Early transcriptome profile of goat peripheral blood mononuclear cells (PBMCs) infected with peste des petits ruminant's vaccine virus (Sungri/96) revealed induction of antiviral response in an interferon independent manner

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52 **Abstract**

53 Sungri/96 vaccine strain is considered the most potent vaccine providing long-term
54 immunity against peste des petits ruminants (PPR) in India. Previous studies in our laboratory
55 highlighted induction of robust antiviral response in an interferon independent manner at 48
56 h and 120 h post infection (p.i.). However, immune response at the earliest time point 6 h p.i.
57 (time taken to complete one PPRV life cycle), in PBMCs infected with Sungri/96 vaccine virus
58 has not been investigated. This study was taken up to understand the global gene expression
59 profiling of goat PBMCs after Sungri/96 PPRV vaccine strain infection at 6 h post infection
60 (p.i.). A total of 1926 differentially expressed genes (DEGs) were identified with 616 -
61 upregulated and 1310 - downregulated. TLR7/TLR3, IRF7/IRF1, ISG20, IFIT1/IFIT2, IFITM3,
62 IL27 and TREX1 were identified as key immune sensors and antiviral candidate genes.
63 Interestingly, type I interferons (IFNα/β) were not differentially expressed at this time point as
64 well. TREX1, an exonuclease which inhibits type I interferons at the early stage of virus
65 infection was found to be highly upregulated. IL27, an important antiviral host immune factor
66 was significantly upregulated. ISG20, an antiviral interferon induced gene with exonuclease
67 activity specific to ssRNA viruses was highly expressed. Functional profiling of DEGs showed
68 significant enrichment of immune system processes with 233 genes indicating initiation of
69 immune defense response in host cells. Protein interaction network showed important innate
70 immune molecules in the immune network with high connectivity. The study highlights
71 important immune and antiviral genes at the earliest time point.

72 **Keywords:** Microarray, PPRV, PBMCs, STRING protein-protein interactions, Ingenuity
73 pathway analysis.

74 **Introduction**

75 Peste-des-petits ruminant's virus (PPRV), is an important viral pathogen of sheep and
76 goats that causes devastating disease Peste-des-petits ruminants (PPR), which is spreading

77 extensively over the last two decades causing significant economic loses in developing
78 countries (Albina et al., 2013; Banyard et al., 2010). PPRV is a single stranded (ss) RNA virus
79 that belongs to the genus *Morbillivirus* and Family *Paramyxoviridae.* The PPRV genome
80 encodes six structural proteins nucleoprotein (N), a viral RNA-dependent polymerase (L), an
81 RNA-polymerase phosphoprotein co-factor (P), a matrix protein (M), a fusion protein (F) and
82 a hemagglutinin protein (H) and two non-structural proteins C and V proteins. The C protein
83 of morbilliviruses mediates efficient viral replication in peripheral blood cells in host by
84 blocking the induction of type I interferons (Escoffier et al., 1999; Boxer et al., 2009). These
85 non-structural proteins (C and V) in morbilliviruses and the paramyxoviruses, play an efficient
86 role in virus replication, its virulence and help the virus in evading the host immune defense
87 mechanisms by blocking type I IFN signaling pathway. Studies by many researchers have
88 shown that the non-structural proteins of the paramyxoviruses inhibit the type I interferon
89 (IFNα/β) response (Garcin et al., 1999, Shaffer et al., 2003 and Ramachandran et al., 2008).
90 Recently, PPRV V protein was found to bind MDA-5 and its overexpression was shown to
91 block IFN-β pathways (Bernardo et al., 2017). Previous transcriptome studies in our
92 laboratory highlighted induction of robust antiviral response in an interferon independent
93 manner at 48 h and 120 h post infection (p.i.) (Manjunath et al., 2017). However, immune
94 response at the earliest time point 6 h p.i. (time taken to complete one PPRV life cycle), in
95 PBMCs infected with Sungri/96 vaccine virus has not been investigated.
96 Interferons (IFNs) are the family of the cytokines, which inhibit viral growth inducing an
97 antiviral state in host cells at the early stage of infection and play a critical role in modulating
98 adaptive immune responses. Type I IFNs (IFN-α and IFN-β) commonly referred as “viral IFNs”
99 are directly induced when the viral conserved molecular patterns called pathogen associated
100 molecular patterns (PAMPs) are recognized by the pattern recognition receptors (PRRs) of
101 the host cells. Type I interferon (IFN) gene induction in host cells against viral infections is a

consequence of activation of virus responsive elements, controlled by key regulators called interferon regulatory factors (IRFs mainly IRF-3 and 7). The induction of type I IFNs as secreted factors bind to the cell surface transmembrane receptors called interferon alpha receptor (IFNAR) on cells adjacent to infected cells and activates downstream JAK-STAT pathway. The activated STAT proteins translocates into the nucleus and binds to interferon stimulated responsive elements (ISREs) present at the upstream of the interferon stimulated genes (ISGs) activating many ISGs, which ultimately exerts the antiviral effect synergistically with other cytokines and chemokines (Pitha-Rowe and Pitha., 2007). Thus, interferon stimulated genes (ISGs) along with other cytokines and chemokines help in establishing an antiviral state in infected host cells. Interestingly, few RNA viruses including PPRV, have developed unique mechanisms to counterattack the host interferon (IFN) responses by subverting the host interferon signaling and thus, establish infection evading host innate and adaptive immune defenses (Bernardo et al., 2017; Nanda and Baron., 2006; Pauli et al., 2008; Nan et al., 2014).

