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Exploration of genetic basis of differential immune response to CSF vaccination in desi (indigenous) piglets using RNA-Seq approach

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ABSTRACT

In the present study, the transcriptome profiling of peripheral blood mononuclear cells (PBMCs) of indigenous piglets against classical swine fever (CSF) vaccination was performed for elucidating the genetic basis of their differential humoral immunity. Piglets were vaccinated with lapinised strain of CSF virus (CSFV) followed by measurement of humoral immune response using c-ELISA at 28th day post vaccination (28dpv). The RNA sequencing data was analysed using established pipeline to determine set of differentially expressed genes (DEGs) in high responder as compared to low responder piglet. The differentially expressed important immune molecules were involved in regulating important pathways including antigen processing and presentation, T cell receptor signalling, B cell development, activation and signaling genes. The genes with differential expression also included TLR 3, 6, 7, 8, 9, and antiviral molecules such as MX, and ISG (Interferon stimulated genes) family members. The protein-protein interaction of the immune genes was extracted for network representation. Most of the immune genes involved showed upregulation except the genes for antigen processing and presentation and T cell receptor signaling that were downregulated in the high responder. The immunologically important genes namely IFIT1, IFIT5, TAPBP, and TLR7 were validated using qRT-PCT and were observed to be in concordance with the RNA Seq results.

Key words: CSFV, Humoral immune response, PBMCs, Pig, RNA Seq

Classical Swine Fever (CSF) or Hog Cholera is a highly contagious and devastating viral disease of pigs worldwide resulting in serious economic losses to the farmers. Classical swine fever virus (CSFV) is a single positive stranded RNA virus belonging to the genus *Pestivirus* within the family *Flaviviridae*. In the past few years sizeable numbers of studies have been conducted to assess the protective immunity to CSFV in terms of both humoral and cell mediated immune response.

Association between the production of serum neutralizing antibodies and protection from the disease are well reported by various workers (Suradhat *et al*. 2007, Graham *et al*. 2012). Singh *et al*. (2016) reported that mean percentage inhibition (PI) of desi piglets for E2 antibodies after CSF vaccination was 62.73% which was significantly lower than the PI of crossbred piglets (97.24%). Inception of immune response against a pathogen depends on antigen presenting cells (APC's) and T cells whose activation and proliferation are regulated by antigen receptor interactions. These cells in turn stimulate the B cells to proliferate and differentiate into immunoglobulin producing subsets of plasma cells. The CSFV shows a predilection for cells of the immune system (Summerfield and Ruggli 2015) and may alter transcription of immune response genes. Considerable variations have been reported among the individuals in response to infectious disease and vaccination, a significant segment of which is contributed by the genetics of the individual (Davies *et al*. 2009, Lewis *et al*. 2008, Pathak *et al*. 2017a). Hence, transcriptional profile during CSF vaccination may facilitate the development of effective strategies for controlling classical swine fever. Additionally, this will also help in exploring the genetic differences in escalating immune response between individuals. The differential immune response of the piglets against CSF vaccination may form the basis of their selection for breeding for natural resistance to CSF disease.

RNA Sequencing (RNA Seq) is a powerful deep sequencing technology to quantify the number of transcripts in a cell. It helps in identification of novel transcripts unlike the hybridisation based approaches that allow transcript detection from existing genome sequences only. The present work envisages the study of transcriptome profile of PBMCs of two vaccinated desi (indigenous) piglets using RNA Seq

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analysis to justify the genetic basis of differences in their humoral immune response. We identified specific transcriptome signatures and pathways that might account for differences in immune response to CSFV vaccine.

MATERIALS AND METHODS

Animals, vaccination and blood collection: The experimental procedures in the present study were approved by Institute Animal Ethics Committee. The desi piglets maintained at Indian Veterinary Research Institute, Izatnagar under AICRP were utilized in present investigation. The IVRI strain of lapinised vaccine was used for vaccination of piglets at 3 months of age through subcutaneous route (field dose) after ensuring absence of their maternally derived antibodies. About 10 ml of blood was collected from anterior vena cava of each piglet at 28 dpv in heparin coated and uncoated vacutainers for PBMCs and serum isolation, respectively.

