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Antiviral Research 99 (2013) 100–107

Contents lists available at SciVerse ScienceDirect

Antiviral Research

journal homepage: www.elsevier.com/locate/antiviral

Effect of chloroquine on feline infectious peritonitis virus infection *in vitro* and *in vivo*

Tomomi Takano, Yasuichiroh Katoh, Tomoyoshi Doki, Tsutomu Hohdatsu *

Laboratory of Veterinary Infectious Disease, School of Veterinary Medicine, Kitasato University, Towada, Aomori 034-8628, Japan

ARTICLE INFO

Article history:
Received 9 January 2013
Revised 22 March 2013
Accepted 24 April 2013
Available online 3 May 2013

Keywords:
Feline infectious peritonitis
Feline coronavirus
Chloroquine

ABSTRACT

Feline infectious peritonitis (FIP) is a feline coronavirus-induced fatal disease in domestic and wild cats. Several studies have investigated potential treatments for FIP. However, there have been no reports on agents that have exhibited a therapeutic effect. Recently, chloroquine has been reported to antiviral effect. We investigated whether chloroquine can be used to treat FIP *in vitro* and *in vivo*. It was demonstrated that chloroquine has inhibitory effect against the replication of FIPV and anti-inflammatory effect *in vitro*. *In vivo* study using cats with experimentally induced FIP, the clinical score of chloroquine-treatment groups were better than in chloroquine-untreated group. However, alanine aminotransferase levels increased in the chloroquine-treated groups. It will be necessary to further investigate the possibility of FIP treatment with a combination of chloroquine and other agents.

1. Introduction

Coronaviruses cause diseases of the respiratory, digestive, and nervous systems in mammals and birds. After severe acute respiratory syndrome coronavirus (SARS-CoV) was newly identified as a pathogen of fatal respiratory disease in 2003 (Ksiazek et al., 2003), new coronavirus species have been isolated from various animals including humans. Recently, it has been suggested that coronavirus may be transmitted from animals to humans (Müller et al., 2012).

Feline infectious peritonitis virus (FIP virus: FIPV), a feline coronavirus (FCoV) of the family *Coronaviridae*, causes a fatal disease called FIP in wild and domestic cat species. Cats that developed FIP were affected in several organs, including the liver, lungs, spleen, and central nervous system, forming lesions accompanied by necrosis and pyogenic granulomatous inflammation (Pedersen, 2009). In some cats, pleural effusion and ascitic fluid accumulated.

Monocytes and macrophages (monocytes/macrophages) play an important role in the pathogenesis of FIP. It has been reported that virus replication in monocytes/macrophages induced interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α production (Regan et al., 2009; Takano et al., 2007b, 2009). FIPV replication and cytokine production are enhanced when monocytes/macrophages are inoculated with FIPV in the presence of anti-FIPV S antibodies (antibody-dependent enhancement: ADE) (Hohdatsu et al., 1991; Corapi et al., 1992; Olsen et al., 1992). The phenomenon of ADE is involved in the aggravation of the pathology of FIP (Pedersen and Boyle, 1980; Takano et al., 2007b).

Over the past forty years, several studies have investigated potential treatments for FIP (Hartmann and Ritz, 2008). Antiviral, immunostimulating, and immunosuppressive drugs have been experimentally used for the treatment of FIP, but none of these have exhibited a sufficient therapeutic effect. Several agents that significantly inhibit FCoV replication *in vitro* have been identified (Balzarini et al., 2006; Barlough and Shacklett, 1994; Hsieh et al., 2010; Kim et al., 2012; Takano et al., 2008); however, whether or not these agents exhibit a therapeutic effect in cats with FIP has not been investigated.

The anti-malarial drug, chloroquine, has been reported to inhibit the replication of human immunodeficiency virus (HIV), influenza A/H5N1 virus, SARS-CoV, and human coronavirus 229E (Kono et al., 2008; Murray et al., 2010; Savarino et al., 2003; Vincent et al., 2005; Yan et al., 2012). Chloroquine also has been used to treat immune-mediated inflammatory disorders (Karres et al., 1998; Landewé et al., 1994; Lesiak et al., 2010). FIP is a viral infection that causes an immune-mediated inflammatory disease. On the basis of these findings, chloroquine may be effective as a therapeutic drug for FIP.

We investigated whether or not chloroquine inhibited FIPV replication *in vitro* and the FIPV-induced ADE activity of monocytes. Furthermore, we investigated the effect of chloroquine for cats with experimentally induced FIP.

* Corresponding author. Tel.: +81 176 23 4371; fax: +81 176 23 8703.
E-mail address: hohdatsu@vmas.kitasato-u.ac.jp (T. Hohdatsu).

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http://dx.doi.org/10.1016/j.antiviral.2013.04.016

2. Materials and methods

2.1. Cell cultures and viruses

Felis catus whole fetus-4 (fcwf-4) cells were grown in Eagles' minimum essential medium containing 50% L-15 medium, 5% fetal calf serum (FCS), and antibiotics. Feline monocytes were cultured in RPMI 1640 medium containing 10% FCS and antibiotics. Strain FIPV 79-1146 was grown in fcwf-4 cells. Strain FIPV 79-1146 was supplied by Dr. M.C. Horzinek (Utrecht University).

2.2. Monoclonal antibodies (MAbs)

MAb 6-4-2 (IgG2a) used in the present study recognizes the Spike (S) protein of type II FIPV, as demonstrated by immunoblotting. It has been reported that MAb 6-4-2 exhibits a neutralizing activity in fcwf-4 cells, and an enhancing activity in feline monocytes/macrophages depending on the reaction conditions (Hohdatsu et al., 1993).

2.3. Recovery of feline monocytes

Feline monocytes were isolated from specific pathogen-free (SPF) cats as previously described by Dewerchin et al. (2005).

2.4. Effect of chloroquine on virus and cytokine production in feline cells

2.4.1. Pre- and post-treatment with chloroquine

Confluent fcwf-4 cell monolayers were cultured in medium containing chloroquine (Wako, Japan) in 24-well multi-plates at 37 °C for 1 h. After washing, FIPV strain 79-1146 (1×10^4 $TCID_{50}$) was added to the culture and adsorbed to the cells at 37 °C for 1 h in the presence of chloroquine. After washing, cells were cultured in medium containing chloroquine, and culture supernatants were collected after 1 day. The experiment using feline monocytes from SPF cats was performed as follows: feline monocytes (2×10^5 cells) were cultured in this medium in 24-well multi-plates at 37 °C for 1 h. After washing, FIPV strain 79-1146 (1×10^4 $TCID_{50}$) reacted with or without MAb 6-4-2 was added to the culture and adsorbed to the cells at 37 °C for 1 h in the presence of chloroquine. After washing, cells were cultured in medium containing chloroquine, and culture supernatants and cells were collected after 2 days. As the virus titer in the culture supernatant, $TCID_{50}$ was calculated by the method of Reed–Muench. Cells were used to measure IL-1β, TNF-α, and IL-6 mRNA, and FCoV N genes. To evaluate the cytotoxic effects of chloroquine in feline cells, cell viability was measured by the WST-8 assay as described before (Takano et al., 2007b). The percent viability was calculated using the following formula: Cell viability (%) = (OD of chloroquine-treated cells/OD of chloroquine-untreated cells) × 100.