Therefore, in the present study, the transcriptomic signatures in PPRV infected PBMCs at 6 h post infection (p.i) was analyzed to identify important innate immune / antiviral genes induced, and candidate genes driving interferon (IFN) evasion at early stages of PPRV infection. The results in the present study along with our previous observations (Manjunath et al., 2017; Manjunath et al., 2015) will add important protective innate immune signatures stimulated by Sungri/96 PPRV vaccine virus from early time point to the later stages of PPRV infection in an interferon independent manner.

2. Materials and Methods

2.1. Animals, blood collection and screening animals for PPRV antibodies: All experimental procedures in the present study were approved by Institute Animal Ethics Committee (I.A.E.C No.F.1.53/2012-13-J.D.). Blood was collected from goat kids (5 months

old, n=5) screened negative for PPRV antibodies using competitive ELISA (c-ELISA) (Singh et al., 2004) kit and serum neutralization test (SNT) (Dhinakar Raj et al., 2000). Blood collected from animals negative for PPRV antibodies with percentage inhibition (PI) values less than 40 was used for isolating Peripheral blood mononuclear cells (PBMCs) and further studies.

2.2. Isolation of PBMCs and PPRV Infection Confirmation Peripheral blood mononuclear cells (PBMCs) from PPRV negative goats were isolated from fresh, heparinized venous blood by density gradient centrifugation on Histopaque-2000 and was washed thrice with sterile phosphate buffered saline (PBS), the cells were finally resuspended in RPMI-1640 supplemented with 10% fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin). Viability of the cells was assessed using trypan blue exclusion test and the cells were found to be more than 95% viable. PBMCs were seeded at a density 1 X 10^6 viable cells/ml in two 6 well plates, one serving as control and the other plate was used for virus infection. Goat PBMCs isolated were infected with purified (Ultracentrifuged) Sungri/96 PPRV vaccine virus at 1.0 MOI (multiplicity of infection) and incubated at 37^0C in 5% CO_2 incubator for 1 h of virus adsorption. After 1 h of adsorption, the virus inoculum was removed, centrifuged to collect the lymphocytes as they are in suspension, washed with fresh RPMI medium and added back to the wells. Fresh RPMI medium was added to wells and incubated upto 6 h post infection (p.i). The control (mock-infected) PBMCs on the other hand just received RPMI medium. Finally, the infected cells along with the control (mock-infected) PBMCs in duplicates were harvested at 6 h post-infection (p.i) and were processed for RNA isolation. Cells from three wells were combined per replicate sample. This time point was chosen to give enough time for the establishment of infection and for the completion of one viral life cycle (for PPRV 6-8 h). Experiments were performed in duplicates using RNA samples from two independently infected cell cultures for analysis. PPRV infection in PBMCs

was confirmed with N gene PCR and qRT-PCR. RNA isolated from control and the infected PBMCs was reverse transcribed to cDNA and N gene was amplified and quantified as previously (Manjunath et al., 2015).

2.3. RNA preparation for microarray analysis: Total RNA was isolated from the PPRV infected and control PBMCs using Trizol reagent (Invitrogen). The quality and the integrity of the RNA samples isolated were determined using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies) and the RNA was quantified on ND-1000 Spectrophotometer. Labeling of the complementary RNA (cRNA) samples was performed as detailed in the agilent one-colour microarray based gene expression analysis protocol. Briefly, 200ng of total RNA was used for amplification and labeling step using Agilent Quick Amp Labelling kit (Agilent Technologies) in presence of Cy3-CTP. Amplified cRNA samples were purified and the dye incorporation rates were measured before hybridization with ND-1000 Spectrophotometer.

2.4. Microarrays and Hybridization: The labeled samples were hybridized according to the one-color microarray based gene expression analysis (part number G4140-90040). In brief, 1.65 µg of Cy3-labelled fragmented cRNA was hybridized overnight (at 65^0 C for 16 h) to 4 x 44K Agilent *Bos taurus* microarrays. Since *Capra hircus* genome was not completely annotated, *Bos taurus* microarrays were used based on the fact that the average identity across exon sequences between *Bos taurus* and *Capra hircus* was 93.77% (Fontanesi et al., 2010). After overnight hybridization, the arrays were washed with Triton X-102 added gene expression hybridization buffer I for 1 min at room temperature followed by wash with pre-warmed gene expression hybridization buffer II for 1 min at 37^0 C. The hybridized microarrays were scanned using standard protocols and fluorescence signals were detected using Agilent's Microarray scanner system (G2505C Scanner, Agilent Technologies, USA). Agilent

feature extraction software (FES) was used to process the scanned raw microarray image files to text files for the data analysis.

2.5. Microarray data analysis and Identification of differentially expressed genes (DEGs): The sample files generated from microarray experiment were processed with agilent feature extraction software and then subjected to bioinformatics analysis using GeneSpring GX10 software (Agilent Technologies). The RMA algorithm conducts background correction, followed by quantile normalization and probe summarization. Overview of the experiment and the microarray analysis followed in the present study is highlighted in Figure 1. Differentially expressed genes (DEGs) statistically significant based on fold change ≥ 3 and *P*-value < 0.05 were identified. A total of 1926 DEGs were identified of which 616 were upregulated and 1310 genes were downregulated.

