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13. ABSTRACT *(Maximum 200 Words)*

Many mechanisms have been proposed to account for the anti-tumor activities of therapeutic antibodies, blockade of signaling pathways, activation of apoptosis and effector-cell-mediated cytotoxicity (ADCC). However we have demonstrated (**Clynes RA,** et al., *Nature Medicine* 6:443-446 (2000)) that engagement of Fcgamma receptors on effector cells is a dominant component of the *in vivo* activity of antibodies against tumors. Engagement of activating Fc receptors (FcRI and/or III) was required for the *in vivo* activity of mouse monoclonal antibodies and vaccines in syngenic melanoma models, as well as of humanized, clinically effective therapeutic mAbs Herceptin and Rituxan in breast cancer and lymphoma xenograft system). Mice deficient in the inhibitory receptor FcgammaRIIB showed much more ADCC; while 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*. Unpublished work from our lab suggests that FcRIII is dispensable for in vivo efficacy of antitumor antibodies suggesting that NK-mediated ADCC is not a requirement. Ongoing studies with FcR deficient mice reconstituted to express FcRs in restricted lineages such as monocytes or NK cells will further clarify the importance of singular FcR bearing effector cell subsets to antitumor antibody efficacy.

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INTRODUCTION

We have completed year three of this funded project on track, having demonstrated in year 1 that monoclonal antitumor antibodies require FcR engagement for full activity in vivo. These findings were published in Nature Medicine. In year two we have generated novel transgenic mice that express Fc receptors in a lineage specific manner. These mice express activating Fc receptors in only myeloid cells or only NK cells. These mice will be invaluable to the investigation of the singular importance of individual Fc receptor- bearing cellular subsets to ADCC in vivo. We continue our mouse breeding and transgenic mouse production to generate genotypically unique mice that can be tested for involvement of specific FcR bearing cell types and specific apoptotic pathways in ADCC in vivo. We have tested these two transgenic lines for antitumor activity in vivo and both lines were unable to offer substantial protection. Further work using cytokine-treated mice may demonstrate that enhances numbers and activity of either NK cells or macrophages may provide protection. In addition, mating of the two lines will produce a third strain that expresses FcRs in both monocyte and NK compartments and will be provide a suitable model to test whether these two lines in combination generate ADCC capacity in vivo.

Statement of Work: Final Report
Cytotoxic Mechanism of Tumor-Specific Antibodies:

1. **Characterization of the role of the cellular receptors for IgG (FcγRI, II and III) and complement in mediating tumor responses induced by anti-melanoma, lymphoma and breast carcinoma monoclonal antibodies.**

a) Analysis of anti-tumor responses in γ -/-, FcγRII -/-, FcγRIII -/- and C3 -/- athymic nude mice using Herceptin, Rituxan and anti-gp75 mAbs in breast cancer, lymphoma and melanoma models (months 1 to 6, 200 mice).

Progress Year 1: In work published in *Nature Medicine* (please see attached manuscript for details) we have determined that the activating Fc receptors (I and III) are required for the in vivo activity of antitumor antibodies. In addition the inhibitory receptor, FcRII, was found to modulate the potency of these antibodies, including the anti-breast cancer antibody, Herceptin. At 10% of the dose that was effective in wild-type mice, FcRII -/- mice were completely protected from tumor growth. The implications from these studies are profound and have provided the catalyst for major efforts in industry to generate anti-tumor antibodies which p[referentially recruit activating Fc receptors at the expense of the inhibitory Fc receptors.

