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14. ABSTRACT Trastuzumab, a monoclonal antibody (mAb) targeting HER-2/neu, kills tumor cells by several mechanisms, including antibody-dependent cellular cytotoxicity (ADCC). Strategies that enhance the activity of ADCC effectors, including natural killer (NK) cells, may improve trastuzumab's efficacy. NK cells that encounter trastuzumabcoated, HER2-overexpressing breast cancer cells become activated and express CD137, a costimulatory receptor. CD137 activation, which is dependent on the FcγRIII receptor, occurred both in vitro and in the peripheral blood of women with HER2-expressing breast cancer following trastuzumab treatment. Stimulation of trastuzumab-activated NK cells with an agonistic mAb against CD137 killed breast cancer cells more efficiently in vitro.					
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INTRODUCTION

Of the 207,000 women diagnosed with breast cancer in the United States in 2010 one quarter have tumors overexpressing the transmembrane receptor tyrosine kinase, HER-2/neu (human epidermal growth factor receptor 2, HER2). These women comprise a disproportionate number of the 40,000 annual breast cancer deaths. Trastuzumab is a humanized monoclonal antibody (mAb) targeting HER2. Despite improving the outcome for this poor prognostic group of patients, response rates in metastatic breast cancer to trastuzumab as monotherapy are limited, approximately 10-15%(1).

Multiple strategies have been investigated to enhance the anti-tumor activity of trastuzumab, which is due, at least in part, to antibody-dependent cell-mediated cytotoxicity (ADCC)(2-5). ADCC is dependent upon immune effector cells, mainly natural killer (NK) cells, binding *via* their Fc receptor (FcγRIII, CD16) to the IgG1 Fc, heavy-chain, portion of trastuzumab(3). This leads to the activation of the NK cells, release of their cytotoxic granules, and lysis of the trastuzumab-bound breast cancer cell(6). Clinical results have shown that patients harboring an FcγRIIIA polymorphism with higher NK affinity for IgG1 have a better response to trastuzumab, further supporting the hypothesis that ADCC, including its mediators, is an important *in vivo* mechanism of trastuzumab action(7, 8). Additional supporting clinical data demonstrated that responders to neoadjuvant trastuzumab exhibited a four-fold increase in antibody-dependent lytic activity from isolated peripheral blood mononuclear cells compared to that of nonresponders(4). Therefore, augmenting ADCC could increase the clinical efficacy of trastuzumab therapy.

Selectively targeting activated NK cells at the tumor site would be an attractive strategy to improve ADCC without incurring the systemic toxicity of global NK cell stimulation, such as that observed with systemic IL-2 or IL-12(9, 10). Recently, it was shown that human NK cells upon Fc-receptor triggering, such as the interaction with antibody-bound tumor cells, upregulate the inducible costimulatory molecule, CD137(11). Once induced to express CD137, we hypothesize that the killing function of these activated

NK cells can be enhanced by their exposure to an agonistic mAb against CD137, leading to improved anti-tumor activity. In the current study we investigate this hypothesis that an agonistic mAb against CD137 can enhance the killing of human breast cancer cells by trastuzumab both in vitro and in vivo.

BODY

Human HER2-expressing tumor cells coated with trastuzumab induce the expression of CD137 on human NK cells. Purified NK cells from healthy human subjects were incubated with trastuzumab and breast cancer cell lines (BT474M1, HER18, or SKBR3) expressing HER2. This resulted in robust upregulation of CD137 expression. By contrast incubation of the same human NK cells in the absence of tumor cells, or in the presence of HER2-expressing tumor cells and a non-binding mAb (rituximab) had little effect on CD137 expression (Figure 1A). No induction of CD137 occurred on NK cells following incubation of breast cancer cell lines with trastuzumab in the presence of a breast cancer cell line which does not overexpress HER2 (MCF7) (Figures 1A-B). Similarly, trastuzumab D265A, a trastuzumab variant which does not bind human FcγRs, abrogated the increase in CD137 expression on NK cells following exposure to trastuzumab-coated HER2-expressing tumor cells. CD137 upregulation occurred preferentially among CD56^{dim} in comparison to CD56^{hi} NK cells (Figure 1C). The induction of CD137 peaked after 24 hours and was associated with a concurrent decrease in the expression of the FcγRIII (CD16)(Figure 1C). Despite a similar decrease in NK cell expression of CD16 following culture with trastuzumab-bound HER2-expressing tumor cells, NK cells from healthy donors with high affinity polymorphisms of the FcγRIIIA-158 (V/V or F/V) expressed increased levels of CD137 compared to NK cells from donors with a low affinity FcγRIIIA-158 (F/F) polymorphism.