2.6. Gene Ontology (GO) and Pathway analysis: The GO category based on biological process was retrieved for all the 1926 differentially expressed genes and also separately for all upregulated (616) and the downregulated genes (1310) using g:profiler (Reimand et al., 2011). Further, analysis of enrichment of differentially expressed genes to canonical pathways was done using Cytoscape plugin ClueGO (Shannon et al., 2003), the enriched pathways were based on KEGG and REACTOME databases.

2.7. Ingenuity Pathway analysis (IPA): The total differentially expressed genes were subjected to IPA analysis tool (IPA; QIAGEN, Redwood, CA). Also, the upregulated and the downregulated genes in isolation were analyzed by IPA. IPA analysis can be used to identify top significant canonical pathways (ranked by the z-score) and enriched networks in given set of input query genes. This tool in addition to identifying significant pathways can also predict downstream effects on the biological process, and activation/inhibition of the transcription factors from gene expression data set (Kramer et al., 2014). The differentially expressed genes (DEGs) identified from the microarray analysis were uploaded into IPA with

gene identifiers (ID) and corresponding fold changes. The same was followed separately for upregulated and the downregulated genes in the DEGs. Here, two parameters were used to calculate the significance between the genes from the input set and the canonical pathway: (1) Ratio which refers to number of genes in list to the total number of genes in the canonical pathway (2) *P*-value calculated by the Fisher's exact test, determining the probability that there is an association between the input genes and the canonical pathway. Top 5 significant canonical pathways for the total DEGs and for upregulated and downregulated genes in isolation were identified and, the ratio and *P*-value were recorded.

2.8. STRING analysis of protein-protein interactions (PPI) of DEGs: The protein-protein interactions among upregulated and the downregulated genes (with fold change ≥ 5) in the study were predicted using STRING database (http://string-db.org/), which has known and predicted protein interactions. The STRING represents the functional association, and the basic interaction unit here gives the specific and productive relationship between the two proteins (Szklarczyk et al., 2015). The STRING new version 10.0 used to detect protein-protein interactions (PPI) in this study covers more than 2031 organisms and for our study we used *Bos taurus* as closely related species to *Capra hircus*. The PPI analysis was carried out separately for upregulated and the downregulated genes. PPI for the upregulated genes consisted of 192 nodes and 171 edges. PPI analysis for the downregulated genes consisted of 392 nodes and 347 edges. Here, nodes represent the proteins and the edges represent the protein-protein associations. Nodes which were not connected were removed from the study.

2.9. Validation of the microarray data with qRT-PCR: To confirm the results of our microarray analysis, among the identified differentially expressed genes (DEGs) some of the candidate DEGs of interest (Table 1) were validated using qRT-PCR. GAPDH, which is the most stable housekeeping genes for PPRV infected PBMCs observed previously (Manjunath

et al., 2015) was used as an endogenous control for the experiment. Total RNA was isolated from control and the infected PBMCs at 6 h p.i using the TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The RNA quality was checked and quantified using nanodrop spectrophotometer (Thermo Scientific, USA). 100 ng of RNA was used to synthesize cDNA using Revert Aid First Strand cDNA synthesis kit. qRT-PCR was performed in Applied Biosystems 7500 fast machine using 2X SYBR green. A melt curve analysis was performed to know the specificity of the qPCR. For the test and endogenous control genes the percentage efficiency ranged between 90% and 100%. All the samples were assayed in triplicates. The relative expression of each sample was calculated using the $2^{-\Delta\Delta CT}$ method with uninfected control group as calibrator (Livak and Schmittgen, 2001). Student's t-test was done in JMP9 (SAS Institute Inc, Cary, USA) and differences between groups were considered significant at $P \leq 0.05$.

3. Results

3.1. Infection confirmation and quantification of PPRV Infected cells: PPRV infection in PBMCs was confirmed by amplifying 'N' gene from the infected PBMCs at 6 h post infection (p.i.). The control cells were negative for N gene (Figure 2A). qRT-PCR quantified N gene expression at 6 h p.i, further confirmed PPRV infection in PBMCs (Figure 2B).

3.2. Effects of early PPRV infection and Overview of differentially expressed genes (DEGS) at 6 h p.i

In response to PPRV infection at 6 h p.i, a total of 1926 genes were found to be differentially expressed in infected PBMCs based on fold change of (Fc) >±3 and *P* <0.05 (see Supplementary Table 1). Among these 1926 significant differentially expressed genes, 616 and 1310 genes were found to be significantly upregulated and downregulated respectively at 6 h p.i. List of top 20 upregulated and downregulated genes are given in (Table 2) with their corresponding fold changes. Among the upregulated genes, SERTAD1 was found to be the

