Mechanisms of Escape from Trastuzumab-mediated ADCC in Esophageal Squamous Cell Carcinoma: Relation to Susceptibility to Perforin-granzyme

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Abstract. *Background: The escape mechanisms leading to trastuzumab-resistance are under investigation, but no report has yet described the mechanisms of escape from trastuzumab-mediated antibody-dependent cellular cytotoxicity (ADCC). In the present study, the mechanisms of escape from trastuzumab-mediated ADCC were elucidated using esophageal squamous cell carcinoma (SCC) cell clones. Materials and Methods: The esophageal SCC cell line TE4, which is highly susceptible to trastuzumab-mediated ADCC, was cloned by limited dilution, resulting in SCC clones with different sensitivities to trastuzumab-mediated ADCC. Results: There was no significant correlation between human epidermal growth factor receptor (HER) 2-expression on the tumor and the sensitivity to trastuzumab-mediated ADCC. Altered major histocompatibility complex (MHC) class I expression treated by IFN-γ or the blocking of natural killer (NK) cell inhibitory receptors did not induce significant changes in sensitivity to trastuzumab-mediated ADCC. However, the tumor clones with a lower sensitivity to trastuzumab-mediated ADCC showed a reduced susceptibility to the perforin-granzyme system compared to those with a greater sensitivity to trastuzumab-mediated ADCC. Conclusion: Lower susceptibility to the perforin-granzyme system is one of the important mechanisms explaining escape from trastuzumab-mediated ADCC.*

The human epidermal growth factor receptor (HER) family of receptor tyrosine kinases consists of four members: epidermal growth factor receptor (EGFR=HER-1), HER-2, HER-3, and HER-4. HER family-related signaling has been reported to play an important role in modulating cell proliferation, survival, migration, and differentiation in malignant cells (1). The *HER-2* proto-oncogene located on chromosome 17 encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity (2, 3). HER-2 overexpression has been identified in a variety of human carcinomas such as breast, ovarian (4), and gastric carcinomas (5), and is correlated with progression in a wide variety of tumors. Furthermore, we have shown that the overexpression of HER-2 was present in 30.3% of esophageal squamous cell carcinomas (SCC) (6).

Many methods have been used to therapeutically target HER-2 in HER-2 overexpression carcinomas (7). The humanized monoclonal antibody (mAb) against the extracellular domain of HER-2, trastuzumab (Herceptin™), has been clinically shown to lead to a survival advantage in metastatic breast cancer patients exhibiting HER-2 overexpression (8).

Many mechanisms are thought to contribute to the anti-tumor activity of trastuzumab, including the blockade of signaling pathways (9), inhibition of tumor cell growth such as the down-regulation of the HER-2 receptor (9), and activation of apoptotic signals of the tumor (10). In addition, we recently reported the application of trastuzumab for esophageal SCC and gastric cancer with analysis of the antibody-dependent cellular cytotoxicity (ADCC) mediated by trastuzumab (11, 12).

However, the majority of patients who achieve an initial response generally acquire resistance within 1 year (13), resulting in tumor re-growth with trastuzumab-resistance. Several reports have described escape mechanisms whereby a decrease in $p27^{kip1}$ levels (14), loss of phosphatase and tensin homolog (PTEN) (15), and insulin-like growth factor-I receptor signaling (16) are implicated in trastuzumab-resistance. These reports focused on the direct action of trastuzumab against HER-2-expressing tumors, including its anti-proliferative effect or apoptosis-inducing activity. However, no report has yet described the escape mechanisms

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from trastuzumab-mediated ADCC, which is one of the important antitumor effects induced by trastuzumab.

In the present study, the escape mechanisms from trastuzumab-mediated ADCC were elucidated using esophageal SCC cell clones with different sensitivities to trastuzumab-mediated ADCC.

Materials and Methods

Esophageal SCC cell line and establishment of tumor cell clones. The esophageal SCC cell line TE4 was a kind gift from Dr. Nishihara (Institute of Development, Aging and Cancer, University of Tohoku, Sendai, Japan). TE4, which is reported to be highly susceptible to trastuzumab-mediated ADCC (12), was cloned by limited dilution. Briefly, 2, 5 or 10 TE4 tumor cells/well in 96-well plates together with 2×10^4/well irradiated (40 Gy) TE4 cells were cultured in RPMI-1640 medium with 5% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mmol/l L-glutamine.

The tumor cells which were growing in fewer than two wells among the 96 wells of one plate were considered as tumor cell clones. Growing tumor cells were expanded and maintained.

Chemicals. The anti-HER-2 mAb, trastuzumab (Herceptin™) and anti-CD20 mAb Rituxan, which is an isotype-matched control mAb, were purchased from Roche (Basel, Switzerland). Human recombinant IFN-γ was purchased from R&D systems (Minneapolis, USA). Human recombinant perforin (PFN) and granzyme B (GrB) were purchased from Kamiya Biomedical Company (Seattle, WA, USA). Streptolysin O (SLO) was purchased from Sigma (Saint Louis, MO, USA).

Flow cytometry. For the analysis of HER-2 expression, a phycoerythrin (PE)-labeled anti-HER2/neu mAb (Becton Dickinson, San Jose, CA, USA) and a PE-labeled mouse immunoglobulin G_1 mAb (Beckman-Coulter, Miami, FL, USA) as a negative control were used.