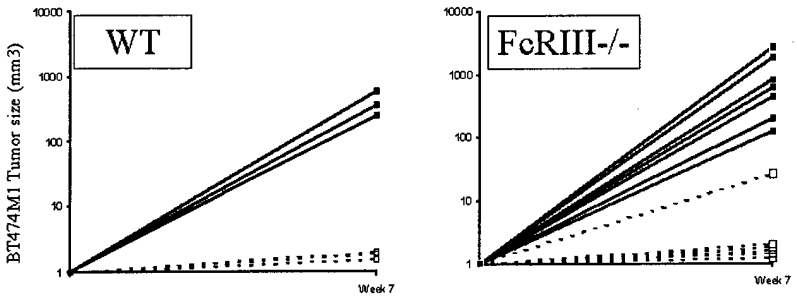
Progress Years 2 and 3: We have bred the FcRIII -/- mice with athymic nu/nu mice to determine which activating Fc receptor (Type I or III) is required for ADCC in vivo. Our results below (Figure 1) shows that FcRIII is not required for Herceptin-mediated antitumor efficacy using

BT474M1 xenografts. These mice still retain functional expression of the remaining activating Fc receptor, FcRI. Given our previous published findings that anti-tumor antibody protection is absent in FcR γ-/- mice which lack both activating Fc receptors FcRI and FcRIII, this new result with FcRIII-/- mice suggests that FcRIII is not necessary and FcRI is sufficient for ADCC in vivo. It however does not dismiss the possibility of redundant pathways in which either FcRI or III is sufficient. A dispensable role for FcRIII was confirmed in another model of antibody-mediated tumor immunity, namely the syngeneic melanoma model and the antibody TA99, an IgG2a specific for TRP-1. In control WT mice complete protection was seen in WT but not in FcRγ-/- mice which lack both FcRI and FcRIII. Mice deficient in FcRIII alone were completely protected by TA99. These provocative results suggest that NK-mediated ADCC is also not necessary for antitumor antibody immunity since FcRIIII is the sole FcR expressed on this cell type.

Figure 1

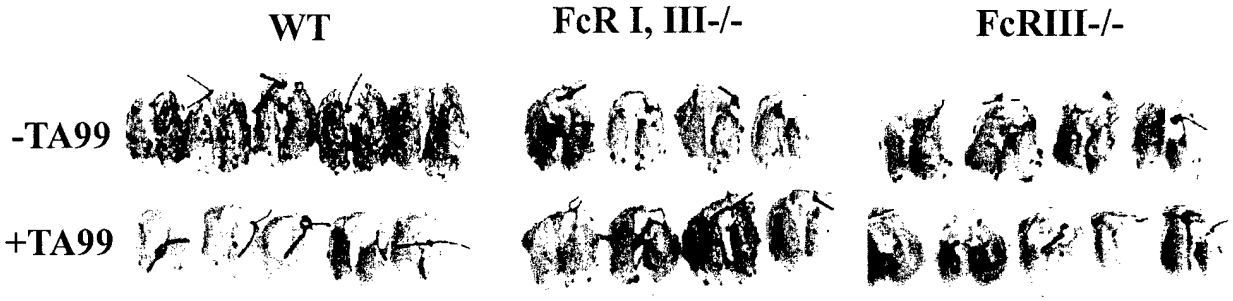
FcRIII is Not Required for Anti-HER2 Mab Tumor Immunity

BT474M11 Breast Cancer Tumor Sizes



--- + Herceptin
___No Herceptin

Figure 2: FcRIII is Dispensable for ADCC in Vivo: Melanoma Metastases are Effectively Treated with mAb TA99 in WT and FcRIII-/- but not in FcRI, III-/-



2. Identification of FcγR-bearing effector cells responsible for ADCC in vivo.

a)Reconstitution of ADCC phenotype by cell transfer of γ -/- with wild-type bone marrow, macrophages and NK cells (months 1 to 6, 120 mice).

b)Reconstitution of ADCC of γ -/- by tissue specific transgenes.

