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Expression of Toll-like receptors 3, 7, 9 and cytokines in feline infectious peritonitis virus-infected CRFK cells and feline peripheral monocytes

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ABSTRACT

Background: The role of Toll-like receptors (TLRs) in a feline infectious peritonitis virus (FIPV) infection is not completely understood.
Objectives: This study examined the expression of TLR3, TLR7, TLR9, tumor necrosis factor-alpha (TNF-α), interferon (IFN)-β, and interleukin (IL)-10 upon an FIPV infection in Crandell-Reese feline kidney (CRFK) cells and feline monocytes.
Methods: CRFK cells and monocytes from feline coronavirus (FCoV)-seronegative cats and FCoV-seropositive cats were infected with type II FIPV-79-1146. At four, 12, and 24 hours post-infection (hpi), the expression of TLR3, TLR7, TLR9, TNF-α, IFN-β, and IL-10, and the viral load were measured using reverse transcription quantitative polymerase chain reaction. Viral protein production was confirmed using immunofluorescence.
Results: FIPV-infected CRFK showed the upregulation of TLR9, TNF-α, and IFN-β expression between 4 and 24 hpi. Uninfected monocytes from FCoV-seropositive cats showed lower TLR3 and TLR9 expression but higher TLR7 expression compared to uninfected monocytes from FCoV-seronegative cats. FIPV-infected monocytes from FCoV-seropositive cats downregulated TLR7 and TNF-α expression between 4 and 24 hpi, and 4 and 12 hpi, respectively. IFN-β was upregulated early in FIPV-infected monocytes from FCoV-seropositive cats, with a significant difference observed at 12 hpi compared to FCoV-seronegative cats. The viral load in the CRFK and FIPV-infected monocytes in both cohorts of cats was similar over time.
Conclusion: TLR7 may be the key TLR involved in evading the innate response against inhibiting TNF-α production. Distinct TLR expression profiles between FCoV-seronegative and FCoV-seropositive cats were observed. The associated TLR that plays a role in the induction of IFN-β needs to be explored further.

Keywords: Feline infectious peritonitis (FIP); Toll-like receptor (TLR); cytokine; monocytes

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Conflict of Interest

The authors declare no conflicts of interest.

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INTRODUCTION

Feline coronavirus (FCoV), an *Alphacoronavirus 1* species within the *Alphacoronavirus* genus, is divided into two biotypes: feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). FCoV can also be divided into types I and II depending on the spike (S) protein, where type I FCoV encodes the natural S protein, while type II FCoV encodes the canine coronavirus (CCV) S protein [1,2]. In naturally-infected cats, type I FECV infections are more prevalent than type II FECV infections, but the cats do not show apparent symptoms other than mild diarrhea [1]. The high genomic similarity between FECV and FIPV suggested that FIPV emerged from small mutational events in FECV within the same host [3]. FECV mainly infects intestinal epithelial cells, where an FIPV infection is mediated mainly by monocytes/macrophages, but the specific mechanism on the entry of FIPV into the monocytes/macrophages is unknown. Eventually, these FIPV-activated monocytes/macrophages express inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, adhesion molecules, and vascular endothelial growth factor, which causes endothelial cells activation, extravasation, and increased vascular permeability due to the net effect of inflammatory responses [4]. In general, strong cell-mediated immunity offers protection against the disease, and only a small percentage of FECV-infected cats (5–10%) that failed to mount a robust CMI would develop a fatal immune-mediated disease called feline infectious peritonitis (FIP) [5-9].

In addition to CMI, the severity of FIP was also correlated with the upregulation of humoral immunity in the host against the virus, a phenomenon known as antibody-dependent enhancement (ADE). Early studies showed that seropositive cats or seronegative cats immunized with FCoV-exposed sera would develop the disease faster than seronegative cats [10-12]. Alveolar macrophages produced high levels of surviving B cells and differentiation factors upon an FIPV infection that promoted B cells differentiation into plasma cells in FIP cats, which was enhanced by anti-FIPV S protein incubation [13]. Despite the reported association of monocytes and macrophages activation with possible hyperglobulinemia in an FIPV infection, the mechanism that shaped its pro-inflammatory response in cats of different serological statuses was not well understood.

Feline monocytes, macrophages, and epithelial cells express different numbers of Toll-like receptors (TLRs) [14,15]. TLR is one of the pattern recognition receptors localized either on the cell surface or in the endosomes that recognize various pathogen-associated molecular patterns and plays an important role in activating the innate immunity [15]. For example, surface TLRs, such as TLR2, TLR4, and TLR5, recognize the cell components of bacteria while endosomal TLRs, such as TLR3, TLR7, TLR8, and TLR9, detect nucleic acid components from viruses. In the context of coronavirus infections, TLR7 recognized the single-stranded RNAs of severe acute respiratory syndrome (SARS)-CoV in plasmacytoid dendritic cells and Middle-East respiratory syndrome (MERS)-CoV in lung epithelial cells, which subsequently caused the swift release of type I interferon (IFN) that suppressed the virus infection [16,17]. In contrast, a pathogenic strain of porcine epidemic diarrhea virus (PEDV) could inhibit the effects of TLR4, TLR7, TLR8, and TLR9 activation, shutting the production of pro-inflammatory cytokines as one of the strategies of the virus to evade the innate immunity [18].

Considering that the TLRs are important receptors expressed in monocytes/macrophages, and epithelial cells, the TLR activation or evasion used by other coronaviruses and the

influence of activated monocytes/macrophages on B cells differentiation, the immune induction through TLR, and its associated cytokines of the serological status of different hosts need to be evaluated further to determine any distinct pattern. Therefore, this paper provides new information on the TLR expressional changes upon an FIPV infection in monocytes from healthy FCoV-seropositive and FCoV-seronegative cats and Crandell-Reese feline kidney (CRFK) cells, which is a cell line derived from feline kidney epithelial cells and is susceptible to FIPV infections.

