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ORIGINAL ARTICLE

RNA Seq analysis for transcriptome profiling in response to classical swine fever vaccination in indigenous and crossbred pigs

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Abstract In present investigation, differential expression of transcriptome after classical swine fever (CSF) vaccination has been explored at the cellular level in crossbred and indigenous (desi) piglets. RNA Sequencing by Expectation-Maximization (RSEM) package was used to quantify gene expression from RNA Sequencing data, and differentially expressed genes (DEGs) were identified using EBSeq, DESeq2, and edgeR softwares. After analysis, 5222, 6037, and 6210 common DEGs were identified in indigenous post-vaccinated verses pre-vaccinated, crossbred post-vaccinated verses pre-vaccinated, and post-vaccinated crossbred verses indigenous pigs, respectively. Functional annotation of these DEGs showed enrichment of antigen processing-cross presentation, B cell receptor signaling, T cell receptor signaling, NF-κB signaling, and TNF signaling pathways. The interaction network among the immune genes included more number of genes with greater connectivity in vaccinated crossbred than the indigenous piglets. Higher expression of *IRF3*, *IL1β*, *TAP1*, *CSK*, *SLA2*, *SLADM*, and *NF-kB* in crossbred piglets in comparison to indigenous explains the better humoral response observed in crossbred piglets. Here, we predicted that the processed CSFV antigen through the T cell receptor signaling cascade triggers the B cell receptor-signaling pathway to finally activate MAPK kinase and NF-κB signaling pathways in B cell. This activation results in expression of genes/transcription factors that lead to B cell ontogeny, auto immunity and immune response through antibody production. Further, immunologically important genes were validated by qRT-PCR.

Keywords CSF vaccination · Functional annotation · Humoral response · Pigs · Transcriptome

Abbreviations

CSF	Classical swine fever
MDA	Maternally derived antibodies
PBMC	Peripheral blood mononuclear cells,
FCS	Fetal calf serum
GTF	Gene transfer file
GO	Gene ontology
RIN	RNA integrity number
DEGs	Differentially expressed genes
cELISA	Competitive enzyme linked immunosorbent assay
BioGRID	Biological General Repository for Interaction Datasets
IRF3	Interferon regulatory factor 3
TAP1	Transport associated protein1
NF-kB	Nuclear factor kappaB,
SLA2	Swine leucocyte antigen 2
IL1β	Interleukin1 β
RSEM	RNA Seq by expectation maximization
BCR	B cell receptor

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Introduction

Classical swine fever (CSF), also called Hog Cholera or European Swine Fever is one of the most important infectious diseases of pigs and wild boar, causing significant economic losses to the pig industry all over the world. It is an acute or chronic, contagious, febrile, highly devastating disease which is characterized by fever, hemorrhage, leucopenia, abortion and high mortality. The disease is caused by a pestivirus named classical swine fever virus (CSFV), an enveloped, single-stranded, positive RNA virus, approximately 12.3 kb in length. The 12.3 kb CSFV genome consists of one large open reading frame (ORF) that encodes an approximately 4000 amino acid polyprotein which is co- and post-translationally processed into 11–12 final cleavage products (NH2, Npro, C, Erns, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B,COOH) using cellular and viral proteases. The ORF is flanked by untranslated regions (UTRs) that are highly conserved among virus isolates (Risatti et al. 2003). The virus is structurally and antigenically related to other members of the genus Pestivirus, such as bovine viral diarrhea virus (BVDV) and border disease virus (BDV). The CSFV has one serotype divided into three major genotypes (1, 2, and 3) and 10 subgenotypes (1.1, 1.2, 1.3; 2.1, 2.2, 2.3; 3.1, 3.2, 3.3, and 3.4). Based on phylogenetic analysis, the Indian isolates are grouped into two subgenotypes, 1.1 and 2.2, with a predominance of subgenotype 1.1. During the past few years, the outbreaks of swine fever have been recorded from the states of Nagaland, Manipur, Tripura, West Bangal, and Tamilnadu (Rahman 2011).

In the highly endemic areas, routine vaccination against CSF is the most common means used for prevention and control. Massive vaccination with live attenuated vaccines, such as C-strain, developed in China in mid-1950s has been implemented routinely as a major control strategy. The C strain, modified live vaccine has been regarded as one of the most effective CSF vaccines that provides complete clinical and virological protection, i.e., sterile immunity, within a week of vaccination (Suradhat et al. 2001; Van Oirschot 2003). Although, modified live CSF vaccine could effectively induce protective immunity in pigs, certain conditions are required to achieve complete viral protection. Piglets are immunized at 8–12 weeks old for developing a greater number of CSFV-specific IFN-γ producing cells and CSFV-specific antibody titer. The CSFV shows a predilection for cells of the immune system and may alter transcription of immune response genes. It has also been shown to evade the host immune system and to establish chronic infection under both natural and experimental conditions (Haller et al. 2007).

Hence, transcriptional profile during CSF vaccination may facilitate the development of effective strategies for controlling classical swine fever. The RNA Seq analysis has been used as a powerful tool to quantify and identify the differentially expressed genes on genome wide scale across the species (Majewska et al. 2017; Conesa et al. 2016; Shi et al. 2015). Again considerable variations have been found among individuals in response to infectious disease and vaccination, a significant proportion of which can be shown to be genetic (Davies et al. 2009). The animals yielding differential immune response may be basis for selecting breeding animal for swine fever natural resistance. In an investigation (Singh et al. 2016) of our laboratory, it was found that mean percentage inhibition (PI) of desi piglets for E2 antibodies after CSF vaccination was 62.73% which was significantly ($P \leq 0.01$) lower than the PI of crossbred piglets (97.24%). Therefore, in present study, transcriptome profile of PBMC isolated from unvaccinated and vaccinated indigenous (desi) and crossbred piglets was investigated to compare the differential humoral immune response in indigenous (desi) and crossbred piglets after CSF vaccination. Further, transcriptional status of important genes was validated by real time PCR (qPCR).

Material and methods

Animals and ethics

The experimental procedures in the present study were approved by Institute Animal Ethics Committee. The piglets maintained at Indian veterinary research institute, Izatnagar, under AICRP were utilized in present investigation. The IVRI strain of lapinized vaccine was used for vaccination. All 08 piglets (2 indigenous and 6 crossbred) were vaccinated at 3 months of age and at the time of vaccination it was ensured that animals had nil maternally derived antibodies. The 02 indegenous piglets were litter of a common farrowing whereas; the 06 crossbred piglets were of another farrowing. The both crossbred and indigenous (desi) pigs were maintained in similar managemental regime.

Blood collection

The permission for blood collection was obtained from Institute Animal Ethics Committee (IAEC). The blood was collected on the day of vaccination and after 4 weeks of vaccination for PBMC and RNA isolation in heparin coated vacutainer vials.

PBMC isolation

The whole blood diluted with PBS (1:1) was layered on 3 ml of histopaque-1077 and was centrifuged at 2200 rpm for 40 min. The interphase layer rich in peripheral blood mononuclear cells (PBMCs) was transferred into a separate tube and was washed three times with PBS at 2500 rpm for 15 min. The final pellet was resuspended in a complete

medium containing RPMI-1640 and 10% fetal calf serum (FCS) followed by centrifugation at 2500 rpm for 15 min. Supernatant was discarded and Trizol was added. Before and after CSF vaccination, PBMC from indigenous (desi) and crossbred piglets were used for RNA sequencing.

Library preparation and RNA sequencing

The PBMC isolated from indigenous (desi) and crossbred piglets before and after vaccination was further processed for RNA isolation. The quality of RNA was checked 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 QC output provided information on raw reads summary and graphs for quality distribution, base distribution and GC distribution.

