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Porcine reproductive and respiratory syndrome virus induces concurrent elevation of High Mobility Group Box-1 protein and pro-inflammatory cytokines in experimentally infected piglets

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ABSTRACT

Porcine Reproductive and Respiratory Syndrome (PRRS), caused by PRRS virus (PRRSV), is one of the most important devastating diseases of pigs, characterized by reproductive failure in sows, and respiratory disease with heavy mortality in piglets. PRRS virus has been reported to elevate the levels of proinflammatory cytokines in the serum of infected pigs. High Mobility Group Box-1 (HMGB-1) protein is a cellular biomolecule belonging to the Danger Associated Molecular Patterns (DAMP) family, which stimulates immune cells to release pro-inflammatory cytokines upon release out of cells. The role of HMGB-1 in the pathogenesis of PRRSV remains largely unknown. In the present study, HMGB-1 levels in serum samples collected from six-week-old piglets infected intra-nasally with $2 \times 10^{5.75}$ $TCID_{50}$/mL of Indian PRRSV (Ind-297221/2013) was estimated by ELISA up to 21 days post infection (dpi). Pro-inflammatory cytokine mRNA (IL-1β, IL-6 and TNF- α) expression in PBL was estimated by SYBR green based real time PCR. Mean HMGB-1 concentration in serum was found to be significantly elevated in PRRSV infected piglets on 6 dpi as compared to uninfected control piglets. At mRNA level, significant increase in expression of HMGB-1 was observed from 4 to 5 dpi and from 11 to 13 dpi. IL-1β and IL-6 mRNA were significantly upregulated between 4 and 6 dpi. Significant increase in TNF-α gene expression was seen only on 7 and 9 dpi. Higher levels of pro-inflammatory cytokines and HMGB-1 could be correlated with fever which was observed within 7 dpi in all the infected piglets and additionally around 13 dpi in the animal that died on 17 dpi. Thus, elevated HMGB-1 level in PRRSV infected piglets could be correlated with concurrent increase in pro-inflammatory cytokine (IL-6) mRNA. *In-vitro* studies were conducted in PRRSV infected Porcine Pulmonary Alveolar Macrophages (PAM) to ascertain HMGB-1 role in PRRS pathogenesis. The results of both *in-vivo* and *in-vitro* studies showed that HMGB-1 plays an important role in mediating the pro-inflammatory cytokine responses in PRRS pathogenesis.

1. Introduction

Porcine Reproductive and Respiratory Syndrome (PRRS) is considered as one of the most important devastating diseases of pigs, characterized by reproductive failure in sows, and respiratory disease with heavy mortality in piglets [34,61]. It is caused by PRRS virus (PRRSV), a small enveloped virus with single stranded non-segmented RNA genome of approximately 15.1–15.5 kb. The virus belongs to family *Arteriviridae*, order *Nidovirale* and classified into two genotypes as genotype 1 (European) and Genotype 2 (North American) based on genetic relatedness [49]. Since 2006, outbreaks of atypical PRRS caused by highly pathogenic PRRSV (HPPRRSV) resulted in heavy neonatal mortality up to 100% in major pig producing area of china and other ASEAN countries with expanded tissue tropism and viral antigen distribution was reported [72,45].

PRRSV has been reported to modulate induction of many pro-inflammatory, anti-viral and T cell immunomodulatory cytokines in infected macrophages and dendritic cells [3,14]. Several studies consistently revealed significantly elevated levels of IL-1, IL-6 and TNF-α in the serum of HPPRRSV infected pigs [31–33,46,84]. In addition, it was also reported that the levels of IL-1β, TNF-α, IL-6 and IL-8 were elevated in the local lung tissues and in the infected-microglia [19,31,32]. There appeared to be a correlation between the virulence of PRRSV strain, the severity of clinical signs and the expression of pro-inflammatory cytokines. In pigs infected with HP-PRRSV, marked inappetence and severe respiratory signs were related to severe

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interstitial pneumonia and high levels of expression of IL-1α in the lungs in comparison with other classical PRRSV strains [5,36]. Higher expression of pro-inflammatory cytokines was observed in septal macrophages of PRRSV infected pigs [27]. Apart from pro-inflammatory cytokines, PRRSV was reported to also stimulate pro-inflammatory chemokine, Regulated upon Activation Normal T-cell Expressed and Secreted (RANTES) in TLR-MyD88-dependent manner [78]. Thus the outcome of infection depends on the levels of proinflammatory, anti-inflammatory and antiviral cytokines.

High Mobility Group Box-1 protein (HMGB-1) belongs to Danger Associated Molecular Patterns (DAMP) family of so called "alarmins" which are the molecules released from dying cells that in turn, amplify inflammation and exacerbate tissue damage [41,58]. HMGB-1 was known to be actively secreted in the extracellular medium by a variety of immune and non-immune cells such as macrophages, monocytes [16,26,69], neutrophils [38], dendritic cells and NK cells [28,62] in response to various stimuli. HMGB-1 was also identified as an important extracellular mediator in inflammation processes [77]. HMGB-1 was reported to stimulate macrophages, monocytes and neutrophils to release proinflammatory cytokines (e.g., TNF, IL-1, IL-6, IL-8, and MIP-1) in a p38 and JNK MAPK dependent mechanism [7,8,43,56]. There is a report which suggested that HMGB-1could be released passively by virus-mediated cytolysis of alveolar endothelial cells or macrophages and speculated to play a role in mediating an injurious pulmonary inflammatory response including neutrophil infiltration, derangement of epithelial barrier, lung oedema, and lung injury [18].

The potential role of HMGB-1 in viral pathogenesis has been shown through increased HMGB1 levels in patients infected with Dengue virus and Hepatitis virus infections [4,48]. In a study using meta-analysis approach to analyze the pig immune response to general stimulus and specific response to PRRSV infection, it was reported that HMGB-1 was the most significant factor potentially involved in the pig specific response to PRRSV infection [9]. In an *in vitro* study, it was found that PRRSV infection triggered HMGB-1 translocation followed by NF-κB activation and subsequent expression of inflammatory cytokines, but it had no effect on PRRSV replication in MARC-145 cells and porcine pulmonary alveolar macrophages (PAM) [22]. But, the *in vivo* involvement of HMGB-1 during PRRSV infection in piglets has not yet been established.

In the present study, we evaluated the serum HMGB-1 levels, expression of pro-inflammatory cytokines in piglets and modulation in proinflammatory cytokine responses were ascertained in *in-vitro* studies by altering the levels of HMGB-1 in PRRSV infected porcine pulmonary alveolar macrophages (PAM).