For the analysis of MIC A/B and Fas expression, a PE-labeled anti-MIC A/B mAb (BD Biosciences Pharmingen, New Jersey, USA) and an FITC-labeled anti-Fas mAb (DakoCytomation, Glostrup, Denmark) were used.

For the analysis of MHC class I expression, a mouse anti-human HLA-ABC mAb (DakoCytomation) as the primary Ab and a FITC-conjugated polyclonal anti-mouse Ab (DakoCytomation) as the secondary Ab were used. As a negative control for the primary Ab, mouse immunoglobulin G_1 mAb (Beckman-Coulter) was used.

Each step of the incubation with mAb was performed at 4˚C for 30 min. After the cells were washed twice in phosphate-buffered saline (PBS), the stained cells were analyzed on a flow cytometer.

Preparation of cells. Peripheral blood mononuclear cells (PBMCs) were separated from peripheral blood obtained from healthy donors by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation.

To prepare natural killer (NK) cells by negative selection, the NK cells were isolated from the PBMCs by centrifugation with Ficoll-Paque after being incubated with RosetteSep antibody cocktail for NK cells (StemCell Technologies Inc., Vancouver, British Columbia, Canada). RosetteSep antibody cocktail was bound in bispecific antibody complexes, which are directed against cell surface antigens on human hematopoietic cells (CD3, CD4, CD19, CD36 and CD66b) and glycophorin A on red blood cells. The unwanted cells, which adhered to the red blood cells, and the desired cells were separated on a Ficoll-Paque density gradient.

Monocytes were prepared by negative selection and were isolated by the Ficoll-Paque density gradient after being incubated with RosetteSep antibody cocktail (CD2, CD3, CD8, CD19, CD56 and C66b) for monocytes, as described above.

Antibody-dependent cell-mediated cytotoxicity assay (ADCC). After the target cells were labeled with 50 μCi of ^{51}Cr for 60 min, target cells (5×10^3/well) and PBMCs, NK cells or monocytes as effector cells were co-incubated at various effector/ target ratios in 200 μl of X-VIVO medium (CAMBREX, East Rutherford, NJ, USA) in a 96-well U-bottomed plate in triplicate with trastuzumab (10 μg/ml) or the control mAb, Rituxan. After 6 h of incubation, the radioactivity of the supernatant (100 μl) was measured with a γ-counter. The percentage of specific lysis=100× (experimental cpm – spontaneous cpm) / (maximum cpm–spontaneous cpm).

In some experiments, the PBMCs were pre-incubated with 10 μg/ml anti-CD158a mAb (Acris Antibodies GmbH, Hiddenhausen, Germany), anti-CD158b mAb (Immunotech, Marseille, France) or anti-159a mAb (Immunotech) or the combination of all three mAb prior to the addition of the PBMCs to the target cells and subjected to ADCC assays.

MTT cell proliferation assay. Each cell clone (2,500 cells) was incubated in 200 μl of X-VIVO with control mAb or trastuzumab (10 μg/ml) in a 96-well flat-bottomed plate in triplicate. After incubation for 96 h at 37˚C, 50 μl of MTT (3(4,5-dimenthylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, 2 mg/ml; Sigma) was added to each well and incubated for 4 h. The supernatant was discarded and the crystal products were eluted with DMSO (50 μl/well; Sigma). Colorimetric evaluation was carried out using a spectrophotometer at 570 nm. The inhibition of proliferation is shown as % cell growth inhibition induced by trastuzumab in comparison with that induced by the control mAb.

Quantitative determination of transforming growth factor (TGF)-beta2 and IL-10. Each cell clone (2×10^5 cells) was cultured in 2 ml of X-VIVO at 37˚C for 24 h in a six-well plate. After incubation, the culture supernatant in each cell was collected and tested for its contents using a Quantikine ELISA kit for human TGF-beta2 or IL-10 (R&D Systems) following the recommendations of the manufacturer.

IFN-γ stimulation of tumor cells. Tumor cell clones were treated with IFN-γ, since IFN-γ is an important cytokine in the regulation of MHC class I (17, 18). The tumor cells were incubated with 100 U/ml of IFN-γ at 37˚C for 24 h. Thereafter, the IFN-γ-treated tumor cells were subjected to flow cytometry or a ^{51}Cr release assay.

Perforin and SLO permeabilization assay. PFN was diluted in HEPES + 1% BSA (20 mM HEPES, 150 mM NaCl, 1% BSA, pH 7.4). Each tumor cell clone (1×10^6 cells) was washed twice in high calcium HEPES buffer (20 mM HEPES, 150 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) and incubated with indicated doses of PFN at 37˚C for 30 min. SLO was diluted in PBS. Each tumor cell clone (1×10^6 cells) was washed twice in PBS. The cell suspension was added to the tubes containing SLO and incubated at 37˚C for 30 min.

