



## SHORT COMMUNICATION

### Age-dependent Cytokine Expression in Response to Foot-and-mouth Disease Virus in Bovine Peripheral Blood Mononuclear Cells

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#### ABSTRACT

The severity of foot-and-mouth disease virus (FMDV) infections differs between calves and cattle. Here, we compared immunological responses to FMDV in bovine peripheral blood mononuclear cells (PBMCs) from juvenile cattle (7–9 months) and calves (3–4 months). PBMCs were collected from cattle and inoculated with serotype O FMDV and incubated for 0, 1, 3, 6, 12, 24, 48, 72, and 96 h. At each time point, total RNA was isolated from PBMCs and the mRNA expression of six cytokines (*IFN-γ*, *TNF-α*, *IL-2*, *IL-4*, *IL-6*, and *IL-10*) was evaluated. Th1 (*IFN-γ* and *IL-2*) and Th2-related (*IL-4* and *IL-10*) cytokine levels were more prominent in juvenile cattle than in calves. In particular, *IL-2* and *IL-10* from juvenile cattle were significantly ( $p < 0.001$ ) higher than those from calves at 48 and 24 hpi, respectively. Therefore, the juvenile cattle showed remarkable Th1 and Th2-related immune response than calves within 48 h after FMDV infection.

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#### INTRODUCTION

Foot-and-mouth disease is a highly contagious viral disease that is caused by the foot-and-mouth disease virus (FMDV). It is associated with high morbidity in cloven-hoofed animals, such as pigs, goats, and cattle. FMDV-infected cattle show obvious clinical signs, including drooling, foot lesions, and fluid-filled vesicles in the mouth. Vesicular lesions are also seen on teats, particularly those of lactating cows, whereas calves usually die before the appearance of vesicles (Kitching, 2002). Clinical aspect and mortality rate also differ markedly between adult cows and calves (Kitching, 2002). High mortality was reported in calves from Egypt (34.3%) between 2014 and 2016 (Zhang *et al.*, 2021b). FMDV affects myocardial cells in calves, causing white grayish spots or stripes on their hearts (Kitching, 2002). However, lesions or viral replication were not observed in the myocardium of adult cattle (Zhang *et al.*, 2021b).

