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1 **Early transcriptome profile of goat peripheral blood mononuclear cells (PBMCs)**
2 **infected with peste des petits ruminant's vaccine virus (Sungri/96) revealed induction**
3 **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

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

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

123 **2. Materials and Methods**

124 **2.1. Animals, blood collection and screening animals for PPRV antibodies:** All
125 experimental procedures in the present study were approved by Institute Animal Ethics
126 Committee (I.A.E.C No.F.1.53/2012-13-J.D.). Blood was collected from goat kids (5 months

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

132 **2.2. Isolation of PBMCs and PPRV Infection Confirmation** Peripheral blood mononuclear
133 cells (PBMCs) from PPRV negative goats were isolated from fresh, heparinized venous blood
134 by density gradient centrifugation on Histopaque-2000 and was washed thrice with sterile
135 phosphate buffered saline (PBS), the cells were finally resuspended in RPMI-1640
136 supplemented with 10% fetal bovine serum and antibiotics (100 IU/ml penicillin, 100 μg/ml
137 streptomycin). Viability of the cells was assessed using trypan blue exclusion test and the
138 cells were found to be more than 95% viable. PBMCs were seeded at a density 1 X 10^6 viable
139 cells/ml in two 6 well plates, one serving as control and the other plate was used for virus
140 infection. Goat PBMCs isolated were infected with purified (Ultracentrifuged) Sungri/96 PPRV
141 vaccine virus at 1.0 MOI (multiplicity of infection) and incubated at 37^0C in 5% CO_2 incubator
142 for 1 h of virus adsorption. After 1 h of adsorption, the virus inoculum was removed,
143 centrifuged to collect the lymphocytes as they are in suspension, washed with fresh RPMI
144 medium and added back to the wells. Fresh RPMI medium was added to wells and incubated
145 upto 6 h post infection (p.i). The control (mock-infected) PBMCs on the other hand just
146 received RPMI medium. Finally, the infected cells along with the control (mock-infected)
147 PBMCs in duplicates were harvested at 6 h post-infection (p.i) and were processed for RNA
148 isolation. Cells from three wells were combined per replicate sample. This time point was
149 chosen to give enough time for the establishment of infection and for the completion of one
150 viral life cycle (for PPRV 6-8 h). Experiments were performed in duplicates using RNA
151 samples from two independently infected cell cultures for analysis. PPRV infection in PBMCs

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

155 **2.3. RNA preparation for microarray analysis:** Total RNA was isolated from the PPRV
156 infected and control PBMCs using Trizol reagent (Invitrogen). The quality and the integrity of
157 the RNA samples isolated were determined using the Agilent RNA 6000 Nano Kit on the
158 Agilent 2100 Bioanalyzer (Agilent Technologies) and the RNA was quantified on ND-1000
159 Spectrophotometer. Labeling of the complementary RNA (cRNA) samples was performed as
160 detailed in the agilent one-colour microarray based gene expression analysis protocol. Briefly,
161 200ng of total RNA was used for amplification and labeling step using Agilent Quick Amp
162 Labelling kit (Agilent Technologies) in presence of Cy3-CTP. Amplified cRNA samples were
163 purified and the dye incorporation rates were measured before hybridization with ND-1000
164 Spectrophotometer.

165 **2.4. Microarrays and Hybridization:** The labeled samples were hybridized according to the
166 one-color microarray based gene expression analysis (part number G4140-90040). In brief,
167 1.65 µg of Cy3-labelled fragmented cRNA was hybridized overnight (at 65^0 C for 16 h) to 4 x
168 44K Agilent *Bos taurus* microarrays. Since *Capra hircus* genome was not completely
169 annotated, *Bos taurus* microarrays were used based on the fact that the average identity
170 across exon sequences between *Bos taurus* and *Capra hircus* was 93.77% (Fontanesi et al.,
171 2010). After overnight hybridization, the arrays were washed with Triton X-102 added gene
172 expression hybridization buffer I for 1 min at room temperature followed by wash with pre-
173 warmed gene expression hybridization buffer II for 1 min at 37^0 C. The hybridized microarrays
174 were scanned using standard protocols and fluorescence signals were detected using
175 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

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

209 **2.8. STRING analysis of protein-protein interactions (PPI) of DEGs:** The protein-protein
210 interactions among upregulated and the downregulated genes (with fold change ≥ 5) in the
211 study were predicted using STRING database (http://string-db.org/), which has known and
212 predicted protein interactions. The STRING represents the functional association, and the
213 basic interaction unit here gives the specific and productive relationship between the two
214 proteins (Szklarczyk et al., 2015). The STRING new version 10.0 used to detect protein-
215 protein interactions (PPI) in this study covers more than 2031 organisms and for our study
216 we used *Bos taurus* as closely related species to *Capra hircus*. The PPI analysis was carried
217 out separately for upregulated and the downregulated genes. PPI for the upregulated genes
218 consisted of 192 nodes and 171 edges. PPI analysis for the downregulated genes consisted
219 of 392 nodes and 347 edges. Here, nodes represent the proteins and the edges represent
220 the protein-protein associations. Nodes which were not connected were removed from the
221 study.