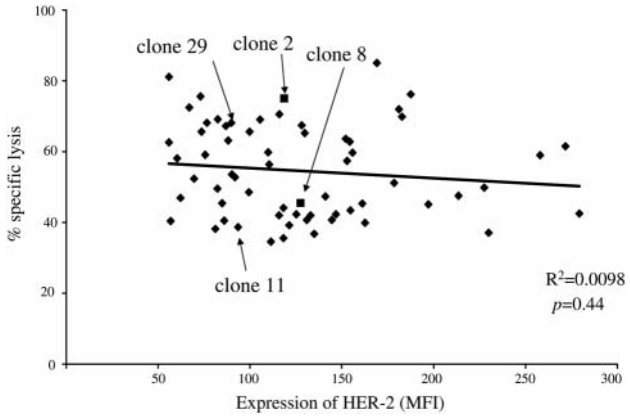

Figure 1. *Correlation between the levels of HER-2 expression and the grade of trastuzumab-mediated ADCC in tumor clones derived from the esophageal squamous cell carcinoma TE4. HER-2 expression was evaluated by flow cytometric analysis and trastuzumab-mediated ADCC (% specific lysis) determined with PBMCs isolated from one healthy donor. MFI=mean fluorescence intensity.*

Thereafter, the tumor cells were stained with propidium iodide (PI) (MBL, Nagoya, Japan), and PFN- or SLO-mediated permeabilization was measured by flow cytometry.

Apoptosis assay. For the analysis of trastuzumab-mediated apoptosis, each tumor cell clone (2×10^5 cells) was incubated in 2 ml of X-VIVO with control mAb or trastuzumab (10 μg/ml) at 37˚C in a 6-well plate. After incubation for 24 h, the level of the apoptosis in each cell was measured by staining with FITC-conjugated annexin-V and PI using a MEBCYTO Apoptosis kit (MBL) following the manufacturer's recommendations.

For analysis of PFN- and GrB-mediated apoptosis, the tumor cells (1×10^6 cells) were washed twice in 2.5 mM calcium HEPES buffer. The cell suspension was added to the tubes containing indicated doses of PFN and GrB and incubated at 37˚C for 2 h. After staining with FITC-conjugated annexin-V and PI, apoptosis was measured by flow cytometry, as described above.

DNA typing of HLA in tumor clones. DNA typing of *HLA-A, -B,* and *-C* gene polymorphisms in clones 2 and 8 was performed by the polymerase chain reaction-sequencing based typing (PCR-SBT) method (19).

Statistics. To evaluate differences between groups, Student's *t*-test was performed. Differences were considered significant at $p<0.05$.

Results

Esophageal SCC cell clones escaping trastuzumab-mediated ADCC. After the selection of tumor clones from the TE4 cell lines, the relationship between the HER-2 expression and the sensitivity to trastuzumab-mediated ADCC was examined using PBMCs isolated from one donor as the effectors. As shown in Figure 1, there were variable levels of HER-2 expression and ADCC. While some of the tumor clones with high HER-2 expression were susceptible to trastuzumab-mediated ADCC, others with high HER-2 expression were less susceptible. There was no significant correlation between HER-2 expression and trastuzumab-mediated ADCC, indicating that the escape mechanisms were not only related to the expression of HER-2, but also related to other factors.

Out of these tumor clones, clones 2 and 8, clone 29 and 11, which had almost equal levels of HER-2 expression, but different levels of ADCC (Figure 1), were selected in order to analyze the mechanisms behind tumor cell escape from trastuzumab-mediated ADCC. Morphological microscopic observation revealed that the clones had the same appearance.

Susceptibility of esophageal SCC cell clones to trastuzumab-mediated ADCC. The trastuzumab-mediated ADCC for tumor cell clones 2 and 8 was determined by PBMCs isolated from several different healthy donors (n=5). As a result, clone 2 was found to be significantly more susceptible to trastuzumab-mediated ADCC in comparison to clone 8 (Figure 2A).

Trastuzumab-mediated ADCC against clones 2 and 8 was also determined with either NK cells or monocytes separated from peripheral blood in the healthy donors (n=5). The ADCC assay with the NK cells indicated that clone 2 was significantly more susceptible to trastuzumab-mediated ADCC in comparison to clone 8 (Figure 2B), while the ADCC assay with the monocytes indicated that the sensitivity of clone 2 to trastuzumab-mediated ADCC was almost equal to that of clone 8 (data not shown). These results indicated that the lower sensitivity to trastuzumab-mediated ADCC of clone 8 was mainly due to the difference in susceptibility to NK cell-mediated ADCC.

Expression of MHC class I antigens, Fas, and MIC A/B on tumor cell clones. The expression of MHC class I on clone 8 was significantly higher than that on clone 2 (Figure 3A). There was no significant difference in Fas expression (Figure 3B) and neither clone showed MIC A/B expression (data not shown). These results suggested that a higher expression of MHC class I on clone 8 was one possible mechanism for the lower sensitivity to trastuzumab-mediated ADCC.

Moreover, DNA typing of *HLA-A, -B* and *-C* gene polymorphisms in clones 2 and 8 revealed that both the genotypes were *HLA-A**0207/110101, *HLA-B**4601/5401, *HLA-C**010201/010201. There was neither haplotype nor allelic loss in either clone.

Tumor cell clones treated with INF-γ. Treatment of clone 2 with IFN-γ resulted in an up-regulation of MHC-class I, corresponding to the levels of clone 8 (Figure 3C). However, the sensitivity of IFN-γ-treated clone 2 to trastuzumab-mediated ADCC was not significantly altered (Figure 3D). Furthermore, the sensitivity of IFN-γ-treated clone 8 to

trastuzumab-mediated ADCC was not significantly altered, although the treatment of clone 8 with IFN-γ induced the up-regulation of MHC class I (Figure 3C and 3D) to a great extent.