An agonistic anti-CD137 mAb increases trastuzumab-mediated NK cell cytokine secretion and trastuzumab-dependent NK cell-mediated cytotoxicity. NK cells from healthy donors were co-cultured with trastuzumab-bound HER2-expressing breast cancer cells for 24 hours. Subsequently, the activated NK cells were re-isolated and combined with breast cancer cells under different antibody conditions. The function of these activated NK cells was investigated by measuring secretion of interferon (IFN)-γ and lysis of breast cancer cells. Anti-CD137 agonistic mAb significantly increased trastuzumab-induced IFN-γ secretion when incubated with two of three HER2-overexpressing breast cancer cell lines (SKBR3 and HER18)(Figures 2A-D). Moreover, the anti-CD137 mAb enhanced the ability of these activated NK cells

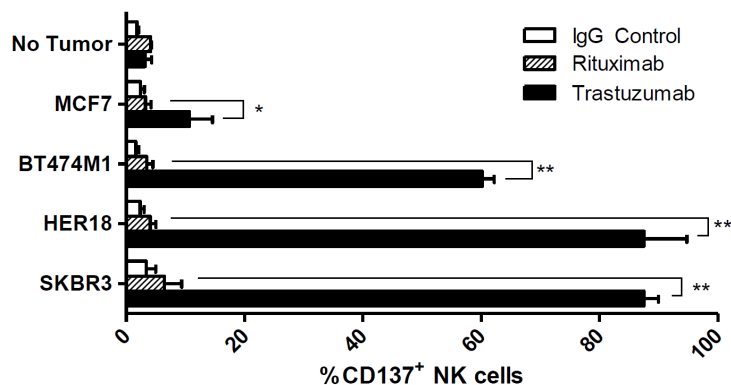
to kill trastuzumab-coated tumor cells (Figures 2E-H). Anti-CD137 mAb alone had minimal effect in the absence of both trastuzumab and HER2-overexpressing breast cancer cells. To confirm the enhanced cytotoxicity, ⁵¹Cr labelled-breast cancer cell lines were cultured with NK cells, both un-purified or purified (Figures 3A-D) following activation, in the presence or absence of trastuzumab and anti-CD137 mAb. Again, the combination of anti-CD137 antibodies with trastuzumab induced enhanced ADCC relative to treatment with trastuzumab alone using both un-purified and purified NK cells. This effect was observed only against HER2-overexpressing breast cancer cell lines, confirming that the killing was due to ADCC, even though the NKs had been pre-activated.

To investigate if augmented antibody-mediated cytotoxicity of activated NK cells is restricted to the antibody-coated tumor cell used for NK cell activation, cytotoxicity against HER2-expressing breast cancer as well as CD20⁺ lymphoma was compared using NK cells activated by trastuzumab-coated breast cancer or rituximab-coated lymphoma. Activated NK cells lysed antibody-coated tumor cells with augmented cytotoxicity independent of antibody-coated tumor cell used for activation. Anti-CD137 mAb significantly increased trastuzumab-mediated breast cancer cytotoxicity of NK cells activated by culture with either trastuzumab-coated breast cancer or rituximab-coated lymphoma. Similar enhancement of rituximab-mediated lymphoma cytotoxicity was observed using NK cells activated by either trastuzumab-coated breast cancer or rituximab-coated lymphoma. Finally, to determine if anti-CD137 mAbs enhance cytotoxicity in the setting of trastuzumab resistance, cytotoxicity against a breast cancer cell line intrinsically resistant to trastuzumab (with maintained expression of HER2, HCC1569) was performed with maintained efficacy observed.

