

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

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Received: 3 December 2008 / Accepted: 23 March 2009 / Published online: 3 April 2009
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Abstract Feline infectious peritonitis (FIP) is a feline coronavirus (FCoV)-induced fatal disease of domestic and wild cats. The infiltration of neutrophils into granulomatous lesions is unusual for a viral disease, but it is a typical finding of FIP. This study aimed to investigate the reason for the lesions containing neutrophils in cats with FIP. Neutrophils of cats with FIP were cultured, and changes in the cell survival rate were assessed. In addition, the presence or absence of neutrophil survival factors was investigated in specimens collected from cats with FIP. Furthermore, it was investigated whether macrophages, one of the target cells of FIPV infection, produce neutrophil survival factors (TNF-alpha, GM-CSF, and G-CSF). We showed that virus-infected macrophages overproduce neutrophil survival factors, and these factors act on neutrophils and up-regulate their survival. These observations suggest that sustained production of neutrophil survival factors by macrophages during FCoV infection is sufficient for neutrophil survival and contributes to development of granulomatous lesions.

Introduction

Feline coronavirus (FCoV) belongs to Group I of the family *Coronaviridae*. FCoV consists of three major proteins: nucleocapsid (N) protein, membrane (M) protein and

peplomer spike (S) protein [23]. FCoV is classified into serotypes I and II according to the amino acid sequence of its S protein [19, 20]. Both serotypes consist of two biotypes: feline infectious peritonitis (FIP) virus (FIPV) and feline enteric coronavirus (FECV). Thus, there are types I and II FECV and FIPV in FCoV. FECV is asymptomatic in cats, but FIPV causes FIP. It has been proposed that FIPV arises from FECV by mutation [8, 29, 37], but the exact mutation and inducing factors have not yet been clarified. FIP is a fatal disease, characterized by vasculitis associated with granulomatous inflammation containing B cells, neutrophils and macrophages. The infiltration of neutrophils into granulomatous lesions is unusual for a viral disease, but it is a typical finding of FIP [28].

Macrophages/monocytes play an important role in the pathogenesis of FIP. It has been reported that the difference in the proliferation of macrophages/monocytes is related to the difference in pathogenicity between FECV and FIPV [4, 31]. The possibility of feline vascular endothelial cell injury caused by metalloproteinase-9 and TNF-alpha produced by FIPV-infected monocytes has also been reported [12]. We reported that virus replication in macrophages induced TNF-alpha production, and the TNF-alpha produced was involved in aggravation of the FIP pathology: TNF-alpha produced by FIPV-infected macrophages was involved in lymphopenia and an increase in the level of the cellular receptor of type II FIPV, aminopeptidase N [34]. TNF-alpha reportedly inhibits neutrophil apoptosis [22], suggesting its involvement in the infiltration of neutrophils into granulomatous lesions in cats with FIP, but this has not yet been clarified. It is also unclear whether FIPV-infected macrophages/monocytes produce factors other than TNF-alpha that are related to the survival of neutrophils.

In this study, we investigated the reason for the granulomatous lesions containing neutrophils in cats with FIP.

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Neutrophils of cats with FIP were cultured, and changes in the survival rate were examined. The presence or absence of neutrophil survival factors in specimens from cats with FIP was also investigated. Furthermore, whether macrophages, one of the target cells of FIPV, produce neutrophil survival factors was assessed.

Materials and methods

Experimental animals

Type II FIPV strain 79-1146 (10^4 TCID₅₀/ml) was administered orally to 6- to 8-month-old SPF cats. Nine cats that developed FIP symptoms (FIP cats), such as fever, weight loss, peritoneal or pleural effusion, dyspnea, ocular lesions, and neural symptoms, and nine 6- to 8-month-old SPF cats administered a medium as mock infection controls were used in this study. FIP diagnoses were confirmed upon postmortem examination, revealing peritoneal and pleural effusions and granulomatous lesions in major organs.

All experiments were performed in accordance with the Guidelines for Animal Experiments of Kitasato University.

Cell cultures and virus

Felis catus whole fetus-4 (Fcwf-4) cells were grown in Eagle's minimum essential medium containing 50% L-15 medium, 5% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Feline neutrophils and alveolar macrophages were maintained in RPMI 1640 growth medium supplemented with 10% FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, and 50 µM 2-mercaptoethanol. Type II FIPV strain 79-1146 was grown in Fcwf-4 cells at 37°C. FIPV strain 79-1146 was supplied by Dr. M. C. Horzinek of State University Utrecht, The Netherlands.