222 **2.9. Validation of the microarray data with qRT-PCR:** To confirm the results of our
223 microarray analysis, among the identified differentially expressed genes (DEGs) some of the
224 candidate DEGs of interest (Table 1) were validated using qRT-PCR. GAPDH, which is the
225 most stable housekeeping genes for PPRV infected PBMCs observed previously (Manjunath

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

238 **3. Results**

239 **3.1. Infection confirmation and quantification of PPRV Infected cells**: PPRV infection in
240 PBMCs was confirmed by amplifying 'N' gene from the infected PBMCs at 6 h post infection
241 (p.i.). The control cells were negative for N gene (Figure 2A). qRT-PCR quantified N gene
242 expression at 6 h p.i, further confirmed PPRV infection in PBMCs (Figure 2B).

243 **3.2. Effects of early PPRV infection and Overview of differentially expressed genes**
244 **(DEGS) at 6 h p.i**

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

251 highly upregulated gene (Fold change 53.3). TREX1, the cytosolic exonuclease that plays a
252 role in inhibiting induction of type I interferons was also highly upregulated (fold change 41.9).
253 Many interferon related genes were found to be upregulated in our study namely, interferon
254 induced protein with tetricopeptides (IFITs) viz. IFIT1 (fold change 17.3), IFIT2 (fold change
255 3.4) and IFIT3 (8.5). Also, IFITM3 - Interferon inducible transmembrane proteins, an important
256 antiviral immune factor against many pathogenic viruses was found to be upregulated (fold
257 change 16.7). ISG20 (Interferon stimulated gene 20) which inhibits replication of many viruses
258 was found to be highly upregulated (fold change 23.7) in PBMCs infected with PPRV at 6 h
259 p.i. Among the interferon regulatory factors (IRFs), IRF1 (fold change 5.5) and IRF7 (fold
260 change 5.8) was found to be induced. IL-27, an antiviral cytokine was significantly upregulated
261 after PPRV infection (fold change 19.3). The innate immune signaling cascade of events
262 leading to the activation of the downstream effectors i.e interferon stimulated genes (ISGs) is
263 initiated when pattern recognition receptors (PRRs) like TLRs are engaged with viral nucleic
264 acids. In our study, TLR3, TLR7 and TLR8 were found to be upregulated after PPRV infection
265 in PBMCs. Among the downregulated genes, IFNAR1 was found to be downregulated with a
266 fold change 3.08. Among the caspases, caspase 8 (fold change 3.72) and caspase 4 (fold
267 change 3.4) were significantly upregulated.

268 **3.3. Gene Annotation of Differentially expressed genes and their pathway analysis**

269 Significantly enriched biological processes among all the differentially expressed genes (i.e
270 1926 DEGs), and among the upregulated and the downregulated genes in isolation were
271 retrieved using g:profiler. Specific biology can be understood by analyzing the functional
272 enrichment for upregulated and downregulated genes in isolation. Biological processes
273 enriched among the 1926 DEGs are shown in Figure 3A, Supplementary table 2, Sheet1. The
274 top significant biological process enriched among all the DEGs (1926) were immune system
275 process (Genes: 233; *P*-value 2.89E-10), response to stress (Genes: 305; *P*-value 1.68E-05),

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

288 **3.4. Significant Pathway analysis of PPRV Infected PBMCs (6 h p.i.)**

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

301 4B, Table 3). IFN-gamma and transcription factors (IRF7 and IRF1) were found to be the
302 significant upstream regulators controlling significant number of upregulated genes. The
303 important network associated with upregulated genes was antimicrobial response,
304 inflammatory response and infectious diseases (score 27). IPA analysis of the downregulated
305 genes did not show any relevant important canonical pathways related to the infection (Figure
306 4C, Table 3). The network of the upregulated genes consisting of important innate immune
307 molecules was generated from IPA and shown in figure 4D.

308 **3.5. Interaction Network Analysis (INA) of upregulated and downregulated genes**

