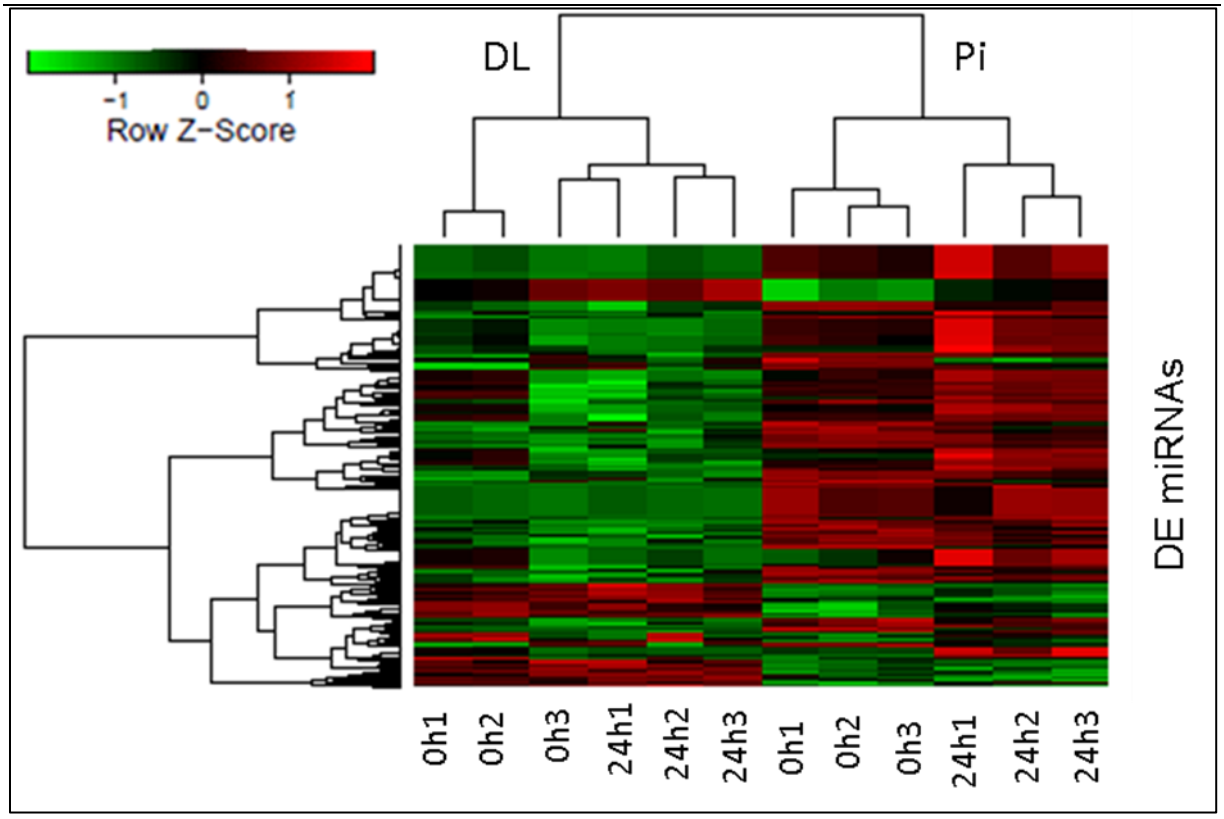

Figure 3. Breed-specific differential expression of miRNAs in PBMCs of DL and Pi pigs after PRRSV vaccination.

5.4.3 Prediction of mRNA targets for the DE miRNAs in PRRSV vaccinated PBMCs

The target genes for deregulated DE miRNAs were predicted with the TargetScan and miRDB tool. Our search yielded a total of 2909 mRNAs which are predicted to be targeted by

20 deregulated miRNAs in PBMCs after PRRSV vaccination. Then, to identify the true target genes of DE miRNAs, we overlaid the predicted target gene list onto the vaccine induced differentially expressed gene (DEGs) list comprised of 2,453 mRNAs (Islam et al. 2016a). After overlapping two lists, a total of 1397 matched mRNAs were found which were considered to be the potential true target genes of DE miRNAs in PBMCs after PRRSV vaccination. The true differentially expressed target genes (TDETGs) obtained from the overlapped results is summarized in Table 3.

Table 3. Top ten significantly down regulated microRNAs and their true differentially expressed target genes (TDETGs) in PBMCs after PRRSV vaccination in DL pigs

DE miRNAs	TDETGs
miR-6762-5p	MAF1, CYP17A1, PRSS8, KCNC1, AGER, TSPAN4, GPR123, ADAMTS14, SOHLH1, PHACTR1, IGLON5, TGFB1, CDCA3, KDR, ARRB1, CSPG4, FGR, CHAC1, XYLB, RAB17, KLHL25, RASSF7, RNF121, CDKN1A, FAM73B, AGPAT1, ZBTB47, TGFB3 & ITGA3
miR-551b-5p	DCTN3, SRC, DIS3L, PPARD, UNC119B, RNF208, WNT7A, MYL3, MRPL38, DIO3, LMO2, SLC25A45, P2RX6, UBAP1, WSCD2, TFEB, SBK1, KLHL31, GLYR1, NFIB, GRK1, BCKDHB, EFHD1, SEC24C, RAB3A, LMF2, PKM & NCS1
miR-125a-5p	ABCG8, PGF, EMP2, RASGRF1, EMILIN3, DIAPH1, PLA2G2D, EPS15L1, ASCC2, ESRRA, RAX2, NOVA2, PIANP, GTPBP2, NAIF1, PRRG4, ROR1, ASB13, SLCO3A1, CSNK1G2, CRLF1, CCDC113, EPN3, TMEM55B, ZNF335 & KAT8
miR-23a-5p	TMEM110, GJA9, COL4A2, EDC3, OXNAD1, TSGA13, SLC35B3, MICAL3, HDAC5, FBXW8, DNAJB13, GMPPB, PPP1R16B, UBL7, MED12L, SETX, PTPN21, BLCAP, SEMA5A, MEA1 & ACADSB
miR-181b	TSTD2, NTRK2, CLYBL, PHLDA1, ARMC8, TBCD, OTUB2, BTBD9, SF3A1, CHSY1, CEP78, KIF1B, MRPL13, NAP1L1, THUMPD3, MALT1, POLR3G, FOXRED2, MINPP1, ACAD11, ATP2B2, PIGN, MAPK14 & FAS
miR-4454	ZNF394, EBPL, RMND5B, CALCOCO2, SEL1L, TROAP, NECAP2, RPS23, RBP4, PIN1, SLC29A3, PDK1, CPE, NLRP6, ALG10, RAD51, ENPP5, JAKMIP2, NKX2-8, TRIM44, ICA1L, TIFA & TREM2
miR-8078	MYLK4, CEP250, SEMA4F, SOX9, POMT1, POLR3D, ATXN1, TRIM25, PIKFYVE, BAZ1B, MUC20, HEYL, RBM8A, MEGF9, GAS2L2, SMAD5, TRIM62, DNMBP, EPHA4 & ACACA
miR-7975	RNF150, PIGO, SLC9A2, SLC44A2, SFRP1, C2CD2, SYNGAP1, CD248, PPAPDC3, SLC35E1, PCGF5, EPAS1, PRKAB1, SHPK, TRPC4, LRIG2, POU2F1, CLSTN2, DPP8, CCDC93, SIN3B, TSHZ2, MAP3K9, STX17, RIPK4, N4BP3, ITPK1, ABHD2 & FBXL19
miR-5617-5p	TMTC4, KIF24, URGCP, DLX2, KRT5, SGCG, IVD, VPS39, CD8B, CERS3, FUZ, PML, SLC30A8, CDH23, NACC1, CUX1, PRR5, DNAJC11, TRPV6, GEMIN4, GAL3ST3, PPP3CB, S1PR2, TSPAN9, OTUB1, TBC1D10C & PLB1
miR-99b-5p	HS3ST2, LRRC8B & PI15

5.4.5 Integrated miRNA-mRNA network for PRRSV vaccine responses in PBMCs

The co-regulatory network was constructed based on down regulated miRNA and their up regulated true mRNA targets profiled in the PBMCs after PRRSV vaccination in DL pigs (Fig 4). The initial miRNA-mRNA interaction was generated between expression profiles of 1397 mRNA and 8 miRNAs. After correlation analysis with this interaction list, we found 289 interaction showing inverse correlation on the expression values of miRNA and mRNAs, and were passed the threshold criteria (Pearson correlation < 0 and FDR <0.05). Finally, those 289 functional integrations were subjected for network visualization. The combined miRNA-mRNA network revealed that that miR-6762, miR-23a-5p, miR-181b-5p, miR-4454, and miR-125-5p are highly interconnected hubs of the network. The closest neighboring nodes of miRNA hubs include SIRT1, FOS, ARNTL, PKM, CD9, WNT1, CDKN1A, ABCG2, VEGFA and TNFAIP3. The hub miRNAs are considered to be the putative regulators of at least in part of PRRSV vaccine induced gene expression changes in PBMCs.

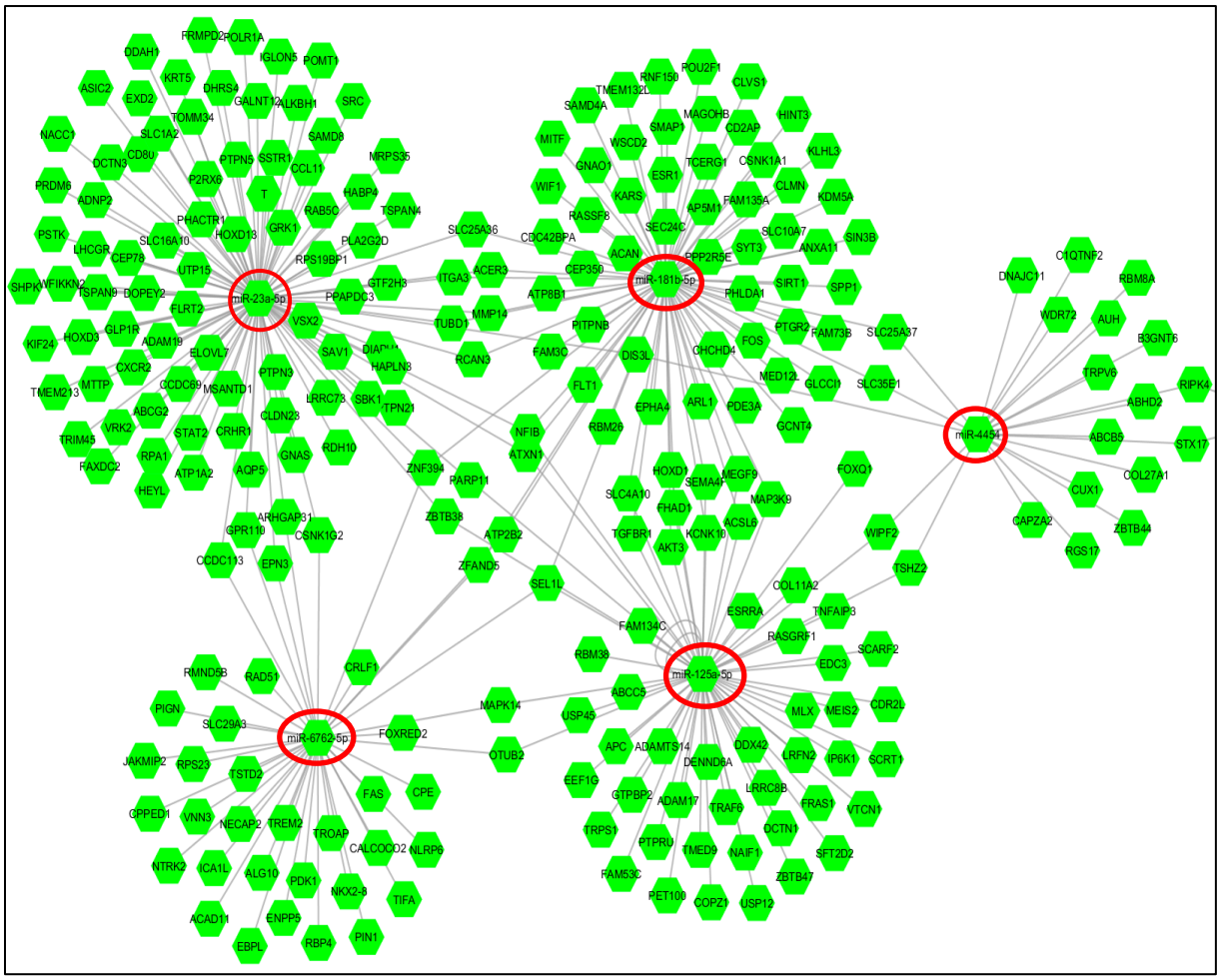

Figure 4. Integrated network for down regulated miRNA and their up regulated mRNA targets profiled in PBMCs following PRRSV vaccination in DL pigs. Red encircled nodes are for miRNAs and rest all green hexagonal nodes for mRNA targets.