2.4.2. Post-treatment with chloroquine

FIPV strain 79-1146 (1×10^4 $TCID_{50}$) was added to the culture and adsorbed to the fcwf-4 cells at 37 °C for 1 h. After washing, cells were cultured in medium containing chloroquine, and culture supernatants were collected after 1 day. The experiment using feline monocytes from SPF cats was performed as follows: FIPV strain 79-1146 (1×10^4 $TCID_{50}$) reacted with or without MAb 6-4-2 was added to the culture and adsorbed to the feline monocytes at 37 °C for 1 h. After washing, cells were cultured in medium containing chloroquine, and culture supernatants and cells were collected after 2 days. The experiment using feline monocytes from cats with FIP (FIP cats) was performed as follows: feline monocytes were cultured in medium containing chloroquine, and culture supernatants and cells were collected after 2 days. As the virus titer in the culture supernatant, $TCID_{50}$ was calculated. Cells were used to measure cytokine mRNA, and FCoV N genes.

2.5. RNA isolation and cDNA preparation

RNA isolation and cDNA preparation were performed employing the method of Takano et al. (2007a).

2.6. Determination of feline cytokine mRNA and FCoV N gene expression levels

cDNA was amplified by PCR using specific primers as shown in Table 3. PCR was performed using the method of Takano et al. (2007a). Band density was quantified under appropriate UV exposure by video densitometry using Image J software (NIH, U.S.A.). Cytokine mRNA and FCoV N genes were quantitatively analyzed in terms of the relative density value to the mRNA of the housekeeping gene GAPDH.

2.7. Treatment with chloroquine on FIP cats

2.7.1. Experimental schedule

Nine SPF cats were randomly assigned to three experimental groups. In accordance with the experimental schedule indicated in Fig. 5. Group A: chloroquine (10 mg/kg) was subcutaneously administered every 3 days from 3 days before FIPV inoculation. Strain FIPV 79-1146 (10^5 $TCID_{50}$/ml) was inoculated orally to cats. Group B: Hanks' balanced salt solution (HBSS) was subcutaneously administered every 3 days from 3 days before FIPV inoculation. Strain FIPV 79-1146 (10^5 $TCID_{50}$/ml) was inoculated orally to cats. HBSS administration was completed 12 days after FIPV inoculation, and chloroquine (10 mg/kg) was subcutaneously administered every 3 days thereafter instead of HBSS. Group C: HBSS was subcutaneously administered every 3 days from 3 days before FIPV inoculation. Strain FIPV 79-1146 (10^5 $TCID_{50}$/ml) was inoculated orally to cats. One animal in the group C died unexpectedly before the challenge of FIPV, and was excluded from this study. Cats were checked daily for clinical signs, and we measured their body temperature and weight. FIP diagnoses were confirmed upon postmortem examination, revealing peritoneal and pleural effusions, and pyogranuloma in the major organs. To evaluate the condition of cats, we monitored Karnofsky's score. Karnofsky's score was calculated in reference to the report by Hartmann and kuffer (1998), Ritz et al. (2007). The protocol for the experiments in the present

Table 1
The cytotoxic effects of chloroquine in fcwf-4 cells and monocytes. Cell viability was evaluated by WST-8 assay.

	Cells	Chloroquine (μM)					
		0.0	0.4	2.0	5.0	10.0	50.0
Cell viability (%)	fcwf-4 cells	100[a]	105.7 ± 3.8[a]	99.4 ± 2.3[a]	N.D.[b]	98.9 ± 6.8[a]	87.1 ± 9.1[a]
	Monocytes	100[a]	N.D.[b]	N.D.[b]	100.9 ± 7[a]	99.8 ± 6[a]	N.D.[b]

[a] Cell viability (%; mean ± S.D.).
[b] Not done.

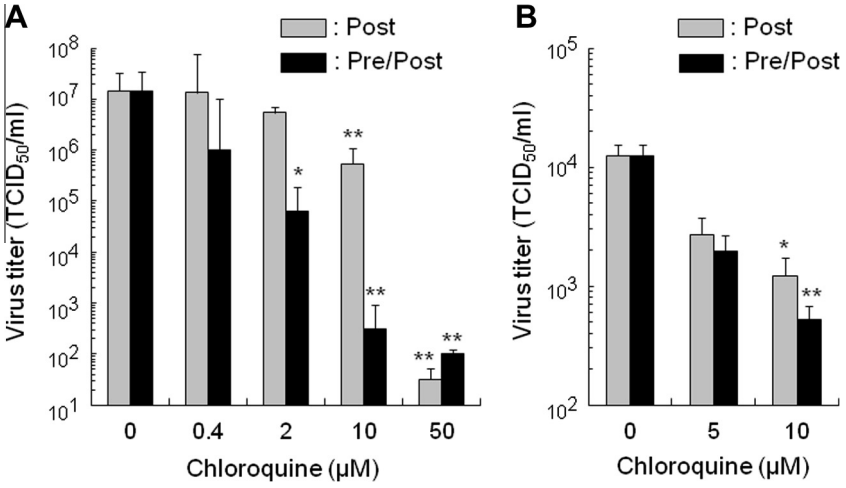

Fig. 1. Effect of chloroquine on FIPV infection in fcwf-4 cells and monocytes. (A) fcwf-4 cells were cultured with chloroquine for 1 h in the Post treatment group (gray bar), but not in the Pre/Post treatment group (black bar), followed by inoculation with FIPV at 37 °C. After 1 h, the supernatant was removed and cells were then treated with chloroquine at 37 °C in the Pre/Post treatment and Post treatment groups. After 1 day, the virus titer in the fcwf-4 cell culture supernatant was measured. $n = 4$. (B) Monocytes were cultured with chloroquine at 37 °C for 1 h in the Pre/Post treatment group, but not in the Post treatment group, followed by inoculation with FIPV at 37 °C. After 1 h, the supernatant was removed and cells were then treated with chloroquine at 37 °C in the Pre/Post treatment and Post treatment groups. After 1 day, the virus titer in the feline primary monocyte culture supernatant was measured. $n = 8$. $^{**}p < 0.01$ vs. without chloroquine (0 μM), $^{*}p < 0.05$ vs. without chloroquine (0 μM).

