

# Atypical Classical Swine Fever Infection Changes Interleukin Gene Expression in Pigs

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## ABSTRACT

Several studies have highlighted the important role of cytokines in disease development of classical swine fever virus (CSFV) infection. In the present study, we examined the changes of body temperature, viral replication and leukocytes, and the kinetics of four porcine interleukins (ILs) in serum from pigs infected with CSFV strains which lead to the atypical CSF during 45 days post infection (dpi). The results showed that compared to those uninfected pigs with CSFV, CSFV-infected pigs had higher temperature and viral replication while showing less leukocytes. Furthermore, using reverse transcription polymerase chain reaction (RT-PCR), the cytokines IL-1, IL-2 and IL-4 in white blood cells of pigs were measured showing increased levels after CSFV infection. However, the cytokine IL-8 level of the CSFV-infected pigs was downregulated before 10 dpi and peaked at 25 dpi. These findings may indicate that IL-1, IL-2, IL-4 and IL-8 are involved in the immune response during CSFV infection.

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**Keywords:** CSFV; RT-PCR; Interleukin; Viral Replication; Temperature; Leukocyte.

## INTRODUCTION

Classical Swine Fever Virus (CSFV), the highly contagious agent of CSF, is a positive-sense single-stranded RNA virus. The infection causes severe economic losses in the pig industry especially in developing countries (1). The virus has a high affinity for the cells of the immune system such as myeloid cells including macrophages and dendritic cells (DCs), and interferes with cellular antiviral defenses (2). Furthermore, CSFV can efficiently evade and compromise the host immune system, causing a severe disease of pigs characterized by fever, hemorrhage, thrombocytopenia and lymphoid organ atrophy severe lymphopenia, particularly in B cells, due to the apoptosis of uninfected lymphocytes (3,4). The invasion of CSFV to the host immune system can cause severe lymphopenia which is the hallmark of CSFV infection, resulting in immunosuppression. At present, many countries including

the European Union (EU) member states have used different strategies for the eradication of CSF in wild boar and in domestic pigs (5). However, some developing countries such as China, India and Africa have pointed out difficult methods to eradicate this complex disease, even though vaccination against CSFV has been applied. In addition, CSF epidemics appeared in these countries with no typical clinical symptoms, leading to the difficulties in accurate diagnosis, monitoring and prevention of this disease (6). Recently, atypical CSF has been found in many areas of China. It suggests that atypical CSF is also caused by CSFV, but compared to CSF, its epidemiology, clinical symptoms, pathological changes are not classical. Furthermore, atypical CSF often occurs in the pigs inoculated with vaccines and its latent period is very long. The previous findings demonstrated that sows in different stages of pregnancy inoculated with a low-virulence

CSFV strain showed no clinical signs of disease, but most of their pigs showed splay-leg with nervous disorders and died. The pigs which survived were immunotolerant but remained unapparent carriers of virus. Virus was transmitted from immunotolerant pigs to susceptible pigs by contact 5 weeks but not 3 months after farrowing (7).

In the present study, during different periods of CSFV infection, the clinical symptoms and the changes of cytokines IL-1, IL-2, IL-4 and IL-8 were investigated, providing the evidence that the observed gene expression profile of these cytokines might explain immunological and pathological changes associated with atypical CSFV infections.

## MATERIALS AND METHODS

### Virus

The wild-type CSFV strain named CSFV-YN-2009 was originally isolated from lymphoid tissue of a pig with naturally occurring atypical CSF. Virus titres were determined and calculated as described previously (8). The virus ( $1.0 \times 10^{5.2}$  TCID<sub>50</sub>/ml) were preserved and stored at -80°C in Laboratory of Viruses, College of Animal Science, Yunnan Agricultural University.

### Animal and treatment

Pigs negative by RT-PCR and seronegative of CSFV, bovine viral diarrhea viruses (BVDV), porcine respiratory and reproductive syndrome virus (PRRSV), porcine circovirus type 2 (PCV2), porcine pseudorabies virus (PRV), porcine parvovirus (PPV) and foot-and-mouth disease virus (FMDV) were selected among 25-day-old Yorkshire pigs. Eight negative pigs were intramuscularly injected with 1 ml atypical CSFV strain ( $1.0 \times 10^3$  TCID<sub>50</sub>/ml), and 8 negative pigs served as controls were injected intramuscularly with 1 ml physiological saline. Body temperature and clinical symptoms were recorded daily post-infection.

