学　位　論　文

Augmentation of Antibody-Dependent Cellular Cytotoxicity with defucosylated monoclonal antibodies in patients with GI-tract cancer

(消化管癌患者における脱フコシル化抗体を用いた ADCC 活性の解析)

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Abstract

Enhancement of antibody-dependent cellular cytotoxicity (ADCC) with some modalities would be a promising approach to enhance the efficacy of therapeutic monoclonal antibodies (mAbs). It has shown that removal of fucose from antibody oligosaccharides (defucosylation) leads to augmentation of ADCC activity. To establish clinically relevant evidence of this mechanism, we evaluated trastuzumab- and cetuximab-mediated ADCC by comparing the defucosylated mAb with conventional mAbs using peripheral blood mononuclear cells (PBMCs) from cancer patients and healthy donors.

PBMCs were isolated from 20 cancer patients and 10 healthy volunteers. ADCC of conventional mAbs (cetuximab and trastuzumab) and those defucosylated versions were measured using PBMCs from donors as effector cells and two gastric cancer cell lines as target cells.

The ADCC was significantly enhanced with defucosylated mAbs compared with conventional mAbs using PBMC from both healthy donors and cancer patients. 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. However, when the defucosylated mAbs used instead of the conventional mAbs, the ADCC activities in the advanced cases were almost comparable to those in early disease or healthy individuals. Furthermore, when natural killer group member D receptor (NKG2D) ligand and major histocompatibility complex (MHC) class I expression on tumor cells were modified toward immunosuppressive status for ADCC activity with a mitogen-activated protein kinase (MAPK) inhibitor, the conventional cetuximab- and trastuzumab-mediated ADCC was down-regulated, while the defucosylated mAbs can overcome the down-regulation of ADCC.

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.

Introduction;

Gastric cancer is the third leading cause of cancer mortality worldwide and the overall survival rate in patients with advanced stage is still poor (1). As for the human epidermal growth factor receptor (EGFR) related 2 (HER2)-overexpressing gastric cancer, Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-oesophageal junction 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.

It has been shown that removal of fucose from antibody oligosaccharides attached to Asn^{297} 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 improve clinical efficacy of therapeutic mAbs. One possible problem is that the past studies used peripheral blood mononuclear cells (PBMCs) from healthy individuals as effector cells. It was unclear whether this activity is functioning for PBMCs from cancer patients whose immune system could be impaired by the immunosuppressing activity of tumor cells. Therefore, it is necessary to verify the effectiveness of the defucosylated antibody in cancer patients or immunosuppressive state. Suzuki et al. showed that the use of the defucosylated antibodies may improve the therapeutic effects of trastuzumab for breast cancer patients (16). In the present study, using PBMCs from gastrointestinal 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 gastrointestinal 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

In all, 20 patients with gastrointestinal tract cancer, who were presented at Fukushima Medical University Hospital (Fukushima, Japan) from February to August in 2016. 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^{TM}, Cosmo Bio Company) with density gradient. All of the patients were pathologically diagnosed and treated at Fukushima Medical University Hospital. None of the patients received radiotherapy, chemotherapy, surgery, or other medical interventions before this study. Patients' characteristics are shown in Table 1. This study was approved by the ethical committee of Fukushima Medical University (approval number: 2353), and informed consent for blood donations was obtained for all individuals.

Cell lines.

MKN-7 (HER-2 overexpressing gastric cancer cell lines) and K562 (myelogenous leukemia cell lines) 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). All cell lines were maintained in RPMI-1640 (Sigma- Aldrich, St. Louis, MO) with 10% fetal bovine serum (Nichirei Biosciences Inc. Tokyo, Japan) and 1% penicillin/streptomycin (Nichirei Biosciences Inc.) at 37 ℃ and 5% CO2.

Antibodies

Anti-HER-2 monoclonal antibody trastuzumab (Herceptin^{R}), anti-human EGFR antibody cetuximab (Erbitax^{R}), and their defucosylated version were obtained from Kyowa Hakko Kirin Co. Ltd. (Tokyo, Japan).