MATERIALS AND METHODS

In Vitro FIPV infection in CRFK cells

CRFK cells (ATCC CCL-94) were seeded at a density of 10^5 cells per well in MEM (Gibco, USA) supplemented with 10% FBS (Gibco) and 1% penicillin-streptomycin-glutamine (Gibco) in 6-well plates. After incubation for 24 h at 37°C and 5% CO_2, type II FIPV 79-1146 (ATCC VR-2202, USA) cells were was inoculated at a multiplicity of infection (MOI)=10 and allowed to adhere for 1 h in 37°C and 5% CO_2. The cells were washed twice with PBS (Sigma-Aldrich, St. Louis, MO), followed by the addition of MEM with 1% FBS and 1% penicillin-streptomycin-glutamine. Each well was incubated for 4, 12, or 24 h at 37°C and 5% CO_2. Three replicates (n = 3) were conducted per experiment. The media was removed at the designated time points, and the cells were washed twice with PBS before RNA extraction and immunofluorescence staining. Uninfected cells were prepared in parallel using empty media instead of the virus.

Isolation of feline monocytes and *ex vivo* infection with type II FIPV 79-1146

Animal ethics approval was obtained from the Institutional Animal Use and Care Committee (IACUC) of Universiti Putra Malaysia before sampling (UPM/IACUC/AUP-R092-2016). A total of 5–10 mL blood samples were collected from healthy, non-FIP shelter- or client-owned cats, kept in an EDTA tube (BD Vacutainer, USA) and screened for FeLV and FIV using a Rapid FeLV Ag/FIV Ab Test Kit (GenBody Inc., Korea) according to the manufacturer's protocols. The negative FIV/FeLV blood samples were then subjected to FCoV antibody titer measurement using an immunocomb antibody test kit (Biogal-Galed Laboratories, Israel). The anti-FCoV antibody titer was based on the intensity of the test color result, which corresponded to the combscale S value, of which cats with antibody titers of S0 or S1 were considered to be FCoV-seronegative cats. Cats with a titer of S2 to S5 were designated as FCoV-seropositive cats [19]. Subsequently, the monocytes from FCoV-seropositive cats (n = 8) and FCoV-seronegative cats (n = 5) were isolated from peripheral blood mononuclear cells (PBMC). PBMC was isolated within 2–3 h after blood collection using Ficoll-paque PLUS (GE Healthcare, USA) according to the manufacturer's recommendations. Briefly, the blood was diluted 1:1 with RPMI media (Gibco) and layered on top of an equal volume of Ficoll-paque PLUS in a 15 mL conical tube. The tubes were centrifuged in a swing-rotor centrifuge at room temperature for 20 min at 1,500 rpm, and the buffer layer containing PBMC was transferred into a new 15 mL conical tube. The PBMCs were washed twice with 10 mL RPMI media at 1,000 rpm for 10 min. Subsequently, the number of cells and cell viability was determined using Trypan Blue exclusion method. The number of cells was then adjusted to a density of 10^5 cells/mL in RPMI media (Gibco), containing 10% FBS and 1% penicillin–streptomycin-glutamine. The PBMCs from each cat were seeded at a density of 10^5 cells/mL in a 24-well plate, and incubated at 37°C in a 5% CO_2 incubator for 2 h before harvesting. Upon harvesting, the monocytes were purified by removing the non-adherent cells, and the adherent cells were washed twice with warm RPMI. The isolated monocytes were inoculated with FIPV 79-1146 at MOI=10 or

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complete RPMI alone for the control wells and incubated for 1 h to allow virus attachment. Subsequently, 1 mL of complete RPMI was added to the virus-inoculated monocytes and control wells and incubated for 4, 12, or 24 h in 37°C and 5% CO_2. The monocytes were also seeded on extra wells to determine the purity of monocytes by flow cytometry, and the adherent cells were detached using cold PBS containing 2% FBS and 2 mM EDTA after washing with warm RPMI. In the wells for reverse transcription quantitative polymerase chain reaction (RT-qPCR), 200 μL Tri Reagent (Zymo Research, USA) was added, mixed, and proceeded for RNA extraction. In the wells for IF, 200 μL of 4% paraformaldehyde in PBS was added to fix the monocytes before IF staining.

Flow cytometry

The purity of the monocytes was determined using anti-human CD14-PE (clone TUK4) (Miltenyi Biotec, USA), which cross-reacted with feline CD14 [20]. The cells were pelleted by centrifugation at 300 ×g for 10 min and resuspended in 100 μL of staining buffer containing 2% FBS and 2 mM EDTA. Ten microliters of antibody were added to the cells and incubated at room temperature. The cells were washed once with staining buffer, resuspended in 1% paraformaldehyde, and stored at 4°C for up to 24 h before flow cytometry. The population of positive cells was determined by gating a similar population of unstained cells. The flow cytometry data of 5,000 events were acquired using BD FACSCanto (BD Biosciences, USA) followed by data analysis using BD FACSDiva software (BD Biosciences). The purity of isolated monocytes was more than 90% (**Supplementary Table 1**, **Supplementary Figs. 1** and **2**).

RNA extraction and cDNA synthesis

For the CRFK experiment, the total RNA was extracted using an easy-BLUE Total RNA Extraction Kit (Intron Biotechnology, Korea) following the manufacturer's recommendations. Upon cell lysis with 1 mL of easy-BLUE reagent (Intron Biotechnology), 200 μL of chloroform was added. The cells were vortexed briefly and centrifuged at 13,000 rpm at 4°C for 10 min before transferring 400 μL of the aqueous phase to a new tube. RNA was precipitated by mixing with 800 μL of 96%–100% ethanol and incubated for 10 min at room temperature. After centrifugation at 13,000 rpm (4°C) for 5 min, the RNA pellet was washed twice with 75% ethanol, each at 10,000 rpm (4°C) for 5 min before removing the supernatant and letting the RNA dry. The RNA was dissolved in 50 μL of RNAse-free water (Macherey-Nagel, Germany). The extracted RNA was treated with rDNAse (Macherey-Nagel) according to the manufacturer's recommendations and re-purified using the phenol-chloroform and ethanol precipitation method. The purity and concentration were measured using Biophotometer (Eppendorf, USA). The RNA purity (260/280) ranged from 1.76 to 2.01, whereas the concentration (ng/μL) ranged from 32.1 to 102.4. For the feline monocytes experiment, the total RNA was extracted using Direct-zol RNA Miniprep PLUS (Zymo Research) according to the manufacturer's recommendations. After lysis with 200 μL TRI Reagent (Zymo Research), the reagent was mixed with an equal volume (200 μL) of absolute ethanol and transferred to Zymo-Spin IIICG Column (Zymo Research) before centrifuging at 10,000 ×g for 30 sec. After binding RNA onto the column, 400 μL of RNA wash buffer was added and centrifuged before incubating with 80 μL of 6U/μL DNAse I in the column for 15 min at room temperature. The column was washed twice with 400 μL of Direct-zol RNA PreWash before a final wash with 700 μL RNA wash buffer and centrifuged at 10,000 ×g for 30 sec. The RNA was eluted with 20 μL DNAse/RNase-free water, and the purity and concentration were measured using Biophotometer (Eppendorf). The RNA purity (260/280) ranged from 1.35 to 2.22, while the concentration (ng/μL) ranged from 5.3 to 72.6. Subsequently, 200 ng (for CRFK) or 20 ng (for monocytes) of the extracted RNA was

immediately converted to cDNA using SensiFAST cDNA Synthesis Kit (Bioline, UK) according to the manufacturer's recommendations. The reaction product was stored at −20°C prior to quantifying the gene expression and viral load using RT-qPCR.

