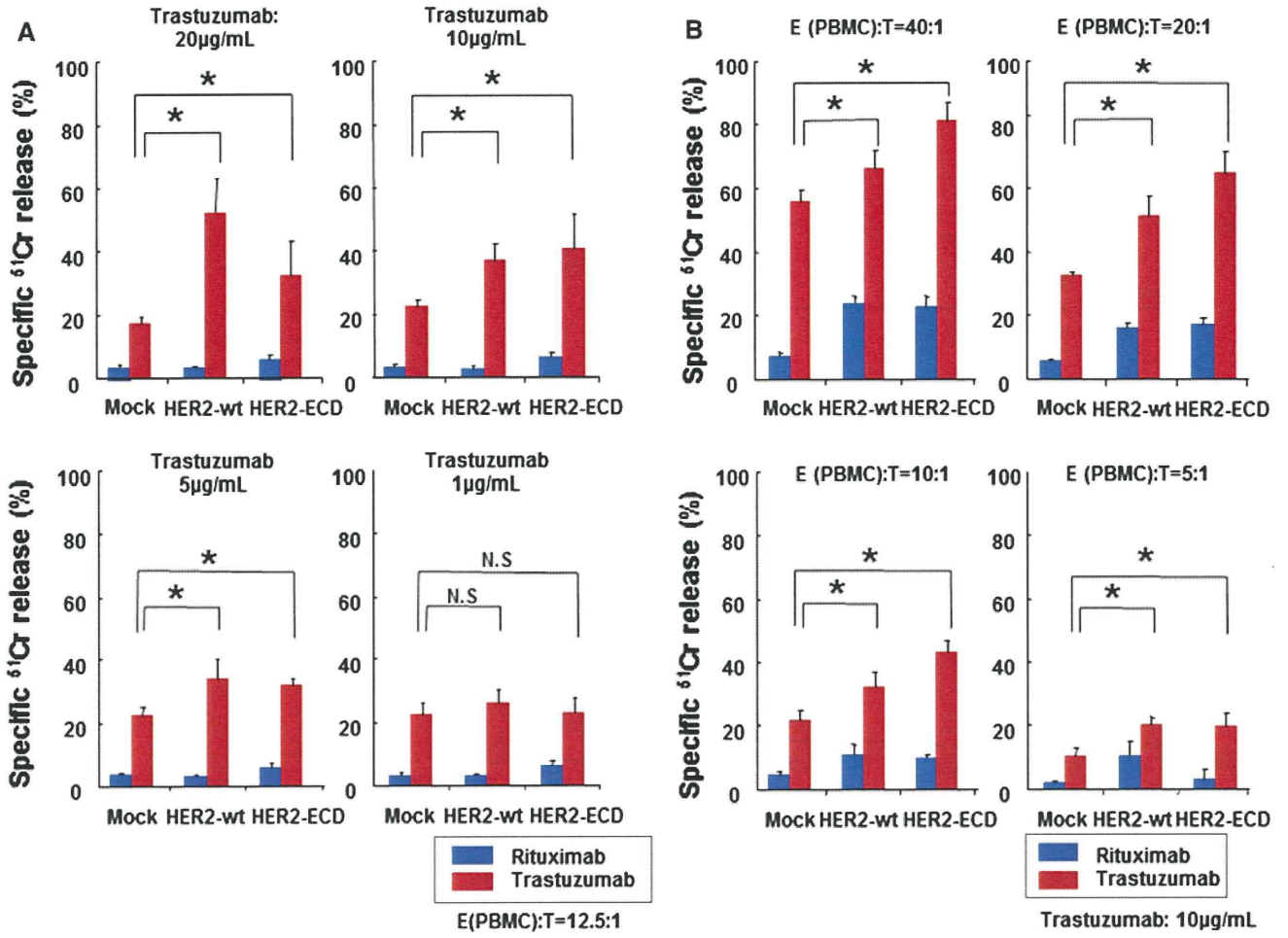

Fig. 3 Trastuzumab-mediated ADCC activity on HER2-ECD-expressing MCF7 cells. **a** The cytotoxic activity against MCF7 human breast cancer cells transfected with vector expressing HER2-wt or HER2-ECD or empty vector (mock) was assessed by a 4-h standard ^{51}Cr-release assay in the presence of the indicated doses of trastuzumab or control rituximab. Data represent the mean ± SD of 3 wells at an E/T ratio of 12.5:1. *$p < 0.05$. **b** A 4-h ^{51}Cr-release assay was also performed against MCF7 cells expressing HER2-wt or HER2-ECD, or mock-treated MCF7 cells in the presence of 10 μg/ml of trastuzumab or control rituximab. *Data* represent the mean ± SD of 3 wells at four different E/T ratios. *$p < 0.05$