Antibody-dependent cell-mediated cytotoxicity (ADCC) assay

Cytotoxicity was determined by the lactate dehydrogenase (LDH) release assay using human PBMCs as effector cells and either MKN-7 cells or MKN-28 cells as target cells. Briefly, target cells (5×10^{3} per well) were distributed into 96-well U-bottomed plates and pre-incubated with mAbs for 1.5 h at 37 ℃, 5% CO2. Then, effector cells were added at indicated doses and incubated for 7 hours. 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) and was measured at 490nm excitation and 650nm 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. Because the spontaneous release of effector cells was approximately zero, we excepted from the formula. 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 (Selleckchemicals) as indicated in our previous report (17). Then, cytotoxicity assays were performed after 48 hours of incubation.

Statistical analysis

Date comparing differences between two groups assessed using unpaired Student's t test and two way ANOVA. Significant differences were considered at $p<0.05$.

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, Figure 1A and 1B). 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 Figure 1A which summarized data from 3 healthy donors at effector: target ratio of 40:1, defucosylated cetuximab-mediated ADCCs 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 reached plateau, even leading to a drop in the present experimental condition, consistent with the previous report (18). 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 (Figure

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 1. 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 found optimal NK activities in each experiment (Supplemental Figure S1), indicating that the PBMCs during the experiments were healthy.

As shown in Figure 2A, the defucosylated cetuximab-mediated ADCCs were markedly enhanced in comparison to conventional cetuximab-mediated ADCCs in healthy donor`s PBMCs. For example, the defucosylated and conventional cetuximab-mediated ADCC at 40:1 ratio were 58.9±7.5 % and 33.5±3.9 %, respectively. Similar observation was also confirmed using the PBMCs from cancer patients (Figure 2B), in which the defucosylated and conventional trastuzumab-mediated ADCC at 40:1 ratio were 52.9±4.0 % and 32.0±2.8 %, respectively.

Also, the enhancement by defucosylated mAbs was confirmed in the trastuzumab-mediated ADCCs in both healthy donors and cancer patients (Figure 2C and 2D).

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 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, 19, 20). Correspondingly, 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 (Figure 3A and 3B). 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 (Figure 3A and 3B) 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 natural killer group member D receptor (NKG2D) ligand and major histocompatibility complex (MHC) class I expression on tumor cells by MAPK inhibitors as indicated in our previous report (17). It is generally accepted that NK cells can react with tumor cells through the balance of inhibitory and stimulatory signals between NKG2D and its ligands on target cells including MHC Class I chain-related A (MICA), MICB, and several UL-16-binding proteins (ULBPs) (21-23). 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 MICA/B (17). As expected, conventional cetuximab- and trastuzumab-mediated ADCC was significantly decreased, when target tumor cells were pre-treated with the MAPK inhibitor (Figure 4A and 4B). 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.

Taken together, defucosylated therapeutic mAbs can efficiently enhance ADCC activities even if the NKG2D ligand and MHC class I expression on tumor cells 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 (24-28), and augmentation of ADCC will be able to enhance the clinical efficacy of the therapeutic mAbs. For example, modification of antibodies to increase binding to FcγR 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 Asn^{297} 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 (19, 29), 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, 19, 20). However, even in such a condition, the defucosylated mAbs can restore the impaired ADCC in advanced diseases.

MAPK inhibitors have been developed for anti-cancer drugs based on their anti-proliferative action against tumor cells (30, 31). In addition, it was previously reported that the MAPK inhibitor could induce up-regulation of HLA Class I and down-regulation of MICA/B expression on tumor cells as well as their original anti-proliferative action (22, 23). Therefore, when the MAPK inhibitors are considered for clinical application, tumor cell killing by NK cell is also expected to be inhibited. In the present study, we confirmed that conventional cetuximab- and trastuzumab-mediated ADCC was decreased, 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.

The study had some limitations. When ADCC was analyzed, we obtained PBMC from patients with several types of cancer, including esophageal, gastric and colorectal cancer exhibiting different clinical stage, histology and differentiation. Accordingly, subgroup analysis could not be conducted due to insufficient number of patients in each specific cancer types. Thus, future studies would be necessary to address the potential effect of defucosylated mAbs in each specific patient subgroups using a larger set of patient-derived PBMC. Also, this study cannot exclude the possibility of differential activities of NK cells owing to the different background between healthy individuals and cancer patients, such as age and gender.

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. The future outlook on this research is that we will prepare tumor-bearing mice and conduct research in vivo.

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Figure legends

Figure 1

The tendency of cytotoxicity depend on various concentrations. Cetuximab-mediated **(A)** and trastuzumab-mediated ADCCs **(B)** were evaluated in various concentrations of conventional and defucosylated mAbs using healthy donor`s PBMCs (n=3). The error bars indicated mean ± SE of cytotoxicities (%) in triplicates at effector: target ratio of 40: 1. *, $p < 0.001$, statistically significant differences of cytotoxicity by two-way ANOVA.

