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Network analysis of key genes regulating humoral immune response against live attenuated C-strain CSF virus immunization in Landly pigs

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Abstract
Classical swine fever (CSF), also known as the hog cholera is an important viral disease of pigs which causes great economic loss to pig industry in the world mainly due to high mortality caused by the disease. Vaccination has been tried and tested method to control and prevent the spread of this disease. The present study was conducted to obtain in depth information on genes mapped to associative SNPs after genome wide association study (GWAS) on humoral immune response to live attenuated C-strain CSF virus immunization in Landly pigs. For the purpose, sampling was done on the day of CSF and after 28 days of vaccination. E2 antibody ELISA was conducted and genotyping by sequencing was performed. After preliminary GWAS analysis, the genes in the vicinity of putative SNPs were mined and network analysis for protein-protein interaction study was conducted. The functional annotation analysis was carried out, which revealed significant enrichment of biological, molecular function and cellular components, wherein, immune system process, defense response, inflammatory response, signalling, positive regulation of cytokine production, response to stimulus, molecular function regulator, protein containing complex and many more related to immuno-pathophysiology were observed. KEGG and Reactome pathway analysis revealed the role of TLR6, TLR5, NFKB, IL1 family and in immune response process and signalling pathways, which can be associated with immune status in CSF vaccinated pigs. Hub gene analysis also linked TLRs as the key gene in the regulation of immune response process against CSF immunization.

Keywords: Immune system, vaccination, protein-protein interaction, Landly pigs, signalling

Introduction
The research on breeding for disease resistance is mostly limited by difficulties with challenge study in large animals and sporadic outbreak of disease (Kumar *et al*., 2020) [10]. In these circumstances, alternative strategy like genetic selection of animals for high level of immune response after vaccination can be a good strategy (Kumar *et al*., 2019a). The understanding of signalling processes behind high, non- or low response, or pathology at the other extreme may help in identifying new targets for immune modulation against diseases causing great economic losses in animal farming (Mehrotra *et al*., 2022) [13]. Classical swine fever (CSF) which is also known as pig plague or hog cholera, remains one of the highly contagious disease of swine, characterized by fever, haemorrhage, leukopenia, abortion and high mortality. It has tremendous impact on animal health and pig industry and is therefore notifiable disease to the World Organization for Animal Health (OIE) (Edwards *et al*., 2000) [7]. The disease occurs due to the infection of classical swine fever virus (CSFV) which belongs to Flaviviridae family and *Pestivirus* genus. Routine vaccination is often promoted as the best possible solution for controlling the CSF in endemically infected regions in Eastern Europe, America, some African countries and Asia including India. In India the incidence of the disease was reported from Maharashtra, Uttar Pradesh, Tamil Nadu, Punjab and the North Eastern states (Bharati *et al*., 2022) [1, 2]. In the highly endemic areas routine vaccination against CSF is the most common method for prevention and control of CSF disease. Series of experiments established CMI (cell-mediated immune) responses in providing defensive immunity against CSF virus infection. Although the modified live CSF vaccines which were commercially available can provide complete protection in immunised pigs, other factors like the age of primary vaccination, maternal immunity, complications caused by other pathogens and vaccination protocol can affect the efficacy of CSF vaccines in the field (Mehrotra *et al*., 2022) [13]. The C strain Lapinized virus vaccine is one of the most effective vaccines that

provide sterile immunity against CSFV within a week of vaccination and is considered as the gold standard vaccine for controlling CSF disease in pigs (Van Oirschot, 2003) [22]. Previous studies have reported the presence of CSF virus specific IFN- γ secreting cells in the peripheral blood mononuclear cells from pigs immunized with the lapinized C strain vaccine, as early as 6 days, which lasted for up to 140 days (Suradhat *et al*., 2001) [19]. These findings highlight the role of vaccine-induced cellular immunity in viral protection during the early phases of immunity. Also, the importance of humoral immune system in protection against CSF virus infection was established along with the CSF virus specific neutralizing antibodies in immunized animals. There exists limited study on host immune response and the signalling pathways regulating these mechanisms. Keeping these aspects in the view, the present study was envisaged with the following objective to explore the putative genes responsible for immune response to CSF immunization in Landly pigs and further gain insights into the key genes and their network of signalling pathways responsible for immune process which provides protection against the deadly CSF virus infection.