Serum separation and ELISA: The blood samples collected were kept at slanting position for 2 h for serum separation and kept overnight at 4°C in the refrigerator for better yield of serum followed by centrifugation at 3000 rpm for 15 min. The serum was kept in 1.5 ml micro centrifuge tubes and stored at –20°C for long term storage. The serological study was done using commercial ELISA kit (Priochek). The percent inhibition (P.I) values were calculated based on a formula PI = 100 – [Corrected OD test sample/Corrected OD reference serum 4]*100 using the optical density (OD) values as measured by the ELISA reader. Based on the differences between the PI values, two desi pigs were chosen for the trancriptome study using their PBMCs.

PBMCs isolation: The whole blood diluted with PBS (1:1) was layered gently on histopaque-1077 (Histopaque®-1077 Sigma) and centrifuged at 2200 rpm for 40 min. The interphase layer above the red blood cell layer rich in peripheral blood mononuclear cells (PBMCs) was transferred into a separate tube and washed three times with PBS with centrifugation at 2500 rpm for 15 min. The final pellet was re-suspended in a complete medium containing RPMI-1640 and antibiotic Streptopenicillin (1×) followed by centrifugation at 2500 rpm for 15 min. Supernatant was discarded and Trizol was added to the pellet. PBMCs from two desi piglets were outsourced for RNA sequencing fraction of which was used for RNA isolation using Trizol.

Library preparation and RNA sequencing: PBMCs were outsourced for sequencing. The quality of RNA was checked using bioanalyzer followed by library preparation and sequencing on Illumina HiSeq Platform. The raw reads generated in form of FASTQ format were subjected to quality test. The Quality Check (QC) report provided information on raw reads summary and graphs for quality distribution, base distribution and GC distribution.

Identification of differentially expressed genes: The low quality reads (phred score < 25 and read length <50) were trimmed using PRINSEQLITE following which the read quality checked using FASTQC. The gtf file downloaded from UCSC genome browser and combined FASTA file of *Sus scrofa* were gunzipped and concatenated to prepare the reference genome. All the quality reads obtained were mapped with the reference genome using aligner BOWTIE2. The pig with PI value 92% (low responder) was assumed as reference control against the one with 98% (high responder) PI. The counts were estimated using RSEM and the differentially expressed genes (DEGs) were determined using different DE packages. The differentially expressed genes were identified using DESeq (Anders and Huber 2010), edgeR (Robinson *et al*. 2010) and EBSeq (Leng *et al*. 2013). The common differentially expressed genes from all the packages were identified by VENNY 2.0 (Oliveros 2007). The common DE genes were mapped to orthologous sequences of the human in g:Orth for obtaining GeneID followed by functional profiling of genes. Then interaction of differentially expressed genes of immunological importance was studied.

BIOGRID (Biological General Repository for Interaction Datasets) is a freely accessible online database that stores and distributes vast array of protein and genetic interactions in its latest version 2.0 containing more than 1,16,000 interactions from certain species (Stark *et al*. 2006). This repository has well defined genetic and protein interactions for *Homo sapiens* but not for *Sus scrofa.* Since the genetic interactions are well conserved across the species (Suthram *et al.* 2006) g:Orth in g:Profiler web server was employed to produce orthology (functionally equivalent genes) predictions between species to facilitate functional and interaction annotation transfer across species (Reimand *et al.* 2011). To construct the interaction network with the differentially expressed genes in the present study, *Sus scrofa* orthologs in human were queried using g:Orth in g:profiler. Customized perl scripts were used to extract interactions involving the differentially expressed genes. The complete interaction network was visualized in Cytoscape 3.0.2 (Shannon *et al*. 2003).

RNA extraction and cDNA synthesis: The RNA was isolated from PBMCs using Trizol reagent (Ambion) and purity and concentration were measured at 260/280 nm and 260/230 nm using a spectrophotometer (Nanodrop 1000, Thermo Scientific). cDNA was synthesized using RevertAid First Strand cDNA Synthesis kit (Thermo Fisher Scientific).

Validation of DE genes by quantitative real time PCR (qRT-PCR): A set of four immunologically important DEGs were selected for validation by qRT-PCR. The primers were designed using Primer3Plus software and Real time PCR (qRT-PCR) was performed on Applied Biosystems 7500 Fast real time PCR using 2× SYBR Green Master mix. GAPDH was used as an endogenous control (Cinar *et al*. 2013). The primer sequences of DEGs used in the study for validation are given in Table 1. A melt curve analysis was performed to know the specificity of the qRT-PCR. All the samples were run in triplicates and the relative expression of each sample was calculated using the $2^{-\Delta\Delta CT}$ method with low responder as calibrator.