Figure 2

Augmented ADCC by defucosylated cetuximab and trastuzumab. ADCC activities mediated by either conventional or defucosylated mAbs using PBMCs from cancer patients (n=20, B and D) and healthy volunteers (n=10, A and C). The error bars indicated mean ±SE. *, $p < 0.001$ (two-way ANOVA).

Figure 3

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 cetuximab-mediated **(A)** and trastuzumab-

mediated ADCCs **(B)**. Net ADCC (%) = ADCC activities (%) – antibody-independent cellular cytotoxicity (AICC; %). The error bars indicated mean ±SE.

Figure 4

ADCC by defucosylated cetuximab and trastuzumab when treated with MAPK inhibitors. When target tumor cells were pre-treated with a MAPK inhibitor (PD0325901), cetuximab-mediated ADCCs **(A)** or trastuzumab-mediated ADCCs **(B)** were evaluated in PBMCs from healthy donor (n=5) in the presence of conventional or defucosylated mAb. The error bars indicated mean ±SE.

Supplemental Figure S1

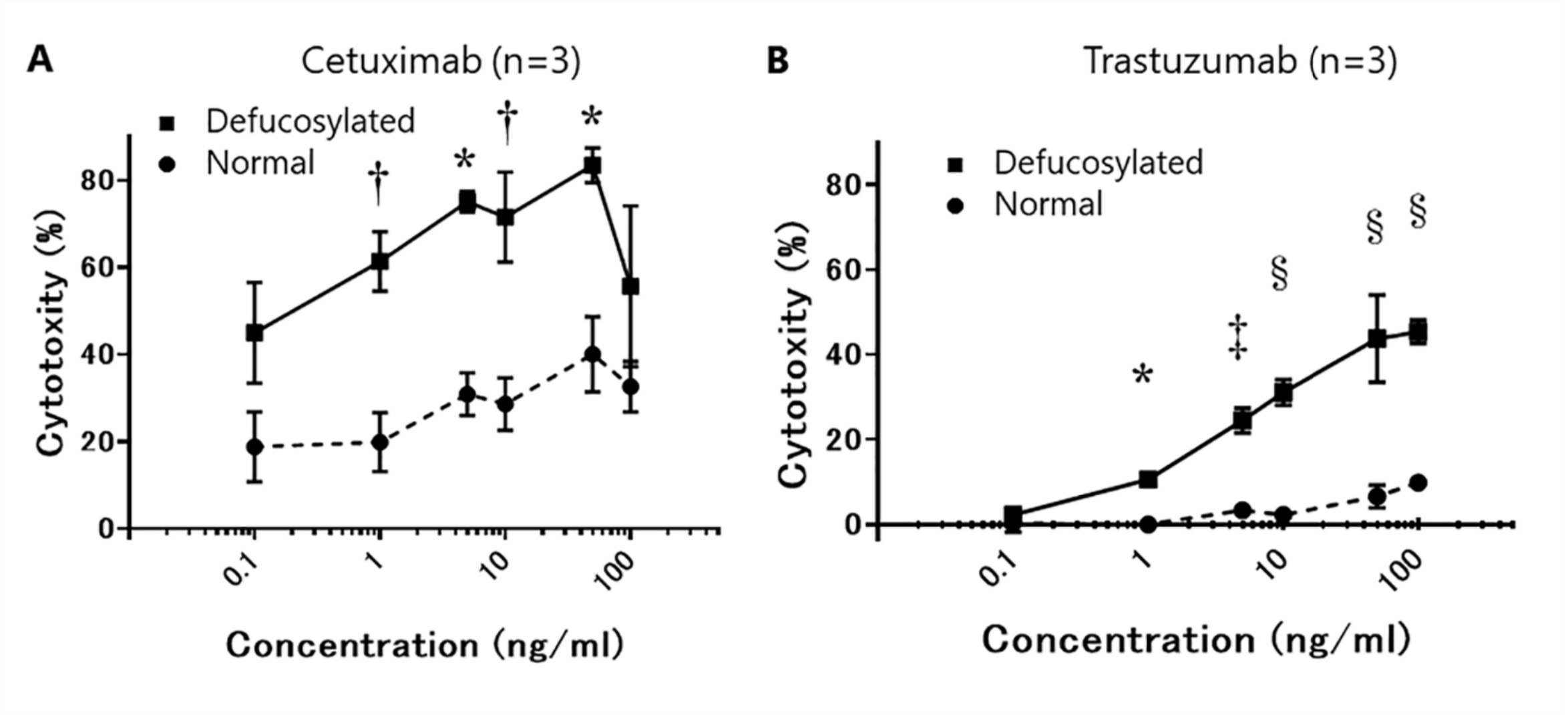
NK cell conditions in PBMCs from healthy donors. NK cell activities targeted for K562 in PBMCs from healthy individuals were evaluated in parallel to the ADCC assay and there were optimal NK activities in this condition. The error bars indicated mean±SE.