Table 1 HER2 expression status of gastric cancer cell lines

Cell lines	HER2 status		
	FACS (MFI)	HercepTest	Western blotting
MKN1	13	–	Weak
MKN7	106	3+	Strong
MKN28	30	–	Negative
MKN45	23	1+	Weak
NUGC3	9	–	Negative
KATO-III	30	2+	Medium

HER2-expressing status of six different gastric cancer cell lines measured by flow cytometry, immunocytochemistry (HercepTest), and Western blotting analysis

MFI mean fluorescence intensity, *FACS* fluorescence-activated cell sorting

Direct antitumor effects of Ad-HER2-ECD on trastuzumab-resistant or low HER2-expressing human cancer cells

Next, we assessed the cell growth pattern of trastuzumab-resistant SKBR3 and BT474 human breast cancer cells and low HER2-expressing MKN1 and MKN28 human gastric cancer cells following Ad-HER2-ECD infection. MCF7 cells that were stably transfected with the HER2-ECD plasmid showed a growth pattern similar to that of parental or control vector-transfected MCF7 cells (Fig. 2a). However, adenovirus-mediated overexpression of HER2-ECD unexpectedly induced a significant suppression of in vitro growth in all cell lines as compared to uninfected cells or cells infected with control dl312 (Fig. 5). These results suggest that Ad-HER2-ECD had a slight but significant direct

