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Generation, characterization and therapeutic potential of anti-feline TNF-alpha MAb for feline infectious peritonitis



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ABSTRACT

Feline infectious peritonitis (FIP) is a lethal infectious disease affecting domestic and wild cats. Several reports suggested that TNF-alpha is related to the progression of FIP. Thus, the administration of a feline TNF-alpha-neutralizing antibody to cats with FIP may reduce the disease progression. In this study, we have prepared nine monoclonal antibodies (MAbs) that recognize feline TNF-alpha. All MAbs neutralized recombinant TNF-alpha. The 50% inhibitory concentrations (IC50) of the MAbs for the cytotoxicity of recombinant TNF-alpha were 5–684 ng/ml. MAb 2–4 exhibited high neutralizing activity against natural TNF-alpha derived from FIPV-infected macrophages, and was confirmed to inhibit the following feline TNF-alpha-induced conditions *in vitro*: (i) an increase in the survival rate of neutrophils from cats with FIP, (ii) aminopeptidase N (APN) mRNA expression in macrophages, and (iii) apoptosis of a feline T-lymphocyte cell line.

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1. Introduction

Tumor necrosis factor (TNF)-alpha is mainly produced as a 26-kDa transmembrane protein (membrane-form TNF-alpha) by activated macrophages. Membrane-form TNF-alpha is released from the cell surface as a 17-kDa non-glycosylated protein (soluble-form TNF-alpha) through the action of the metalloproteinase, TNF-alpha-converting enzyme (TACE), and enters the circulation as a 55-kDa homotrimer (Moss et al., 1997; Smith and Baglioni, 1987). Homotrimer TNF-alpha binds to cell surface TNF receptors (TNFR) and induces various physiological activities (Vandenabeele et al., 1995; Reinhard et al., 1997). For example, when it binds to cell surface TNFR-1, caspase is activated and induces apoptosis, and when it binds to cell surface TNFR-2, transcription factors, such as NF-kB and c-Jun, are activated that promote cell proliferation and induce the expression of cytokines involved in immunity and inflammation.

TNF-alpha induces the necrosis and apoptosis of tumor cells and activates lymphocytes and macrophages. However, the overproduction of TNF-alpha can lead to acute inflammation and immune system abnormalities in human and other animals. It was reported that TNF-alpha is closely related to the progression of inflammatory disease, such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease (Kollias et al., 1999; Brotas et al., 2012; Wang

and Fu, 2005). Moreover, previous studies have described aggravation of the pathologies of viral infections (such as human immunodeficiency virus, influenza A virus, and dengue virus infections) due to increased TNF-alpha production (Fauci, 1993; Maury and Lähdevirta, 1990; Poli et al., 1990; Uchide et al., 2012; Yen et al., 2008).

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 cats. Several organs, including the liver, lungs, spleen, and central nervous system, are affected in cats that develop FIP, and the formation of lesions in these organs is accompanied by necrosis and pyogenic granulomatous inflammation (Pedersen, 2009). Pleural effusion and ascitic fluid was shown to accumulate in some FIP cats. Macrophages/monocytes play an important role in the pathogenesis of FIP. For example, differences in the proliferation of macrophages/monocytes were shown to be related to differences in pathogenicity between feline enteric coronavirus (FECV) and FIPV (Dewerchin et al., 2005; Stoddart and Scott, 1989). We previously showed that virus replication in macrophages induced TNF-alpha production. 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 (APN) (Takano et al., 2007a,b). Moreover, it was reported that neutrophil apoptosis in cats with FIP was inhibited by TNF-alpha. This finding suggests that neutrophilia in cats with FIP due to TNF-alpha-induced neutrophil survival (Takano et al., 2009).

Over the past forty years, several studies have investigated potential treatments for FIP (Hartmann and Ritz, 2008). Antiviral, immunostimulating, and immunosuppressive drugs have been

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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., 2013); however, whether or not these agents exhibit a therapeutic effect in cats with FIP has not been investigated.

In humans, a human TNF-alpha activity-neutralizing antibody has been used as a therapeutic drug for rheumatoid arthritis and inflammatory bowel disease, and sufficient therapeutic effects were achieved (Tracey et al., 2008). These findings suggest that FIP symptoms may also be reduced by a feline TNF-alpha-neutralizing antibody to cats with FIP. However, no feline TNF-alpha-neutralizing antibody has been developed.

We attempted to prepare monoclonal antibodies (MAbs) that recognize feline TNF-alpha (anti-feline TNF-alpha MAbs) and investigated whether these MAbs inhibited feline TNF-alpha activities. Furthermore, we investigated the application of an anti-feline TNF-alpha MAb as a therapeutic drug for FIP *in vitro*.

