

# Defucosylated Anti-Epidermal Growth Factor Receptor Monoclonal Antibody 134-mG<sub>2a</sub>-f Exerts Antitumor Activities in Mouse Xenograft Models of Dog Epidermal Growth Factor Receptor-Overexpressed Cells

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The epidermal growth factor receptor (EGFR) is a type I transmembrane protein, which is a member of the human epidermal growth factor receptor (HER) family of receptor tyrosine kinases. EGFR is a crucial mediator of cell growth and differentiation and forms homodimers or heterodimers with other HER family members to activate downstream signaling cascades. We previously established an anti-human EGFR (hEGFR) monoclonal antibody (mAb), clone EMab-134 (mouse IgG<sub>1</sub>), by immunizing mice with the ectodomain of hEGFR. In this study, the subclass of EMab-134 was converted from IgG<sub>1</sub> to IgG<sub>2a</sub> (134-mG<sub>2a</sub>) and further defucosylated (134-mG<sub>2a</sub>-f) to facilitate antibody-dependent cellular cytotoxicity (ADCC). Although 134-mG<sub>2a</sub>-f was developed against hEGFR, it was shown to cross-react with dog EGFR (dEGFR) using flow cytometry. The dissociation constant ( $K_D$ ) of 134-mG<sub>2a</sub>-f against dEGFR-overexpressed CHO-K1 (CHO/dEGFR) cells was determined by flow cytometry to be  $3.3 \times 10^{-9}$  M, indicating that 134-mG<sub>2a</sub>-f possesses a high binding affinity to dEGFR. Analysis *in vitro* revealed that 134-mG<sub>2a</sub>-f contributed to high levels of ADCC and complement-dependent cytotoxicity (CDC) in experiments targeting CHO/dEGFR cells. Furthermore, the *in vivo* administration of 134-mG<sub>2a</sub>-f significantly inhibited the development of CHO/dEGFR in comparison with the results observed in response to control mouse IgG. Taken together, the findings of this study demonstrate that 134-mG<sub>2a</sub>-f could be useful as part of a therapeutic regimen for dEGFR-expressing canine cancers.

**Keywords:** EGFR, monoclonal antibody, ADCC, CDC, antitumor activity

## Introduction

THE EPIDERMAL GROWTH factor receptor (EGFR) is a transmembrane glycoprotein that constitutes one of the four members of the EGFR family of tyrosine kinase receptors.<sup>(1)</sup> The EGFR family consists of four different receptors with a common structure: HER1 (EGFR/ErbB1), HER2 (ErbB2/neu), HER3 (ErbB3), and HER4 (ErbB4).<sup>(2,3)</sup> The downstream signal pathway of EGFR includes Src kinase, signal transducers and activators of transcription, Ras/Raf/mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/Akt pathway, which promote division, migration, antiapoptosis, oncogenesis, and/or cell cycle progression.<sup>(4)</sup> EGFR is overexpressed and contributes to poor

prognosis in many cancers.<sup>(5-8)</sup> Therefore, several anti-EGFR monoclonal antibodies (mAbs) have been developed and are used in clinical practice. Two anti-EGFR mAbs, cetuximab (Erbix) and panitumumab (Vectibix), have been used to treat unresectable advanced or recurrent colorectal cancer. Although there have been significant advances in treatment with mAbs for targets, including EGFR in humans like this, mAb therapy for animal species has not been largely established.

In dogs, EGFR is overexpressed in multiple tumors as well as humans.<sup>(9-14)</sup> Survival analysis showed that several dog mammary tumors with EGFR overexpression decreased disease-free and overall survival.<sup>(10)</sup> *In vitro* analysis exhibited that vandetanib (caprelsa), a tyrosine kinase inhibitor targeting EGFR with antitumor effects, dose dependently

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inhibited EGFR phosphorylation and PI3K/Akt activation to inhibit EGF-induced proliferation and invasion in dog mammary carcinoma cell lines.<sup>(15)</sup> Thus, the development of a novel anti-EGFR mAb for therapeutic and/or diagnostic use for dog cancers is essential, but no mAb targeting dog EGFR (dEGFR) is available yet. By contrast, the sequences of human EGFR (hEGFR) and dEGFR are 91% identical; thus, Singer *et al.* investigated whether cetuximab can also exert an effect on dEGFR.<sup>(16)</sup> Consequently, cetuximab was shown to exert effects on cell viability and cell cycle in a dEGFR-expressing canine mammary carcinoma cell line, P114. For the establishment of dog cancer treatment, we must investigate whether anti-hEGFR mAbs are useful against dEGFR.