RT-qPCR

Gene expression and the viral load were measured using SensiFast Probe No-ROX kit (Bioline) and CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) with a slight modification. The primers and probe for viral load quantification were obtained from Gut et al. [21], while TNF-α and IL-10 were measured according to Kipar et al. [22]. The primers and probes for GAPDH, β-2-microglobulin TLR3, TLR7, TLR9, and IFN-β were designed using PrimerQuest Tool (Integrated DNA Technologies, USA) based on *Felis catus* mRNA accession ID obtained from the National Center for Biotechnology Information (NCBI). The primers were tested by conventional PCR using a representative sample. The PCR products were purified by gel electrophoresis, sequenced, and analyzed using NCBI BLAST to determine the specificity (**Supplementary Fig. 3**). All primers and probes used in this study had efficiencies ranging from 95% to 110%, and **Table 1** lists their respective annealing temperatures. Briefly, a single tube of 20 μL reaction contained 10 μL of SensiFast Probe No-ROX mix (Bioline), 0.8 μL (400 nM) F primer, 0.8 μL (400 nM) R primer, 0.4 μL (200 nM) probe, 1 μL DMSO, 6 μL deionized water, and 1 μL cDNA. The samples were run in duplicate for CRFK cells, whereas the monocyte samples were run in triplicate. No template control and no reverse transcription control were included in duplicate for each qPCR run.

Table 1. List of primers and probes, sources, and annealing temperature, T_a (°C) used in this study

Gene	Sequence (5′–3′)	Reference	T_a (°C)
TLR3	F: AAACTGTTGGAGGGTCTTGAG R: AGAATGTGGAGGTGAGAAAGAC P: ACGGCTATGGAAACGTGCAAACC	NM_001079829.1	62
TLR7	F: GTCTCCCCTTTCTGTTTCTGT R: GAGTAGAAGCACGATTCCTCTG P: CCTTATGTCCTCTCCTTGCCGCC	NM_001080133.1	56
TLR9	F: CCTGGAGTACCTGCTATTGTCCTAC R: CGGCAGTTTCCACCCACATC P: AACCACATCATCACCCTGGCACCTG	NM_001009285.1	60
TNF-α	F: CTTCTCGAACTCCGAGTGACAAG R: CCACTGGAGTTGCCCTTCA R: TAGCCCATGTAGTAGCAAACCCCGAAGC	Kipar et al., 2001 [22]	60
IFN-β	F: CCACTGTTGAGAACCTCCTTG R: CAGGTTCAGAAGGGTCGTATTG P: ACTGGCAGAAGGAACACCTGGAAA	NM_001009297.1	63
IL-10	F: TGCACAGCATATTGTTGACCAG R: ATCTCGGACAAGGCTTGGC P: ACCCAGGTAACCCTTAAGGTCCTCCAGCA	Kipar et al., 2001 [22]	60
GAPDH	F: CATCAATGGAAAGCCCATCAC R: CCCAGTAGACTCCACAACATAC P: CCCATTTGATGTTGGCGGGATCTC	NM_001009307.1	62
β-2-microglobulin	F: TCTGGTCCACACCGAATTTAC R: ACATGTCTCGATCCCACTTAAC P: TCCCACTGTCGAAGATGAGTATAGCTGC	NM_001009876.1	53
FIPV 7b gene	F: GATTTGATTTGGCAATGCTAGATTT R: AACAATCACTAGATCCAGACGTTAGCT P: TCCATTGTTGGCTCGTCATAGCGGA	Gut et al., 1999 [21]	56

TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha; IFN, interferon; IL, interleukin.

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FCoV IF Staining

IF staining was performed using FIPV3-70 (Custom Monoclonals Inc., USA) as the primary antibody that targeted the nucleocapsid (N) protein of FCoV to confirm the productive FIPV 79-1146 infection on both CRFK cells and monocytes. Alexa Fluor 488 goat anti-mouse IgG (H+L) (Invitrogen, USA) was used as the secondary antibody to detect the primary antibody. After 4% paraformaldehyde fixation, the cells were permeabilized with 0.25% Triton X-100 in PBST (0.1% Tween in PBS) and washed three times with PBS, each for 5 min. The cells were incubated with the blocking buffer (5% goat serum in PBST and 1% FBS) for 30 min at room temperature, and the supernatant was discarded. The cells were incubated with the primary antibody at 37°C for 1 h and washed three times with PBS. The cells were incubated with the secondary antibody at room temperature for 1 h and washed three times with PBS. The cells were incubated with 1 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 10 min to stain the nucleus, followed by visualization under fluorescence microscopy (Nikon, USA). The percentage of virus-positive cells was determined by dividing the total number of cells with the virus antigen by the total number of cells indicated by DAPI staining in the same frame.

Data analysis

Viral load was measured by absolute quantification based on the standard curve constructed from virus cDNA. The uninfected monocytes in the FCoV-seronegative and FCoV-seropositive cats were compared by measuring the gene expression of uninfected monocytes after 24 h culture using relative quantification according to the $2^{-\Delta\Delta Ct}$ method of which the geometric mean Ct value from two reference genes (GAPDH and β-2-microglobulin) were used for normalization and was calibrated with 4 h culture [23,24]. The relative gene expression of the FIPV-infected CRFK and FIPV-infected monocytes were measured using the same formula described above with uninfected CRFK cells or uninfected monocytes at the same time point as a calibrator.