Transcriptome quantification and functional analysis of DEGs

Raw reads were first processed by prinseq-lite to remove the reads of low quality (mean phred score <25 and read length <50). The "rsem prepare reference" script of RSEM package was used to generate reference transcript sequences by using gene annotation file (GTF format) and the full genome sequence (FASTA format) of *Sus scrofa* version 10.2. Then, all the quality reads of different samples (pre-vaccination and post-vaccination reads of indigenous and crossbred piglets) were mapped to generated reference transcript sequences by using RSEM in built Bowtie-2 program (Langmead et al. 2009) to identify the identity between cDNA sequences and its corresponding genomic exons that manifests the regions of exact matches. The "rsem calculate expression" script of RSEM handles both the alignment of reads against reference transcript sequences and the calculation of relative abundances. Then, genes resulted were compared in three different ways: post-vaccinated and pre-vaccinated genes of indigenous piglets, post-vaccinated and pre-vaccinated genes of crossbred piglets, post-vaccinated genes of crossbred and indigenous piglets. All the three combination of genes were used further for identifying DEGs. The differentially expressed genes were identified using DESeq (Anders and Huber 2010), edgeR (Robinson and Oshlack 2010) and EBSeq (Leng et al. 2013) at P value ≤0.05 and ±5 fold change. The numbers of common differentially expressed genes (DEGs identified by all three softwares, i.e., EBSeq, DESeq2, and edgeR) from all the three algorithms were identified by VENNY 2.0.1 (Oliveros 2007). Common DE genes were mapped to orthologous sequences of the Human in g:Orth (A tool for mapping orthologous genes in related organisms) (http://biit.cs.ut.ee/gprofiler/gorth.cgi) for getting GeneID followed by functional profiling of genes. Then, interaction of DEGs of immunological importance was studied.

Interaction network among the DEGs

The Biological General Repository for Interaction Datasets (BioGRID) is a curated biological database of protein-protein and genetic interactions (Stark et al. 2011). It provides a comprehensive resource of protein–protein and genetic interactions for all major model organism species. BioGRID currently holds 7,88,169 interactions (genetic and protein) curated from both high-throughput data sets and individual focused studies derived from over 54,430 publications in the primary literature. In this repository, genetic interactions in human are well defined and those in *Sus scrofa* are very few. Since genetic interactions have been shown to be well-conserved across 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 DEGs 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 DEGs. The complete interaction network was visualized in Cytoscape 3.0.2 (Shannon et al. 2003).

Validation of DEGs by quantitative real time PCR (qRT-PCR)

RNA was isolated from whole blood of same indigenous and crossbred piglets during both pre- and post-vaccination stage by Trizol method. The purity of the RNA was assessed by nanodrop measuring the absorbance of the RNA solution at 260 nm and 280 nm. The QuantiTect Reverse Transcription Kit was used for cDNA synthesis according to the manufacturer's instructions and real time RT-PCR was performed using CFX™ 96 BioRad real time PCR using 2X SYBR Green Master mix. The GAPDH was used as an endogenous control. A melt curve analysis was performed to know the specificity of the qPCR. All the samples were run in triplicates. The relative expression of each sample was calculated using the $2^{-\Delta\Delta CT}$ method with control group as calibrator. One-way ANOVA was done in JMP9 (SAS Institute Inc., Cary, USA) and differences between groups were considered significant at $P < 0.05$.

Results

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. Again cELISA was done 28 days after vaccination to detect the presence of CSFV specific antibodies in indigenous and crossbred piglets. The mean percentage inhibition in

indigenous pigs (n = 2) was 95% and in crossbred piglets (n = 6) was 98.33% which further revealed a lower percentage inhibition in indigenous pigs. RNA isolated from PBMCs of indigenous and crossbred piglets before and after vaccination was subjected to quality check on Bioanalyzer and the RNA Integrity Number (RIN) was found to be satisfactory for RNA Seq Analysis. The cDNA library was subjected to RNA sequencing on Illumina Hiseq-2000 platform. The cDNA libraries prepared from these RNA samples were quality checked for average size of the library and concentration. The library was found to be of average length of 100 bp with good concentration. Over 70 million paired end (PE) reads were generated for each sample. NGS-QC was performed for the raw illumina data. The FASTQ files of raw reads were checked for various parameters like number of reads, Phred score, GC content of reads, mean read length, and distribution of bases. The mean Phred score of reads of indigenous pigs was 33.42 for pre-vaccinated and 34.33 for post-vaccinated sample. The mean phred score of reads of crossbred was 32.36 for pre- vaccinated status and 33.66 for post-vaccinated sample. The Phred score was above 30 in more than 85% reads in each sample, indicating good quality of reads.

DEGs, functional annotation, and interaction network enriched in immune system processes of indigenous (desi) post-vaccinated versus pre-vaccinated pigs

A total 5547, 7587, and 8770 DEGs were identified using EBSeq, DESeq2, and edgeR, respectively, (5% FDR) and 5222 genes were common DEGs, predicted by all three softwares. Out of 5222 significant DE genes, 2836 genes were found to be upregulated and 2386 genes were downregulated. In post-vaccinated pigs mostly immune genes conferring humoral immunity were found to be significantly expressed as compared to pre-vaccinated control pigs. The relative quantification of mRNA transcripts revealed that *TNFα*, *IL12RB2*, *TNFAIP3*, *PDL1*, *TLR4*, *IL1β*, *IL1α*, *REL*, *CXCL8*, *OSM*, *PSEN2*, *NF-kB IZ*, *SAMSN1*, *GZMH*, *FOXJ1*, *CCL4*, *TNFSF9*, and *BCL3* were significantly upregulated in 28dpv pigs as compared to same unvaccinated control pigs. Out of these genes as evidenced from gene ontology *TNFα* is responsible for activation of MAPK activity humoral immune response, intrinsic apoptotic signaling pathway and positive regulation of *NF-kB* import into nucleus. The role of *IL12RB2* is cytokine receptor activity and interferon-gamma production. The *TNFAIP3* is another significantly unregulated gene reported to be involved in negative regulation of B cell activation, endothelial cell apoptotic process, I-kappaB kinase/NF-kappaB signaling innate immune response and *IL-1β, IL-2* and *IL-6* production. The *IL1β* and *IL1α* gene is involved in cytokine-mediated signaling pathway, positive regulation of I-kappaB kinase/NF-kappaB signaling and positive regulation of JNK cascade. The *BCL3* is involved in regulation of NF-kB import into nucleus and response to virus. The significantly downregulated DEGs were *PF4*, *IGJ*, *OASL*, *SDHAF4*, *TLR10*, and *LTF* in 28dpv Indigenous pigs. The *PF4* is responsible for eliciting immune response through leukocyte chemotaxis and platelet activation.

The DEGs of indigenous piglets after vaccination were distributed among more than 1499 categories belonging to the three branches of ontology namely biological process, molecular function and cellular component. According to the GO project for biological processes, significant enrichment of DEGs was seen for immune system processes, gene expression, viral process apart from general cellular and metabolic process (Fig. 1). Among the 5222 DEGs a total of 923 genes were found to be associated to the immune system processes exhibiting a significant ($p < 0.05$) enrichment. Further, to categorize differentially expressed gene function, biological pathways enriched in the vaccinated transcriptome were analyzed using KEGG and Reactome database. A total of 1195 genes were mapped to 42 statistically significant ($p < 0.05$) categories among the documented canonical pathways found in KEGG. T cell receptor signaling pathway, B cell receptor signaling pathway, NF-Kappa B signaling pathway, Antigen processing and presentation pathway and TNF signaling pathway were among the top ten annotated pathways with 55, 39, 48, 39 and 50 genes respectively. Out of the 5222 differentially expressed, a total of 426 genes could also be mapped to 216 significant ($p < 0.05$) categories in the Reactome database. Among these categories, viral mRNA translation, activation of NF-kB in B cell and regulation of activated PAK-2p34 by proteasome mediated degradation were found to be over-represented with 49, 36 and 28 genes respectively. The enrichment of TNF- signaling, T cell receptor signaling pathway, NF-Kappa B (Dev et al. 2011) and B cell receptor signaling pathway indicated the involvement of immune response regulatory pathways in CSF vaccination in indigenous piglets.