### PBMC isolation

At -1, 0, 1, 2, 4, 7, 10, 13, 17, 21, 25, 30, 35, 40 and 45 dpi, 10 ml blood was collected from the anterior vena cava of the pigs and placed into sodium heparin-CPT tubes and EDTA. Following collection, blood sample with EDTA anticoagulant was mixed with 5 ml hydroxypropylmethyl cellulose, and washed three times with phosphate buffered saline (PBS) at 4°C, and centrifuged at 1500 rpm/min for 10 minutes.

After centrifugation, further investigations were carried out in two ways: (1) 0.1 mol/l hydrochloric acid was added to dilute the leukocyte preparation (1:20), and mononuclear leukocytes were counted using light microscopy (Olympus, Japan); (2) the buffy coat layer was transferred to a 15 ml RNase free tube, diluted with an equal volume of PBS, and centrifuged at 1500 rpm/min for 20 min at room temperature. The supernatant was discarded and peripheral blood mononuclear cells (PBMCs) were diluted with 9 ml red cell lysate, and centrifuged at 1000 rpm/min for 10 min at room temperature after placement in an ice bath for 2-3 min. The supernatants were discarded and PBMCs were diluted with PBS three times. PBMCs were retained and used to for the detection of IL-1, IL-2, IL-4 and IL-8 expression.

The *in vivo* experiment in this study was carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by Experimental Animal Center of Yunnan Province (Permit Number: 13-268). All surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

### RT-PCR

Expression of IL-1, IL-2, IL-4 and IL-8 in PBMCs was determined by RNA preparation and reverse transcription polymerase chain reaction (RT-PCR) using SYBR Premix Ex Taq™ kit (TaKaRa, Osaka, Japan). Briefly, total cellular RNA was isolated from PBMCs by the guanidine thiocyanate/phenol-chloroform extraction method using TRIZOL reagent following the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). RNA quality was assessed by agarose gel electrophoresis and complementary DNA (cDNA) was synthesized with random hexamer (TaKaRa, Osaka, Japan). RT-PCR was performed using the ABI Prism 7500 Sequence Detector (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. The PCR reaction was carried out at 1 cycle of 50°C for 3 min and 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 55°C for 45 s and 72°C for 30 s. We used β-actin expression as an internal control. Quantification of gene expression was performed by the  $2^{-\Delta\Delta Ct}$  method. Specific primer sequences were designed using Oligo 6.0 software and synthesized in BIOSUNE Biological Technology Corp (Shanghai, China), and the sequences of the primers were shown in Table 1.

## Quantification of viral replication

Viral RNA was extracted using the MiniBEST Viral RNA/DNA Extraction Kit Ver. 4.0 (TaKaRa, Osaka, Japan), and the synthesis of cDNA was performed using the PrimeScript®RT reagent Kit (TaKaRa, Osaka, Japan) according to the manufacturer's protocol. Real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was used to detect virus copies as described in previous study (9).

## Statistical analysis

The data were analyzed by one-way analysis of variance and by Student's t-test with Bonferroni correction using SPSS15.0 software. All numerical data were collected from at least three separate experiments. The results were expressed as means  $\pm$  standard deviation of the means. The differences were considered significant at a level of  $P<0.05$ .

## RESULTS

### Clinical symptoms after CSFV infection

At 1-5 dpi, the appetite and the mental state of pigs were normal. After 5 dpi, the body temperature began to increase accompanied by mild mental depression, slight conjunctival flushing and food-consumption reduction. By 9 dpi of

