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Inhibitory Fc receptors modulate *in vivo* cytoxicity against tumor targets

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Inhibitory receptors have been proposed to modulate the *in vivo* cytotoxic response against tumor targets for both spontaneous and antibody-dependent pathways[1]. Using a variety of syngenic and xenograft models, we demonstrate here that the inhibitory FcγRIIB molecule is a potent regulator of antibody-dependent cell-mediated cytotoxicity *in vivo*, modulating the activity of FcγRIII on effector cells. Although many mechanisms have been proposed to account for the anti-tumor activities of therapeutic antibodies, including extended half-life, blockade of signaling pathways, activation of apoptosis and effector-cell-mediated cytotoxicity, we show here that engagement of Fcγ receptors on effector cells is a dominant component of the *in vivo* activity of antibodies against tumors. Mouse monoclonal antibodies, as well as the humanized, clinically effective therapeutic agents trastuzumab (Herceptin®) and rituximab (Rituxan®), engaged both activation (FcγRIII) and inhibitory (FcγRIIB) antibody receptors on myeloid cells, thus modulating their cytotoxic potential. Mice deficient in FcγRIIB showed much more antibody-dependent cell-mediated cytotoxicity; in contrast, mice deficient in activating Fc receptors as well as antibodies engineered to disrupt Fc binding to those receptors were unable to arrest tumor growth *in vivo*. These results demonstrate that Fc-receptor-dependent mechanisms contribute substantially to the action of cytotoxic antibodies against tumors and indicate that an optimal antibody against tumors would bind preferentially to activation Fc receptors and minimally to the inhibitory partner FcγRIIB.

Passive and active protection against pulmonary metastasis in the syngenic B16 melanoma model has been demonstrated to require the presence of activation Fc receptors[2] on effector cells, such as natural killer (NK) cells. To determine whether the inhibitory molecule FcγRIIB (Genome DataBase designation, Fcgr2b) is a factor in determining the *in vivo* anti-tumor activity of monoclonal antibody TA99 (ref. 2), a protective immunoglobulin (Ig)G2a antibody specific for the melanoma differentiation antigen gp75, we crossed C57Bl/6 mice to an FcγRIIB-deficient strain and then back-crossed to establish a syngenic strain. Metastases of B16 melanoma cells in the FcγRIIB-deficient background were identical to those in wild-type mice (Fig. 1), demonstrating that the inhibitory receptor was not involved in tumor growth or spread. In contrast, when FcγRIIB-deficient mice received the protective IgG2a antibody, there was much more activity of this antibody than in mice wild-type for FcγRIIB (Fig. 1). Quantification of the tumor nodules in excised lungs showed that wild-type, treated mice reduced tumor load by three-fold (300 ± 30 compared with 100 ± 10) whereas antibody treatment of $Fc\gamma RIIB^{-/-}$ mice resulted in a 100-fold reduction (300 compared to 3). As shown before[2], deletion of the activation γ subunit eliminated the *in vivo* protective effect of this antibody (Fig. 1). NK cells, a principal cell type involved in antibody-dependent cell-mediated cytotoxicity (ADCC), express the activation Fcγ receptor, FcγRIII (Genome DataBase designation, Fcgr3), but do not express the inhibitory counterpart, FcγRIIB. Thus, the increase seen in FcγRIIB-deficient mice cannot be attributed to NK cell hyper-responsiveness. Instead, monocytes and macrophages, which express both FcγRIII and FcγRIIB, may therefore function as the dominant effector cell in this antibody-dependent protection *in vivo*. Thus the activity attributed to the protective IgG2a antibody in a wild-type animal represents the sum of the opposing activation and inhibitory pathways contributed by NK cells, monocytes and macrophages.

