

Augmentation of antibody-dependent cellular cytotoxicity with defucosylated monoclonal antibodies in patients with GI-tract cancer

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Abstract. Enhancement of antibody-dependent cellular cytotoxicity (ADCC) with some modalities may be a promising approach to enhance the efficacy of therapeutic monoclonal antibodies (mAbs). It has previously been demonstrated that the removal of fucose from antibody oligosaccharides (defucosylation) leads to augmentation of ADCC activity. To establish clinically relevant evidence of this procedure, the present study evaluated trastuzumab- and cetuximab-mediated ADCC by comparing defucosylated mAbs with conventional mAbs using peripheral blood mononuclear cells (PBMCs). PBMCs were isolated from 20 patients with gastrointestinal tract cancer and 10 healthy volunteers. ADCCs were measured using PBMCs as effector cells and two gastric cancer cell lines as target cells. ADCCs were significantly enhanced with defucosylated mAbs compared with conventional mAbs using PBMC from the healthy donors and patients with cancer. The results confirmed that the cetuximab- and trastuzumab-mediated ADCCs in advanced disease were impaired in comparison to those in early disease or healthy individuals. However, when the defucosylated mAbs were used instead of the conventional mAbs, the ADCC activities in the advanced cases were almost comparable with those in early disease or healthy individuals. Furthermore, the expression of ADCC associated molecules were modified toward immunosuppressive status with a mitogen-activated protein kinase inhibitor *in vitro*, the conventional cetuximab- and trastuzumab-mediated ADCC was downregulated, and the defucosylated mAbs overcome the downregulation of ADCC. In conclusion, defucosylated

therapeutic mAbs may enhance ADCC activities in patients with cancer, which may lead to more effective anti-cancer treatments.

Introduction

Although improvement of systemic chemotherapy has been demonstrated in gastrointestinal (GI) tract cancer including gastric and colon cancer, the overall survival rate in patients with advanced stage is still poor (1). Therefore, it would be desirable to develop molecular target therapy more efficiently in GI-tract cancer. For example, for the human epidermal growth factor receptor (EGFR) related 2 (HER2)-overexpressing gastric cancer, Trastuzumab in combination with chemotherapy vs. chemotherapy alone for treatment of HER2-positive advanced gastric cancer (ToGA) trial concluded that anti-HER2 monoclonal antibody (trastuzumab) plus chemotherapy is a standard treatment option, in which trastuzumab plus chemotherapy showed better survival in comparison to chemotherapy alone (2).

It is generally accepted that trastuzumab can act on gastric cancer cells through both anti-proliferative function directly to cancer cells and antibody-dependent cellular cytotoxicity (ADCC) activity via immune cells (3,4). It has been reported that Trastuzumab-mediated ADCC can be influenced by several factors including single nucleotide polymorphisms (SNPs) in the Fc gamma receptor (FcγR) genes (5-7) or natural killer (NK) cell dysfunction (8). In fact, the SNPs can alter the FcγR binding affinity to the therapeutic monoclonal antibodies (mAbs) and consequently resulted in impairment of the ADCC activity. Of importance, a clinical trial showed that therapeutic efficacy of trastuzumab against HER2-positive breast cancer was significantly different between patients with and without certain SNPs in the FcγR genes (9). Furthermore, the same observation was also confirmed in colorectal cancer treated with anti-EGFR antibody, cetuximab (10). These results strongly suggest that enhancement of ADCC with some modalities would be a promising approach to enhance the efficacy of therapeutic mAbs.