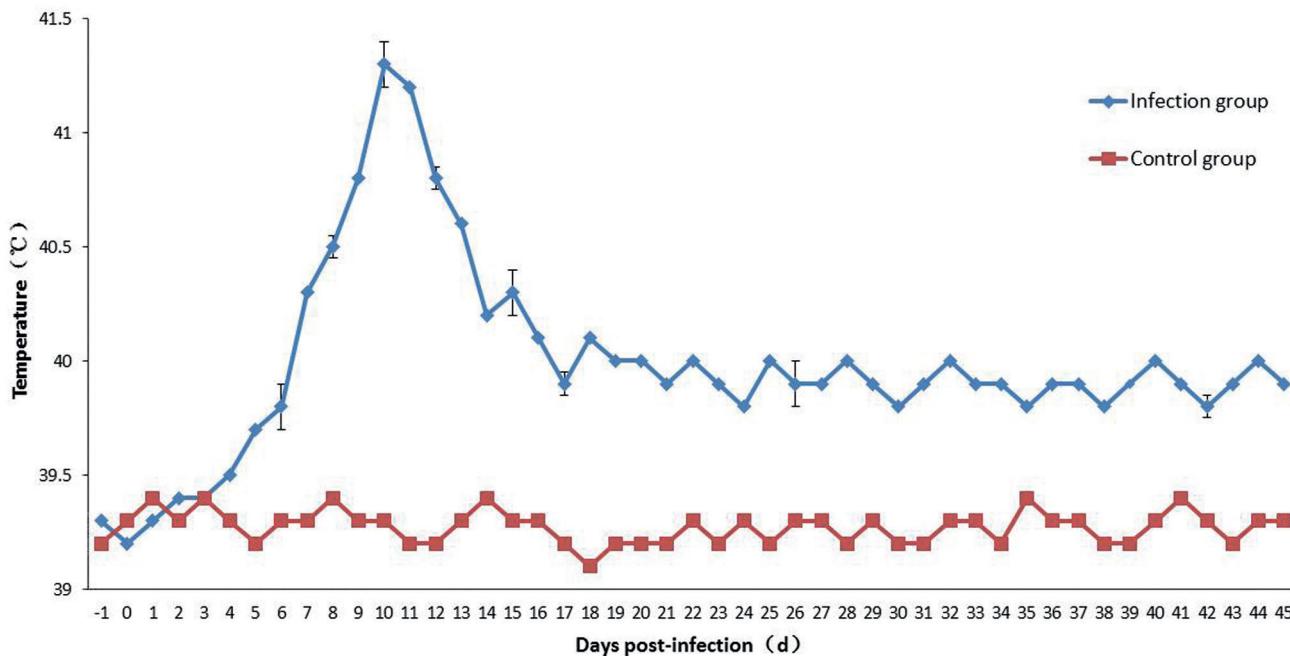
**Table 1:** Nucleotide sequences of primers for real-time PCR

Gene	Sequence of primers 5'-3'	Size (bp)	GenBank Number
$\beta$ -actin	F: CCTGACCCCTCAAGTACCCCA R: GCTCGTTGAGAAGGTGTGGTG	89	U16368.1
IL-1 $\beta$	F: ACCAGGGTTACTGACTATGGC R: GTTGAGGCAGGAAGGAGAT	337	NM_214029.1
IL-2	F: GCACTCATGGCAAACGGTGCA R: GTTACTGTCTCATCATCATATT	362	FJ543109.1
IL-4	F: AACCCCTGGTCTGCTTACTG R: CATAATCGTCTTACGCCTTTC	352	NM_214123.1
IL-8	F: AGCCCCTGTCAACATGACTTCC R: GAAGTTGTGTTGGCATCTTTA	394	NM_213867.1

atypical CSFV, body temperature was elevated to 41.3°C, and decreased significantly in the following days. By 45 dpi, body temperature of pigs was decreased to 39.8°C, which was still higher than that of uninfected pigs. As a control, the uninfected pigs did not show the clinical symptoms, and body temperature remained at 39.2-39.4°C (Fig.1).

### Changes of leukocytes and viral replication

As shown in Fig.2A, the average total number of leukocytes in CSFV infected pigs declined from  $2.08 \times 10^7$  cells/ml to  $1.94 \times 10^7$  cells/ml at 2 dpi, and to  $1.39 \times 10^7$  cells/ml at 4 dpi ( $P<0.05$ ). A rapid onset of mononuclear lymphocytopenia



**Figure 1:** Changes of the rectal temperature of the infected pigs. Following infection with CSFV isolate, clinical signs and body temperature were recorded. Results are expressed as the mean data for each group of pigs and error bars represent standard deviation.