2. Materials and methods

2.1. Ethics and bio-safety statement

The animal experiments were carried out at the Biosafety level-3 containment facility of National Institute of High Security Animal Diseases, Bhopal, India, after due approvals from Institute Animal Ethical Committee (Approval no. 83/IAEC/HSADL-14), Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forests and Climate change, Government of India (Approval no. 25/51/2015-CPCSEA) and Institute Bio-safety Committee (Approval no. HSADL/IBSC/2013-14/121). Piglets were acclimatized for 5 days in containment facility before starting the experiment. Infected tissues and other bio wastes were safely disposed as per biosafety norms. Prior to experimental infection, all piglets were reconfirmed to be free of PRRS virus and the antibodies by methods described previously [53].

2.2. PRRS virus

PRRS virus (Ind-297221/2013; GenBank accession no. KM668213) isolated from porcine serum samples collected during the first outbreak of PRRS in India was used in the study. The virus was isolated in porcine pulmonary alveolar macrophages as described in *OIE* terrestrial manual, 2015 from suspected sample and supernatant from third passage was tested by RT-PCR. PRRSV antigen in infected cells were stained by PRRSV specific antibodies by Immuno Peroxidase Monolayer Assay (IPMA). PRRSV positive supernatants were passed through MARC-145 cell line for more passages. At passage 7, MARC 145 cells were screened using IPMA and the supernatant was tested negative for swine influenza virus (H1N1), Transmissible gastro enteritis virus, porcine Circo virus -2, Classical swine fever virus by RT-PCR.

2.3. Virus titration

The titre of Ind-297221 virus was assessed at passage 7 when CPE was observed in MARC-145 cells, which had been seeded into 96 well plate 24 h before infection. Virus containing cell culture supernatants were serially diluted ten-fold in Eagle's Minimal Essential Medium (EMEM) and added 50 µl to wells. Titration assays were conducted in five replicates for each dilution. Cells were incubated for 5 days at 37 °C in 5% CO2 incubator. The 50% tissue culture infective dose was calculated [59].

2.4. Inoculation of animals and experimental design

Piglets were screened for diseases viz. PRRS, swine influenza, classical swine fever, transmissible gastroenteritis by RT-PCR and for PRRSV antibodies by IPMA and procured from Livestock Farm, Veterinary College, Jabalpur, Madhya Pradesh. Twelve healthy (aforesaid diseases free) Large White Yorkshire (LWY) x local non-descript cross piglets of age 4–5 weeks were used in the present study. All the animals were dewormed using albendazole (7.5 mg/kg body weight) and given a course of antibiotic injection (Tetracycline LA, Pfizer, India) during quarantine period (seven days). Pre-infection baseline data for haematological, immunological and clinical parameters were collected.

Nine piglets of either sex were inoculated intranasally with $4 \times 10^{5.75}$ $TCID_{50}$ PRRSV (Ind- 297221, Genbank accession no. KM668213). Three piglets were kept as uninfected controls that received equal volume of sterile uninfected cell culture supernatant. Blood samples were collected in vacutainers, with and without EDTA (BD Biosciences, USA), by anterior venacava venepuncture. Serum separated was stored at −80 °C until further use. Total leucocyte count and blood samples in EDTA were processed immediately for total leucocyte count as per standard procedure [12]. Piglets were necropsied either on death or after euthanasia by barbiturate overdosing on 7, 14 or 21 dpi (one uninfected control and three PRRSV infected piglets per interval). Lung tissues were collected in RNAlater (Ambion, USA) and stored at −80 °C.

2.5. In-vitro studies to assess the role HMGB-1 in altering levels of proinflammatory cytokines

Porcine pulmonary alveolar macrophages (PAM) were seeded in 24 well plates and grown in the presence of EMEM containing 10% FBS at 37 °C for 24 h. Cells were divided into six groups as healthy cell control, PRRSV infected, HMGB-1 supplementation, PRRSV + HMGB-1 supplementation, HMGB-1 depletion, PRRSV + HMGB-1 depletion. In groups that required PRRSV infection, cells were infected with PRRSV Ind-297221 (Genbank accesion no. KM668213) at a multiplicity of infection (MOI) of 0.1. After adsorption of PRRSV for 1 h at 37 °C, the inoculum was replaced with medium containing respective treatment, wherever necessary. In HMGB-1 supplemented group of cells, recombinant HMGB-1 (R&D systems, USA; Cat. No. CF1690-HM025) was added at a concentration of 0.2 µg/mL. For HMGB-1 depletion, ethyl pyruvate was added to 24 h PAM cultures at a final concentration of 5 mM. All groups

Table 1
List of oligonucleotide primers used.

S. no	Primer name	Sequence (5′→3′)	Expected product size	Reference
1.	IL 1β F	AAAGGGGACTTGAAGAGAG	286	[20]
	IL 1β R	CTGCTTGAGAGGTGCTGATGT		
2.	IL6 F	CTGGCAGAAAACAACCTGAACC	94	[65]
	Il6 R	TGATTCTCATCAAGCAGGTCTCC		
3.	IL8 F	GGGTGGAAAGGTGTGGAATG	75	[73]
	IL8 R	GGCTGCAGTTCTGGCAAGAG		
4.	TNF α F	CACCACGCTCTTCTGCCTAC	132	[10]
	TNF α R	ACGGGCTTATCTGAGGTTTGAG		
6.	IFN α F	TGGTGCATGAGATGCTCCA	55	[23]
	IFN α R	GCCGAGCCCTCTGTGCT		
7.	IFNβ F	TGCAACCACCACAATTCC	80	[10]
	IFNβ R	CTGAGAATGCCGAAGATCTG		
8.	IL4 F	GCCGGGCCTCGACTGT	68	[21]
	IL4 R	TCCGCTCAGGAGGCTCTTC		
9.	IL2 F	GAGCCATTGCTGCTGGATTT	111	[21]
	IL2 R	GTAGCCTGCTTGGGCATGTAA		
10.	HMGB1 F	TTGCCGGGAGGAGCATAAGAAGAA	136	[79]
	HMGB1 R	GTCCGCCTTTGCCATGTCTTCAAA		
11.	AMCF F	GCTCGTGTCAACATGACTTCCA	77	[29]
	AMCF R	GCCTCACAGAGAGCTGCAGAA		
12.	18s rRNA F	CCCCAACTTCTTAGAGGGACAA	70	[84]
	18s rRNA R	GGGCATCACAGACCTGTTATTG		
13.	IFN γ F	CATGAACACCATCAAGGAACAAAT	85	[83]
	IFN γ R	TTTGAATCAGGTTTTTGAAAGCC		

were maintained in triplicates and supplemented with polymyxin B at 140 IU/mL for every 12 h to inhibit the residual LPS activity if present in the media. The cells were harvested, processed for RNA extraction at 6 h, 24 h and 48 h and supernatant was centrifuged at 12,000 RPM for 10 min at 4 °C and stored at −80 °C until use.