We previously developed a novel anti-hEGFR mAb, EMab-134,<sup>(17)</sup> by immunizing mice with the purified recombinant ectodomain of hEGFR (hEGFRec) from culture supernatants of hEGFRec-overexpressed LN229 cells. This mAb is useful for flow cytometry, Western blotting, and immunohistochemical analyses to detect hEGFR. Furthermore, the IgG<sub>2a</sub> version of EMab-134, 134-mG<sub>2a</sub>, has antitumor activities in mouse xenograft models of hEGFR-overexpressing oral squamous cell carcinoma.<sup>(18)</sup> In this study, we defucosylated 134-mG<sub>2a</sub> (134-mG<sub>2a</sub>-f) to reinforce antibody-dependent cellular cytotoxicity (ADCC). Then, we determined whether 134-mG<sub>2a</sub>-f exhibits ADCC and complement-dependent cytotoxicity (CDC) in dEGFR-overexpressed cells and antitumor activities in mouse xenograft models of dEGFR-overexpressed cells.

## Materials and Methods

### Antibodies

Anti-hEGFR mAb EMab-134 (mouse IgG<sub>1</sub>, kappa) was developed as previously described.<sup>(17)</sup> To generate 134-mG<sub>2a</sub>, V<sub>H</sub> cDNA of EMab-134 and C<sub>H</sub> mouse IgG<sub>2a</sub> were subcloned into the pCAG-Ble vector, and V<sub>L</sub> and C<sub>L</sub> cDNAs of EMab-134 were subcloned into the pCAG-Neo vector (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), respectively.<sup>(18)</sup> Vectors were transfected into BINDS-09 cells (FUT8-deficient ExpiCHO-S cells) using the ExpiCHO Expression System (Thermo Fisher Scientific, Inc., Waltham, MA).<sup>(19)</sup> The resulting mAb, 134-mG<sub>2a</sub>-f, was purified with Protein G-Sepharose (GE Health Care Bio-Sciences, Pittsburgh, PA). Mouse IgG (cat. no. I8765) and IgG<sub>2a</sub> (cat. no. M7769) were purchased from Sigma-Aldrich (St. Louis, MO).

### Cell lines

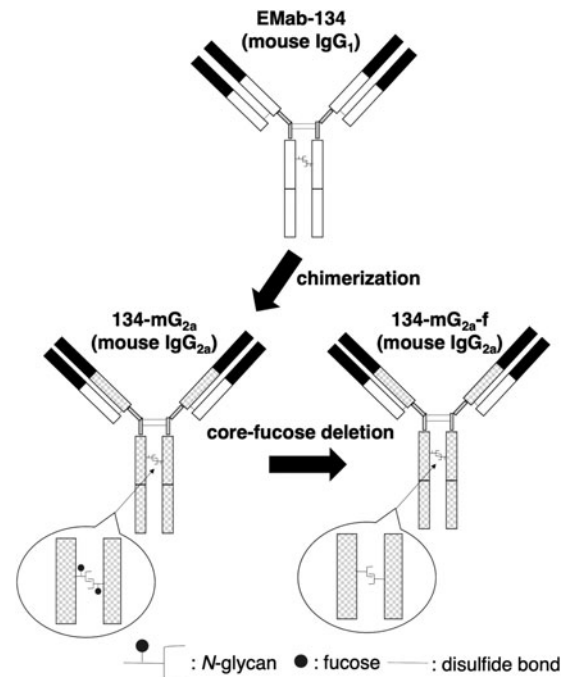
The CHO-K1 cell line was obtained from the American Type Culture Collection (Manassas, VA). dEGFR-overexpressed CHO-K1 cells (CHO/dEGFR) were established by the transfection of pCAG/3xRIEDL-dEGFR into CHO-K1 cells using the Neon transfection system (Thermo Fisher Scientific, Inc.).<sup>(20)</sup> The amino acid sequence of 3xRIEDL comprises 15 amino acids (RIEDLRIEDLRIEDL). CHO-K1 and CHO/dEGFR were cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Nacalai Tesque, Inc., Kyoto, Japan), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Thermo Fisher Scientific, Inc.), 100 U/mL of penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B (Nacalai Tesque, Inc.). Cells were cultured at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### Animals