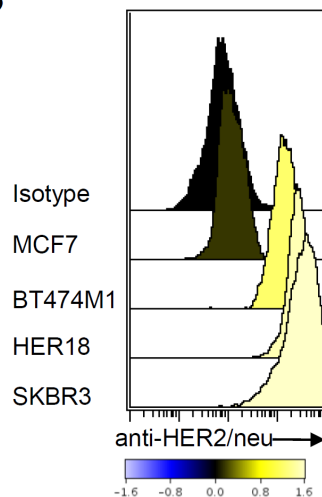
FIGURES

FIGURE 1

A



B



C

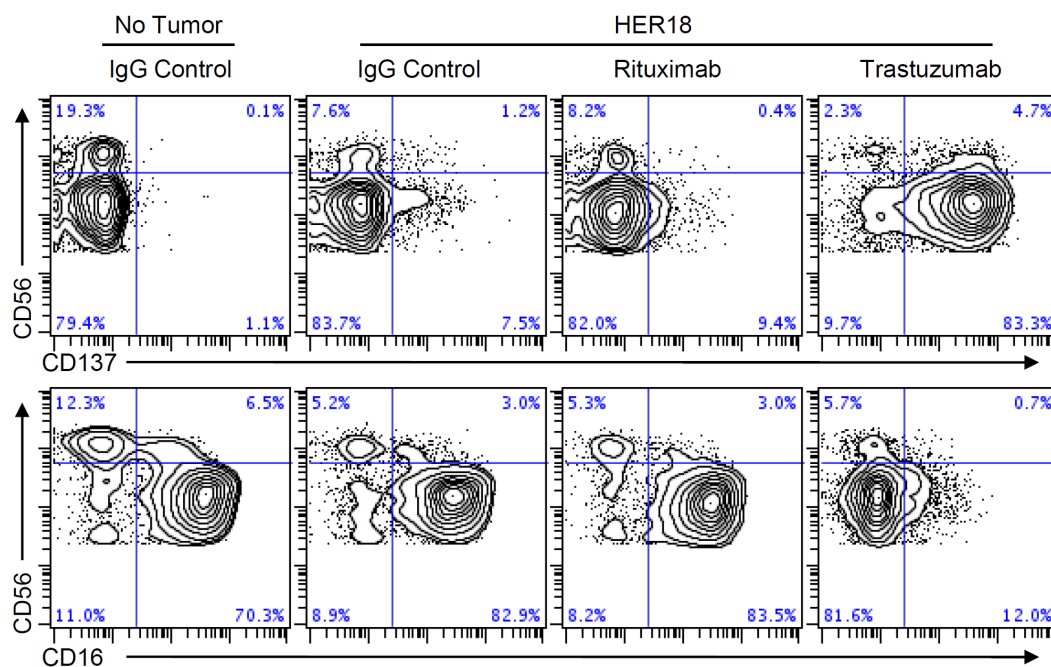


Figure 1

Trastuzumab induces CD137 upregulation on human NK cells following exposure to HER2-overexpressing tumor cells. Purified NK cells from the peripheral blood of three healthy donors were analyzed for CD137 expression on after 24 hour culture with breast cancer cell lines or no tumor and IgG control, rituximab, or trastuzumab. (A) shows the percentage of CD137⁺ cells among CD3⁺CD56⁺ NK cells from three healthy donors cultured with MCF7, BT474M1, HER18, and SKBR3 breast cancer cell lines (**p*=0.046, ***p*<0.001). (B) shows HER2 surface expression on breast cancer cell lines with histograms colored according to the log₁₀-fold increase in MFI of breast cancer cell line relative to isotype. (C) shows CD137 and CD16 expression on NK cell subsets CD3⁺CD56^{high} and CD3⁺CD56^{dim} from a representative healthy donor after 24 hour culture with IgG control alone, HER18 and IgG control, HER18 and rituximab, or HER18 and trastuzumab.