Materials and Methods

Sampling and Animals

A total of 96 Landly crossbred piglets developed by crossing indigenous Ghurrah pigs of Uttar Pradesh with exotic Landrace pigs at AICRP on Pig farm located at ICAR-IVRI, Bareilly, U.P, India was used in the study. The total exotic inheritance of Landrace in the Landly pig is 75%. All the piglets were housed under similar environmental conditions and were administered live attenuated C-strain vaccine of CSF at 12 weeks of age as a routine farm practice. The methodology of the study is depicted diagrammatically in Figure 1. Briefly, about 5 ml blood was collected from the anterior venacava of each piglet in a sterile BD vacutainer with EDTA and without anti-coagulant for serum. The first blood sample was collected from each piglet on day of vaccination and second blood samples was collected from each piglet on 21 days post vaccination (DPV). Presence of antibodies was measured using commercialized PRIOCHECK CSFV Antibody Serum ELISA Test Kit of Thermo Fisher Scientific and the O.D. was measured at 450 nm within 15 minutes after colour development. The ELISA for each sample was done in duplicate and the average PI value was used as the phenotype for association with the SNP data.

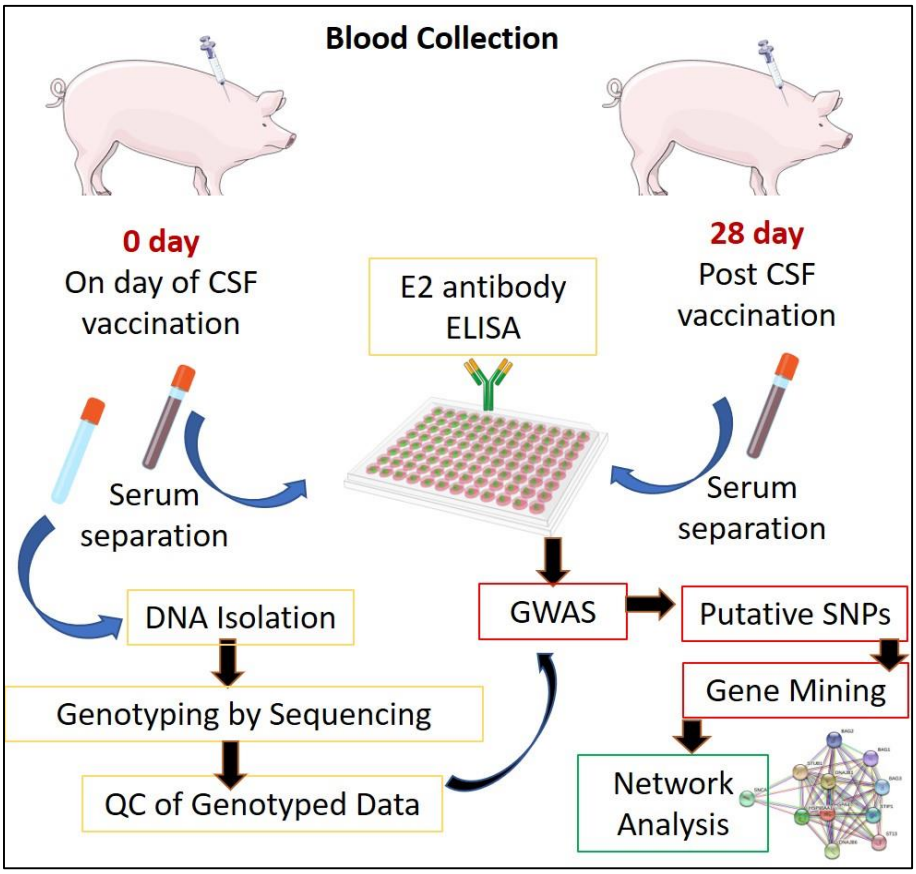

Fig 1: The diagrammatic representation of the methodology used in the present study.