These results indicated that MHC class I expression was not the main reason for the difference in sensitivity to trastuzumab-mediated ADCC between clones 2 and 8.

Trastuzumab-mediated ADCC after treatment with anti-CD158 a/b mAb and CD159a mAb. In order to further analyze the involvement of MHC class I expression in trastuzumab-mediated ADCC, the NK cell inhibitory receptors were blocked by mAbs in the ADCC assay. The treatment with anti-CD158 a/b or CD159a mAbs did not enhance the trastuzumab-mediated ADCC activity (Figure 4A). Both the mAbs showed functionally confirmed blocking capacity, as they enhanced the NK cell activity in the control experiment (Figure 4B).

These results further supported the suggestion that MHC class I expression was not the main reason for the difference in sensitivity to trastuzumab-mediated ADCC between clones 2 and 8.

Sensitivity to PFN and GrB of tumor cell clones. The PFN-induced permeabilization analyzed by flow cytometry with PI staining in clone 2 was significantly higher compared to that in clone 8 (Figure 5A). On the other hand, the permeabilization by SLO was not different between clones 2 and 8 (Figure 5B). These results indicated that the difference in sensitivity to permeabilization in both clones was PFN specific.

Furthermore, the treatment with PFN and GrB in clone 2 induced noticeable levels of apoptosis, while in clone 8 it did not (Figure 6). These results indicated that clone 2 was more sensitive and susceptible to PFN combined with GrB than clone 8. Different sensitivities to PFN and GrB were also confirmed between clone 29 and 11 (Figure 6) and clone 29 was more susceptible to trastuzumab-mediated ADCC than clone 11 (Figure 1).

Quantitative determination of TGF-beta 2 and IL-10. Although the quantity of TGF-beta$_2$ produced by clone 2 was significantly less than that by clone 8 (Table I) by statistical analysis, such a difference of TGF-beta$_2$ would usually not affect the ADCC activity. None of the cell clones produced detectable levels of IL-10 (data not shown).

The anti-proliferative activity and apoptosis-inducing activity of trastuzumab in the tumor clones. The direct actions including the anti-proliferative and apoptosis-inducing activity induced by trastuzumab in clones 2 and 8 were assessed by MTT and Annexin-PI assays.

The spontaneous cell growth did not differ between clones 2 and 8 (Table I). The inhibition of tumor cell growth induced

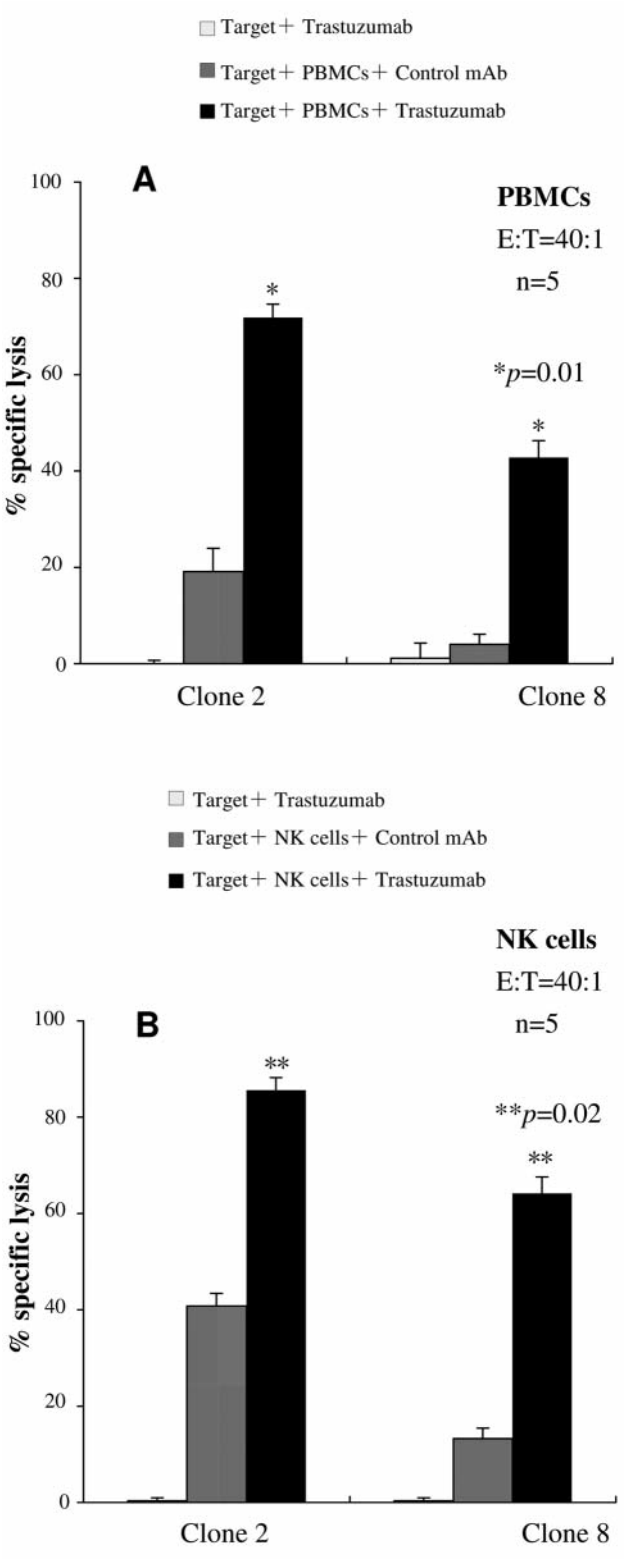

Figure 2. *Susceptibility of esophageal SCC cell clones to trastuzumab-mediated ADCC. Trastuzumab-mediated ADCC in tumor cell clones 2 and 8 was determined with PBMCs from healthy donors (n=5) (A) and NK cells negatively selected from healthy donor PBMCs (n=5) (B). E:T=effector:target.*