All animal experiments were performed following relevant guidelines and regulations to minimize animal suffering and distress in the laboratory. Animal experiments for ADCC and antitumor activity were approved by the Institutional Committee for Experiments of the Institute of Microbial Chemistry (permit no. 2021–019 for ADCC assays, 2021–006 for antitumor experiments). Mice were maintained in a specific pathogen-free environment (23°C ± 2°C, 55% ± 5% humidity) on an 11-hour light/13-hour dark cycle with food and water supplied *ad libitum* across the experimental period. Mice were monitored for health and weight every 2 or 5 days during the 3-week period of each experiment. The loss of original body weight to a point >25% and/or a maximum tumor size >3000 mm<sup>3</sup> were identified as humane endpoints for euthanasia. Mice were euthanized by cervical dislocation; death was verified by respiratory and cardiac arrest.

### Flow cytometry

CHO-K1 and CHO/dEGFR cells were harvested by brief exposure to 0.25% trypsin/1 mM ethylenediamine tetraacetic acid (EDTA; Nacalai Tesque, Inc.). After washing with blocking buffer: 0.1% bovine serum albumin in phosphate-buffered saline (PBS), cells were treated with 1 µg/mL of 134-mG<sub>2a</sub>-f or control blocking buffer for 30 minutes at 4°C followed by Alexa Fluor 488-conjugated anti-mouse IgG at a dilution of 1:1000 (cat no. 4408S; Cell Signaling Technology, Inc., Danvers, MA) for 30 minutes at 4°C. Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp., Tokyo, Japan).



**FIG. 1.** Production of 134-mG<sub>2a</sub> (mouse IgG<sub>2a</sub>) and 134-mG<sub>2a</sub>-f (core-fucose-deficient mouse IgG<sub>2a</sub>) from EMab-134 (mouse IgG<sub>1</sub>).