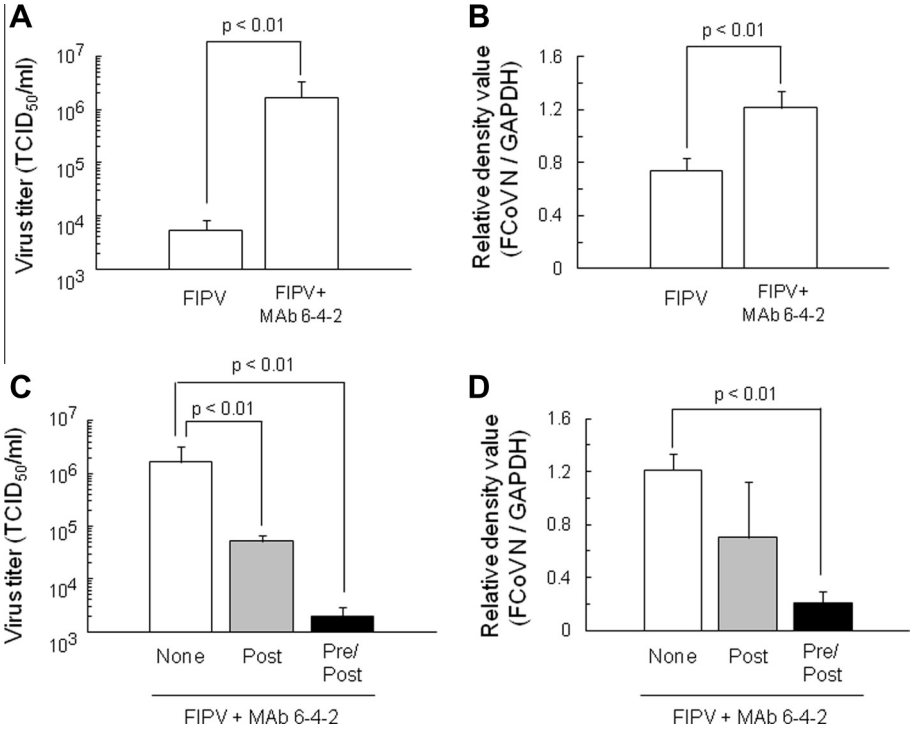

Fig. 2. Effect of chloroquine on the ADE of FIPV infection in monocytes. (A, B) Monocytes were treated with a reaction mixture of FIPV and anti-FIPV-S antibodies (FIPV + MAb 6-4-2) or FIPV alone (FIPV). After 2 days, the virus titer in the cell culture supernatant (A) or the intracellular FCoV N gene level (B) was measured. $n = 8$. (C, D) Monocytes were cultured with chloroquine for 1 h in the Pre/Post treatment group (black bar), but not in the None group (white bar) or Post treatment group (gray bar), followed by treatment with a reaction mixture of FIPV and anti-FIPV-S antibodies (FIPV + MAb 6-4-2) for 1 h. Then, the supernatant was removed and cells were treated with chloroquine at 37 °C in the Pre/Post treatment and Post treatment groups, but not in the None group. After 2 days, the virus titer in the cell culture supernatant (C) or the intracellular FCoV N gene level (D) was measured. The FCoV N gene was quantitatively analyzed in terms of the relative density value to the mRNA of the housekeeping gene GAPDH. $n = 8$.

study using cats was approved by the Ethics Committee of Kitasato University, School of Veterinary Medicine.

2.7.2. Blood chemistry and hematological analyses

Blood (1 ml) collected from cats using a syringe coated with EDTA were used for the collection of samples for blood biochemistry and hematological analyses. A half milliliter of the blood sample was centrifuged (800*g*) and the supernatant was used as a plasma sample for blood biochemistry. The remainder of blood samples was used for hematological analyses.

2.7.3. Separation of PBMC

Heparinized blood (5 ml) was 2-fold diluted with phosphate-buffered saline (PBS), and subjected to Ficoll-Hypaque

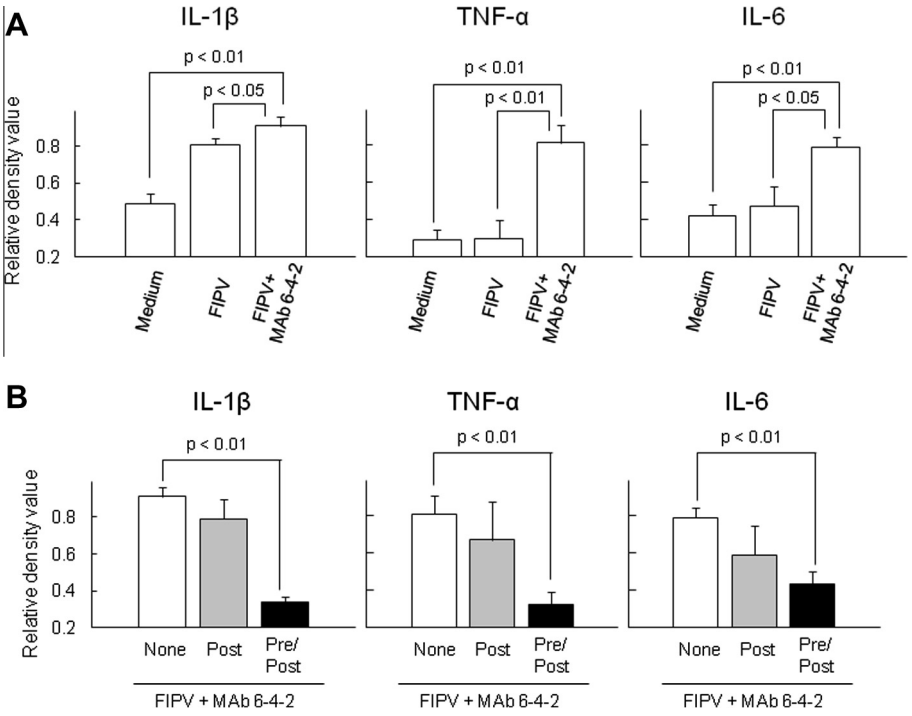

Fig. 3. Effect of chloroquine on IL-1β, TNF-α, and IL-6 mRNA expression levels in FIPV-infected monocytes. (A) Monocytes were treated with a reaction mixture of FIPV and anti-FIPV-S antibodies (FIPV + α-FIPV S MAb), FIPV alone (FIPV), or medium only (Medium). After 2 days, intracellular IL-1β, TNF-α, and IL-6 mRNA levels were measured. $n = 8$. (B) Monocytes were cultured with chloroquine for 1 h in the Pre/Post treatment group (black bar), but not in the None (white bar) and Post treatment groups (gray bar), followed by treatment with a reaction mixture of FIPV and anti-FIPV-S antibodies (FIPV + α-FIPV S MAb) for 1 h. Then, the supernatant was removed and cells were treated with chloroquine at 37 °C in the Pre/Post treatment and Post treatment groups, but not in the None group. After 2 days, intracellular IL-1β, TNF-α, and IL-6 mRNA levels were measured. Cytokine mRNAs were quantitatively analyzed in terms of the relative density value to the mRNA of the housekeeping gene GAPDH. $n = 8$.