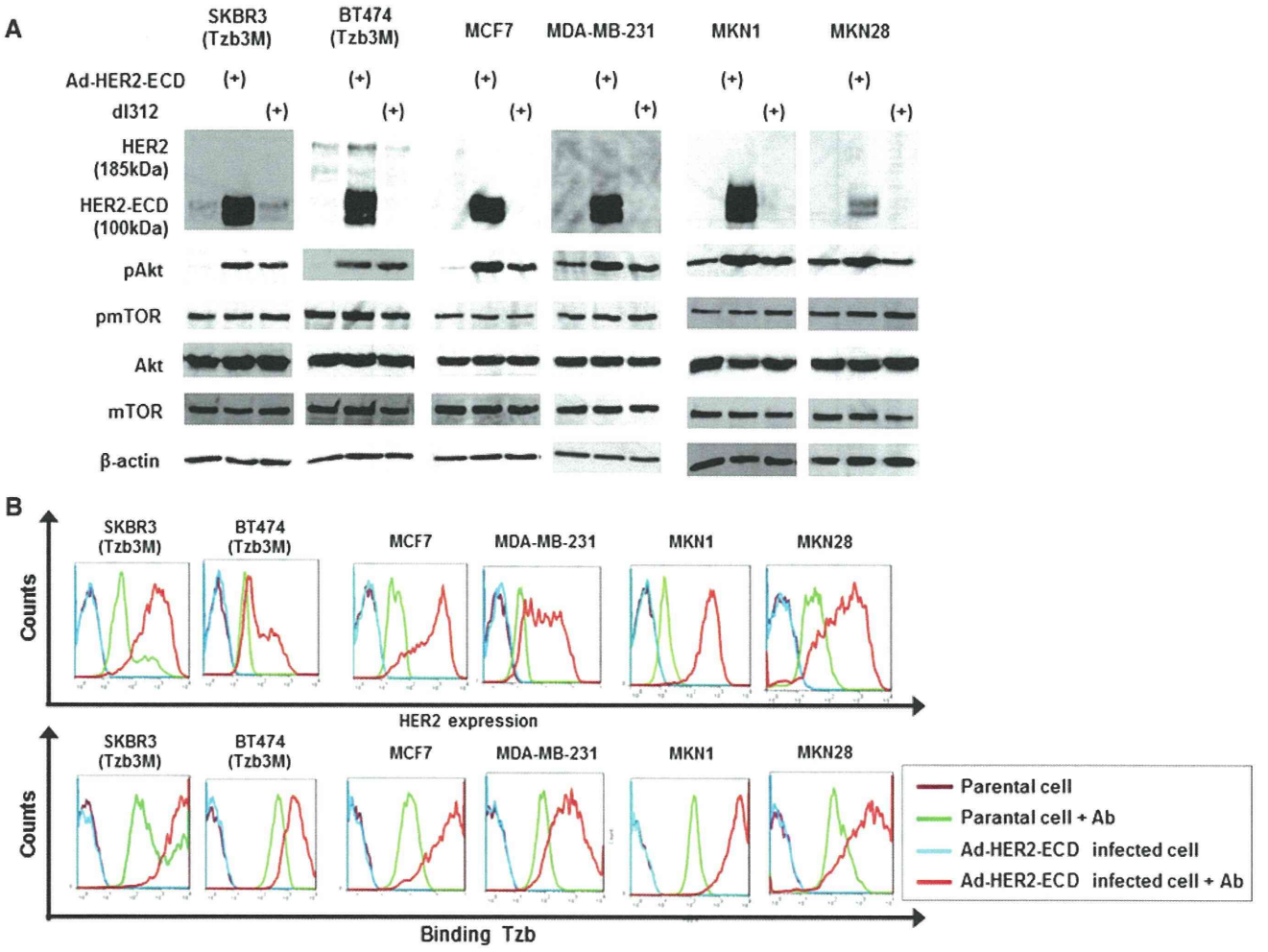

Fig. 4 Efficient HER2-ECD overexpression in human cancer cell lines by a recombinant replication-deficient adenovirus vector. **a** Western blot analysis of HER2-wt (185 kDa), HER2-ECD (100 kDa), and representative HER2-related signaling proteins in various types of human cancer cells. Trastuzumab-resistant breast cancer cells (SKBR3 and BT474), low HER2-expressing breast cancer cells (MCF7 and MDA-MB-231), and low HER2-expressing gastric cancer cells (MKN1 and MKN28) were infected with replication-deficient adenoviral vector expressing exogenous HER2-ECD (Ad-HER2-ECD) or replication-deficient control adenovirus (dl312) at an MOI of 20 for 36 h. **b** Flow cytometric analysis of HER2 expression and the amount of bound trastuzumab in cells 36 h after Ad-HER2-ECD infection at an MOI of 20

antitumor effect on trastuzumab-resistant and low HER2-expressing human cancer cell lines in vitro.

Adenovirus-mediated HER2-ECD overexpression sensitizes trastuzumab-resistant or low HER2-expressing human cancer cells to trastuzumab-mediated ADCC

Finally, we examined whether Ad-HER2-ECD infection could overcome acquired resistance to trastuzumab-mediated ADCC in SKBR3 and BT474 human breast cancer cells. Enhancement of ADCC activity by Ad-HER2-ECD infection was also assessed in low HER2-expressing human breast and gastric cancer cell lines. Following Ad-HER2-ECD infection, trastuzumab-resistant (Fig. 6a) as well as low HER2-expressing cells (Fig. 6b, c) were more efficiently killed by ADCC, and a significant difference was detected at all effector/target ratios in all cell lines, except trastuzumab-resistant SKBR3 cells, as compared to mock- or control dl312-infected cells. Thus, Ad-HER2-ECD-mediated exogenous expression of HER2-ECD could sensitize trastuzumab-resistant HER2-downregulated cells or low HER2-expressing cells to trastuzumab through ADCC activation in vitro.

Discussion

The nature of acquired resistance to trastuzumab is an area of active research in both the laboratory and the clinic. In the present study, we exposed HER2-positive breast cancer cells to trastuzumab continuously in vitro to induce resistance against this antibody and investigate the mechanisms