To determine the generality of this pathway of antibody-mediated cytotoxicity mediated by FcγRIIB, we investigated other well-defined tumor models for which therapeutic antibodies against tumors have been developed. Antibodies against the HER2/neu growth factor receptor prevent the growth of breast carcinoma cells *in vitro* and *in vivo*[3]. Similarly, antibodies against the CD20 antigen on B cells arrest the growth of non-Hodgkin's lymphoma[4]. These antibodies were developed based on their ability to interfere with tumor cell growth *in vitro* and are representative of a class that includes those with specificities for the epidermal growth factor receptor[5], interleukin-2 receptor[6] and others[7]. Trastuzumab (Herceptin®), a humanized IgG1 antibody specific for the cellular proto-oncogene p185HER-2/neu (refs. 8,9), and rituximab (Rituxan®), the chimeric monoclonal IgG1 antibody specific for the B-cell marker CD20 (ref. 10), were recently approved for the treatment of HER-2 positive breast cancer and B-cell lymphoma, respectively. Some *in vitro* studies have indicated that the essential mechanisms responsible for the anti-tumor activities of trastuzumab and its mouse 'parent' IgG1 antibody against HER2, 4D5, are due to receptor–ligand blockade[11,12]; others have indicated that factors such as ADCC may be important[9,12]. *In vitro* studies with rituximab and its mouse 'parent' antibody 2B8 have indicated a direct pro-apoptotic activity may be associated with this antibody[13].

To determine the contribution of interactions between the Fc domain and effector cell FcγRs to the *in vivo* activities of trastuzumab and rituximab, we modified the orthotopic athymic nude mouse tumor model to generate a suitable model to address the role of FcγRIIB and FcγRIII in the anti-tumor response. Mice deficient in the common γ chain ($FcR\gamma^{-/-}$) (14), lacking the activation Fcγ receptors FcγRI and FcγRIII, and mice deficient in FcγRIIB (ref. 15) were each mated with athymic nude mice (nu/nu) to generate $FcR\gamma^{-/-}/nu/nu$ and $Fc\gamma RIIB^{-/-}/nu/nu$ mice for

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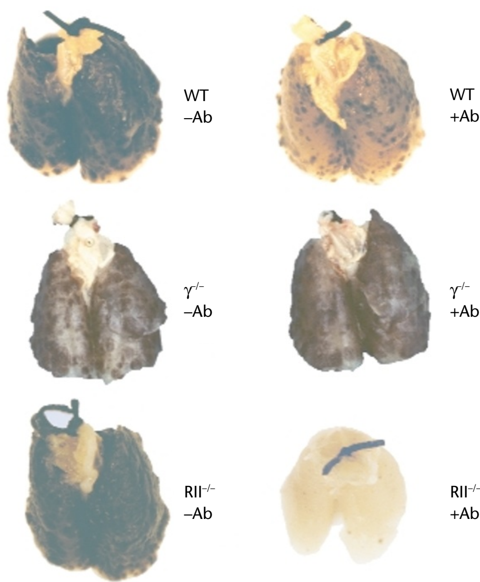

Fig. 1 Passive protection from pulmonary metastasis is increased considerably in FcγRIIB-deficient mice. Mice were injected intravenously with B16 melanoma cells on day 0 and with antibody TA99 on days 0, 2, 4, 7, 9 and 11. Lungs were collected on day 14 WT, wild-type; -Ab, without antibody; +Ab, with antibody; $\gamma^{-/-}$, *FcRγ*$^{-/-}$; RII$^{-/-}$, *FcγRIIB*$^{-/-}$.

use in xenograft human tumor models. We then studied the anti-tumor activity of trastuzumab and 4D5 in preventing the growth of the human breast carcinoma BT474M1, which overexpresses p185/HER-2/neu, in *FcRγ*$^{-/-}$ and *FcRγ*$^{+/+}$ athymic nude mice (Fig. 2*a*–*d*). Tumor growth, measured as volume, was identical in homozygous *FcRγ*$^{-/-}$/*nu*/*nu* and *FcRγ*$^{+/+}$/*nu*/*nu* mice injected subcutaneously with 5×10^6 BT474M1 cells. In *FcRγ*$^{+/+}$ mice, a single intravenous dose of 4 µg/g antibody, followed by weekly intravenous injections of 2 µg/g antibody, resulted in near-complete inhibition of tumor growth (tumor mass reductions of 90 and 96% in mice treated with 4D5 and trastuzumab, respectively) with only 4 of 17 mice developing palpable tumors. However, this protective effect of trastuzumab and 4D5 was reduced in *FcγR*$^{-/-}$ mice. Tumor mass in antibody-treated *FcRγ*$^{-/-}$ mice was reduced by 29 and 44%, respectively, by trastuzumab and 4D5, and 14 of 15 mice developed palpable tumors. We obtained similar results with the *FcRγ*$^{-/-}$/*nu*/*nu* xenograft model for the mechanism by which rituximab inhibits B-cell lymphoma growth *in vivo*. Tumor growth of the human B-cell lymphoma cell line Raji was indistinguishable in *FcRγ*$^{-/-}$/*nu*/*nu* and *FcRγ*$^{+/+}$/*nu*/*nu* mice (Fig. 2*e* and *f*). However, the protective effect of weekly, intravenous, 10-µg/g doses of rituximab seen in *FcRγ*$^{+/+}$/*nu*/*nu* mice was reduced in *FcRγ*$^{-/-}$/*nu*/*nu* mice. Treatment of wild-type athymic mice with rituximab resulted in reductions of tumor mass of more than 99%, and no wild-type mice developed palpable tumors. In contrast, in *FcRγ*$^{-/-}$ mice, little protection was afforded by rituximab; six of seven mice developed palpable tumors, and tumor mass reductions averaged just 23%.