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

323 **3.6. Validation of microarray data by qRT-PCR**

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

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

331 **3.7. Immune signaling pathway of Sungri/96 vaccine virus updated**

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

337 **4. Discussion:**

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

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

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

359 Although different TLRs are activated when engaged to different PAMPs, they cross
360 path to transcriptionally activate downstream interferon regulatory factors (like IRF3, IRF7)
361 and NFKB, which when phosphorylated translocate into the nucleus activating type I
362 interferons. Interestingly, in our study type I interferons (IFN-α and IFN-β) were not
363 differentially expressed, although, interferon induced genes were found to be induced. This
364 supports the recent observation that the paramyxovirus (including PPRV) non-structural
365 proteins (C and V proteins) play a role in inhibiting type I IFN production (Bernardo et al.,
366 2017; Andrejeva et al., 2004). In the present study, at 6 h p.i. we hypothesized that interferon
367 induced genes must have been activated in an interferon (IFN) independent manner as
368 observed at 48 and 120 h p.i. (Manjunath et al., 2017). TREX1, which was highly upregulated
369 in the study is an exoribonuclease, besides being an exonuclease (Yuan et al., 2015). TREX1
370 may act as negative regulator of induction of type I interferons in PPRV infected PBMCs.
371 Similar effect of TREX1 was observed recently in HIV infection (Wheeler et al., 2016). TREX1
372 could be one of the breaks, having inhibitory effect on type I IFN signaling pathway, allowing
373 virus to replicate in the initial stages of the infection. Further, functional studies need to be
374 done to study the effect of TREX1 on PPRV replication. IRFs and interferon induced genes
375 that were upregulated in this study were IRF7, IRF1, IFIT2, IFIT1, IFIT3, IFITM3, OAS2 and

376 OAS1Z. ISG20, an interferon induced gene with exonuclease activity specific against ssRNA
377 viruses (Espert et al., 2003) was found to be upregulated. ISG 20 was linked to other important
378 innate immune genes in the interaction network asserting its crucial role in immune defense.
379 Cells exhibiting increased expression of ISG20 are resistant to RNA virus infection and thus,
380 play an important role in host antiviral innate immune defense (Espert et al., 2005 and Zhou
381 et al., 2011). The increased expression of ISG20 highlights its specific activity against PPRV
382 (ssRNA virus) at the early stages of the infection. Recently, it was shown ISG20 inhibits
383 replication of influenza A virus by interacting with nucleoprotein (Qu et al., 2016) and inhibits
384 Hepatitis B virus replication by binding directly to epsilon stem loop structure of viral RNA (Liu
385 et al., 2017). IRF3 and IRF7 are the main regulators of immune responses to viruses (Lazear
386 et al., 2013). Upregulation of IRF7 in our study indicated host cell responses to trigger
387 downstream signaling cascades against the invading virus. IRF7 is known to induce the
388 interferon stimulated genes (ISGs) by stimulating type I interferons (Pulit-Penaloza et al.,
389 2012). However, IRF7 upregulation, induction of interferon induced genes and inhibition of
390 type I interferon response in PPRV infected PBMCs highlighted induction of ISGs in an IFN
391 independent manner at the early time point of infection. This may be due to the high degree
392 of homology between the ISRE and IRF binding element (IRF-E) consensus sequences,
393 IRF7/IRF3 may bind directly to induce ISGs (Morin et al., 2002; Schmid et al., 2010;
394 Manjunath et al., 2017). Thus, the data suggests activation of interferon induced genes in
395 PPRV infected PBMCs in the absence of type I interferons induction. Similar IFR7 activation
396 of interferon induced restriction factor BST2 was observed in absence of type 1 interferon
397 signaling in Parainfluenza virus V5 protein infection (Bego et al., 2012).
398 Viral infections in host cells induce pro-inflammatory cytokines and chemokines
399 response (Mogensen and Paludan, 2001). Increased expression of interleukins viz. IL-27, IL-
400 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

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

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

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

635 **Figure 7: Updated immune signaling pathway in goat PBMCs infected with PPRV:** PPR
636 virus being lymphotropic infects PBMCs through SLAM/CD46 receptor. The virus enters the
637 cells and gets uncoated, releasing viral nucleic acids in the cytoplasm, which then undergoes
638 replication. Also, the virus enters into endosomes where the viral nucleic acids are released.
639 PPRV ssRNA in the cytoplasm undergoes replication to establish infection in the cells,
640 whereas the excess ssRNA not replicating are chewed by exoribonuclease TREX1, which
641 could otherwise induce a strong type I IFN response in infected cells through RIG1. TREX1
642 significantly upregulated in the present study may probably inhibit type I IFN response in
643 PBMCs. The virus entering the endosomes releases the ssRNA, gets engaged to TLRs (TLR
644 – 3, 7 & 8) and activates interferon regulatory factors (IRFs- 3,7 & 9) with the help of adaptor(s)
645 - TRIM14/21. Activated and phosphorylated IRFs translocate into the nucleus and bind to
646 interferon stimulated responsive elements (ISREs) activating interferon stimulated genes
647 (ISGs). ISGs along with other immune molecule exert a strong antiviral response in PPRV

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

651 **Legends to Supplementary Files:**

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

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

662 **Supplementary file 3:** List of 1926 differentially expressed genes identified in this study with
663 their fold changes. The list also compares the log fold changes of 1926 DEGs presence in
664 our pervious study i.e 48 h p.i. and 120 h p.i. The file shows the candidate genes identified in
665 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-Y	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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