5.4.6 Functional annotation of the miRNA-mRNA network

The list of 289 up regulated mRNAs engaged in the combined network was subjected for functional annotation with GO and pathway enrichment analysis using the InnateDB pathway analysis tool (Breuer et al. 2013). The significantly GO and pathways include protein kinase binding, response to toxic substances and regulation of MAPK kinase activity, TGF-beta signalling, signal transduction, Ap1 transcription factor network and toll-like receptor pathway (Fig. 5). This was an indication for involvement of miRNA-mRNA pairs in the development of immune response in PBMCs following PRRSV vaccination in pigs.

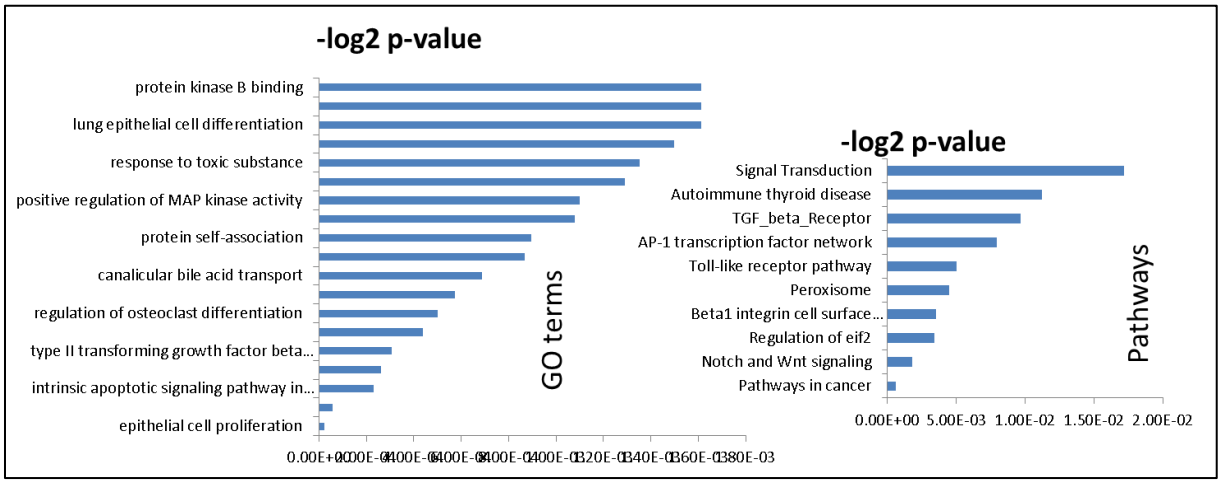

Figure 5. Significantly enriched pathway and gene ontology (GO) terms and enriched by true target genes of DE miRNAs in PBMCs after PRRSV vaccination in DL pigs.

5.5 Discussion

To investigate in parallel the expression dynamics of miRNAs and mRNAs following PRRSV vaccination in pigs, we employed Affymetrix microarray platform for the global miRNA and mRNA transcriptome profiling of PBMCs collected longitudinally at four time points (0, 6, 24 and 72 hpv) relative to the primary vaccination. We observed major changes in miRNA expression profiles after PRRSV vaccination. The temporal changes in miRNAs expression profiles of PBMCs (Fig 1) within the first three days indicated the PRRSV vaccine potential for influencing transcriptional mechanism of innate immune response following vaccination. Moreover, the integrative analysis of paired miRNAs and mRNA expression profiled in the identical samples allowed us to explore more robust miRNA-mRNA co-regulatory networks potentially to be involved with the immune response to PRRSV vaccine in PBMCs. In comparison, similar studies have reported that porcine miRNA can intricately engage itself in host-PRRSV interaction networks in PAMs (Cong et al. 2014, Li et al. 2015b).

The miRNA profiles of PBMCs differed between DL and Pi pigs (Fig.1, Table 2, Fig 3). It was expected as the mRNA expression profiles of matched PBMCs to this miRNAs profiles

also differed greatly between DL and Pi pigs (Chapter 4 of this thesis). Through overlapping the predicted target genes of deregulated miRNAs with that of vaccine induced DEGs profiled in PBMCs, we have identified a set true differentially expressed target genes (TDETGs) of deregulated miRNAs. This research showed 289 differentially expression mRNA targets whose expression was inversely correlated with the expression of their corresponding top ten deregulated miRNAs in PBMCs of DL pigs (Table 3). The correlation between expression levels of miRNAs and their predicted target genes were relatively high and inverse in pattern, which suggested that the prediction algorithms used were reliable (Endale Ahanda et al. 2012). Functional analysis revealed the TDETGs found to be associated with the biological process like include protein kinase binding, response to toxic substances and regulation of MAPK kinase activity, TGF-beta signalling, signal transduction, Ap1 transcription factor network and toll-like receptor pathway (Fig. 5), which are known to be involved with development of immune response.

The miRNA-mRNA co-regulatory network revealed that miR-6762, miR-23a-5p, miR-181b-5p, miR-4454, and miR-125-5p are like the putative miRNA regulators of the PRRSV vaccine induced differential gene expression in PBMCs (Fig 4). In comparison, the miRNA-mRNA interactive network profiled in the PAMs infected with the H- and N-PRRSV identified differential expression of some cellular miRNAs including ssc-miR-10a, 10b, 125a, 99b, 4332, 320, 1285, 210, 503 (Cong et al. 2014). The tissue-specific expression and regulatory networks of pig microRNAome identified different miRNA families in the WBC including miR-15, miR-17, miR-181, miR-23, mir-27 and miR-29 families (Martini et al. 2014). Among them, the miR-17 family comprised of miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92-1; and miR-29 family comprised of miR-29a, miR-29b and miR-29c showed the most pronounced expression in WBC of pigs (Martini et al. 2014).

Among the functional miRNA-mRNA pairs, miR-23a-5p showed the most significantly down regulated in PBMCs at all three time points after PRRSV vaccination (Table 2). miR-23 is a conserved miRNA family implicated in antiviral innate immunity, restricting the PRRSV replication in PAMs (Zhang et al. 2014). Another member (miR-23b) of miR-23 family was also reported to produces by IL-17, TNFα and IL-1β mediated proinflammatory response through triggering TAB2, TAB3 and IKKα (Zhu et al. 2012). Our result underscores the relevance of miR-23 family in immune response to PRRSV vaccine in PBMCs. Concordantly, the member of miR-23 family was previously reported as a host cellular miRNA that inhibits

PRRSV replication by directly targeting PRRSV RNA and possibly by upregulating type I interferon (Zhang et al. 2014). The overexpression of miR-23 rendered the cells more competent in IFN-α and IFN-β expression during PRRSV infection but did not mediate the activation of IFNs in the absence of PRRSV infection (Zhang et al. 2014), suggesting that the activation of IFNs by miR-23 requires other signaling activated by PRRSV. The IRF3/IRF7 signaling was eventually confirmed to be critical for miR-23-mediated induction of IFNs response during PRRSV infection (Zhang et al. 2014). Therefore, PBMCs could have have the potential for developing interferon response at least partly, following in vivo PRRSV vaccination in DL pigs.

At least four members of the miR-181-5p miRNA family were deregulated in PRRSV vaccinated PBMCs (Table 2) and found to be one of the potential hubs of the integrated miRNA-mRNA network (Fig 4). Research have shown that host miRNAs can inhibit viral replication by directly targeting viral genomic RNA, which has been regarded as a new mechanism of host antiviral defense (Lecellier et al. 2005, Li and Ding 2005, Otsuka et al. 2007, Pedersen et al. 2007, Song et al. 2010) or a new way to change the viral life cycle (Chen et al. 2011, Gottwein and Cullen 2008, Huang et al. 2007). The miR-181 was expressed at a much higher level in peripheral blood monocytes and peritoneal macrophages than in PAMs (about 10- and 42-fold higher than that in PAMs, respectively), and the total cumulative expression of miRNAs which could potentially target PRRSV in peripheral blood monocytes and peritoneal macrophages was also higher than that in PAMs (Guo et al. 2013). The miR-181 has been confirmed to regulate CD163 mRNA in blood monocytes to suppress PRRSV infection (Gao et al. 2013), suggesting that miR-181 may further influence PRRSV tropism also by direct targeting of CD163 mRNA. Therefore, it is likely that members of the miR-181 family contribute to immune response in PBMCs following vaccine exposure.

The PRRSV infection has been reported to regulate the expression of the miR-125 family member miR-125b, which reduced PRRSV replication in both Marc-145 cell line and PAMs (Wang et al. 2013). The miR-125b has also implicated in innate immunity to HIV infection (Huang et al. 2007). Our research showed that three members of miR-125 family including miR-125a-5p, 125a-3p and 125a were differentially expressed in PBMCs after vaccination (Table 2). This reduction in viral replication was attributed to the modulation of NF-kB expression by miR-125b. MiR-125b targets kB-RAS2 which serves as a negative regulator of NF-kB. Thus it was speculated that PRRSV infections induce the downregulation of miR-

125b, which subsequently results in increased kB-RAS2 expression and ultimately reduced NF-kB expression. This may serve as part of the immune evasion strategy of PRRSV by limiting the NF-kB signaling response to infection. Taken together, these data support the importance of these miRNAs for the function of the immune system.

5.6 Conclusions

This study characterized the microRNA expression profiles of PBMCs following PRRSV vaccination in DL and Pi pigs. Results of the present study suggest that 20 down regulated miRNAs and their 289 inversely correlated true target genes were involved in the process of post transcriptional protein modification, apoptosis, protein transport, small molecule metabolic process and innate immune response. The miRNA and gene co-regulatory network revealed that miR-6762, miR-23a-5p, miR-181b-5p, miR-4454, and miR-125-5p are putative regulators of the PRRSV vaccine induced differential gene expression in PBMCs. The inversely correlated mRNA targets of down regulated miRNA are involved with cell proliferation, MAPK signaling and apoptosis pathway. Taken together, these results indicated that differential expression of these miRNAs may contribute in the immune response developed from PRRSV vaccination in PBMCs by regulating the expression of mRNA targets involving critical cellular functions. Further functional studies of these miRNAs could be a good basis for the identification and analysis of potential immuno-modulatory effectors in PRRS.

5.7 Declarations

Conflict of interest

Authors declare there is no conflict of interest

Author contributions

MAI, CN, MJU and KS conceived and designed the experiments; CN and DT arranged kits and reagents; MAI, MH and MP performed field experiment and blood sampling; MAI and SAR performed the microarray experiments, MAI analyzed and interpreted the data; MAI drafted the manuscript. MJU, CN, KS reviewed and edited the manuscripts.

Acknowledgements

Authors are thankful to the stuffs of Teaching and Research station at Frankenfrost for the animal husbandry support. We also thankful to the funding body, “Umweltvertragliche und

Standortgerechte Landwirtschaft" (USL) programme of North Rhine-Westphalia, Germany with the grant number 17-02.04.01-4/15.