highly upregulated gene (Fold change 53.3). TREX1, the cytosolic exonuclease that plays a role in inhibiting induction of type I interferons was also highly upregulated (fold change 41.9). Many interferon related genes were found to be upregulated in our study namely, interferon induced protein with tetricopeptides (IFITs) viz. IFIT1 (fold change 17.3), IFIT2 (fold change 3.4) and IFIT3 (8.5). Also, IFITM3 - Interferon inducible transmembrane proteins, an important antiviral immune factor against many pathogenic viruses was found to be upregulated (fold change 16.7). ISG20 (Interferon stimulated gene 20) which inhibits replication of many viruses was found to be highly upregulated (fold change 23.7) in PBMCs infected with PPRV at 6 h p.i. Among the interferon regulatory factors (IRFs), IRF1 (fold change 5.5) and IRF7 (fold change 5.8) was found to be induced. IL-27, an antiviral cytokine was significantly upregulated after PPRV infection (fold change 19.3). The innate immune signaling cascade of events leading to the activation of the downstream effectors i.e interferon stimulated genes (ISGs) is initiated when pattern recognition receptors (PRRs) like TLRs are engaged with viral nucleic acids. In our study, TLR3, TLR7 and TLR8 were found to be upregulated after PPRV infection in PBMCs. Among the downregulated genes, IFNAR1 was found to be downregulated with a fold change 3.08. Among the caspases, caspase 8 (fold change 3.72) and caspase 4 (fold change 3.4) were significantly upregulated.

3.3. Gene Annotation of Differentially expressed genes and their pathway analysis

Significantly enriched biological processes among all the differentially expressed genes (i.e 1926 DEGs), and among the upregulated and the downregulated genes in isolation were retrieved using g:profiler. Specific biology can be understood by analyzing the functional enrichment for upregulated and downregulated genes in isolation. Biological processes enriched among the 1926 DEGs are shown in Figure 3A, Supplementary table 2, Sheet1. The top significant biological process enriched among all the DEGs (1926) were immune system process (Genes: 233; *P*-value 2.89E-10), response to stress (Genes: 305; *P*-value 1.68E-05),

defense response (Genes: 135; *P*-value 2.24E-05), cell surface receptor signaling pathway (Genes: 226; *P*-value 2.49E-05), cytokine production (Genes: 74; *P*-value 0.000643). Biological process retrieved for 616 upregulated DEGs showed enrichment of immune system process, cytokine production, innate immune response and other immune defense processes (Figure 3B, Supplementary table 2 Sheet 2). Enriched biological processes of the downregulated genes showed enrichment of the normal cellular processes (Figure 3B, Supplementary table 2 Sheet 3). ClueGO analysis showed significant enrichment of antiviral mechanism by ISGs and interferon signaling among 1926 DEGs, and among the upregulated genes antiviral mechanism, interferon signaling, cytokine signaling, toll like receptor signaling pathway, etc., were found to be enriched (Figure 3C and 3D). Overall, the functional enrichment of DEGs at 6 h p.i. reflected that the infected PBMCs responded to the PPR virus infection by initiating the immune defense against the virus by alarming the immune sensors.

3.4. Significant Pathway analysis of PPRV Infected PBMCs (6 h p.i.)

All the 1926 DEGs identified were uploaded to Ingenuity pathway analysis (IPA). The top canonical pathways enriched were Interferon (IFN) signaling (*P*=2.82E-08, ratio=0.417); role of BRAC1 in DNA damage response (*P*=1.65E-06, ratio=0.256); hepatic stellate cell activation (*P*=3.28E-06, ratio=0.182), role of JAK family kinases in IL-6 type cytokine signaling (*P*=9.37E-06, ratio=0.4) and glucocorticoid receptor signaling (*P*=2.36E-06, ratio= 0.15) (Figure 4A, Table 3). Further, the network analysis of these DEGs (1926) involved 25 networks with top significant pathway being cell death and survival (score 40). This was followed by cellular assembly and organization, cellular function and maintenance with a score of 38. We then subjected upregulated (616) and downregulated DEGs (1310) to IPA. Statistically significant canonical pathways enriched in upregulated genes were interferon signaling (*P*=3.81E-12, ratio=0.361), T-helper cell differentiation (*P*=4.09E-08, ratio=0.183), communication between innate and adaptive immune cells (*P*=6.4E-07, ratio=0.146) (Figure

4B, Table 3). IFN-gamma and transcription factors (IRF7 and IRF1) were found to be the significant upstream regulators controlling significant number of upregulated genes. The important network associated with upregulated genes was antimicrobial response, inflammatory response and infectious diseases (score 27). IPA analysis of the downregulated genes did not show any relevant important canonical pathways related to the infection (Figure 4C, Table 3). The network of the upregulated genes consisting of important innate immune molecules was generated from IPA and shown in figure 4D.

3.5. Interaction Network Analysis (INA) of upregulated and downregulated genes

Figure 5A and 5B shows predicted protein interaction networks of the upregulated and the downregulated genes encoding proteins. The network analysis of the upregulated genes showed average node degree 1.78 and clustering co-efficient to be 0.795. The network showed interaction between the early immune genes interferon regulatory factors - IRF1, IRF7; interferon stimulated gene - ISG20; and IFN-induced protein with tetricopeptide repeats - IFIT1, IFIT3 TLR7, IFNG and IL27 that play a vital role in early immune defense against the invading virus in the host cell. There were other proteins, which were linked to this network of early innate immune proteins viz. DDX58 – an innate immune receptor that act as a cytosolic sensor for viral nucleic acids stimulating downstream immune signaling molecules for an effective antiviral response; and CXCL10, also known as IP10 (interferon inducible protein 10) is involved in the regulation of lymphocyte recruitment in many viral infections and inhibits viral replication. DDX58 and CXCL10 were found to be connected with IFNG, IRF1, IRF7, and ISG20 - immune network. The network analysis of downregulated genes showed average node degree 1.77 and the clustering co-efficient to be 0.764.