Antibodies

MAb 6-4-2 (IgG2a) used in the present study recognizes the S protein of type II FIPV, as demonstrated by immunoblotting. It has been reported that MAb 6-4-2 has virus-neutralizing activity in assays carried out in Fcwf-4 and CrFK cells, but an enhancing activity in feline macrophage cultures, depending on the reaction conditions [10].

Specimens from SPF and FIP cats

Blood collected from SPF and FIP cats using a heparinized syringe was centrifuged at 3,000 rpm for 10 min, and the supernatant was used as a plasma sample. Ascites fluid was

collected from FIP cats using a heparinized syringe and centrifuged at 3,000 rpm for 10 min, and the supernatant was collected.

Separation of neutrophils

Heparinized blood (10 ml) from SPF and FIP cats was diluted in twofold steps with phosphate-buffered saline (PBS) and subjected to Ficoll-Hypaque density gradient centrifugation at 1,700 rpm for 20 min. After the removal of peripheral blood mononuclear cells and supernatant by aspiration from the top layer, the pellets were mixed with an equal volume of saline containing 6% dextran for granulocyte separation and allowed to stand for 45 min at 37°C. The top clear layer was centrifuged at 400g for 10 min, and the pellet was mixed with 4 ml of 0.2% NaCl for 2 min to eliminate contaminating erythrocytes and then mixed with 4 ml of 1.6% NaCl. The cells were washed three times with PBS and resuspended with growth medium. Cell purity was assessed to be more than 98% neutrophils by the examination of a smear stained with Wright/Giemsa solutions.

Culture of neutrophils with specimens from FIP cats

To examine the effect of specimens from cats on the survival rate of neutrophils, feline neutrophils (1×10^5 cells/100 µl) were seeded into 96-well plates and cultured in the presence of FIP-cat-derived ascites fluid (final concentration of 1:20), plasma (final concentration of 1:20), and SPF-cat-derived plasma (final concentration of 1:20) for 24 h. Prior to and after incubation, 10 µl of WST-8 solution (WST-8 cell proliferation assay kit; Kishida Chemical Co., Ltd, Japan) was added. WST-8 is a tetrazolium salt that reacts with mitochondrial dehydrogenases, forming the formazan dye. Expansion of viable cell numbers results in an increase in the activity of the mitochondrial dehydrogenases in the cells, corresponding to an increase in formazan dye metabolism. After the cells were returned to the incubator for 4 h, the absorbance of the formazan produced was measured at 450 nm with a 96-well spectrophotometric plate reader, as described by the manufacturer. The percent viability was calculated using the following formula: Cell viability (%) = (after incubation OD/prior to incubation OD) × 100.

Recovery of alveolar macrophages

Feline alveolar macrophages were obtained from SPF and FIP cats by broncho-alveolar lavage with Hank's balanced salt solution (HBSS) as described previously by Hohdatsu et al. [9].

RNA isolation and cDNA preparation

RNA isolation and cDNA preparation were performed employing the method of Takano et al. [34].

Determination of levels of feline GAPDH mRNA, TNF-alpha mRNA, G-CSF mRNA, GM-CSF mRNA, and FCoV N gene expression

cDNA was amplified by PCR using primers specific for feline GAPDH mRNA, TNF-alpha mRNA, G-CSF mRNA, GM-CSF mRNA, and FCoV N genes. The primer sequences are shown in Table 1. PCR was performed using the method of Takano et al. [35].

The band density was quantified under appropriate UV exposure by video densitometry using Scion Image software (Scion Corporation, USA). TNF-alpha mRNA, G-CSF mRNA, GM-CSF mRNA, and FCoV N genes were quantitatively analyzed in terms of the relative density value compared to the mRNA for the housekeeping gene GAPDH.

Plaque assay

Confluent Fcwf-4 cell monolayers in 24-well multi-plates were inoculated with 100 μ l of the sample dilutions. After virus adsorption at 37°C, the cells were washed with HBSS, and 1 ml of growth medium containing 1.5% carboxymethyl cellulose was added to each well. The cultures were incubated at 37°C for 2 days, fixed in 10% buffered formalin, and stained with 1% crystal violet.