Table1. Self designed primers for qRT-PCR validation using Primer 3 plus software

Gene	Primer sequence	Amplicon length (bp)	Annealing temperature (°C)
IFIT1	FP:TCAGAGGTGAGAAGGCTGGT RP:GCAAGTGTCCCTCACCTTGT	108	58
IFIT5	FP:ACAGGTCTTCTCTGCGTACC RP:CCCGCTCATAGTACTCAGCA	159	58
TAPBP	FP:AGGGACTCAACGCAAAAGAA RP:GCTCAGTGGAGAGAGGTTGG	148	56
TLR7	FP:AACTCTGCCCTGTGATGTCA RP:TCCAGGAATCGCTGTCAAGT	88	58

RESULTS AND DISCUSSION

Competitive ELISA: The piglets used for collecting blood were screened for CSFV maternally derived antibodies (MDA) and all the animals had 0% (NIL) percentage inhibition (PI) on day of vaccination. The PI values of the two desi piglets were 92% and 98%, respectively. Thus one of the pig (92%) had comparatively low humoral response than second pig (98%). In order to investigate the reason for the difference in humoral immune response of these two piglets, RNA-Seq was done to unravel the differences at transcriptome level.

Library preparation and data quality: The mean read length was found to be 100 bp with a Phred score of 34.8 and 33.23, 35.21 and 33.93 for paired end reads (R1, R2) of the two samples respectively.

Differentially expressed genes (DEGs) and functional annotation: A total 5,869, 10,000 and 22,861 were DEGs mined using EBSeq, edgeR and DESeq2 respectively. The common DEGs across all the packages were 5563. Functional annotation of common DEGs was carried out using g-profiler (Reimand *et al*. 2011). The gene ontology of the common DEGs was defined in three domains namely biological process, cellular component and molecular function. The ontologies showed significant enrichment of immune related processes. The highest number (215) of immune pathways were enriched in biological process. The top 10 upregulated pathways among the significantly enriched immune genes included viral process, immune system process, positive regulation of biological process, immune response-activating signal transduction, positive regulation of cellular process, lymphocyte activation, leukocyte activation, immune response, positive regulation of immune system process and immune response-regulating signaling pathways. In the cellular component domain, the top ten upregulated pathways included intracellular part, cytoplasm, intracellular, intracellular organelle part, membrane-bounded organelle, intracellular organelle, intracellular membrane-bounded organelle, organelle part, organelle and nuclear part. The molecular function comprised of protein binding, poly(A) RNA binding, RNA binding, heterocyclic compound binding, organic cyclic compound binding. Significant ($P<0.05$) categories among the canonical pathways KEGG (Kyoto Encyclopaedia of Genes and Genomes) included antigen processing and presentation, B cell receptor and signaling pathway, T cell receptor and signaling pathway.

The protein-protein interaction network of total 1181 DE genes (differentially expressed genes) with the available human interactions in BIOGRID database resulted in dense hairball. The interactions were narrowed down to DE-DE (differentially expressed) interactions. The network was constructed using 3621 interactions and 742 nodes (proteins) corresponding to protein- protein interactions between them. A total of 209 genes with $\pm 0.5 \log_2$ fold change in expression with a degree 15 were finally obtained to represent network (Fig. 1). These genes were named as Differentially Expressed Highly Connected (DEHC) genes.