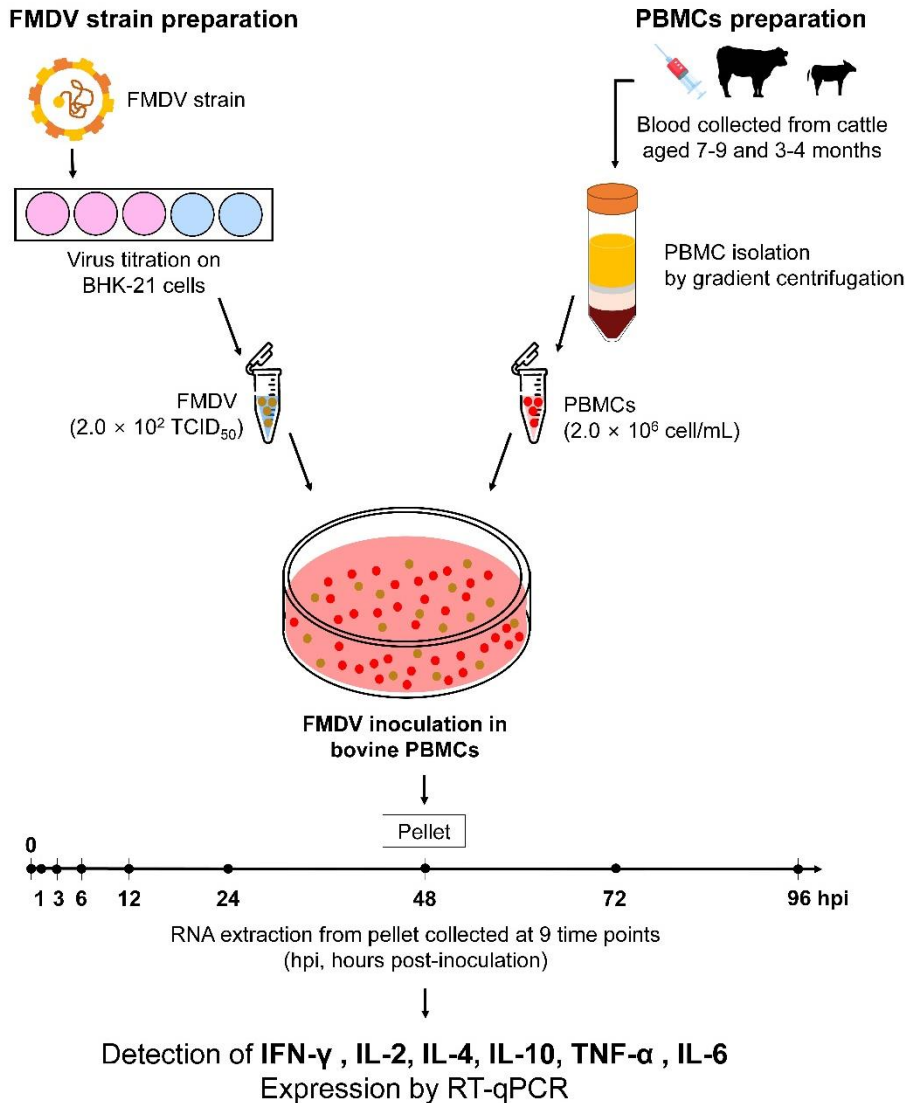
Immune responses to FMDV infection are strongly related to cell-mediated immunity, which can induce humoral immunity and viral clearance (Jung *et al.*, 2014). Subsets of CD4<sup>+</sup> class II major histocompatibility-

restricted T cells respond to viruses by producing T helper type 1 and 2 (Th1 and Th2) responses (Golde *et al.*, 2008). Antiviral CD8<sup>+</sup> T cell responses have also been observed in animals infected with FMDV (Guzman *et al.*, 2008). We hypothesized that the immune responses of cattle to FMDV infection are age-dependent, which causes discrepancies in mortality rates between adult cattle and calves.

However, to our knowledge, the function of immune responses in the early phase of FMDV infection in cattle of different ages remains unexplored. We aimed to compare immunological responses to FMDV in bovine PBMCs obtained at different ages from cattle.

#### MATERIALS AND METHODS

Three juvenile Red Sindh cattle (aged 7–9 months) and three calves (aged 3–4 months) were acquired from the same commercial farm in Vietnam. These animals were confirmed serologically negative for antibodies against FMDV. Fig. 1 shows the overall experimental scheme. Briefly, bovine PBMCs were isolated from each cattle and counted as described earlier (Roh *et al.*, 2021). The cell density was adjusted to  $2.0 \times 10^6$  cells/mL and cells were



**Fig. 1:** Experimental scheme of the study. Bovine PBMCs were isolated from juvenile cattle (7–9) and calves (3–4 months old) and seeded in 24-well plates with 500  $\mu$ L per well ( $2 \times 10^6$  cell/mL). PBMCs were infected with  $2 \times 10^2$  TCID<sub>50</sub> of type O FMDV, and incubated for 0, 1, 3, 6, 12, 24, 48, 72, and 96 h. cDNA synthesized from the RNAs of each cell, and real-time qPCR analysis was conducted to detect the expression of six cytokines (IFN- $\gamma$ , TNF- $\alpha$ , IL-2, IL-4, IL-6, and IL-10)."

seeded in 24-well plates with 500  $\mu$ L of the cell suspension per well. The FMDV strains (serotype O) were used for inoculation to each cell; the strain information and inoculation methods have been previously described (Roh *et al.*, 2021). Except for the negative control, all of the collected PBMCs were infected with  $2.0 \times 10^2$  TCID<sub>50</sub> of FMDV in RPMI 1640 and incubated for varying time points (0, 1, 3, 6, 12, 24, 48, 72 and 96 h). The incubation was performed in a humidified incubator with 5% CO<sub>2</sub> at 37°C.