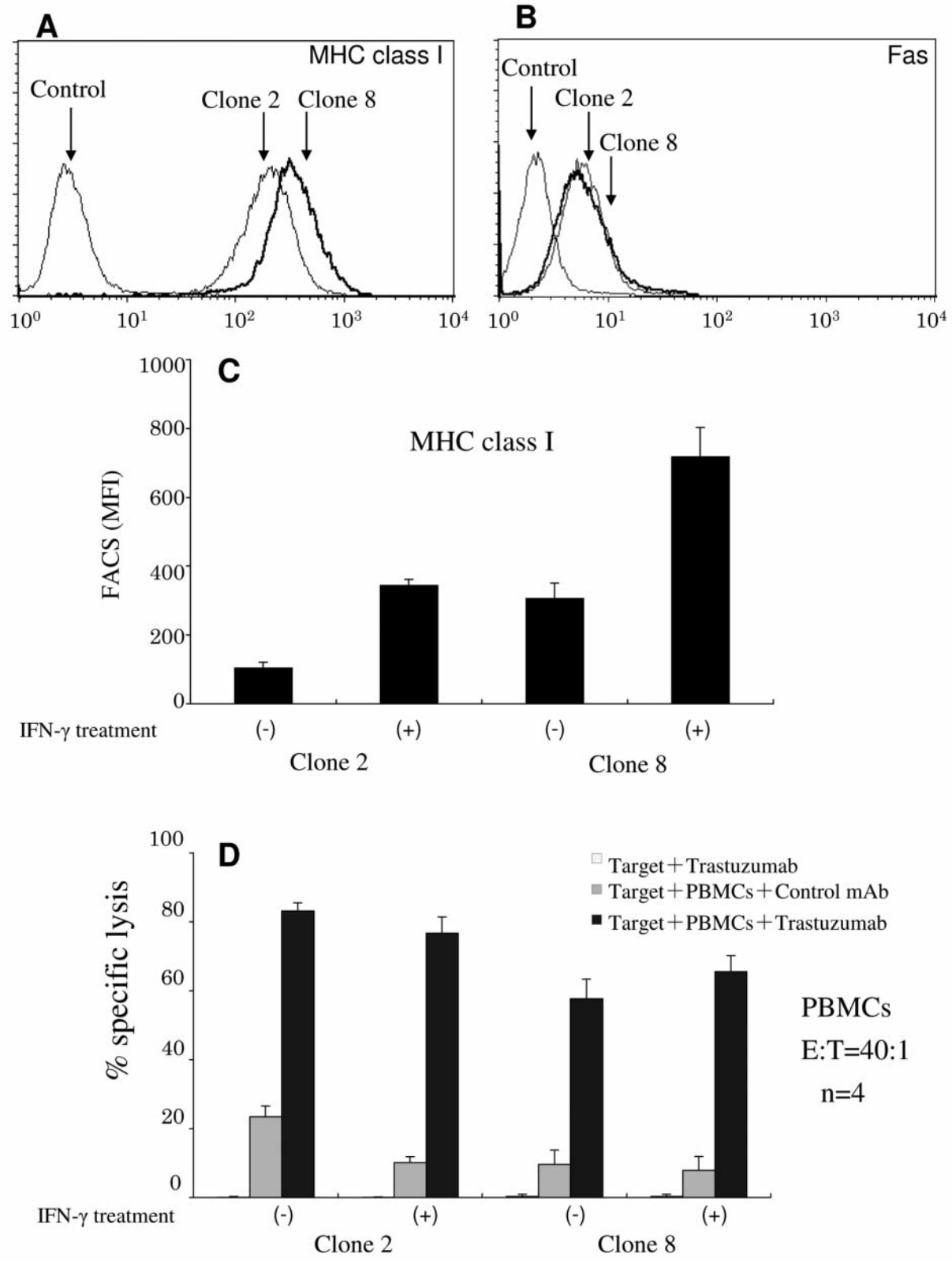

Figure 3. *MHC class I expression on tumor cell clones 2 and 8 and the effect of treatment with INF-γ. MHC class I antigen (A) and Fas (B) expression on tumor cell clones 2 and 8 evaluated by flow cytometric analysis. Flow cytometry of MHC class I expression (C) and trastuzumab-mediated ADCC assay (% specific lysis)(D) after INF-γ treatment. E:T= effector:target.*