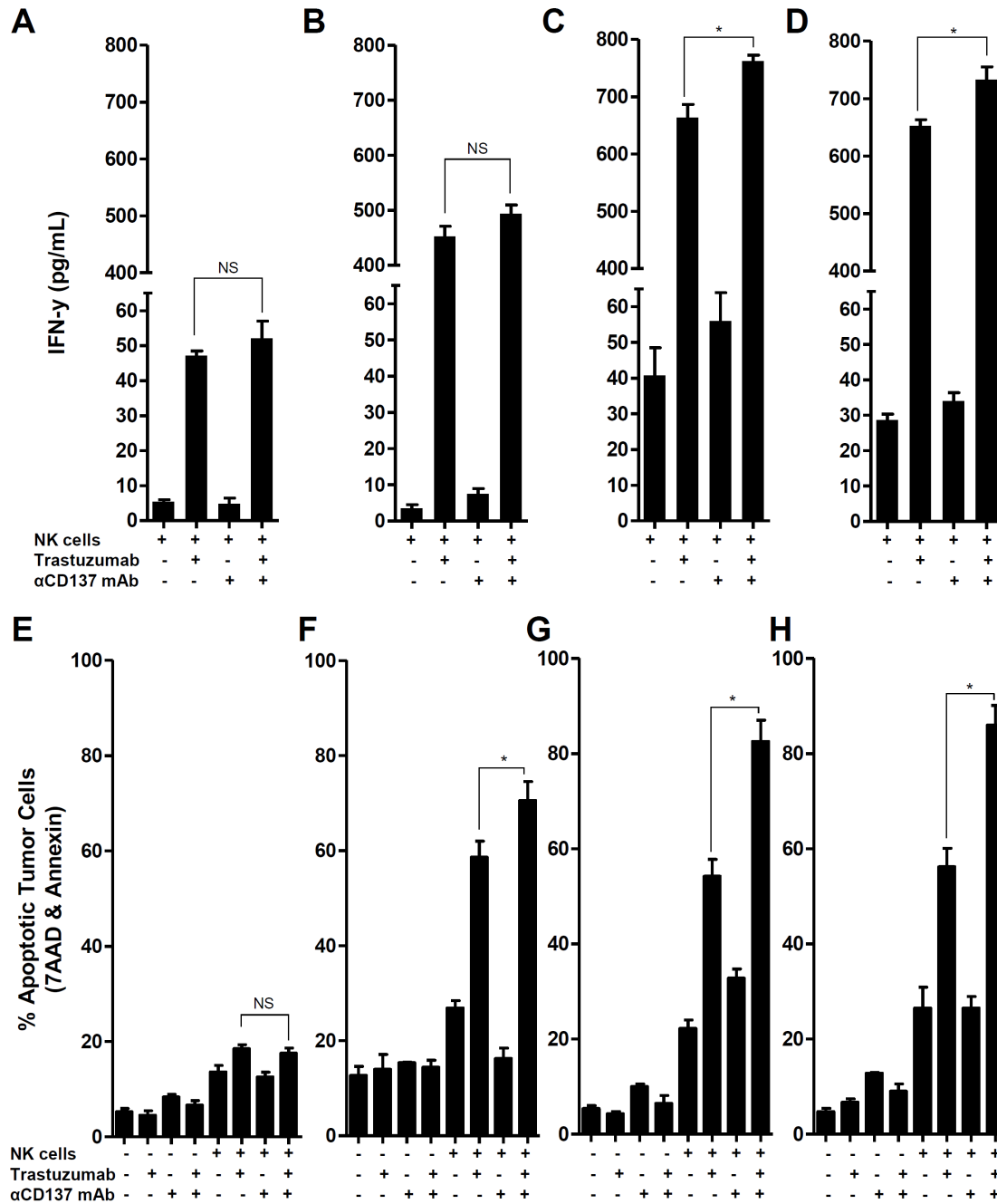


Figure 2

Anti-CD137 agonistic mAb increases trastuzumab-mediated NK cell cytokine secretion and cytotoxicity on tumor cells as assayed by cell viability. To evaluate NK cell function, purified NK cells were isolated from three independent, healthy donor PBMCs and cultured for 24 hours together with trastuzumab (10 $\mu\text{g/mL}$) and irradiated (5,000 rads) breast cancer cells (SKBR3) at a ratio of 1:1. After 24 hours, NK cells were isolated by negative selection and assessed for purity ($>90\%$ purity as defined by $\text{CD}3^+\text{CD}56^+$ flow cytometry) and activation ($>50\%$ expression of CD137). Breast cancer cell lines including MCF7 (A and E), BT474M1 (B and F), HER18 (C and G), and SKBR3 (D and H) were cultured for 18 hours with pre-activated, purified NK cells in media alone, or with anti-CD137 mAb (BMS-663513, 10 $\mu\text{g/mL}$) alone, trastuzumab (10 $\mu\text{g/mL}$) alone, or trastuzumab plus anti-CD137 mAbs (both at 10 $\mu\text{g/mL}$) and supernatant was harvested and analyzed by ELISA for interferon- γ (A, MCF7 $p=.39$; B, BT474M1 $p=.16$; C, HER18 $*p=.017$; D, SKBR3 $*p=.034$). Cells were washed and incubated with annexin V and 7-AAD to determine percent apoptotic tumor cells by annexin V and 7-AAD staining (E, MCF7 $p=.43$; F, BT474M1 $*p=.031$; G, HER18 $*p<.001$; H, SKBR3 $*p<.001$).