The viral load and gene expression changes between the time points post-infection and between FCoV-seronegative and FCoV-seropositive cats were analyzed using GraphPad Prism version 8.0.2 (GraphPad Software, Inc., USA). The distribution of these qRT-PCR data was first assessed using a Shapiro–Wilk test to determine the normality of which all data were not normally distributed. The relative gene expression of uninfected monocytes from FCoV-seronegative and FCoV-seropositive cats were analyzed using a Mann–Whitney test. In addition, a Mann–Whitney test was also used to compare the relative gene expression of FIPV-infected monocytes between FCoV-seronegative and FCoV-seropositive cats at 4 hpi, 12 hpi, and 24 hpi. The relative expression of TLR and cytokines genes for FIPV-infected CRFK cells and FIPV-infected monocytes among the different time points were assessed with Kruskal–Wallis test using a Dunn's *post hoc* test. A p value of ≤ 0.05 for the Kruskal–Wallis test (p^a) and the Dunn's *post hoc* test (p^b) indicated a statistically significant difference among or between the different time points, respectively. The Mann–Whitney test (p^c) indicated a comparison between the FCoV-seronegative and seropositive cats.

RESULTS

Viral load quantification and antigen expression in FIPV-infected CRFK cells

The productive viral replication in FIPV-infected CRFK cells was determined by measuring the intracellular viral load was measured by RT-qPCR. Antigen expression was detected

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Table 2. Intracellular viral load in CRFK cells infected with FIPV 79-1146 at 4, 12, and 24 hpi

Hours post-infection (hpi)	Viral load, log10 copy number (mean ± SD)
4	12.016 ± 0.120
12	13.708 ± 0.082
24	13.744 ± 0.264

The data were represented as mean ± SD and analyzed using the Kruskal–Wallis test with Dunn's *post hoc* test where no significant difference was detected.

by IF staining at 4, 12, and 24 hpi. The viral load showed an increasing trend ($p = 0.050$) from $\log10^{12.016 \pm 0.120}$ to $\log10^{13.744 \pm 0.264}$ copy number, but no significant difference was detected between each time point post-infection in FIPV-infected CRFK cells (**Table 2**). The percentage of CRFK cells expressing the virus antigen (mean ± SD) increased steadily from 4 hpi (no detection) to 12 hpi (mean ± SD: 53.4 ± 4.3%) to 24 hpi (mean ± SD: 75.4 ± 5.4%) (**Supplementary Fig. 4**), which was consistent with the data from a previous study [25]. The uninfected control CRFK cells showed no virus RNA or virus antigen expression (data not shown).

TLRs and cytokines gene expression in FIPV-infected CRFK cells

The transcriptional changes of TLR and cytokine genes upon FIPV infection in CRFK cells were determined by calculating the relative expression of FIPV-infected CRFK cells to uninfected CRFK cells using the $2^{-\Delta\Delta Ct}$ method at each time point post-infection. Although TLR3 expression showed a decreasing expression trend ($p^a = 0.050$), there was no significant difference between the time points post-infection (**Fig. 1**). The FIPV-infected CRFK cells showed the significant upregulation of TLR9, TNF-α, and IFN-β between 4 and 24 hpi ($p^b = 0.034$, 0.022, and 0.022 respectively) (**Fig. 1**). There were no significant changes in IL-10 expression in FIPV-infected CRFK cells when comparing the different time points. TLR7 mRNA, which detects single-stranded RNA motifs, was not detected in both FIPV-infected and uninfected CRFK cells at all time points (data not shown).

Viral load and viral antigen protein expression in FIPV-infected monocytes from FCoV-seropositive and FCoV-seronegative cats

The viral load and viral antigen protein expression were measured to determine the productive viral replication in FIPV-infected monocytes from FCoV-seropositive cats (n = 8) and FCoV-seronegative cats (n = 5). No significant changes in the viral load were observed among the different time points for the FIPV-infected monocytes from each cohort of cats (**Table 3**). On the other hand, when comparing the FIPV-infected monocytes between FCoV-seronegative and seropositive cats at each time point, a significantly higher viral load in the FCoV-seropositive cats was observed at 12 hpi ($p = 0.030$) between the two cohorts of cats. The virus antigen expression was detected at a small percentage of monocytes through FIPV N viral protein immunostaining ranging from 0.385–22.5% (**Table 4**, **Supplementary Fig. 5**). For the FIPV-infected monocytes from the FCoV-seropositive cats, 4/8 (50.0%) cats showed positive detection at 4 hpi, 7/8 (87.5%) at 12 hpi, and 6/8 (75.0%) at 24 hpi. For FIPV-infected monocytes from FCoV-seronegative cats, 1/5 (20.0%) cats showed positive detection at 4 hpi, 4/5 (80.0%) at 12 hpi, and 3/5 (60.0%) at 24 hpi. The monocytes from five cats (Cats 3, 6, 9, 11, and 13) showed positive detection at all time points. The monocytes from four cats (Cat 1, 2, 7, and 8) showed positive detection at 12 and 24 hpi. The monocytes from two cats (Cat 4 and 12) showed positive detection at only 12 hpi. Monocytes from one FCoV-seronegative and one FCoV-seropositive cat (Cat 5 and 10) showed no detection at any of the time points. Uninfected monocytes of both FCoV-seropositive and FCoV-seronegative cats showed no viral RNA and antigen (data not shown).