5.8 References

Adler M, Murani E, Brunner R, Ponsuksili S, Wimmers K (2013): Transcriptomic response of porcine PBMCs to vaccination with tetanus toxoid as a model antigen. PLoS ONE 8(3), e58306

Ait-Ali T, Wilson AD, Carre W, Westcott DG, Frossard JP, Mellencamp MA, Mouzaki D, Matika O, Waddington D, Drew TW, Bishop SC, Archibald AL (2011): Host inhibits replication of European porcine reproductive and respiratory syndrome virus in macrophages by altering differential regulation of type-I interferon transcriptional response. Immunogenetics 63, 437-448

Akira S, Uematsu S, Takeuchi O (2006): Pathogen recognition and innate immunity. Cell 124, 783-801

Albina E (1997): Epidemiology of porcine reproductive and respiratory syndrome (PRRS): an overview. Vet Microbiol 55, 309-316

Bartel DP (2004): MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281-297

Benjamini Y, Hochberg Y (1995): Controlling the false discovery rate: A practial and powerful approach to multiple testing. Journal of the Royal Statistical Society Series B 57, 289-300

Breuer K, Foroushani AK, Laird MR, Chen C, Sribnaia A, Lo R, Winsor GL, Hancock RE, Brinkman FS, Lynn DJ (2013): InnateDB: systems biology of innate immunity and beyond- recent updates and continuing curation. Nucleic Acids Res 41 (Database), D1228-D1233

Chen CZ, Schaffert S, Fragoso R, Loh C (2013): Regulation of immune responses and tolerance: the microRNA perspective. Immunol Rev 253, 112-128

Chen Y, Shen A, Rider PJ, Yu Y, Wu K, Mu Y, Hao Q, Liu Y, Gong H, Zhu Y, Liu F, Wu J (2011): A liver-specific microRNA binds to a highly conserved RNA sequence of hepatitis B virus and negatively regulates viral gene expression and replication. Faseb J 25, 4511-4521

Cline MS, Smoot M, Cerami E, Kuchinsky A, Landys N, Workman C, Christmas R, Avila-Campilo I, Creech M, Gross B, Hanspers K, Isserlin R, Kelley R, Killcovne S, Lotia S, Maere S, Morris J, Ono K, Pavlovic V, Pico AR, Vailva A, Wang PL, Adler A,

Conklin BR, Hood L, Kuiper M, Sander C, Schmulevich I, Schwikowsky B, Warner GJ, Ideker T, Bader GD (2007): Integration of biological networks and gene expression data using Cytoscape. Nat Protoc 2, 2366–82

Coll M, El Taghdouini A, Perea L, Mannaerts I, Vila-Casadesus M, Blaya D, Rodrigo-Torres D, Affo S, Morales-Ibanez O, Graupera I, Lozano JJ, Najimi M, Sokal E, Lambrecht J, Gines P, van Grunsven LA, Sancho-Bru P (2015): Integrative miRNA and Gene Expression Profiling Analysis of Human Quiescent Hepatic Stellate Cells. Sci Rep 5:11549, doi:10.1038/srep11549

Cong P, Xiao S, Chen Y, Wang L, Gao J, Li M, He Z, Guo Y, Zhao G, Zhang X, Chen L, Mo D, Liu X (2014): Integrated miRNA and mRNA transcriptomes of porcine alveolar macrophages (PAM cells) identifies strain-specific miRNA molecular signatures associated with H-PRRSV and N-PRRSV infection. Mol Biol Rep 41, 5863-5875

Contreras J, Rao DS (2012): MicroRNAs in inflammation and immune responses. Leukemia 26, 404-413

Cullen BR (2006): Viruses and microRNAs. Nat Genet 38, S25-30

Cullen BR (2011): Herpesvirus microRNAs: phenotypes and functions. Curr Opin Virol 1, 211-215

De Paz X, Vega D, Duran CO, Angulo J (2015): PRRS prevalence in Europe: Perception of the Pig Producers. ESPHM annual conference on 22-24 April, 2015, Nantes, France

Endale Ahanda ML, Fritz ER, Estelle J, Hu ZL, Madsen O, Groenen MA, Beraldi D, Kapetanovic R, Hume DA, Rowland RR, Lunney JK, Rogel-Gaillard C, Reecy JM, Giuffra E (2012): Prediction of altered 3'- UTR miRNA-binding sites from RNA-Seq data: the swine leukocyte antigen complex (SLA) as a model region. PLoS ONE 7(11), e48607

Farh KK, Grimson A, Jan C, Lewis BP, Johnston WK, Lim LP, Burge CB, Bartel DP (2005): The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. Science 310, 1817-1821

Gao L, Guo XK, Wang L, Zhang Q, Li N, Chen XX, Wang Y, Feng WH (2013): MicroRNA 181 suppresses porcine reproductive and respiratory syndrome virus (PRRSV) infection by targeting PRRSV receptor CD163. J Virol 87, 8808-8812

Genini S, Delputte PL, Malinverni R, Cecere M, Stella A, Nauwynck HJ, Giuffra E (2008): Genome-wide transcriptional response of primary alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. J Gen Virol 89, 2550-2564

Gennarino VA, Sardiello M, Avellino R, Meola N, Maselli V, Anand S, Cutillo L, Ballabio A, Banfi S (2009): MicroRNA target prediction by expression analysis of host genes. Genome Res 19, 481-490

Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, Hornik K, Hothorn T, Huber W, Iacus S, Irizarry R, Leisch F, Li C, Maechler M, Rossini AJ, Sawitzki G, Smith C, Smyth G, Tierney L, Yang JY, Zhang J (2004): Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5, 15

Gottwein E, Cullen BR (2008): Viral and cellular microRNAs as determinants of viral pathogenesis and immunity. Cell Host Microbe 3, 375-387

Guo XK, Zhang Q, Gao L, Li N, Chen XX, Feng WH (2013): Increasing expression of microRNA 181 inhibits porcine reproductive and respiratory syndrome virus replication and has implications for controlling virus infection. J Virol 87, 1159-1171

Holtkamp DJ, Kliebenstein JB, Neumann EJ (2013): Assessment of the economic impact of porcine reproductive and respiratory syndrome virus on United States pork producers. J Swine Health Prod 21, 72-84

Hou J, Wang P, Lin L, Liu X, Ma F, An H, Wang Z, Cao X (2009): MicroRNA-146a feedback inhibits RIG-I-dependent Type I IFN production in macrophages by targeting TRAF6, IRAK1, and IRAK2. J Immunol 183, 2150-2158

Huang J, Wang F, Argyris E, Chen K, Liang Z, Tian H, Huang W, Squires K, Verlinghieri G, Zhang H (2007): Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. Nat Med 13, 1241-1247

Hussain M, Asgari S (2010): Functional analysis of a cellular microRNA in insect host-ascovirus interaction. J Virol 84, 612-620

Islam MA, Grosse-Brinkhaus C, Proll MJ, Uddin MJ, Rony SA, Tesfaye D, Tholen E, Holker M, Schellander K, Neuhoff C (2016a): Deciphering transcriptome profiles of peripheral blood mononuclear cells in response to PRRSV vaccination in pigs. BMC Genomics 17, 016-2849

Islam MA, Grosse-Brinkhaus C, Proll MJ, Uddin MJ, Rony SA, Tesfaye D, Tholen E, Holker M, Schellander K, Neuhoff C (2016b): PBMCs transcriptome profiles identifies the potential candidate genes and functional networks controlling the innate and adaptive immune response to PRRSV vaccination in Pietrain pig. PLoS ONE (Under review).

Jing L, Hou Y, Wu H, Miao Y, Li X, Cao J, Brameld JM, Parr T, Zhao S (2015): Transcriptome analysis of mRNA and miRNA in skeletal muscle indicates an

important network for differential Residual Feed Intake in pigs. Sci Rep 5:11953, doi: 10.1038/srep11953

Julie AH, Yoo D, Liu HC (2013): Characterization of the microRNAome in porcine reproductive and respiratory syndrome virus infected macrophages. PLoS ONE 8(12), e82054

Kim VN (2005): MicroRNA biogenesis: coordinated cropping and dicing. Nat Rev Mol Cell Biol 6, 376-385

Krek A, Grun D, Poy MN, Wolf R, Rosenberg L, Epstein EJ, MacMenamin P, da Piedade I, Gunsalus KC, Stoffel M, Rajewsky N (2005): Combinatorial microRNA target predictions. Nat. Genet 37, 495–500

Lecellier CH, Dunoyer P, Arar K, Lehmann-Che J, Eyquem S, Himber C, Saib A, Voinnet O (2005): A cellular microRNA mediates antiviral defense in human cells. Science 308, 557-560

Lewis BP, Burge CB, Bartel DP (2005): Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120, 15–20

Li H, Wu B, Geng J, Zhou J, Zheng R, Chai J, Li F, Peng J, Jiang S (2015a): Integrated analysis of miRNA/mRNA network in placenta identifies key factors associated with labor onset of Large White and Qingping sows. Sci Rep 5:13074, doi: 10.1038/srep13074

Li HW, Ding SW (2005): Antiviral silencing in animals. FEBS Lett 579, 5965-5973

Li HY, Xi QY, Xiong YY, Liu XL, Cheng X, Shu G, Wang SB, Wang LN, Gao P, Zhu XT, Jiang QY, Yuan L, Zhang YL (2012): Identification and comparison of microRNAs from skeletal muscle and adipose tissues from two porcine breeds. Anim Genet 43, 704-713

Li J, Chen Z, Zhao J, Fang L, Fang R, Xiao J, Chen X, Zhou A, Zhang Y, Ren L, Hu X, Zhao Y, Zhang S, Li N (2015b): Difference in microRNA expression and editing profile of lung tissues from different pig breeds related to immune responses to HP-PRRSV. Sci Rep 5:9549, doi: 10.1038/srep09549

Li Y, Chan EY, Li J, Ni C, Peng X, Rosenzweig E, Tumpey TM, Katze MG (2010): MicroRNA expression and virulence in pandemic influenza virus-infected mice. J Virol 84, 3023-3032

Li Y, Li J, Fang C, Shi L, Tan J, Xiong Y, Bin F, Li C (2016): Genome-wide differential expression of genes and small RNAs in testis of two different porcine breeds and at two different ages. Sci Rep 6:26852, doi: 10.1038/srep26852

Martini P, Sales G, Brugiolo M, Gandaglia A, Naso F, De Pitta C, Spina M, Gerosa G, Chemello F, Romualdi C, Cagnin S, Lanfranchi G (2014): Tissue-specific expression and regulatory networks of pig microRNAome. PLoS One 9(4), e89755

McDaneld TG, Smith TP, Harhay GP, Wiedmann RT (2012): Next-generation sequencing of the porcine skeletal muscle transcriptome for computational prediction of microRNA gene targets. PLoS One 7(7), e42039

Meulenberg JJ, Hulst MM, de Meijer EJ, Moonen PL, den Besten A, de Kluyver EP, Wensvoort G, Moormann RJ (1993): Lelystad virus, the causative agent of porcine epidemic abortion and respiratory syndrome (PEARS), is related to LDV and EAV. Virology 192, 62-72

Miranda K, Huynh T, Tay Y, Ang YS, Tam WL, Thomson AM, Lim B, Rigoutsos I (2006): A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes. Cell 126, 1203–1217

Nathan W, Xiaowei W (2015): miRDB: an online resource for microRNA target prediction and functional annotations. Nucleic Acids Res. 43(D1), D146-152

Neumann EJ, Kliebenstein JB, Johnson CD, Mabry JW, Bush EJ, Seitzinger AH, Green AL, Zimmerman JJ (2005): Assessment of the economic impact of porcine reproductive and respiratory syndrome on swine production in the United States. J Am Vet Med Assoc 227, 385-392

O'Connell RM, Rao DS, Chaudhuri AA, Baltimore D (2010): Physiological and pathological roles for microRNAs in the immune system. Nat Rev Immunol 10, 111-122

O'Neill LA, Sheedy FJ, McCoy CE (2011): MicroRNAs: the fine-tuners of Toll-like receptor signalling. Nat Rev Immunol 11, 163-175

Otsuka M, Jing Q, Georgel P, New L, Chen J, Mols J, Kang YJ, Jiang Z, Du X, Cook R, Das SC, Pattnaik AK, Beutler B, Han J (2007): Hypersusceptibility to vesicular stomatitis virus infection in Dicer1-deficient mice is due to impaired miR24 and miR93 expression. Immunity 27, 123-134

Pedersen IM, Cheng G, Wieland S, Volinia S, Croce CM, Chisari FV, David M (2007): Interferon modulation of cellular microRNAs as an antiviral mechanism. Nature 449, 919-922

Podolska A, Kaczkowski B, Kamp Busk P, Sokilde R, Litman T, Fredholm M, Cirera S (2011): MicroRNA expression profiling of the porcine developing brain. PLoS One 6, e0014494

Reiner G, Willems H, Pesch S, Ohlinger VF (2010): Variation in resistance to the porcine reproductive and respiratory syndrome virus (PRRSV) in Pietrain and Miniature pigs. J Anim Breed Genet 127, 100-106

Ruike Y, Ichimura A, Tsuchiya S, Shimizu K, Kunimoto R, Okuno Y, Tsujimoto G (2008): Global correlation analysis for micro-RNA and mRNA expression profiles in human cell lines. J Hum Genet 53, 515-523

Selbach M, Schwanhausser B, Thierfelder N, Fang Z, Khanin R, Rajewsky N (2008): Widespread changes in protein synthesis induced by microRNAs. Nature 455, 58-63

Sharbati S, Friedlander MR, Sharbati J, Hoeke L, Chen W, Keller A, Stahler PF, Rajewsky N, Einspanier R (2010): Deciphering the porcine intestinal microRNA transcriptome. BMC Genomics 11, 1471-2164

Siegrist C-A (2012): General aspects of vaccinations. In: Vaccines, 6th ed. Elsevier Health Sciences Saunders, USA. pp. 17-36

Siengdee P, Trakooljul N, Murani E, Schwerin M, Wimmers K, Ponsuksili S (2013): Transcriptional profiling and miRNA-dependent regulatory network analysis of longissimus dorsi muscle during prenatal and adult stages in two distinct pig breeds. Anim Genet 44, 398-407

