Shiraz University

IJVR

ISSN: 1728-1997 (Print)
ISSN: 2252-0589 (Online)

Vol. 21 No.4 Ser. No.73 2020

IRANIAN JOURNAL OF VETERINARY RESEARCH

Original Article

Cytotoxicity effect of trastuzumab on canine peripheral blood mononuclear cells

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(Received 11 Aug 2020; revised version 11 Oct 2020; accepted 24 Oct 2020)

Abstract

Background: Trastuzumab is an antibody drug used to treat human epidermal growth factor receptor 2 (HER2) overexpressing human metastatic breast cancer. Antibody-dependent cellular cytotoxicity (ADCC) is considered to be the major mechanism of cytotoxicity of the drug. However, its ability to induce an ADCC response in canine peripheral blood mononuclear cells (PBMCs) is not well established. **Aims:** We aimed to evaluate the ability of trastuzumab in enhancing the cytotoxicity of PBMCs against canine tumor cells. **Methods:** We used canine tumor cell lines isolated from metastatic mammary gland tumors (CHMm and CIPm) and thyroid adenocarcinoma (CTAC). The binding of trastuzumab to the cells was confirmed using flow cytometry analysis. Peripheral blood mononuclear cells obtained from healthy beagles and lymphokine-activated killer (LAK) cells, generated by interleukin-2 (IL-2) stimulation of PBMCs, were used as effector cells. Standard lactate dehydrogenase (LDH) release assay was used to measure the cytotoxicity of the LAK cells against tumor cell lines in the presence of trastuzumab. **Results:** Trastuzumab enhanced the cytotoxicity of PBMCs against CHMm. Moreover, LAK cells killed CHMm synergistically in the presence of trastuzumab. However, the presence of trastuzumab did not produce such a synergistic effect when LAK cells acted against CIPm and CTAC. **Conclusion:** We confirmed the ability of trastuzumab to induce an ADCC response in canine PBMCs and determined its synergistic effect with LAK cells. Although the *in vitro* system in the present study did not show the induction of trastuzumab-mediated ADCC response against all canine tumor cell lines, the results of this study indicate the potential antitumor activity of trastuzumab in canines.

Key words: Canine, Cytotoxicity, HER2, Peripheral blood mononuclear cells, Trastuzumab

Introduction

Molecularly targeted drugs have attracted attention in antitumor therapy because of the recent developments in molecular biological analysis, which have uncovered various abnormalities associated with signal transduction processes in tumors. Antibody drugs are emerging molecularly targeted drugs with demonstrated efficacy in veterinary medicine (London, 2013).

Trastuzumab is a humanized monoclonal antibody used as a drug for human metastatic breast cancer (Slamon *et al*., 2001). It specifically recognizes the transmembrane glycoprotein of human epidermal growth factor receptor 2 (HER2) encoded by the *HER2/neu* proto-oncogene. HER2 expression has been reported in human cancers, including breast, ovary, and stomach (Slamon *et al*., 1989; Takehana *et al*., 2002). The potential antitumor activities of trastuzumab have been demonstrated to be mediated by HER2-associated pathways leading to the induction of apoptotic cell death (Carter *et al*., 1992; Sliwkowski *et al*., 1999; Cuello *et al*., 2001). Moreover, trastuzumab has been shown to exert antibody-dependent cellular cytotoxicity (ADCC) against HER2 overexpressing cancer cells through its Immunoglobin G1 (IgG1) Fc domain (Cooley *et al*., 1999; Sliwkowski *et al*., 1999).

Lymphokine-activated killer (LAK) cells are immune effector cells generated by interleukin-2 (IL-2) stimulation of peripheral blood mononuclear cells (PBMCs). LAK cells exhibit enhanced cytotoxicity and other characteristics suitable for ADCC (Grimm *et al*., 1982; Yamaguchi *et al*., 2005). In addition, low dose administration of recombinant IL-2 *in vivo* has been reported to produce significant immunological advantages and negligible toxicities through the generation of LAK cells, not only in humans but also in canine models (Ohnishi *et al*., 1993; Meropol *et al*., 1998).