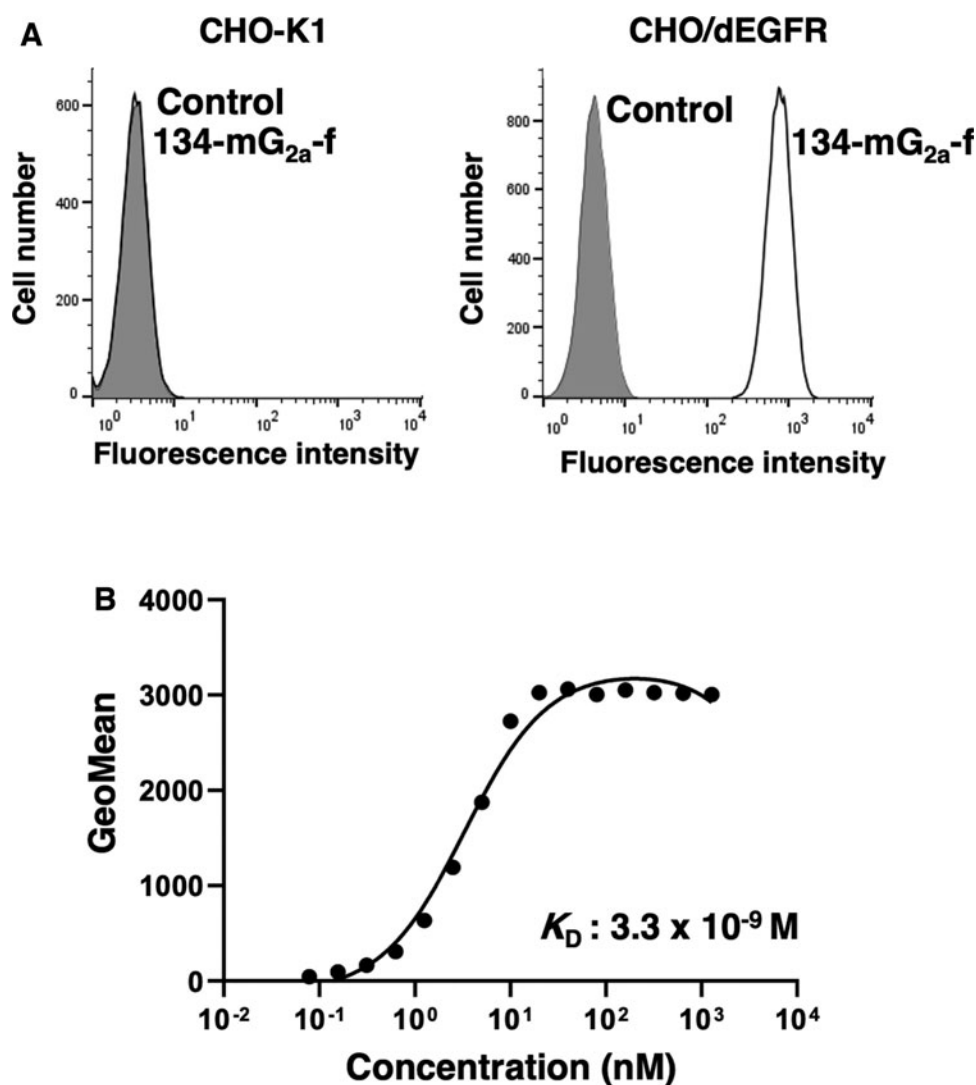
*Determination of the binding affinity*

CHO/dEGFR cells were suspended in 100  $\mu$ L of serially diluted 134-mG<sub>2a</sub>-f (0.006–100  $\mu$ g/mL) followed by the addition of Alexa Fluor 488-conjugated anti-mouse IgG (1:200; Cell Signaling Technology, Inc.). Fluorescence data were collected using the EC800 Cell Analyzer (Sony Corp.). The dissociation constant ( $K_D$ ) was calculated by fitting binding isotherms to built-in one-site binding models in GraphPad Prism 8 (GraphPad Software, Inc., La Jolla, CA).

*Antibody-dependent cellular cytotoxicity*

A total of five female 5-week-old BALB/c nude mice (weighing 14–17 g) were purchased from Charles River Laboratories, Inc., (Kanagawa, Japan). Spleen cells from five mice were used as the source of NK cells for the evaluation of ADCC, which has been reported previously.<sup>(18)</sup> Following euthanasia by cervical dislocation, the spleens were removed

aseptically and single-cell suspensions were obtained by forcing spleen tissues through a sterile cell strainer (352360; BD Falcon, Corning, New York, NY) using a syringe. Erythrocytes were lysed with a 10 s exposure to ice-cold distilled water. Splenocytes were washed with Dulbecco's modified Eagle's medium (DMEM; Nacalai Tesque, Inc.) and resuspended in DMEM with 10% FBS; this preparation was used as effector cells. Target tumor cells were labeled with 10  $\mu$ g/mL Calcein AM (Thermo Fisher Scientific, Inc.) and resuspended in the same medium. The target cells ( $2 \times 10^4$  cells/well) were plated in 96-well plates and mixed with effector cells (effector/target cell ratio, 100), 100  $\mu$ g/mL of 134-mG<sub>2a</sub>-f, or control mouse IgG<sub>2a</sub>. Following a 5-h incubation at 37°C, the release of Calcein into the supernatant was measured in each well. The fluorescence intensity was determined using a microplate reader (Power Scan HT; BioTek Instruments, Inc., Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm.



**FIG. 2.** Flow cytometry using 134-mG<sub>2a</sub>-f. **(A)** CHO/dEGFR and CHO-K1 cells were treated with 134-mG<sub>2a</sub>-f or buffer control, followed by secondary antibodies. Fluorescence data were collected using the EC800 Cell Analyzer. **(B)** Determination of the binding affinity of 134-mG<sub>2a</sub>-f for CHO/dEGFR cells using flow cytometry. CHO/dEGFR cells were suspended in 100  $\mu$ L of serially diluted 134-mG<sub>2a</sub>-f, followed by the addition of Alexa Fluor 488-conjugated anti-mouse IgG. Fluorescence data were collected using the EC800 Cell Analyzer. EGFR, epidermal growth factor receptor.