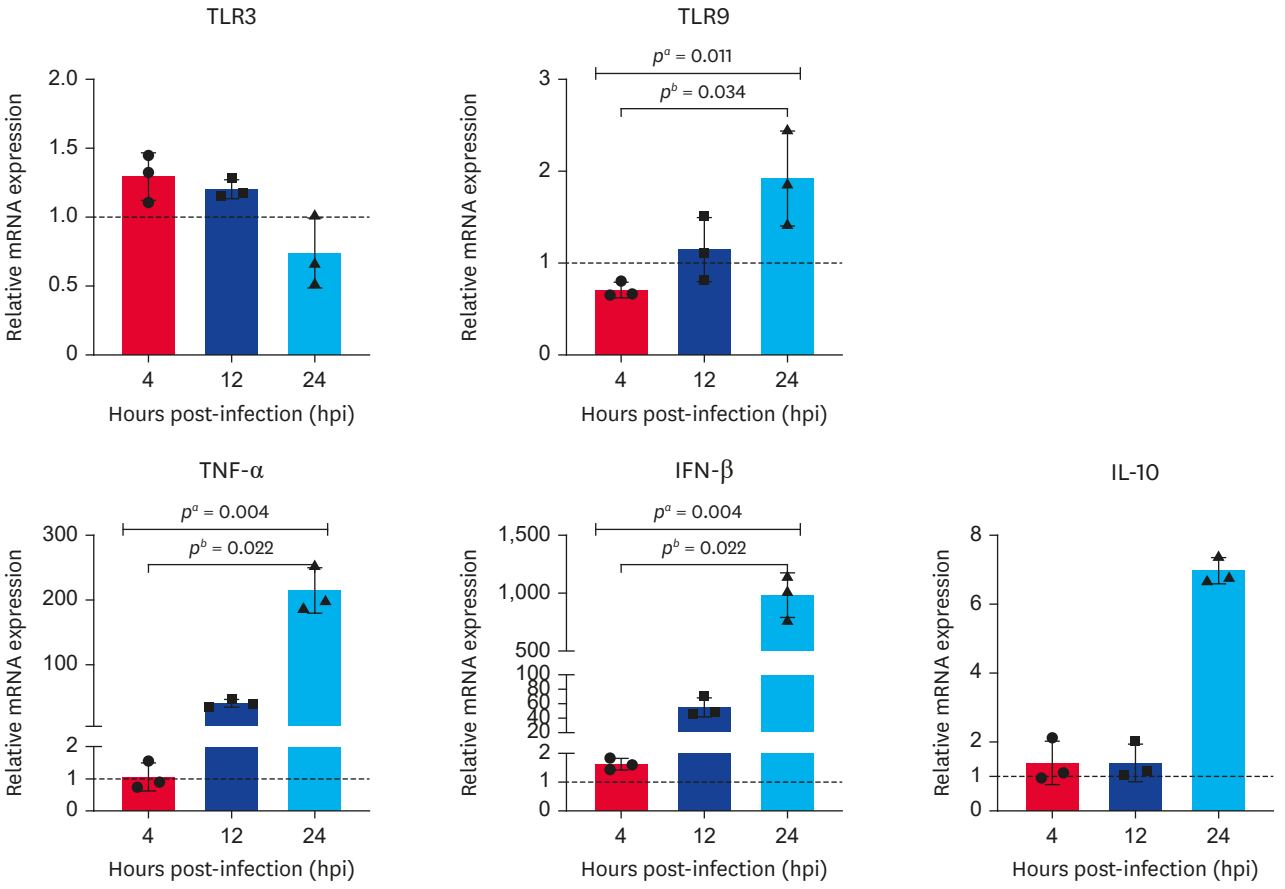

Fig. 1. Relative expression of TLRs and cytokines in CRFK cells infected with FIPV 79-1146 at 4, 12, and 24 hpi. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method using GAPDH and β-2-microglobulin as reference genes for normalization and the expression of uninfected CRFK cells at the same time point as calibrator (horizontal dashed line). The data were reported as mean (top of the bar) ± SD (error bar) and analyzed using Kruskal–Wallis test with Dunn's *post hoc* test. The *p* values, p^a and p^b for Kruskal–Wallis and Dunn's *post hoc* tests, respectively, were considered significant among the different time points (4, 12, and 24 hpi) if $p \leq 0.05$.
TLR, Toll-like receptor; TNF-α, tumor necrosis factor-alpha; IFN, interferon; IL, interleukin; CRFK, Crandell-Reese feline kidney; FIPV, feline infectious peritonitis virus.

Table 3. Intracellular viral load in monocytes from FCoV-seronegative and FCoV-seropositive cats infected with FIPV 79-1146 at 4, 12, and 24 hpi

Hours post-infection (hpi)	Viral load, log10 copy number (mean ± SD)	
	FCoV-seronegative	FCoV-seropositive
4	10.234 ± 1.104	11.462 ± 0.642
12	11.045 ± 0.551[a]	11.985 ± 0.461[a]
24	10.937 ± 0.870	11.648 ± 0.510

The data were represented as mean ± SD and analyzed using Kruskal–Wallis test with Dunn's *post hoc* test where no significant difference was detected.
FCoV, feline coronavirus; FIPV, feline infectious peritonitis virus.
The alphabet [a] indicated significant difference ($p \leq 0.05$) between FCoV-seronegative and FCoV seropositive cats using the Mann–Whitney test.

TLRs and cytokines gene expression in uninfected monocytes from FCoV-seronegative and FCoV-seropositive cats

The relative expression of TLR and cytokine genes of uninfected monocytes from FCoV-seronegative cats and FCoV-seropositive cats were measured after a 24 h short-term culture (**Fig. 2**). The levels of TLR3 and TLR9 expression in the uninfected monocytes from FCoV-seronegative cats were higher than the uninfected monocytes from the FCoV-seropositive cats (p^c = 0.008 and 0.045, respectively). In contrast, TLR7 expression was higher in the uninfected monocytes from the FCoV-seropositive cats than those of the FCoV-seronegative cats (p^c = 0.002). There were no significant differences in the expression of cytokines (TNF-α, IFN-β, and IL-10) between the two cohorts of cats.

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Table 4. Percentages of virus antigen-positive monocytes after infection with FIPV 79-1146 at 4, 12, and 24 hpi for FCoV-seronegative and FCoV-seropositive cats

Cat ID	Hours post-infection (hpi)		
	4 hpi	12 hpi	24 hpi
FCoV-seronegative			
1	-	+ (22.5%)	+ (8.33%)
2	-	+ (2.86%)	+ (4.55%)
3	+ (5.21%)	+ (4.11%)	+ (7.12%)
4	-	+ (3.23%)	-
5	-	-	-
FCoV-seropositive			
6	+ (3.04%)	+ (1.19%)	+ (4.88%)
7	-	+ (1.16%)	+ (1.05%)
8	-	+ (0.877%)	+ (3.26%)
9	+ (0.826%)	+ (0.385%)	+ (1.47%)
10	-	-	-
11	+ (5.59%)	+ (10.6%)	+ (9.79%)
12	-	+ (0.629%)	-
13	+ (1.15%)	+ (5.618%)	+ (0.985%)

"-" indicates no detection, "+" indicates positive detection, and "()" indicates the percentage of virus antigen-positive monocytes.
FIPV, feline infectious peritonitis virus; FCoV, feline coronavirus.