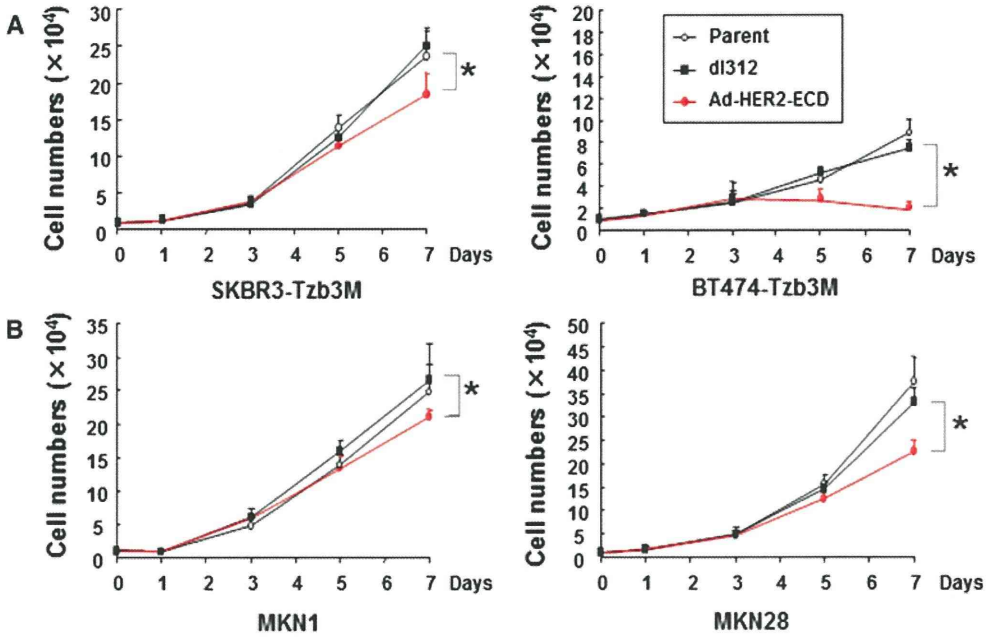

Fig. 5 Antitumor effects of Ad-HER2-ECD on trastuzumab-resistant or low HER2-expressing human cancer cells. Trastuzumab-resistant SKBR3 and BT474 breast cancer cells (**a**) and low HER2-expressing MKN1 and MKN28 gastric cancer cells (**b**) cultured as a monolayer were infected with Ad-HER2-ECD or control dl312 at an MOI of 20. The cell growth was determined by counting cell numbers each day after infection. The mean ± SD of three different wells is shown.
*$p < 0.05$