Table 1

Patients' characteristics (n=20)

Age (median), y	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

Fig. 1


A
Cetuximab (n=3)
Defucosylated
Normal
Cytotoxity (%)
0
20
40
60
80
0.1
1
10
100
Concentration (ng/ml)
B
Trastuzumab (n=3)
Defucosylated
Normal
Cytotoxity (%)
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20
40
60
80
0.1
1
10
100
Concentration (ng/ml)

Fig. 2

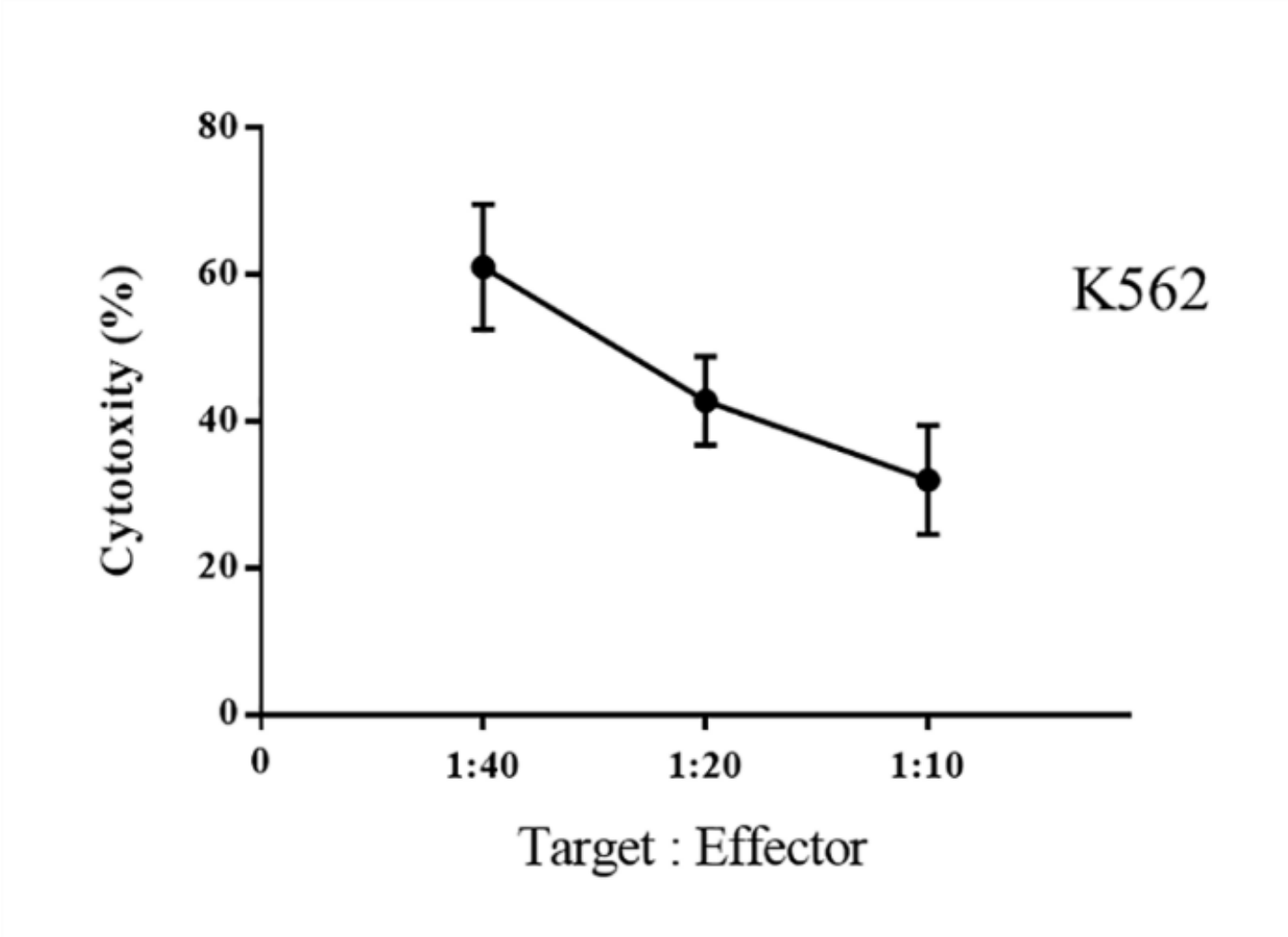


Fig. 3

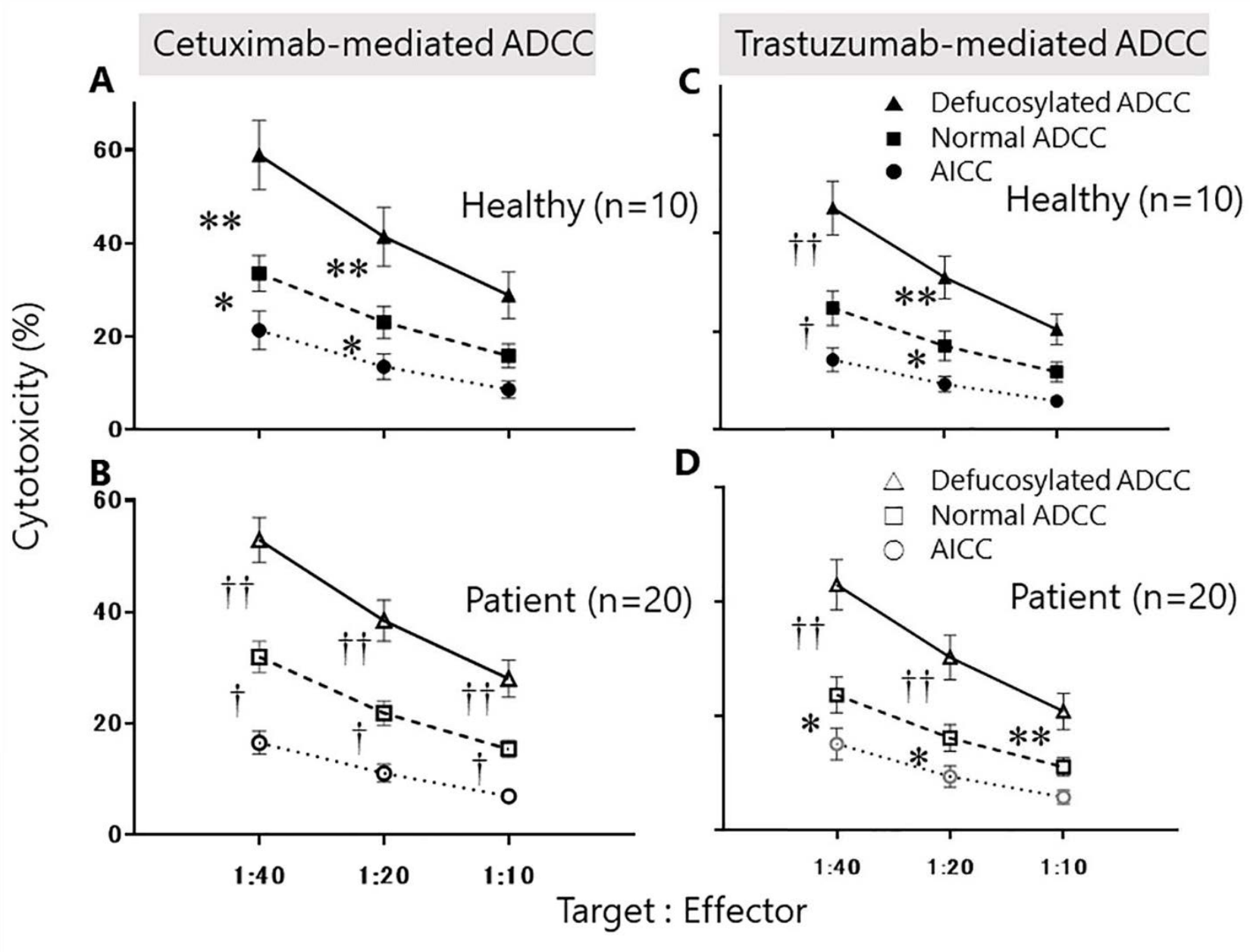

Cetuximab-mediated ADCC
Trastuzumab-mediated ADCC
A
B
C
D
Cytotoxicity (%)
60
40
20
0
Healthy (n=10)
Patient (n=20)
Defucosylated ADCC
Normal ADCC
AICC
1:40
1:20
1:10
Target : Effector