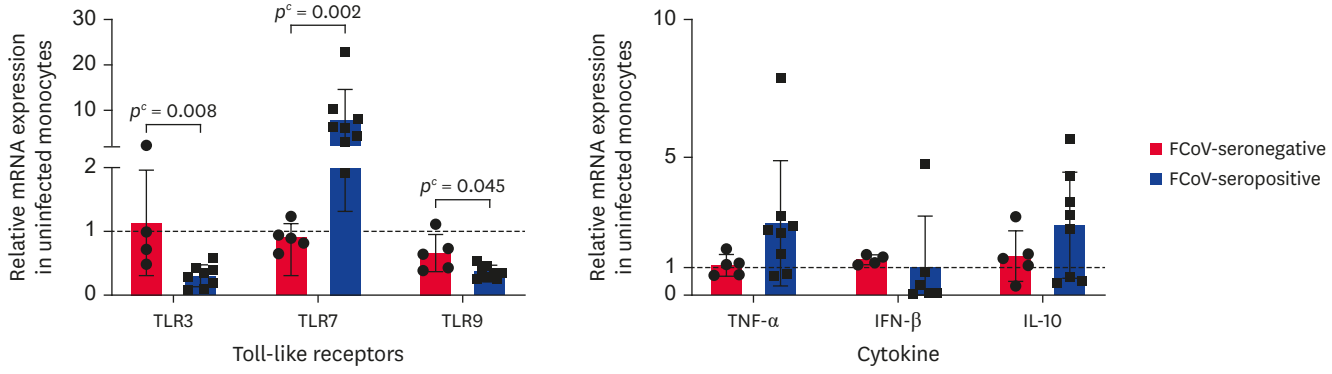

Fig. 2. Relative expression of TLRs and cytokines in uninfected monocytes from FCoV-seronegative (white) and FCoV-seropositive (grey) cats cultured for 24 h. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method using GAPDH and β-2-microglobulin as reference genes for the normalization and expression of uninfected monocytes at 4 hpi as calibrator (horizontal dashed line). The data were reported as the mean (top of the bar) ± SD (error bar) and analyzed using Mann–Whitney test where the *p* value, $p^c \leq 0.05$ was considered significant between the different groups of cats.
TLR, Toll-like receptor; FCoV, feline coronavirus; TNF-α, tumor necrosis factor-alpha; IFN, interferon; IL, interleukin.

TLRs and cytokines gene expression in FIPV-infected monocytes from FCoV-seronegative and FCoV-seropositive cats

The relative expression of TLR and cytokine genes of FIPV-infected monocytes from FCoV-seronegative and FCoV-seropositive cats were measured at each time point post-infection (**Fig. 3**). For FIPV-infected monocytes from FCoV-seronegative cats, there were no significant changes in any of the genes tested (TLR3, TLR7, TLR9, TNF-α, IFN-β, and IL-10) among the different time points post-infection. By contrast, FIPV-infected monocytes from FCoV-seropositive cats showed significant downregulation of TLR7 expression (p^a = 0.001) with *post-hoc* analysis showing a significant result between four and 24 hpi (p^b = 0.001). TNF-α also downregulated expression among the FCoV-seropositive cats (p^a = 0.022) with a significant result observed between 4 and 12 hpi (p^b = 0.018). There were no differences in the TLR3, TLR9, IFN-β, and IL-10 expression between the different time points post-infection.

Comparisons of the changes in gene expression of FIPV-infected monocytes were also made between the FCoV-seronegative and FCoV-seropositive cats. FIPV-infected monocytes from FCoV-seropositive cats had a significantly higher level of TLR7 than FCoV-seronegative cats