2. Materials and methods

2.1. Cell cultures and virus

FO mouse myeloma cells (ATCC CRL-1646), and hybridoma cells producing the antibody to feline TNF-alpha were maintained in Dulbecco's modified Eagle's minimum essential medium supplemented with 10% FCS and antibiotics. Alveolar macrophages, neutrophils, WEHI-164 murine sarcoma cells (ATCC CRL1751), and Fet-J feline T-lymphocyte cells were maintained in RPMI 1640 growth medium supplemented with 10% FCS, antibiotics, 50 μ M 2-mercaptoethanol, and 2 μ g/ml of polybrene. FO mouse myeloma cells and WEHI-164 murine sarcoma cells were obtained from the American Type Culture Collection. Fet-J cells were kindly provided by Dr. Yamamoto of the University of Florida, USA. Type II FIPV strain 79-1146 was grown in *Felis catus* whole fetus-4 cells at 37 °C. FIPV strain 79-1146 was supplied by Dr. Horzinek of State University Utrecht, the Netherlands.

2.2. Production of antibody-secreting hybridomas

BALB/c mice, approximately 4 weeks of age, were inoculated intraperitoneally with a mixture of 10 μ g of commercial recombinant feline TNF-alpha (R&B SYSTEMS., USA) and complete Freund's adjuvant. Mice received an intraperitoneal booster dose of 1 μ g of recombinant TNF-alpha every three or four weeks. The boost was repeated four or five times, and spleen cells were obtained three days after the final immunization. Fusion was carried out by essentially the same method described by Köhler and Milstein (Köhler and Milstein, 1975). Polyethyleneglycol-4000 (Merck, Germany) was used as a fusing agent and the ratio of mouse spleen cells and mouse myeloma cells (FO) was 3:1. The selective medium contained hypoxanthine (10^{-4} M), aminopterin (4×10^{-7} M), and thymidine (1.6×10^{-5} M). Fused cells, at a concentration of 1×10^6 cells/ml, were dispensed in 100 μ l volumes into the wells of 96-well, flat-bottomed microplates and incubated at 37 °C in a humid atmosphere containing 5% CO₂. After incubation for 2 weeks, the wells were examined and those that contained hybridoma cultures were screened for the production of recombinant TNF-alpha specific antibodies by an enzyme-linked immunosorbent assay (see below). Colonies in antibody-positive wells were passaged in 24-well multiplates and incubated in medium containing hypoxanthine (10^{-4} M) and thymidine (1.6×10^{-5} M). The cells were then cloned by the limit dilution method.

2.3. Enzyme-linked immunosorbent assay

ELISA plates (Sumitomo Bakelite Co., Ltd., Japan) were coated overnight at 4 °C with recombinant TNF-alpha (50 ng/100 μ l/well) diluted with carbonate buffer (0.05 M, pH 9.6). After washing with phosphate buffered saline (PBS) containing 0.02% Tween-20, the plates were blocked with a blocking buffer containing 0.5% skim milk in PBS at 37 °C for 60 min. Each well of the plates then received 100 μ l of the hybridoma culture supernatant. After 60 min incubation at 37 °C, the plate was washed and 100 μ l of the mixture of peroxidase (POD) conjugate goat anti-mouse IgG1 (Southern Biotechnology Associates Inc., USA) and POD conjugate goat anti-mouse IgG2a (Southern Biotechnology Associates Inc., USA) were then added to each well of the plate. After incubation at 37 °C for 30 min, the plate was washed and each well received 100 μ l of substrate solution followed by incubated at 25 °C for 20 min in the dark. The substrate solution was prepared by dissolving o-phenylenediamine dihydrochloride at a concentration of 0.4 mg/ml in 0.1 M citric acid and 0.2 M Na₂HPO₄ buffer (pH 4.8) and adding 0.2 μ l/ml of 30% H₂O₂. The reaction was stopped with 3 N H₂SO₄ solution, and the optical density (O.D.) at 492 nm was determined.

2.4. Determination of the antibody class and subclass

The hybridoma culture supernatant was used to determine the antibody class and subclass with a mouse MAb isotyping test kit (Serotec Ltd., UK) according to the product manual.

2.5. Purification of monoclonal antibodies (MAbs)

MAbs were purified from the hybridoma culture supernatant with Protein G Sepharose (GE Healthcare., USA) according to the product manual. After purification, the buffer of MAbs was exchanged to PBS (pH 7.4) by Amicon Ultra-15 centrifugal filter devices (NMWL 30,000; Millipore., USA). The concentrations of purified MAbs were assayed by the Bradford method.

2.6. Western immunoblotting assay

Recombinant TNF-alpha or FIPV-infected macrophage culture supernatants that contained natural TNF-alpha were run using 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and a transferred nitrocellulose membrane. Protein markers (Precision Plus Protein All Blue Standards) were purchased from Bio-Rad. The blot was blocked with 5% nonfat dry milk powder in TBST (20 mM Tris-HCl, pH 8.0, 0.88% NaCl, 0.05% Tween-20) for 1 h at 37 °C, followed by 1 h incubation at 37 °C with each anti-feline TNF-alpha MAb. Following washing, the membrane was incubated with horseradish peroxidase conjugated goat anti-mouse IgG1 for 1 h at 37 °C, and then visualized in the substrate for 10 min.