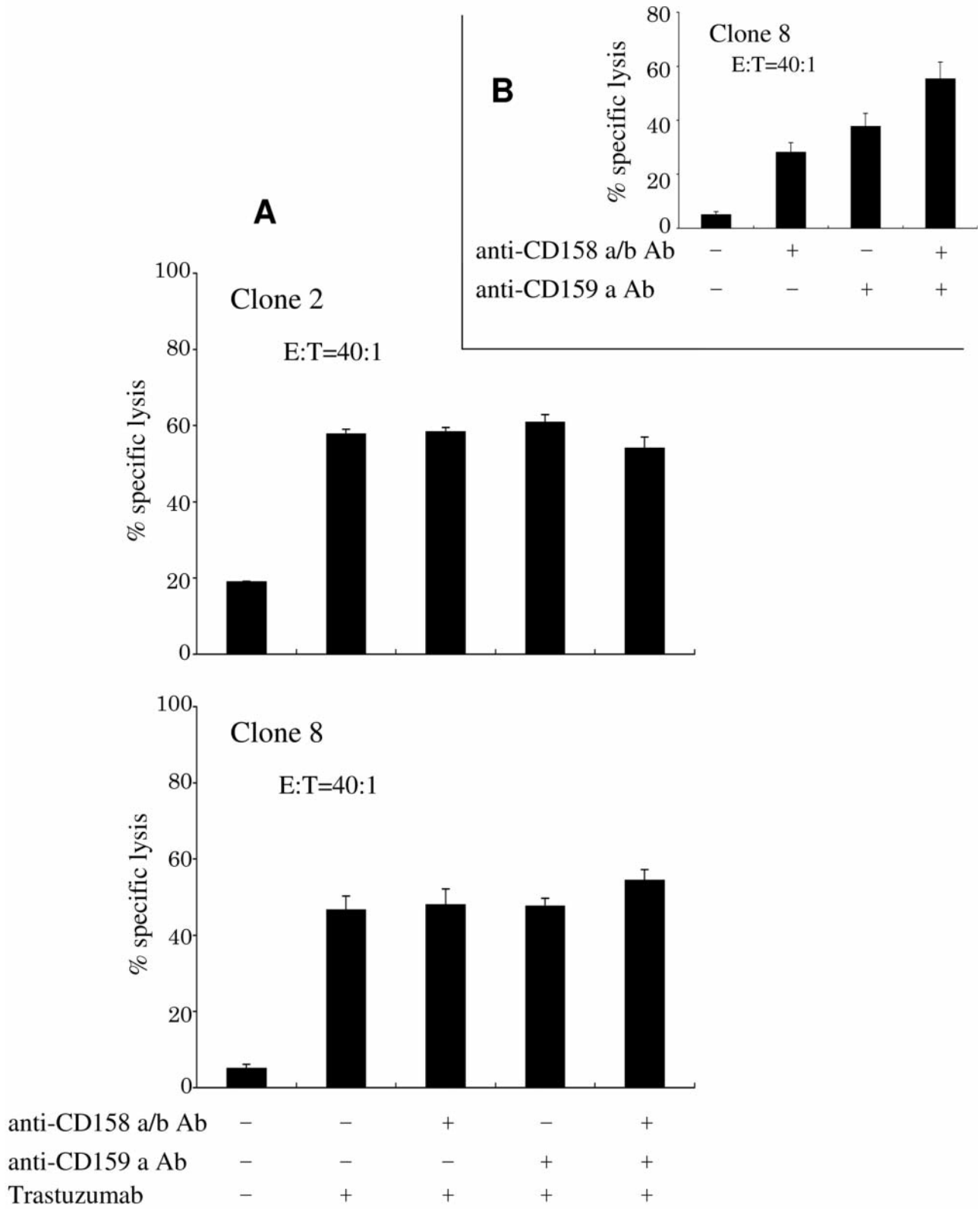

Figure 4. *Trastuzumab-mediated ADCC after treatment with anti-CD158 a/b and CD159a mAbs. NK cell inhibitory receptors were blocked by anti-CD158 a/b or/and CD159a mAbs prior to the addition of the PBMCs to the target cells and the ADCC assay, (A). Control experiment, NK cell activity with clone 8 after blocking with both mAbs, (B). E:T= effector:target.*