https://doi.org/10.4142/jvs.21225

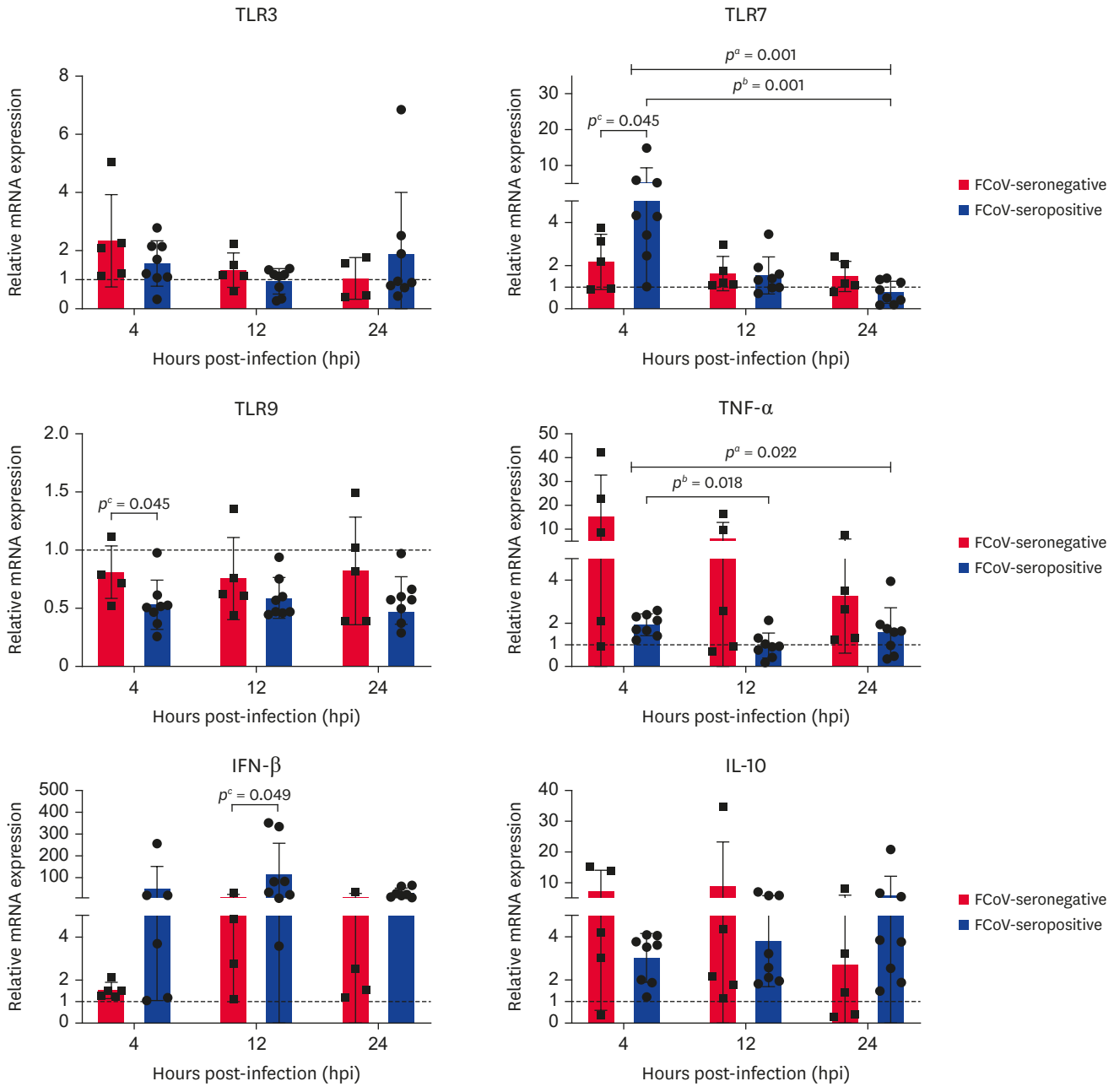

Fig. 3. Relative expression of TLRs and cytokines in monocytes from FCoV-seronegative (white) and FcoV-seropositive (grey) cats infected with FIPV 79-1146 at 4, 12, and 24 hpi. The relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method using GAPDH and β-2-microglobulin as reference genes for normalization and the expression of uninfected cells at the same time point, as calibrator (horizontal dashed line). The data were represented as mean (top of the bar) ± SD (error bar) and analyzed using the Kruskal-Wallis test with Dunn's *post hoc* test. The *p* values, p^a and p^b for Kruskal-Wallis and Dunn's *post hoc* tests, respectively, $p \leq 0.05$ was considered significant between the different time points (4, 12, and 24 hpi). The data was also analyzed using Mann–Whitney test where the *p* value, p^c of ≤ 0.05 was considered significant between the different groups of cats.
TLR, Toll-like receptor; FCoV, feline coronavirus; TNF-α, tumor necrosis factor-alpha; IFN, interferon; IL, interleukin.

at 4 hpi (p^c = 0.045) (**Fig. 3**). In contrast, TLR9 expression in FIPV-infected monocytes from FCoV-seropositive cats was significantly lower than FCoV-seronegative cats at 4 hpi (p^c = 0.045). IFN-β was upregulated early in the FCoV-seropositive cats at 4 hpi compared to the FCoV-seronegative cats at 12 hpi, of which a significant difference was observed at 12 hpi (p^c = 0.049). On the other hand, there were no differences in TLR3, TNF-α, and IL-10 gene expression between the two cohorts of cats.

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DISCUSSION

The roles of humoral immunity and monocytes-induced inflammatory reaction associated with ADE in promoting FIP have been described [10-13,26]. On the other hand, the TLRs, which are important receptors of monocytes/macrophages that may be involved in orchestrating the immune response of FCoV serological status of different cats, are not well understood. This study measured the gene expression of TLR3, 7, and 9 *in vitro* using CRFK cells, a well-known cell line susceptible to FIPV 79-1146 infections and in FIPV-infected peripheral monocytes from FCoV-seronegative and FCoV-seropositive cats. The downstream cytokines involved in inflammation (TNF-α), antiviral response (IFN-β), and anti-inflammation (IL-10), which were immune mediators in FIP development, were also measured to determine how the TLRs modulated cytokines expression.

From the pattern of the TLRs tested, the FIPV 79-1146 involved in downregulating TLR7 expression by monocytes from FCoV-seropositive cats. This is highlighted by the significantly higher level of TLR7 expression in the uninfected cells of the FCoV- seropositive cats *vs.* seronegative cats. On the other hand, upon the FIPV 79-1146 infection, the TLR7 expression was downregulated significantly between 4 hpi and 24 hpi, indicating that viral replication may block the expression of the TLR7 gene. Inhibition of the TLR7 signaling pathway has been reported to be mediated by the ORF1a-encoded papain-like protease of SARS-CoV [27]. Unfortunately, CRFK cells lacked TLR7 expression. Therefore, the consistency of TLR7 induction both in CRFK and monocytes could not be established [13]. Nevertheless, TLR9 gene expression was upregulated in the FIPV-infected CRFK cells. On the other hand, the level of TLR9 gene expression in the FIPV-infected monocytes in FCoV-seronegative and seropositive cats was relatively lower than the basal level, i.e., the uninfected cells indicated that the viral infection did not trigger activation of TLR9 expression. Although the TLR9 expression in FIPV-infected monocytes from FCoV-seronegative cats was significantly higher than the seropositive cats at 4 hpi (**Fig. 3**), it was unlikely because of the effect of FIPV infection as the uninfected cells from the two cohorts of cats showed a similar level of expression with FIPV-infected monocytes (**Fig. 2**). Indeed, these findings partly confirmed the observation that TLR9 was downregulated in cats with FIP, indicating that TLR9 may play a role in the protective immune response towards the host [28].

TLR induction will lead to the activation of a NF-κB signaling pathway or IFN regulatory factor (IRF) with the outcome of cytokines production mainly to induce inflammation and control viral replication, respectively [15]. TNF-α is one of the pro-inflammatory cytokines produced through the activation of NF-κB signaling. Based on these results, there were no significant changes in TNF-α expression of the uninfected cells between FCoV-seronegative and FCoV-seropositive cats (**Fig. 2**). Upon FIPV infection, there were varying increases in TNF-α expression in the infected monocytes of FCoV-seronegative cats compared to the uninfected cells (calibrator). In contrast to the FCoV-seronegative cats, there was a minimal increase in TNF-α expression in the FIPV-infected monocytes of FCoV-seropositive cats, while a significant decrease in TNF-α expression was observed from 4 hpi to 12 hpi, suggesting that TNF-α was possibly suppressed in this cohort of cats. TLR7 expression was downregulated in response to the pathogenic PEDV infection *in vivo*, causing the inhibition of NF-κB signaling and a decrease in TNF-α expression [18]. Considering that TLR7 was also downregulated in FIPV-infected monocytes from seropositive cats, TNF-α induction through NF-κB signaling may have been suppressed. In contrast, increases in TNF-α mRNA expression and protein production were observed in multiple FIP studies [28-30]. The increased level of TNF-α

also promoted the upregulation of feline aminopeptidase N (fAPN), which acts as an FIPV receptor, making the cells more susceptible to infection [31]. Whether or not the TLR7-mediated TNF-α suppression was used by FIPV as an innate evasion strategy in FCoV-seropositive cats could be investigated further by studying the effects of TLR7 agonist or antagonist in the presence of FIPV infection.