Inoculation of feline alveolar macrophages with FIPV

Viral suspension (FIPV strain 79-1146, 2×10^3 TCID₅₀/0.1 ml) and MAb 6-4-2 solution were mixed in an equal volume ratio and allowed to react at 4°C for 1 h, and 0.1 ml of this reaction solution was used to inoculate feline alveolar

macrophages (2×10^6 cells) cultured in each well of 24-well multi-plates. As controls, medium alone and virus suspension alone were added to feline alveolar macrophages. After virus adsorption at 37°C for 1 h, the cells were washed with HBSS and 1 ml of growth medium. The cells and culture supernatant were collected every 24 h thereafter. The cells were used for measurement of the TNF-alpha mRNA, G-CSF mRNA, GM-CSF mRNA, and FCoV N genes. TNF-alpha mRNA, G-CSF mRNA, GM-CSF mRNA, and FCoV N genes were quantitatively analyzed in terms of the relative density value compared to the mRNA for the housekeeping gene GAPDH. The culture supernatant was employed for determination of the virus titer.

Statistical analysis

Data were analyzed by Student's *t* test. The data in Fig. 1a, b were also analyzed using the Mann–Whitney test. *P* values < 0.05 were considered to indicate a significant difference between compared groups.

Results

Neutrophil counts in the blood of SPF and FIP cats

The neutrophil counts in peripheral blood of FIP cats were examined and compared with those of uninfected SPF cats. The count of neutrophils at the time of blood sampling is shown in Fig. 1. The blood neutrophil counts in SPF and FIP cats were $5,252 \pm 1,493 \mu\text{l}^{-1}$ (mean \pm SD) and $9,425 \pm 3,996 \mu\text{l}^{-1}$, and median values were 4,921 and $8,093 \mu\text{l}^{-1}$, respectively (*P* < 0.05).

Survival rate of neutrophils of SPF and FIP cats

To investigate the cause of increases in the neutrophil counts in FIP cats, neutrophils were isolated from SPF and

Table 1 Sequences of PCR primers for feline GAPDH, TNF-alpha, GM-CSF, G-CSF, and FCoV N

	Orientation	Nucleotide sequence	Location	Length (bp)	References
GAPDH	Forward	5'-AATTCACGGCACAGTCAAGG-3'	158–178	97	Avery and Hoover [2]
	Reverse	5'-CATTTGATGTTGGCGGGATC-3'	235–254		
TNF-alpha	Forward	5'-TGGCCTGCAACTAATCAACC-3'	195–214	251	Avery and Hoover [2]
	Reverse	5'-GTGTGGAAGGACATCCTTGG-3'	426–445		
GM-CSF	Forward	5'-CACTGTGGTCTGCAGCATCT-3'	30–49	198	GenBank Accession No. AY878357
	Reverse	5'-AGGTGAGTCTGCAGGCATTT-3'	208–227		
G-CSF	Forward	5'-GCTATCAACATCTGGCAGCA-3'	462–481	205	GenBank Accession No. AB042552
	Reverse	5'-ACTCGTGAGGGAGGGTTTTT-3'	647–666		
FCoV N	Forward	5'-CAACTGGGGAGATGAACCTT-3'	876–895	788	Takano et al. [34]
	Reverse	5'-GGTAGCATTTGGCAGCGTTA-3'	1,644–1,663		

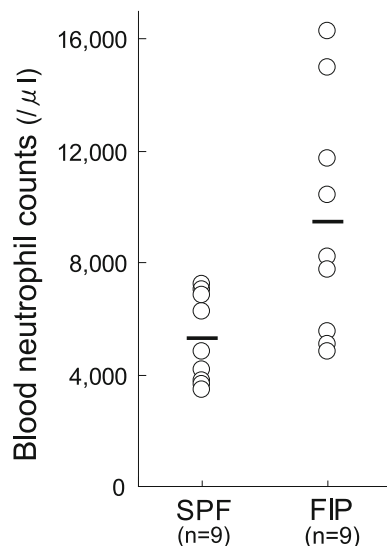


Fig. 1 Neutrophil counts in the blood of SPF and FIP cats. Horizontal lines represent the median for each group

FIP cats, and the survival rates after 24-h culture were compared. The survival rate of neutrophils from FIP cats was increased, but not significantly ($P = 0.122$), compared to that of SPF cats (Fig. 2).