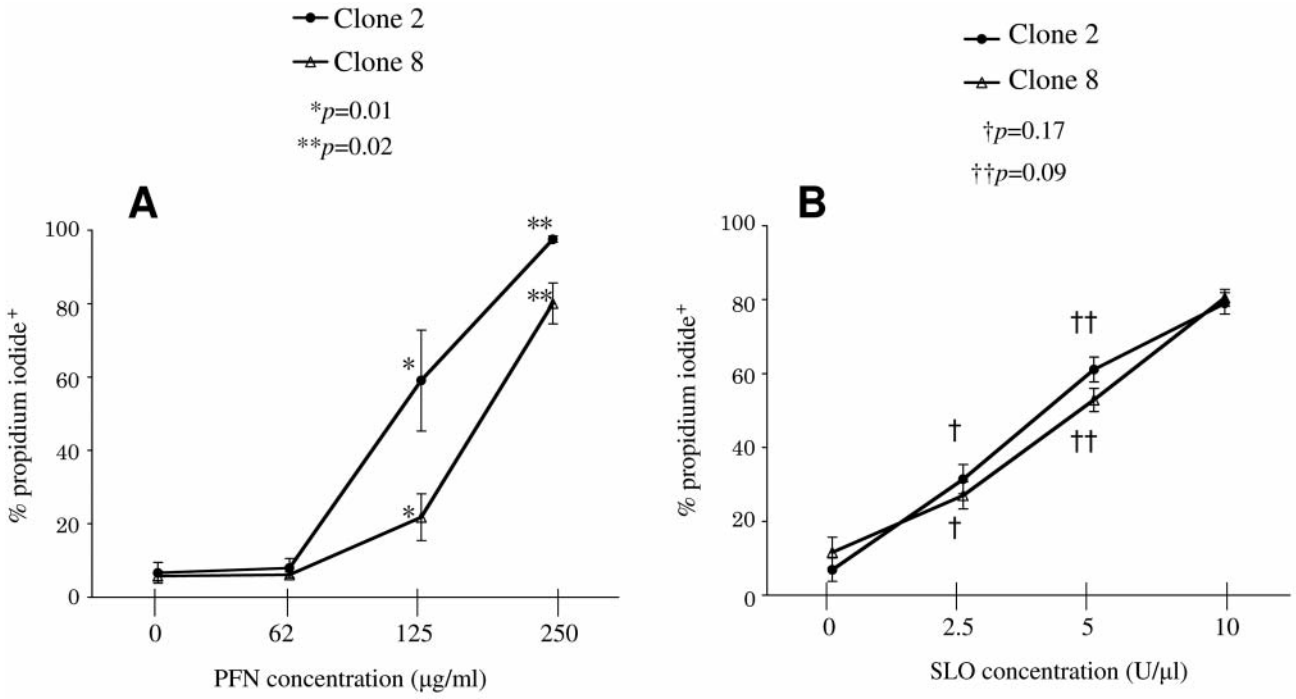

Figure 5. *The degree of permeabilization induced by PFN or SLO in tumor cell clones. Flow cytometric analysis with PI staining of PFN-induced permeabilization in clone 2 and clone 8 (A). Permeabilization by SLO in clones 2 and 8 (B).*

Table I. *Anti-proliferative activity and apoptosis-inducing activity of trastuzumab.*

	Clone 2	Clone 8	
HER-2 expression FACS (MFI)	119	128	NS
Spontaneous cell growth rate (OD 570 nm)	0.20±0.01	0.21±0.01	NS
% Growth inhibition induced by trastuzumab (MTT)	1.9±4.9	1.3±3.3	NS
Apoptosis (%)			
Medium+Control mAb	5.1±1.3	5.1±1.7	NS
Medium+Trastuzumab	3.2±1.2	2.8±0.8	NS
TGF-β_2 (pg/ml)	146±18	340±41	$p<0.05$

FACS, Fluorescence-activated cell sorting; MFI, mean fluorescence intensity; OD, optical density; MTT, 3- (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NS, not significant.

by trastuzumab was not significant in either clones 2 and 8 (Table I). Moreover, significant levels of apoptosis induced by trastuzumab were not observed in clones 2 and 8 (Table I).

Discussion

The present study using clones with different sensitivities to trastuzumab-mediated ADCC contains important findings relevant to the mechanisms of escape from trastuzumab-mediated ADCC. Firstly, there was no significant correlation between HER-2 expression on the tumor and the sensitivity to trastuzumab-mediated ADCC. Secondly, using esophageal SCC cell clones with different sensitivities to trastuzumab-mediated ADCC and equal expression of HER-2, one of the reasons for the reduced sensitivity to trastuzumab-mediated ADCC was found to be a lower susceptibility to the perforin-granzyme system.

Various mechanisms behind the reduced sensitivity to trastuzumab-mediated, NK cell-derived ADCC such as deficient expression of HER-2 (20), up-regulation of MHC class I expression (21), agonistic effect on NK cell inhibitory receptors (22), the acquisition of cell death resistance (23), and the production of immunosuppressive cytokines such as TGF-beta and IL-10 (24-26) have been considered.