2.6. Estimation of HMGB-1 in PAM culture supernatant and serum

The porcine HMGB-1 protein in PAM culture supernatant and serum of experimental piglets was estimated by ELISA kit for porcine HMGB-1 (Cat. No. SEA399Po, Cloud clone Corp., USCN life sciences, USA) as per manufacturer's instructions. Briefly, a standard curve was prepared by using standards provided in the kit, viz., 1,000 pg/mL, 500 pg/mL, 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL, and 0 pg/mL. Undiluted PAM culture supernatant and serum samples diluted (1:200) in PBS were added to each well in duplicates and incubated for 2 h at 37 °C. Standards were included in each plate. After incubation, the well contents were discarded and added with 100 µl of reagent A to each well and incubated for 1 h at 37 °C. The plate was washed three times using wash buffer. One hundred µL of Reagent B was added to each well and incubated for 30 min at 37 °C. Then, 90 µl of Substrate solution was added to each well after washing and incubated for 15–25 min at 37 °C in dark. Fifty µL of stop solution was added to each well and the absorbance was measured at 450 nm using ELISA microplate reader (Berthold, Germany). The porcine HMGB-1 protein in the sample was extrapolated based on mean A_{450} using the standard curve prepared. The HMGB-1 protein in the supernatant of PAM culture of supplemented group were analyzed after reducing the amount of recombinant protein added in the supernatant.

2.7. Total RNA extraction from tissues, porcine pulmonary alveolar macrophages and peripheral blood leukocytes

Total RNA was extracted from tissue samples frozen at −80 °C in RNAlater (Ambion, USA) using TriReagent solution (Ambion, USA) following manufacturers recommendations. Briefly, about 50 mg of tissue was placed in a DNase and RNAse free 1.5 ml microcentrifuge tube containing 1 ml of TriReagent solution and homogenized using a bead beater (Ez-lyzer, Genetix, India) for one minute at 20 and 30 Hz frequency for soft and hard tissues, respectively. For peripheral blood leukocytes (PBL) and harvested pulmonary alveolar macrophages (PAM) samples, total RNA was extracted from PBL and PAM samples frozen at −80 °C in TriReagent solution (Ambion) following manufacturer's recommendations. Homogenization was done by repeated pipetting directly after thawing of samples. The homogenate was incubated at room temperature for 5 min and 200 µl of chloroform was added and vortexed. The mixture was incubated at room temperature for 15 min and centrifuged at 12000*g* for 15 min at 4 °C. RNA from aqueous phase was recovered and precipitated by adding 500 µl of isopropanol. After a final ethanol wash, the RNA pellet was air dried, resuspended in 50 µl of nuclease free water (Promega, USA). Total RNA was treated with RNase-free DNase (Thermo scientific, USA) for 30 min at 37 °C. DNase was inactivated by 5 mM EDTA at 65 °C for 10 min. The quality and quantity of extracted RNA was assessed by Nano spectrophotometer (Nano Spec, VWR, USA). The resultant product from tissues/PBL was used directly for virus quantification by real time RT-PCR. For quantification of cytokine expression in PBL and lungs, total RNA extracted was used for first strand cDNA synthesis.

2.8. First strand complementary DNA (cDNA) synthesis

Reverse transcription of total RNA was carried out using Revert Aid H-minus first strand cDNA synthesis kit (Thermo Scientific, USA) following manufacturer's protocol using random hexamer primers and MMLV-RT. The cDNA was stored at −20 °C until further use.

Complementary DNA synthesized from total RNA extracted under identical conditions was used for relative quantification of cytokine mRNA by SYBR green based real time PCR. The results were expressed as mean fold change after normalization using 18 s rRNA expression. For sequential data obtained for cytokine mRNA expression in PBL samples, where comparison with 0 day value of the same animal was possible, values were compared with the 0 day values.

2.9. Amplification of porcine cytokine genes

Prior to carrying out SYBR green based real time PCR for cytokine mRNA estimation, conventional PCR was optimized for each cytokine studied (IL-1β, IL-6, TNF-α, HMGB-1, IL-8, IL-2, IL-4, Interferons α and β). Amplification of porcine cytokines was done by conventional PCR using Platinum™ PCR super mix (Invitrogen, USA). Each 25 µl reaction

consisted of 1x reaction mix, 12 pmol each of forward and reverse primers, 1.5 µl of cDNA template. The list of primers used in this study is given in Table 1. Thermal cycling condition optimized for a single annealing temperature is as follows: initial denaturation at 95 °C for 2 min followed by 40 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension step of 72 °C for 10 min. PCR products were resolved by electrophoresis on 1.5% agarose gels to confirm the expected size. The bands of expected size were gel purified using QiaQuick gel extraction kit (Qiagen, Germany) for further confirmation of respective cytokine genes by sequencing.

2.10. Cloning and sequencing of cytokine genes

Purified PCR products were cloned into a TA cloning vector pTZ57R/T vector (Thermo Scientific, USA) using InsT/Aclone ligation kit (Fermentas, USA) as per manufacturer's protocol. Inserts were confirmed by restriction enzyme digestion using *ECoR*I and *Pst*I (NEB, USA) enzymes and by nucleotide sequencing using Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) and ABI 3130 automatic DNA sequencer (ABI, USA). The online tool BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to confirm the cytokine genes sequenced.

2.11. Relative quantification of cytokine mRNA

SYBR Green based real time PCR was done using GoTaq qPCR master mix (Promega, USA) and cytokine specific primers in a real time PCR machine (Light Cycler 480 II, Roche, Germany). Each reaction mixture consisted of 1X reaction master mix, 10 pmol each of cytokine specific forward and reverse primers, 1.5 µl of cDNA and nuclease free water to make up the volume to 15 µl. All reactions were carried out in duplicates and no template control (NTC) was included for each primer set. Cycling conditions involved initial denaturation at 95 °C for 10 min, followed by 40 cycles of amplification (denaturation at 95 °C for 15 s, annealing and extension at 55 °C for 45 s). At the end of amplification cycles, melt curve analysis was carried out by gradual increment of temperature from 50 °C to 95 °C and melting temperature (Tm) of all amplicons were optically read with an increment of 0.3 °C. Ability to generate a specific amplification curve along with a single specific Tm product was taken as the criteria to ascertain the specificity of cytokine mRNA amplification. Threshold cycle (C_T) values were normalized using 18 s rRNA as the housekeeping gene. Relative quantification of cytokine mRNA expression was done by the comparative threshold method $2^{-\Delta\Delta Ct}$ as described previously [57] by using Microsoft Excel 2010 software.