Total RNA was isolated and RNA concentrations were determined according to our previous study (Roh *et al.*, 2021). From the extracted RNA, cDNA was synthesized by reverse transcription using an RT<sup>2</sup> First Strand Kit (Qiagen, Germany) containing random primers. To eliminate genomic DNA, 2  $\mu$ L of Buffer GE (Qiagen, Germany) was added to 8  $\mu$ L of RNA (<1  $\mu$ g), which was incubated at 42 °C for 5 min using a Veriti 96-well thermal cycler (Applied Biosystems, USA). Then, 10  $\mu$ L of reverse transcription mixture containing 4  $\mu$ L Buffer BC3, 1  $\mu$ L control P2, 2  $\mu$ L buffer RE3, and 3  $\mu$ L RNase-free water was added to the sample. The mixture was incubated at 42 °C for 15 min, at 95 °C for 5 min, and at 4°C for 20 min. Subsequently, in each sample 91  $\mu$ L RNase-free water was added. At each time point post infection, the relative expression levels of genes in PBMCs were quantified. Real-time PCR analysis was performed using primer sets targeting Th1-related

genes (IFN- $\gamma$  and IL-2), Th2-related genes (IL-4 and IL-10), inflammation-related genes (TNF- $\alpha$  and IL-6), and an internal control gene (*GAPDH*) with a Real-Time PCR System (ABI7500, Thermo Fisher Scientific, USA). Primers and thermal profiles used are presented in Table 1. Relative cytokine mRNA expression levels were quantified using the  $2^{-\Delta\Delta C_t}$  method, with target gene expression normalized to that of *GAPDH*.

All quantitative results are presented as the mean  $\pm$  standard error (SE) and all experiments were performed at least three times. For statistical analysis, 18 groups were generated according to the nine different time points and two age groups. To assess significance by comparing cytokine mRNA expression between calves (3–4) and juvenile cattle (7–9 months old) at the same time point, One-way analysis of variance with Bonferroni alpha correction was used.  $P < 0.002$  (0.05/18) indicate statistical significance.