Smyth GK (2005): Limma: linear models for microarray data. In: Gentleman R, Carey V, Dudoit S, Irizarry R, Huber W, editors. Bioinformatics and computational biology solutions using r and bioconductor. Springer New York, pp. 397–342

Song L, Liu H, Gao S, Jiang W, Huang W (2010): Cellular microRNAs inhibit replication of the H1N1 influenza A virus in infected cells. J Virol 84, 8849-8860

Taganov KD, Boldin MP, Chang KJ, Baltimore D (2006): NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. Proc Natl Acad Sci USA 103, 12481-12486

Tang Z, Yang Y, Wang Z, Zhao S, Mu Y, Li K (2015): Integrated analysis of miRNA and mRNA paired expression profiling of prenatal skeletal muscle development in three genotype pigs. Sci Rep 5:15544, doi:10.1038/srep15544

Tian Z, Greene AS, Pietrusz JL, Matus IR, Liang M (2008): MicroRNA-target pairs in the rat kidney identified by microRNA microarray, proteomic, and bioinformatic analysis. Genome Res 18, 404-411

Timoneda O, Balcells I, Nunez JI, Egea R, Vera G, Castello A, Tomas A, Sanchez A (2013): miRNA expression profile analysis in kidney of different porcine breeds. PLoS One 8(1), e55402

Trobaugh DW, Gardner CL, Sun C, Haddow AD, Wang E, Chapnik E, Mildner A, Weaver SC, Ryman KD, Klimstra WB (2014): RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. Nature 506, 245-248

Wang D, Cao L, Xu Z, Fang L, Zhong Y, Chen Q, Luo R, Chen H, Li K, Xiao S (2013): MiR-125b reduces porcine reproductive and respiratory syndrome virus replication by negatively regulating the NF-kappaB pathway. PLoS ONE 8(2), e55838

Wang P, Hou J, Lin L, Wang C, Liu X, Li D, Ma F, Wang Z, Cao X (2010): Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. J Immunol 185 (10), 6226-6233

Wu J, Peng X, Zhou A, Qiao M, Wu H, Xiao H, Liu G, Zheng X, Zhang S, Mei S (2014): MiR-506 inhibits PRRSV replication in MARC-145 cells via CD151. Mol Cell Biochem 394, 275-281

Van Breedam W, Delputte PL, Van Gorp H, Misinzo G, Vanderheijden N, Duan X, Nauwynck HJ (2010): Porcine reproductive and respiratory syndrome virus entry into the porcine macrophage. J Gen Virol 91, 1659-1667

Xiaowei W (2016): Improving microRNA target prediction by modeling with unambiguously identified microRNA-target pairs from CLIP-Ligation studies. Bioinformatics 32(9), 1316-1322

Xing J, Xing F, Zhang C, Zhang Y, Wang N, Li Y, Yang L, Jiang C, Wen C, Jiang Y (2014): Genome-wide gene expression profiles in lung tissues of pig breeds differing in resistance to porcine reproductive and respiratory syndrome virus. PLoS One 9(1), e86101

Zhang Q, Guo XK, Gao L, Huang C, Li N, Jia X, Liu W, Feng WH (2014): MicroRNA-23 inhibits PRRSV replication by directly targeting PRRSV RNA and possibly by upregulating type I interferons. Virology 451, 182-195

Zhu S, Pan W, Song X, Liu Y, Shao X, Tang Y, Liang D, He D, Wang H, Liu W, Shi Y, Harley JB, Shen N, Qian Y (2012): The microRNA miR-23b suppresses IL-17-associated autoimmune inflammation by targetting TAB2, TAB3 and IKK-alpha. Nat Med 18 (7), 1077-86

Chapter 6: General discussion and conclusions

Genetic control of PRRS through improvement of host resistance by marker assisted selection breeding has been a debate for last couple of years. The disease resistance traits are likely to be polygenic and influenced by multiple factors. Moreover, in most of the large-scale intensive settings of pig production, disease resistance phenotype is difficult to estimate; hence an indirect approach through measuring the vaccine mediated host immunocompetence is recommendable (Rowland et al. 2012). To this end, we have implemented a PBMCs transcriptome model to characterize the molecular genetics of host immune response to PRRSV vaccination in clinically healthy pigs. Numerous studies have investigated the transcriptome profiles of pulmonary alveolar macrophages (PAMs) after in vivo or in vitro PRRSV infection (Badaoui et al. 2013). However, given that intramuscular vaccination, PRRSV vaccine antigen can reach faster the blood circulation through bypassing the lungs (Siegrist 2012). Vaccine antigen initiates the T and B cell (major components of PBMCs) directed immune response soon after getting into the bloodstream. Therefore, PBMCs-based investigation of host transcriptional response would provide deeper insights into the complex molecular events of host-vaccine interaction in a relatively rapid and efficient manner. Moreover, from a genetic selection standpoint, it would be desirable to select on a trait in uninfected pigs (i.e. before PRRSV infection) that is correlated with a response after infection (Lunney et al. 2016).

Transcriptome is referred the full range of RNAs (including mRNA and miRNAs) expressed in specific tissue or cell at a given time point. Different omics approaches such as microarray, RNA-seq, differential display (DD), suppression subtractive hybridization (SSH), and serial analysis of gene expression (SAGE) have been used to identify differentially expressed RNA transcripts during the course of immune responses in pigs (Tuggle et al. 2007, Tuggle et al. 2010, Lunney and Chen 2010, Schroyen and Tuggle 2015). The present dissertation project performed the microarray-based global expression profiling of mRNA and miRNA transcriptome in PBMCs collected from Landrace and Pietrain pigs. The microarray platform used in this study, has ensured maximum coverage of transcriptome through amplifying both poly-A and non-poly A tailed end of the transcripts derived from a gene during hybridization (Eklund et al. 2006). Moreover, three individual biological replications provided the statistical power of the transcriptome datasets. Therefore, functional networks of PRRSV vaccine response and their key regulatory genes identified as well as breed differences regulating the vaccine response, could provide better insights of host immunocompetence mechanism and thereby help to understand the genetic control of PRRS.

6.1 Expression dynamics of mRNAs profiles in PBMCs after PRRSV vaccination

The current study showed that massive gene expression changes occurred in PBMCs following PRRSV vaccination in pigs. These findings are suggestive for the potential of PBMCs to induce immune response to PRRSV vaccine in vivo. Previous studies revealed that porcine PBMCs are likely to be involved with the expression changes of immune response candidate genes after in vitro PRRSV infection (Feng et al. 2003, Zhuge et al. 2012). Whereas, little information on transcriptome modification of PBMCs after in vivo PRRSV infection or vaccination are available. However, porcine PBMC-transcriptome profiles have been shown to be altered by tetanus toxoid vaccine antigen indicated by differential expression of more than 5000 genes within three days following in vivo vaccination (Adler et al. 2013). The first experiment of this thesis performed a global mRNA expression profiling of PBMCs from PRRSV vaccinated and age-matched unvaccinated German Landrace pigs at right before (0 h), and at 6, 24 and 72 h post vaccination using the Affymetrix GeneChip Porcine Gene 1.0 ST array.

The genome-wide comparison of PBMCs transcriptome profiles between vaccinated and unvaccinated pigs revealed a distinct host innate immune transcriptional response to PRRSV vaccine. There was a significant temporal variation in transcriptional responses of PRRSV vaccine in PBMCs accounting 542, 2,263 and 357 DEGs at 6, 24 and 72 h post vaccination, respectively compared to the time point before vaccination (Chapter 2, Fig 4). The global picture of gene expression changes in lung tissues following PRRSV infection revealed the temporal variation (Genini et al. 2008, Xiao et al. 2010). For instances, a total of 1409 differentially expressed transcripts were identified by analysis of variance in the microarray data, of which 2, 5, 25, 16 and 100 differed from controls by a minimum of 1.5-fold at 1, 3, 6, 9 and 12 h post PRRSV infection to pulmonary alveolar macrophages, respectively (Genini et al. 2008). The transcriptome profiles of porcine lung tissue showed 4,520 genes differentially expressed at 96 h and 168 h after in vivo infection with highly pathogenic PRRSV (Xiao et al. 2010). Zhou et al (2011) have employed Affymetrix microarrays to investigate the gene expression patterns of porcine alveolar macrophages (PAMs) isolated from Tongcheng piglets (a Chinese indigenous breed) after infection with HP-PRRSV. A total of 12,775 transcripts (53% of all probesets) were expressed in the infected and non-infected PAMs. After quantile normalization, 321 genes were identified as differentially expressed (DE) genes, with 219 being upregulated and 102 being downregulated, under the threshold of fold change (FC) of 1.5 or greater and a false discovery rate (FDR) of approximately 5% (Zhou et al. 2011).

Therefore, the current study with the snap shot of global mRNA expression pattern in PBMCs may provide understanding on the mRNA transcriptome alterations in response to PRRSV vaccination.

6.2 Transcripts signature for innate and adaptive immunity to PRRSV vaccine

The immune system is a complex network of cells and organs which is typically divided into two categories: innate and adaptive immunity; although these distinctions are not mutually exclusive. Following any viral infection, adequate activation of the host innate immune system is critical to prevent viral replication and invasion into mucosal tissues and, importantly, in initiation of the strong adaptive immune response to fight against intracellular pathogens (Koyama et al. 2008). Thus, synergistic efforts of the innate and the adaptive immunity are the key for developing strong, durable immunity from PRRSV vaccination. The innate immune system as the first line of host defense against viral infections occurs within hours of exposure and may persist up to few days (Beutler 2004). To understand the innate immune transcriptional activity in PBMCs, the global gene expression profiles of PBMCs were analyzed within first three days of PRRSV vaccination. It revealed that the highest mRNA transcriptome alterations occur at 24 h post vaccination, and altered transcripts are involved with activation of basic innate immune response pathways including interferon signaling, cytokine signaling and proinflammatory reaction; which indicated the occurrence of innate immune response (Chapter 2, Table 2). The innate immune related pathways such as TREM1, Toll-like receptor and hypercytokinemia signaling were also reported to be involved with PRRSV induced host immune response (Badaoui et al. 2013).

The current study suggested that APP, TRAF6, PIN1, FOS, CDKN1A and TNFAIP3 could be considered as potential candidate genes for PRRSV vaccine responses at early stage post vaccination (Chapter 2, Fig 8). The type I interferons (IFNα, β) are the most important cytokines responsible for antiviral innate immunity. The PRRSV infection is a poor inducer of IFNα, and its level remains low throughout the course of infection thereby activation of adaptive immunity is delayed and dampened (reviewed by Loving et al. 2015). The modulation of cytokines in vaccinated pigs appeared to be more dependent on vaccination or infection condition than on stimulation by different isolates; changes in production of IL-10 appear to be more relevant than those of TNFα at gene and protein levels (reviewed by Lunney et al. 2016). Sun et al (2012) reviewed data that affirmed that nsp1, nsp2, and nsp11 are early proteins, and N protein, a late protein, are involved in controlling gene expression pathways for IFNα suppression and NF-kB regulation of adaptive immunity. Recent efforts

have compared immunity to type 1 PRRSV isolates varying in virulence. Infection with virulent type 1 (Lena) PRRSV resulted in a more severe disease than with other type 1 (Belgium A or Lelystad = LV) strains (Weesendorp et al. 2013). Lena caused more severe pathology, with increased IL-1α production in the lungs and lymph nodes and a leukocyte influx (neutrophils, monocytes) into the bronchoalveolar lavage (BAL) fluid. By five weeks post infection, BAL from all infected pigs had a higher percentage of CD8+ T cells and higher levels of IFNγ-producing cells compared with controls. Infection with Lena PRRSV resulted in increased levels of IL-1β, IFNα, IL-10, IL-12, TNFα, and IFNγ mRNA during the first week of infection (Weesendorp et al. 2014).

We found that, TGFβ1, IL7R, RAD21, SP1 and GZMB genes are likely to be predictive for the adaptive immune transcriptional response to PRRSV vaccine in PBMCs at 4 weeks post primary vaccination. Viruses evade host immunity by promoting the secretion of immunosuppressive cytokines IL-10 and transforming growth factor-β (TGF-β), which antagonize induction of strong cell mediated immune response. PRRSV infection induced a strong immunosuppressive response, resulting in delayed onset of a Th1 immune response (Johnsen et al. 2002, Suradhat et al. 2003, Renukaradhya et al. 2010). Immunomodulatory properties of PRRSV N protein resulted in upregulation of the frequency of Foxp3+ T-regulatory cells (Tregs) and IL-10 production (Wongyanin et al. 2012). Both live and inactivated PRRSV significantly increased IL-10 gene expression (Suradhat et al. 2003); an increased concentration of IL-10 was found in pig lungs even after clearance of viremia (Johnson et al. 2002, Renukaradhya et al. 2010). A coordinated immunosuppressive function of PRRSV was shown to likely be mediated by the cytokines IL-10 and TGF-β and Tregs (Johnsen et al. 2002, Suradhat et al. 2003, Renukaradhya et al. 2010). All these studies pointed out the contribution of dysregulated expression of immune molecules following PRRSV infection, resulting in weakened adaptive immunity. Induced Tregs could suppress antiviral immunity and thus facilitate establishment of PRRSV infection, although the data are inconsistent.

