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# Generation, characterization and therapeutic potential of anti-feline TNF-alpha MAbs 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.



## 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 μM 2-mercaptoethanol, and 2 μg/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 μg of commercial recombinant feline TNF-alpha (R&B SYSTEMS., USA) and complete Freund's adjuvant. Mice received an intraperitoneal booster dose of 1 μg 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 sells and mouse myeloma cells (FO) was 3:1. The selective medium contained hypoxanthine ($10^{-4}$ M), aminopterin ($4 \times 10^{-7}$ M), and thymidine ($1.6 \times 10^{-5}$ M). Fused cells, at a concentration of $1 \times 10^6$ cells/ml, were dispensed in 100 μl volumes into the wells of 96-well, flat-bottomed microplates and incubated at 37 °C in a humid atmosphere containing 5% $CO_2$. 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 ($1.6 \times 10^{-5}$ 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 μl/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 μl of the hybridoma culture supernatant. After 60 min incubation at 37 °C, the plate was washed and 100 μl 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 μl 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 $Na_2HPO_4$ buffer (pH 4.8) and adding 0.2 μl/ml of 30% $H_2O_2$. The reaction was stopped with 3 N $H_2SO_4$ 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 $1 \times 10^6$ cells/ml in the dilution medium containing 1 μg/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 μl in the wells of a 96-well plate. Fifty microliters of the mixture was then added into each well.