- Engineering of NK cell and Macrophage specific γ expressing plasmids (months 1 to 3)
- Demonstration of tissue specific reconstitution in transient and stable γ -/- transfectants (months 3 to 5).
- Generation of transgenic mice bearing tissue-specifc transgenes (months 5-12).
- Confirmation of tissue specific expression in transgenic mice (months 12-14)
- Breeding and analysis of TA99 anti-tumor antibody responses in transgenic mice (months 12-18, 40 mice)
- Generation of γ –/– nu/nu mice bearing γ transgenes by two rounds of mating with founder lines (months 12- 18)
- Breeding and analysis of Harceptin and Rituxan anti-tumor responses in γ –/– nu/nu mice bearing γ transgenes (months 18 to 36, 80 mice)
- Generation of transgenic mice bearing γ/humanFcγRIIIA transgenes and analysis in xenograft nu/nu models (months 24 to 48, 80 mice)

We have concentrated on genetic reconstitution as a first priority. We have successfully generated expression constructs which target lineage specific expression to NK cells and myeloid cells. These constructs have been injected into embryos and transgenic founder lines generated. Transgenic mice have been generated which harbor the FcRγ gene driven by either a granzyme promoter (NK cell specific) or the CD11b promoter (myeloid cell specific).

Identification of Cell Type and Receptor Class Responsible for ADCC in Vivo

Lineage Specific Reconstitution of FcR γ -/- Mice

Transgenic Construct	Lineage	FcR Expression
CD11b promoter | γ chain	Monocytes	mFcRI,III
Granzyme promoter | γ chain	NK cells	mFcRIII

Three founder Tg+ lines of each construct have been screened for lineage-specific expression. One of three lines of each transgenic construct exhibited no evidence of functional expression of FcRIII by flow cytometry and functional assays while the other two lines were positive expressors and were further screened. Functional analysis of the higher expressor of the remaining two lines is shown in Figure 3 for granzyme-γ and Figure 4 for CD11b-γ.

Granzyme promoter	γ chain

NK Cell Mediated ADCC is
Reconstituted in Granzyme-γ Transgenic

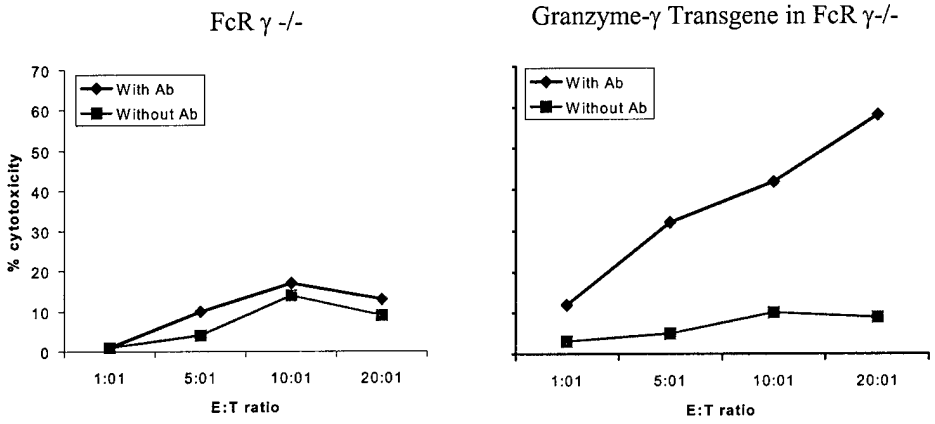


Figure 3: NK cells cultured from 10 day cultures of IL-2 stimulated nylon-wool non-adherent splenocytes were incubated with Chromium labeled TNP-derivitized EL-4 cells in the presence or absence of anti-TNP IgG2b antibodies for 4 hrs and % cytotoxicity determined.