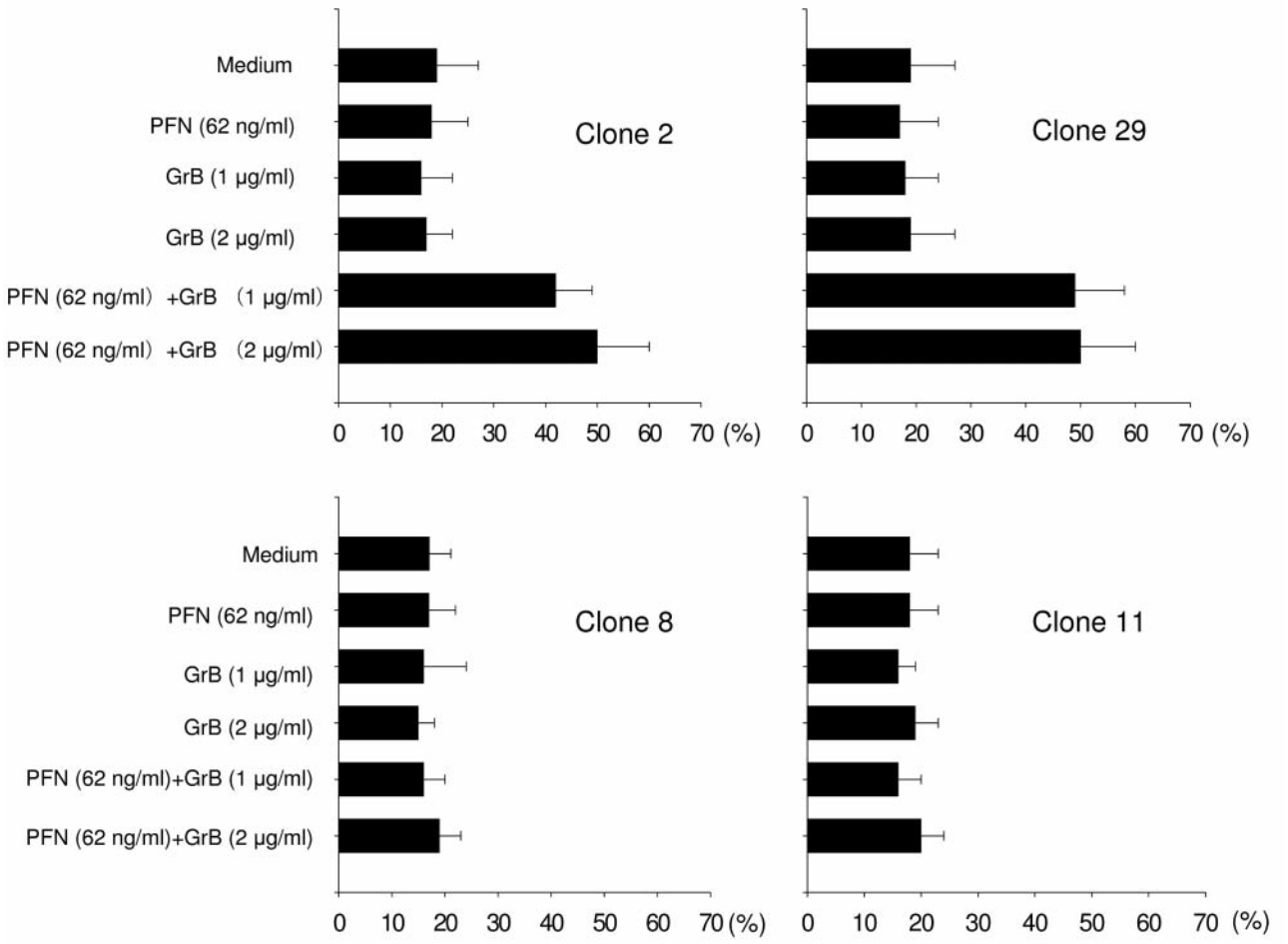

Figure 6. *Apoptosis induced by the combination of PFN with GrB in tumor cell clones analyzed by the Annexin-V/PI assay.*

NK cell-mediated ADCC activities are influenced by MHC class I, Fas, or MIC A/B expressions on targets cells (27). It is well known that a decreased expression of MHC class I antigens on tumor cells is associated with enhanced NK cell cytotoxicity (21), and NK cell functions are inhibited by the interaction of NK cell inhibitory receptors with MHC class I molecules (28). However, in the present *in vitro* system, altered MHC class I expression or blocking of NK cell inhibitory receptors did not induce significant changes in sensitivity to trastuzumab-mediated ADCC, indicating that altered MHC class I expression was not the main reason for the reduced sensitivity to trastuzumab-mediated ADCC.

CD158a/b is an NK cell inhibitory receptor of the killer Ig-like receptor type, which binds to different alleles of HLA-C molecules (29, 30). CD159a is an NK cell inhibitory receptor, belonging to the C-type lectin family, which associates with CD94 to form a disulfide link. The CD94/CD159a receptor binds to the non-classical MHC class I molecule HLA-E (31, 32). Therefore, anti-CD158 a/b and CD159a mAbs were used, in the present study in order to block HLA-C and -E, since NK cell functions are inhibited by the interaction of NK cell inhibitory receptors with MHC class I molecules (28). Blocking of the NK cell inhibitory receptors did not induce significant changes in sensitivity to trastuzumab-mediated ADCC. However, the relationship between other unknown NK cell inhibitory receptors and different HLA molecules might be involved in the sensitivity to trastuzumab-mediated ADCC. It has been shown that NK cell activity is also regulated by the interaction between NKG2D activating receptor and its ligands, MIC A/B (33). However, in the present study, neither tumor clone showed detectable MIC A/B.

NK cells mainly induce apoptosis in target cells by two pathways, the Fas-Fas ligand system and the PFN/GrB system (34, 35). ADCC activity was reported to be related to the sensitivities to PFN and GrB (36). It has been reported that the PFN/GrB system is crucial for the

elimination of tumor cells, whereas the Fas pathway seems less important in tumor-bearing mouse models (37, 38). The present results indicated that the lower sensitivity to PFN/GrB of the tumor clones was related to reduced trastuzumab-mediated ADCC. Since both clones revealed the same sensitivity to another pore-forming protein, SLO, the difference in sensitivity between clones seemed to be PFN/GrB system-specific. Since the precise pathway of PFN binding and pore formation is still under investigation, it is unknown which factors in the tumor clones are involved in PFN/GrB system resistance.

In conclusion, lower susceptibility to the perforin-granzyme system is one of the important mechanisms explaining escape from trastuzumab-mediated ADCC. A better understanding of the underlying mechanism of escape from trastuzumab-mediated ADCC may lead to more effective trastuzumab therapy.

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