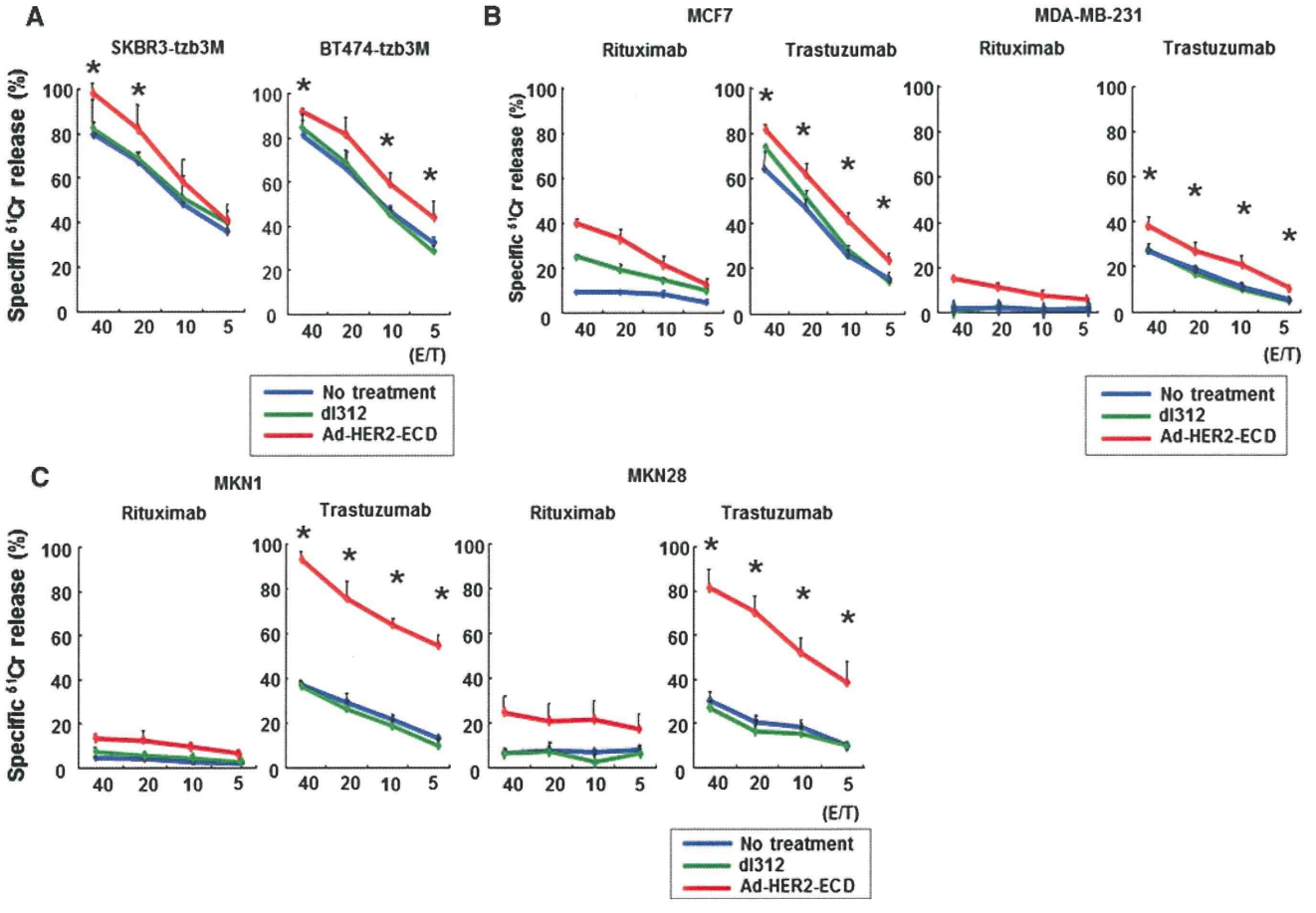

Fig. 6 Molecular sensitization of human cancer cells to trastuzumab by Ad-HER2-ECD-mediated exogenous expression of HER2-ECD. The cytotoxic reactivity of PBMCs against HER2-downregulated SKBR3 or BT474 cells (**a**), low HER2-expressing MCF7 or MDA-MB-231 human breast cancer cells (**b**), or low HER2-expressing MKN1 or MKN28 human gastric cancer cells (**c**) was assessed after Ad-HER2-ECD or dl312 infection in the presence of 10 μg/ml of trastuzumab or control rituximab by a 4-h ^{51}Cr-release assay. *Data* represent the mean ± SD of 3 wells at four different E/T ratios

responsible for this resistance. Some studies indicated that trastuzumab treatment does not alter the cell-surface HER2 expression status [30, 31]. However, we have demonstrated that continuous exposure to trastuzumab results in HER2 downregulation in HER2-overexpressing breast cancer cell lines in vitro. Previous studies also showed that alternative receptor tyrosine kinase signaling may play a role in trastuzumab resistance [18–20]. In fact, trastuzumab-exposed SKBR3 cells exhibited upregulated IGF-1R expression, suggesting that an alternative signaling pathway was enhanced to protect cells from trastuzumab-mediated HER2 signaling inhibition.

We also found that trastuzumab-exposed HER2-overexpressing breast cancer cells developed impaired trastuzumab-mediated ADCC activity in vitro. The ability of trastuzumab to mediate ADCC activity is strictly related to HER2 density [7]. In addition, Mimura et al. [32] previously reported that the HER2 status determined by flow cytometry is well correlated with trastuzumab-mediated ADCC activity in esophageal squamous cell carcinoma cell lines in vitro. Taking into account these reports, we conclude that the impaired trastuzumab-mediated ADCC activity in trastuzumab-exposed HER2-positive human cancer cells was due to the downregulation of HER2 expression on the cell surface. These results led us to examine whether exogenous expression of the HER2 receptor on the cell surface could re-sensitize HER2-downregulated human cancer cells to trastuzumab via ADCC re-activation.