2.12. Quantification of virus by qRT-PCR

PRRSV copy numbers in PAM cultures collected at various intervals was estimated by TaqMan based one step qRT-PCR using SuperScript III Platinum One-Step qRT-PCR system (Invitrogen, USA) in Light Cycler® 480 Real Time PCR System II (Roche, Germany). Each reaction mix consisted of 1x reaction mix, 0.25 µl of SuperScript III RT/Platinum Taq Mix, 1.0 µl of template RNA, 10 µM each of forward and reverse primers [81], 10 µM of in house designed Taqman probe (5′-FAM-CGCGAAGA ACTGTGATAACAACGCTGA-TAMRA-3′), 0.25 µl of ROX dye and nuclease free water were to make up the volume to 12.5 µl. No template control (NTC), no probe control (NPC) and positive control were included in every run along with the test samples. *In vitro* transcribed (IVT) RNA prepared from linearized plasmid containing PRRSV (Ind-297221) genome using T7 RNA polymerase using mMessage mMachine kit (Life Technologies, USA Cat. No. AM1344) was used as the positive control. Each reaction was carried out in duplicate. The cycling condition is as follows: Reverse transcription for 15 min at 50 °C, RT inactivation for 2 min at 95 °C, followed by 40 cycles of amplification (denaturation at 95 °C for 15 s and annealing/extension at 58 °C for 30 s). Fluorescence was acquired at the end of each annealing/extension step. Viral copy number was calculated from a standard curve prepared using ten-fold serial dilutions of known quantity of IVT RNA, plotting CT value versus RNA copy numbers.

2.13. Statistical analysis

For sequential data obtained for cytokine mRNA expression in PBL samples, where comparison with 0 day value of the same animal was possible, values were compared with the 0 day values. For cytokine mRNA expression in lung tissue samples, values of infected piglets in each time interval were compared with that of the control animals. Changes were expressed either mean or mean per cent change or as mean fold change and were plotted against the time interval post infection. The data obtained from control and treatment groups at different intervals were analyzed by one way ANOVA and multiple comparison was done using Tukey's test for statistical significance using software (SPSS 16.0, USA). The Pearson correlation coefficient test was used to evaluate associations between two quantitative variables. *p*-value (< 0.05) was considered statistically significant [66].

3. Results

The aim of the study was to understand the role of HMGB-1, proinflammatory cytokines in PRRS pathogenesis in piglets. We studied expression of various cytokines and HMGB-1 in peripheral blood leucocytes (PBL), serum HMGB-1 level in PRRS virus infected piglets and *in vitro* studies were carried by altering the levels of HMGB-1 in PRRSV infected pulmonary alveolar macrophages (most susceptible primary culture) to confirm its role in modulation of pro-inflammatory cytokine response.

3.1. Relative quantification of HMGB-1 and cytokine mRNA in PBL

A sharp and significant ($p = 0.008$) increase in expression of HMGB-1 mRNA was observed on 1 day post infection (dpi) (up to 77.57 ± 35.24 fold). A significant ($p = 0.031$) upregulation in HMGB-1 mRNA expression was observed on 4, 5 dpi and on 11, 13 dpi, out of which the maximum increase was seen on 13 dpi (52.9 ± 30.5 fold) (Fig. 1). The expression levels were close to the pre-infection level on 21 dpi.

Amongst proinflammatory cytokines, there was about 70 fold increase in the IL-1β mRNA expression on 2 dpi itself, significant increase

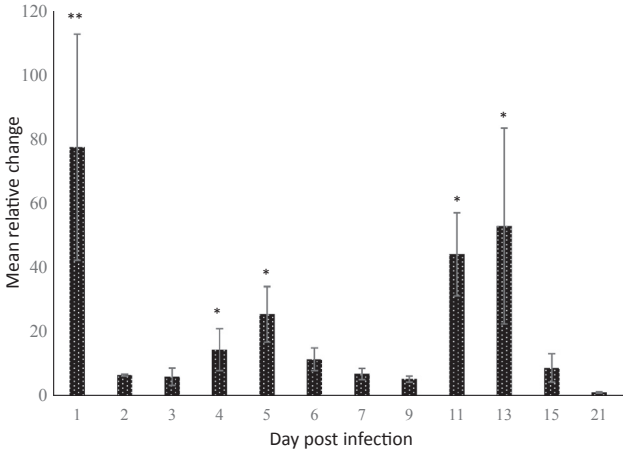

Fig. 1. Relative expression of HMGB-1 mRNA in PBL of PRRSV infected piglets (n = 9). Data are means ± SD, statistical significance ($^{*}p \leq 0.05$, $^{**}0.01$) versus pre-infection values. No significant difference was observed between the intervals with common symbol.

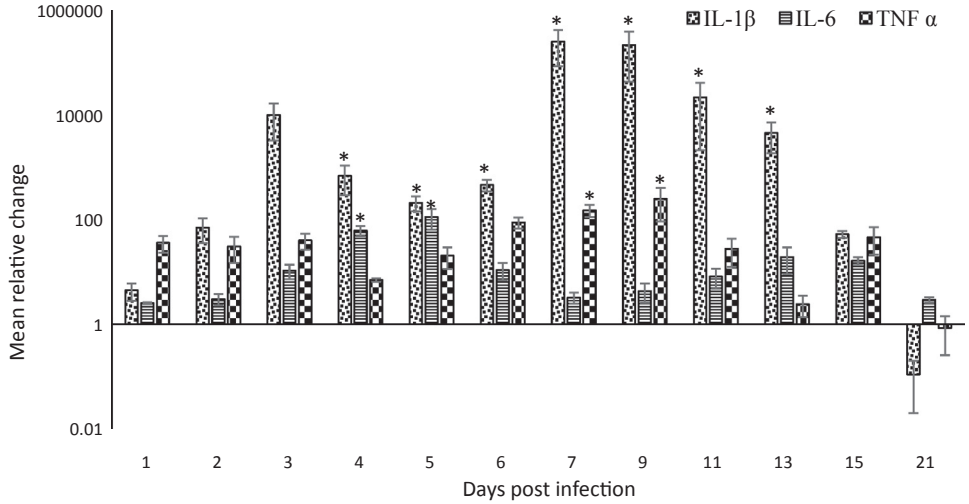

Fig. 2. Relative expression of pro-inflammatory cytokines (Il-1β, IL-6 and TNF-α) mRNA in PBL of PRRSV infected piglets (n = 9). Data are means ± SD, statistical significance ($^{*}p \leq 0.05$) versus pre-infection values. No significant difference was observed between the intervals with common symbol.

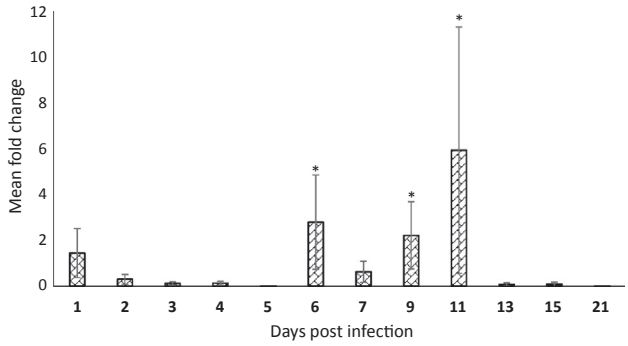

Fig. 3. Relative mRNA expression of IL-8 in PBL of PRRSV infected piglets (n = 9). Data are means ± SD, statistical significance ($^{*}p \leq 0.05$) versus pre-infection values. No significant difference was observed between the intervals with common symbol.