6.3 Cell type specific gene expression after PRRSV vaccination

The PBMCs are heterogeneous population of immune cells in the blood that include lymphocytes (T, B and NK cells), monocytes and dendritic cells. The proportion of lymphocytes are typically in the range of 70-90 % of PBMCs, monocytes ranges from 10-30 % of PBMCs, while dendritic cells are rare, being only 1-2 % of PBMCs. Among the PBMC-

subsets, lymphocytes are accounted the height proportion. The frequencies of cell types within the lymphocyte population include 70–85% CD3+ T cells (45 – 70% of PBMCs), 5–20% B cells (up to 15% of PBMCs), and 5–20% NK cells (up to 15% of PBMCs).

The frequencies of sub cellular populations of PBMCs may vary across individuals (Fairbairn et al. 2013). The vaccine induced cellular activation and differentiation of cells resulted changes in the proportion of each sub types of PBMCs which likely might contribute to gene expression changes (Palmer et al. 2006). Therefore, the current dataset has the limitation in evaluating the cell type specific contribution on vaccine responses. There are two possible option to address this cellularity issue, one is to fluorescent activated cell sorting of PBMCs samples followed by expression profiling (Christopher et al. 2015), another is bioinformatics approach of gene expression deconvolution (Steuerman and Gat-Viks 2016). In fact, the reports on, and option for, specific cell subset of PBMCs are limited in swine and mostly due to the relative lack of immune-tagged reagents critical for such detail phenotyping (reviewed by Shroyen ad Tuggle 2015). In the current study, the unfractionated PBMCs model was used in this microarray study as a rapid and convenient model to evaluate host transcriptional response to PRRSV vaccination. To cope up with the limitation of cellularity issue, we performed the cell type enrichment analysis using an online bioinformatics tool called CTen (cell type enrichment); (Shoemaker et al. 2012). The CTen platform implements a highly expressed, cell specific (HECS) gene database comprises of 10,058 genes of human and mouse origin.

The cell type enrichment analysis revealed that PRRSV vaccine induced mRNA transcripts specifically expressed in CD4+ T cells, CD8+ T cells, CD14+ and CD33+ monocytes during early stage; and lymphnode, thymus, BDCA4+ dendritic cells, CD4+ T cells and CD8+ T cells in later stage of vaccine immunity (Chapter 3, Fig 7). This could indicate that the variation in expression patterns of the genes were not solely due to vaccine mediated transcriptional events but possibly also due to a difference in demographics of PBMCs subsets recruited into the blood circulation. Shimizu et al. observed a remarkable decrease in CD4+ T cells after 3 days PRRSV infection in pigs (Shimizu et al. 1996); and this study also reported slight decreases in CD8+ T cells at 3 dpi, followed by substantially increased levels (Shimizu et al. 1996), while at the same time, the ratios of CD4+/CD8+ T cells were significantly lower between day 3 and 28 post-inoculation compared with that of day 0 (Shimizu et al. 1996). However, the proportion of CD4+ and CD8+ T cells were found to be significantly decreased

for a few days shortly after PRRSV infection, but returned to pre-infection levels on 8-10 days post infection (Nielsen and Botner 1997). Renukaradhya et al (2010) performed a comprehensive analysis of innate and adaptive immune responses in dual-virus infected pigs and found that reduced innate NK-cells population along with increased frequencies of CD4+ T cell, CD8+ T cells and myeloid cells resulted from PRRSV infection in pigs. The PRRSV infection were reported to cause an increase in CD14+ expression throughout the early stage of infection, due to a rise in CD14+ monocytes that differentiate to macrophages and migrate to bronchoalveolar spaces (Van Gucht et al. 2004). Silva-Campa et al (2012) observed that PRRSV infection increases the frequency of T cell regulatory cells (Tregs) with the phenotype CD4+, CD8+, CD25+ and Foxp3+ high. Therefore, this information on cell-type specific contribution to vaccine immunity could be an important add-on for PRRS research.

The T lymphocytes (CD3+ T cells) are composed of CD4+ (25–60% of PBMC) and CD8+ T cells (5–30% of PBMCs), in a roughly 2:1 ratio. Both CD4+ and CD8+ T cells are further subdivided into naïve, and the antigen-experienced central memory, effector memory, and effector subtypes that exist in resting or activated states. T lymphocytes play the central rote in cell mediated adaptive immunity through cytotoxic apoptosis. Cross-reactivity against divergent PRRSV can show a different intensity and be differently associated with cytotoxic CD8+ IFNγ as well as CD8− IFNγ+ cells (Lunney et al. 2016). Especially after infection, a different immune reactivity was evident upon stimulation with various virus isolates in terms of frequency and CD8 phenotype of PRRSV specific IFNγ-producing cells. Using IFNγ ELISPOT assays, Xiao et al (2004) demonstrated that PRRSV-specific T cells were observed as early as 2 weeks pi, with no significant difference in these T cells in lymphoid tissues during or post PRRSV infection. Viral loads were shown to be decreased by 3–4 logs in persistent infection primarily in tonsils and sternal and inguinal lymph nodes. However, there was no apparent correlation of tissue viral levels and PRRSV-specific T-cell frequencies (Xiao et al. 2004). When the IFNγ-secreting CD8+ T-cell response was evaluated, a late and low virus-specific response was observed (Ferrari et al. 2013). Overall, the effect of PRRSV infection on specific CD8+ T-cell frequencies in lymphoid tissues has not been established. There are limited indications of effective CD8+ cytotoxic T cells (CTLs) controlling primary PRRSV infection, as only after clearance of viremia, the anti-PRRSV-targeted CTLs were detected (Costers et al. 2009). FoxP3+ T cells may also be involved (Wongyanin et al. 2010, Silva-Campa et al. 2012). Apoptosis in B- and T-cell areas may also be a factor, but must be affirmed (Gomez-Laguna et al. 2013); with HP PRRS, apoptosis may be an even greater

factor (Wang et al. 2014). However, more basic immune reagents (pig-specific monoclonal antibodies, major histocompatibility complex antigen tetramers, and well-characterized cell lines) are required to elucidate these complex immune regulatory issues (Loving et al. 2015).

The natural killer (NK) cell is another innate lymphocyte subset of PBMCs that helps in nonspecific clearance of any virus-infected cell from the body. In younger pigs, the NK cell is small to medium sized and lacks adequate intracellular granules (Gerner et al. 2009). Thus, in spite of having higher frequency of NK cells, nursery pigs have reduced NK cell cytotoxic activity as compared with young pigs. Nursery pigs suffer from PRRSV infection more than adult animals (Klinge et al. 2009), owing to their poorly developed innate immune system as well as limited response to counter viral immune evasion strategies. Stimulation of IFNα has been shown in vitro to be down regulated mainly by viral nonstructural proteins (nsp1, 2, 4, 11); (Chen et al. 2010). Genetic studies indicate that all PRRSV-infected pigs have detectable IFNα in serum by 4 dpi. In vitro stimulation of porcine monocytes and macrophages with low levels of IFNα stimulates the expression of sialoadhesin (Sn/CD169), a putative PRRSV receptor in macrophages. Interestingly, such a subtle stimulation of macrophages during the first 2 dpi is sufficient to enhance the efficiency of PRRSV infection by nearly 20-fold (Delputte et al. 2007). The gene knockout technology has affirmed that intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of PRRSV (Prather et al. 2013). Similar studies using gene knockout and editing (e.g., CRISPR, Talen) techniques are under way to assess the role of CD163 in PRRS infections (Carlson et al. 2013). In a study involving 50 PRRSV-infected pigs maintained under field conditions, secretion of low levels of IFNα early pi coincided with detection of viremia from day 2 pi in most pigs (Dwivedi et al. 2012). Thus, to establish clinical disease in pigs, PRRSV modulates the host innate immunity through dysregulation of NK cell function and IFNα production.

Overall, the information on cell-type specific contribution to PRRSV vaccine induced gene expression divergence obtained from the current study could be an important add-on for PRRS research. However, there is a major need to evaluate the different roles of effector versus memory T-cell populations in anti-PRRSV responses, and in turn to stimulate protective versus pathologic responses. As more cell specific markers and immune reagents become available, more detailed research will be possible to address these important cellularity issues.

6.4 Antibody mediated adaptive immunity to PRRSV vaccine

Antibodies are the principal effector of vaccine-mediated adaptive immunity, produced by B lymphocytes, and are capable of binding specifically to a toxin or pathogen (Cooper and Nemerow 1984). Among the antibodies, neutralizing antibody (NAb) is one of the important determinants for vaccine derived adaptive immunity. Production of protective levels of NAbs usually requires multiple vaccinations since a high titre is necessary for durable protection. NAbs are usually specific for the vaccine strain (homologous), with lower/no titers of cross-neutralizing (heterologous) antibodies (Vu et al. 2011, Zhou et al. 2012). In the current study, the antibody response appeared to start at 2 weeks of primary vaccination and reached a steady state over 4-6 weeks following primary vaccination (Fig 1, Chapter 2; Fig 9, Chapter 3). This finding reflects the previous reports stating that PRRSV specific antibodies begin to appear in the infected pigs as early as 7-10 days post infection with a low viral titer followed by delayed production of neutralizing antibody (NAb) between 2-4 weeks post infection (Loemba et al. 1996). The early appearance of antibody response even after 2 weeks post vaccination does not guarantee for protection of reinfection (Lopez et al. 2007). Time of onset and raising at peak titre may vary with type of antibodies, for instance the PRRSV-specific IgM could be detected at 7 days post infection (PI), with titre peaking between 14 and 21 day post infection and decreasing to undetectable levels around 40 days PI (Loemba et al. 1996). It has been reported that anti-nsp antibodies are found early after infection (De Lima et al. 2006). Conversely, the anti-PRRSV IgG peaks at day 21 to 28 days PI and the level remain elevated through the persistent phase of infection (Nelson et al. 1997). The earliest antibodies detected are directed against the 15kDa N protein (Loemba et al. 1996) which seems unable to provide sufficient protection (Yoon et al. 1994). On the other hand, serum neutralizing antibodies (NAbs) appeared typically more than 28 dpi (Yoon et al. 1994), have a positive correlation with the level of protection against PRRSV infection (Li et al. 2014). There may be several reasons behind this unwanted long delay in PRRSV-specific antibody response in systemic circulation. As reviewed by Lunney et al., 2016; the potential mechanisms responsible for delayed NAbs include (i) glycan shielding effects of N-linked glycosylation in GPs (Ansari et al. 2006, Chand et al. 2012); (ii) presence of an immunodominant decoy epitope in GP5 upstream of the neutralizing epitope (Ostrowski et al. 2002); (iii) antibody-dependent enhancement of viral entry into target cells (Cancel-Tirado et al. 2004); (iv) suppression of innate immune responses (Sang et al. 2011); and (v) prevention of normal B cell repertoire development (Butler et al. 2014).

In commercial settings, breeding sows are vaccinated against PRRS before pregnancy occurred. The IgG antibodies are actively transferred through the placenta, via the FcRn receptor, from the maternal to the fetal circulation (Simister 2003). The passively acquired immunity through maternally derived antibody (MDA) provides a short-term protection to PRRS disease in young piglets during the suckling period. We observed a decreasing plasma level PRRSV-specific antibody of maternal origin which reached near to zero at the time of primary vaccination, and postulated that it would not interfere with the vaccine induced antibody titre (Chapter 2, Fig 2; Chapter 3, Fig 1). The influences of MDA on vaccine mediated antibody response are two dimensional. On the one hand, the MDA may allow a certain degree of priming for induction of memory B cells and enhancement of the T cell responses upon vaccine priming (Gans et al. 1999, Rowe et al. 2004). On the other hand, MDA may adversely affect the vaccination success by interfering with vaccine induced antibody response, and increasing disease severity through antibody dependent enhancement process (Yoon et al. 1996). Following primary vaccination of young piglets, the maternal antibodies bind to their specific epitopes at the vaccine antigen surface, competing with infant B cells and thus limiting B cell activation, proliferation and differentiation (Albrecht et al. 1977). This inhibition is epitope-specific, such that infant responses to non-immunodominant maternal epitopes may still be elicited (Jelonek et al. 1996). The inhibitory influence of maternal antibodies is antibody titre dependent, or rather reflects the ratio of maternal antibodies to vaccine antigen (Siegrist et al. 2004). It has been reported that antibody responses were only elicited when maternal antibodies reached a threshold of 300-400 mIU/mL in human infant (Dagan et al. 2000). Therefore the primary vaccination is recommended at the time when low serum antibody titre of maternal origin is present. However, the maternal antibody titer at which the vaccine mediated antibody responses may be elicited can only be defined experimentally by comparing antibody responses in piglets stratified according to maternal antibody titre at the time of primary vaccination.