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It has been shown that removal of fucose from antibody oligosaccharides attached to Asn²⁹⁷ of the heavy chain (defucosylation) significantly enhanced FcγR binding affinity between FcγR on NK cells and the mAbs, in comparison to that of conventional antibody, leading to augmentation of ADCC activity (11-15). Thus, the defucosylation technology could be one of the most powerful approaches to enhance clinical efficacy of therapeutic mAbs. There is, however, still limited information describing the clinical usefulness of the defucosylated therapeutic antibody on the ADCC, except for one report showing that the use of the defucosylated antibodies may improve the therapeutic effects of trastuzumab for breast cancer patients (16). Thus, it is necessary to draw solid conclusion for the effectiveness of the defucosylated antibody in cancer patients or immunosuppressive state. In the present study, using PBMCs from GI tract cancer patients and healthy donors, we evaluated trastuzumab- and cetuximab-mediated ADCC by comparing the defucosylated mAbs with conventional mAbs. This is the first report using PBMCs from patients of GI tract cancer. In addition, when ADCC-related molecules are modulated by mitogen-activated protein kinase (MAPK) inhibitors, the trastuzumab- and cetuximab-mediated ADCC were also evaluated.

Materials and methods

Preparation of human effector cells. Twenty patients with histologically diagnosed GI tract cancer, who were treated at Fukushima Medical University Hospital (Fukushima, Japan) from February to August in 2016, were enrolled. PBMCs were isolated from esophageal (n=4), gastric (n=9), and colon cancer patients (n=7), and healthy individuals (n=10, 34.8±7.8 years old, Male: Female=9:1). PBMCs were separated by lymphocyte separation solution (Lymphoprep™, Cosmo Bio Company) with density gradient. None of the patients received radiotherapy, chemotherapy, surgery, or other medical interventions before this study. Patients' characteristics are shown in Table I. This study was approved by the ethical committee of Fukushima Medical University (approval no. 2353), and informed consent for blood donations was obtained for all individuals.

Cell lines. MKN-7 (HER-2 overexpressing gastric cancer cell lines; cat. no. JCRB1025) and K562 (myelogenous leukemia cell lines; cat. no. JCRB0019) were purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). MKN-28 (EGFR overexpressing gastric cancer cell line) was obtained from the American Type Culture Collection (Rockville, MD, USA). The MKN28 cell line has previously been reported to be a mixed gastric cancer type, with MKN74 (an EGFR overexpressing cancer cell line) contamination (17). However, this contamination is not thought to have affected the results of the present study as MKN28 and MKN74 share similar characteristics in terms of EGFR overexpression, as described previously (18). All cell lines were maintained in RPMI-1640 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) with 10% fetal bovine serum (Nichirei Biosciences, Inc., Tokyo, Japan) and 1% penicillin/streptomycin (Nichirei Biosciences, Inc.) at 37°C and 5% CO₂.

Antibodies. Anti-HER-2 monoclonal antibody trastuzumab and anti-human EGFR antibody cetuximab were used as clinical

Table I. Characteristics of the patients (n=20).

Characteristic	Number of patients (n)
Age, years (median, range)	54-80 (65)
Male:female	17:3
Location of carcinoma	
Esophagus	4
Stomach	9
Colon	7
Clinical stage (TNM classification)	
0	2
1	6
2	2
3	8
4	2

TNM, tumor-node-metastasis classification of malignant tumors.

grade products, and their defucosylated version were provided by Kyowa Hakko Kirin Co., Ltd. (Tokyo, Japan), which were designed according to the known amino acid sequences (19,20) and produced with parent or α-1,6-fucosyltransferase knockout Chinese hamster ovary cells (21).

Antibody-dependent cellular cytotoxicity (ADCC) assay. Cytotoxicity was determined by the lactate dehydrogenase (LDH) release assay using PBMCs as effector cells and either MKN-7 cells or MKN-28 cells as target cells. Briefly, target cells (5×10³ per well) were distributed into 96-well U-bottomed plates and pre-incubated with mAbs for 1.5 h at 37°C, 5% CO₂. Then, effector cells were added at indicated doses and incubated for 7 h. Assays were performed in triplicate with or without antibodies. The supernatant LDH activity was measured using a nonradioactive cytotoxicity assay kit (Cytotoxicity Detection kit^{PLUS}; Roche Diagnostics, Basel, Switzerland) and was measured at 490 nm excitation and 650 nm emission wavelengths using spectrometer. Percentage cytotoxicity was calculated according to the formula: Cytotoxicity (%) = 100 × (Experimental release - Spontaneous release) / (Maximum release - Spontaneous release). The maximum release was prepared with target cells lysed with the lysis solution. In the several pre-test run, the spontaneous release of effector cells was approximately zero. Net ADCC was calculated according to the formula: net ADCC (%) = ADCC activities (%) - antibody-independent cellular cytotoxicity (AICC; %), where AICC is the nonspecific cytotoxicity in the absence of antibodies.