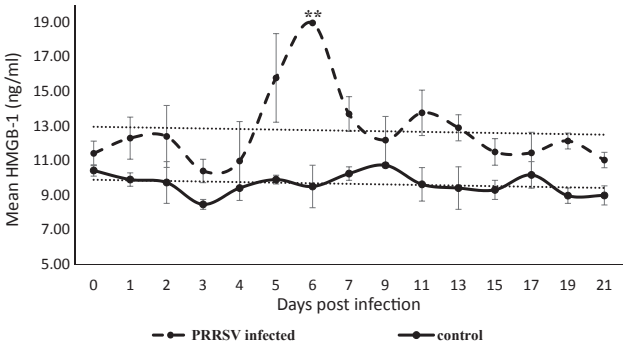

Fig. 4. Mean serum HMGB-1 levels in PRRSV infected and control piglets (n = 9). Data are means ± SD, statistical significance ($^{**}p \leq 0.01$) versus control subjects.

($p = 0.033$) in mean fold expression started from 3 dpi and remained significantly elevated up to 13 dpi (Fig. 2). Values for relative expression of IL-6 gene in PBL ranged from 2 to 111 fold, with statistically significant ($p = 0.041$) upregulation in the mRNA levels on 4 and 5 dpi, as compared to other dpi and pre-infection level. The mean fold change peaked on 5 dpi (111.66 ± 47.44). Although relative expression of TNF-α mRNA was slightly higher than pre-infection level from 1 to 4 dpi (6 to 88 fold mean increase), the significant increase ($p = 0.039$) in TNF-α gene expression was seen on 7 and 9 dpi (up to 250 fold mean increase). There was a sharp increase in IL-8 mRNA on 1 dpi following which the levels declined up to 5 dpi. There was a significant ($p = 0.028$) increase in expression of IL-8 mRNA in PRRSV infected piglets on 6, 9 and 11 dpi, with zenith on 11 dpi (Fig. 3).

3.2. Serum HMGB-1 protein

Serum HMGB-1 protein level was estimated for control and infected animals using a commercial ELISA kit. Mean HMGB-1 level was significantly ($p = 0.008$) higher in PRRSV infected piglets on 6 dpi as compared to uninfected control piglets. The level was highest in piglet no.10 on 5 dpi as 22.6 ng/ml. HMGB-1 level was significantly elevated from 5 to 13 dpi in piglet no 13 which died on 17 dpi. Mean HMGB-1 level ranged between 10 and 19 ng/mL in infected animals whereas in control animals it varied between 9.5 and 10.5 ng/ml during the period of study (Fig. 4).

3.3. Correlation between levels of HMGB-1 and proinflammatory cytokines during PRRSV infection

A moderate positive correlation between the serum HMGB-1 level and expression of proinflammatory cytokines was observed as IL-1β (R = 0.23), IL8 (R = 0.41), TNFα (R = 0.25). A significant positive correlation (R = 0.55) was seen between level of serum HMGB-1 and IL-6 expression. A negative correlation (R = −0.34) was observed between serum HMGB-1 level and WBC count (data not shown) of PRRSV infected piglets.

3.4. Relative quantification of cytokines and HMGB-1 mRNA in lungs of experimental animals

Expression pattern of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α), chemokines like (IL8, alveolar macrophage chemotactic factor (AMCF)), interferons viz., interferon β and γ, IL-2, IL-4 and HMGB-1 in lungs of PRRSV infected piglets was studied.

Mild upregulation of HMGB-1 mRNA expression was observed (maximum of up to 5 fold) on 7 dpi and it was found to be significantly downregulated on 21 dpi; but in one piglet (no. 9) it was upregulated up to 3 fold as compared to the control piglets on 21 dpi. Similarly, all the proinflammatory cytokines mRNA expression was upregulated significantly on 7 dpi. Relative expression of TNF α mRNA was upregulated significantly ($p = 0.024$) (2740 mean fold) followed by IL-1β, which reached up to 150 fold in a piglet (no. 9) and IL-6. Expression of IL-6 was highest in piglet no. 13 which died on 17 dpi but its expression

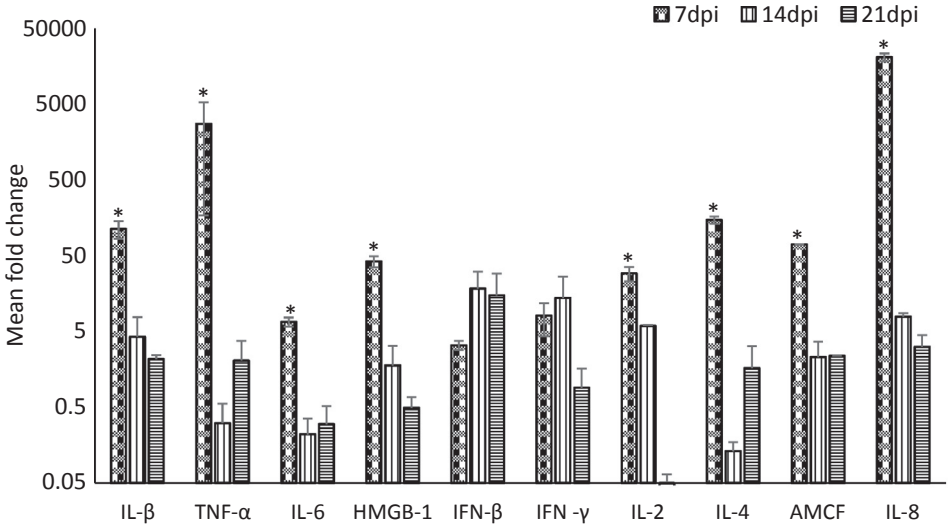

Fig. 5. Relative mRNA expression of HMGB-1 and various cytokines in porcine lungs during the course of experimental PRRSV infection (n = 9), control piglets (n = 3). Data are means ± SD, statistical significance ($^{*}p \leq 0.05$) versus other intervals. No significant difference was observed between the intervals with common symbol.

was downregulated in other animals euthanized of 21 dpi interval, when compared to control piglets. mRNA expression of chemokines like AMCF and IL-8 mRNA was increased significantly ($p = 0.031$) on 7 dpi (70 and 20,850 mean fold, respectively) and their expression was reduced in later intervals.