The factors governing innate immune response such as TLR's and interferon regulatory factors expressed differently. *TLR1, TLR2, TLR3, TLR4, TLR6, TLR9* were upregulated, *RIG-1* and interferon regulatory factor such as *IRF1, IRF3* were downregulated in high responder piglet; whereas, *IFN-g* receptor 1 (*IFNGR1*) expression was upregulated. Some of the T cell receptor signalling molecules *Lck, CD4, Zap70, Lat2, SH2B3, GRAP2, ITK* and *PLCG1* along with molecules involved in antigen processing and presentation like *TAP1, TAP2, CALR, B2M, CREB3, RFX1* showed significant downregulation; whereas *TAPBP* was upregulated. Antiviral molecules *MX1, IFIT1 (ISG56), IFIT2(ISG54), IFIT3(ISG60), IFIT5(ISG58)* showed upregulation in high responder. A total of 62 genes (Fig. 2) governing B cell activation from gene ontology of g-profiler that were differentially expressed included *RC3H, KIT, MEF2C, CD86, CD24, CR2, IKZF3, LYN, HHEX, MSH6, ZBTB1, INPP5D, TLR9, SWAP70, TNFAIP3, PRKCD, CD180, ATP11C, RBPJ, BMI1, HSPD1, TPD52, ID2, APLF, PRKDC, LIG4, MALT1, CD38, GPR183, FOXP1, CD40, PLCL2, BCL3, PTPN6, JAK3, LFNG, LYL1, LAT2, TRDC, FAS, LEF1, MLH1, IL7R, BST1, CTLA4, SASH3, MFNG, POLM, NHEJ1, BAD, TBX21, MIF, CLCF1, TNIP2, MSH2, CD40LG, BATF, MZB1, ADA, TBC1D10C, FOXP3.* Out of these, 237 were upregulated, and 25 were downregulated. Besides these, genes of B cell receptor (BCR) signalling pathway such as *SYK, LYN, SYN,*