SNP Identification and Genotyping of SNPs by Genotyping by Sequencing (GBS)

SNPs related to humoral mediated immune response was identified and genotyped by the double digestion GBS technique. PCR amplification was conducted, followed by Next-generation sequencing in parallel high throughput Illumina HiSeq sequencing technologies. After the quality check, the raw sequence data were filtered and mapped to *Sus scrofa* version 11.1 reference genome using Burrows Wheeler Aligner algorithm. The dDOCENT pipeline was used to call SNPs from Raw FASTQ files and the SNPs were called from read mapped using FreeBayes version v0.9.10. PLINK 1.9 was used for the quality control of SNP genotypes which was mapped to their respective autosomes for establishing genome wide association study with the humoral immune response. Both the genic and intergenic region flanking around the obtained SNPs was screened using UCSC Genome Browser assembly ID: susScr11 pig assembly and the genes around significant SNPs were identified (Mehrotra *et al*., 2022) [13].

Network Analysis

To explore the functions of genes in the vicinity of significant SNPs, STRING database (https://string-db.org/) was used to obtain association network of key genes regulating immunity in the present study (Szklarczyk *et al*. 2021) [20]. The protein-protein interaction (PPI) network based on sus scrofa protein with predictions limited to the top 20 interactors in the first and second shell of interaction. The minimum required interaction score was kept at medium confidence (0.400), Since the parent network was dense, the network was centred around TLR6, since it was observed to be a key gene regulating immune system. The TLR6 cluster were then studied in detail for gene ontology (GO) annotation viz. the biological process (BP), molecular functions (MF), cellular components (CC) and the KEGG and Reactome pathway analysis employing enrichment strength and false discovery rate (FDR) p-values (p<0.05) corrected for multiple testing within each category using the Benjamini–Hochberg procedure (Bharati *et al*., 2021) [3]. The specialized immune system process pathway and the associated genes were analysed in order to gain specific insight into the humoral immune system associated pathways behind response to CSF vaccination. For the purpose, the parent network was imported from STRING into Cytoscape (Shannon *et al*., 2003) [18] and hub gene analysis was conducted in Cytohubba plugin (Chin *et al*., 2014) [5] and the top 10 hubba nodes in the network were ranked by eccentrity (Bharati *et al*., 2022) [1, 2].

Results and Discussion

The candidate genes which were present in nearby vicinity (1Mb) of significant SNPs mapped to immune associated genes like *TLR5, TLR6, IL9R, CD101, CXCL13* and *TAS2R4*. Toll-like receptors (*TLRs*) are well known first line of immune defence against microorganisms and play a critical adaptive role in immune system process (Kumar *et al*., 2019b). In the present study, the network analysis revealed two TLR genes namely *TLR5* and *TLR6*, which were found nearby the significant SNPs associated with CSF virus vaccination in crossbred pigs. The TLR6 cluster consisted of 101 nodes and 1954 edges constituting the cluster network with average node degree: 38.7; average local clustering coefficient: 0.723 and PPI enrichment p-value: <1.0e-16 (Figure 2). TLR6 Cluster was further comprised of three sub-clusters, each with 40, 33 and 28 gene count respectively in each sub-cluster. The GO analysis revealed BP associated with defence response, immune response, inflammatory response, regulation of cytokine production and immune system process (Figure 3a). The enriched MF included cytokine receptor binding, protein binding, toll like receptor binding, pattern recognition receptor activity and interleukin1 receptor binding (Figure 3b). The major CC in the cluster were receptor complex, membrane raft, autophagosome, plasma membrane signalling receptor complex (Figure 3c). KEGG pathway analysis revealed involvement of signalling pathways like NF-kappa beta, toll like receptor and NOD like receptor (Figure 4a) whereas REACTOME pathway analysis revealed innate immune system, cytokine signalling, MyD88 family signalling, IL1 signalling, TRAF6 mediated induction of NF-kappa beta and MAP kinases (Figure 4b).