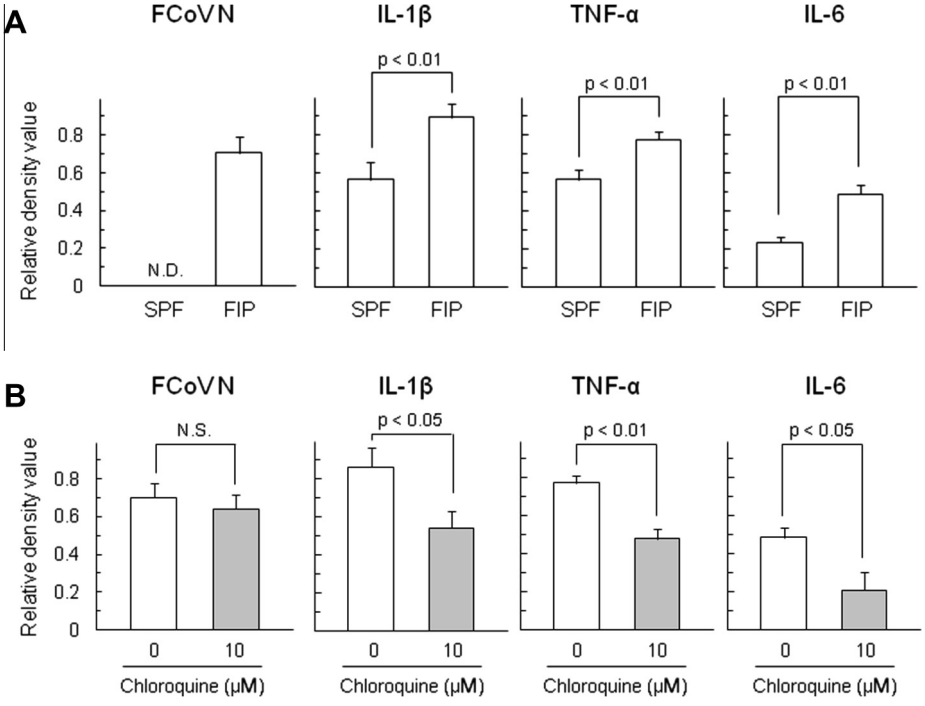

Fig. 4. Effect of chloroquine on IL-1β, TNF-α, and IL-6 mRNA and FCoV N gene expression levels in the monocytes of cats with FIP. (A) Monocytes were collected from FIP and SPF cats, and the FCoV N gene and IL-1β, TNF-α, and IL-6 mRNA were detected by RT-PCR. (B) Monocytes from cats with FIP were treated with chloroquine at 37 °C. After 2 days, intracellular IL-1β, TNF-α, and IL-6 mRNA and FCoV N gene levels were measured. Cytokine mRNAs and the FCoV N gene were quantitatively analyzed in terms of the relative density value to the mRNA of the housekeeping gene GAPDH. $n = 6$. N.D.: not detected. N.S.: not significant.

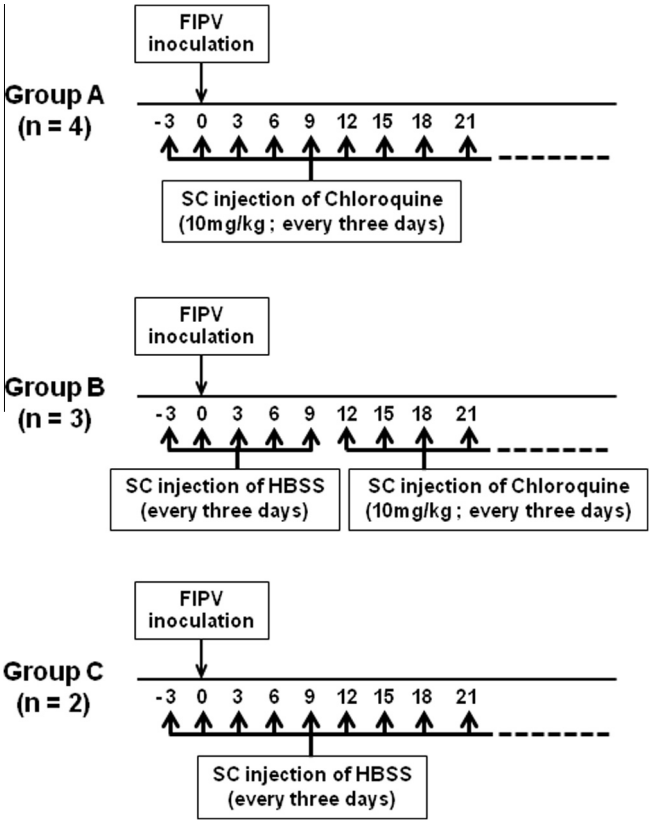

Fig. 5. Experimental schedule of the chloroquine treatment for FIPV-infected cats.

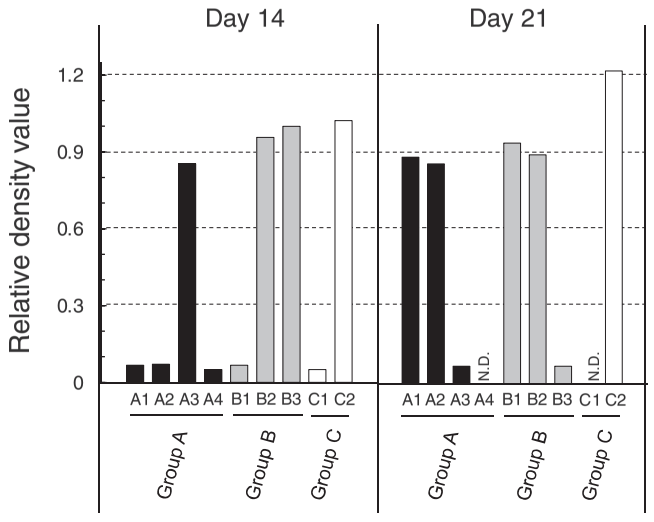

Fig. 6. FCoV N gene expression levels of PBMC in FIPV-infected cats treated with chloroquine. After inoculation with FIPV 79-1146 for 14 and 21 days, PBMCs were collected from cats. Intracellular FCoV N gene levels were measured. FCoV N gene was quantitatively analyzed in terms of the relative density value to the mRNA of the housekeeping gene GAPDH. N.D.: not done.

density gradient centrifugation at 540*g* for 20 min. The PBMC layer was collected, washed twice with PBS, and resuspended with growth medium at 2×10^6 cells/ml.