In contrast, *FcγRIIB*$^{-/-}$ mice were more effective at arresting BT474 growth in this nude mouse model (Fig. 3). At a sub-therapeutic dose of antibody (a 0.4-µg/g loading dose and 0.2 µg/g given weekly), tumor growth in FcγRIIB-deficient mice was arrested, demonstrating the involvement of the inhibitory FcγRIIB pathway in this model as well. Nude mice have increased numbers of NK cells, leading to the presumption that antibody protection in those mice are not representative of the protection seen in syngenic systems, as in human disease. The observation that deletion of FcγRIIB increases protection in nude mice indicates the involvement of effector cells other than NK cells, such as monocytes and macrophages, in the protective response and further indicates that the Fc-receptor-dependent pathways are not restricted to an system biased to NK cells, but, as in the syngenic melanoma system, is likely to be relevant in other syngenic systems as well.

To further demonstrate the involvement of interactions between Fc and Fcγ receptors in the protective response, we engineered a modification of 4D5 to disrupt the ability of the antibody to engage cellular Fcγ receptors while retaining its affinity for its cognate antigen p185 HER-2/neu. We systematically mutated the CH2 and CH3 domains of mouse IgG1 Fc sequence, replacing each amino acid, in turn, with alanine (alanine scanning). We then expressed each mutant antibody thus generated and determined its binding to mouse Fc receptors. Based on this alanine-scanning mutagenesis mapping, a single amino-acid replacement at residue 265 in the C_H2 domain of the mouse IgG1 heavy chain reduced binding of IgG1-containing immune com-

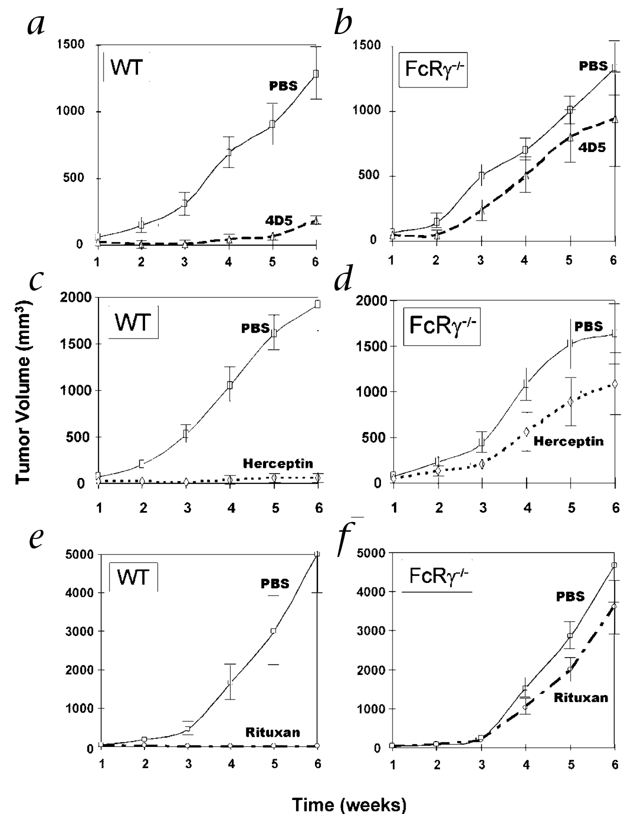

Fig. 2 Anti-tumor activities of 4D5, trastuzumab and rituximab require activation Fcγ receptors. Nude mice (n = 6–10 per group) were injected with BT474M1 cells (*a*–*d*) or Raji B cells (*e* and *f*), followed by weekly injections of 4D5 (***a*** and ***b***), trastuzumab (***c*** and ***d***) or rituximab (***e*** and ***f***). PBS, phosphate buffered saline (control). The antibody-dependent tumor protection seen in BALB/c nude mice (WT; *a*, *c* and *e*) is absent in *FcRγ*$^{-/-}$ nude mice (*b*, *d* and *f*). All experiments were repeated three times with similar results.