Among interferons, the upregulation of IFN β and γ mRNA expression on 7 dpi was only mild and the levels were found to be higher on 14 dpi (18 and 13 mean fold increase), as compared to control piglets. Fold change in IFN β and γ increased up to 30 and 26 fold in piglet no. 13 and 8, respectively on 14 dpi. Significant ($p = 0.048$) upregulation of IL-2 gene expression was observed on 7 dpi, while the levels were found to be reduced or downregulated on 14 and 21 dpi. Relative expression of IL-2 was upregulated up to 35 fold in piglet no. 9 on 7 dpi. The relative expression of IL-4 mRNA was maximum on 7 dpi (164 fold increase), after which they remained either slightly downregulated or close to control animal values. The mean fold changes in relative expression of target genes expressed in lungs of PRRSV infected piglets is given in Fig. 5.

3.5. Relative quantification of cytokines in PRRSV infected porcine pulmonary alveolar macrophages

Expression of HMGB-1 mRNA was significantly ($p = 0.008$) higher at 6 h interval in HMGB1 + PRRSV (H + V) group as compared to other groups (Fig. 6).

Expression of IL-1β mRNA (Fig. 7) was significantly ($p < 0.05$) higher in HMGB-1 only supplemented group up to 12.6 and 148 mean fold change respectively at 6 and 24 h post infection (hpi) respectively. A significant ($p < 0.01$) increase in expression of IL-1 β was PRRSV infected PAM supplemented with HMGB-1 at 24 and 48hpi as 204 and 245 mean fold change in comparison with other groups.

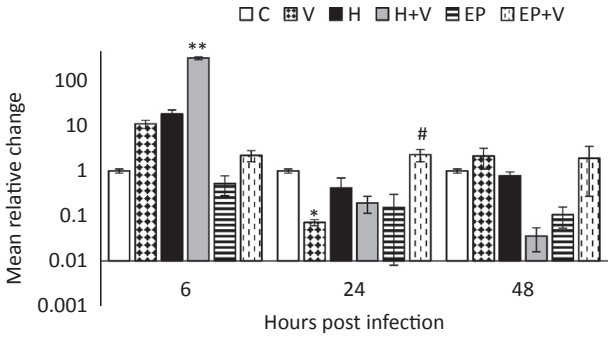

Fig. 6. Relative mRNA expression of HMGB-1 in PRRSV infected porcine pulmonary alveolar macrophages with various treatments and their respective controls. Data are means ± SD, statistical significance ($^{*}p \leq 0.05$, $^{**}0.01$) versus other groups. No significant difference was observed in the groups of same interval with common symbol.

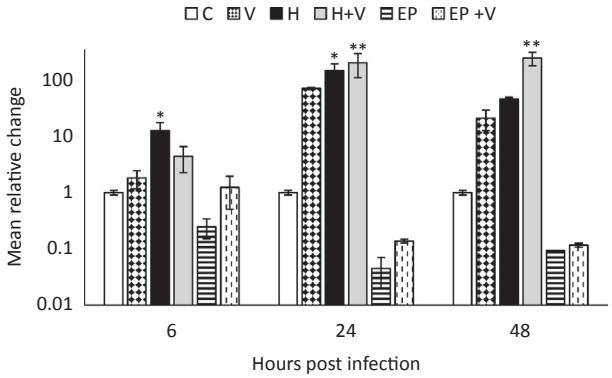

Fig. 7. Relative mRNA expression of IL-1β in PRRSV infected porcine pulmonary alveolar macrophages with various treatments and their respective controls. Data are means ± SD, statistical significance ($^{*}p \leq 0.05$, $^{**}0.01$) versus other groups. No significant difference was observed in the groups of same interval with common symbol.

Significant ($p < 0.05$) upregulation of IL-6 mRNA at 6, 24 and 48 hpi interval was observed in V, H and H + V group as compared to control and HMGB-1 depleted groups (Fig. 8). A significant ($p < 0.05$) down regulation of TNF- α mRNA expression was noticed at 6 h in HMGB-1 depleted groups as compared to others. A significant upregulation of TNF- α (Fig. 9) expression was observed in V, H and H + V group at 24 hpi as compared other groups.

There was significant ($p = 0.005$) upregulation of IL-8 mRNA at 6 h in PRRSV infected cells supplemented with recombinant HMGB-1. At 24 and 48 hpi, there was significant ($p < 0.05$) upregulation of IL-8 expression in PRRSV infected group only as compared other treatments of the study. The expression pattern of IL-8 is given in Fig. 10.

3.6. Effect of HMGB-1 on PRRSV replication in PAM cultures

PRRSV increased to 77,900 copies (mean) by 24 hpi in infected PAM cells. In HMGB-1 supplemented group the virus replication was less (a mean of 33,000 copies) than that of virus only group at 24 hpi, while at 48 hpi, it was slightly more. At 24 and 48 hpi there was a significant ($p < 0.01$) increase (mean of 506,000 copies) in virus copy numbers in

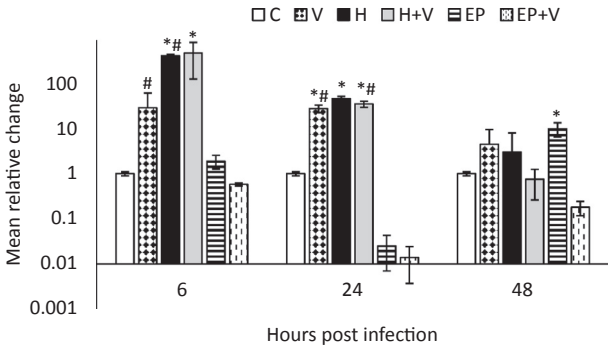

Fig. 8. Relative mRNA expression of IL-6 in PRRSV infected porcine pulmonary alveolar macrophages with various treatments and their respective controls. Data are means ± SD, statistical significance ($^{*/\#}p \leq 0.05$) versus other groups. No significant difference was observed in the groups of same interval with common symbol.

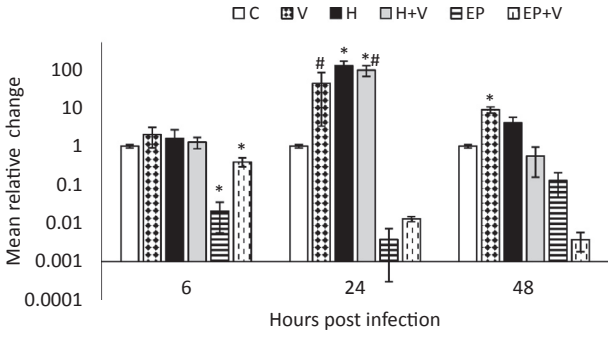

Fig. 9. Relative mRNA expression of TNF-α in PRRSV infected porcine pulmonary alveolar macrophages with various treatments and their respective controls. Data are means ± SD, statistical significance ($^{*/\#}p \leq 0.05$) versus other groups. No significant difference was observed in the groups of same interval with common symbol.