2.7. Neutralization test of MAbs against feline TNF-alpha using WEHI-164 cells

WEHI-164 cells were suspended at a density of 1×10^6 cells/ml in the dilution medium containing 1 μ g/ml of Actinomycin D (Sigma Lab., USA) and pre-incubated at 37 °C for 3 h. Serially diluted MAbs were mixed with 40 ng/ml recombinant TNF-alpha or FIPV-infected macrophage culture supernatants that were used as natural feline TNF-alpha samples (final concentration of 1:20). The mixture was incubated at 37 °C for 1 h. Pre-incubated cells were seeded in a volume of 50 μ l in the wells of a 96-well plate. Fifty microliters of the mixture was then added into each well.

After incubation at 37 °C for 24 h, 10 µl of WST-8 solution (WST-8 cell proliferation assay kit; Kishida Chemical Co., Ltd., Japan) was added, and the cells were returned to the incubator for 1 h. The absorbance of formazan produced was measured at 450 nm with a 96-well spectrophotometric plate reader, as described by the manufacturer. The percent neutralization was calculated by the following formula: Neutralization (%) = (O.D. of wells containing MAb and samples – O.D. of wells containing samples alone)/O.D. of wells without MAb and samples × 100.

2.8. 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 as controls were used in this study. FIP diagnosis was confirmed upon postmortem examination, revealing peritoneal and pleural effusions, and pyogranuloma in the major organs. All experiments were performed in accordance with the Guidelines for Animal Experiments of Kitasato University.

2.9. Separation of neutrophils

The separation of feline neutrophils was performed as described by Takano et al. (2009). Briefly, heparinized blood (10 ml) from cats was diluted 2-fold with PBS, and subjected to Ficoll-Hypaque density gradient centrifugation at 540g for 20 min. After removal of peripheral blood mononuclear cells and the supernatant by aspiration from the top layer, the pellets were mixed with an equal volume of saline containing 6% dextran for the separation of granulocytes, and allowed to stand for 45 min at 37 °C. The top clear layer was centrifuged at 400g for 10 min. 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. 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.

2.10. Culture of neutrophils with specimens from FIP cats

Feline neutrophils (1×10^5 cells/100 µl) were seeded into 96-well plates and cultured in the presence of recombinant TNF-alpha (final concentration 100 ng/ml or 10 ng/ml) and/or anti-feline TNF-alpha MAb (final concentration 10 µg/ml) for 24 h. After incubation, 10 µl of WST-8 solution was added, and the cells were returned to the incubator for 4 h. Absorbance was measured as described above. The percent viability was calculated by the following formula: Cell viability (%) = (after incubation O.D./prior to incubation O.D.) × 100.

2.11. RNA isolation and cDNA preparation

Total cellular RNA was extracted from macrophages using a High Pure RNA Isolation Kit (Roche Diagnostics, Switzerland) according to the instructions of the manufacturer. RNA was dissolved in the elution buffer. Using total cellular RNA as a template, cDNA was synthesized using Ready-to-Go RT-PCR beads (GE

Healthcare Life Sciences, USA). Reverse transcription was performed in a 50 µl final volume containing 0.5 µg oligo(dT)_{12–18} primers. The resulting solution was incubated at 42 °C for 1 h to synthesize cDNA.

2.12. Determination of feline GAPDH and fAPN mRNA expression levels

cDNA was amplified by PCR using specific primers for feline GAPDH and fAPN. Primer sequences are shown in Table 1. PCR was performed in a total volume of 50 µl. One microliter of sample cDNA was mixed with a 10-fold concentrated reaction buffer (TaKaRa, Japan), 4 µl of the deoxynucleotide mix (TaKaRa, Japan) containing 2.5 mM each, 2 µl of 20 µM primer mix, 0.25 µl of Ex Taq polymerase (1000 U; TaKaRa, Japan), and 37.75 µl of distilled water. Using a PCR Thermal Cycler Dice (TaKaRa, Japan), DNA was amplified at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 55 °C for 45 s, and synthesis at 72 °C for 45 s, with a final extension at 55 °C for 5 min. The PCR products were resolved by electrophoresis on 2% agarose gels. The gels were incubated with SYBR Green I Nucleic Acid Gel Stain (Roche Diagnostics, Switzerland), and bands were visualized using a UV transilluminator at 312 nm and photographed. Band density was quantified under appropriate UV exposure by video densitometry using Image J (NIH, USA) fAPN mRNA was quantitatively analyzed in terms of the relative density value to mRNA for the housekeeping gene GAPDH.