Cell treatment with MAPK signal inhibitors. Tumor cells were cultured in a 6-well plate and exposed to the MAPK signal inhibitor, PD0325901 (Selleck Chemicals, Houston, TX, USA) as indicated in our previous report (22). Then, cytotoxicity assays were performed after 48 h of incubation.

Statistical analysis. Data comparing differences between two groups assessed using unpaired Student's t-test and two way

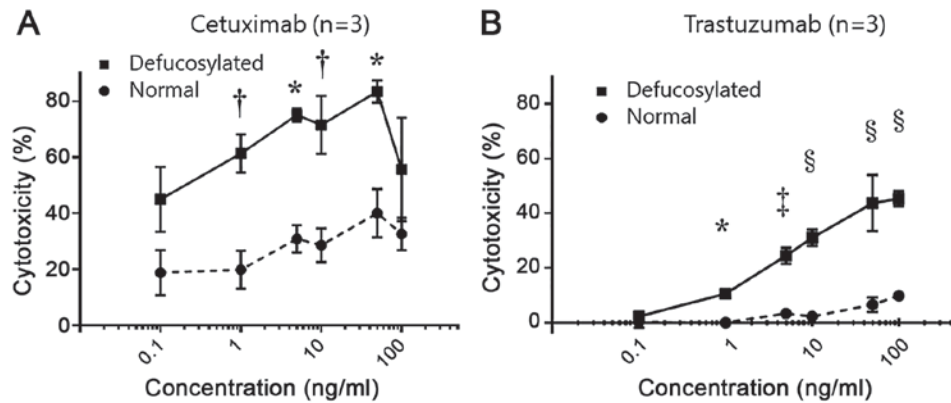


Figure 1. Optimal concentration of defucosylated mAbs. (A) Cetuximab-mediated and (B) trastuzumab-mediated antibody-dependent cellular cytotoxicity were evaluated in various concentrations of conventional (Normal) and defucosylated mAbs using peripheral blood mononuclear cells from healthy donors (n=3). Data are presented as the mean \pm standard deviation of the cytotoxicity (%) in triplicates at effector: target ratio of 40:1. *P<0.05, †P<0.005, ‡P<0.0005 and §P<0.00005 vs. Normal. mAbs, monoclonal antibodies.

ANOVA. P<0.05 was considered to indicate a statistically significant difference.

Results

Optimal condition of defucosylated therapeutic mAbs for ADCC activity. Cetuximab-mediated and trastuzumab-mediated ADCCs were evaluated in various concentrations of conventional and defucosylated mAbs using healthy donor's PBMCs (n=3, Fig. 1A and B). The EGFR-positive MKN28 gastric cancer cell line was used for cetuximab-mediated ADCC and the HER2-positive MKN7 gastric cancer was used for trastuzumab-mediated ADCCs, and the overexpression of EGFR or HER2 on tumor cells were repeatedly confirmed by flow cytometry (data not shown).

As shown in Fig. 1A, defucosylated cetuximab-mediated ADCCs at effector: Target ratio of 40:1 were significantly higher than conventional cetuximab-mediated ADCCs in each concentration. We observed a dose-dependent increase from 0.1 to 50 ng/ml and thereafter the ADCC led to a drop in the present experimental condition, consistent with the previous report (21). Therefore, 50 ng/ml of defucosylated and conventional cetuximab were used for subsequent experiments as optimal doses.