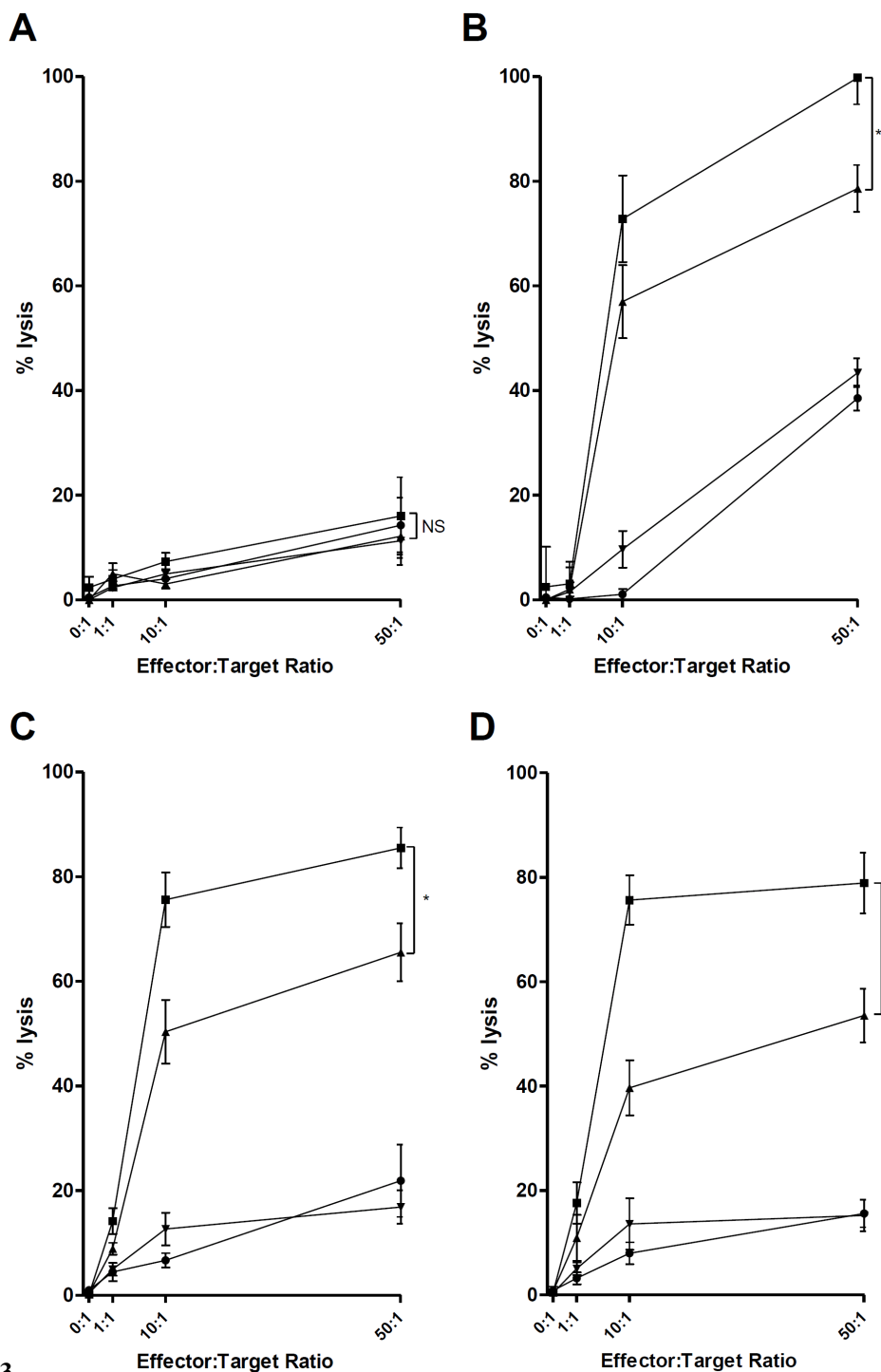


Figure 3

Anti-CD137 agonistic mAb increases trastuzumab-mediated NK cell cytotoxicity on tumor cells as assayed by chromium release. To evaluate NK cell cytolytic function, healthy PBMCs were cultured for 24 hours together with trastuzumab (10 μ g/mL) and irradiated (5,000 rads) breast cancer cells (SKBR3) at a ratio of 1:1. After 24 hours, NK cells were isolated by negative selection and assessed for purity (>90% purity as defined by CD3⁺CD56⁺ flow cytometry) and activation (>50% expression of CD137). Chromium-labeled breast cancer cell lines including MCF7 (A), BT474M1 (B), HER18 (C), and SKBR3 (D) were cultured for 4 hours with preactivated, purified NK cells in media alone, or with anti-CD137 mAb (BMS-663513, 10 μ g/mL) alone, trastuzumab (10 μ g/mL) alone, or trastuzumab plus anti-CD137 mAbs. Shown is percent lysis of target cells by chromium release at varying effector (activated NK cells):target cell ratios cultured with media alone (●), anti-CD137 (▼), trastuzumab (▲), or trastuzumab and anti-CD137 (■) antibodies (A p = .67; B p = .006; C p = .041; D p = .031).

KEY RESERCH ACCOMPLISHMENTS

1- Human HER2-expressing tumor cells coated with trastuzumab induce the expression of CD137 on human NK cells.

2- An agonistic anti-CD137 mAb increases trastuzumab-mediated NK cell cytokine secretion and trastuzumab-dependent NK cell-mediated cytotoxicity.