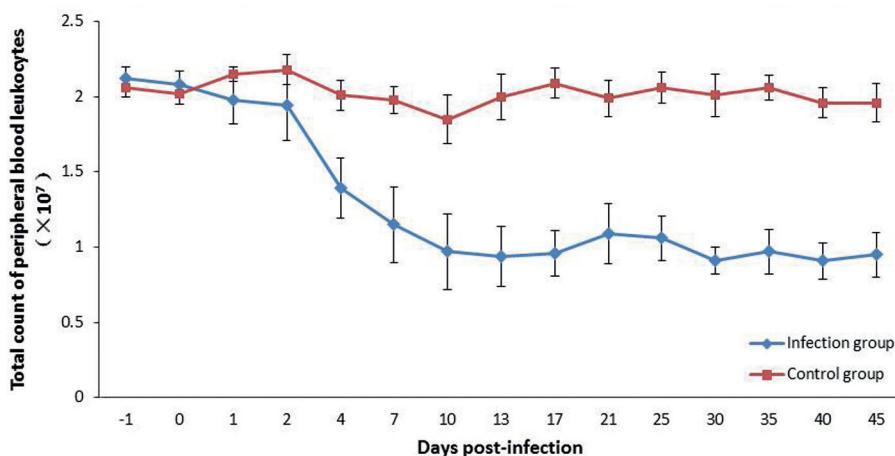
was also detected at 7 dpi in infected pigs, the number of leukocytes declined to  $1.15 \times 10^7$  cells/ml ( $P < 0.05$ ). There was a slight increase in the number of leukocytes at 10 dpi which stabilized at 21 dpi. The number of leukocytes of pigs in the control group was maintained at  $1.85-2.18 \times 10^7$  cells/ml. This result confirmed the depletion of mononuclear leukocytes in atypical CSFV infected pigs. The findings of CSFV replication detected by qRT-PCR indicated that viral replication increased rapidly after challenge of CSFV in pigs and reached the highest peak at 10 dpi, and decreased in the following days (Fig. 2B).

### Changes of interleukin expression in PBMC

The PBMC cytokine secretion in response to CSFV stimulation was measured by RT-PCR, and the results are shown in Figures 3-6. Results in Figure 3 showed that, compared to the control (virus uninfected pigs), IL-1 $\beta$  mRNA transcription level began to increase at 1 dpi. It peaked at 17 dpi, which was 259 times as many as that of the control. Then it slightly decreased, but still remained 100 times higher than that of the control (Figure 3) indicating that CSFV infection induced IL-1 $\beta$  mRNA expression significantly ( $P < 0.05$ ).

Compared to the controls after CSFV infection the mRNA transcript level of IL-2 was increased sharply starting from 1 dpi, and followed by a slight decrease. However, at 10 dpi, IL-2 expression increased again and reached a peak of 230 times greater than the control and continued to stay at high levels. After 35 dpi, IL-2 levels were still 100 times as higher than the controls, indicating that CSFV upregulated IL-2 mRNA transcription level significantly in the early stages of virus infection (Figure 4).

As illustrated in Figure 5, compared with the control, IL-4 mRNA expression was induced in the early stages of CSFV infection. It started to increase rapidly by 5-7 times from 1 dpi, and was slightly downregulated at 7 dpi. At the beginning of 9 dpi, a sharp increase was observed, and a peak (increased by 231 times) was found at 25 dpi. In the



**Figure 2:** Leukocyte counts and viral replication in peripheral blood of pigs in infection and control groups. (A) After infection with CSFV isolate, leukocyte numbers in peripheral blood of pigs were measured. (B) Viral replication in blood was determined by qRT-PCR. Results are expressed as the mean data for each group of pigs and error bars represent standard deviation.

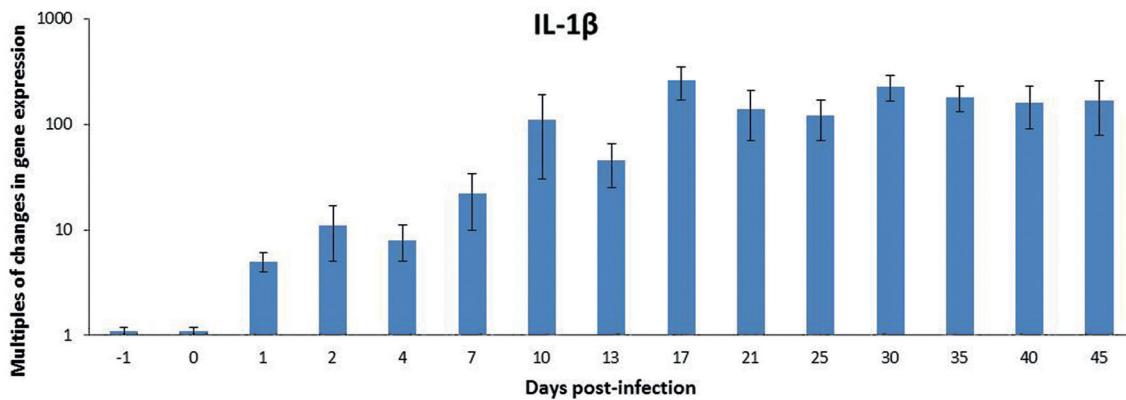
following days, IL-4 levels were decreased but still kept at high levels ( $P < 0.05$ ).