2.13. Recovery of alveolar macrophages

Feline alveolar macrophages were obtained by bronchoalveolar lavage with Hank's balanced salt solution (HBSS) from SPF cats, as previously described by Hohdatsu et al. (1991).

2.14. Effect of anti-fNTF-alpha MAb on fAPN mRNA expression levels in TNF-activated macrophages

Feline alveolar macrophages (2×10^6 cells) were cultured in medium containing recombinant TNF-alpha (10 ng/ml) and/or anti-feline TNF-alpha MAb (4 µg/ml). The cells were collected after culturing for 3 days, and fAPN mRNA expression levels were measured.

2.15. Effect of anti-feline TNF-alpha MAb on TNF-induced apoptosis in the T-lymphocyte cell line

Recombinant TNF-alpha (20 ng/ml) and hybridoma culture supernatants (final concentration of 1:8) were mixed and incubated 37 °C. Fet-J cells (2×10^6 cells) were cultured for 4 h in the mixture, and examined for their apoptosis-inducing activities.

2.16. Detection of apoptosis by TUNEL

Apoptotic cells were detected by TUNEL according to the method of Takano et al. (2007a).

2.17. 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 1
Sequence of PCR primers for feline GAPDH and APN.

	Orientation	Nucleotide sequence	Location	Length	Reference
GAPDH	Forward	5'-AATTCCACGGCAGTCAAGG-3'	158–178	97	Avery and Hoover (2004)
	Reverse	5'-CATTGTGATGTTGGCGGATC-3'	235–254		
APN	Forward	5'-AGGCTACAGATGCTCGGAAA-3'	614–635	248	GenBank Accession No. U96104
	Reverse	5'-ACTGGGAGCTCTTGCTCCA-3'	863–882		

3. Results

3.1. Production and characterization of MAb against feline TNF-alpha

Cell fusion was conducted 3 times using the spleen cells of mice immunized with recombinant TNF-alpha. Screening by ELISA revealed that 9 hybridomas were formed. The immunoglobulin isotype and polypeptide specificity of MABs secreting hybridomas are shown in Table 2 and Fig. 1.

All MABs were tested for their reactivity with recombinant TNF-alpha using Western blotting. Five of the MABs were able to detect

the 17-kDa band (Fig. 1). Reactivity with natural TNF-alpha contained in FIPV-infected macrophage culture supernatants was similarly confirmed. Seven of the MABs were able to detect the 55-kDa band (Fig. 1).

3.2. Neutralizing activity of MABs against feline TNF-alpha

All MABs were tested for their neutralizing activity against recombinant TNF-alpha by the WEHI-164 cytotoxicity assay system. The dose required to inhibit 50% of cell death (the IC50 value) was used as a parameter to evaluate the neutralizing activity of MABs.

Table 2
Characterization of MABs against feline TNF-α.

Mab	Subclass	ELISA O.D. value against rTNF-α	Western blot analysis of MAB reactivity with fTNF-α		50% inhibitive concentration of MAB against WEHI-164 cytotoxicity induced by fTNF-α (ng/ml)	
			rTNF-α	nTNF-α in FIPV-infected macrophage culture supernatant	rTNF-α	nTNF-α in FIPV-infected macrophage culture supernatant
1-1	IgG1/κ	1.389	17 kDa	50 kDa	11 ± 5	N.D.
1-3	IgG1/κ	1.470	17 kDa	50 kDa	682 ± 206	664 ± 450
1-4	IgG1/κ	1.425	17 kDa	50 kDa	142 ± 25	N.D.
2-3	IgG1/κ	2.748	17 kDa	50 kDa	5 ± 1	N.D.
2-4	IgG1/κ	2.875	17 kDa	50 kDa	8 ± 2	13 ± 8
2-1	IgG1/κ	2.613	-	50 kDa	107 ± 21	N.D.
3	IgG1/κ	2.592	-	50 kDa	38 ± 14	315 ± 98
1-2	IgG1/κ	1.498	-	-	664 ± 160	N.D.
2-2	IgG1/κ	2.679	-	-	22 ± 6	N.D.

* mean ± S.E. N.D.: Not done.