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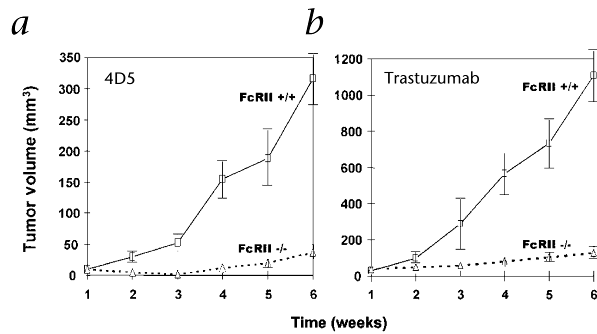

Fig. 3 Anti-breast tumor activity of 4D5 and trastuzumab is enhanced FcγRIIB-deficient mice. Nude mice (n = 8 per group) were injected with BT474M1 cells and treated with a 0.4-μg/g loading dose and 0.2 μg/g weekly (a sub-therapeutic dose for wild-type mice) of 4D5 (***a***) or trastuzumab (***b***). There is complete inhibition of tumors in FcγRIIB-deficient mice (dotted lines) at sub-therapeutic antibody doses.

plexes to both FcγRIIB and FcγRIII in a receptor-coated plate assay (Fig. 4*a*). This residue was located at a site within the Fc portion of the IgG molecule thought to interact directly with surfaces of Fc receptors. We put the mutation of Asp to Ala at residue 265 into the 4D5 IgG1 heavy chain gene and expressed this in parallel with the wild-type 4D5 IgG1 heavy chain in A293 cells along with the 4D5 kappa chain to produce 4D5 and mutant (D265A) antibodies. As the mutation would not be expected to disrupt antibody–antigen interactions, as predicted, both 4D5 and D265A antibodies purified from transfected-cell supernatants bound cellular p185HER-2/neu with equivalent avidity and had similar *in vitro* growth inhibitory activity when added to BT474M1-expressing breast carcinoma cells in tissue culture (Fig. 4*b*). However, although D265A retained the wild-type characteristics of *in vivo* half-life (data not shown), antigenic targeting and functional p185HER-2/neu receptor blockade, the *in vitro* ADCC capacity of the mutant was lost as a consequence of its reduced affinity for FcγRIII on effector cells (Fig. 4*c*). *In vivo*, D265A, when tested in the breast carcinoma BT474M1 xenograft model, had less anti-tumor activity than 4D5 (Fig. 4*d*). Palpable tumors developed in all wild-type athymic mice treated with D265A but developed in only two of five mice treated with 4D5. D265A treatment reduced tumor volumes by 30%, compared with a reduction of 85% with 4D5. The attenuated anti-tumor responses of D265A correlate with its impaired ability to activate Fc-receptor-bearing effector cells despite its ability to inhibit tumor growth *in vitro*, supporting the conclusion that Fc receptor engagement is a substantial contributing component of anti-tumor activity *in vivo*.