In recent years, HER2 targeting treatment in canine tumor medicine has been expected due to the accumulated reports of HER2 expression in canine tumors (Pena *et al*., 2014; Yoshimoto *et al*., 2019; Veloso *et al*., 2020). Previous studies have shown the ability of trastuzumab to bind with canine tumor cells to suppress canine tumor cell proliferation and stimulate ADCC response in selectively expanded canine Natural Killer (NK) cell-like cells (Singer *et al*., 2012; Kim *et al*., 2019). In humans, it is reported that trastuzumab can induce enough ADCC response in LAK cells (Yamaguchi *et al*., 2005). Therefore, the combined

treatment with trastuzumab and cytotoxic stimulatory cytokines may exert a significant antiproliferative effect through ADCC in canine tumor medicine. However, it has not been described whether trastuzumab can stimulate ADCC in canine PBMCs and LAK cells. In this study, we evaluated the effects of trastuzumab and IL-2 stimulation on cytotoxicity of PBMCs in *in vitro* systems.

Materials and Methods

Cell lines and reagents

Cell lines used in this study included two canine mammary gland tumor cell lines (CHMm and CIPm) which were gifted by Dr. Nakagawa, Laboratory of Veterinary Surgery, Graduate School of Agricultural and Life Sciences, the University of Tokyo, Japan, and a canine thyroid adenocarcinoma cell line (CTAC, purchased from ECACC, Salisbury, UK). Cultures of cell lines were maintained using Roswell Park Memorial Institute 1640 (RPMI1640) medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin (GIBCO, NY, USA). Trastuzumab (Herceptin®), a humanized IgG1 anti-HER2 antibody reagent, was purchased from Chugai Pharmaceutical (Tokyo, Japan). Human IgG (hIgG) was used as an isotype-matched control antibody (Sigma-Aldrich, Missouri, USA).

Measurement of *HER2* mRNA expression

Total RNA was extracted from cell lines using TRIzol (Invitrogen, CA, USA). The reverse transcription reaction was performed at 42°C for 50 min using SuperScript II reverse transcriptase and oligo dT primer (Invitrogen, CA, USA). The primers used for polymerase chain reaction (PCR) amplification of *HER2* were designed based on the gene sequence registered in GenBank (ID: AB008451.1). The *GAPDH* gene was selected as a positive control, and primers were similarly designed. The designed primers were as follows: for *HER2*, 5′-GAC ACC TTC GAA TCC ATG CC-3′ and 5′-GGC AAA CTC CTG GAT GTT CG-3′; for *GAPDH*, 5′-GAT GGG CGT GAA CCA TGA G-3′ and 5′-TCA TGA GGC CCT CCA CGA T-3′. Real time RT-PCR was performed using Step One$_{TM}$ PLUS Real-Time PCR System and POWER SYBR Green PCR Master Mix (Thermo Fisher Scientific, CA, USA) following the manufacturer's instructions. Confirming the specificity of PCR, a single peak in the melt curve analysis was obtained. All samples were tested in duplicates. The expression level of *HER2* mRNA was calculated as a relative value compared to *GAPDH* mRNA.

Detection of trastuzumab binding to cells

The cell lines (1×10^5) were treated with 10 µg/ml trastuzumab or hIgG at 4°C for 30 min and washed. Then, Fluorescein IsoThioCyanate (FITC)-labeled sheep anti-human IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, USA) staining was similarly performed. The stained cells were analyzed using Cytomics FC500 (Beckman Coulter), and data collection was set to stop when 1,000 events were properly gated. Following this, forward and side scatter were analyzed. Then, data analysis was performed using FlowJo (TREE STAR). The ratio of mean fluorescence intensities of trastuzumab and hIgG (trastuzumab/hIgG MFIR) was used to measure the binding of trastuzumab to target cells.

PBMCs collection and generation of LAK cells

Six healthy beagles from the Laboratory Animal Facility of Kitasato University School of Veterinary Medicine were used in this study. The experiments were performed with the approval of the Kitasato University Laboratory Animal Ethics Committee. The animals were not exposed to any drug for 6 months before the experiments. Whole blood was obtained from the jugular vein and immediately heparinized. Peripheral blood mononuclear cells were separated from heparinized blood using the method described below. Blood diluted 2-fold with physiological saline was gently layered on SEPARATE-L (specific gravity: 1.077, Muto Pure Chemicals, Tokyo, Japan) and centrifuged at 400 × g for 30 min. Then, the PBMC layer was collected, suspended in phosphate buffered saline (PBS), and washed by centrifugation (300 × g, 10 min) twice. The obtained PBMCs were suspended in the Alys505S medium (Cell Science & Technology Institute, Sendai, Japan) containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin, followed by 2 h culture under 5% CO_2 and 37°C. Only non-adherent PBMCs were collected. Furthermore, to generate LAK cells, the collected PBMCs were cultured with the Alys505S medium containing 500 U/ml IL-2, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin for 6 days under 5% CO_2 and 37°C. Fresh medium was added 2 and 4 days after the start of culture. Non-adherent PBMCs and LAK cells were used as effector cells.