Cytolytic activity (% lysis) was calculated using the equation  $\% \text{ lysis} = (E - S)/(M - S) \times 100$ , where “E” is the fluorescence measured in combined cultures of target and effector cells, “S” is the spontaneous fluorescence of target cells only, and “M” is the maximum fluorescence measured following the lysis of all cells with a buffer containing 0.5% Triton X-100, 10 mM Tris-HCl (pH 7.4), and 10 mM of EDTA.

#### Complement-dependent cytotoxicity

The cells ( $2 \times 10^4$  cells/well) were plated in 96-well plates and mixed with rabbit complement (final dilution 1:10; Low-Tox-M Rabbit Complement; Cedarlane Laboratories, Hornby, Ontario, Canada) together with 100  $\mu\text{g}/\text{mL}$  of 134-mG<sub>2a</sub>-f or control mouse IgG<sub>2a</sub>. Following 5 hours of incubation at 37°C, MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; inner salt] assay was performed using a CellTiter 96 Aqueous assay kit (Promega, Madison, WI).

#### Antitumor activity of 134-mG<sub>2a</sub> in xenografts of CHO/dEGFR cells

A total of 16 female BALB/c nude mice (5 weeks old, weighing 14–17 g) were purchased from Charles River Laboratories, Inc., and used in experiments once they reached 7 weeks of age. CHO/dEGFR cells ( $0.3 \text{ mL}$  of  $1.33 \times 10^8$  cells/mL in DMEM) were mixed with 0.5 mL BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, CA); 100  $\mu\text{L}$  of this suspension ( $5 \times 10^6$  cells) was injected subcutaneously into the left flanks of the mice. On day 6 postinoculation, 100  $\mu\text{g}$  of 134-mG<sub>2a</sub>-f ( $n=8$ ), or control mouse IgG ( $n=8$ ) in 100  $\mu\text{L}$  PBS was injected intraperitoneally. Additional antibody inoculations were performed on days 14 and 21. At 25 days following cell implantation, all mice were euthanized by cervical dislocation; tumor diameters and volumes were determined as previously described.<sup>(18)</sup>

#### Statistical analyses

All data are expressed as mean  $\pm$  standard error of the mean (SEM). Statistical analysis was conducted with Welch's *t* test for ADCC and CDC, ANOVA and Sidak's multiple comparisons tests for tumor volume and mouse weight, and Welch's *t* test for tumor weight. All calculations were performed using GraphPad Prism 7 (GraphPad Software, Inc.). A *p*-value of  $<0.05$  was considered statistically significant.

## Results

#### Production and characterization of 134-mG<sub>2a</sub>-f, a mouse defucosylated IgG<sub>2a</sub>-type anti-EGFR antibody