Also, the same tendency was observed in trastuzumab-mediated ADCCs (Fig. 1B) and 50 ng/ml of defucosylated and conventional trastuzumab were used for subsequent experiments as optimal doses.

Augmented ADCC by defucosylated cetuximab and trastuzumab. ADCC activities mediated by either conventional or defucosylated mAbs using PBMCs from cancer patients (n=20) and healthy volunteers (n=10) were evaluated. The patient's background is shown in Table I. In order to confirm the condition of PBMCs as effector cells, NK cell activities targeted for K562 were also evaluated in parallel to the ADCC assay and we confirmed condition of NK status in each experiment (Fig. 2).

As shown in Fig. 3A, the defucosylated cetuximab-mediated ADCCs were markedly enhanced in comparison to conventional cetuximab-mediated ADCCs in healthy donor's

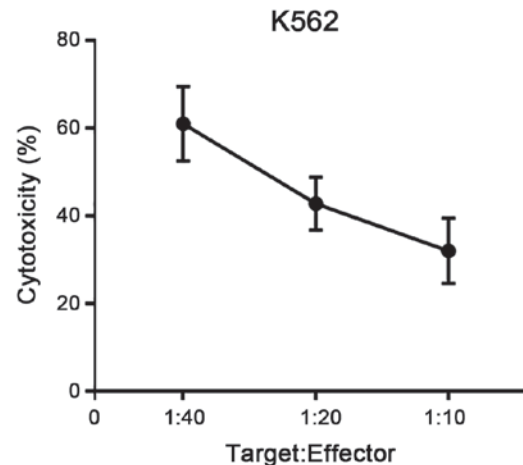


Figure 2. Confirmation of the optimal NK cell activities. NK cell activities targeted for K562 in the peripheral blood mononuclear cells of healthy individuals (n=10) were evaluated in parallel to the antibody-dependent cellular cytotoxicity assay; the optimal NK activities under these conditions were identified. Data are presented as the mean \pm standard deviation. NK, natural killer.

PBMCs. For example, the defucosylated and conventional cetuximab-mediated ADCC at 40:1 ratio were 58.9 ± 7.5 and $33.5 \pm 3.9\%$, respectively. Similar observation was also confirmed using the PBMCs from cancer patients (Fig. 3B), in which the defucosylated and conventional trastuzumab-mediated ADCC at 40:1 ratio were 52.9 ± 4.0 and $32.0 \pm 2.8\%$, respectively.

Also, the enhancement by defucosylated mAbs was confirmed in the trastuzumab-mediated ADCCs in both healthy donors and cancer patients (Fig. 3C and D).

Taken together, the defucosylated therapeutic mAbs can enhance the ADCC activities in comparison to the conventional mAbs using PBMCs from both healthy donors and cancer patients.

Defucosylated cetuximab- and trastuzumab-mediated ADCC in advanced cancer cases. Based on the UICC-TNM classification, we classified the cancer patients into advanced disease corresponded to stage III and IV, or into early disease