3.6. Validation of microarray data by qRT-PCR

Microarray analysis of the viral infected cells in comparison to the control cells yielded large number of differentially expressed genes. It is important to identify candidate genes, which

play an important role in our experimental study and validate them by qRT-PCR. We validated nine candidate differentially expressed genes predicted to be antiviral and are important innate immune molecules against PPRV in PBMCs at the early stage of infection (6 h p.i). These DEGs expression in qRT-PCR was in concordance with microarray results except IFN-Ɣ (Figure 6).

3.7. Immune signaling pathway of Sungri/96 vaccine virus updated

The pathway predicted at 6 h post infection was also found to be IFN independent similar to our previously mentioned pathway (Manjunath *et al*., 2017). Based on the transcriptome analysis at 6 h p.i. and qRT-PCR validation of important candidate genes, we updated our previously mentioned pathway with unique innate immune molecules that were predicted at this earliest time point of infection (supplementary table 3).

4. Discussion:

Global gene expression profiling helps to identify candidate genes involved in host-virus interactions and host immune defense molecules activated or inhibited under viral infection. In our previous study, we reported the transcriptional profile and immune response mechanism(s) at 120 h p.i, and 48 h vs 120h response in PBMCs infected with Sungri/96 vaccine strain (Manjunath et al., 2015; Manjunath et al., 2017). The immune protection mechanisms induced by Sungri/96 vaccine virus at an earliest time point (6 h p.i.) vis -a vis the transcriptional signatures have not been explained. Therefore, in the present study the transcriptional response of goat PBMCs at 6 h p.i., which is the time required to complete one complete life cycle of PPRV (Naveen Kumar et al., 2013) has been explored.

At 6 h p.i., 1926 significant differentially expressed genes (FC > 3) that were mostly associated with the immune and defense responses were identified. Innate immunity acts as a first line of defense against invading pathogens and is activated when pathogen associated molecular patterns (PAMPs) are engaged to pattern recognition receptors (PRRs) like TLRs

(Thompson et al., 2011). Toll like receptors (TLRs) - TLR7, TLR3, TLR8 and TLR2 were significantly upregulated in this study. TLR 7 was the most highly upregulated PRR in our study along with TLR 8, both of which are involved in the recognition of single stranded (ss) RNA viruses (Jansen and Thomson, 2012; Lund et al., 2004). These upregulated TLRs -3,7 and 8, were identified to further initiate downstream immune signaling cascades induced by Sungri/96 vaccine virus. Similarly, previous studies with Hepatitis C Virus (HCV – ssRNA virus) and other ssRNA viruses identified these TLRs (TLR7 and TLR8) in initiating the antiviral response (Zhang et al., 2016; Lund et al., 2004; Deibold et al., 2004).

Although different TLRs are activated when engaged to different PAMPs, they cross path to transcriptionally activate downstream interferon regulatory factors (like IRF3, IRF7) and NFKB, which when phosphorylated translocate into the nucleus activating type I interferons. Interestingly, in our study type I interferons (IFN-α and IFN-β) were not differentially expressed, although, interferon induced genes were found to be induced. This supports the recent observation that the paramyxovirus (including PPRV) non-structural proteins (C and V proteins) play a role in inhibiting type I IFN production (Bernardo et al., 2017; Andrejeva et al., 2004). In the present study, at 6 h p.i. we hypothesized that interferon induced genes must have been activated in an interferon (IFN) independent manner as observed at 48 and 120 h p.i. (Manjunath et al., 2017). TREX1, which was highly upregulated in the study is an exoribonuclease, besides being an exonuclease (Yuan et al., 2015). TREX1 may act as negative regulator of induction of type I interferons in PPRV infected PBMCs. Similar effect of TREX1 was observed recently in HIV infection (Wheeler et al., 2016). TREX1 could be one of the breaks, having inhibitory effect on type I IFN signaling pathway, allowing virus to replicate in the initial stages of the infection. Further, functional studies need to be done to study the effect of TREX1 on PPRV replication. IRFs and interferon induced genes that were upregulated in this study were IRF7, IRF1, IFIT2, IFIT1, IFIT3, IFITM3, OAS2 and