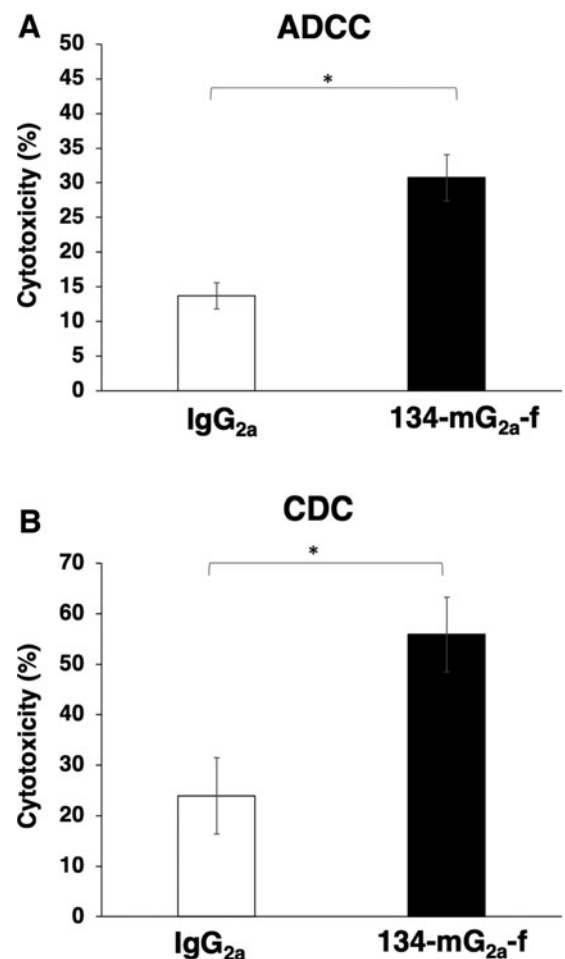
A mouse IgG<sub>2a</sub> version of the IgG<sub>1</sub> EMab-134<sup>(17)</sup> was previously generated by subcloning V<sub>H</sub> cDNA of EMab-134 and C<sub>H</sub> mouse IgG<sub>2a</sub> into the pCAG-Ble vector, and V<sub>L</sub> and C<sub>L</sub> cDNAs of EMab-134 into the pCAG-Neo vector (Fig. 1). The IgG<sub>2a</sub> version of EMab-134 was named 134-mG<sub>2a</sub>.<sup>(18)</sup> The vectors of 134-mG<sub>2a</sub> were transfected into BINDS-09 cells,<sup>(19)</sup> and the resulting defucosylated 134-mG<sub>2a</sub> was named 134-mG<sub>2a</sub>-f (Fig. 1). The sensitivity of 134-mG<sub>2a</sub>-f in

CHO/dEGFR was analyzed by flow cytometry. As shown in Figure 2A, 134-mG<sub>2a</sub>-f detected CHO/dEGFR, but not parental CHO-K1 cells.

A kinetic analysis of the interactions of 134-mG<sub>2a</sub>-f with CHO/dEGFR cells was performed by flow cytometry. As shown in Figure 2B, the  $K_D$  for the interaction of 134-mG<sub>2a</sub>-f with CHO/dEGFR cells was  $3.3 \times 10^{-9}$  M, suggesting that 134-mG<sub>2a</sub>-f shows high affinity to CHO/dEGFR cells.

#### 134-mG<sub>2a</sub>-f-mediated ADCC and CDC in CHO/dEGFR cells

We investigated whether 134-mG<sub>2a</sub>-f was capable of mediating ADCC against CHO/dEGFR cell lines. As shown in Figure 3A, 134-mG<sub>2a</sub>-f showed ADCC (31% cytotoxicity;  $p < 0.05$ ) against CHO/dEGFR cells more effectively than control mouse IgG<sub>2a</sub> (14% cytotoxicity). We then investigated whether 134-mG<sub>2a</sub>-f could mediate CDC against CHO/dEGFR cell lines. As shown in Figure 3B, 134-mG<sub>2a</sub>-f