The protein–protein interaction network between 5222 DEGs of indigenous piglets after vaccination and 923 immune genes resulted in 417 nodes and 2109 edges. Then, interaction network of the differentially expressed 342 immune genes from important pathways with the immune genes was constructed at degree 10 and fold change 0.5. It resulted in 104 nodes and 281interactions (Fig. 2). *RELA* that activates NF-kB pathway was the most highly connected gene (degree = 710) and was upregulated. *BLK* and *PLCϒ* involved in B cell receptor signaling pathway were upregulated with connectivity 13 and 70, respectively. The BIRC3 and UBC that regulates NF-kB activation was upregulated with connectivity 274 and 614 respectively. JUN and FOS promoting antibody production were upregulated with 327 and 177 interactions, respectively.

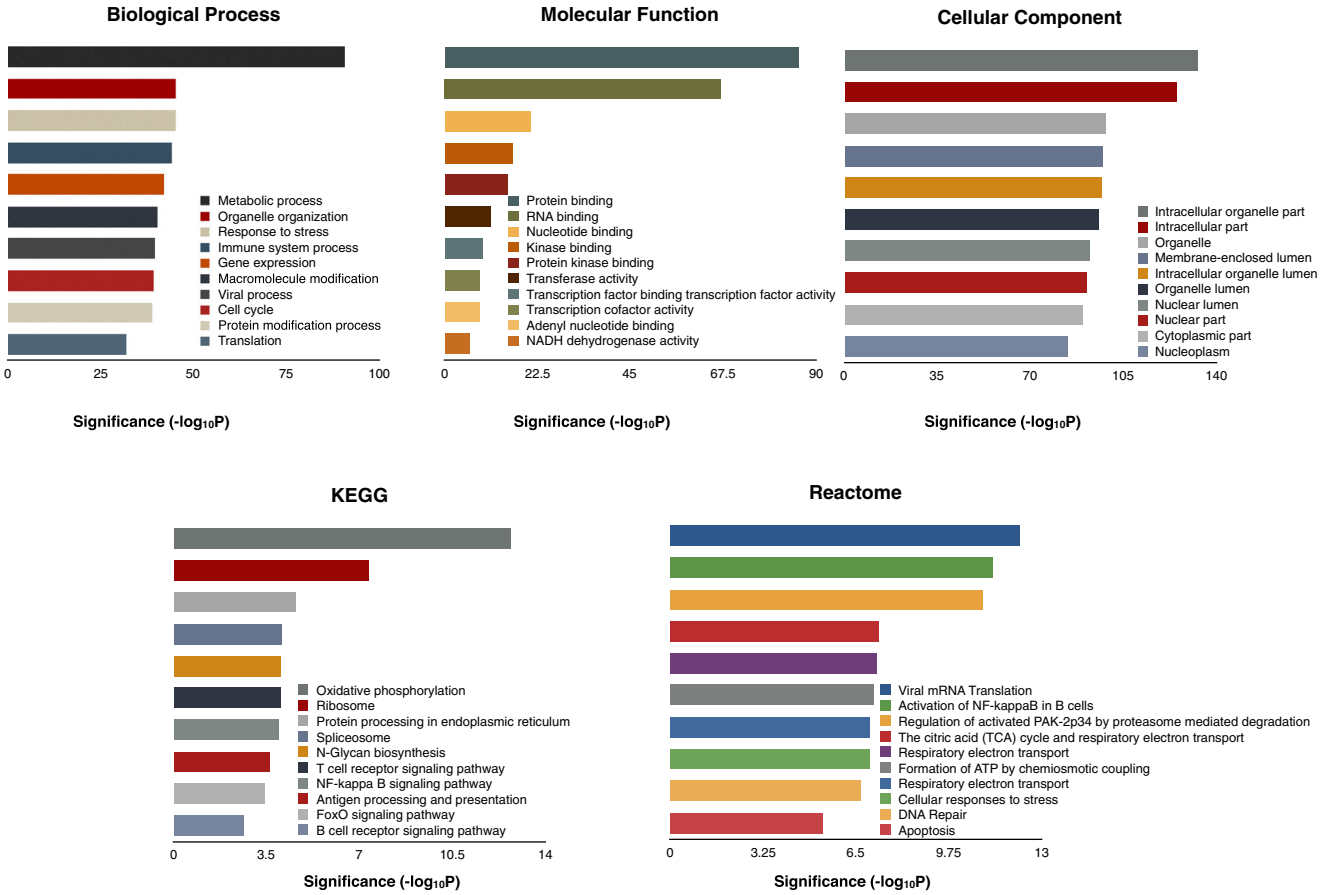

Fig. 1 Functional annotation was carried out by using bioinformatics tools, g:profiler. Gene ontology (GO) terms were retrieved using g:profiler. The significantly ($P < 0.05$) enriched GO terms in biological process, cellular component, and molecular function branches are shown. Significant ($p < 0.05$) categories among the canonical pathways found in KEGG and REACTOME databases are shown

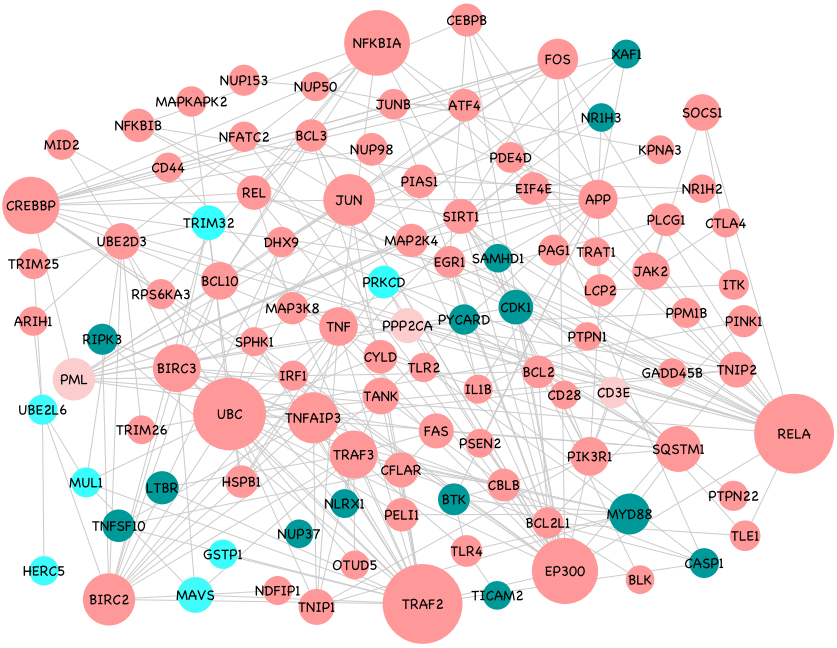

Fig. 2 Protein-protein interaction network of immune genes in indigenous post-vaccinated verses pre-vaccinated

DEGs, functional annotation, and interaction network enriched in immune system processes of crossbred post-vaccinated versus pre-vaccinated pigs

After analysis a total 6345, 7771, and 9149 DEGs were identified using EBSeq, DESeq2 and edgeR respectively (5% FDR) and 6037 genes were common during prediction by all three softwares. Out of these 6037 significant DE genes, 3669 genes were found to be upregulated and 2368 genes were downregulated. The analysis of RNA Seq data revealed that more number of immunity genes were involved in provoking immunity among crossbred pigs as compared to indigenous (indigenous) pigs after CSF vaccination in pigs. In post-vaccinated crossbred pigs, differentially expressed most important upregulated immune genes were *IRF3, TNFα, SLA-DRB2, SLA-DQB1, SLA-DRB5, SLA-DMB, SLA, NF-kB IE, CTSH, PNP, TYROBP, SMAD2, IDO1, IL1B, IL1A, LIF, OSM, MIF, PSEN2, CD14, IL10, CCL20, CCL4, CCL2, CCL8, CCL22, TNFSF9, SEPX1, TICAM1, BCL3, RELB*,*CD4, TAP1, GZMB, TYROBP, FCER1G, S100A10, IDO1, ZP3, CXCL8, PYCARD, IL1β, CXCL2, CXCL10, MIF, AQP3, SAC3D1, CD14, GZMH*, and *ECM1* whereas the *CX3CR1* gene was significantly downregulated as compared to pre-vaccinated crossbred pigs.