6.5 Breed-specific transcriptome signature for PRRSV vaccine responses

The variation of clinical outcomes following PRRSV infection is influenced by a complex set of interactions between the virus and the pig host. Breed differences clearly play an important role in determining resistance/susceptibility to PRRS; various studies have affirmed that lines or breeds with improved reproductive traits, e.g., Meishan or Large White, are more resistant to the effects of PRRS (Lunney and Chen 2010). Identification of potential innate immune candidate genes will likely assist in improving our understanding not just of resistance to PRRS but also of protective immune mechanisms and thus vaccine development (reviewed by

Loving et al. 2015). In the present study, genome-wide comparisons of the microarray gene expression profiles revealed the breed specific transcriptome signatures for PRRSV vaccine response in PBMCs of DL and Pi pigs. These results are particularly comparable with the findings of Ait-Ali et al. (2011), who observed the transcriptional differences in infected pulmonary alveolar macrophages (PAMs) between Landrace and Pietrain pigs. They found a higher number of PRRSV-regulated transcripts in PAMs of Landrace pigs than in those of Pietrain pigs, which was an indication for Landrace PAMs having a reduced susceptibility to PRRSV infection compared to that of Pietrain pigs (Ait-Ali et al. 2011). Some other previous studies also support this breed differences as they have shown that detrimental impact of PRRSV infection on growth varies between and within lines and breeds (Greiner et al. 2000, Petry et al. 2005, Doeschl-Wilson et al. 2009).

The current study identified a set of potential breed specific transcriptome signature which might be predictive for the PRRSV vaccine response in Landrace and Pietrain pigs. Among the vaccine induced DEGs in PBMCs of both breeds, many of them had relatively high expression values in vaccinated Landrace pigs as compared with that of Pietrain which would imply that Landrace pigs are more susceptible to PRRSV than Pietrain pigs. The breed specific transcriptome signature for host response to PRRSV infection has also been identified in Dapulian pigs (a Chinese indigenous breed) and Duroc×Landrace×Yorkshire pigs recently (Xiao et al. 2015). The PRRS Host Genetics Consortium (PHGC) in USA conducted detailed studies of genetic resistance to PRRSV infection using a nursery-pig model and commercial crossbred pigs (Lunney et al. 2011, Rowland et al. 2012). It has been reported that inheritance of specific alleles within the swine major histocompatibility or swine leukocyte antigen (SLA) complex (SLA on SSC7) positively influences disease and vaccine responses (Lunney et al. 2009).

In addition to breed differences, there might have individual variation in host response to PRRSV (Arceo et al. 2012, Schroyen et al. 2015). In this case, the PRRSV induced immune responses are likely to be influenced by the immune status of individual pigs (reviewed by Loving et al. 2015). Given that stimulated with the same dose of virus load, innate immune responses to PRRSV could even be variable within a swine population (Xiao et al. 2004) since the disparity of individual host genetics of the same species may even attribute to variation in their resistance to infections (Ardia et al. 2011). Furthermore, the age of individual animals is another potential determinant influencing the immune response to

pathogen. The age of around four weeks, shortly after weaning, has been proved to be suitable for investigating the role of pig genome in response to PRRSV through a 'nursery pig model' (Boddicker et al. 2012).

6.6 Expression dynamics of miRNAs in PBMCs following PRRSV vaccination

MicroRNAs regulate the gene expression at post-transcriptional level through recognition of complementary sequence target elements followed by either inhibiting mRNA translation or inducing mRNA degradation (Bartel 2004). Emerging evidence suggest that miRNAs are tightly involved in many pathophysiological process of virus-host interaction including virus replication (Li et al. 2010, Guo et al. 2013, Trobaugh et al. 2014) and host immune responses (Hussain and Asgari 2010, Chen et al. 2013). MicroRNAs affect the replication of various viruses either through binding to the genome of the viruses or regulating host antiviral pathways (Li et al. 2010, Trobaugh et al. 2014). The current study performed the global miRNA expression profiling within the same PBMCs sample as used for mRNA expression profiling.

The global microRNA profiles of PBMCs identified 5, 134 and 11 differentially expressed (DE) mRNAs in DL; and 13, 222 and 37 DE miRNAs in Pietrain at 6, 24 and 72 h post vaccination, respectively. Several studies have pointed out the complex roles of microRNAs: miR-181 downregulates CD163 expression, miR-23 induces type I interferon expression through IRF3/IRF7 activation, miR-125b regulates the NF-κB pathway, and miR-24-3p suppresses hemeoxygenase-1 expression (Gao et al. 2013, Wang et al. 2013, Zhang et al. 2014, Xiao et al. 2015). Others suggested that miRNAs contribute to the pathogenesis of PRRSV infection (Cong et al. 2014). Numerous studies are under way, using samples generated in vivo and in vitro and RNA-seq analyses, to pinpoint novel pathways and genes involved in regulating PRRSV infection processes and subsequent effects on PRRS control, pathology, and persistence (Wysocki et al. 2012, Schroyen et al. 2015). Therefore, information generated from this study on the molecular bases for miRNA regulated gene expression in PRRSV vaccinated PBMCs will help to select the potential gene candidate for exploring single nucleotide polymorphisms and DNA marker associated with PRRS resistance.

6.7 Conclusions and future perspectives

We established a PBMCs transcriptome model for the evaluation of host immunogenomic response to PRRSV vaccination in pigs. As PBMCs are one of the readily accessible

biological samples, this model could be implemented for biomarker discovery involving larger pig populations. This study identified some potential candidate genes which are likely to be predictive for the PRRSV vaccine responses in peripheral blood, however, further validation is needed to affirm their association with host immune response to PRRSV vaccine. The current study could have substantial impact on the pig breeding. Germany is the third largest pork producers globally, where PRRS is of high concern for poor growth performance, reproductive impairment and animal welfare issues. The Landrace and Pietrain are two leading breeds for commercial pig production through out the world including Germany. Therefore, the results of the present study could be an add-on for the pig breeding strategy through genetic control of PRRS. However, for enhanced genetic resistance to disease to be useful for marker-assisted selection or genome-wide selection, more careful planning is required as Mellencamp et al (2008) pointed out. The stage is now set for deeper probing of the role of alleles and haplotypes involved in controlling specific antiviral responses, and for determining specific genes and their SNPs that are associated with antiviral innate immunity and vaccine responses. Moreover, selection using genomic markers that can be measured in uninfected pigs is advantageous. The present PBMCs transcriptome model has opened opportunities to expand genetic selection to a larger number of traits, simultaneously monitoring numerous phenotypes and integrating health information with growth traits.

Future studies should aim to verify whether marker-assisted selection for improved viral resistance will be effective in commercial pig production. In continuation of the current research as well as to resolve the questions yet to be answered, several experiments could be followed:

- Functional validation of the candidate genes identified in this transcriptome analysis in an independent and larger pig population
- Investigating whether the polymorphisms involved with the candidate genes identified in PBMCs are linked to PRRSV vaccine responses
- Investigate the immune potential of PBMCs in fighting off the concurrent secondary bacterial infection
- Investigating the correlation between the early stage gene expression patterns in PBMCs and vaccine mediated plasma antibody responses
- Integrated network of miRNA-mRNA for exploring the regulatory mechanism of gene expression during PRRSV vaccine mediated immunity in PBMCs

- Identifying genes associated with viral persistence and host tolerance could be of two additional targets for future work using PBMCs model

6.8 References

Adler M, Murani E, Brunner R, Ponsuksili S, Wimmers K (2013): Transcriptomic response of porcine PBMCs to vaccination with tetanus toxoid as a model antigen. PLoS ONE 8(3), e58306

Ait-Ali T, Wilson AD, Carre W, Westcott DG, Frossard JP, Mellencamp MA, Mouzaki D, Matika O, Waddington D, Drew TW, Bishop SC, Archibald AL (2011): Host inhibits replication of European porcine reproductive and respiratory syndrome virus in macrophages by altering differential regulation of type-I interferon transcriptional response. Immunogenetics 63, 437-448

Albrecht P, Ennis FA, Saltzman EJ, Krugman S (1977): Persistence of maternal antibody in infants beyond 12 months: mechanism of measles vaccine failure. J Pediatr 91, 715-718

Ansari IH, Kwon B, Osorio FA, Pattnaik AK (2006): Influence of N-linked glycosylation of porcine reproductive and respiratory syndrome virus GP5 on virus infectivity, antigenicity, and ability to induce neutralizing antibodies. J Virol 80, 3994-4004

Arceo ME, Ernst CW, Lunney JK, Choi I, Raney NE, Huang T, Tuggle CK, Rowland RR, Steibel JP (2012): Characterizing differential individual response to porcine reproductive and respiratory syndrome virus infection through statistical and functional analysis of gene expression. Front Genet 3:321, doi: 10.3389/fgene.2012.00321

Ardia DR, Parmentier HK, Vogel LA (2011): The role of constraints and limitation in driving individual variation in immune response. Functional Ecology 25, 61-73

Badaoui B, Tuggle CK, Hu Z, Reecy JM, Ait-Ali T, Anselmo A, Botti S (2013): Pig immune response to general stimulus and to porcine reproductive and respiratory syndrome virus infection: a meta-analysis approach. BMC Genomics 14, 1471-2164

Bartel DP (2004): MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281-297

Beutler B (2004): Innate immunity: an overview. Mol Immunol 40, 845-859

Boddicker N, Waide EH, Rowland RR, Lunney JK, Garrick DJ, Reecy JM, Dekkers JC (2012): Evidence for a major QTL associated with host response to porcine reproductive and respiratory syndrome virus challenge. J Anim Sci 90, 1733-1746

Butler JE, Lager KM, Golde W, Faaberg KS, Sinkora M, Loving C, Zhang YI (2014): Porcine reproductive and respiratory syndrome (PRRS): an immune dysregulatory pandemic. Immunol Res 59, 81-108

Cancel-Tirado SM, Evans RB, Yoon KJ (2004): Monoclonal antibody analysis of porcine reproductive and respiratory syndrome virus epitopes associated with antibody-dependent enhancement and neutralization of virus infection. Vet Immunol Immunopathol 102, 249-262

Carlson DF, Tan W, Hackett PB, Fahrenkrug SC (2013): Editing livestock genomes with site-specific nucleases. Reprod Fertil Dev 26, 74-82

Chand RJ, Trible BR, Rowland RR (2012): Pathogenesis of porcine reproductive and respiratory syndrome virus. Curr Opin Virol 2, 256-263

Chen CZ, Schaffert S, Fragoso R, Loh C (2013): Regulation of immune responses and tolerance: the microRNA perspective. Immunol Rev 253, 112-128

Chen Z, Lawson S, Sun Z, Zhou X, Guan X, Christopher-Hennings J, Nelson EA, Fang Y (2010): Identification of two auto-cleavage products of nonstructural protein 1 (nsp1) in porcine reproductive and respiratory syndrome virus infected cells: nsp1 function as interferon antagonist. Virology 398, 87-97

Cong P, Xiao S, Chen Y, Wang L, Gao J, Li M, He Z, Guo Y, Zhao G, Zhang X, Chen L, Mo D, Liu X (2014): Integrated miRNA and mRNA transcriptomes of porcine alveolar macrophages (PAM cells) identifies strain-specific miRNA molecular signatures associated with H-PRRSV and N-PRRSV infection. Mol Biol Rep 41, 5863-5875

Christopher PC, Danielle P, Ings CB, Sylwia K, Werner K, Tomasz IM (2015): Immune cell subsets and their gene expression profiles from human PBMC isolated by Vacutainer Cell Preparation Tube (CPT™) and standard density gradient. BMC Immunol 16, 48

Cooper NR, Nemerow GR (1984): The role of antibody and complement in the control of viral infections. J Invest Dermatol 83 (1 Suppl), 121s-127s

Costers S, Lefebvre DJ, Goddeeris B, Delputte PL, Nauwynck HJ (2009): Functional impairment of PRRSV-specific peripheral CD3+CD8high cells. Vet Res 40, 16

Dagan R, Amir J, Mijalovsky A, Kalmanovitch I, Bar-Yochai A, Thoelen S, Safary A, Ashkenazi S (2000): Immunization against hepatitis A in the first year of life: priming despite the presence of maternal antibody. Pediatr Infect Dis J 19, 1045-1052