Fig. 4

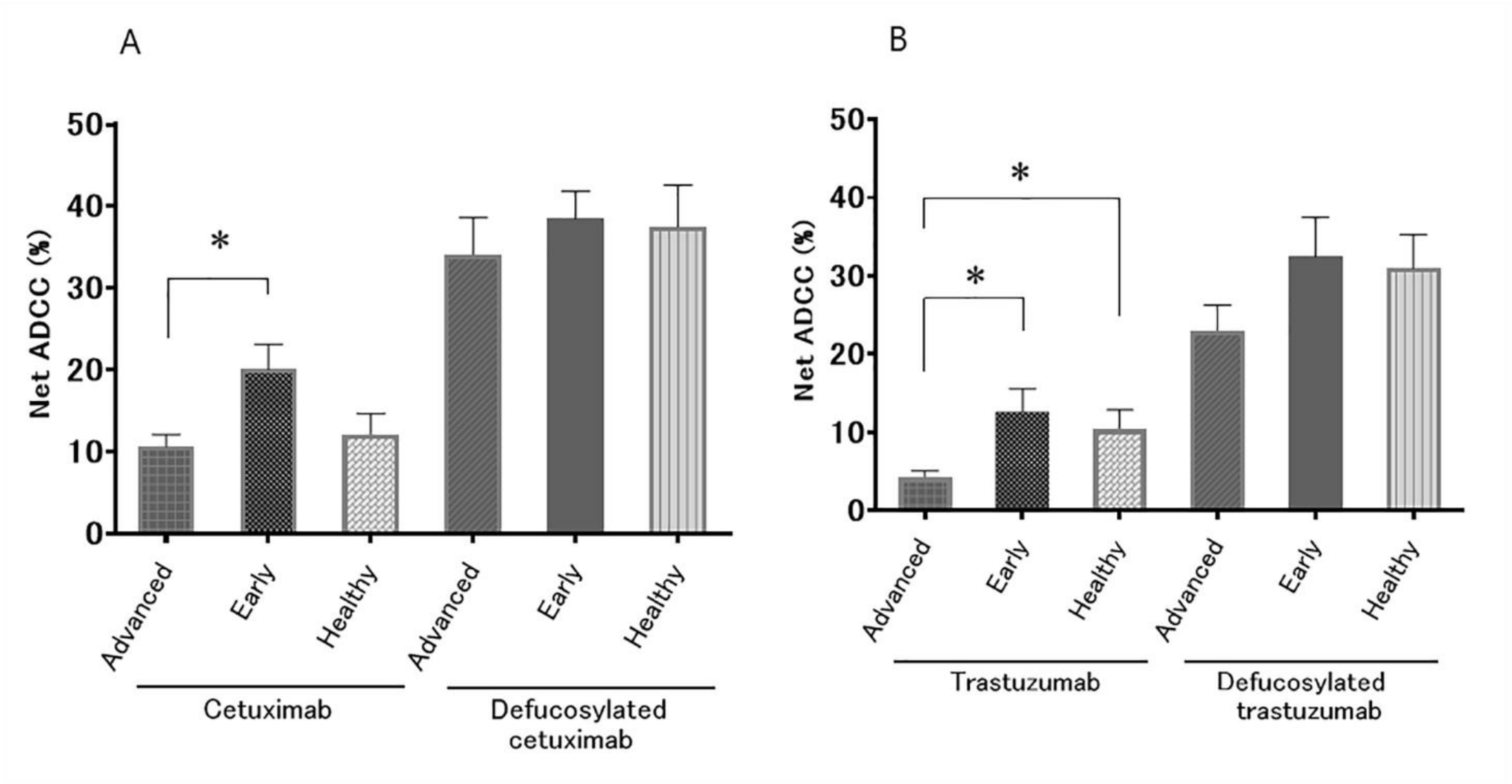
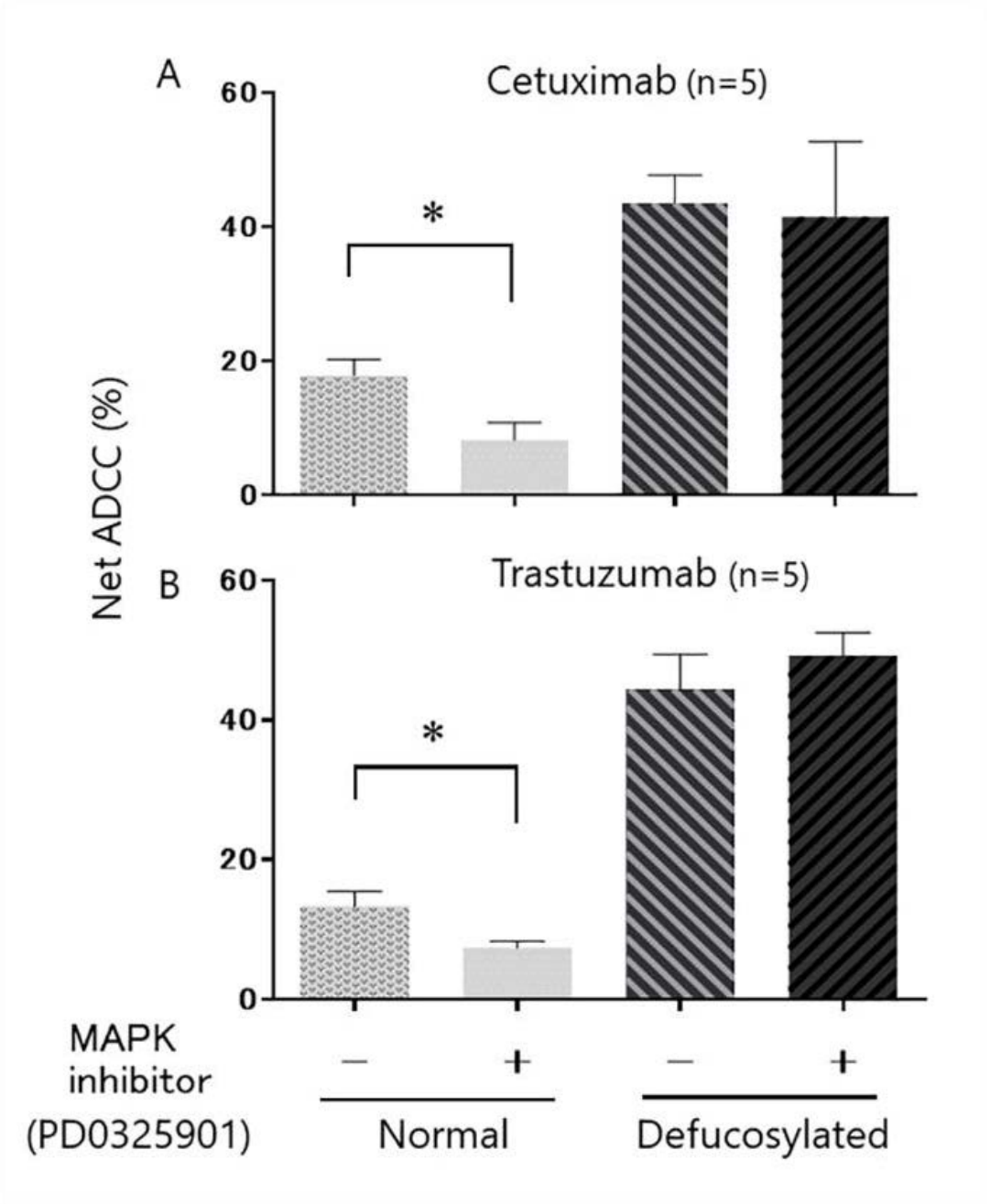

A
Net ADCC (%)
50
40
30
20
10
0
*
Advanced
Early
Healthy
Advanced
Early
Healthy
Cetuximab
Defucosylated cetuximab
B
Net ADCC (%)
50
40
30
20
10
0
*
*
Advanced
Early
Healthy
Advanced
Early
Healthy
Trastuzumab
Defucosylated trastuzumab

Fig. 5


A
Cetuximab (n=5)
60
40
20
0
*
Net ADCC (%)
B
Trastuzumab (n=5)
60
40
20
0
*
MAPK inhibitor (PD0325901)
–
+
–
+
Normal
Defucosylated