## RESULTS AND DISCUSSION

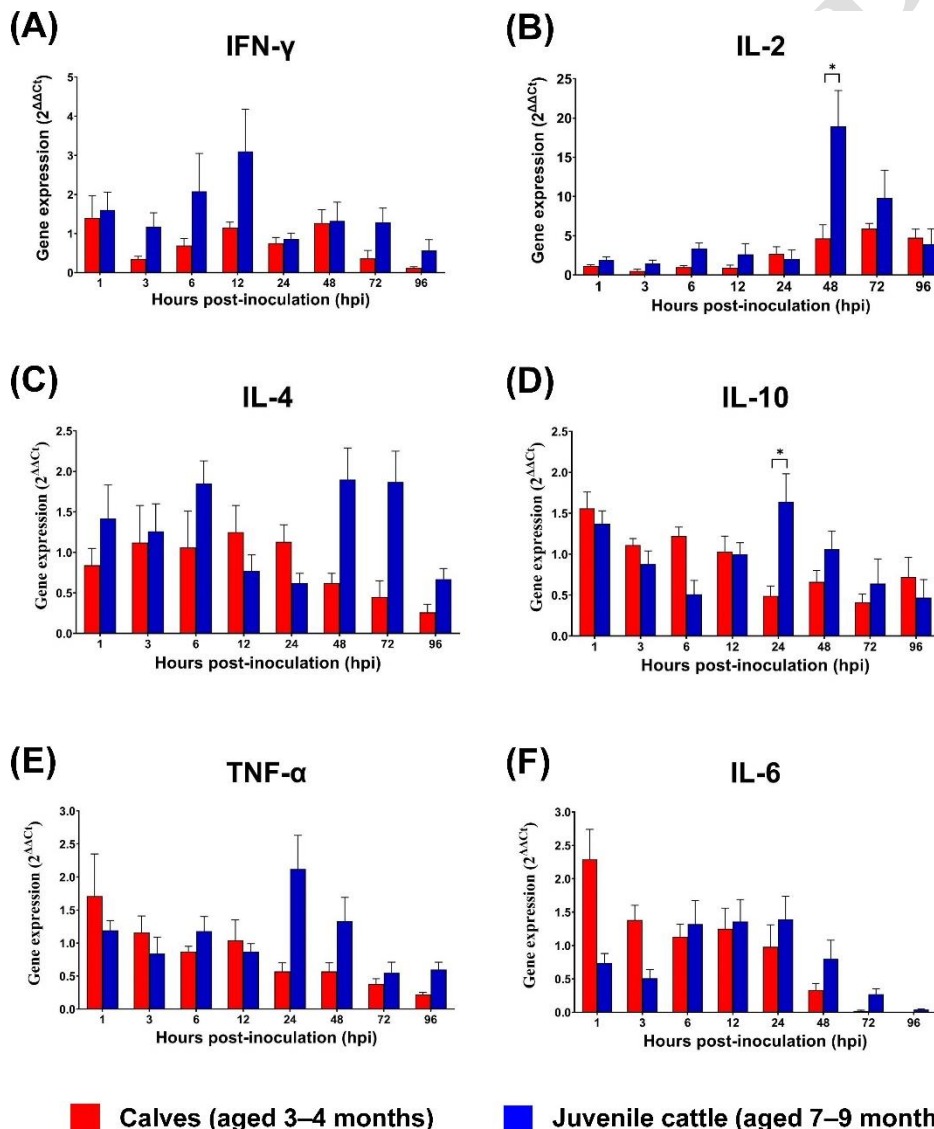
IFN- $\gamma$  expression mean fold induction in the FMDV-infected juvenile cattle PBMCs increased rapidly from 3 ( $1.17 \pm 0.36$ ) to 12 h post infection (hpi;  $3.10 \pm 1.08$ ) and declined at 24 hpi ( $0.86 \pm 0.15$ -fold; Fig. 2A). In calves, it increased from 3 ( $0.35 \pm 0.07$ ) to 48 hpi ( $1.27 \pm 0.34$ ) and

then declined at 96 hpi ( $0.12 \pm 0.03$ -fold). *IL-2* mRNA expression in PBMCs from juvenile cattle peaked at 48 hpi ( $18.94 \pm 4.57$ ) and progressively declined until 96 hpi ( $3.91 \pm 1.97$ ), whereas in those from calves, it gradually increased from 12 ( $0.91 \pm 0.32$ ) to 72 hpi ( $5.89 \pm 0.68$ ; Fig. 2B). In particular, *IL-2* expression was significantly higher in the PBMCs from juvenile cattle than in those from calves at 48 hpi ( $P < 0.001$ ). A cellular immune response is evoked by  $CD8^+$  cytotoxic and  $CD4^+$  helper T cells and Th1 cells secrete *IFN- $\gamma$*  and *IL-2* (Kim *et al.*, 2020). *IFN- $\gamma$*  regulates immune responses towards the infection and inhibits

*in vitro* replication (Rodriguez-Habibe *et al.*, 2020). *IL-2* aids in adjusting the signaling pathway involved in FMDV recognition by cellular immunity and promotes B-cell proliferation (Li *et al.*, 2021). In this study, the *IFN- $\gamma$*  and *IL-2* mRNA expression in the PBMCs of juvenile cattle was more pronounced than in those of calves after FMDV infection. Hence, strong immune responses were activated in juvenile cattle within 48 h, whereas calves showed a relatively weak response. Mice vaccinated with adjuvants containing *IFN- $\gamma$* , *TNF- $\alpha$* , and *IL-2* exhibited a 100% survival rate, implying that these cytokines are important