2.8. Statistical analysis

Data from two groups were analyzed by the Student's *t* test, and multiple groups were analyzed by a one-way ANOVA.

Table 2
Body temperatures, hematological findings, blood biochemical findings, and the index of the Karnofsky score in FIPV-infected cats treated with chloroquine.

Parameter	Unit	Day of post inoculation									
		0			14			21			
		Group A Mean (Range)	Group B Mean (Range)	Group C Mean (Range)	Group A Mean (Range)	Group B Mean (Range)	Group C Mean (Range)	Group A Mean (Range)	Group B Mean (Range)	Group C Mean (Range)
Body temperature	°C	38.1 (37.8–38.6)	38.4 (37.8–39.0)	38.5 (38.2–38.7)	39.0 (38.1–39.9)	39.0 (38.9–39.0)	39.3 (39.2–39.3)	39.1 (38.8–39.5)	38.8 (38.6–39.0)	38.2 (38.2)
Lymphocytes	$\times 10^3/\mu l$	6.07 (5.12–7.49)	5.65 (4.45–5.95)	6.08 (5.24–6.92)	2.10 (0.98–3.92)	1.67 (1.50–2.07)	0.26 (0.20–0.31)	0.91 (0.11–2.49)	0.16 (0.13–0.20)	0.11 (0.11)
Neutrophils	$\times 10^4/\mu l$	6.71 (5.13–8.04)	7.06 (6.00–8.11)	6.33 (5.65–7.01)	4.61 (3.20–5.12)	6.03 (5.06–7.10)	7.13 (6.22–8.04)	5.33 (2.20–10.18)	4.73 (3.13–6.21)	5.20 (5.20)
TP	g/dl	5.90 (5.50–6.00)	6.17 (5.50–6.60)	6.05 (5.70–6.40)	5.90 (5.40–6.40)	6.80 (6.20–7.40)	7.10 (6.80–7.40)	6.60 (6.20–7.00)	6.87 (6.20–7.40)	7.80 (7.80)
A/G		0.63 (0.57–0.69)	0.69 (0.63–0.74)	0.67 (0.62–0.71)	0.62 (0.53–0.80)	0.47 (0.35–0.57)	0.41 (0.37–0.45)	0.44 (0.40–0.47)	0.45 (0.34–0.57)	0.39 (0.39)
GGT	U/l	5.4 (5.0–5.7)	5.1 (4.6–5.5)	5.2 (4.8–5.5)	5.3 (4.7–6.0)	5.0 (4.4–5.8)	4.5 (4.1–4.9)	7.7 (5.9–9.0)	8.0 (6.4–9.5)	10.3 (10.3)
ALP	U/l	88 (77–92)	80 (67–88)	76 (63–89)	85 (43–107)	116 (73–142)	156 (151–161)	97 (76–111)	127 (70–150)	152 (152)
ALT	U/l	109 (82–135)	117 (73–128)	121 (106–136)	96 (57–126)	98 (69–129)	65 (51–79)	152 (98–248)	169 (99–288)	114 (114)
D-Bil	mg/dl	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.1 (0.1)	0.9 (0.3–1.9)	1.1 (0.4–1.5)	1.9 (1.9)
Karnofsky's score	%	100 (100)	100 (100)	100 (100)	60 (20–100)	60 (40–100)	50 (30–70)	40 (0–100)	50 (20–80)	10 (0–20)

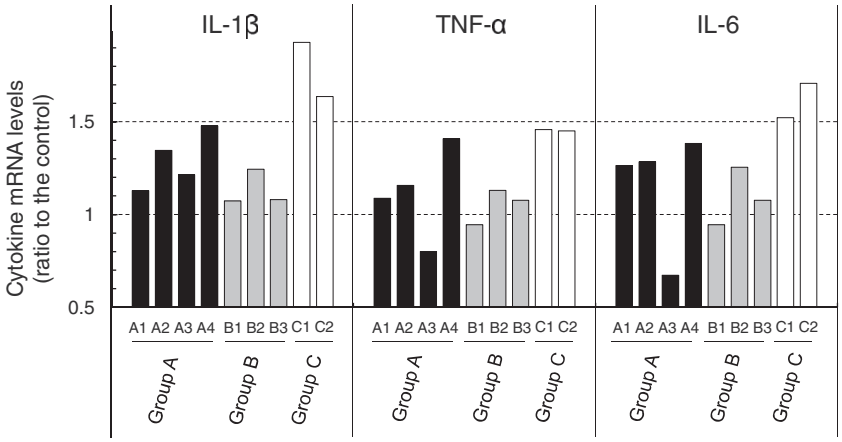

Fig. 7. IL-1β, TNF-α, and IL-6 mRNA expression levels of PBMC in FIPV-infected cats treated with chloroquine. After inoculation with FIPV 79-1146 for 18–21 days, PBMCs were collected from cats. Intracellular IL-1β, TNF-α, and IL-6 mRNA levels were measured. Cytokine mRNAs were quantitatively analyzed in terms of the relative density value to the mRNA of the housekeeping gene GAPDH. Data are expressed as a ratio of the control value (at the day of inoculation with FIPV).

3. Result

3.1. Effects of chloroquine on FIPV infection in feline cells

The effect of chloroquine on FIPV infection in fcwf-4 cells and SPF cat-derived monocytes was investigated. The cytotoxic effect by chloroquine in feline cells was showed in Table 1. In fcwf-4 cells, FIPV replication was inhibited in a chloroquine concentration-dependent manner in both Pre/Post and Post treatment groups (Fig. 1A). Similarly, concentration-dependent inhibition of viral replication was noted in feline monocytes treated with chloroquine (Fig. 1B). However, the antiviral effect of chloroquine was slightly lower in the Post treatment group than in the Pre/Post treatment group.