The DEGs of crossbred piglets after vaccination were distributed among more than 1528 categories, belonging to the three branches of ontology namely biological process, molecular function and cellular component. According to the GO project for biological processes, significant enrichment of DE genes was seen for immune system processes, gene expression, viral process, regulation of immune system process, positive regulation of immune response apart from general cellular and metabolic process (Fig. 3). Among the 6037 DEGs, a total of 105 genes were found to be associated to the immune system processes exhibiting a significant enrichment ($p < 0.05$). Further, to categorize differentially expressed gene function, biological pathways enriched in the vaccinated transcriptome were analyzed using KEGG and Reactome database. A total of 1254 genes were mapped to 47 statistically significant categories ($p < 0.05$) among the documented canonical pathways found in KEGG. T cell receptor signaling pathway, B cell receptor signaling pathway, NF-kB signaling

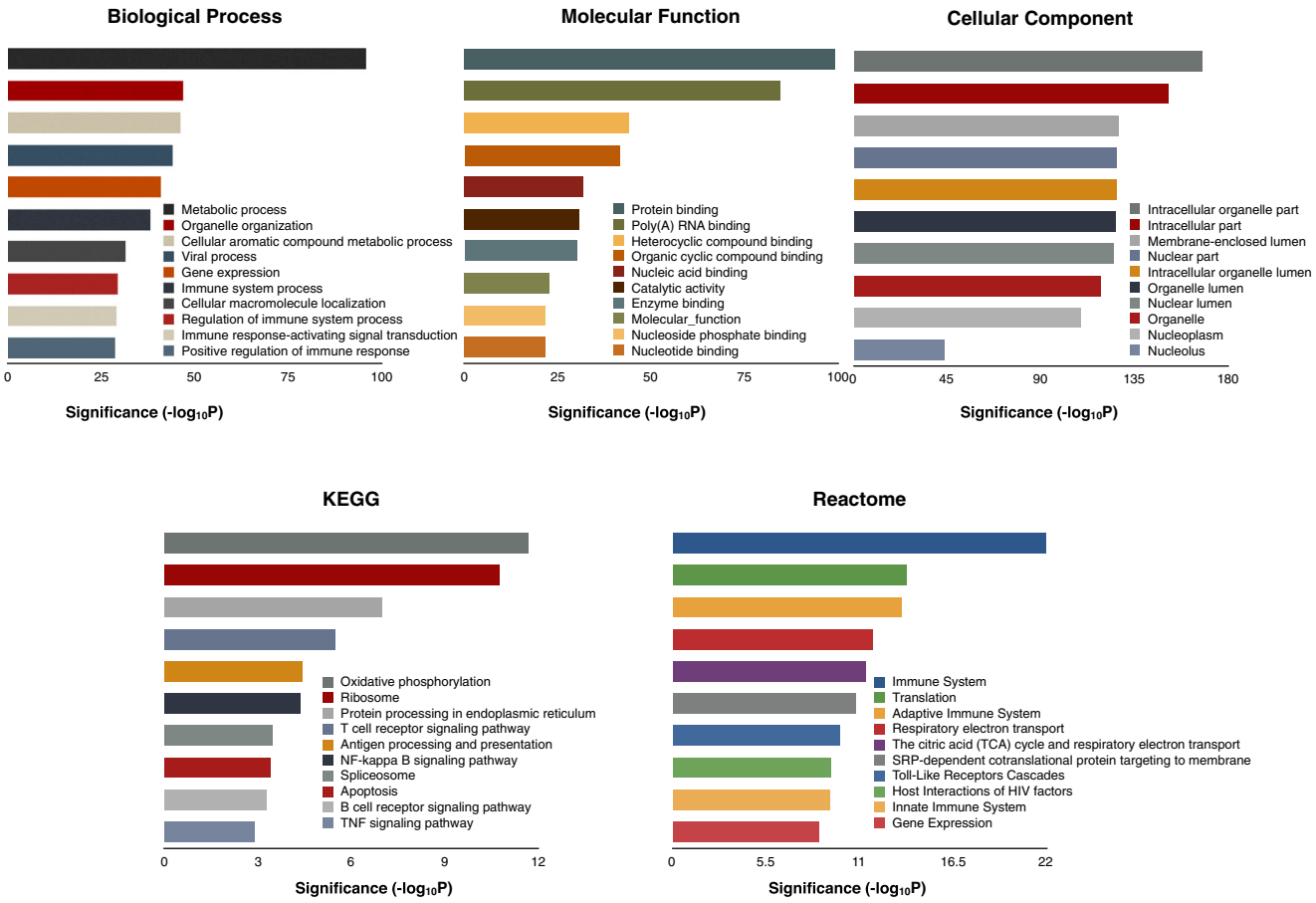

Fig. 3 Functional annotation was carried out by using bioinformatics tools, g:profiler. Gene ontology (GO) terms were retrieved using g:profiler. The significantly ($P < 0.05$) enriched GO terms in biological process, cellular component, and molecular function branches are shown. Significant ($p < 0.05$) categories among the canonical pathways found in KEGG and REACTOME databases are shown

pathway, antigen processing and presentation pathway and TNF signaling pathway were among the top ten annotated pathways with 62, 43, 52, 43, and 58 genes, respectively. Out of the 6037 differentially expressed, a total of 537 genes could also be mapped to 205 significant ($p < 0.05$) categories in the Reactome database. Among these categories immune system, innate immune system, adaptive immune system and Toll like receptor cascade were found to be over-represented with 555, 308, 310 and 87 genes respectively. The enrichment of TNF-signaling, T cell receptor signaling pathway, NF-kB signaling (Dev et al. 2011), Toll-like receptor cascade, and B cell receptor signaling pathway indicated the involvement of immune response regulatory pathways in CSF vaccination in crossbred piglets.

The interaction network of 6037 DEGs of crossbred piglets with the immune genes resulted in 417 nodes and 2109 interactions. Then interaction network of the differentially expressed 359 immune genes from important pathways with the immune genes was constructed at degree 10 and fold change 0.3. It resulted in 192 nodes and 831 interactions (Fig. 4). *GRB2* and *SHC1* genes associated with Ras activation pathway were upregulated and highly connected with degree 874 and 829 respectively. *SOS1* and *SOS2* were downregulated with 118 and 24 interactions, respectively. *RELA, IKBKB* and *NF-kB IA* involved in NF-kB signaling pathway were upregulated and highly connected. The *CSK* and *PAG* involved in B cell receptor signaling pathway were upregulated and downregulated, respectively. The *JUN* and *FOS* promoting antibody production were upregulated with 408 and 144 interactions, respectively.

DEGs, functional annotation, and interaction network enriched in immune system processes of crossbred versus indigenous (desi) post-vaccinated pigs

A total 6501, 8832 and 9096 DEGs were identified using EBSeq, DESeq2, and edgeR, respectively, (5% FDR) and 6210 DEGs were common. Out of these 6210 significant DEGs, 3139 genes were found to be upregulated and 3071 genes were downregulated. The analysis of RNA Seq data revealed that more number of immunity genes were involved in provoking immunity among crossbred pigs as compared to indigenous (desi) pigs after CSF vaccination in pigs. The differentially expressed significantly upregulated important immune genes were *IRF3, TNFα, TAP1, NF-kB IE, CTSH, IDO1, LIF, OSM, MIF, CD14, IL10, GZMH, PF4, CD4, SLA-DMB, SLA, GZMB, CCL22, TYROBP, FCER1G, S100A9, IDO1, ZP3, PYCARD, IL1β, SH2D6, CXCL10, MIF, AQP3, SAC3D1, MATK, NLRX1, CCL, CCL23, CCL8, CXCL2, CCL2, SEPX1, TICAM1, TNFSF9, CCL4, BCL3, BPI, ECM1, SERPINB9, TMP-SLA-5* and *LST1g* whereas the *CX3CR1, CXCL8, SAMSN1*, and *MID2* genes were significantly downregulated in crossbred versus indigenous post-vaccinated pigs.