OAS1Z. ISG20, an interferon induced gene with exonuclease activity specific against ssRNA viruses (Espert et al., 2003) was found to be upregulated. ISG 20 was linked to other important innate immune genes in the interaction network asserting its crucial role in immune defense. Cells exhibiting increased expression of ISG20 are resistant to RNA virus infection and thus, play an important role in host antiviral innate immune defense (Espert et al., 2005 and Zhou et al., 2011). The increased expression of ISG20 highlights its specific activity against PPRV (ssRNA virus) at the early stages of the infection. Recently, it was shown ISG20 inhibits replication of influenza A virus by interacting with nucleoprotein (Qu et al., 2016) and inhibits Hepatitis B virus replication by binding directly to epsilon stem loop structure of viral RNA (Liu et al., 2017). IRF3 and IRF7 are the main regulators of immune responses to viruses (Lazear et al., 2013). Upregulation of IRF7 in our study indicated host cell responses to trigger downstream signaling cascades against the invading virus. IRF7 is known to induce the interferon stimulated genes (ISGs) by stimulating type I interferons (Pulit-Penaloza et al., 2012). However, IRF7 upregulation, induction of interferon induced genes and inhibition of type I interferon response in PPRV infected PBMCs highlighted induction of ISGs in an IFN independent manner at the early time point of infection. This may be due to the high degree of homology between the ISRE and IRF binding element (IRF-E) consensus sequences, IRF7/IRF3 may bind directly to induce ISGs (Morin et al., 2002; Schmid et al., 2010; Manjunath et al., 2017). Thus, the data suggests activation of interferon induced genes in PPRV infected PBMCs in the absence of type I interferons induction. Similar IFR7 activation of interferon induced restriction factor BST2 was observed in absence of type 1 interferon signaling in Parainfluenza virus V5 protein infection (Bego et al., 2012).

Viral infections in host cells induce pro-inflammatory cytokines and chemokines response (Mogensen and Paludan, 2001). Increased expression of interleukins viz. IL-27, IL-19, IL-6, IL-10 and IL-21 and chemokines - CCL8, CCL3, CCL25 and CCL4 in response to

401 PPRV infection was observed in this study. IL-27, an IL-12 family of cytokines was found to
402 be highly upregulated and may be a host immune factor produced in response to PPRV
403 infection. Its role as host immune factor has also been identified in Influenza A virus infection
404 (Liu et al., 2012). IL-27 has both pro and anti-inflammatory properties and is known to play
405 an important role in bridging innate and adaptive immune response (Villarino et al., 2004). IL-
406 27 also synergizes with other interleukins viz. IL-12 to trigger IFN-γ production of naïve $CD4^+$
407 T cells promoting Th1 differentiation (Hunter 2005; Yoshida and Miyazaki, 2008). IFN-γ was
408 found to be significantly upregulated in the present study. Recently, Sungri/96 vaccine was
409 shown to induce strong IFN-γ production and higher number of $CD4^+$ T cells specifically
410 responding to the virus (Hodgson et al., 2018). Thus, in the present study high expression of
411 IL-27 represents its possible antiviral role in PPRV infection. In addition, STAT proteins viz.
412 STAT1, STAT2 and STAT3 showed increased expression in this study. Therefore, the
413 findings from the present and previous studies highlights mechanism of host immune
414 response induced by Sungri/96 vaccine and emphasize the importance of stimulating type I
415 interferon response lacking in present vaccine, which may otherwise provide longer duration
416 of immune protection in hosts.

417 **4.1 Updated pathway after inclusion of early immune signaling molecules that were**
418 **uniquely expressed at an early time point in Sungri/96 vaccine virus infected PBMCs**

419 Microarray analysis of PPRV infected PBMCs at 6 h p.i., followed by qRT-PCR validation of
420 key candidate genes helped to include early immune signaling molecules that would aid in
421 triggering robust antiviral response (Figure 7). This study also confirmed the inhibition of type
422 I interferons at the earliest time point (6 h p.i.), which corroborated with our previous
423 observations at 48 h and 120 h p.i. (Manjunath et al., 2017). Lymphotropic PPR virus enters
424 the PBMCs via SLAM receptor (Adombi et al., 2011) or other alternate receptors expressed
425 on the surface of PBMCs. PPR virus (negative sense ssRNA) after entering the host cell

426 uncoats releasing viral nucleic acid, which is transcribed in the cytoplasm to establish infection
427 in host cells. The residual ssRNA genome not replicating in cytoplasm are strong inducers of
428 type I interferon response at initial stages of the infection (Yan et al., 2010). TREX1, an
429 exoribonuclease degrades ssRNA (Yuan et al., 2015), which could otherwise strongly trigger
430 type I IFN response in host cells on being recognized by RIG1. TREX1 in our study was
431 significantly upregulated (FC = 41.9) indicating its role in inhibiting type I IFN response in early
432 PPRV infection by degrading ssRNA. This TREX1 was observed only at 6 h p.i. and was not
433 found in our previous transcriptome studies at 48 h and 120 h p.i. (Manjunath et al., 2017).
434 Alternatively, virus is endocytosed and the viral PAMPs gets engaged to PRRs i.e TLRs
435 located on the endosomes. TLR7/8 on the endosomal surface recognize ssRNA to initiate
436 downstream immune signaling cascade. These were upregulated in the present study. TLRs
437 activates interferon regulatory factors called IRFs via adaptor TRIM21 which was also
438 upregulated in the present study. Activated TLR7/8 induces the activation of IRF7 during virus
439 infection. IRF7 normally expressed at low levels is upregulated in response to virus infection
440 (Liang et al., 2007). IRF7 was significantly upregulated in our study. Activation of TLR7, IRF7
441 and TRIM21 was also observed at later time points of infection in our previous study
442 (Manjunath et al., 2017). IRF7 once activated translocates into the nucleus binding directly to
443 the interferon stimulated responsive elements (ISREs) due to the homology between the two
444 and thus, activates transcription of interferon induced genes (Ning et al., 2005; Schmid et al.,
445 2010). The interferon induced genes after transcription are translated in the cytoplasm, the
446 translated interferon induced genes along with other antiviral proteins act synergistically to
447 exert antiviral response. ISG20 and IFIT1 were the unique interferon induced genes
448 significantly upregulated at 6 h p.i. along with IL-27, an important antiviral host immune factor.
449 These molecules along with the innate immune molecules identified in our previous study

450 (Manjunath et al., 2017) help in triggering a robust antiviral response in an interferon
451 independent manner in PBMCs infected with Sungri/96 vaccine virus.