**Table 1:** List of primers specific to bovine cytokines in this study

Target	Sequence (5'→3')	Size (bp)	No. of PCR cycles (conditions)
<i>IFN-<math>\gamma</math></i>	F: CTCGCGCCTAACTCTCTCTCT	175	35 (20 s at 95°C; 20 s at 56°C; 30 s at 72°C)
	R: AGGCCACCCCTTAGCTACAT		
<i>TNF-<math>\alpha</math></i>	F: CCATCAACAGCCCTCTGGTT	138	35 (20 s at 95°C; 20 s at 60°C; 30 s at 72°C)
	R: CCATGAGGGCATTGGCATACT		
<i>IL-2</i>	F: TTTTACGTGCCCAAGGTTAA	217	40 (15 s at 95°C; 30 s at 54°C; 45 s at 72°C)
	R: CGTTTACTGTTGCATCATCA		
<i>IL-4</i>	F: CAAAGAACAACAAGTGAAGAAG	181	40 (15 s at 95°C; 30 s at 56°C; 30 s at 72°C)
	R: AGGTCTTTCAGCGTACTTGT		
<i>IL-6</i>	F: TGAGTGTGAAAGCAGCAAGGA	137	40 (15 s at 95°C; 1 min at 56°C; 30 s at 72°C)
	R: TCGCCTGATTGAACCCAGATT		
<i>IL-10</i>	F: CTTTAAGGGTTACTGGGTTGC	239	40 (15 s at 95°C; 30 s at 60°C; 45 s at 72°C)
	R: CTCACATGGCTTTGTAGACAC		
<i>GAPDH</i>	F: GCGTGAACCACGAGAAGTATAA	194	-
	R: CCCTCCACGATGCCAAAGT		



**Fig. 2:** Comparison of mRNA transcription dynamics of cytokines in PBMCs from juvenile cattle (aged 7–9 months) and calves (aged 3–4 months) inoculated with FMDV antigens. The results are presented as fold changes in cytokine mRNA transcription in FMDV-inoculated cells as compared to non-inoculated control cells in media. As an internal control, *GAPDH* was used, and mRNA expression at 0 h was used for calibration. mRNA expression of (A) *IFN- $\gamma$* ; (B) *IL-2*; (C) *IL-4*; (D) *IL-10*; (E) *TNF- $\alpha$* ; and (F) *IL-6*. \* $p < 0.002$ .

for preventing FMDV infection (Kim *et al.*, 2020). Therefore, our finding of lower Th1-related cytokine expression in the FMDV-inoculated PBMCs of calves may explain the observed age-dependent severity of FMDV in infected cattle. By contrast, the *IFN- $\gamma$*  and *IL-2* mRNA expression in PBMCs from unvaccinated juvenile cattle (aged 6–8 months Zebu) peaked in the initial phase of infection (6 and 2 hpi, respectively; Dar *et al.*, 2015). This discrepancy may be attributed to the difference in host subspecies. Moreover, our results confirmed that FMDV antibodies were absent in all animals; although Dar *et al.* (2015) used unvaccinated animals, they did not confirm the seronegativity of FMDV antibodies.