The DEGs between crossbred and indigenous (desi) piglets after vaccination were distributed among more than 1409 categories belonging to the three branches of ontology namely biological process, molecular function and cellular component. According to the GO project for biological processes, significant enrichment of DE genes was seen for viral processes, gene expression, translation and protein

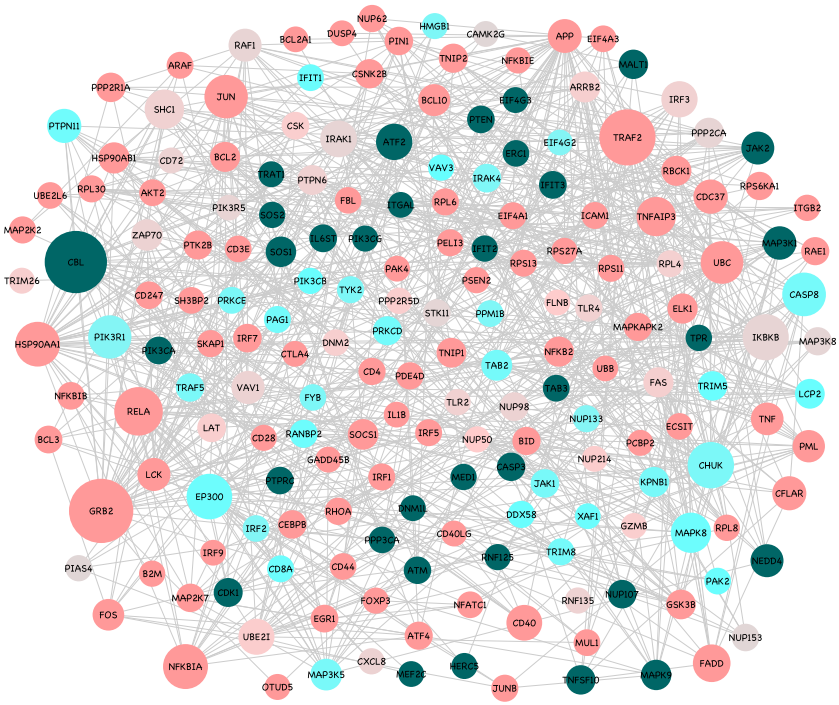

Fig. 4 Protein-Protein Interaction network of immune genes in crossbred post-vaccinated verses pre-vaccinated

localization apart from general cellular and metabolic process (Fig. 5). Among the 6210 DEGs, a total of 1036 genes were found to be associated to the immune system processes exhibiting a significant enrichment ($p < 0.05$). To further categorize differentially expressed gene function, biological pathways enriched in the vaccinated transcriptome were analyzed using KEGG and Reactome database. A total of 1451 genes were mapped to 34 statistically significant categories ($p < 0.05$) among the documented canonical pathways found in KEGG. T cell receptor signaling pathway, B cell receptor signaling pathway, NF-kB signaling pathway and antigen processing and presentation pathway were among the top ten annotated pathways with 55, 42, 53, and 44 genes, respectively. Out of the 6210 DEGs, a total of 1041 genes could also be mapped to 197 significant ($p < 0.05$) categories in the Reactome database. Among these categories immune system, adaptive immune system and class I MHC mediated antigen processing and presentation were found to be over-represented with 573, 325 and 140 genes respectively. The enrichment of T cell receptor signaling pathway, NF-kB signaling pathway (Dev et al. 2011), B cell receptor signaling pathway and class I MHC mediated antigen processing and presentation pathway indicated the involvement of immune response regulatory pathways to vaccine response in cross-bred piglets compared to indigenous (desi).

The interaction network of the differentially expressed 619 immune genes from important pathways with the immune genes was constructed at degree 10 and fold change 0.5. It resulted in 147 nodes and 674 interactions (Fig. 6). The *BLNK, ATF2* and *BAD* involved in B cell receptor signaling pathway were downregulated with 48, 133, and 11 interactions, respectively. *BIRC3* and *UBC* that regulates *NF-kB* activation was upregulated with connectivity 129 and 455 respectively. The *CXCL8* was downregulated with 4 interactions. The *LCK* and *FYN* activating T cell receptor signaling pathway was upregulated with 79 and 84 interactions, respectively. *LYN* necessary for TLR4-dependent NF-kB and MAPK activation was upregulated with 6 interactions in the network. The *JUN* and *FOS* responsible for regulation of antibody production were downregulated and upregulated respectively with 395 and 115 interactions. The *IRF3* was upregulated with connectivity 154 and *IRF7* was downregulated

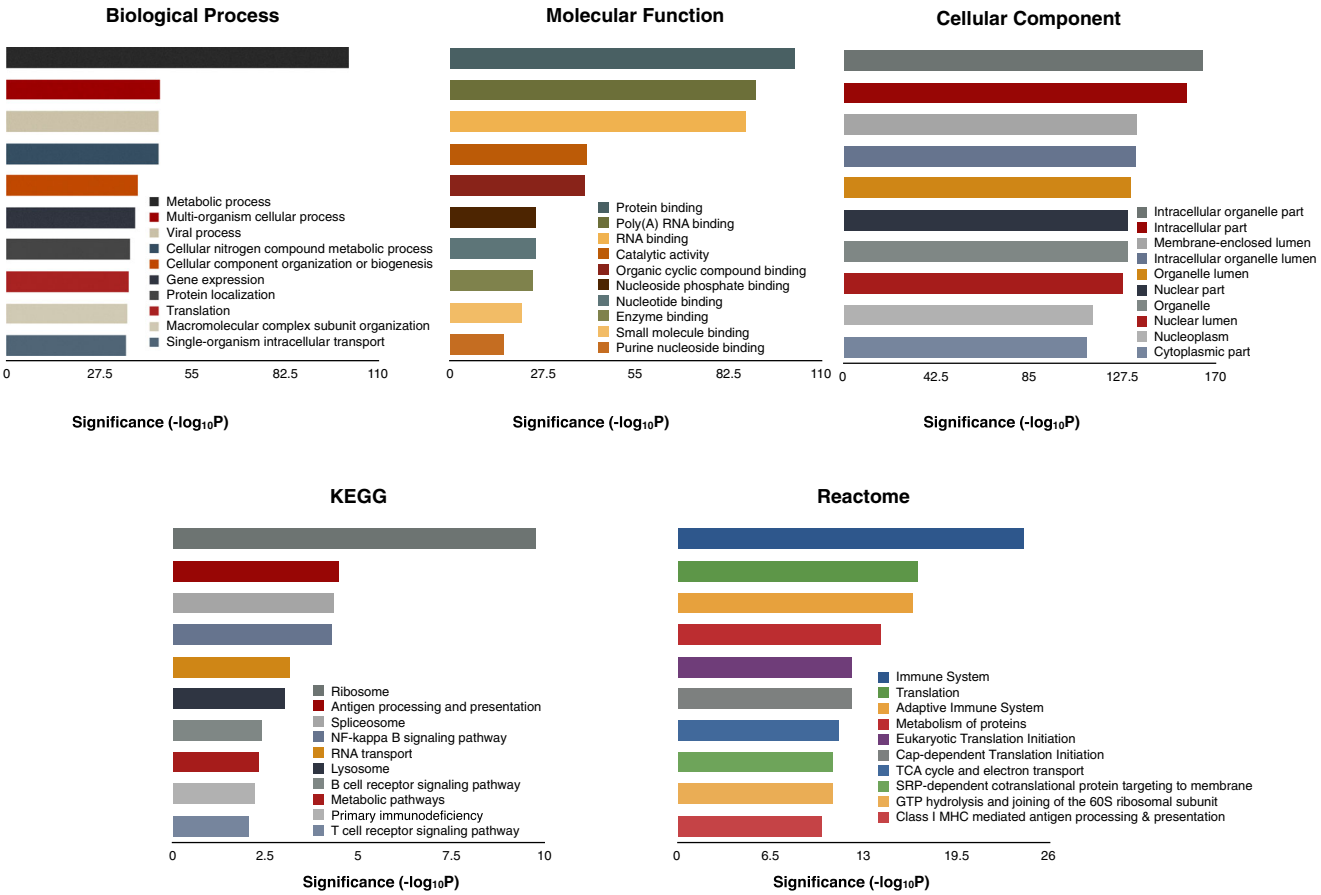

Fig. 5 Functional annotation was carried out by using bioinformatics tools, g:profiler. Gene ontology (GO) terms were retrieved using g:profiler. The significantly ($P < 0.05$) enriched GO terms in biological process, cellular component and molecular function branches are shown. Significant ($p < 0.05$) categories among the canonical pathways found in KEGG and REACTOME databases are shown