Survival rate of neutrophils in specimens from SPF and FIP cats

The survival rate of neutrophils in the presence of specimens from FIP cats was significantly higher than those of neutrophils cultured with SPF cat-derived plasma and medium (Fig. 3).

TNF- α , GM-CSF, and G-CSF mRNA and FCoV N gene expression levels in macrophages of SPF and FIP cats

TNF- α , GM-CSF, and G-CSF mRNA and FCoV N gene expression levels were increased in alveolar macrophages derived from FIP cats (Fig. 4).

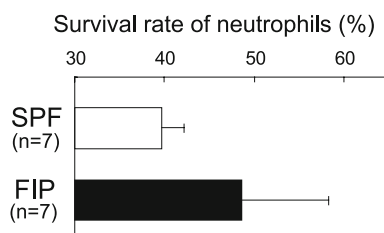


Fig. 2 Survival rate of neutrophils in SPF and FIP cats. The neutrophils of SPF cats ($1 \times 10^6 \text{ ml}^{-1}$) were cultured at 37°C for 24 h in medium, and cell viability was assessed by the WST-8 assay

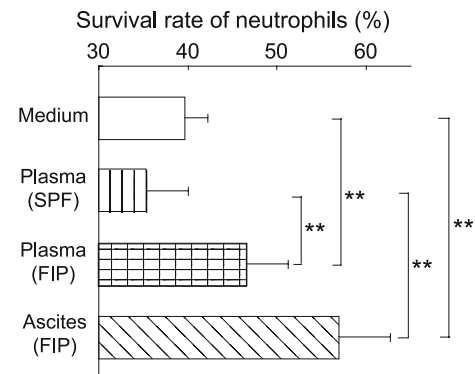


Fig. 3 Survival rate of neutrophils in specimens from SPF and FIP cats. The neutrophils of SPF cats ($1 \times 10^6 \text{ ml}^{-1}$) were cultured at 37°C for 24 h in the presence of the plasma of SPF and FIP cats and ascites of FIP cats. Cell viability was assessed by the WST-8 assay. $n = 7$, $**P < 0.01$

The culture supernatant of FIPV-infected macrophage promotes neutrophil survival

The virus titer was significantly higher in the culture supernatant of macrophages infected with a mixture of FIPV and MAb 6-4-2 than in that of macrophages cultured with medium and FIPV alone. The neutrophil survival rates were significantly increased in the presence of the culture supernatant of macrophages infected with the mixture of FIPV and MAb 6-4-2 compared to those in the presence of other supernatants (Fig. 5).

Relationship between TNF- α , GM-CSF, and G-CSF mRNA expression and FIPV replication in macrophages

When SPF-cat-derived alveolar macrophages were infected with a mixture of FIPV and MAb 6-4-2, the intracellular

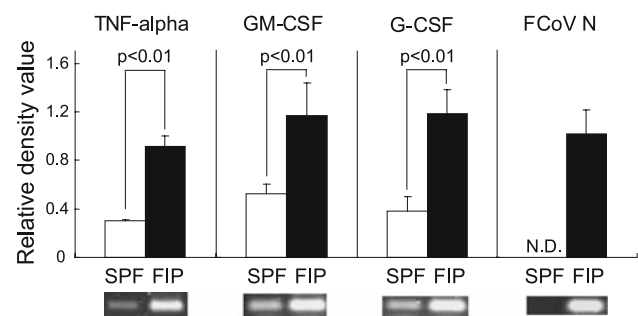


Fig. 4 TNF- α , GM-CSF, and G-CSF mRNA and FCoV N gene expression levels in macrophages of SPF and FIP cats. Alveolar macrophages (2×10^6 cells) were collected from SPF and FIP cats, and TNF- α , GM-CSF, and G-CSF mRNA and FCoV N gene expression was detected by RT-PCR. TNF- α , GM-CSF, and G-CSF mRNA and FCoV N gene expression levels were quantitatively analyzed in terms of the relative density value to mRNA for the housekeeping gene GAPDH. $n = 7$, ND not detected