The *IL-4* expression mean fold induction in PBMCs from juvenile cattle increased quickly from 3 ( $1.26\pm 0.34$ ) to 6 hpi ( $1.85\pm 0.28$ ) and from 24 ( $0.62\pm 0.12$ ) to 48 hpi ( $1.90\pm 0.39$ ) but declined at 12 ( $0.33\pm 0.20$ ) and 96 hpi ( $0.67\pm 0.13$ -fold; Fig. 2C). Meanwhile, the *IL-4* expression in the calves' PBMCs increased slightly until 12 hpi ( $1.25\pm 0.33$ ) and declined at 96 hpi ( $0.26\pm 0.10$ ). The expression of *IL-10* in juvenile cattle peaked at 24 hpi ( $1.64\pm 0.34$ ) and declined at 96 hpi ( $0.47\pm 0.22$ ), whereas it generally decreased from 1 ( $1.56\pm 0.20$ ) to 72 hpi ( $0.41\pm 0.10$ ) in the PBMCs of calves (Fig. 2D). Particularly, the fold induction of *IL-10* expression at 24 hpi differed significantly ( $p < 0.001$ ) between the two age groups. Th2 cell-produced *IL-4* and *IL-10* promote B-cell proliferation and plasma cell differentiation. These cytokines can initiate antibody secretion and promote humoral immune responses (Zhang *et al.*, 2021a). Upregulated expression of *IL-4* and *IL-10* was observed in the PBMCs of calves during the early phase (until 24 and 12 hpi, respectively), whereas it was observed in juvenile cattle during the late phase of infection (peaking at 48–72 and 24 hpi, respectively). These findings suggest that Th2 cells are activated early after FMDV infection in calves. Th2 cells are induced by *IL-4* and *IL-10* to generate FMDV specific antibodies and downregulate the expression of Th1 cytokines (Liu *et al.*, 2020). Therefore, our results imply that the Th1 cell-mediated delayed immune response in calves may render them more susceptible to the virus owing to their weaker immune responses in the early phase of infection.

The *TNF- $\alpha$*  mean fold induction in the juvenile cattle's PBMCs increased rapidly from 12 ( $0.87\pm 0.12$ ) to 24 hpi ( $2.12\pm 0.51$ ) and reduced at 72 hpi ( $0.55\pm 0.16$ ) (Fig. 2E). The *TNF- $\alpha$*  fold values in calves peaked at 1 hpi ( $1.19\pm 0.15$ ), but progressively decreased until 96 hpi ( $0.22\pm 0.03$ ). Furthermore, *TNF- $\alpha$*  expression was markedly higher in the PBMCs of juvenile cattle than in those of calves at 24 hpi. Additionally, *IL-6* expression fold induction in juvenile cattle increased rapidly from 3 ( $0.51\pm 0.13$ ) to 6 hpi ( $1.32\pm 0.35$ ) and declined at 96 hpi ( $0.04\pm 0.01$ ), whereas it decreased until 96 hpi ( $0.01\pm 0.003$ ) after rapidly peaking at 1 hpi ( $2.29\pm 0.45$ ) in calves (Fig. 2F). *TNF- $\alpha$*  plays a crucial role in the maturation of dendritic cells, which bridge innate and adaptive immunity after viral infection, aiding the inflammatory cell recruitment and activation leading to immune reactions (Su *et al.*, 2008). *IL-6* promotes the production of immunoglobulin in activated B cells and regulates the expression of Th1-associated cytokines (Su *et al.*, 2008). Hence, these cytokines are considered to be effective

molecular adjuvants that can augment antigen-specific cell-mediated responses towards FMDV vaccines (Su *et al.*, 2008). In this study, the steady decline in *TNF- $\alpha$*  expression in PBMCs of calves suggests that they lack strong host defense mechanisms towards FMDV infection. Zhang *et al.* (2021b) reported that, in various cardiac-resident cells, picornavirus infection induces *IL-6* expression. Further, calf cardiomyocytes are more susceptible to FMDV due to acute inflammation. Therefore, the *IL-6* expression upregulation in PBMCs of calves in the initial stages of infection (until 3 hpi), may help explain the age-dependent pathogenicity of FMDV infections.

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**Author contributions:** SO conceived and designed the study. SY and SO write manuscript. SY, NB, VB, DD, and SO performed the experiments. HL, YJ, TH, and SO performed the data analyses. The data interpretation was done by all authors, who also critically revised the manuscript for important intellectual content and granted their approval to the final version.

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