The PPI analysis of *TLR6* cluster associated genes and pathways indicates the critical role of this gene in regulation of different immune system process. These results also depict the importance *TLR6* gene located on SSC8, encoding for *TLR6* protein which is a type I transmembrane protein and aids in recognition of bacterial ligands. TLR6 protein recognizes molecular patterns derived by *Mycoplasma hyopneumoniae* derived (Kumar *et al*., 2009) [9]. The differential expression of *TLR6* was found in two pig population in response to *Mycoplasma hyopneumoniae* vaccination in pigs (Régia Silva Sousa, *et al*. 2011) [17]. This gene was also associated with cytokine production and altered signalling along with variation in susceptibility to diseases in humans (Misch and Hawn, 2009) and affects the tetanus antibody production in pigs (Uddin *et al*. 2010) [21]. *TLR6* gene play crucial role in the recognition of *M. hyopneumoniae* in alveolar macrophages of pig and in understanding the innate immunity against *M. hyopneumoniae* (Muneta *et al*. 2003) [15]. The activation of *TLR6* gene by Dengue viral NS1 protein play important role in the immunopathogenesis of infection by Dengue virus and blocking of *TLR6* caused reduced production of IL-6 and TNF-α (Chen *et al*. 2015) [4].

TLR5 gene located on SSC10 encoding a member of toll-like receptor family, which have important role in recognition of pathogen and thus activate innate immune responses. The hub gene analysis also revealed the network of genes wherein TLR5 had a key role along with interleukin genes. *IL9R* (interleukin 9 receptor) gene located on SSC3 is a protein encoding gene which encodes for cytokine receptor which mediates biological effects of multifunctional cytokine interleukin 9 that control the function of many humoral immune cells. The activation of *TLR5* causes mobilization of nuclear factor NF-kappaB as depicted in functional annotation and gene ontology study of TLR cluster of genes. Mutations in *TLR5* was associated with resistance/susceptibility to the Legionnaire disease and systemic lupus erythematosus disease. SNPs in *TLR5* gene caused impaired functionality associated with intestinal microbiota in pigs (Pieper *et al*. 2020) [16]. *TLR5* expression pattern in jejunum and duodenum suggested that it has important regulatory role in the invasion of *E. coli* and may act as new candidate marker gene for screening *E. coli* resistance in pigs. In a similar note, *TLR5* and *TLR6* can be associated with immune response in pigs and their differential expression can be used for determining the high, low or medium responder to the immunization against CSF vaccination in pigs. Nevertheless, this study is supported by a computational prediction and analysis, hence a further study in larger population is required for a more convincing result.