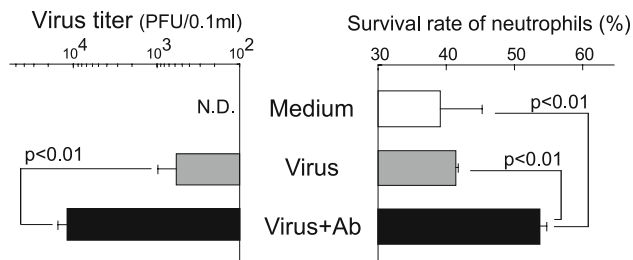


Fig. 5 The culture supernatant of FIPV-infected macrophages promotes neutrophil survival. SPF-cat-derived alveolar macrophages (2×10^6 cells) were cultured with medium alone, FIPV, or FIPV and MAb 6-4-2. The culture supernatant was collected after 72 h. The virus titer in the culture supernatant was measured by the plaque assay method (*left*). The neutrophils of SPF cats ($1 \times 10^6 \text{ ml}^{-1}$) were cultured at 37°C for 24 h in the presence of each culture supernatant (final dilution of 1:5), and cell viability was assessed by the WST-8 assay (*right*). $n = 6$. ND not detected

TNF-alpha, GM-CSF, and G-CSF mRNA levels increased (Fig. 6).

Discussion

Neutrophils are important for host defense against pathogens. Viral infection generally reduces the number of

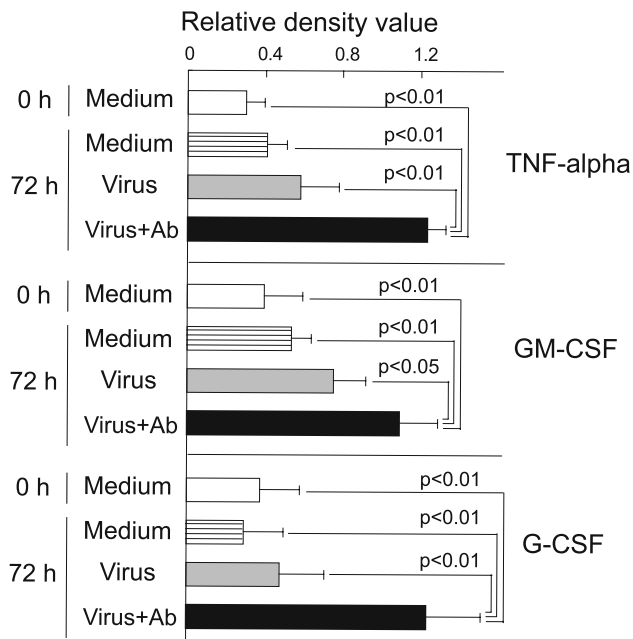


Fig. 6 Relationship between TNF-alpha, GM-CSF, and G-CSF mRNA expression and FIPV replication in macrophages. SPF-cat-derived alveolar macrophages (2×10^6 cells) were cultured with medium alone, FIPV, or FIPV and MAb 6-4-2. The cells were collected at 0 h (as a control) and 72 h. The intracellular TNF-alpha, GM-CSF, and G-CSF mRNA expression levels were measured by RT-PCR. TNF-alpha, GM-CSF, and G-CSF mRNA were quantitatively analyzed in terms of the relative density value to mRNA for the housekeeping gene GAPDH. $n = 6$

neutrophils. The transfer of peripheral blood neutrophils to marginal tissues, destruction of neutrophils due to excess antibody production, and direct cell death caused by viral infection are considered to be the causes of neutropenia [3, 16, 18, 19]. In human immunodeficiency virus and canine parvovirus infections, a reduced neutrophil count has been suggested to allow severe bacterial infection [14, 15]. Feline immunodeficiency virus and feline panleucopenia virus infections also affect stromal cells in the bone marrow, thus leading to a decreased production of neutrophils [17, 26]. In contrast, severe acute respiratory syndrome (SARS) coronavirus, which belongs to the same family as FIPV, can also cause neutrophilia [39]. Neutrophilia has been also reported in cats with FIP [25]. Paltrinieri [24] reported that cytokines accelerate the delivery of neutrophils to the inflamed lesions, thereby prolonging the lifespan of circulating neutrophils. He also reported that the apoptosis of neutrophils is delayed by cytokines, thus increasing the life of neutrophils in lesions. It is likely that neutrophilia in cats with FIP is associated with the infiltration of neutrophils into granulomatous lesions. However, there seems to be no established theory to explain the infiltration of neutrophils into granulomatous lesions in cats with FIP.