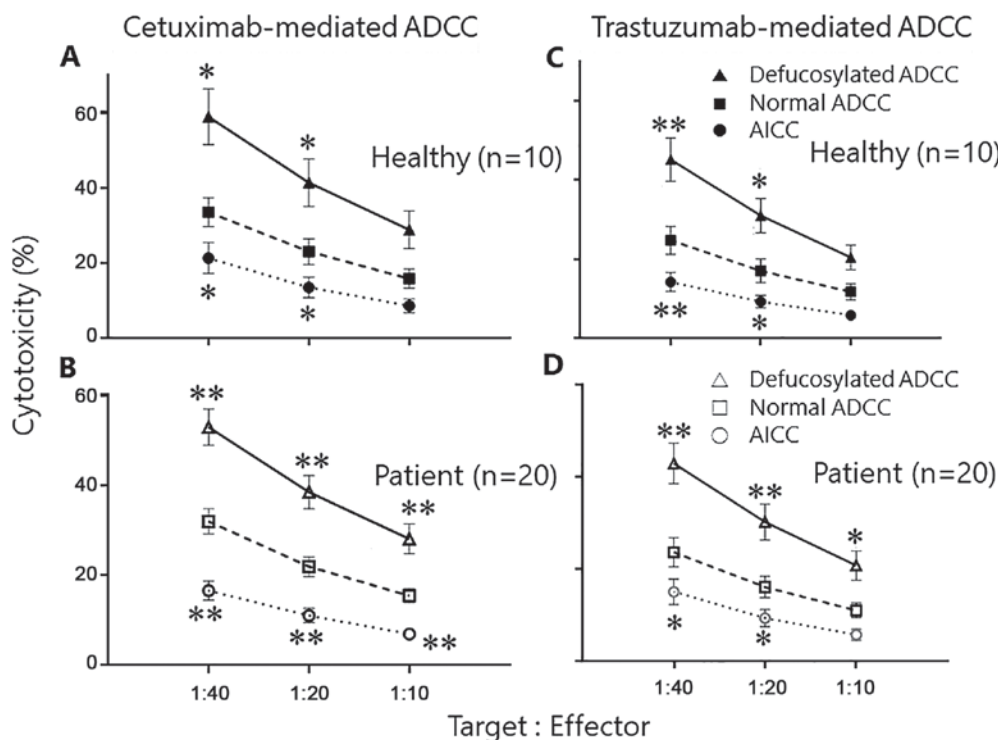


Figure 3. Augmented ADCC by defucosylated cetuximab and trastuzumab. ADCC activities mediated by either conventional or defucosylated monoclonal antibodies using peripheral blood mononuclear cells from (B and D) cancer patients (n=20) and (A and C) healthy volunteers (n=10). Data are presented as the mean \pm standard deviation. * $P<0.05$ and ** $P<0.005$ vs. Normal ADCC. ADCC, antibody-dependent cellular cytotoxicity; AICC, the nonspecific cytotoxicity in the absence of antibodies.

corresponded to stage 0, I, and II. It has been already reported that the ADCC activities in advanced cancer patients were impaired due to several mechanisms, including NK cell dysfunction or immunosuppressive factors (8,23,24). As expected, we confirmed that the cetuximab-mediated and trastuzumab-mediated ADCCs in advanced disease were impaired in comparison to those in early disease or healthy individuals (Fig. 4). However, when the defucosylated mAbs were used instead of the conventional mAbs, the ADCC activities in the advanced cases were almost comparable to those in early disease or healthy individuals (Fig. 4) and this observation was confirmed in both defucosylated cetuximab and trastuzumab.

Thus, the defucosylated therapeutic mAbs can rescue the impaired ADCC in advanced disease.

ADCC by defucosylated cetuximab and trastuzumab when treated with MAPK inhibitors. In order to further investigate defucosylated therapeutic mAbs-mediated ADCC, we modified the expression of natural killer group 2 member D receptor (NKG2D) ligand and major histocompatibility complex (MHC) class I on tumor cells by MAPK inhibitors as indicated in our previous report (22). It is generally accepted that NK cells can react with tumor cells through the balance of inhibitory and stimulatory signals. The interaction between the killer immunoglobulin-like receptor family on NK cells and MHC class I molecules results in inhibitory signals, whereas activating signals by NKG2D ligands expressed on targets induce stimulatory signals leading to target cell killing (25-27). We have shown that treatment of target tumor cells with MAPK inhibitors can decrease ADCC activities through upregulation

of MHC class I and down-regulation of NKG2D ligands such as MICA/B (22). As expected, conventional cetuximab- and trastuzumab-mediated ADCC was significantly decreased, when target tumor cells were pre-treated with the MAPK inhibitor (Fig. 5). However, when the defucosylated mAbs were used instead, ADCC activities did not alter even if the target cells were pre-treated with the MAPK inhibitor. In the current experiment condition, MAPK inhibitors do not have any direct anti-proliferative effect on target cancer cells (data not shown).