Cell viability assay

The influence of trastuzumab on the viability of tumor cell lines was evaluated. The cell lines (2×10^5 cells/well) were seeded on a 96-well flat-bottom microplate and cultured overnight under 5% CO_2 at 37°C. Trastuzumab or hIgG was added to each well at a concentration of 1-100 µg/ml, and cell lines were further cultured for 24 or 48 h. The experiment was performed in triplicate, and the wells in which medium was added, not the antibody reagent, were used as a negative control. The cell viability was determined by measuring the absorbance optical density (OD) at 490 nm using CellTiter 96® AQueous One Solution (Promega, Madison, WI, USA), according to the manufacturer's instructions. The viability was calculated as follows:

Cell viability (%) = 100 × experimental OD/negative control OD

ADCC assay

The cytotoxic activity of PBMCs and LAK cells against cell lines were evaluated by standard lactate

dehydrogenase (LDH) release assay, in which the LDH released due to cell lysis is quantified. The cell lines were used as a target and were seeded at a density of 4 × 10^3 cells/well in a 96-well U-bottom microplate. In the presence or absence of 10 μg/ml trastuzumab or hIgG, mixtures of the effector cells and the target cells at an effector target (E/T) ratio of 40, 20, and 10 were cultured under 5% CO_2 at 37°C for 6 h. The cytotoxicity to the target cells was quantified using CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI, USA) and the formula below according to the manufacturer's instructions and the procedure reported previously (Lin *et al*., 2010). After culturing, the culture medium was harvested, the LDH production was analyzed, and the absorbance at 490 nm was measured. The experiment was performed in triplicate. The cytotoxic activity was calculated as follows:

Cytotoxicity (%) = 100 × (experimental release - effector spontaneous release - target spontaneous release) / (target maximum release - target spontaneous release)

Statistical analysis

Results are expressed as mean ± standard deviation (SD) and analyzed using Student's t-test. Differences with $P<0.05$ were considered statistically significant.

Results

HER2 mRNA expression and the binding ability of trastuzumab to cell lines

First, the expression of *HER2* mRNA in the tumor cell lines was detected using real time RT-PCR. The expression levels in the two canine mammary gland tumor cell lines are shown in Fig. 1. Interestingly, *HER2* mRNA expression was also observed in CTAC. Then, we confirmed the target-specific binding of trastuzumab to HER2 expressing cell lines using flow cytometry analysis. Although trastuzumab was found to bind to tumor cell lines, including CTAC, the weak binding affinity was observed in all cell lines (CHMm, CIPm, and CTAC: 4.8 ± 0.7, 1.5 ± 0.5, and 4.2 ± 0.3, respectively) (Fig. 2).

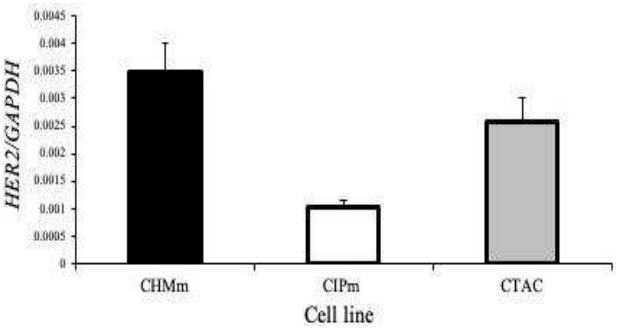


Fig. 1: *HER2* mRNA expression in cell lines. The expression levels are shown as a relative value to that of *GAPDH* mRNA

Cell viability assay

The influence of trastuzumab on the viability of tumor cell lines was evaluated. The growth of all cell lines incubated for 24 h in the presence of trastuzumab was not suppressed at any concentration. Similar results were obtained even when the culture period was extended to 48 h (Fig. 3).