HER2 overexpression is a significant prognostic factor in terms of nodal status, tumor grade, overall survival and probability of relapse in breast cancer patients [33, 34]. Although reports are conflicting, some studies have suggested that HER2-positive status in gastric cancer is associated with poor outcomes and aggressive disease [12, 13]. As expected, human cancer cells transfected with the full-length functional HER2 showed accelerated cell growth as compared to parental cells, whereas the cell growth pattern of HER2-ECD-transfected low HER2-expressing human cancer cells was similar to that of parental cells. Furthermore, we showed that HER2-ECD transfection of low HER2-expressing human cancer cells did not enhance the HER2/HER3 signaling pathway, which is the major oncogenic signal in HER2-overexpressing breast tumors [35, 36]. Although transfection of HER2-ECD-expressing plasmid did not influence cell growth, adenovirus-mediated exogenous HER2-ECD expression significantly suppressed the tumor cell growth in vitro, suggesting that the growth inhibition associated with HER2-ECD overexpression might be due to its levels on the cell surface. Therefore, Ad-HER2-ECD infection showed slightly enhanced cytotoxic activity against some types of human cancer cells even with the control antibody rituximab in the ^{51}Cr release assay. The mechanism of Ad-HER2-ECD-mediated cell growth inhibition is unclear; however, it is likely to be caused by the restriction of other HER family receptors through the formation of heterodimers with exogenously expressed HER2-ECD that lacks the downstream signaling pathway.

Some previous studies demonstrated that primary or acquired resistance to trastuzumab often results from preventing the binding of antibody to the HER2 protein by proteins such as membrane-associated glycoprotein mucin-4 [37, 38]. In our study, even after a long-term exposure to trastuzumab, trastuzumab-mediated ADCC activity on stably HER2-ECD-expressing MCF7 cells was significantly enhanced compared to mock-treated MCF7 cells, and, furthermore, HER2-downregulated or low HER2-expressing human cancer cells could be re-sensitized to trastuzumab via re-activation of trastuzumab-mediated ADCC. These results indicate that the degree of antibody-mediated ADCC activity is likely to be correlated with the cell-surface expression levels of HER2. These results suggest that the HER2-downregulated or low HER2-expressing human cancer cells exogenously overexpressing HER2-ECD is hard to develop resistance to trastuzumab in terms of the importance of ADCC activity in antitumor effects of this antibody.

A previous study has demonstrated that heterogeneity and incomplete membranous immunoreactivity for HER2 were more common in gastric cancer than in breast cancer [39], suggesting that the gastric tumors diagnosed as HER2-positive by immunohistochemistry or fluorescent in situ hybridization are more likely to be residual and regrow under trastuzumab treatment. Therefore, molecular sensitization to trastuzumab through the expression of HER2-ECD is thought to be effective even against HER2-positive gastric cancer. We would like to examine whether the ADCC activation by exogenous HER2-ECD expression functions in vivo; however, since murine NK cells do not recognize trastuzumab, which is a humanized antibody, the in vivo experiments are hard to be performed. The genetically engineered fluorescent tumor cells as well as the whole-body fluorescent imaging technology may be available for such kinds of in vivo studies [40, 41].

Although the strategy for molecular sensitization to trastuzumab via ADCC activation by using an adenoviral vector is considered to be effective, some limitations exist; for example, there are variations in the efficiency of viral infection and the expression levels of exogenous HER2-ECD. As we used a replication-deficient adenovirus vector, the viral spread might be less than ideal after intratumoral administration. We previously developed a telomerase-specific oncolytic adenovirus that causes cell death in human cancer cells with telomerase activities. These oncolytic viruses engineered to replicate in tumor cells but not in normal cells could be used as tumor-specific vectors carrying

therapeutic genes such as HER2-ECD. Moreover, ADCC activity of PBMCs from cancer patients is likely to be impaired due to immunosuppression and NK cell dysfunction, as previously reported for gastric cancer patients [42, 43]. The immunosuppressive state is associated with immunosuppressive cytokines such as IL-10 and TGF-β. These cytokines are produced within the tumor microenvironment and suppress the activity of NK cells, monocytes, and T cells [43–46]. Therefore, to sufficiently enhance the effect of trastuzumab-mediated ADCC activity in cancer patients, supportive immunotherapy such as the administration of immune-stimulating cytokines may be required.

In conclusion, our data demonstrate that HER2 downregulation and impaired ADCC activity may be one mechanism of trastuzumab resistance. We also show that exogenous overexpression of non-signaling HER2-ECD could sensitize HER2-downregulated or HER2-negative human cancer cells via ADCC activation, an outcome that has important implications for the treatment of human cancers.

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Conflict of interest All authors state that they have no potential conflicts of interest.

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