Taken together, defucosylated therapeutic mAbs can efficiently enhance ADCC activities, even if the NKG2D ligand and MHC class I expression on tumor cells, which are corresponding to immune suppressive status, were modified.

Discussion

The present study provide an important finding relevant to clinical cancer treatment with therapeutic mAbs. First, we showed the augmentation of ADCC by defucosylated therapeutic mAbs using PBMCs from healthy donors and cancer patients. Second, although the ADCC activities were impaired in advanced disease, the defucosylated mAbs can restore the impaired ADCC to the levels of healthy individuals. Finally, the defucosylated therapeutic mAbs can enhance ADCC activities even if the NKG2D ligand and MHC class I expression on tumor cells were modified to induce immunosuppressive environment.

There is accumulating evidence that ADCC is an important antitumor mechanism when the therapeutic mAbs showed the clinical benefit (28-32), and augmentation of ADCC will be

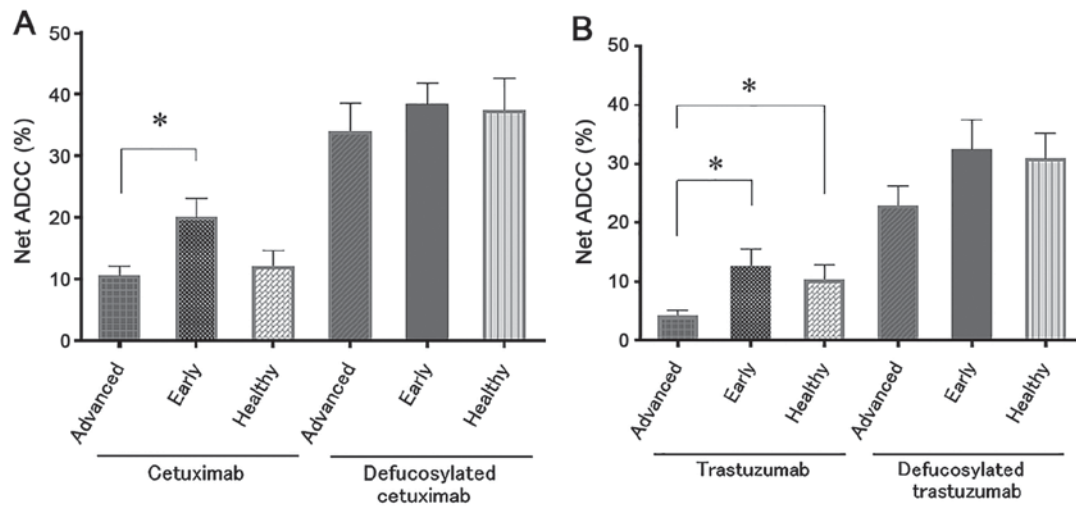


Figure 4. Defucosylated cetuximab- and trastuzumab-mediated ADCC in advanced cancer cases. Net ADCC in advanced disease (n=10), early disease (n=10) and healthy individuals (n=10) were evaluated in (A) cetuximab-mediated and (B) trastuzumab-mediated ADCCs. Net ADCC (%)=ADCC activities (%)–antibody-independent cellular cytotoxicity (%). Data are presented as the mean \pm standard deviation. * $P<0.05$, as indicated. ADCC, antibody-dependent cellular cytotoxicity.