ADCC assay

We evaluated whether trastuzumab could enhance the cytotoxicity of PBMCs or LAK cells against tumor cell lines. In all the experimental systems, the effector cells were found to kill the target cells in a E/T ratio-dependent manner. As shown in Fig. 4A, the cytotoxicity of PBMCs against CHMm was significantly enhanced in the presence of trastuzumab compared to hIgG. The cytotoxicity of LAK cells against CHMm in the presence of trastuzumab was also significantly higher than that in the presence of hIgG. Moreover, the cytotoxicity of LAK cells against CHMm in the presence of trastuzumab was significantly higher than that of PBMCs. However, no significant difference in cytotoxicity against hIgG-treated CHMm was found between PBMCs and LAK cells. In CIPm, there was no difference in the cytotoxicity of

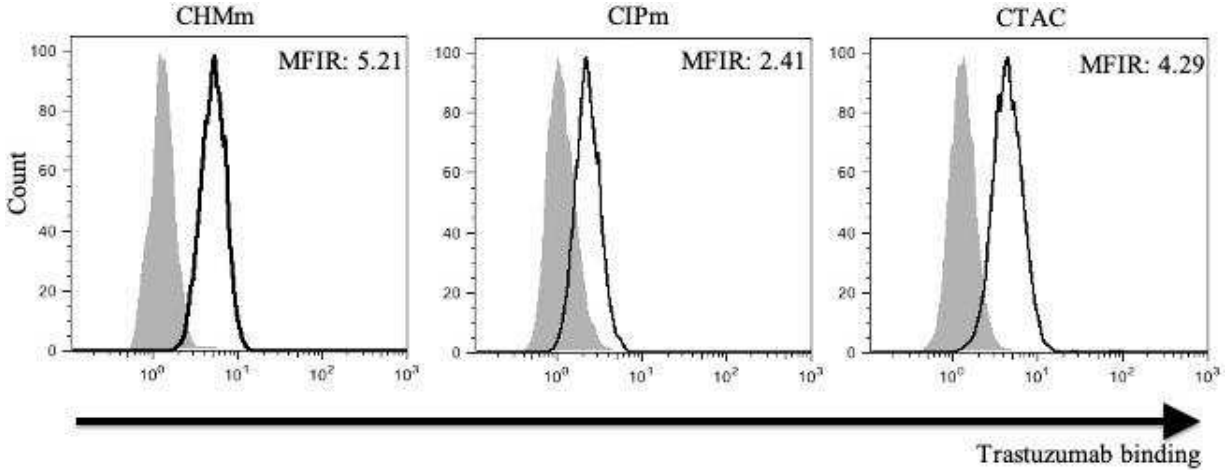


Fig. 2: The binding strength of trastuzumab to CHMm, CIPm, and CTAC. Black lines and filled histograms represent trastuzumab and hIgG staining, respectively. Results are representative of three experiments

effector cells in any condition (Fig. 4B). Against CTAC, the cytotoxicity of LAK cells was significantly enhanced compared to that of PBMCs; however, trastuzumab did not affect the cytotoxicity of PBMCs and LAK cells against CTAC (Fig. 4C).