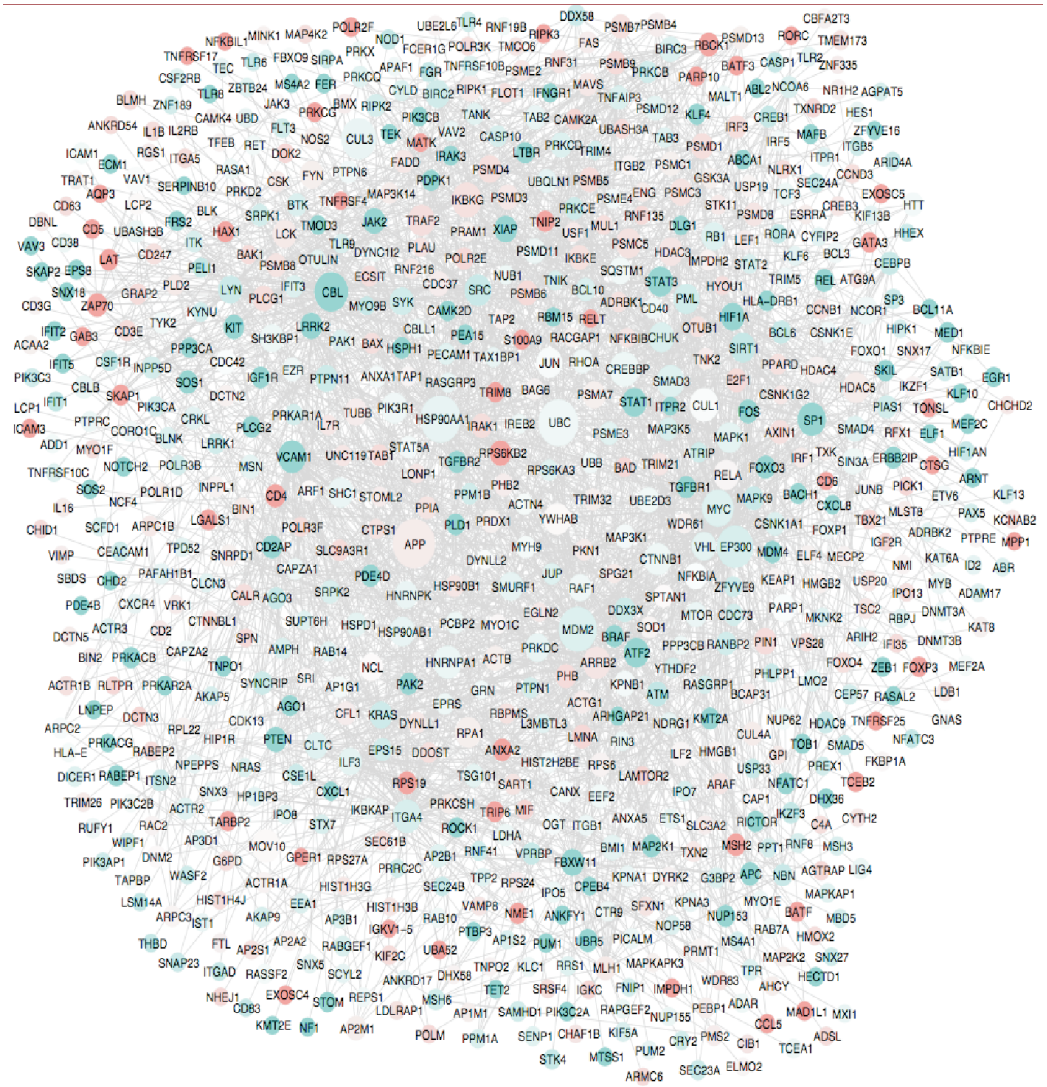

Fig. 1. Gene network showing protein-protein interaction of DEHC (Differentially Expressed Highly Connected) genes of high responder piglet using Cytoscape (3.4.0). The green nodes indicate upregulation and red nodes indicate downregulation of genes. The size of node indicates its degree of connectivity to the other immune genes/molecules.

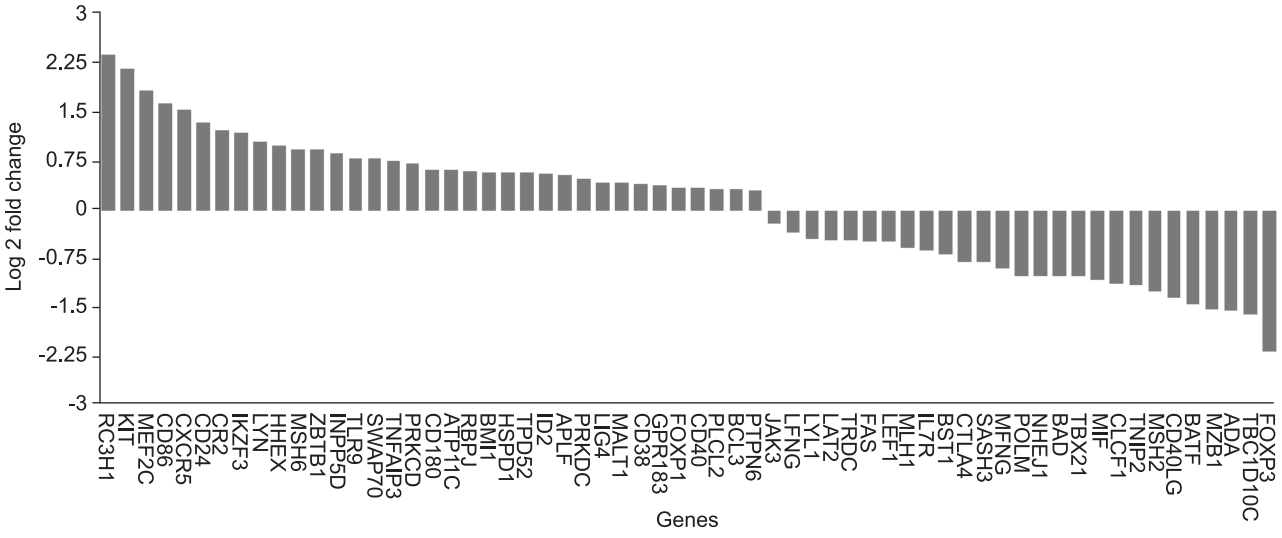

Fig. 2. Graphical representation of B cell activation genes in terms of their log 2 fold changes. Out of 62 genes, 35 were upregulated and 27 were downregulated in the high responder piglet.

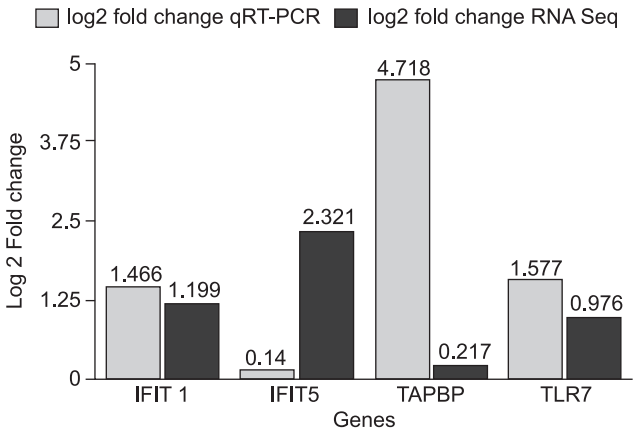

Fig. 3. The change in gene expression levels of IFIT1, IFIT5, TAPBP and TLR7 was measured by qRT-PCR and RNA-Seq. For qRT-PCR GAPDH used as reference gene for normalization and fold change was calculated by the ΔΔCt method. The concordance graphs are plotted for the qRT-PCR and RNA seq analysis results.