Through the IRF-signaling pathway, type I IFNs, either IFN-α or IFN-β, were induced and activated the Janus kinase signal transducer and activator of transcription (JAK-STAT) and signal transduction leading to the antiviral state in the cells. These findings suggest that IFN-β expression in uninfected monocytes from both cohorts of cats was at the basal level. On the other hand, the induction of IFN-β expression in monocytes from FCoV-seropositive cats upon an FIPV infection could be observed as early as 4 hpi compared to 12 hpi in FCoV-seronegative cats. Other TLR, such as TLR8, were capable of inducing IFN-β production via the IRF-signaling pathway triggered by FIPV infection, as reported by other FCoV studies because there was no similar pattern between the TLRs investigated in this study with IFN-β induction [28,32]. Furthermore, IFN-β production mediated by TLR3 induction inhibited type I FIPV replication in Fcwf-4 cells [33]. On the other hand, there were no significant changes in TLR3 gene expression in both CRFK and monocytes from the two cohorts of cats, possibly due to the different types of cells used in this study. Nevertheless, given the importance of IFN-β in limiting viral replication in the infected host, studies investigating TLR8 and its possible association with the production of IFN-β are warranted.

The expression of cytokines (TNF-α, IFN-β, and IL-10) was similar in uninfected monocytes from both cohorts, which was expected because cytokines are produced in response to stimuli [34]. On the other hand, the level of TLR expression in healthy uninfected monocytes was either downregulated (TLR3 and TLR9) or upregulated (TLR7) in FCoV-seropositive cats. M1 macrophage profile, an inflammatory type of macrophage, was observed from ascitic peritoneal macrophages in experimentally-induced FIP cats with limited mRNA expression of genes associated with M2 macrophage (anti-inflammatory) activation [35]. TLR signaling was one of the pathways observed in the profiling, suggesting that the macrophage profile in the FCoV-seropositive cats may be of the M1 type. This was supported by the non-significant changes in IL-10, an anti-inflammatory cytokine associated with the characteristics of M2 macrophages, observed in the FCoV-seropositive cats. Nevertheless, future studies should examine the macrophage type or markers in a FIP infection.

The viral load in FIPV-infected monocytes from the FCoV-seropositive cats was slightly higher than that in the FCoV-seronegative cats at 12 hpi. This could be due to the influence of FCoV antibody exposure as a result of the previous FCoV infection, which might have pre-sensitized the monocytes of FCoV-seropositive cats to FIPV. Several reports demonstrated the impact of ADE and sustainable FIPV infection to induce a remarkable immune reaction, which led to FIP development [6,9,26,36]. The monocytes of FCoV-seropositive cats may have immune memory [37,38], which shapes the response against FIPV infection, and TLR7 could be the key for an antiviral immune response because it was highly elevated in uninfected monocytes but suppressed upon infection. Despite the high efficiency of virus internalization into monocytes [39], the virus replication and production in FIPV-infected monocytes was restricted in both cohorts of cats. Two studies also reported the phenomena of intrinsic resistance displayed by monocytes against FIPV infection, where the amount of virus production was less than 1% when infected with MOI 5 or 100 [38,40]. On the other hand, it is unclear if TLR plays a role during this mechanism.

In conclusion, this study provided new insights on the involvement of TLR7 in FIPV-infected monocytes. Despite other reports of TLR induction in FIPV, this was the first study that identified the possible involvement of TLR7 in suppressing TNF-α expression upon an FIPV infection. In addition, some elements of immune responses induced by monocytes from FCoV-seropositive and FCoV-seronegative cats during FIPV infection were different. Nevertheless, the immune response from monocytes of both groups resulted in suppressed viral production, indicating that monocytes showed a certain degree of resistance towards an FIPV infection [38]. In addition, the associated TLR that induces the production of IFN-β, an important IFN involved in the antiviral immune response, needs to be explored further. These data provide information on the use of TLR as new immunotherapeutic strategies for the treatment of FIP symptoms in cats.

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SUPPLEMENTARY MATERIALS

Supplementary Table 1

Summary of cats sampled and monocytes purity

Click here to view

Supplementary Fig. 1

Purity analysis of monocytes isolated from FCoV-seropositive cat (Cat ID: 11) as representative. Cells were stained with anti-human CD14-PE (clone TUK4) antibody as described in methods section and compared with unstained cells of same cat for gating analysis.

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Supplementary Fig. 2

Purity analysis of monocytes isolated from FCoV-seropositive cat (Cat ID: 11) as representative. Cells were stained with anti-human CD14-PE (clone TUK4) antibody as described in methods section and compared with unstained cells of same cat for gating analysis. FCoV, feline coronavirus.

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Supplementary Fig. 3

Representative of reverse transcription quantitative polymerase chain reaction product of TLRs and cytokines genes run on 1.5% w/v agarose gel. Lanes 1 and 11: 100 bp ladder; lane 2: β-2-microglobulin; lane 3: GAPDH; lane 4: TLR3; lane 5: TLR7; lane 6: TLR9; lane 7: TNF- α; lane 8: IFN-β; lane 9: IL-10; lane 10: FIPV 7b gene. The bands were gel purified, sequenced, and analyzed using Basic Local Alignment Search Tool (BLAST) to confirm the specificity of genes tested.

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Supplementary Fig. 4

The detection of viral nucleocapsid (N) protein expression in CRFK cells at 4 hpi (A), 12 hpi (B), and 24 hpi (C). The blue staining (4′,6-diamidino-2-phenylindole, DAPI) indicated cell nucleus and green staining (Alexa Fluor 488) indicated virus antigen. Image was captured under 100× magnification.

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Supplementary Fig. 5

Representative image of viral nucleocapsid (N) protein expression in FIPV 79-1146-infected monocytes from cat 11 (FCoV-seropositive) (A-C) and cat 3 (FCoV-seronegative) (D-F) at 4 hpi (A and D), 12 hpi (B and E), and 24 hpi (C and F). The blue staining (4′,6-diamidino-2-phenylindole, DAPI) indicated cell nucleus and green staining (Alexa Fluor 488) with arrows indicated viral protein antigen. Image was captured under 200× magnification.

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