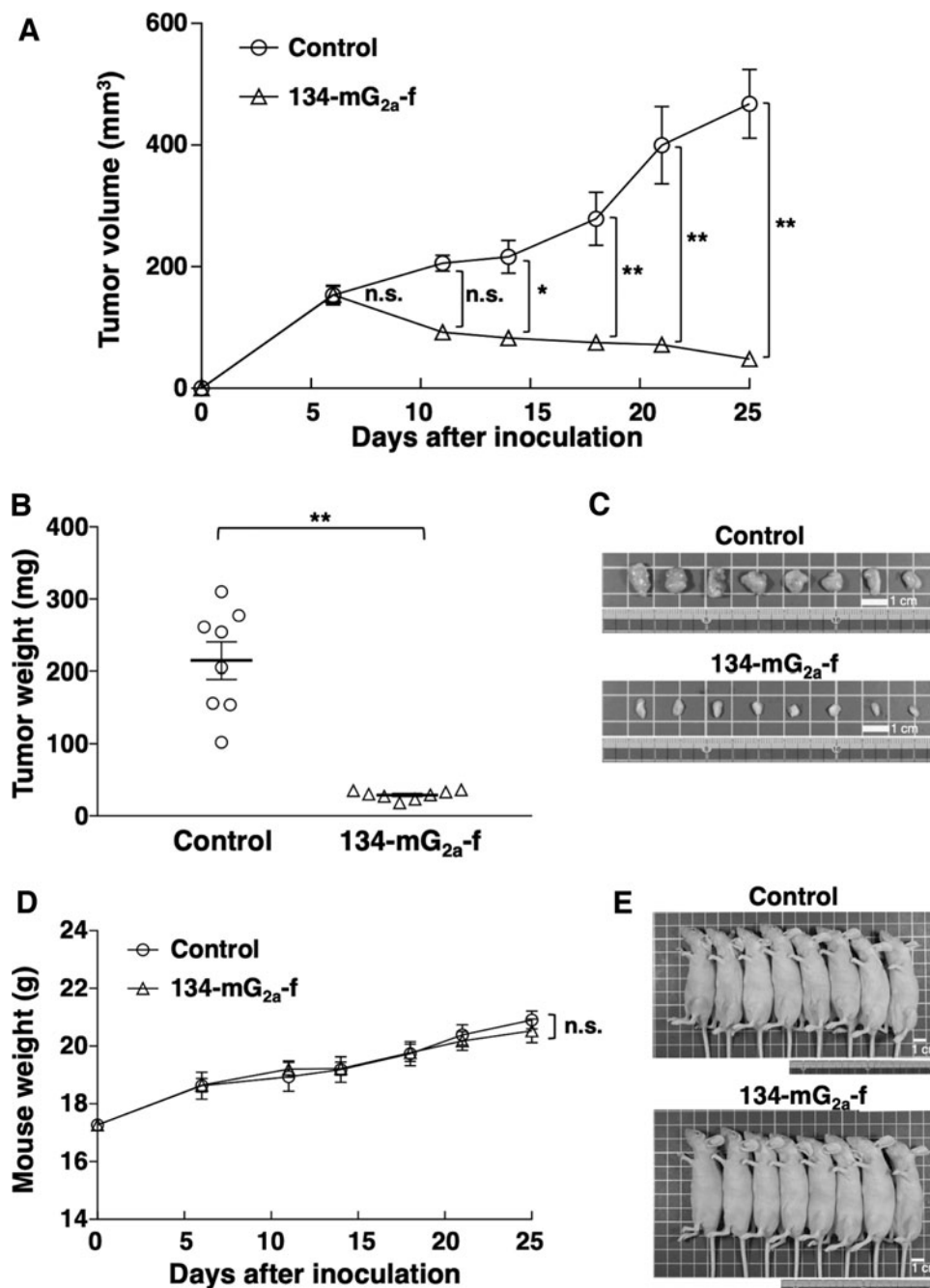


**FIG. 3.** Evaluation of ADCC and CDC elicited by 134-mG<sub>2a</sub>-f. (A) ADCC elicited by 134-mG<sub>2a</sub>-f or control mouse IgG<sub>2a</sub> targeting CHO/dEGFR cells. (B) CDC elicited by 134-mG<sub>2a</sub>-f or control mouse IgG<sub>2a</sub> targeting CHO/dEGFR cells. The values shown are the means  $\pm$  SEM. Asterisks indicate statistical significance ( $*p < 0.05$ ; Welch's *t* test). ADCC, antibody-dependent cellular cytotoxicity; CDC, complement-dependent cytotoxicity.

elicited a higher degree of CDC (56% cytotoxicity;  $p < 0.05$ ) in CHO/dEGFR cells compared with that elicited by control mouse IgG<sub>2a</sub> (24% cytotoxicity). These results demonstrated that 134-mG<sub>2a</sub>-f promoted significantly higher levels of ADCC and CDC against dEGFR-overexpressed cells.

#### Antitumor activities of 134-mG<sub>2a</sub>-f in the mouse xenografts of CHO/dEGFR cells

In the CHO/dEGFR xenograft models, 134-mG<sub>2a</sub>-f (100  $\mu$ g) and control mouse IgG (100  $\mu$ g) were injected intraperitoneally into the mice on days 6, 14, and 21 following