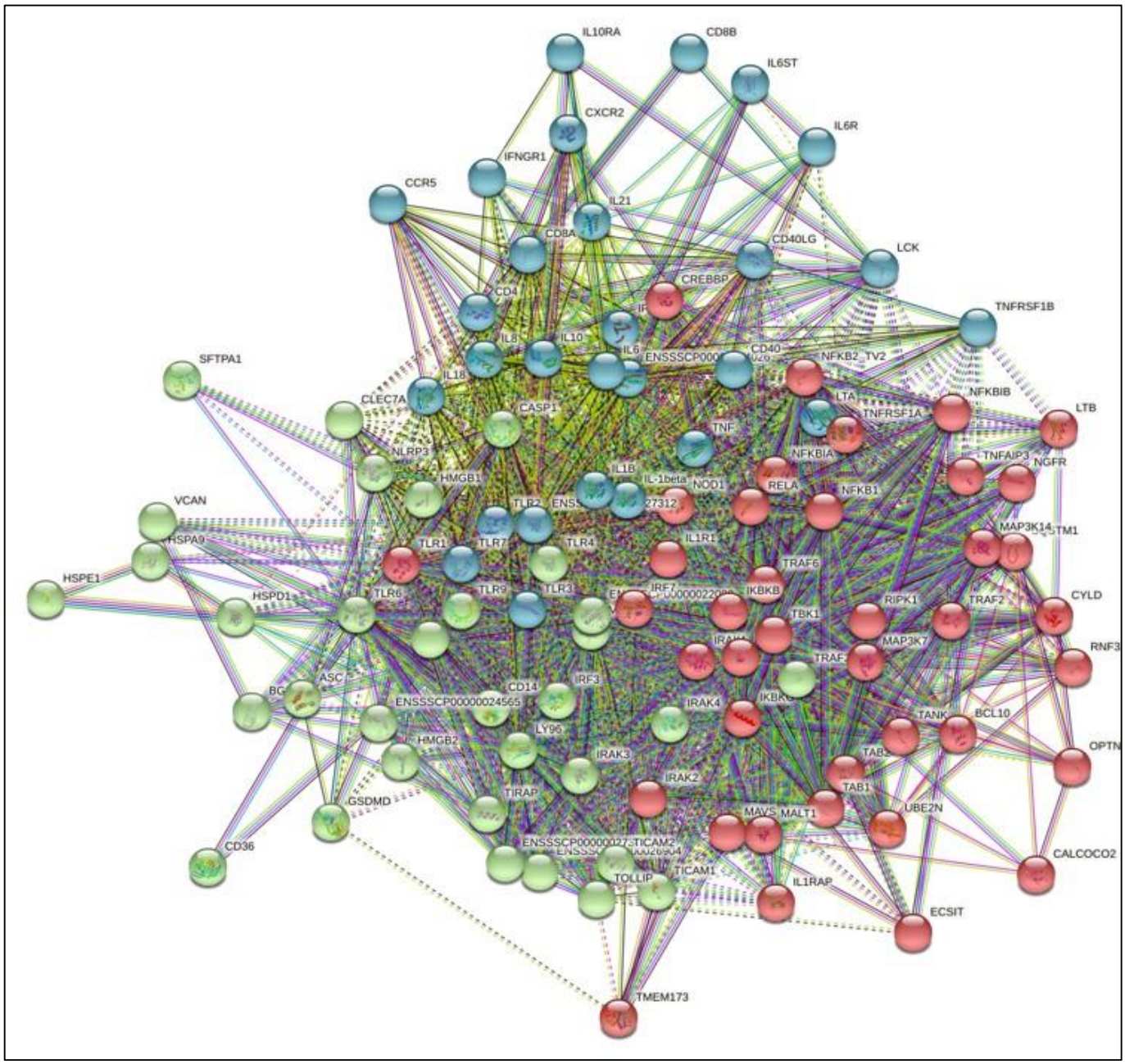

Fig 2: The protein-protein interaction network of genes regulating immune system process in the study.