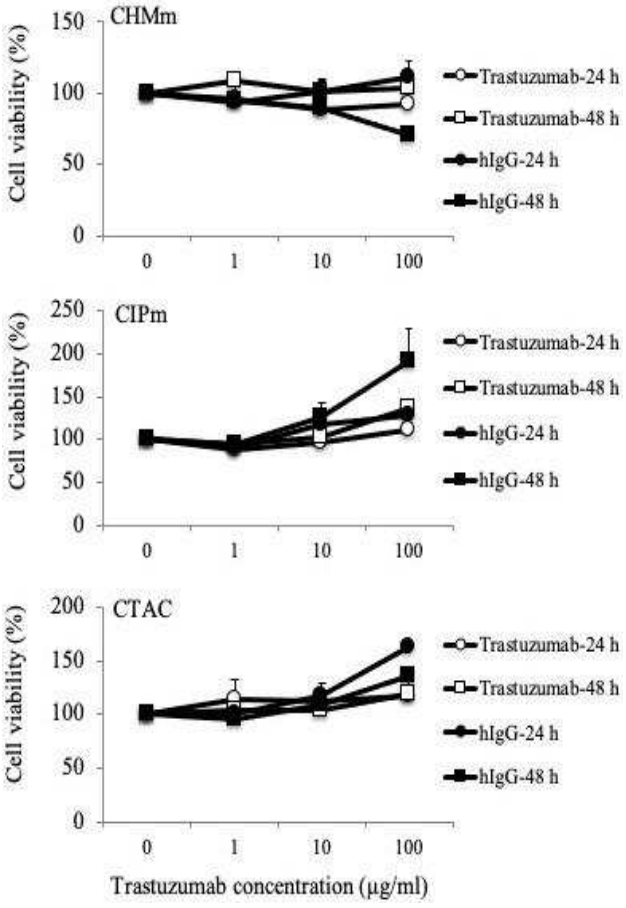
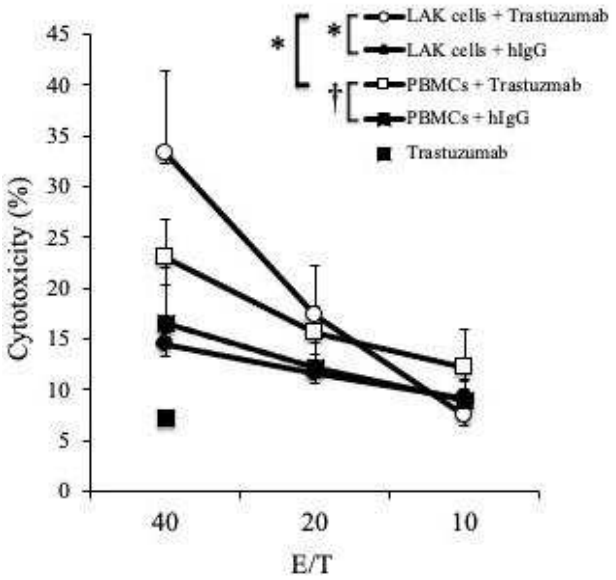
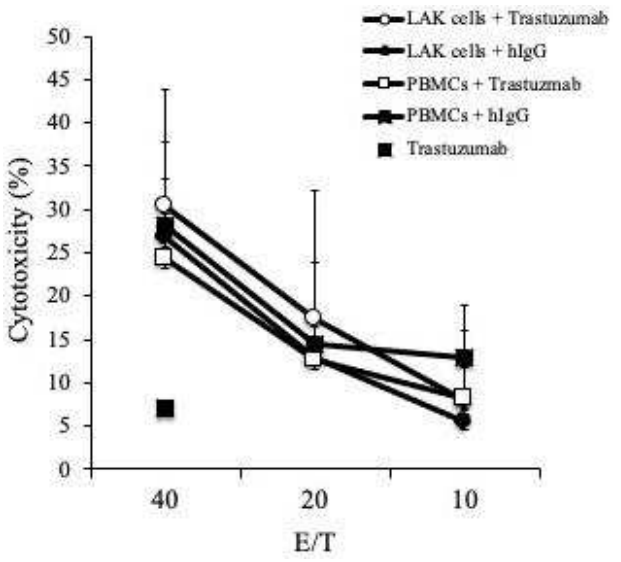


Fig. 3: The direct effect of trastuzumab on the viability of the tumor cell lines. Representative data of at least two experiments performed in triplicates are shown

A



B



C

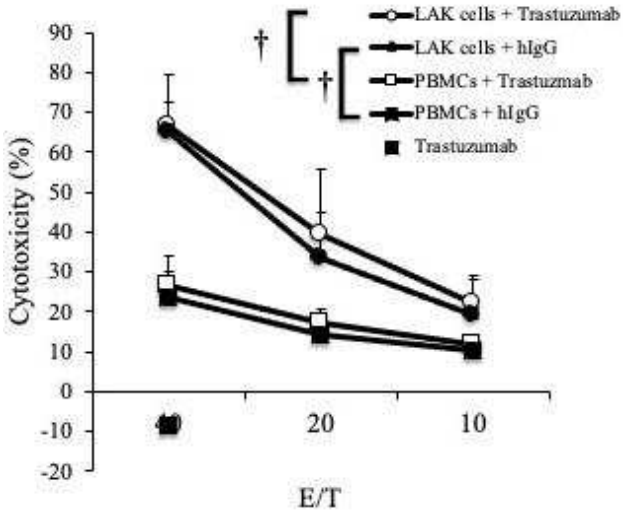


Fig. 4: The cytotoxicity of PBMCs or LAK cells against CHMm (**A**), CIPm (**B**), and CTAC (**C**) in the presence of trastuzumab or hIgG. * P<0.05, and †: P<0.01

Discussion

In this study, using canine tumor cell lines, we showed that trastuzumab exerts an antitumor effect when combined with PBMCs. We also demonstrated that the cytotoxicity was enhanced when PBMCs stimulated with IL-2 were used as effector cells.