**FIG. 4.** Evaluation of antitumor activity of 134-mG<sub>2a</sub>-f in CHO/dEGFR xenografts. **(A)** CHO/dEGFR cells ( $5 \times 10^6$  cells) were injected subcutaneously into the left flank. After day 6, 100  $\mu$ g of 134-mG<sub>2a</sub>-f or control mouse IgG in 100  $\mu$ L PBS was injected intraperitoneally into mice; additional antibodies were then injected on days 14 and 21. The tumor volume was measured on days 6, 11, 14, 18, 21, and 25 after the injection. Values shown are the mean  $\pm$  SEM. Asterisks indicate statistical significance (\*\* $p < 0.01$ , \* $p < 0.05$ ; n.s., not significant; ANOVA and Sidak's multiple comparisons test). **(B)** Tumors of CHO/dEGFR xenografts were resected from 134-mG<sub>2a</sub>-f and control mouse IgG groups. Tumor weight on day 25 was measured from excised xenografts. Values are mean  $\pm$  SEM. Asterisk indicates statistical significance (\*\* $p < 0.01$ , Welch's  $t$ -test). **(C)** Resected tumors of CHO/dEGFR xenografts from control mouse IgG and 134-mG<sub>2a</sub>-f groups on day 25. Scale bar, 1 cm. **(D)** Body weights of the mice implanted with CHO/dEGFR xenografts were recorded on days 6, 11, 14, 18, 21, and 25 (n.s.: not significant). **(E)** Body appearance of mice on day 25. Scale bar, 1 cm.

the injection of CHO/dEGFR cells. The tumor volume was measured on days 6, 11, 14, 18, 21, and 25 after the injection. The administration of 134-mG<sub>2a</sub>-f resulted in a significant reduction in tumor development on days 14 ( $p < 0.05$ ), 18 ( $p < 0.01$ ), 21 ( $p < 0.01$ ), and 25 ( $p < 0.01$ ) compared with that of the control mouse IgG (Fig. 4A). The administration of 134-mG<sub>2a</sub>-f resulted in a 90% reduction in tumor volume compared with that of the control mouse IgG on day 25 postinjection. Furthermore, tumors from the 134-mG<sub>2a</sub>-f-treated mice weighed significantly less than those from the control mouse IgG-treated mice (87% reduction;  $p < 0.01$ , Fig. 4B). Tumors that were resected from mice on day 25 are demonstrated in Figure 4C. Total body weights did not differ significantly among the two groups (Fig. 4D). The body appearances of mice on day 25 are demonstrated in Figure 4E. Taken together, these results indicated that the administration of 134-mG<sub>2a</sub>-f effectively reduced the growth of CHO/dEGFR xenografts.

### Discussion

In human disease treatment, various types of mAbs are available and great consequences have been achieved so far.<sup>(21–23)</sup> By contrast, there is only one commercially available mAb, lokivetmab (Cytopoint) targeting interleukin-31, which is used to treat atopic dermatitis in dogs.<sup>(24,25)</sup> Lokivetmab treatment is effective in reducing itching and the severity of skin disease in dogs with a good safety profile.<sup>(26)</sup> Thus, developing effective and useful mAbs for applying to veterinary medicine is desirable.

Gene amplification or protein overexpression of EGFR has been detected in a wide range of human tumors and is significantly associated with tumor progression and poor prognosis.<sup>(27–32)</sup> The aberrant activation of EGFR initiates multiple intracellular pro-oncogenic signaling pathways, including RAS/MAPK and Akt/PI3K, leading to cell proliferation, differentiation, and migration.<sup>(33)</sup> Consequently, EGFR-targeted therapies are clinically applied as single agents or in combination with chemotherapy for several human cancers.<sup>(34–37)</sup> Interestingly, EGFR is highly conserved between humans and dogs, and it can enhance the malignant property in several canine cancers.<sup>(15,38)</sup> These results prompted us to develop a novel anti-EGFR mAb for therapeutic and/or diagnostic use for canine cancers.

The most important aim of this study was to demonstrate the cytotoxic activity of 134-mG<sub>2a</sub>-f against dEGFR-expressing cells. Indeed, this novel anti-EGFR mAb caused significant growth inhibition against CHO/dEGFR cells *in vivo* (Fig. 4), suggesting that 134-mG<sub>2a</sub>-f is applicable for antibody therapy against canine cancers. Since the cells examined in this study were only dEGFR-overexpressed cells, further studies using various types of dEGFR-expressing canine cancers are required to develop effective antibody therapies for canine cancers.

### Author Disclosure Statement

The authors have no conflict of interests.

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