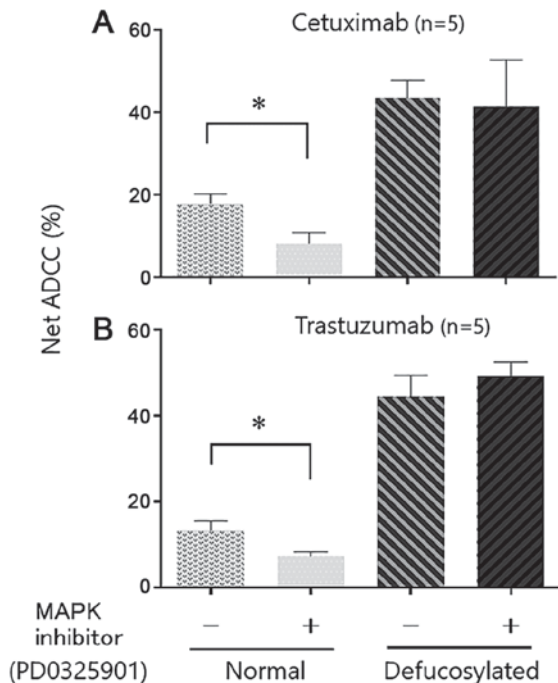


Figure 5. ADCC is induced by defucosylated cetuximab and trastuzumab when treated with MAPK inhibitors. Target tumor cells were pretreated with a MAPK inhibitor (PD0325901), and (A) cetuximab-mediated ADCCs or (B) trastuzumab-mediated ADCCs were evaluated in peripheral blood mononuclear cells from healthy donors (n=5) in the presence of conventional or defucosylated monoclonal antibodies. Data are presented as the mean \pm standard deviation. * $P<0.05$, as indicated. ADCC, antibody-dependent cellular cytotoxicity; MAPK, mitogen-activated protein kinase.

able to enhance the clinical efficacy of the therapeutic mAbs. For example, modification of antibodies to increase binding to Fc γ R has been pursued in order to augment ADCC (11,12). It has finally been reported that removal of the α -1,6 fucose moiety on the N-glycan at Asn²⁹⁷ of the heavy chain, which is called defucosylation technology, significantly enhanced ADCC in comparison to that of conventional antibody.

Previously, there is only one report describing the efficacy of defucosylated trastuzumab using PBMCs from breast cancer patients (16). Herein, our present study is the first report indicating usefulness of defucosylated cetuximab and trastuzumab for ADCC using the PBMCs of GI-tract cancer patients. Our observation and the previous report using clinical samples clearly confirmed the defucosylation technology efficiently can enhance the therapeutic mAbs-mediated ADCC.

In line with several previous reports (23,33), we confirmed that conventional therapeutic mAbs-mediated ADCC in advanced disease was impaired in comparison to those in early disease or healthy donors. It is generally accepted that NK cells in cancer-bearing hosts are impaired by many mechanisms, including their reduced number, imbalances in their activating and inhibitory receptor, impaired activation signaling cascade as well as immunosuppressive cytokines (8,23,24). However, even in such a condition, the current study clearly indicated that the defucosylated mAbs can restore the impaired ADCC in advanced diseases.

MAPK inhibitors have been originally developed for anti-cancer drugs based on their anti-proliferative action against tumor cells (34,35). In addition, we and others previously reported that the MAPK inhibitor can induce up-regulation of HLA Class I and down-regulation of MICA/B expression on tumor cells, leading to less NK sensitivity (26,27). Therefore, in the present study, we tried to mimic immunosuppressive status specific for NK-killing by modulating ADCC-related molecules with MAPK inhibitors. As a result, we confirmed that conventional cetuximab- and trastuzumab-mediated ADCC was impaired when target tumor cells were pre-treated with the MAPK inhibitor. Of importance, the defucosylated therapeutic mAbs can enhance ADCC activities even if the NKG2D ligand and MHC class I expression on tumor cells were modified by the MAPK inhibitor into immunosuppressive status. Taking it into consideration, the defucosylated therapeutic mAbs have a strong capability to enhance ADCC activities.

Theoretically, it would be likely that the defucosylated therapeutic mAbs has no ADCC activity against normal

tissues, since the therapeutic potential of the mAbs are dependent on the level of target antigens expressed on the surface of target cells. When we consider the clinical application of the defucosylated therapeutic mAbs described in the present study, further study to exclude potential toxicities of the defucosylated mAbs to tissues expressing low levels of such target antigens will be needed.

In conclusion, the defucosylated therapeutic mAbs can restore the impaired ADCC activities in advanced stage of cancer patients, leading to more effective anti-cancer treatments.

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