De Lima M, Pattnaik AK, Flores EF, Osorio FA (2006): Serologic marker candidates identified among B-cell linear epitopes of Nsp2 and structural proteins of a North

American strain of porcine reproductive and respiratory syndrome virus. Virology 353, 410-421

Delputte PL, Van Breedam W, Barbe F, Van Reeth K, Nauwynck HJ (2007): IFN-α treatment enhances porcine arterivirus infection of monocytes via upregulation of the porcine arterivirus receptor sialoadhesin. J Interf Cytokine Res. 27, 757–66

Doeschl-Wilson AB, Kyriazakis I, Vincent A, Rothschild MF, Thacker E, Galina-Pantoja L (2009): Clinical and pathological responses of pigs from two genetically diverse commercial lines to porcine reproductive and respiratory syndrome virus infection. J Anim Sci 87, 1638-1647

Dwivedi V, Manickam C, Binjawadagi B, Linhares D, Murtaugh MP, Renukaradhya GJ (2012): Evaluation of immune responses to porcine reproductive and respiratory syndrome virus in pigs during early stage of infection under farm conditions. Virol J 9, 9-45

Eklund AC, Turner LR, Chen P, Jensen RV, deFeo G, Kopf-Sill AR, Szallasi Z (2006): Replacing cRNA targets with cDNA reduces microarray cross-hybridization. Nat Biotechnol 24(9), 1071-83

Fairbairn L, Kapetanovic R, Beraldi D, Sester DP, Tuggle CK, Archibald AL, Hume DA (2013): Comparative analysis of monocyte subsets in the pig. J Immunol 190, 6389-6396

Feng WH, Tompkins MB, Xu JS, Zhang HX, McCaw MB (2003): Analysis of constitutive cytokine expression by pigs infected in-utero with porcine reproductive and respiratory syndrome virus. Vet Immunol Immunopathol 94, 35-45

Ferrari L, Martelli P, Saleri R, De Angelis E, Cavalli V, Bresaola M, Benetti M, Borghetti P (2013): Lymphocyte activation as cytokine gene expression and secretion is related to the porcine reproductive and respiratory syndrome virus (PRRSV) isolate after in vitro homologous and heterologous recall of peripheral blood mononuclear cells (PBMC) from pigs vaccinated and exposed to natural infection. Vet Immunol Immunopathol 151, 193-206

Gans HA, Maldonado Y, Yasukawa LL, Beeler J, Audet S, Rinki MM, DeHovitz R, Arvin AM (1999): IL-12, IFN-gamma, and T cell proliferation to measles in immunized infants. J Immunol 162, 5569-5575

Gao L, Guo XK, Wang L, Zhang Q, Li N, Chen XX, Wang Y, Feng WH (2013): MicroRNA 181 suppresses porcine reproductive and respiratory syndrome virus (PRRSV) infection by targeting PRRSV receptor CD163. J Virol 87, 8808-8812

Genini S, Delputte PL, Malinverni R, Cecere M, Stella A, Nauwynck HJ, Giuffra E (2008): Genome-wide transcriptional response of primary alveolar macrophages following infection with porcine reproductive and respiratory syndrome virus. J Gen Virol 89, 2550-2564

Gerner W, Kaser T, Saalmuller A (2009): Porcine T lymphocytes and NK cells--an update. Dev Comp Immunol 33, 310-320

Gomez-Laguna J, Salguero FJ, Pallares FJ, Carrasco L (2013): Immunopathogenesis of porcine reproductive and respiratory syndrome in the respiratory tract of pigs. Vet J 195, 148-155

Greiner LL, Stahly TS, Stabel TJ (2000): Quantitative relationship of systemic virus concentration on growth and immune response in pigs. J Anim Sci 78, 2690-2695

Guo XK, Zhang Q, Gao L, Li N, Chen XX, Feng WH (2013): Increasing expression of microRNA 181 inhibits porcine reproductive and respiratory syndrome virus replication and has implications for controlling virus infection. J Virol 87, 1159-1171

Hussain M, Asgari S (2010): Functional analysis of a cellular microRNA in insect host-ascovirus interaction. J Virol 84, 612-620

Jelonek MT, Maskrey JL, Steimer KS, Potts BJ, Higgins KW, Keller MA (1996): Maternal monoclonal antibody to the V3 loop alters specificity of the response to a human immunodeficiency virus vaccine. J Infect Dis 174, 866-869

Johnsen CK, Botner A, Kamstrup S, Lind P, Nielsen J (2002): Cytokine mRNA profiles in bronchoalveolar cells of piglets experimentally infected in utero with porcine reproductive and respiratory syndrome virus: association of sustained expression of IFN-gamma and IL-10 after viral clearance. Viral Immunol 15, 549-556

Klinge KL, Vaughn EM, Roof MB, Bautista EM, Murtaugh MP (2009): Age-dependent resistance to Porcine reproductive and respiratory syndrome virus replication in swine. Virol J 6, 6-177

Koyama S, Ishii KJ, Coban C, Akira S (2008): Innate immune response to viral infection. Cytokine 43, 336-341

Li X, Galliher-Beckley A, Pappan L, Trible B, Kerrigan M, Beck A, Hesse R, Blecha F, Nietfeld JC, Rowland RR, Shi J (2014): Comparison of host immune responses to homologous and heterologous type II porcine reproductive and respiratory syndrome virus (PRRSV) challenge in vaccinated and unvaccinated pigs. Biomed Res Int 416727, doi: 10.1155/2014/416727

Li Y, Chan EY, Li J, Ni C, Peng X, Rosenzweig E, Tumpey TM, Katze MG (2010): MicroRNA expression and virulence in pandemic influenza virus-infected mice. J Virol 84, 3023-3032

Loemba HD, Mounir S, Mardassi H, Archambault D, Dea S (1996): Kinetics of humoral immune response to the major structural proteins of the porcine reproductive and respiratory syndrome virus. Arch Virol 141, 751-761

Lopez OJ, Oliveira MF, Garcia EA, Kwon BJ, Doster A, Osorio FA (2007): Protection against porcine reproductive and respiratory syndrome virus (PRRSV) infection through passive transfer of PRRSV-neutralizing antibodies is dose dependent. Clin Vaccine Immunol 14, 269-275

Loving CL, Osorio FA, Murtaugh MP, Zuckermann FA (2015): Innate and adaptive immunity against Porcine Reproductive and Respiratory Syndrome Virus. Vet Immunol Immunopathol 167, 1-14

Lunney JK, Chen H (2010): Genetic control of host resistance to porcine reproductive and respiratory syndrome virus (PRRSV) infection. Virus Res 154, 161-169

Lunney JK, Fang Y, Ladinig A, Chen N, Li Y, Rowland B, Renukaradhya GJ (2016): Porcine Reproductive and Respiratory Syndrome Virus (PRRSV): Pathogenesis and Interaction with the Immune System. Annu Rev Anim Biosci 4, 129-154

Lunney JK, Fritz ER, Reecy JM, Kuhar D, Prucnal E, Molina R, Christopher-Hennings J, Zimmerman J, Rowland RR (2010): Interleukin-8, interleukin-1beta, and interferon-gamma levels are linked to PRRS virus clearance. Viral Immunol 23, 127-134

Lunney JK, Ho CS, Wysocki M, Smith DM (2009): Molecular genetics of the swine major histocompatibility complex, the SLA complex. Dev Comp Immunol 33, 362-374

Lunney JK, Steibel JP, Reecy JM, Fritz E, Rothschild MF, Kerrigan M, Trible B, Rowland RR (2011): Probing genetic control of swine responses to PRRSV infection: current progress of the PRRS host genetics consortium. BMC Proc 3, 1753-6561

Mellencamp MA, Galina-Pantoja L, Gladney CD, Torremorell M (2008): Improving pig health through genomics: a view from the industry. Dev Biol 132, 35-41

Nielsen J, Botner A (1997): Hematological and immunological parameters of 4 1/2-month old pigs infected with PRRS virus. Vet Microbiol 55, 289-294

Ostrowski M, Galeota JA, Jar AM, Platt KB, Osorio FA, Lopez OJ (2002): Identification of neutralizing and nonneutralizing epitopes in the porcine reproductive and respiratory syndrome virus GP5 ectodomain. J Virol 76, 4241-4250

Palmer C, Diehn M, Alizadeh AA, Brown PO (2006): Cell-type specific gene expression profiles of leukocytes in human peripheral blood. BMC Genomics 7, 115

Petry DB, Holl JW, Weber JS, Doster AR, Osorio FA, Johnson RK (2005): Biological responses to porcine respiratory and reproductive syndrome virus in pigs of two genetic populations. J Anim Sci 83, 1494-1502

Prather RS, Rowland RR, Ewen C, Trible B, Kerrigan M, Bawa B, Teson JM, Mao J, Lee K, Samuel MS, Whitworth KM, Murphy CN, Egen T, Green JA (2013): An intact sialoadhesin (Sn/SIGLEC1/CD169) is not required for attachment/internalization of the porcine reproductive and respiratory syndrome virus. J Virol 87, 9538-9546

Renukaradhya GJ, Alekseev K, Jung K, Fang Y, Saif LJ (2010): Porcine reproductive and respiratory syndrome virus-induced immunosuppression exacerbates the inflammatory response to porcine respiratory coronavirus in pigs. Viral Immunol 23, 457-466

Rowe J, Poolman JT, Macaubas C, Sly PD, Loh R, Holt PG (2004): Enhancement of vaccine-specific cellular immunity in infants by passively acquired maternal antibody. Vaccine 22, 3986-3992

Rowland RR, Lunney J, Dekkers J (2012): Control of porcine reproductive and respiratory syndrome (PRRS) through genetic improvements in disease resistance and tolerance. Front Genet 3, doi: 10.3389/fgene.2012.00260

Sang Y, Rowland RR, Blecha F (2011): Interaction between innate immunity and porcine reproductive and respiratory syndrome virus. Anim Health Res Rev 12, 149-167

Shoemaker JE, Lopes TJ, Ghosh S, Matsuoka Y, Kawaoka Y, Kitano H (2012): CTen: a web-based platform for identifying enriched cell types from heterogeneous microarray data. BMC Genomics 13, 1471-2164

Schroyen M, Steibel JP, Koltes JE, Choi I, Raney NE, Eisley C, Fritz-Waters E, Reecy JM, Dekkers JC, Rowland RR, Lunney JK, Ernst CW, Tuggle CK (2015): Whole blood microarray analysis of pigs showing extreme phenotypes after a porcine reproductive and respiratory syndrome virus infection. BMC Genomics 16, 015-1741

Schroyen M, Tuggle CK (2015): Current transcriptomics in pig immunity research. Mamm Genome 26, 1-20

Shimizu M, Yamada S, Kawashima K, Ohashi S, Shimizu S, Ogawa T (1996): Changes of lymphocyte subpopulations in pigs infected with porcine reproductive and respiratory syndrome (PRRS) virus. Vet Immunol Immunopathol 50, 19-27

Siegrist CA, Pihlgren M, Tougne C, Efler SM, Morris ML, AlAdhami MJ, Cameron DW, Cooper CL, Heathcote J, Davis HL, Lambert PH (2004): Co-administration of CpG

oligonucleotides enhances the late affinity maturation process of human anti-hepatitis B vaccine response. Vaccine 23, 615-622

Silva-Campa E, Mata-Haro V, Mateu E, Hernandez J (2012): Porcine reproductive and respiratory syndrome virus induces CD4+CD8+CD25+Foxp3+ regulatory T cells (Tregs). Virology 430, 73-80

Simister NE (2003): Placental transport of immunoglobulin G. Vaccine 21, 3365-3369

Steuerman Y, Gat-Viks I (2016): Exploiting Gene-Expression Deconvolution to Probe the Genetics of the Immune System. PLoS Comput Biol 12(4), e1004856.