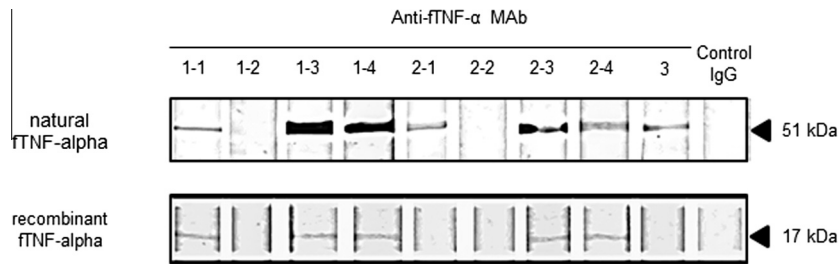


Fig. 1. Reactivity of MABs with feline TNF-alpha in Western blotting analysis. Recombinant TNF-alpha or FIPV-infected macrophage culture supernatants that contained natural TNF-alpha were run using 15% SDS-PAGE and a transferred nitrocellulose membrane. The blot was blocked with 5% nonfat dry milk powder for 1 h at 37 °C, followed by 1 h incubation at 37 °C with each anti-feline TNF-alpha MAB. Following washing, the membrane was incubated with horseradish peroxidase conjugated goat anti-mouse IgG1 for 1 h at 37 °C, and then visualized in the substrate for 10 min. Control IgG: Anti-glutathione S-transferase mouse MAB.

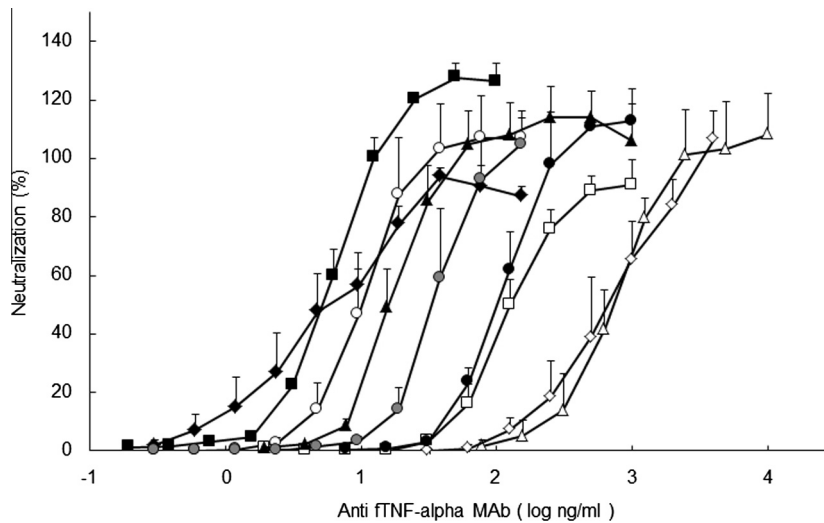


Fig. 2. Neutralization dose-response curve against recombinant fTNF-alpha. The neutralizing activities of MABs against fTNF-alpha-induced cytotoxicity on WEHI-164 cells were measured. WEHI-164 cells were treated with mixtures of serial dilutions of MABs and fTNF-alpha, and the level of TNF-alpha-induced cytotoxicity was measured after 24 h. White circle; MAB 1-1, White triangle; MAB 1-2, White square; MAB 1-3, White rhombus; MAB 1-4, Black circle; MAB 2-1, Black triangle; MAB 2-2, Black square; MAB 2-3, Black rhombus; MAB 2-4, Gray circle; MAB 3.

All MABs neutralized recombinant TNF-alpha activity in a concentration-dependent manner. Of these MABs, MAB 1-1, 2-3, and 2-4 showed markedly higher neutralizing activities (Table 2, Fig. 2).

The natural TNF-alpha-neutralizing activity of the MABs was evaluated. FIPV-infected macrophage culture supernatants were used for natural TNF-alpha. Three MABs that were selected based on their recombinant TNF-alpha-neutralizing activity were analyzed to determine whether they inhibited natural TNF-alpha cytotoxicity on WEHI-164 cells. MAB 2-4 exhibited strong neutralizing activity, which indicated that it exhibited strong neutralizing activity against both recombinant and natural TNF-alpha (Table 2, Fig. 3).

3.3. Influence of anti-feline TNF-alpha MAB on the survival rate of FIP cat-derived neutrophils cultured with recombinant TNF-alpha

A TNF-alpha-induced increase in the survival rate of neutrophils in cats with FIP has been previously reported (Takano et al., 2009). An increase in neutrophils has also been suggested to aggravate the pathology of FIP. Based on these reports, we investigated whether MAB 2-4 inhibited recombinant TNF-alpha-induced elevations in the survival rate of neutrophils. The survival rate of cells was significantly higher in the presence of 100 ng/ml of recombinant TNF-alpha than in that of the medium alone (Fig. 4). On the other hand, the survival rate of cells was significantly lower in the presence of 100 ng/ml of recombinant TNF-alpha and MAB 2-4 than in that of recombinant TNF-alpha alone.