BTK, FCGR2B, PLCG2, BLNK (SLP-65/BASH), NFAT, BAD and *ATF-2* were upregulated.

qRT-PCR: The RNA-Seq analysis results were validated by qRT-PCR (Fig. 3). *IFIT1*, *IFIT5*, *TAPBP* and *TLR7* genes were selected for qPCR validation keeping in view their significant role in antiviral and antigen processing activity. The results expressed as log 2 fold change, were in concordance with the RNA Seq results as the selected genes *IFIT1*, *IFIT5*, *TAPBP* and *TLR7* showed upregulation (Fig. 3). However, differences in the fold change were higher for *IFIT5* and *TAPBP* genes.

The CSFV is a potential pathogen by virtue of its powerful strategy to evade the host immune system. Thus the role of host immunity in combating the viral pathogenesis is crucial component to formulate the preventive strategy against this deadly disease. After visualising the differences in humoral immune response, transcriptome study elucidated immune genes which may be responsible for these differences. The significant enrichment of TNF-signaling, T cell receptor signaling pathway (Medzhitov and Janeway 2000), NF-Kappa B (Dev *et al.* 2011, Pathak *et al.* 2017b) and B cell receptor signaling pathway indicated the involvement of immune response regulatory pathways in response to CSF vaccination in desi pigs.

The *TLR 3,7,8,9* recognize the viral nucleic acid which are released in the endosomes after their phagocytosis and lysis in the cell (Kawai *et al*. 2006). Out of all the TLR's with positive enrichment *TLR3, TLR7, TLR8, TLR9* and *TLR6* showed a higher fold change in high responder. But, the *TLR3* was upregulated and *IRF3* was downregulated. Similar findings wherein, Npro mediated degradation of *IRF3* to prevent type I IFN induction by CSFV has been studied previously by various workers (Seago *et al*. 2007, Chen *et al*. 2007, Hilton *et al*. 2006, Summerfield and Ruggli 2015). Cao *et al*. (2015) reported activation of *TLR2, TLR4, and TLR7* in response to CSFV virulent and vaccine strains whereas *TLR3* showed no change in virulent and downregulation against vaccine virus. In humans, memory B cells constitutively expressed specific TLR's like *TLR9* which resulted in persistent innate immune response across the time (Pulendran *et al*. 2006). Consequently, it provided prolonged neutralizing antibody titres. The significant upregulation of TLR's at 28 dpv accounts for higher antibody response in high responder.

Non-TLR viral sensors such as *RIG1* were downregulated at 28 dpv in high responder. The *IFNGR1* which is the major receptor for *IFN-g* showed upregulation. Virus specific *IFN-g* responses in CSF challenged and vaccinated pigs reported to increase up to 21d post vaccination (Graham *et al*. 2010).

Some key molecules involved in antigen processing and presentation such as *TAP1, TAP2, CALR, B2M, CREB3* and *RFX1* were downregulated; whereas *TAPBP, CANX, NFAT*, and C3 were upregulated in high responder piglet. The genes for T cell receptor signaling such as *LCK, CD4, ZAP70, LAT2, SH2B3, GRAP2, PLCG1, AKT, ITK* showed downregulation. The antigen triggers antigen presenting cells (APCs) namely dendritic cells and macrophages which undergo maturation and finally migrate to lymph nodes to activate T and B cells. The reason of downregulation of major APC molecule in present study may be due to the role and expression of these genes at earlier phase of immune response.