REPORTABLE OUTCOMES

1. **Kohrt, H**, Houot, R, Weiskopf, K, Goldstein, M, Lund, P, Czerwinski, D, Sunwoo, J, Tedder, T, Chen, L, and R Levy (2012). "Stimulation of natural killer cells with an anti-CD137 antibody enhances the efficacy trastuzumab, cetuximab, and rituximab in HER2-expressing breast cancer, EGFR+ head and neck cancer, and CD20+ lymphoma." Proceedings of the 103rd Annual Meeting of the American Association for Cancer Research.

CONCLUSION

Trastuzumab, a monoclonal antibody (mAb) targeting HER-2/neu, kills tumor cells by several mechanisms, including antibody-dependent cellular cytotoxicity (ADCC). Strategies that enhance the activity of ADCC effectors, including natural killer (NK) cells, may improve trastuzumab's efficacy. NK cells that encounter trastuzumab-coated, HER2-overexpressing breast cancer cells become activated and express CD137, a costimulatory receptor. CD137 activation, which is dependent on the FcγRIII receptor, occurred both in vitro and in the peripheral blood of women with HER2-expressing breast cancer following trastuzumab treatment. Stimulation of trastuzumab-activated NK cells with an agonistic mAb against CD137 killed breast cancer cells more efficiently in vitro.

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