3.4. Inhibitory effect of anti-feline TNF-alpha MAB on fAPN mRNA expression in feline alveolar macrophages cultured with recombinant TNF-alpha

TNF-alpha elevates fAPN expression levels in macrophages, and FIPV infectivity and viral production were shown to be increased in macrophages (Takano et al., 2007b). We investigated whether MAB 2-4 inhibited recombinant TNF-alpha-induced increases in fAPN mRNA expression levels. fAPN mRNA expression levels in feline alveolar macrophages were significantly higher in the presence of recombinant TNF-alpha than in that of the medium alone (Fig. 5). On the other hand, fAPN mRNA expression levels were significantly

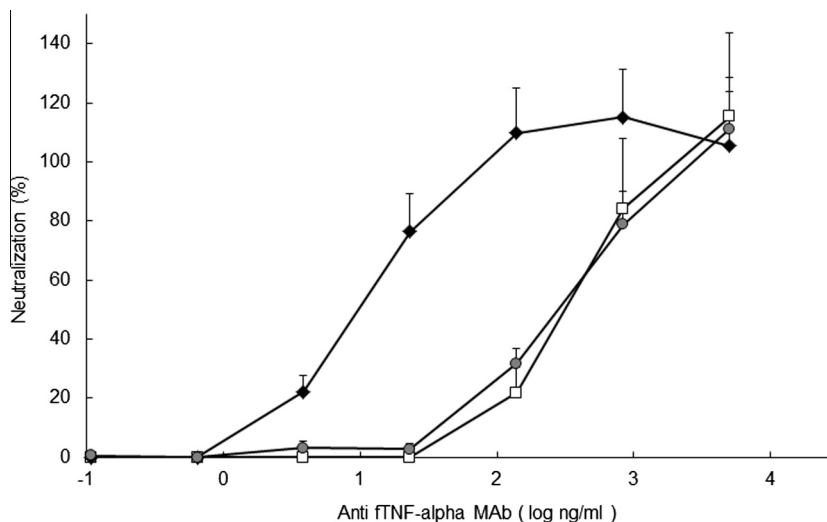


Fig. 3. Neutralization dose-response curve against natural TNF-alpha in FIPV-infected macrophage culture supernatants. The neutralizing activities of MABs against FIPV-infected macrophage culture supernatant-induced cytotoxicity on WEHI-164 cells were measured. WEHI-164 cells were treated with mixtures of serial dilutions of MABs and FIPV-infected macrophage culture supernatants, and the level of TNF-alpha-induced cytotoxicity was measured after 24 h. White square; MAB 1-3, Black rhombus; MAB 2-4, Gray; MAB 3.

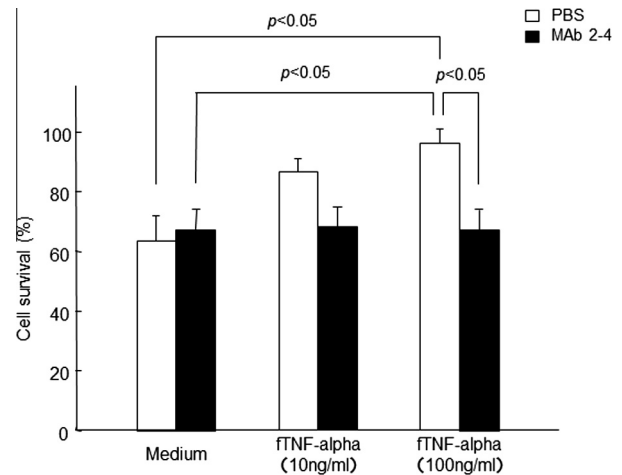


Fig. 4. Influence of anti-feline TNF-alpha MAB on the survival rate of neutrophils derived from FIP cats cultured with recombinant TNF-alpha. Purified feline neutrophils were treated with a mixture of MAB and medium or TNF-alpha (10 or 100 ng/ml). As a control, neutrophils were treated with PBS instead of MAB 2-4. Cell viability was assessed after 24 h of incubation by the WST-8 assay.

lower in the presence of recombinant TNF-alpha and MAB 2-4 than in that of recombinant TNF-alpha alone.

3.5. Effect of anti-feline TNF-alpha MAB on TNF-alpha-induced apoptosis in the T-lymphocyte cell line

TNF-alpha induces apoptosis in lymphocytes, especially T lymphocytes, resulting in decreased cell-mediated immunity (Takano et al., 2007a). We investigated whether MAB 2-4 inhibited recombinant TNF-alpha-induced apoptosis of lymphocytes using the feline T-lymphocyte cell line, Fet-J cells. A histogram of the fluorescence intensity of Fet-J cells measured using flow cytometry is shown in Fig. 6A. A bimodal peak was noted in the histogram of Fet-J cells reacted with recombinant TNF-alpha, which indicated that apoptosis was induced. The positive rate of apoptotic cells was significantly higher in the presence of recombinant TNF-alpha than in that of the medium alone. On the other hand, the positive