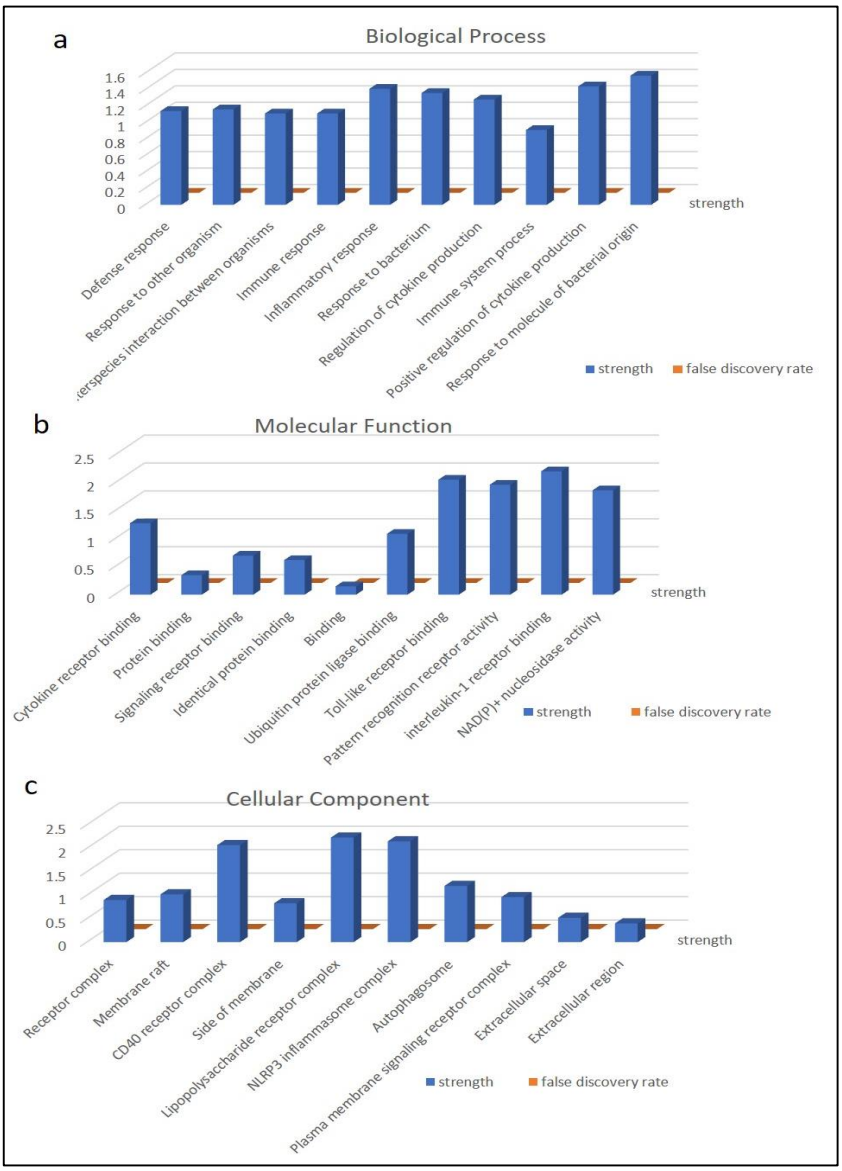

Fig 3: The gene ontology analysis of the key genes regulating immune process signalling in the network. (a) Biological Process (b) Molecular Function (c) Cellular component