In addition, IL-8 mRNA expression was changed after infection of CSFV. It demonstrated that IL-8 level was decreased by 2.89 times and 9.29 times, respectively at 4 dpi and 7 dpi. At 10 dpi, IL-8 mRNA expression began to increase and reached their peak at 25 dpi (about 41 times), and continued to stay at high expression levels in the following days (Figure 6).

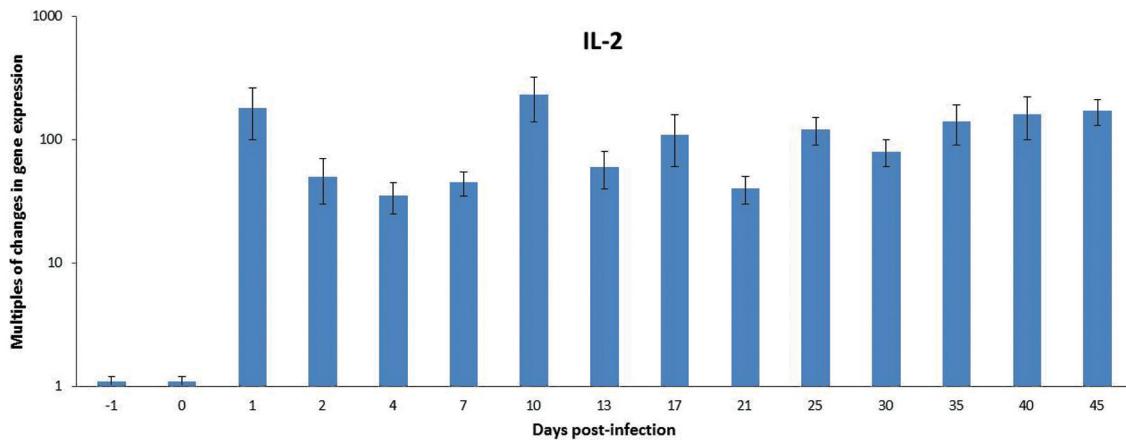
## DISCUSSION

Classical swine fever (CSF), a highly contagious hemorrhagic viral disease of pigs, not only runs an acute, sub-acute, chronic or late onset course, but also may be subclinical (10). CSF outbreaks often lead to extensive epidemics in areas with a high density of pigs (11). In this study, we investigated the changes of body temperature, lymphocyte counts, and the kinetics of porcine IL-1, IL-2, IL-4 and IL-8 in PBMCs isolated from pigs infected with CSFV strain which lead to the atypical CSF during 45 dpi.