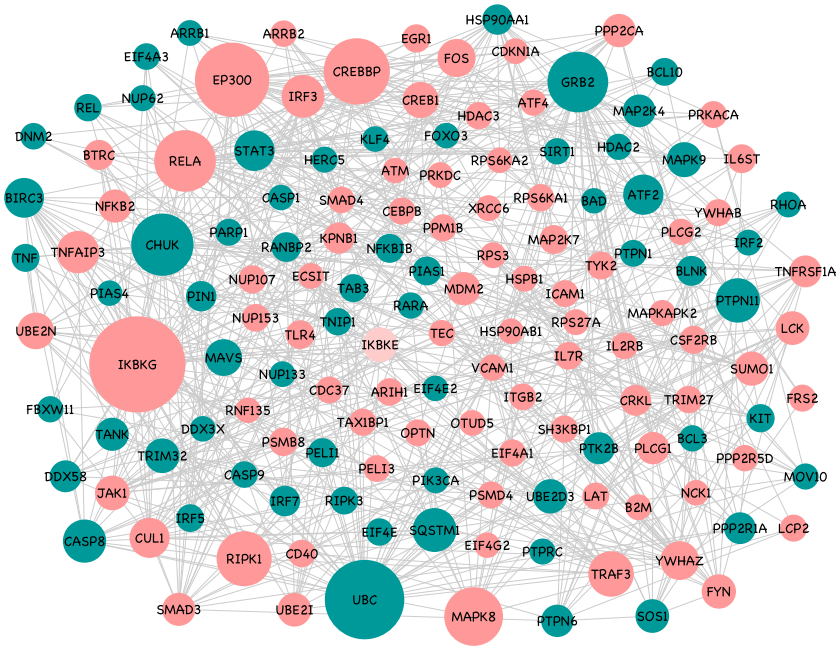

Fig. 6 Protein-Protein Interaction network of Immune genes in crossbred verses indigenous post-vaccinated

with 53 interactions. It suggests that blocking effect of Npro fraction of CSF vaccine virus was more evident in indigenous as compared to crossbred. The *TAB1* and *TAB3*, activators of NF-kB were downregulated with connectivity 61 and 32 respectively.

Among the pathways enriched in the DEGs, it was observed that across all the comparisons (indigenous post-verses re-vaccinated, crossbred post- verses pre-vaccinated and post-vaccinated crossbred verses indigenous (desi) 38, 24, 65, 28, and 3 genes were commonly involved in antigen processing and presentation pathway, T cell receptor signaling pathway, B cell receptor signaling pathway, NF-kB signaling pathway and TNF signaling pathway, respectively (Table 1).

Table 1 Common enriched pathway in different groups

Pathway	Indigenous post verses pre-vaccinated	Crossbred post verses pre-vaccinated	Crossbred verses indigenous post-vaccinated
Antigen processing and presentation pathway	44	51	49
T cell receptor signaling pathway	34	35	73
B cell receptor signaling pathway	95	98	106
NFkB signaling pathway	48	52	53
TNF signaling pathway	50	57	14

Validation of DEGs of immunological importance by qPCR

DEGs were identified through RNASeq analysis and four genes of immunological importance were validated by quantitative real time PCR across all the comparisons (indigenous post verses re-vaccinated, crossbred post verses pre-vaccinated and post-vaccinated crossbred verses indigenous (desi). These genes included *IRF3, TAP1, IL1β* and *NF-kB* (Fig. 7). The *IRF3* gene was upregulated in crossbred post-vaccination verses pre-vaccination by 6.36 fold change as well as in post-vaccinated crossbred verses indigenous group by 7.06 fold change. *TAP1* was found upregulated in indigenous post-vaccinated verses pre-vaccinated by 1.69 fold change and in crossbred post-vaccinated verses pre-vaccinated by 1.89 fold change. *IL1β* gene was upregulated in indigenous post-vaccinated verses pre-vaccinated by 11.75 fold change

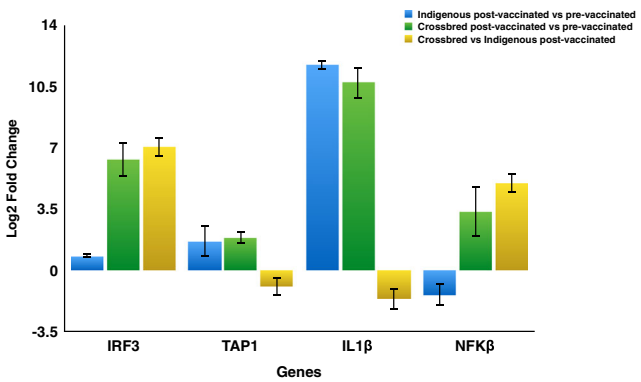

Fig. 7 Validation of immunologically important genes after CSF vaccination

and in crossbred post-vaccinated verses pre-vaccinated by 10.73 fold change. *NF-kB* gene was found upregulated in crossbred post-vaccinated verses pre-vaccinated by 3.37 fold change and in post-vaccinated crossbred verses indigenous by 5.01 fold change.

Discussion

In present investigation, we analyzed the transcriptome data in CSFV vaccinated indigenous and crossbred piglets to identify the DEGs which are ultimately leading to differential immune response between the indigenous and crossbred piglets. The blood samples were collected before vaccination and on 28th day post vaccination and transcriptome data generated was analyzed to identify the DEGs in both indigenous and crossbred piglets. The DEGs were functionally annotated and gene network analysis was carried out to identify the reasons behind the differential adaptive immune response to CSFV vaccination in indigenous and crossbred piglets. The humoral immune response measured by cELISA was found to be better in crossbred piglets than indigenous. Similarly, Singh et al. (2016) reported significantly lower percentage inhibition in desi pigs (62.73%) than crossbred pigs (97.24%).

A total 5222, 6037, and 6210 common DEGs were identified by EBSeq, DESeq2, and edgeR in indigenous post-vaccinated versus pre-vaccinated, crossbred post-vaccinated versus pre-vaccinated and post-vaccinated crossbred versus post-vaccinated indigenous, respectively. The key pathways enriched in the DEGs of all combinations were antigen processing-cross presentation, B cell receptor signaling, T cell receptor signaling, NF-κB signaling, and TNF signaling pathways.