452 **5. Conclusion**

453 The study highlighted key early immune sensors and antiviral molecules like IL-27, IFIT1 and
454 ISG20 at the earliest time point - 6 h p.i. (time taken to complete one PPRV life cycle), in
455 PBMCs infected with Sungri/96 vaccine virus. The study also confirms the inhibition of type I
456 interferon response at this time point supporting our observation at 48 h and 120 h p.i. TREX1
457 is predicted to be the possible molecule responsible for type I interferon inhibition.

458 **Conflict of Interest**

459 The authors declare no conflict of interest

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603 **Figure legends:**

604 **Figure 1: Overview of the experiment and microarray analysis:** Depicts the experimental
605 method followed to generate microarray data and further analysis. Steps include identification
606 of the differentially expressed genes (DEGs), functional enrichment of the DEGs, gene
607 interaction network analysis, pathway analysis and validation of the candidate DEGs.

608 **Figure 2: PPRV infection confirmation: A.** PPRV infection confirmation at 2h p.i. (Lane 2)
609 and 6 h p.i. in PPRV infected PBMCs showing N gene amplicon of 351 bp (Lane 3) and the
610 uninfected cells showed no N gene amplification (Lane 1). **B.** Fold changes for N gene
611 expression quantified by qRT-PCR after normalization at 6 h p.i in comparison to 2h p.i..

612 **Figure 3: Gene Ontology (GO) of differentially expressed genes (DEGs): A.** GO category
613 in terms of biological processes for all the DEGs (1926) was retrieved using g:profiler,
614 significant biological processes ($P < 0.05$) are shown and the number represents the genes
615 involved in the particular process. **B.** Significant biological processes retrieved separately for
616 the upregulated and the downregulated genes are shown. **C** and **D.** Pathways enriched by
617 clueGO analysis in total DEGs (1926) and the upregulated genes (616) respectively. The
618 number on the graph represents the number of genes belonging to a particular term.

619 **Figure 4: Ingenuity pathway analysis (IPA): A, B and C.** IPA analysis showing top five
620 canonical pathways enriched in all DEGs (1926), upregulated genes (616) and
621 downregulated genes (1310) respectively. **D.** Gene interaction network generated by IPA of
622 upregulated transcripts at 6 h p.i. The interaction network showed the relationship between

important innate immune related genes. In the figure, the genes are displayed with various shapes, which actually represents the IPA defined functional class of gene product as indicated. The solid line indicates the direct interaction and the dotted line indicates the indirect interaction.

Figure 5: STRING analysis of protein-protein Interaction networks: A and B. Protein-protein interaction networks of upregulated and the downregulated genes respectively. The nodes represent the proteins and the edges represent the interactions between them.

Figure 6: Validation of microarray results by qRT-PCR of selected genes: Nine candidate genes from the microarray analysis were validated with quantitative real time PCR (qRT-PCR). Fold changes ($2^{-\Delta\Delta Ct)}$ for each gene is represented, calculated with control sample as the calibrator with standard error bar. Here levels not connected with same letter are significantly different.

Figure 7: Updated immune signaling pathway in goat PBMCs infected with PPRV: PPR virus being lymphotropic infects PBMCs through SLAM/CD46 receptor. The virus enters the cells and gets uncoated, releasing viral nucleic acids in the cytoplasm, which then undergoes replication. Also, the virus enters into endosomes where the viral nucleic acids are released. PPRV ssRNA in the cytoplasm undergoes replication to establish infection in the cells, whereas the excess ssRNA not replicating are chewed by exoribonuclease TREX1, which could otherwise induce a strong type I IFN response in infected cells through RIG1. TREX1 significantly upregulated in the present study may probably inhibit type I IFN response in PBMCs. The virus entering the endosomes releases the ssRNA, gets engaged to TLRs (TLR – 3, 7 & 8) and activates interferon regulatory factors (IRFs- 3,7 & 9) with the help of adaptor(s) - TRIM14/21. Activated and phosphorylated IRFs translocate into the nucleus and bind to interferon stimulated responsive elements (ISREs) activating interferon stimulated genes (ISGs). ISGs along with other immune molecule exert a strong antiviral response in PPRV

infected PBMCs. Upregulated and downregulated genes are indicated by up (↑) and down (↓) arrows respectively in the figure. The unique candidate genes expressed at early time point are coloured in blue.

Legends to Supplementary Files:

Supplementary file 1: List of differentially expressed genes and their fold change. This Supplementary file shows list of 1926 differentially expressed genes and their corresponding fold changes associated with them.