3.2. Effects of chloroquine on the ADE of FIPV infection in feline monocytes

The effect of chloroquine on the antibody-dependent enhancement of FIPV infection in monocytes was investigated. The virus titer and intracellular expression level of the FIPV N gene were significantly higher in the culture supernatant of monocytes inoculated with a mixture of FIPV and MAb 6-4-2 than in monocytes cultured with FIPV alone (Fig. 2A and B). In monocytes inoculated with a mixture of FIPV and MAb 6-4-2, the virus titer was significantly lower in the Post and Pre/Post treatment groups than in the None group (Fig. 2C). The intracellular expression level of the FIPV N gene was significantly lower in the Pre/Post treatment group than in the None group (Fig. 2D). Intracellular IL-1β, TNF-α, and IL-6 mRNA expression levels were significantly higher in monocytes inoculated with a mixture of FIPV and MAb 6-4-2 than in monocytes cultured with medium or FIPV alone (Fig. 3A). In monocytes inoculated with a mixture of FIPV and MAb 6-4-2, IL-1β, TNF-α and IL-6 mRNA expression levels were significantly lower in the Pre/Post treatment group than in the None group (Fig. 3B). However, no significant differences in these mRNA expression levels were noted between the Post treatment and the None groups (Fig. 3B).

3.3. Effects of chloroquine on inflammatory cytokine mRNA and FCoV N gene expression levels in the monocytes of cats with FIP

The influence of chloroquine on cytokine mRNA and FCoV N gene expressions was investigated in monocytes from cats with FIP. IL-1β, TNF-α, and IL-6 mRNA expression levels were significantly higher, with an increase in the level of FCoV N gene expression, in monocytes from cats with FIP than in monocytes from SPF cats (Fig. 4A). These mRNA expression levels were significantly lower in monocytes from cats with FIP cultured in medium containing 10 μM of chloroquine than in monocytes cultured without chloroquine (Fig. 4B). However, no significant difference was noted in the level of FCoV N gene expression with or without chloroquine.

3.4. Effects of chloroquine on cats with FIP

Based on the *in vitro* results, the therapeutic effect of chloroquine was investigated using cats with experimentally induced FIP. The experiment was performed following the schedule shown in Fig. 5. The FCoV N gene and cytokine mRNA were detected and clinical parameters were measured after FIPV inoculation until the onset of FIP.

PBMC was collected 14 and 21 days after FIPV inoculation, viral mRNA expression level was measured. The viral mRNA expression level was measured by semiquantitative RT-PCR. On day 14, viral mRNA expression level was slightly lower in group A than in group B and C. On day 21, viral mRNA expression level was slightly lower in group A and B (chloroquine-treated groups) than in group C. (Fig. 6). Inflammatory cytokine mRNA expression levels in PBMC on day 18–21 after FIPV inoculation were measured, and the ratios of mRNA expression levels to those before inoculation were determined (Fig. 7). Inflammatory cytokine mRNA expression ratios were slightly lower in group A and B (chloroquine-treated groups) than in group C.

Changes in body temperature after FIPV inoculation were measured (Table 2). No major change in body temperature was noted in any group. Lymphocyte and neutrophil counts in peripheral blood decreased in all cats on day 14 after FIPV inoculation (Table 2), but the level of this reduction was smaller in chloroquine-treated groups than in group C. Increases in total protein (TP), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), and direct bilirubin (D-Bil) levels were smaller in group A than in the other groups (Table 2). On the other hand, alanine aminotransferase (ALT) levels increased in the chloroquine-treated groups. When the clinical condition of the cats in each group was evaluated using the Karnofsky score, the score was high in the chloroquine-treated groups (Table 2). The mean number of survival days were 34.3, 31.7, and 21.0 in groups A, B, and C, respectively. Unfortunately, one animal in the group C died before the challenge of FIPV, statistical significance was not shown for any of the differences between

Table 3
Sequences of PCR primers for feline GAPDH, IL-1β, TNF-alpha, IL-6, and FCoV N.

	Orientation	Nucleotide sequence	Location	Length (bp)
GAPDH	Forward	5′-AATTCCACGGCACAGTCAAGG-3′	158–178	97
	Reverse	5′-CATTTGATGTTGGCGGGATC-3′	235–254	
IL-1β	Forward	5′-CTGGTGCTGTCTGGCTCATA-3′	441–460	280
	Reverse	5′-TTCCCGTCTTTCATCACACA-3′	601–620	
TNF-alpha	Forward	5′-TGGCCTGCAACTAATCAACC-3′	195–214	251
	Reverse	5′-GTGTGGAAGGACATCCTTGG-3′	426–445	
IL-6	Forward	5′-GCAGAAAACAACCTGAATCTTCCG-3′	247–270	426
	Reverse	5′-GAGAAAGGAATGCCCGTGAAC-3′	601–620	
FCoV N	Forward	5′-CAACTGGGGAGATGAACCTT-3′	876–895	788
	Reverse	5′-GGTAGCATTTGGCAGCGTTA-3′	1644–1663	

the chloroquine-treatment groups and the group C in the days of survival.

4. Discussion

It has been reported that chloroquine is useful for the treatment for viral infections (Kono et al., 2008; Murray et al., 2010; Savarino et al., 2003; Vincent et al., 2005; Yan et al., 2012). However, most of studies were obtained by *in vitro* experiments; only a few studies have investigated the antiviral effect of chloroquine using experimental animals (Pallister et al., 2009; Vigerust and McCullers, 2007). We investigated the effect of chloroquine as a therapeutic drug for FIP both *in vitro* and *in vivo*.

FIPV was enhanced proliferation in monocytes treated with a reaction mixture of FIPV and anti-FIPV-S antibodies. In contrast, virus proliferation was inhibited in chloroquine-treated monocytes, i.e., chloroquine inhibited the FIPV-induced ADE activity of monocytes. In addition, a significant inhibitory effect on FIPV gene expression was noted in cells with Pre/Post treatment. In contrast, no significant inhibitory effect was noted in cells with Post treatment, and the treatment of FIPV-infected monocytes collected from FIP cats with chloroquine did not change FIPV N gene expression, suggesting that the treatment of cells already infected with FIPV with chloroquine induces no antiviral effect.

FIPV-infected monocytes/macrophages are major component cells that produce the inflammatory cytokines involved in pathological deterioration (Regan et al., 2009; Takano et al., 2007b, 2009). In this study, chloroquine significantly inhibited inflammatory cytokine mRNA expression levels in FIPV-infected monocytes. The mechanism of inhibiting inflammatory cytokine mRNA expression by chloroquine is still unclear. Weber and Levitz (2000) suggested the presence of lysosomotropic and non-lysosomotropic mechanisms in the chloroquine-induced inhibition of TNF-α production. The former inhibits TNF-α release from cells and the latter inhibits TNF-α mRNA expression and transcription, which suggests that the significant inhibition of inflammatory cytokine mRNA expression levels by chloroquine in FIPV-infected monocytes involves the non-lysosomotropic mechanism. However, in a more recent paper by Jang et al. (2006), it was shown that chloroquine does inhibit TNF-α release, but does not change the TNF-α mRNA levels or the synthesis of the TNF-α precursor. The reason for this discrepancy is unclear. Furthermore, in addition to the lowering of TNF-α mRNA expression, the expression of IL-1β and IL-6 mRNA was decreased. Jang et al. (2006) reported that the stability of IL-1β and IL-6 mRNA was decreased by chloroquine. In the present study, the same mechanisms might decrease the expression of cytokine mRNA.