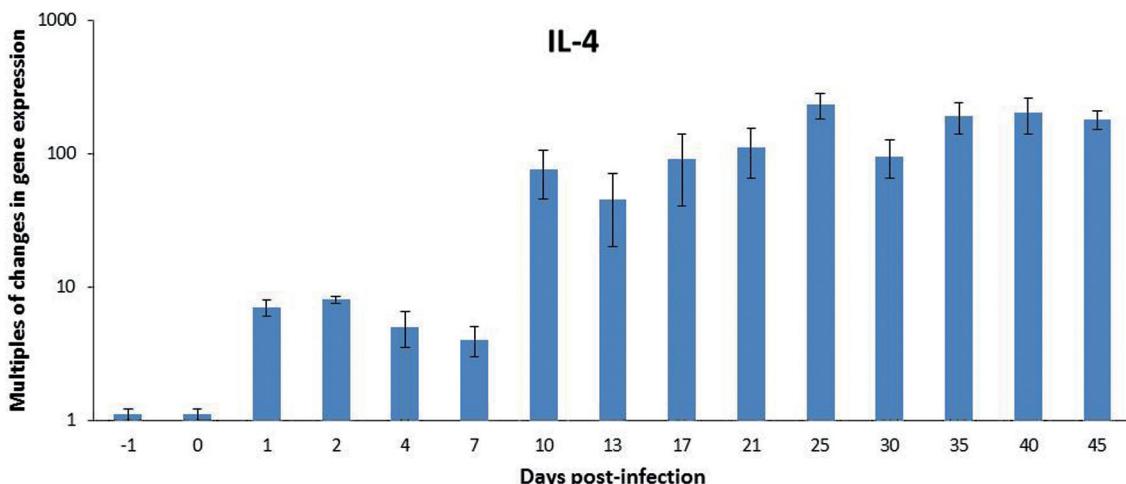
In our study, we demonstrated that body temperature of CSFV infected pigs was only 39.4°C at 3 dpi and reached the highest 41.3°C at 10 dpi, then decreased gradually. At 15 dpi, the temperature showed a stable trend, and was 0.5-0.7°C higher than that of the control group. As reported by Li *et al.* (2006), treatment with the same dose of virulent Shimen strain virus the body temperature of pigs reached 41.4°C at 3 dpi, and the highest temperature was 42.5°C (12). This discrepancy probably was due to the virulence of CSFV.



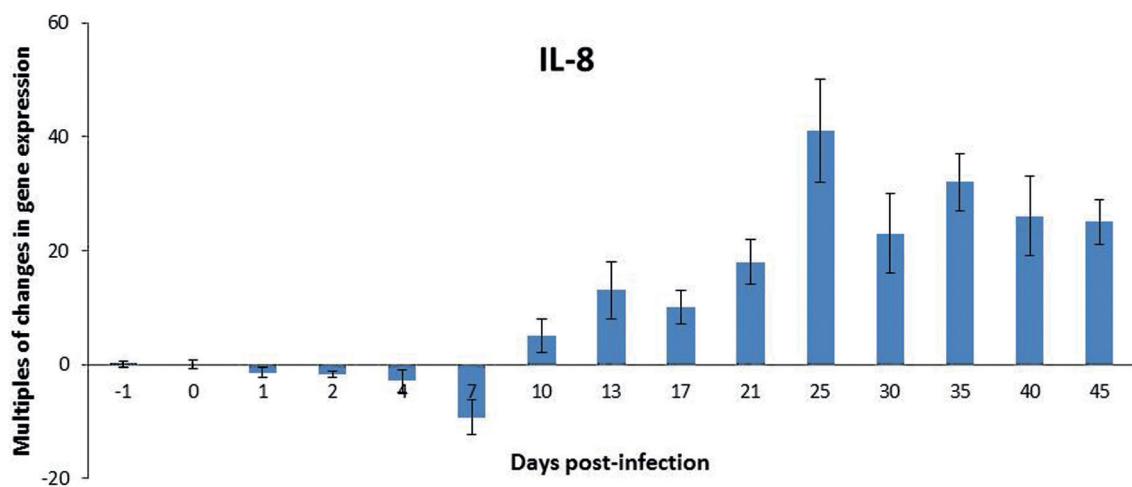
**Figure 3:** The concentrations of IL-1 $\beta$  in PBMCs of inoculated pigs (n=8) and pigs inoculated with CSF virus (n=8 pigs). Analyses of peripheral blood cell cytokine responses by stimulation of CSFV isolate. PBMC were isolated and cytokine IL-1 $\beta$  expression was analyzed using RT-PCR. Results are expressed as the mean data for each group of pigs and error bars represent standard deviation.



**Figure 4:** The kinetics of IL-2 mRNA in PBMCs of pigs. Analyses of peripheral blood cell cytokine responses by stimulation of CSFV isolate. PBMC were isolated and cytokine IL-2 expression was analyzed using RT-PCR. Results are expressed as the mean data for each group of pigs and error bars represent standard deviation.



**Figure 5:** The kinetics of IL-4 mRNA in PBMCs of pigs. Analyses of peripheral blood cell cytokine responses by stimulation of CSFV isolate. PBMC were isolated and cytokine IL-4 expression was analyzed using RT-PCR. Results are expressed as the mean data for each group of pigs and error bars represent standard deviation.