The protein-protein interaction network among the DEGs of all comparisons resulted in "hairball" networks with many nodes and edges that could not be represented clearly and interpreted. Therefore, genes involved in the key pathways antigen processing-cross presentation, B cell receptor signaling, T cell receptor signaling, NF-κB signaling, and TNF signaling pathways were considered for further analysis and interpretation. A total of 342, 359, and 619 genes were found in indigenous post-vaccinated versus pre-vaccinated, crossbred post-vaccinated versus pre-vaccinated and post-vaccinated crossbred versus indigenous, respectively and the interactions between these unique genes were extracted using customized perl scripts from the BioGRID data. On analyzing and evaluating all the representative networks, it was found that several genes were specifically involved in antigen processing and presentation, B cell receptor signaling pathway, and T cell receptor signaling pathway. Classical class II molecules are involved in antigen presentation to CD4+ T cells whereas classical class I genes have a double function of antigen presentation to CD8+ T cells and regulation of natural killer cell cytotoxicity by interacting with NK receptors such as *NKG2D* (Shi and Van Kaer 2006). In pig, downregulation of *MHC* genes has been reported in vivo in the spleen of animals infected by H. parasuis (Chen et al. 2009) and in vitro in PK15 cells infected by the pseudorabies virus (Flori et al. 2008). In our study, we found that *SLA2* - the *MHC* class I molecule involved in antigen processing and presentation was downregulated in indigenous but upregulated in crossbred after vaccination. Innate cytokine responses are important mediators of early defense against infections. Tamura et al. (2014) reported that Npro of classical swine fever virus contributes to pathogenicity in pigs by preventing type I interferon induction at local replication sites. In the context of viral infections, interferons alpha and beta (*IFN-*$\alpha\backslash\beta$) are induced to high levels and help to mediate and regulate immuneresponses most effective against this class of agents. O'Brien et al. (2014) reported that vaccination with recombinant adenoviruses expressing Ebola virus glycoprotein elicits protection in the interferon alpha/beta receptor knock-out mouse. In our study, we found that *IFN-*α and *IFN-*β was upregulated in crossbred after vaccination. Several genes associated with proteasome complex like *PSMC5, PSMB8, PSME2, PSMA4, PSMD4, PSME1, PSMA6*, and *PSMB5* were upregulated in crossbred after vaccination. The mannose receptor (MRC2) play a role in antigen uptake and presentation by immature dendritic cells in the adaptive immune system. *PSME3* encodes the gamma subunit of the 11S regulator of immunoproteasome which is a modified proteasome for processing of MHC classI peptides. The *MRC2* and *PSME3* were differentially upregulated in post-vaccinated crossbred versus post-vaccinated indigenous which suggested effective viral antigen presentation and processing. The *TAP1* that is involved in the transport of antigens from the cytoplasm to the endoplasmic reticulum for association with MHC class I molecules and acts as a molecular scaffold for the final stage of MHC class I folding was also upregulated after vaccination in both genetic groups. The expression of *TAP1* was higher in crossbred than in indigenous suggesting better antigen presentation leading to better immunity. In our study, we found B cell receptor signaling pathway enriched in all three comparisons, i.e., indigenous post-vaccinated versus pre-vaccinated, crossbred post-vaccinated versus pre-vaccinated and post-vaccinated crossbred versus indigenous. *PSME3, PIK3CD, NCK1, CD19, CALM2, PRKCB*, and *CREB1* were found to be upregulated in crossbred versus indigenous post-vaccinated pigs. Protein encoded by *PIK3CD* is a class I PI3K found primarily in leukocytes. Like other class I PI3Ks (p110-alpha p110-beta, and p110-gamma), the encoded protein binds p85 adapter proteins and GTP-bound RAS. Vogan (2013) reported that *PIK3CD* mutation cause immunodeficiency. *CREB1* is a Phosphorylation-dependent transcription factor that stimulates transcription upon binding to the DNA, cAMP response element (CRE), a sequence present in many

viral and cellular promoters. It has been suggested that overexpression of *CREB1* can cause repression of HCMV replication and may contribute to the development of new anti-HCMV strategies (Chia et al. 2014). *CSK* that inhibits src family kinases was found to be downregulated in indigenous and upregulated in crossbred. But, *PAG* required for translocation of *CSK* from cytosol to plasma membrane was upregulated in indigenous and downregulated in crossbred explaining the better immune response in the crossbred to CSFV vaccination. Npro - a Classical swine fever virus coded protein interacts with *IRF3* and promotes its degradation. From RNA Seq analysis *IRF3* was found downregulated in indigenous and upregulated in crossbred post-vaccination verses pre-vaccination as well as post-vaccinated crossbred verses indigenous group. The *VCAM* is a proinflammatory and antigen presenting molecule which was upregulated in crossbred indicating that the crossbred may have a better inflammatory response in comparison to indigenous as evident from the significantly higher expression of *IL-6ST, IL-4R,* and *IL1B* in crossbred pigs. The cytokine milieu is required for the development of the immune response following B cell–T cell interactions. They also play important role for the development and proliferation of B cell. The *IL-4* also known as B cell activating or differentiation factor1 acts on B cells to induce activation and differentiation. The *IL-6* is a critical growth factor for differentiation of B cells to antibody forming cells (AFC). In our study, *IL-6ST* needed for *IL-6R* activity and signal transduction of the *IL-6* related cytokines was found upregulated in crossbred after vaccination in comparison to indigenous piglets where as in indigenous *IL-6R* was found to be downregulated which can be correlated with the comparatively low antibody titer observed in indigenous piglets. Hence, it can be concluded that although after CSF vaccination both indigenous and crossbred pigs are capable to produce protective antibody titer but immune response processes are more enriched in cossbred piglets and revealed better adaptive immune response compared to indigenous.

On basis of these key genes, differential humoral response in two genetic group of pigs (indigenous verses crossbred) may be explained. The key adaptive immunity genes of MHCII belonging to the swine leukocyte antigen family, LOC100513292 (ENSSSCG00000001472), *SLA-DMB* (ENSSSCG00000001978) and *SLA-DOA* (ENSSSCG00000001979) were having significantly higher number of mRNA transcripts in post-vaccinated crossbred pigs as compared to post-vaccinated indigenous pigs which may be responsible for conferring better humoral immune response in crossbred pigs. Further, although in both genetic group the level of mRNA for *IL1β* increased significantly but it was significantly higher in crossbred post vaccinated pigs as compared to post vaccinated indigenous pigs. The significantly downregulated DEGs were *CXCL8, SAMSN1,* and *MID2* in 28dpv indigenous pigs. The gene ontology revealed that *CXCL8* is a chemokine responsible for chemokine mediated signaling pathways and neutrophil activation. The *MID2* is responsible for negative release of viral release from host cell and negative regulation of viral transcription. The *SAMSN1* is known to be involved in negative regulation of adaptive immune response and B cell activation. Upregulation of *CXCL5* (7.47- and 6.11-fold at 24 and 48 hpi, respectively), *IL-8* (9.09- and 6.44-fold at 24 and 48 hpi, respectively), *CCL7* (3.68-fold at 24 hpi) and *CCL8* (3.64-fold at 24 hpi) in swine macrophages has been reported (Gladue et al. 2010).

Based on the observations in the present study and comprehensive knowledge available in the literature, pathway involving antigen presentation and antibody response has been predicted. The CSFV virus after entry into the cell is processed, presented, and recognized by B cell receptor which triggers B cell receptor signaling pathway. Initially the src family kinases viz. *Lyn, Fyn* and *Blk* (expressed in our present study) phosphorylate *ITAM* to initiate the signal transduction. The *CSK* that inhibits src family kinases was found to be downregulated in indigenous and upregulated in crossbred. The *PAG* required for translocation of *CSK* from cytosol to plasma membrane was upregulated in indigenous and downregulated in crossbred explaining the better humoral immune response in the crossbred to CSF vaccination. This is followed by activation of *Syk* kinase on binding to the doubly phosphorylated src family kinases. This activated *Syk* kinase is essential to couple the B-cell receptor to distal signal transduction elements. The activated *Syk* kinase recruits *BLNK* to the plasma membrane which along with BTK activates *PLCGϒ2*. The recruitment and activation of *PLCϒ2* and elevation of [Ca2+]i appears to be a pivotal in BCR-mediated signal transduction. The *PLCϒ2* was found to be upregulated in crossbred reaffirming the antibody titer in the crossbreds in comparison to indigenous. The activated *PLC-γ* and *Syk* initiate *NF-kB* and *MAPK* signaling pathways through *PKC* and *RAS*, respectively. The activation of *MAPK* and *NF-kB* signaling would lead the expression of genes/transcription factors that would lead B cell ontogeny, auto immunity, immune response and Ig production (Fig. 8). *IκB* degradation allows dimeric NF-κB transcription factors composed largely of *RelA* (p65) and *NF-κB1* (p50) subunits to accumulate in the nucleus and drive expression of a large number of pro-inflammatory genes (Newton and Dixit 2012). *NF-κB* also induces genes that limit the duration and magnitude of the inflammatory response, such as *Tnfaip3* and *NF-kBIA* (the latter encodes *IκBα* and thus forms a negative-feedback loop).