Chloroquine is administered orally or intravenously for the treatment of malaria. However, oral or intravenous administration of chloroquine is a burden on veterinarians and/or owners. Thus, in the present study, we chose a subcutaneous route. Unfortunately, there are no pharmacokinetic data on chloroquine after subcutaneous administration. Therefore, we must rely on pharmacokinetic data on chloroquine after intravenous administration examined under similar conditions to our study. Aderounmu et al. (1986) reported pharmacokinetic data after a single intravenous dose of 3 mg/kg chloroquine. In our present study, the subcutaneous dose of chloroquine was 2.5–3.3 mg/kg. Thus, pharmacokinetic data on chloroquine after subcutaneous administration to cats may be similar to those reported by Aderounmu et al. (1986).

We administered chloroquine when there were some clinical symptoms in cats after inoculation with FIPV strain 79-1146. The timing of administration was established based on the report of de Groot-Mijnes et al. (2005) and our previous experiments (Takano and Hohdatsu, unpublished data). Therefore, the CQ treatment timing in group B was set to 12 days after inoculation with FIPV strain 79-1146.

The clinical score of chloroquine-treatment groups were better than in chloroquine-untreated group, which suggests that chloroquine has therapeutic effect against FIP. If chloroquine has antiviral effect against FIPV *in vivo*, this drug may be useful for the treatment of FIP. To obtain the antiviral effect, it is necessary to increase the chloroquine dosage. However, as mentioned above, when the chloroquine concentration is increased, a severe side effect may be induced. Combining chloroquine with other "anti-FIPV agents" overcomes this problem. Hsieh et al. (2010) succeeded in inhibiting FIPV replication in fcwf-4 cells using the low-cytotoxic antiviral agents. It will be necessary to further investigate the possibility of FIP treatment with a combination of chloroquine and other agents.

In conclusion, it was demonstrated that chloroquine has inhibitory effect against the replication of FIPV and anti-inflammatory effect. It was also noted experimentally that chloroquine may be an effective drug for the treatment of FIP. The present experiments only examined whether CQ is effective for treating FIP. Future studies are necessary to collect preclinical data using the pharmacokinetics data of CQ reported by Aderounmu et al. (1986) and others (Aderounmu and Fleckenstein, 1983; Gustafsson et al., 1983; Ducharme and Farinotti, 1996).

Acknowledgements

This work was in part supported by KAKENHI (Grants-in-Aid for Young Scientists (B), no. 22780285) from the Ministry of Education, Culture, Sports, Science and Technology, and Kitasato University Research Grant for Young Researchers (2012).

References

Aderounmu, A.F., Fleckenstein, L., 1983. Pharmacokinetics of chloroquine diphosphate in the dog. J. Pharmacol. Exp. Ther. 226, 633–639.
Aderounmu, A.F., Salako, L.A., Lindström, B., Walker, O., Ekman, L., 1986. Comparison of the pharmacokinetics of chloroquine after single intravenous