The cell lines used in the present study, including mammary gland tumors and thyroid adenocarcinoma, were shown to express *HER2* mRNA with real time RT-PCR, and the interaction between trastuzumab and the cell lines using flow cytometry analysis. The two mammary gland tumors were isolated from metastatic lesions and have been reported to express HER2 protein (Nakagawa *et al*., 2006). Additionally, canine thyroid adenocarcinoma has been recently reported to express HER2. Thus, HER2 can be used for targeted treatment

against canine cancer (Yoshimoto *et al*., 2019). The amino acid homology of HER2 between humans and canines is known to be 92%. This indicated the possibility of potential antitumor activity of trastuzumab against canine tumor cells (Singer *et al*., 2012).

No direct inhibitory effect of trastuzumab on the growth of any of the tumor cell lines was observed in this study, consistent with the report by Kim *et al*. (2019). However, ADCC plays an important role in the antitumor activity of trastuzumab because the tumor growth was not inhibited even when the anti-HER2 antibody treatment was performed on the Fc receptor-deficient mouse model transplanted with HER2-overexpressing tumor cells (Clynes *et al*., 2000). Therefore, to optimize the antitumor activity of trastuzumab in canines, we focused on its cytotoxic effect in combination with PBMCs.

The ADCC assay in this study showed that trastuzumab enhanced the cytotoxicity of PBMCs. Moreover, the combination of IL-2 stimulation and trastuzumab synergistically enhanced the cytotoxicity of PBMCs. It must be emphasized that NK cells included in PBMCs and LAK cells played an important role in exerting ADCC in this study. Although macrophages also have Fc receptors and exert ADCC, macrophages were removed from the effector cells during the isolation of PBMCs in our study. A previous report has shown that trastuzumab mediated ADCC was induced in selectively expanded NK cell-like cells (Kim *et al*., 2019). Meanwhile, the present study suggested that trastuzumab does not necessarily require selective isolation and expansion of NK cells to induce ADCC response in canines, which may be enhanced by IL-2 treatment.

However, a concern remained in trastuzumab mediated ADCC in the present study. Although all cell lines used in this study were confirmed to bind with trastuzumab, ADCC was observed only in the cytotoxicity against CHMm, and the cytotoxicities of the effector cells against CIPm and CTAC were not affected by trastuzumab. Additionally, even though there was a statistically significant difference in the cytotoxicity of effector cells against CHMm when trastuzumab was present and absent, whether this difference could strongly support the biological significance is questionable. Even a previous study reporting trastuzumab mediated ADCC in selectively expanded canine NK cell-like cells showed similar results (Kim *et al*., 2019). Meanwhile, it has been reported that trastuzumab induced ADCC response against tumor cell lines with HER2-non-amplified or low expression (Cooley *et al*., 1999; Mimura *et al*., 2005; Collins *et al*., 2012). There is probably a threshold of HER2 expression below which the addition of trastuzumab to the system (tumor + PBMCs or LAK cells + trastuzumab) would not make much therapeutic difference. Moreover, some ADCC resistance mechanisms in tumor cells, including the insusceptibility to cytotoxic granules released from immune effector cells and the release of immunosuppressive cytokines, such as TGF-β, may also be involved in the system in this study (Mimura *et al*., 2005; Kawaguchi *et al*., 2009). Therefore, even at the same HER2 expression level, a sufficient threshold to overcome ADCC suppression may differ between tumor cell lines.

In conclusion, this study showed the potential of trastuzumab in enhancing the cytotoxicity of canine PBMCs through ADCC response. Furthermore, cytotoxicity could be increased by IL-2 stimulation of PBMCs. However, it was also suggested that even canine tumor cells capable of binding trastuzumab did not sufficiently stimulate ADCC response in the system in this study. Therefore, further investigations are necessary to improve trastuzumab-mediated ADCC of PBMCs against various canine HER2-expressing tumors.

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