**Figure 6:** The kinetics of IL-8 mRNA in PBMCs of pigs. Analyses of peripheral blood cell cytokine responses by stimulation of CSFV isolate. PBMC were isolated and cytokine IL-6 expression was analyzed using RT-PCR. Results are expressed as the mean data for each group of pigs and error bars represent standard deviation.

CSFV infection caused severe leukopenia, particularly affecting the lymphocytes. The target cells for CSFV in the peripheral blood appeared to be mainly monocytes, lymphocytes and granulocytic cells, but all leukocyte populations may be depleted during CSFV infection (13). Our study also suggested that the number of leukocytes in CSFV infected pigs was reduced, which might predispose the pigs to secondary diseases. Peripheral blood mononuclear cells are a heterogeneous population of blood cells that include monocyte and lymphocytic immune cells consisting of T-cells, B-cells and natural killer (NK) cells. These blood cells represent a critical component in the immune system for fighting infection and adapting to intruders. So, PBMCs have emerged as a critical resource for immune responses to CSFV, and CSFV infection is demonstrated to strongly affect the function of PBMCs (14). Therefore, after challenge of CSFV, changes of porcine IL-1, IL-2, IL-4 and IL-8 expression were observed in this study.

Inflammation is a protective response of the body to ensure the removal of detrimental stimuli. Moderate inflammatory responses are favorable for the repair of the damaged tissues, and also critical for the pathogenesis of diseases (15). The inflammatory response is orchestrated by proinflammatory cytokines such as TNF, IL-1, and IL-8. These cytokines are pleiotropic proteins that regulate the cell death of inflammatory tissues, modify vascular endothelial permeability, recruit blood cells to inflamed tissues, and induce the production of acute-phase proteins (16). Inflammatory cytokine IL-1 $\beta$  is essential in antiviral host

defences. Despite its essential role in host defense, high levels of IL-1 $\beta$  are also responsible for side-effects like fever, hypotension, vasodilatation or acute lung injury by fluid accumulation in response to viral infection (17). IL-8, formerly known as neutrophil-activating peptide-1 (NAP-1), is important in the initiation and development of inflammatory processes through its capacity to attract and activate neutrophils (18). The previous studies suggested that CSFV infection had a more pronounced effect on cytokine secretion of macrophages or PBMCs. An increase in mRNA levels of IL-1 $\beta$  (19) and IL-6 (20) was observed in CSFV-infected macrophages (21). Additionally, IFN- $\alpha$ , IL-8 and TNF- $\alpha$  levels were involved in the immune responses during CSFV infection with strains of different virulence (22).

IL-2 is a potent T-cell growth factor that induces lymphokine-activated killer activity, mediates activation-induced cell death and is an essential factor for the development of regulatory T-cells (23). IL-4 is produced by T helper (Th) 2 cells. IL-4 is a pleiotropic cytokine and plays a number of important roles, including the regulation of inflammation (24), acting as an autocrine growth factor to promote the differentiation of naive T cells to Th2 cells and inhibiting the differentiation of naive T cells to Th1 cells as well as inhibiting cytokine production by Th1 cells (25).

Recent studies demonstrated that CSFV could replicate in all lymph nodes (21) and mouse bone marrow-derived immature dendritic cells (BM-imDCs) effectively and were found to promote the proliferation of allogeneic naive T cells, and

induce a stronger Th1 response (26). After pigs inoculated with the Alfort 187 CSFV isolate, quantitative changes in the T-lymphocyte population such as CD3(+), CD4(+) and CD8(+) and cytokines IL-2, IL-4 and IFN- $\gamma$  secreted by these cells in serum, thymus and spleen were observed (27). As indicated in the results of this study, the production of IL-1 $\beta$ , IL-2, IL-4 and IL-8 was significantly increased after infection with CSFV in pigs, which suggested a possible mechanism for this virus to evade the host's immune and cause a persistent infection in pigs.

In summary, our observations provide new evidences that an immune response is activated at the early stage of CSFV infection with upregulated production of interleukins IL-1 $\beta$ , IL-2, IL-4 and IL-8. The increased expression of these cytokines may in consort be the underlying cause of the observed clinical symptoms in pigs infected with CSFV. Defining the relationships between ILs expression and the pathology and clinical manifestations of atypical CSF may help to shed light on the molecular pathogenesis of atypical CSF infections.

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