In present investigation, we also validated the expression profile of some genes of immunological importance viz. *IRF3, TAP1, IL1β,* and *NF-kB*. Host recognition of viral infection through Pattern recognition receptors (PRRs) normally initiate signaling pathways that can lead to the activation of transcription factor *IRF3* which often culminate in the induction of an array of antiviral cytokines, including type I IFN and

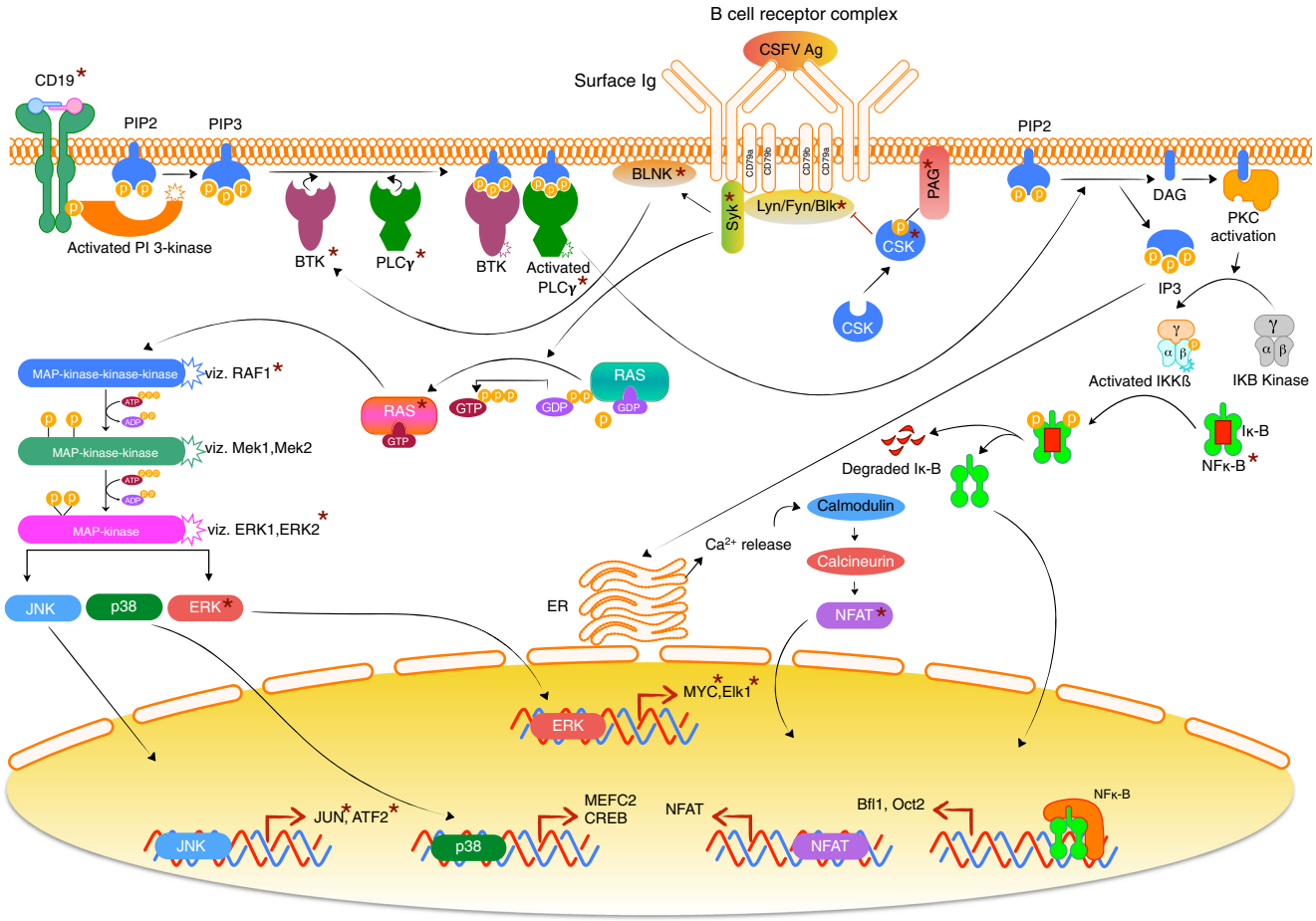

Fig. 8 Predicted pathway for immune response induced by CSF vaccine virus in crossbred verses indigenous Pigs. *Differentially expressed in our investigation

proinflammatory cytokines such as *TNF, IL-6* and *IL-8*. The results of previous studies showed that N (pro) protein in pestiviruses appeared to be essential for the virus-mediated degradation of IRF-3 by proteasomes and thus prevented *IRF3* from activating transcription from the *IFN-β* promoter (Summerfield and Ruggli 2015). In qPCR study, we found that the *IRF3* gene was upregulated in crossbred post-vaccination verses pre-vaccination as well as post-vaccinated crossbred verses indigenous group. The higher expression of *IRF3* in crossbred after vaccination compared to indigenous may support better immune response in crossbred piglets. Cao et al. (2015) reported that Shimen strain infection of CSF resulted in a significant suppression of *IRF3*. In qPCR study, *TAP1* was found upregulated in indigenous post-vaccinated verses pre-vaccinated by 1.69 fold change and in crossbred post-vaccinated verses pre-vaccinated by 1.89 fold change. By qRT-PCR analysis, Li et al. (2010) reported that this gene was upregulated after treatments with mimic viral and bacterial infection (polyriboinosinic-polyribocytidylic acid (poly(I:C)) and lipopolysaccharide (LPS), respectively). In addition they reported elevated *TAP1* expression after porcine reproductive and respiratory syndrome virus (PRRSV) infection in porcine white blood cells (WBCs). More recently Dong et al. (2013) reported that CSFV improved expression of *RIG-I and MDA5* leading to the nuclear translocation of activated IRF-3 and NFκB and finally *IFN-α, IFN-β, IL-1β, IL-6,* and *TNF-α* were upregulated in a dose-dependent way within 24 h post-infection (hpi) in porcine alveolar macrophage. Li et al. (2016) reported levels of *IL-4*, *IL-6*, *IL-8*, *TGF-β1,* and *IL-12* were significantly higher in LW pigs at the early stage of PCV2 infection. In our study, *IL1β* gene was upregulated in indigenous and crossbred piglets after vaccination. In agreement with our finding, Sun et al. (2014) reported that *IL1β* expression was on peak on 17 dpi in pigs after infection with CSF virus and remained elevated till 45 dpi. In an investigation during CSF virus challenge in swine macrophage, the cytokine genes *IL-1β, IL-1α, IL-6,* and *IL-12p35*, showed significantly higher levels of expression (Borca et al. 2008). Transcriptional response studies using microarray of swine macrophages infected with the Brescia strain revealed increased expression of *IL1β* mRNA (Zaffuto et al. 2007). In agreement with present study, the transcripts of *IL1α* have been reported to be upregulated in vivo in lymphoid tissues of pigs (Sanchez-Cordon et al. 2005). Expression of *IL-6*

increased in CSFV-infected macrophages in vitro (Borca et al. 2008) and in vivo (Sanchez-Cordon et al. 2005) after CSF virus challenge. It has been reported that CSFV infection inhibits the transcription of *SLA-2, TAP1, SLA-DR, Ii, IFN-α,* and *IFN-β* in PK-15 cells (Feng et al. 2012). From RNASeq analysis, it was observed that expression profile of *IRF3*, *TAP1*, *IL1β*, *SLA2*, *SLADR*, *CSK*, *PAG*, *IL-6ST*, *NFκB*, *Lyn*, *Fyn*, *Jun*, *Fos*, *Syk*, *BLNK*, and various other genes were ascertaining to breed differences in humoral immune response of two different genetic group of piglets under investigation.

Hence, it can be concluded that immune response processes are more enriched in crossbred piglets and they show better adaptive immune response compared to indigenous (desi) following CSF vaccination.

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