The *CXCR5* (chemokine receptor) was highly upregulated which is instrumental in B lymphocyte migration to lymphoid tissue for their interaction with follicular dendritic cells and T cells for antibody response (Payne *et al*. 2009). Further, *Hhex* a transcription factor for B cell development was upregulated in our study. It is reported that loss of *Hhex* gene in mouse model led to the progressive loss of B lymphocytes in circulation along with complete loss of B cell lymphoid progenitors in bone marrow and B cell subsets in spleen (Jackson *et al*. 2014). Three key signaling mechanisms playing interesting role in early B cell development are *c-KIT, FLT3* and *IL-7*. Out of these, *KIT* gene, a homolog of *c-KIT* was upregulated. When bound to ligand *SF, KIT* gene induces activation of several signaling pathways such *RAS/Erk, PI3K, MAPK* orchestrating the recruitment of various kinases and phosphatases like *JAK2, MATK, SHP-1, SHP-2* terminating in the activation of *STAT 1, 3* and *5* whose translocation to the nucleus is instrumental in activating transcription factors essential for B cell development (Maddaly *et al*. 2010).

Apart from these, *IKZF3* member of Ikaros family Zinc Finger 3 which is another important transcription factor for early B cell progenitors was also significantly upregulated in high responder. The B cell formation is characterized by a series of stages highlighted by stage specific differentiation cell surface markers. The *CD24* which is a persistent cell surface marker from Late pro-B cell stage to mature B cell stage (Maddaly *et al*. 2010) was also upregulated. Besides this, *CR2/CD21* an important B cell co-receptor was upregulated. These all molecules which

are key players in B cell development were found to be up regulated in high responder which indicates more robust B cell development in high responder and thus supports the differences in humoral immune response between high and low responder piglets.

The genes involved in BCR signalling also showed upregulation. Genes *SYK, LYN, SYN, BTK, FCGR2B, BLNK (SLP65/BASH), PLCγ2, NFAT, BAD* and *ATF-2* showed upregulation in high responder. The *LYN, SYN* are kinases that play important role in initiating the signal transduction by phosphorylating *ITAM*. *SYK* kinases connect the BCR to distal signal transduction elements by coupling *BLNK* and *BTK* to activate *PLCγ2*. *PLCγ2* is an important molecule of BCR-mediated signal transduction. *NFAT* is a distal effector molecule of BCR signalling that mediates cytokine production and activates other effector molecules (Woyach *et al*. 2012).

The role of *MX1* as a potent antiviral molecule has been suggested by various workers in pigs as well as humans (Mitchell *et al*. 2013). In our study, *MX1* and *MX2* both were upregulated but *MX2* had a higher fold change, though till date the mechanism of *MX* to exert antiviral effects is unknown. In addition to *MX*, other ISG's also reported in the study which are responsible for antiviral activity in several species. The swine *MX1* confers resistance against a number of viruses like vesicular stomatitis virus (Zhang *et al*. 2013) and influenza virus (Palm *et al*. 2010). The Swine *Mx1* was able to block the proliferation of CSFV in PK-15 cell line (He *et al*. 2014). *IFITs* protein expression is one of the most highly induced expression in response to interferons and viruses (Fenstrel *et al*. 2011).

Studies have shown that *IFIT1* removes viral RNA's containing 5'PPP with the help of a protein complex composed of several *IFITs* (Pichlmair *et al*. 2011) . In our study, the most highly expressed *IFIT* was *IFIT5/ISG58* followed by expression of *IFIT1/ISG56, IFIT2/ISG54*, and *IFIT3/ISG60*. Some reports suggest that the translation of hepatitis c virus positive strand RNA genome is disrupted in presence of *ISG56 (*Jiang *et al*. 2008).The expression of these ISG's family supports certain level of anti-viral host response in high responder. Apart from this, *Viperin/RSAD2* another *ISG* which is known to be used by Human Cytomegalo Virus (HCMV) to enhance its infectivity by disrupting host cell metabolism (Seo *et al*. 2011) is known to inhibit the expression of several other viruses including HCMV, Sindbis virus and Influenza A virus (Jiang *et al*. 2008, 2010; Zhang *et al*. 2007, Wang *et al*. 2007) was found to be upregulated in our study with a higher fold change. *OAS1, OAS2, ADAR* were also upregulated. But the expression of *IFITM 1/2/3 ISG* group was downregulated in the study.

Our current study on the effect of CSF vaccine on pig PBMCs transcriptome depicts significant upregulation of genes involved in B cell development and B cell receptor signaling pathway which support the higher antibody titre in high responder piglet. Further investigation of the effect of these genes on immune responses to viral vaccines may lead to important findings regarding genetic control of immune responses and the ability to use such information in designing new vaccine candidates.

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