Many mechanisms have been proposed for the ability of antibodies against tumors to mediate their effects *in vivo*. The data presented here indicate that Fcγ receptor binding contributes substantially to *in vivo* activity. This Fcγ-recptor dependence seems to apply to more than a single antibody, as it has been seen in both syngenic and xenograft models for the three unrelated tumors and target antigens presented here. Fcγ receptor engagement involves both activation and inhibitory receptors and thus indicates involvement of monocytes and macrophages in the effector cell component of the protective response. Supportive evidence for this interpretation is found in the ability of trastuzumab to mediate ADCC *in vitro* and the ability of antibodies against Fc receptor to inhibit some of the *in vivo* activity of antibodies against CD20 (ref. 16). Although the studies presented here demonstrate the importance of interactions between Fc and Fcγ receptors, triggering the growth and apoptotic regulatory pathways by antibody engagement of p185HER2/neu and CD20 may still contribute to the total *in vivo* efficacy of antibodies against tumors. Support for this interpretation can be seen in the partial protection in $FcR\gamma^{-/-}$ mice treated with antibodies against HER2/neu (Fig. 2), in which the anti-tumor activity of these antibodies against the BT474M1 breast carcinoma cells was reduced but not ablated. Similarly, previous studies showed that the 225 antibody against epidermal growth factor receptor was able to reduce the epithelial tumor cell A431 growth *in vivo* as an F(ab′)$_2$, although with only 50% of the activity shown by the intact antibody[17]. Blocking the signaling on tumor cells by antibodies may also act synergistically with immune effector responses by rendering the tumor cells more susceptible to immune effector cell triggered apoptotic or lytic cell death[18]. Our results thus indicate the importance of selection and engineering of therapeutic antibodies against tumor to maximize their interactions with FcγRIII and minimize their interaction with FcγRIIB, which along with the appropriate antigenic target will potentiate their therapeutic capacity. In addition, these studies emphasize the fundamental

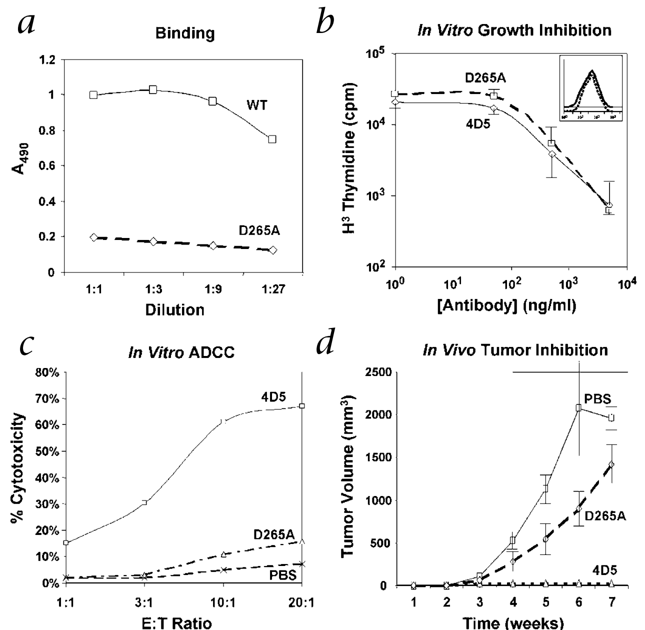

Fig. 4 *In vitro* and *in vivo* properties of the D255A mutant antibody. ***a***, FcγRIII binding. Both wild-type and mutant Fc fragments were grafted onto an anti-human IgE Fab fragment. Solid-phase binding assays used hexameric complexes of human IgE and anti-human IgE and plates coated with recombinant FcγRIIIs. A_{490}, absorbance at 490 nm. ***b***, Growth inhibition of BT474M1 cells. Inset, Fluorescence-activated cell sorting analysis of BT474M1 cells demonstrates equivalent avidities of 4D5 (solid line) and D265A (dotted line) for cell surface p185 HER2/neu. Main graph, ^{3}H-thymidine incorporation of BT474M1 cells, measured in the presence of either 4D5 or D265A. ***c***, NK-cell ADCC of chromium-labeled tumor targets. Chromium-labeled SKBR-3 cells were incubated with NK effector cells (effector:target (E:T) ratios, horizontal axis), and release of label was quantified. ***d***, *In vivo* growth of breast carcinoma cells. Athymic BALB/c nu/nu mice were implanted with BT474M1 xenografts and their growth in response to treatment with 4D5, D265A or PBS was measured.

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importance of the inhibitory pathways *in vivo* and indicate that individual responses to antibodies against tumors may depend on the expression of these inhibitory pathways.

Methods

Melanoma metastasis model. Mice were injected intravenously with 1 × 10^6 B16 melanoma cell on day 0 and with either phosphate-buffered saline (PBS) or 20 µg purified TA99 intraperitoneally on days 0, 2, 4, 7, 9 and 11. In previous experiments[2], a dose of 200 µg of monoclonal antibody TA99 induced a reduction of more than 90% in tumor metastasis in wild-type but not $FcR\gamma^{-/-}$ mice. However, at this lower dose of TA99 (20 µg), only limited protection was provided against tumor metastasis in wild-type mice. Mice were killed on day 14 and surface lung metastasis were counted under a dissecting microscope.