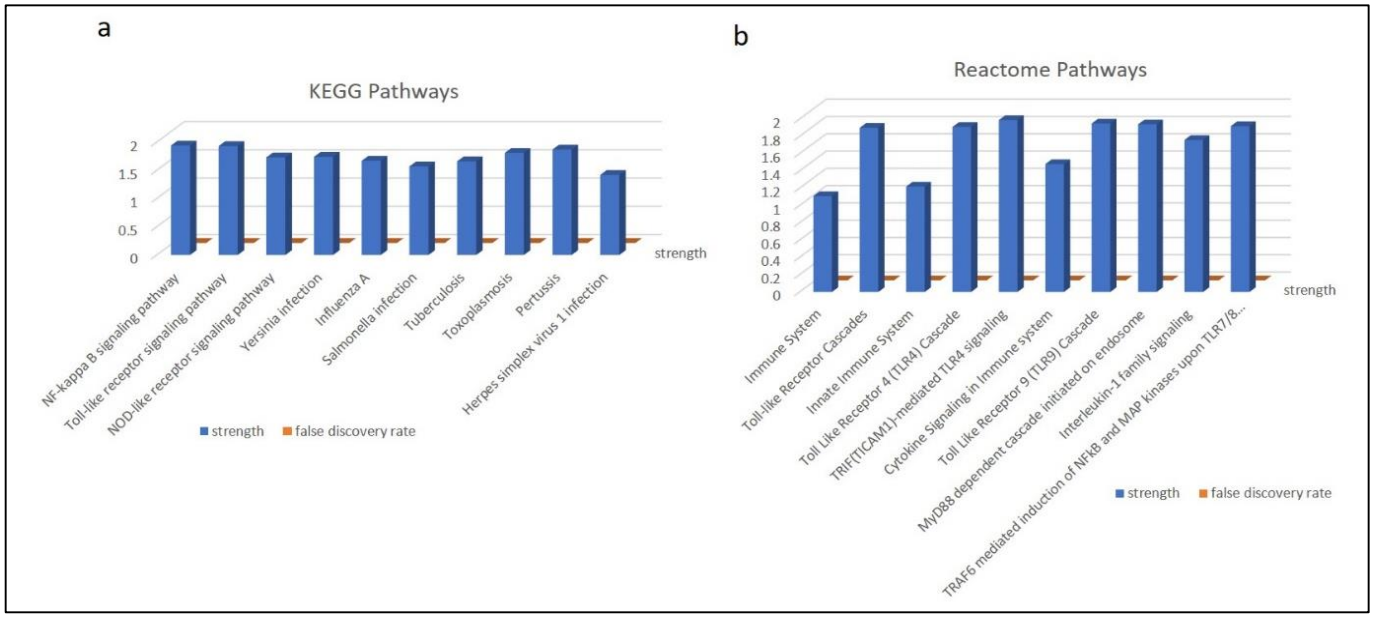

Fig 4: Pathway enrichment analysis. (a) KEGG pathway; (b) Reactome pathways of genes in the protein-protein interaction network of TLR6 cluster. Shown are enrichment strength and FDR (false discovery rate) p-values (p<0.05) corrected for multiple testing within each category using the Benjamini–Hochberg procedure.

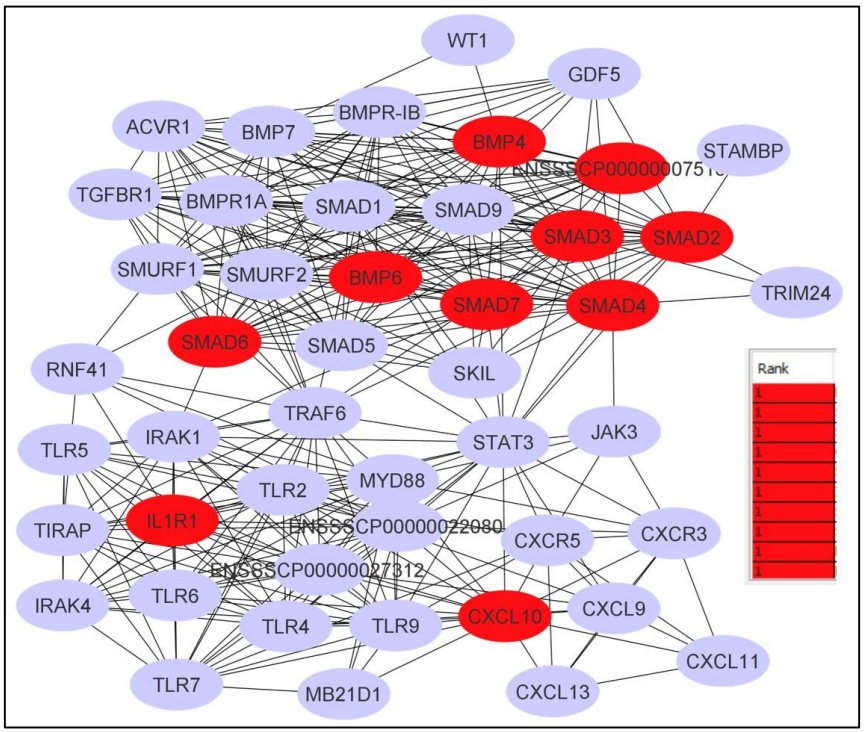

Fig 5: Visualization of protein-protein interaction network with highly connected Cytohubba nodes (top 10) according to eccentrity score ranking method. Highlighted in red (maximum centrality).

Conclusion

The TLRs form a key component of network of signalling pathways regulating immune response against CSF vaccination in Landly pigs, which needs further exploration.

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Data availability statement

All data generated or analysed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

AK - Conceptualization and funding acquisition; AK, BB and SB -provided resources; SK- Investigation and formal analysis; JB - Bioinformatics and functional annotation analysis. SK - Writing original draft. SK, AK, SoK, PJD, JB, SB and MP reviewed and edited the original draft. All authors discussed the results, edited the manuscript, and approved the final version of the manuscript for publication.

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