Supplementary file 2: GO in terms of biological process retrieved from g-profiler for 1926 differentially expressed genes (Sheet 1), upregulated genes (Sheet 2) and downregulated genes separately (Sheet 3). This Supplementary file Sheet 1, Sheet 2 and Sheet 3 shows the significantly enriched processes among the biological processes in g-profiler for total 1926 DEGs, upregulated genes and downregulated genes respectively. The genes involved in each process and their significant *p*-value has been indicated in this file. The minus log P values were used to construct the GO Fig.s.

Supplementary file 3: List of 1926 differentially expressed genes identified in this study with their fold changes. The list also compares the log fold changes of 1926 DEGs presence in our pervious study i.e 48 h p.i. and 120 h p.i. The file shows the candidate genes identified in this study and validated, some of which are unique to early stages of PPRV infection.

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673 **Table 1**: Primers Sequences used for qRT-PCR

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Genes	Primer Sequence
IFIT3	Forward: AAGGGTGGACACTGGTCAAG Reverse: AGGGCCAGGAGAACTTTGAT
ISG20	Forward: TGCATGCACAGACATCCC Reverse: CTAACAGTCATCAGAGTGTAGCC
IFN-Ɣ	Forward: CAGGAGCTACCGATTTCAGC Reverse: AGGCCCACCCTTAGCTACAT
IFNα	Forward: CAGCCTGGTCCTTACTCCTG Reverse: CTGCTCTGACAACCTCCCAG
IFNβ	Forward: GTGTCTCTCCACCACAGCTC Reverse: CGGAGGTAACCTGTTAGGCTC
TREX1	Forward: GCATCTACTGGAACCAACCC Reverse: CAGGAAGGCCAGAAGGC
IL-27	Forward: CTGCTTCCTCTCCCTGACAC Reverse: TTCCTCCTCATTCTCGTGCT
TLR7	Forward: GCAGCCTGTTCTGGAAAATC Reverse: GAAGGGGCTTCTCAAGGAAT
IRF7	Forward: GACACGCCCATCTTTGACTT Reverse: ACTGTCCAGGGAGGACACAC

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679 **Table 2:** List of top 20 upregulated and the downregulated genes in the present study with their
680 corresponding fold change

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Top 20 Upregulated genes	Fold Change	Top 20 Downregulated genes	Fold Change
SERTAD1	53.4	EFHD1	-3937.9
TREX1	41.9	MTURN	-1405.9
FN1	30.6	SYNE4	-180.6
TNFRSF11B	29.3	KCNK5	-72.9
TSGA10IP	26.4	TMEM39A	-56.4
ISG20	23.8	VPS13A	-15.6
TNFRSF11B	22.0	ITGAD	-15.4
MAP1B	21.5	NUPR1L	-13.8
LAG3	19.6	ITGAD	-13.0
IL27	19.3	ABCA7	-12.9
IFNW1	18.3	PLD4	-12.0
EMC7	17.7	FAM92A1	-11.9
IFIT1	17.4	PON3	-11.8
IFITM3	16.7	CRABP2	-11.5
ERAP1	15.5	TGFBI	-11.2
AKAP11	14.4	IFT122	-11.0
ACADM	14.2	KCNB2	-10.8
DDX58	14.2	RNF6	-10.8
FAM170B	14.1	ZNF280D	-10.6

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687 **Table 3:** Ingenuity Pathway Analysis (IPA) of 1926 DEGs, upregulated and downregulated genes with
688 top significant canonical pathways and upstream regulators for each category

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IPA analysis of 1926 DEGs with the top canonical pathways and upstream regulators				
Top Cannonical Pathways	*P-value*	Ratio	Upstream Regulators	*P-value*
Intereferon (IFN) Signaling	2.82E-08	0.417	TGFB1	4.34E-26
Role of BRCA1 in DNA damage response	1.65E-06	0.256	TNF	3.70E-22
Hepatic stellate cell activation	3.28E-06	0.182	B-Estradiol	8.35E-21
Role JAK family kinases in IL6 type cytokine signaling	9.37E-06	0.4	LPS	4.29E-19
Glucocorticoid receptor signaling	2.36E-05	0.15		
IPA analysis of Upregulated DEGs with the top canonical pathways and upstream regulators				
Top Cannonical Pathways	*P-value*	Ratio	Upstream Regulators	*P-value*
Intereferon (IFN) Signaling	3.81E-12	0.361	IFNG	2.19E-35
Th cell differentiation	4.09E-08	0.183	IFN-alpha	3.09E-33
Type1 Diabetes Mellitus Signaling	2.24E-07	0.136	IRF7	6.87E-30
Communication between innate and adaptive immune cells	6.40E-07	0.146	IRF1	9.16E-25
Role of JAK1, JAK2 and TYK2 in IFN signalling	2.11E-06	0.292		
IPA analysis of Downregulated DEGs with the top canonical pathways and upstream regulators				
Top Cannonical Pathways	*P-value*	Ratio	Upstream Regulators	*P-value*

Transcriptional regulatory network in embryonic stem cells	1.07E-05	0.275	TGFB1	1.00E-10
Role of BRCA1 in DNA damage response	4.73E-04	0.167	B-Estradiol	1.07E-09
Calcium signaling	1.41E-03	0.118	ESR2	1.26E-06
cAMP mediated signaling	2.10E-03	0.109	TNF	1.77E-06
UVC induced MAPK signaling	2.39E-03	0.19		

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