and intramuscular administration in healthy Africans. Br. J. Clin. Pharmacol. 22, 559–564.
Balzarini, J., Keyaerts, E., Vijgen, L., Vandermeer, F., Stevens, M., De Clercq, E., Egberink, H., Van Ranst, M., 2006. Pyridine N-oxide derivatives are inhibitory to the human SARS and feline infectious peritonitis coronavirus in cell culture. J. Antimicrob. Chemother. 57, 472–481.
Barlough, J.E., Shacklett, B.L., 1994. Antiviral studies of feline infectious peritonitis virus in vitro. Vet. Rec. 1135, 177–179.
Corapi, W.V., Olsen, C.W., Scott, F.W., 1992. Monoclonal antibody analysis of neutralization and antibody-dependent enhancement of feline infectious peritonitis virus. J. Virol. 66, 6695–6705.
Dewerchin, H.L., Cornelissen, E., Nauwynck, H.J., 2005. Replication of feline coronaviruses in peripheral blood monocytes. Arch. Virol. 150, 2483–2500.
Hartmann, K., Ritz, S., 2008. Treatment of cats with feline infectious peritonitis. Vet. Immunol. Immunopathol. 123, 172–175.
Ducharme, J., Farinotti, R., 1996. Clinical pharmacokinetics and metabolism of chloroquine. Focus on recent advancements. Clin. Pharmacokinet. 31, 257–274.
de Groot-Mijnes, J.D., van Dun, J.M., van der Most, R.G., de Groot, R.J., 2005. Natural history of a recurrent feline coronavirus infection and the role of cellular immunity in survival and disease. J. Virol. 79, 1036–1044.
Gustafsson, L.L., Walker, O., Alván, G., Beermann, B., Estevez, F., Gleisner, L., Lindström, B., Sjöqvist, F., 1983. Disposition of chloroquine in man after single intravenous and oral doses. Br. J. Clin. Pharmacol. 15, 471–479.
Hartmann, K., Kuffer, M., 1998. Karnofsky's score modified for cats. Eur. J. Med. Res. 1998 (3), 95–98.
Hohdatsu, T., Nakamura, M., Ishizuka, Y., Yamada, H., Koyama, H., 1991. A study on the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection in feline macrophages by monoclonal antibodies. Arch. Virol. 120, 207–217.
Hohdatsu, T., Yamada, H., Ishizuka, Y., Koyama, H., 1993. Enhancement and neutralization of feline infectious peritonitis virus infection in feline macrophages by neutralizing monoclonal antibodies recognizing different epitopes. Microbiol. Immunol. 37, 499–504.
Hsieh, L.E., Lin, C.N., Su, B.L., Jan, T.R., Chen, C.M., Wang, C.H., Lin, D.S., Lin, C.T., Chueh, L.L., 2010. Synergistic antiviral effect of *Galanthus nivalis* agglutinin and nelfinavir against feline coronavirus. Antiviral Res. 88, 25–30.
Jang, C.H., Choi, J.H., Byun, M.S., Jue, D.M., 2006. Chloroquine inhibits production of TNF-alpha, IL-1beta and IL-6 from lipopolysaccharide-stimulated human monocytes/macrophages by different modes. Rheumatology 45, 703–710.
Karres, I., Kremer, J.P., Dietl, I., Steckholzer, U., Jochum, M., Ertel, W., 1998. Chloroquine inhibits proinflammatory cytokine release into human whole blood. Am. J. Physiol. 274, R1058–R1064.
Kim, Y., Mandadapu, S.R., Groutas, W.C., Chang, K.O., 2012. Potent inhibition of feline coronaviruses with peptidyl compounds targeting coronavirus 3C-like protease. Antiviral Res.. http://dx.doi.org/10.1016/j.antiviral.2012.11.005, pii: S0166-3542(12)00260-4.
Kono, M., Tatsumi, K., Imai, A.M., Saito, K., Kuriyama, T., Shirasawa, H., 2008. Inhibition of human coronavirus 229E infection in human epithelial lung cells (L132) by chloroquine: involvement of p38 MAPK and ERK. Antiviral Res. 77, 150–152.
Ksiazek, T.G., Erdman, D., Goldsmith, C.S., Zaki, S.R., Peret, T., Emery, S., Tong, S., Urbani, C., Comer, J.A., Lim, W., Rollin, P.E., Dowell, S.F., Ling, A.E., Humphrey, C.D., Shieh, W.J., Guarner, J., Paddock, C.D., Rota, P., Fields, B., DeRisi, J., Yang, J.Y., Cox, N., Hughes, J.M., LeDuc, J.W., Bellini, W.J., Anderson, L.J., SARS Working Group, 2003. A novel coronavirus associated with severe acute respiratory syndrome. N. Engl. J. Med. 348, 1953–1966.
Landewé, R.B., Goei Thè, H.S., van Rijthoven, A.W., Breedveld, F.C., Dijkmans, B.A., 1994. A randomized, double-blind, 24-week controlled study of low-dose cyclosporine versus chloroquine for early rheumatoid arthritis. Arthritis Rheum. 37, 637–643.
Lesiak, A., Narbutt, J., Sysa-Jedrzejowska, A., Lukamowicz, J., McCauliffe, D.P., Wózniacka, A., 2010. Effect of chloroquine phosphate treatment on serum MMP-9 and TIMP-1 levels in patients with systemic lupus erythematosus. Lupus 19, 683–688.
Müller, M.A., Raj, V.S., Muth, D., Meyer, B., Kallies, S., Smits, S.L., Wollny, R., Bestebroer, T.M., Specht, S., Suliman, T., Zimmermann, K., Binger, T., Eckerle, I., Tschapka, M., Zaki, A.M., Osterhaus, A.D., Fouchier, R.A., Haagmans, B.L., Drosten, C., 2012. Human coronavirus EMC does not require the SARS-coronavirus receptor and maintains broad replicative capability in mammalian cell lines. MBio 3. http://dx.doi.org/10.1128/mBio.00473-12, pii: e00473-12.
Murray, S.M., Down, C.M., Boulware, D.R., Stauffer, W.M., Cavert, W.P., Schacker, T.W., Brenchley, J.M., Douek, D.C., 2010. Reduction of immune activation with chloroquine therapy during chronic HIV infection. J. Virol. 84, 12082–12086.
Olsen, C.W., Corapi, W.V., Ngichabe, C.K., Baines, J.D., Scott, F.W., 1992. Monoclonal antibodies to the spike protein of feline infectious peritonitis virus mediate antibody-dependent enhancement of infection of feline macrophages. J. Virol. 66, 956–965.
Pallister, J., Middleton, D., Crameri, G., Yamada, M., Klein, R., Hancock, T.J., Foord, A., Shiell, B., Michalski, W., Broder, C.C., Wang, L.F., 2009. Chloroquine administration does not prevent Nipah virus infection and disease in ferrets. J. Virol. 83, 11979–11982.
Pedersen, N.C., 2009. A review of feline infectious peritonitis virus infection: 1963–2008. J. Feline Med. Surg. 11, 225–258.
Pedersen, N.C., Boyle, J.F., 1980. Immunologic phenomena in the effusive form of feline infectious peritonitis. Am. J. Vet. Res. 41, 868–876.
Regan, A.D., Cohen, R.D., Whittaker, G.R., 2009. Activation of p38 MAPK by feline infectious peritonitis virus regulates pro-inflammatory cytokine production in primary blood-derived feline mononuclear cells. Virology 384, 135–143.
Ritz, S., Egberink, H., Hartmann, K., 2007. Effect of feline interferon-omega on the survival time and quality of life of cats with feline infectious peritonitis. J. Vet. Intern. Med. 21, 1193–1197.
Savarino, A., Boelaert, J.R., Cassone, A., Majori, G., Cauda, R., 2003. Effects of chloroquine on viral infections: an old drug against today's diseases? Lancet Infect. Dis. 3, 722–727.
Takano, T., Azuma, N., Satoh, M., Toda, A., Hashida, Y., Satoh, R., Hohdatsu, T., 2009. Neutrophil survival factors (TNF-alpha, GM-CSF, and G-CSF) produced by macrophages in cats infected with feline infectious peritonitis virus contribute to the pathogenesis of granulomatous lesions. Arch. Virol. 154, 775–781.
Takano, T., Hohdatsu, T., Hashida, Y., Kaneko, Y., Tanabe, M., Koyama, H., 2007a. A "possible" involvement of TNF-alpha in apoptosis induction in peripheral blood lymphocytes of cats with feline infectious peritonitis. Vet. Microbiol. 119, 121–131.
Takano, T., Hohdatsu, T., Toda, A., Tanabe, M., Koyama, H., 2007b. TNF-alpha, produced by feline infectious peritonitis virus (FIPV)-induced macrophages, upregulates expression of type II FIPV receptor feline aminopeptidase N in feline macrophages. Virology 364, 64–72.
Takano, T., Katada, Y., Moritoh, S., Ogasawara, M., Satoh, K., Satoh, R., Tanabe, M., Hohdatsu, T., 2008. Analysis of the mechanism of antibody-dependent enhancement of feline infectious peritonitis virus infection: aminopeptidase N is not important and a process of acidification of the endosome is necessary. J. Gen. Virol. 89, 1025–1029.
Vincent, M.J., Bergeron, E., Benjannet, S., Erickson, B.R., Rollin, P.E., Ksiazek, T.G., Seidah, N.G., Nichol, S.T., 2005. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. Virol. J. 2, 69.
Vigerust, D.J., McCullers, J.A., 2007. Chloroquine is effective against influenza A virus in vitro but not in vivo. Influenza Other Respir. Viruses 1, 189–192.
Weber, S.M., Levitz, S.M., 2000. Chloroquine interferes with lipopolysaccharide-induced TNF-alpha gene expression by a nonlysosomotropic mechanism. J. Immunol. 165, 1534–1540.
Yan, Y., Zou, Z., Sun, Y., Li, X., Xu, K.F., Wei, Y., Jin, N., Jiang, C., 2012. Anti-malaria drug chloroquine is highly effective in treating avian influenza A H5N1 virus infection in an animal model. Cell Res.. http://dx.doi.org/10.1038/cr.2012.165.