In humans, the lifespan of neutrophils is short. When neutrophils are isolated from peripheral blood and cultured in vitro, apoptosis is induced, and about half of the cells die within 24 h [27]. When neutrophils isolated from peripheral blood of SPF cats were cultured for 24 h, more than 60% died, suggesting that feline neutrophils also die due to apoptosis, similar to human neutrophils. Furthermore, co-culture with specimens from FIP cats increased the survival rate of neutrophils, suggesting that the production of factors involved in the survival of neutrophils is enhanced in FIP cats, and these factors act on neutrophils and prolong their lifespan.

The TNF-alpha, GM-CSF, and G-CSF mRNA levels were increased in macrophages of FIP cats. These cytokine mRNA levels were also elevated in macrophages infected with FIPV and MAb 6-4-2, clarifying the presence of neutrophil survival factors in the macrophage culture supernatant. It was suggested that: (1) FIPV-infected macrophages release TNF-alpha, GM-CSF, and G-CSF in response to virus replication, and (2) these cytokines act on neutrophils and prolong their survival. We previously reported that FIPV-infected macrophages produced TNF-alpha and B-cell differentiation/survival factors, and these factors may have been involved in lymphopenia and hypergammaglobulinemia [33–35]. Kipar et al. [12] suggested that FIPV-infected monocytes are involved in feline vascular endothelial cell injury. Cytokines and chemical mediators produced by FIPV-infected macrophages/monocytes may play an important role in the pathogenesis of FIP in cats.

TNF- α , GM-CSF, and G-CSF are cytokines inhibiting the apoptosis of neutrophils [5, 13, 21, 22]. These cytokines are secreted by macrophages and T cells. When these cytokines act on neutrophils, the intracellular apoptosis-inhibitory protein level increases, whereas the apoptosis-promoting protein level decreases, prolonging the survival of neutrophils [5, 38]. In inflammatory diseases, such as cystic fibrosis and Kawasaki disease, the pathological condition is aggravated as the survival time of neutrophils is prolonged [5, 36]. Moreover, the pathological aggravation of inflammatory diseases is associated with protease and reactive oxygen species produced by neutrophils [6, 32]. These mediators may also be produced in excess and aggravate the granulomatous lesions in FIP cats. We are now progressing with studies on the expression of apoptosis-inhibitory and -promoting proteins in neutrophils of FIP cats.

The enhancement of cathepsin B production in macrophages by elastase produced by neutrophils has been reported [7], while FIPV reportedly requires cathepsin B to invade cells [30], suggesting that neutrophils are also involved in a viral replication enhancement mechanism, different from antibody-dependent enhancement (ADE), in FIPV infection. Addie et al. [1] reported that FCoV re-infection of anti-FCoV antibody-positive domestic cats might not result in the development of ADE. The involvement of neutrophils in the enhancement of FIPV production in macrophages and the influence of macrophages on neutrophil survival should be investigated further.

Virus production and TNF- α , GM-CSF, and G-CSF mRNA expression were markedly enhanced in macrophages infected with FIPV and MAb 6-4-2 compared to macrophages infected with the virus alone. We used immunofluorescence to detect FIPV antigen: 10–20% of cells were positive when SPF cat-derived alveolar macrophages were infected with FIPV and MAb 6-4-2, whereas 1–2% of cells were positive when alveolar macrophages were infected with FIPV [9, 11]. It seems that the increased number of virus-infected macrophages leads to the over-expression of neutrophil survival-factor-associated mRNA in macrophages.

In cats with FIP, virus can be detected in macrophages of granulomatous lesions. To elucidate the mechanism of the infiltration of neutrophils into granulomatous lesions, we used alveolar macrophages, which can be readily collected in appropriate numbers. In addition, alveolar macrophages can be cultured without activation treatment, unlike monocytes.

In this study, we showed that virus-infected macrophages overproduce neutrophil survival factors, and these factors act on neutrophils and up-regulate their survival. These observations suggest that sustained production of

neutrophil survival factors by macrophages during FCoV infection is sufficient for neutrophil survival and contributes to the development of granulomatous lesions. These findings may be important for elucidating the pathogenesis of FIP.

Acknowledgments This work was supported by Grant for Encouragement of Young Scientists (No. E0801) from the School of Veterinary Medicine, Kitasato University.

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