Sun Y, Han M, Kim C, Calvert JG, Yoo D (2012): Interplay between interferon-mediated innate immunity and porcine reproductive and respiratory syndrome virus. Viruses 4, 424-446

Suradhat S, Thanawongnuwech R, Poovorawan Y (2003): Upregulation of IL-10 gene expression in porcine peripheral blood mononuclear cells by porcine reproductive and respiratory syndrome virus. J Gen Virol 84, 453-459

Trobaugh DW, Gardner CL, Sun C, Haddow AD, Wang E, Chapnik E, Mildner A, Weaver SC, Ryman KD, Klimstra WB (2014): RNA viruses can hijack vertebrate microRNAs to suppress innate immunity. Nature 506, 245-248

Tuggle CK, Bearson SM, Uthe JJ, Huang TH, Couture OP, Wang YF, Kuhar D, Lunney JK, Honavar V (2010): Methods for transcriptomic analyses of the porcine host immune response: application to Salmonella infection using microarrays. Vet Immunol Immunopathol 138, 280-291

Tuggle CK, Wang Y, Couture O (2007): Advances in swine transcriptomics. Int J Biol Sci 3, 132-152

Van Gucht S, Labarque G, Van Reeth K (2004): The combination of PRRS virus and bacterial endotoxin as a model for multifactorial respiratory disease in pigs. Vet Immunol Immunopathol 102, 165-178

Van Reeth K, Labarque G, Nauwynck H, Pensaert M (1999): Differential production of proinflammatory cytokines in the pig lung during different respiratory virus infections: correlations with pathogenicity. Res Vet Sci 67, 47-52

Vincent AL, Thacker BJ, Halbur PG, Rothschild MF, Thacker EL (2005): In vitro susceptibility of macrophages to porcine reproductive and respiratory syndrome virus varies between genetically diverse lines of pigs. Viral Immunol 18, 506-512

Vu HL, Kwon B, Yoon KJ, Laegreid WW, Pattnaik AK, Osorio FA (2011): Immune evasion of porcine reproductive and respiratory syndrome virus through glycan shielding involves both glycoprotein 5 as well as glycoprotein 3. J Virol 85, 5555-5564

Wang D, Cao L, Xu Z, Fang L, Zhong Y, Chen Q, Luo R, Chen H, Li K, Xiao S (2013): MiR-125b reduces porcine reproductive and respiratory syndrome virus replication by negatively regulating the NF-kappaB pathway. PLoS ONE 8(2), e55838

Wang G, He Y, Tu Y, Liu Y, Zhou EM, Han Z, Jiang C, Wang S, Shi W, Cai X (2014): Comparative analysis of apoptotic changes in peripheral immune organs and lungs following experimental infection of piglets with highly pathogenic and classical porcine reproductive and respiratory syndrome virus. Virol J 11, 11-12

Weesendorp E, Morgan S, Stockhofe-Zurwieden N, Popma-De Graaf DJ, Graham SP, Rebel JM (2013): Comparative analysis of immune responses following experimental infection of pigs with European porcine reproductive and respiratory syndrome virus strains of differing virulence. Vet Microbiol 163, 1-12

Weesendorp E, Rebel JM, Popma-De Graaf DJ, Fijten HP, Stockhofe-Zurwieden N (2014): Lung pathogenicity of European genotype 3 strain porcine reproductive and respiratory syndrome virus (PRRSV) differs from that of subtype 1 strains. Vet Microbiol 174, 127-138

Wongyanin P, Buranapraditkul S, Yoo D, Thanawongnuwech R, Roth JA, Suradhat S (2012): Role of porcine reproductive and respiratory syndrome virus nucleocapsid protein in induction of interleukin-10 and regulatory T-lymphocytes (Treg). J Gen Virol 93, 1236-1246

Wongyanin P, Buranapraditkun S, Chokeshai-Usaha K, Thanawonguwech R, Suradhat S (2010): Induction of inducible CD4+CD25+Foxp3+ regulatory T lymphocytes by porcine reproductive and respiratory syndrome virus (PRRSV). Vet Immunol Immunopathol 133, 170-182

Wysocki M, Chen H, Steibel JP, Kuhar D, Petry D, Bates J, Johnson R, Ernst CW, Lunney JK (2012): Identifying putative candidate genes and pathways involved in immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) infection. Anim Genet 43, 328-332

Xiao S, Mo D, Wang Q, Jia J, Qin L, Yu X, Niu Y, Zhao X, Liu X, Chen Y (2010): Aberrant host immune response induced by highly virulent PRRSV identified by digital gene expression tag profiling. BMC Genomics 11, 1471-2164

Xiao S, Wang X, Ni H, Li N, Zhang A, Liu H, Pu F, Xu L, Gao J, Zhao Q, Mu Y, Wang C, Sun Y, Du T, Xu X, Zhang G, Hiscox JA, Goodfellow IG, Zhou EM (2015): MicroRNA miR-24-3p promotes porcine reproductive and respiratory syndrome virus replication through suppression of heme oxygenase-1 expression. J Virol 89, 4494-4503

Xiao Z, Batista L, Dee S, Halbur P, Murtaugh MP (2004): The level of virus-specific T-cell and macrophage recruitment in porcine reproductive and respiratory syndrome virus infection in pigs is independent of virus load. J Virol 78, 5923-5933

Yoon IJ, Joo HS, Goyal SM, Molitor TW (1994): A modified serum neutralization test for the detection of antibody to porcine reproductive and respiratory syndrome virus in swine sera. J Vet Diagn Invest 6, 289-292

Yoon KJ, Wu LL, Zimmerman JJ, Hill HT, Platt KB (1996): Antibody-dependent enhancement (ADE) of porcine reproductive and respiratory syndrome virus (PRRSV) infection in pigs. Viral Immunol 9, 51-63

Zhang Q, Guo XK, Gao L, Huang C, Li N, Jia X, Liu W, Feng WH (2014): MicroRNA-23 inhibits PRRSV replication by directly targeting PRRSV RNA and possibly by upregulating type I interferons. Virology 451, 182-195

Zhou L, Ni YY, Pineyro P, Sanford BJ, Cossaboom CM, Dryman BA, Huang YW, Cao DJ, Meng XJ (2012): DNA shuffling of the GP3 genes of porcine reproductive and respiratory syndrome virus (PRRSV) produces a chimeric virus with an improved cross-neutralizing ability against a heterologous PRRSV strain. Virology 434, 96-109

Zhou P, Zhai S, Zhou X, Lin P, Jiang T, Hu X, Jiang Y, Wu B, Zhang Q, Xu X, Li JP, Liu B (2011): Molecular characterization of transcriptome-wide interactions between highly pathogenic porcine reproductive and respiratory syndrome virus and porcine alveolar macrophages in vivo. Int J Biol Sci 7, 947-959

Zhuge ZY, Zhu YH, Liu PQ, Yan XD, Yue Y, Weng XG, Zhang R, Wang JF (2012): Effects of Astragalus polysaccharide on immune responses of porcine PBMC stimulated with PRRSV or CSFV. PLoS ONE 7(1), e29320

7. Acknowledgements

PhD is the highest scientific degree that one can achieve. I am really excited to earn my PhD at the University of Bonn, Germany. This dissertation represents not only my work at the pipette and the keyboard; it is a milestone for about three decades of love and care for animals. The story, my childhood essay, begins at a remote village of Bangladesh, where I was a close witness of how farmer cries on the death of his only cow, the mean of livelihood. Later in life, this fascination was rekindled during my bachelor studies (Doctor of Veterinary Medicine) at the Bangladesh Agricultural University (BAU), where I got the hands-on training for clinical care and management of animals. Technically, this thesis presents the genetic grounds of immune response to PRRSV vaccination in pig. Overall, this is the accumulation of many experiences I have encountered at the Institute of Animal Science (ITW), University of Bonn, from dozens of remarkable individuals whom I wish to acknowledge.

First and foremost, I wish to express my sincere gratitude to my supervisor Prof. Dr. Karl Schellander, Chair, Animal Breeding and Husbandry group, ITW, University of Bonn. I am grateful for his acceptance, valuable time and insightful advices. I was blessed with a stunning research project and I thank him very much for believing that I could take on such a work. I sincerely appreciate the encouragement, freedom and the excellent working atmosphere he has provided me to grow as an independent research scientist. Prof. Schellander has supported me not only academically but also emotionally throughout the doctoral period. He is the most influential person I have ever met in my academic career. I could not have imagined having a better advisor than him for my doctoral research. Hat's off to Prof. Schellander.

I would like thank my co-supervisor Prof. Dr. Brigitte Petersen, Chair, Preventive Health Management group, ITW; University of Bonn for her valuable suggestions and comments. Besides my supervisors, I would like to thank the rest of my dissertation committee: Prof. Dr. Karl-Heinz Südekum, Animal Nutrition group, ITW; and Prof. Dr. Wolfgang Büscher, Institute of Agricultural Engineering; both from the University of Bonn for serving as valued member of the jury for thesis evaluation and defense, even at hardship. I also want to thank them for letting my defense be an enjoyable moment, and for the brilliant comments and suggestions. My sincere thanks go to ADir Dr. Earnst Tholen and PD Dr. David Tesfaye, ITW, University of Bonn for their scientific advices and comments on the manuscripts.

I would like to thank my research group leader Dr. Christiane Neuhoff, who was always willing to help, organize stuffs in time and to give her best suggestions. Her continuous guidance enabled me to successfully complete the thesis. My sincere thanks also go to Dr. Muhammed Jasim Uddin, who was the primary resource for getting my science questions answered on air. I sincerely appreciate his time for practical suggestions and comments on the manuscripts. I do extend my sincere thanks to Dr. Christin Grosse-Brinkhaus for her technical suggestions in dry lab works particularly in bioinformatics analysis. Thanks to Dr. Maren Julia Pröll for supporting the wet lab works and social affairs.

The thesis would not have come to a successful completion without the support services I received from staff of the Teaching and Research Station at Frankenfrost, University of Bonn. I would like to thank Dr. Michael Hoelker, Mr. Buschen, Mrs. Eckert and their colleagues for animal husbandry support for this in vivo experiment and helping in blood sampling. I would like to thank all the administrative members of the Institute of Animal Science, particularly Mrs. Bianca Peters for her prompt response with all secretarial documents and Mr. Peter Müller for his talent service in computer stuffs. Thanks go to the technical team, Mrs. Helga Brodeßer, Mrs. Birgit Koch-Fabritius, Mrs. Stephanie Fuchs and Mrs. Nadine Leyer for their quick response regarding kit, chemicals and equipment settings. Thanks also go to Ms. Nadine Fricker from Life and Brain Centre, Institute of Human Genetics, University of Bonn for assisting me in performing microarray hybridization and scanning.

My sincere thanks to all the members of Prof. Schellander's group, truly a multinational research group, I have had the pleasure to work with such a giant team. My fellow colleagues, Dr. Mohammad Bozlur Rahman, Dr. Dessie Salilew Wondim, Dr. Asep Gunawan, Dr. M. Ariful Islam, Dr. MMH Sohel, Dr. Ijaj Ahmed, Dr. Sina Seifi, Dr. Sally Rashad Elsaid Ibrahim, Dr. Xeqi Qu, Dr. Sigit Prastowo, Dr. Rhui Zhang, Dr. Qin Yang, Dr. Eryk Andreas, Mr. Samuel Etay, Ms. Julia Welzenbach, Mr. Mohammed Zidane, Mr. Hari Om Pandey, Ms. Esther Heuss, Mr. Mikhael Poirier and Ms. Tsige Hailay Hagos, have all extended their support in a very special way, and I gained a lot from them through personal and scholarly interaction over the last three years and a half.

I would like to recall my former academic mentors: Prof. Dr. M.A. Samad, Department of Medicine, Faculty of Veterinary Science, Bangladesh Agricultural University, Bangladesh; and Prof. Dr. Ir. Dirk Berkvens, Department of Biomedical Sciences, Institute of Tropical

Medicine, Antwerpen, Belgium. Bunch of very foresighted advice from both of them were the guidelines for my higher education. My sincere gratitude is extended to them as well as all of my former teachers.

I would like to thank the Bangladesh Agricultural University, my Alma Mater, for granting the study leave to pursue my doctoral research abroad. My sincere regards to fellow colleagues at the Department of Medicine, Faculty of Veterinary Science, BAU for their inspiration and motivation. Cheers to my friends, too many to enlist, but all you were, you are and you will be in my heart. Thanks also go to the Bangladeshi student community and family friends living here in Bonn for their social support in making my time joyful out side the lab.

I am very much affectionate to my family, my beloved wife, Sharmin Aqter Rony, and our lovely sons Amin Saifullah and Amin Taifullah; the two jewel of my heart washed away all my tiredness. Including a tiny family time, I found Rony as a best friend, fellow coworker and the first proof reader for 24/7, which altogether shaped my working time double to the routine office hours. Thanks for being with me through the good times and bad, for the sleepless nights we were working together before deadlines. I love you so much. Now it is your turn to complete your Ph.D. dissertation. I am extremely indebted to my parents, who longed to see my achievement come true. In spite of being far away, their affection and blessings kept me fresh. Love to my younger brothers and sister, I missed them a lot.

I would like to express my sincere thanks to the Ministry of Climate Protection, Environment, Agriculture, Conservation and Consumer Protection of the State of the North Rhine-Westphalia (MKULNV) for the financial support of this research project through the programme “Umweltverträgliche und Standortgerechte Landwirtschaft (USL)”.

Finally, all praises are to Allah, The Almighty, for granting me the wisdom to undertake the research task and scripting the dissertation.