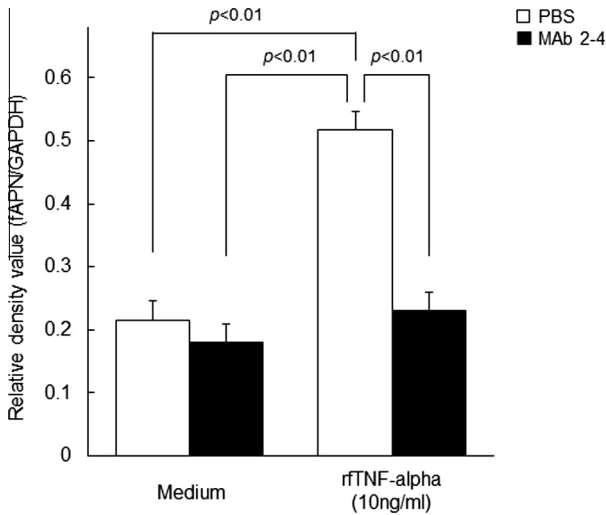


Fig. 5. Effect of anti-fTNF-alpha MAb on fAPN mRNA expression in feline alveolar macrophages cultured with recombinant TNF-alpha. Feline macrophages were treated with a mixture of MAb 2-4 and medium or TNF-alpha (10 ng/ml). As a control, macrophages were treated with PBS instead of MAb 2-4. The expression level of fAPN mRNA was assessed after 72 h of incubation.

rate of apoptotic cells was significantly lower in the presence of recombinant TNF-alpha and Mab 2-4 than in that of recombinant TNF-alpha alone (Fig. 6B).

4. Discussion

No feline TNF-alpha-neutralizing MAb has previously been prepared. We have prepared nine monoclonal antibodies (MAbs) that recognize feline TNF-alpha by immunizing mice with recombinant feline TNF-alpha. All of MAbs produced exhibited different reactivities between the recombinant and natural feline TNF-alpha contained in FIPV-infected macrophage culture supernatants using Western blotting. On Western blotting, TNF-alpha was degenerated by treatment with 2-mercaptoethanol, for which MAb 1-2 and 2-2 did not react with either recombinant or natural TNF-alpha. This result suggests that these MAbs recognized conformation epitope

of TNF-alpha. In contrast, MAb 2-1 and 3 react with natural TNF-alpha only; however, the details of their antigen-recognizing properties could not be clarified. Recombinant TNF-alpha forms a monomer (17-kDa), whereas natural TNF-alpha forms a homotrimer (55-kDa), which suggests that recombinant TNF-alpha is monomeric form while natural TNF-alpha is trimetric form (R&B Systems Product data sheet). Therefore, MAb 2-1 and 3 may recognize only trimetric TNF-alpha. Saijo et al. described the preparation of the trimetric human TNF-alpha-recognizing MAb 3F2 (Saijo et al., 1995). They showed that the weak reactivity of MAb 3F2 was observed with monomeric TNF-alpha dissociated from trimetric TNF-alpha by detergent. MAb 2-1 and 3 reacted with immobilized monomeric TNF-alpha on ELISA. Smith and Baglioni. showed that monomeric TNF-alpha bound to one another to form trimetric TNF-alpha in a solution that did not contain a degenerating agent, which indicated that recombinant TNF-alpha also formed trimer in a solution containing no detergent and reacted in ELISA (Smith and Baglioni, 1987). Therefore, these findings suggest that MAb 2-1 and 3 possess similar properties to those of MAb 3F2.

Although all prepared MAbs possessed recombinant TNF-alpha-neutralizing activity, the strength of this activity varied among the MAbs. MAb 2-3 and 2-4 possessed the strongest recombinant TNF-alpha-neutralizing activity, at levels 80–100 times higher than those of MAb 1-2 and 1-4. The IC50 values of MAb 2-3 and 2-4 for recombinant TNF-alpha were 5 ± 1 and 8 ± 2 ng/ml, respectively. The IC50 value of an anti-human TNF-alpha antibody used as a therapeutic drug, adalimumab, has been reported to be 24.4 ng/ml, which suggests that MAb 2-3 and 2-4 possess neutralizing activity equivalent to or stronger than that of adalimumab (Song et al., 2008).

We measured the natural TNF-alpha-neutralizing activities of the MAbs. Three MAbs were selected based on their recombinant TNF-alpha-neutralizing activities: MAb 2-4 and 1-3 with high and medium recombinant TNF-alpha-neutralizing activities and MAb 3 with intermediate activity between that of MAb 2-4 and 1-3.