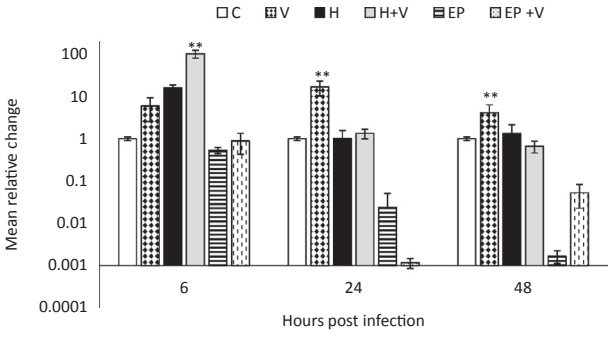

Fig. 10. Relative mRNA expression of IL-8 in PRRSV infected porcine pulmonary alveolar macrophages with various treatments and their respective controls. Data are means ± SD, statistical significance ($^{**}p \leq 0.01$) versus other groups. No significant difference was observed in the groups of same interval with common symbol.

HMGB-1 depleted (EP + V) group as compared other groups. The pattern of virus replication in PRRSV infected PAM with or without HMGB-1 supplementation/depletion is depicted in Fig. 11.

3.7. HMGB-1 protein estimation in PRRSV infected PAM culture supernatants

Porcine HMGB-1 protein (Fig. 12) in the supernatant of PAM cultures were estimated using commercial ELISA kit (USCN life sciences, USA). There was a sharp increase in HMGB-1 release in the supernatant

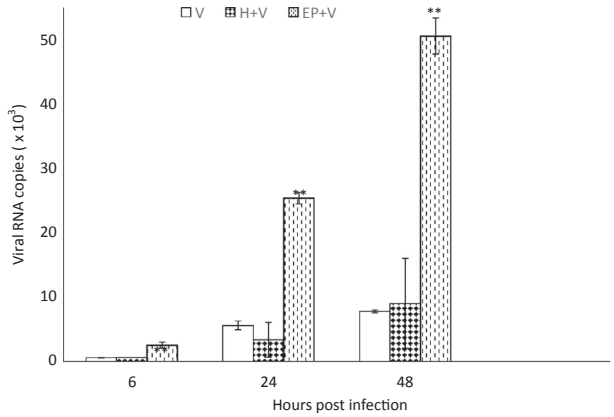

Fig. 11. Quantification of viral RNA in PRRSV infected porcine pulmonary alveolar macrophages with various treatments and their respective controls. Data are means ± SD statistical significance ($^{**}p \leq 0.01$) versus other groups. No significant difference was observed in the groups of same interval with common symbol.

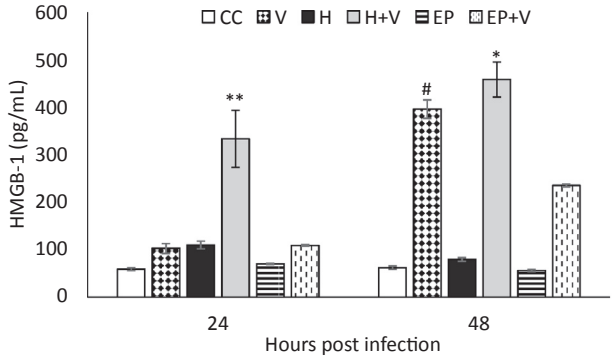

Fig. 12. Quantification of HMGB-1 release in supernatant of PRRSV infected porcine pulmonary alveolar macrophages with various treatments and their respective controls. Data are means ± SD, statistical significance ($^{*}p \leq 0.05$, $^{**}0.01$) versus other groups. No significant difference was observed in the groups of same interval with common symbol.

of PRRSV infected PAM at 48 h and its level was significantly ($p < 0.05$) higher in infected cells supplemented with HMGB-1 at both 24 and 48 hpi.

4. Discussion

Cytokines play a central role in regulating the host immune system by mediating inflammatory process, protective responses against pathogens and also by suppressing inflammatory over responses that may lead to unwanted tissue damage. The ability to examine cytokine responses has been vital in understanding both effector, regulatory responses and outcome elicited during infection. There are several *in vitro* studies on cytokine response to PRRSV infection with limited studies on *in vivo* responses. There is no report on HMGB1 level during PRRS infection in piglets. Hence, cytokine mRNA expression in PBL and target organ (lungs) of PRRSV infected piglets were studied with special focus on HMGB1 to understand the involvement ant its role in PRRSV infection in pigs.

High-mobility group box 1 (HMGB-1), a highly conserved non-histone chromosomal protein, has its physiological role in maintenance of nucleosome structure and regulation of gene transcription [37]. Of late it is considered as a potent proinflammatory cytokine and a “late” mediator in induction of systemic inflammatory response [44]. HMGB-1 once released out of the cell interacts with TLR2 and TLR4 could trigger intracellular signaling cascades in macrophages involving

activation of p38 mitogen-activated protein kinase (MAPK), c-Jun NH (2)-terminal kinase (JNK), and nuclear factor-κB (NF-κB) [76]. Activation of these signals consequently lead to the release of proinflammatory cytokines in monocytes including TNF-α and IL-1β [82]. Hence, HMGB1 has been proposed to be a crucial mediator in the pathogenic processes of many diseases, including sepsis [77], autoimmunity [15], acute lung inflammation [2] and several severe viral infections [4,24,42].

In the present study, significant upregulation of HMGB-1 mRNA in PBL with corresponding increases in pro-inflammatory cytokines like IL-1 β, IL-6, TNF-α , IL-8 and fever was observed. Similarly, upregulation was observed in lungs on 7 dpi and it was either downregulated or almost unchanged on 14 and 21 dpi which correlated with damage and active inflammatory process in lungs by histopathology [63]. Importantly, there was a significant positive correlation between levels of serum HMGB1 and IL-6 expression and *vice versa* was observed with WBC counts during PRRSV experimental infection. Our findings were supported by an *in-vitro* study, in which the release of HMGB-1 in PRRSV infected cells and corresponding elevation of pro-inflammatory cytokines was reported [22]. Hence, HMGB-1 once released out of the cells may have performed a multifunctional role in enhancing the pro-inflammatory cytokine levels by stimulating macrophages, monocytes in PRRS pathogenesis as reported earlier in other infections [7,8,43,55]. There are chances for more leakage of HMGB-1 from PRRSV infected or bystander cells due to increase in proinflammatory cytokines as reported earlier in other studies [30,67].