Tumor xenograft models. For breast carcinoma xenograft experiments, 5 × 10^6 BT474MI cells (BT474 subclone derived at Genentech, South San Francisco, California) were injected subcutaneously on day 1 in 0.1 ml PBS mixed with 0.1 ml Matrigel (Collaborative Research, Bedford, Massachusetts). BALB/c nude mice, $FcR\gamma^{-/-}$ BALB/c nude mice or $Fc\gamma RII^{-/-}$ BALB/c nude mice 2–4 months old were injected subcutaneously with 17β-estradiol 60-day release pellets (0.75 mg/pellet; Innovative Research of America, Sarasota, Florida) 24 h before tumor cell injection. Therapeutic antibodies (obtained from clinical material, in vials; Genentech, South San Francisco, California) were injected intravenously beginning on day 1 at a loading dose of 4 µg/mg, with weekly injections of 2 µg/mg for BALB/c nude and $FcR\gamma^{-/-}$ BALB/c nude. A dose 10% of this (0.4 µg/mg, loading; 0.2 µg/mg, weekly) was used for the experiments in Fig. 3. For B-cell lymphoma xenograft experiments, BALB/c nude mice or $FcR\gamma^{-/-}$ BALB/c nude mice 2–4 months old were irradiated with 3.0 cGy before subcutaneous injection of 5 × 10^6 Raji B-lymphoma cells. Rituximab (Rituxan®; IDEC Phamaceuticals, San Diego, California) was given at a dose of 10 µg/g weekly. Tumor measurements were obtained weekly.

Engineering of D254A mutant antibody and binding assays. Site-directed mutagenesis was accomplished using the QuikChange Mutagenesis Kit (Stratagene, La Jolla, California). Mutant antibody was transiently expressed in A293 cells in the pRK expression vector, and conditioned supernatants were collected and purified by protein G affinity column chromatography. The ability of various mutants to bind recombinant FcγRs was measured using an *in vitro* binding assay[19]. Microtiter plates were coated with 100 ng/well of a fusion protein of recombinant FcγRIII and glutathione S-transferase in PBS. Plates were washed with PBS supplemented with 0.05% Tween-20 (wash buffer) then blocked for 1 h at room temperature with 0.5% BSA, 50 mM Tris-buffered saline, 0.05% Tween 20, 2mM EDTA, pH 8.0 (ELISA buffer). The IgG1 Fc fragment of murine 4D5 as well as D265A was grafted onto the Fab of anti-human IgE (monoclonal antibody E27) and recombinant antibody was produced as described above. The addition of human IgE to E27 with wild-type or mutant Fc domains in a molar ratio of 1:1 in ELISA buffer led to the formation of homogeneous hexameric complexes. Complexes were added to the plates, washed five times in wash buffer, and were detected by the addition of goat $F(ab')_2$ antibody against mouse IgG, with subsequent colorimetric development.

Growth inhibition assays. BT474MI cells were plated at a density of 1 × 10^4 cells per well and allowed to adhere for 24 h. Antibody was added for 48 h, followed by a 14-hour pulse with ^{3}H-thymidine. Cells were collected onto filter mats and incorporated radioactivity was counted in a Wallac Microbeta scintillation counter. BT474M1 cells were incubated with 4D5 or D265A, and stained with FITC-conjugated goat antibody against mouse IgG. Fluorescence intensity was measured on a FACScan flow cytometer (Becton-Dickinson, San Jose, California).

***In vitro* ADCC assay**. Adherent NK effector cells were obtained from interleukin-2-stimulated (250 U/ml; Sigma), 14-day cultures of splenocytes nonadherent to nylon-wool. Four-hour ADCC reactions used as target cells 5 × 10^4 chromium-labeled, HER2-overexpressing, SK-BR3 breast carcinoma cells (American Type Culture Collection, Rockville, Maryland) in 96-well plates in the presence or absence of 10 µg/ml antibody. Percent cytotoxicity is expressed as [counts in supernatant–spontaneous release (without effectors)]/[total counts incorporated–spontaneous release]. Data are expressed as the mean of three replicate wells..

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