To compare the recombinant and natural TNF-alpha-neutralizing activities of each MAb, natural TNF-alpha was used at a concentration exhibiting a specific activity level similar to the cytotoxicity of recombinant TNF-alpha (10 ng/ml) on WEHI-164 cells. The natural TNF-alpha-neutralizing activity levels of MAb 1-3 and 3

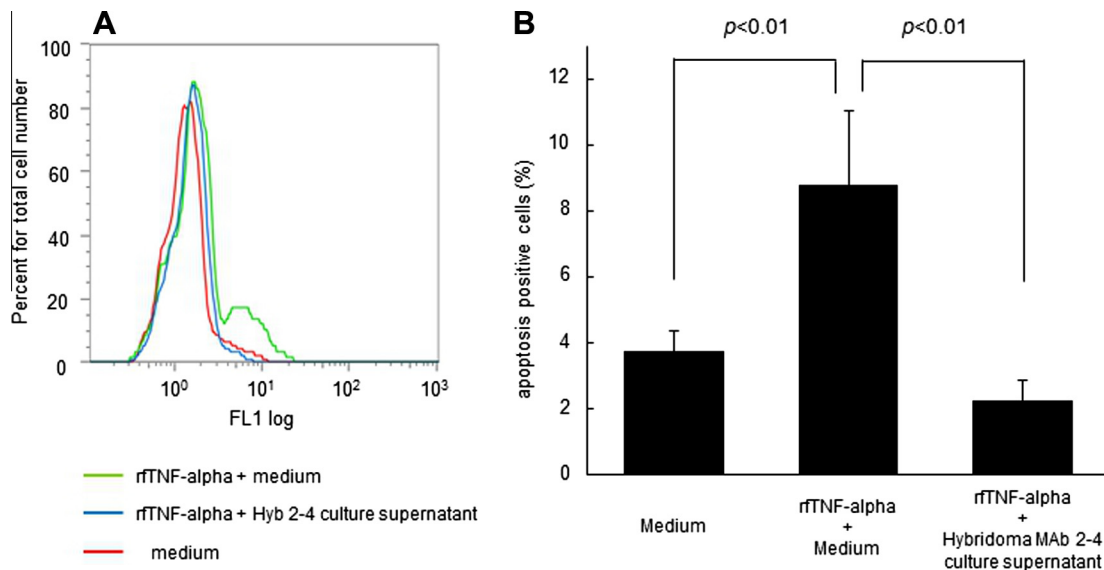


Fig. 6. Effect of MAb 2-4 on TNF-induced apoptosis in the T-lymphocyte cell line. Fet-J cells were cultured for 4 h in a mixture of TNF-alpha and hybridoma culture supernatants, and were examined for their apoptosis-inducing activities by TUNEL. (A) Representative histogram obtained by flow cytometry. (B) Percentages of apoptosis cells to the total number of Fet-J cells.

decreased to approximately 1/5–1/10 that of the recombinant TNF-alpha-neutralizing activity level, whereas the activity of MAB 2–4 decreased to only approximately 1/2, which shows that the decrease in neutralizing activity among the 3 MABs was the smallest in MAB 2–4. Therefore, these results demonstrate that MAB 2–4 efficiently neutralized feline TNF-alpha at a lower dose than those of the other MABs in cats with FIP.

Neutrophilia, an increase in APN on macrophages, and lymphopenia are induced in cats with FIP by TNF-alpha, which is produced by FIPV-infected macrophages. (Takano et al., 2007a,b, 2009) We investigated the effect of MAB 2–4 on this pathology of FIP *in vitro*, and confirmed that the neutralization of feline TNF-alpha activity with MAB 2–4 inhibited: (i) an increase in the survival rate of neutrophils in cats with FIP, (ii) APN mRNA expression in macrophages, and (iii) apoptosis of the feline T-lymphocyte cell line. Several drugs to treat FIP have been reported, but these mostly aim at an antiviral action (Hartmann and Ritz, 2008; Balzarini et al., 2006; Barlough and Shacklett, 1994; Hsieh et al., 2010; Kim et al., 2013). However, FIP is a viral infection that causes an immune-mediated inflammatory disease. The combination of MAB 2–4 that we prepared using already reported anti-FIPV agent(s) may eliminate the virus while inhibiting inflammatory reactions. Currently, steroids are mainly used to inhibit inflammatory reactions in FIP; however, steroids also inhibit the immune system useful for the elimination of FIPV. Since MAB 2–4 specifically inhibits TNF-alpha, a factor aggravating the pathology of FIP, it may be more appropriate than steroids.

MAB 2–4 is a mouse-derived protein. The administration of a large dose of a mouse antibody, which is xenogeneic for cats, may induce anaphylactic reactions. Umehashi et al. reported that the administration of a MAB with a constant region converted to the feline form (mouse-feline chimeric antibody) induced no anaphylactic reaction (Umehashi et al., 2002). To reduce this side effect, modification of MAB 2–4 to a mouse-feline chimeric antibody employing recombinant DNA techniques is needed.

In conclusion, the anti-feline TNF-alpha MABs prepared by us appear to be useful for the treatment of FIP *in vitro*. The modification of MABs to a mouse-feline chimeric antibody and further investigations on the *in vivo* effects of these MABs may lead to their application as a new therapeutic drug for FIP.

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