In the present study, serum HMGB-1 was found to be higher in PRRSV infected animals as compared to their corresponding controls. Mean HMGB-1 level was significantly ($p = 0.008$) higher on 6 dpi in PRRSV infected piglets. Upregulation of pro-inflammatory cytokines was paralleled by increased HMGB-1 mRNA expression, serum HMGB-1 protein level, viremia, high fever and increased serum Lactate dehydrogenase activity [63] in PRRS infection in piglets. Elevation of serum HMGB-1 was accompanied with excessive tissue damage by the virus was observed as peak viremia between 4 and 6 dpi, severe inflammatory response in lungs and it was consistently elevated in dead piglets during the study [63]. This is also supported by previous study indicating the contribution of HMGB-1 in mortality of mice by Influenza A virus [35]. Elevation of HMGB-1 is also reported in viral infections such as influenza, Hepatitis and SARS in humans [13,39,80]. Thus, the serum HMGB-1 level could be implicated as critical mediator of PRRS pathogenesis and severity of the disease.

As reported in a study by Chen et al. [17], it can be speculated that exacerbation of pulmonary injury may be due to virus-mediated cytolysis of macrophages and HMGB-1 mediated pulmonary inflammatory response such as neutrophil infiltration, derangement of epithelial barrier, lung oedema and lung injury in PRRSV infection or through enhancing pro-inflammatory property of secreted cytokines [1,64].

The results of our study showed that there was significant ($p = 0.033$) upregulation of IL-1β mRNA in PBL of PRRSV infected piglets between 6 and 9 dpi. Relative mRNA expression of IL-6 was significantly ($p = 0.041$) higher on 5 and 6 dpi whereas TNF α was significantly upregulated on 9 dpi. These increase in the relative expression of pro-inflammatory cytokine (IL-1β, IL-6 and TNF α) mRNA could be correlated with viraemia, severity of symptoms like fever and other clinical signs and lesions. There are contradicting results on the effect of PRRSV on pro-inflammatory cytokines in infected animals. Several studies consistently revealed significantly elevated levels of IL-1, IL-6 and TNF-α in the serum of HPPRRSV infected pigs [31–33,46,84]. In contrast, low or suppressed expression of pro-inflammatory cytokines (IL-1, IL-6 and TNF-α) was observed in both type 1 and 2 classical PRRSV infection [25,27,47,60,70,71,74]. In lungs, we observed significant upregulation of all pro-inflammatory cytokine (IL-1, IL-6 and TNF-α) mRNA on 7 dpi and also high levels were observed in piglets which died on 3 and 17 dpi correlating with the severe effects of inflammatory cytokines. It is possible that the elevation of proinflammatory cytokines in lungs of dead piglets could be due to viremia during the initial period post infection and it might have been complicated with secondary bacterial infections later. Thus concurrent elevation of HMGB-1 and proinflammatory cytokines during HPPRRSV infection may play a vital role in pathogenesis and exacerbation of disease outcome.

In lungs, IFN β and IFN γ were highest on 14 dpi indicating its role in clearance by reduced or minimal viral copies detected in lungs during later phase, i.e., 14 and 21 dpi whereas, in contrast to PBL, IL-2 and IL-4 expression was higher on 7 dpi. These findings are in concordance with earlier report showing significant increase in IFN γ, IL-2, IL-4 in bronchio alveolar lavage fluid and tracheobronchial lymph node in HPPRRSV infected piglets [31].

Upregulation of HMGB-1, IL-1, IL-6 and IL-8 mRNA in PRRSV infection in PAM culture. The relative expression of pro-inflammatory cytokines such as IL-1 β, IL-6, IL-8 was significantly upregulated in early phase (i.e. 6 h) of PRRS virus infection in PAM PRRSV infected PAM with or without recombinant HMGB1 supplementation as compared to HMGB-1 depleted cells. Relative mRNA expression of TNFα was found to be upregulated in later phase of PRRSV infection (i.e. 24 h) in supplemented cells as compared to other treatments. Similar findings were reported by earlier workers as increase in expression of pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6, IL-8, and MIP-1 by p38 and JNK MAPK mediated mechanism on exogenous HMGB-1 stimulation of monocytes, macrophages and neutrophils [7,8,43,55]. Role of HMGB-1 was also reported in pathogenesis of other viral infections like West Nile virus [11], dengue virus [54], HIV [52], influenza [51], hepatitis C virus [40].

The HMGB1 release in the supernatant was estimated by commercial ELISA also showed similar results as observed in transcriptional response. Higher levels of HMGB-1 protein in the supernatants of virus infected cells was observed in this study were significant. These findings were also supported by the only previous report on release of HMGB-1 in supernatant after 24 h of PRRSV infection in MARC-145 cells and PAM [22]. The late increase in HMGB-1 protein in supernatant was observed even though the HMGB-1 mRNA occurred much earlier. This late release of HMGB-1 was supported by earlier workers that the process of HMGB-1 shuttling from nucleus to cytoplasm, accumulation in cytoplasm and active secretion by immune cells take at least 8 h [6,26,77].

Effect of PRRSV replication on HMGB-1 level was assessed by viral copy number quantification by real time RT-PCR. There was significant ($P < 0.05$) increase in viral copies in HMGB-1 depleted cells at later intervals (24 and 48 h). Depletion of HMGB-1 release could have reduced host pro-inflammatory response to check virus replication. Apart from blocking HMGB1 release, a report by van Zoelen et al. [75] suggested that EP also has anti-inflammatory property by decreasing TNF-α level in human mononuclear cells. It is further supported by a delayed TNF-α transcriptional response with corresponding peak in viral copies in later phase in PRRSV infected PAM in our study. Similar finding was also reported in PRRSV infected PBMC derived macrophages by Subramaniam et al. [68]. Thus, down regulation of TNF-α mRNA transcription in later phase in EP supplemented cells could have benefitted virus for its replication by circumventing host response. Thus, our study shows that HMGB-1 blocking favours PRRSV replication. Similar results were reported as augmentation of Hepatitis C virus infection by blocking HMGB1 with antibody and reduction in propagation by supplementation [40]. The data supports the interpretation that HMGB1 proteins secreted from PRRSV-infected cells block the rapid replication of virus, at least partially. Upregulation of IL-8 in HMGB-1 supplemented groups were observed which draws support from a report on increased secretion of IL-8 in upper way epithelial cells to curtail virus replication [50].

In conclusion, our results that in PRRSV infected piglets, pro-inflammatory cytokines (IL-1 and IL-6) was excessively upregulated in initial course of disease which was correlated with HMGB-1 mRNA

expression, serum HMGB1 level, high fever, severity, extent of tissue damage. The role of HMGB-1 in modulation of proinflammatory cytokine response was also confirmed by increasing and decreasing its level in Porcine PAM during PRRSV infection in invitro experiments. Hence, HMGB-1 as danger associated molecular pattern (DAMP) was found to be an important biomolecule which could be released either actively or passively from the PRRSV infected or bystander immune cells and may have implicit role in early PRRS pathogenesis. It provide the basis for the development of novel strategies for disease management, control and assessment of prognosis. Results also demonstrate that HMGB-1 targeted therapeutics has potential in development of treatment and management of the disease in pigs.

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Conflict of interest

The authors express no conflict of interest.

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