Granzyme-γ NK cells were found to express FcRIII on 15% of IL-2 stimulated NK cells by flow cytometry, whereas FcRγ-/- NK cells were devoid of FcRIII expression and WT NK cells exhibited 90% expression (data not shown). However, despite this incomplete reconstitution of FcR expression in the NK population these cells were as good or better at mediating ADCC *in vitro* as WT NK cells (Figure 2 and data not shown). Lineage restricted expression in granzyme-γ transgenic mice has been confirmed by analysis of monocyte, neutrophil and B lymphocyte populations which do not express γ-chain by Western analysis (data not shown). Thus granzyme-γ transgenic mice are expected to provide an invaluable reagent to explore the consequences of NK-mediated ADCC *in vivo*, in particular antibody-mediated tumor immunity. They are successfully breeding and will be challenged with B16 melanoma +/- the anti-melanoma antibody TA99. The ability of TA99 to prevent lung metatstases in this model will define the unique contribution of NK cell FcR-mediated ADCC *in vivo*. Flow cytometric analysis of isolated NK cell populations demonstrates that although FcRIII is reexpressed on activated IL-2 stimulated NK cells, resting NK cells in the blood and spleen had no detectable expression of FcRIII. We have examined TA99-mediated protection in a small number of animals and no protection was seen (data not shown). Our future studies will include systemic treatment with IL-2 to enhance the number and cellular cytotoxic function of NK cells and to induce the expression of FcRIII on NK cell populations.

CD11b promoter	γ chain

CD11b-γ Transgene Expression Restores Antibody-Mediated Phagocytosis in γ -/-Macrophages

Figure 4: Thioglycollat-elicited macrophages were incubated with IgG-coated SRBCs for 30 minutes and uningested RBCs lysed by osmotic shock. CD11b-γ macrophages (left) but not FcR γ -/- (right) macrophages exhibited WT levels of phagocytosis.

Analysis of CD11b-γ transgenic mice revealed that FcR-mediated phagocytosis had been reconstituted in FcRγ-/- macrophages but not in granzyme-γ tg animals. In contrast NK cells from CD11b-γ transgenic mice did not express FcRIII and were unable to perform ADCC demonstrating that this myeloid-specific promoter was not being expressed in NK cells (data not shown). Western analysis using rabbit-anti γ IgG revealed WT levels of gamma expression in CD11b-γ macrophages but no detectable expression in lymphoid cells. Thus these mice will be useful to address the specific contribution of FcR-mediated macrophage activation to antitumor antibody efficacy *in vivo*. These mice have been tested for the contribution of macrophages in antitumor antibody mediated prevention of B16 melanoma lung metastases. There was little protection afforded by re-expression of activating Fc receptors in the Mac-1+ lineage. Future experiments will include systemic treatment with pro-inflammatory cytokines expected to enhance macrophage cytotoxic function including IFN-γ and GM-CSF.

Figure 5: Monocyte reconstitution of activating FcRs fails to protect against melanoma

3. Dependence of antibody-mediated cytotoxicity on Fas-mediated target cell apoptosis.

- Analysis of Herceptin and Rituxan anti-tumor responses in athymic nude gld/gld and perforin-deficient mice (months 1 to 12, 80 mice)
- Construction of fas and anti-fas expression constructs (months 1 to 3)
- Selection of fas and anti-fas stable transfectants using B16F10, Daudi and BT474M1 cell lines (months 4 to 8)
- Tumor susceptibility studies of fas and anti-fas stable transfectants with Herceptin, Rituxan and TA99 antitumor antibodies (months 8 to 20, 120 mice)
- Analysis of fas up-regulation BT474M1 breast carcinoma by cytotoxic agents and radiotherapy (months 1 to 6)
- Established tumor response studies with combined therapy: Herceptin and cytotoxic agents (months 6 to 24, 100 mice).

We have had breeding problems with groups of gld/gld nu/nu mice to test the role of Fas in mediating the anti-tumor effects of Herceptin. Indeed our entire nude mouse colony with deficiencies in C3, FcRIII, FcRII and fasL (gld) were sacrificed due to MHV infection.

We have failed to generate stable cell lines expressing fas and anti-fas genes in all lines tested implying these constructs were toxic. Therefore we have approached this issue through alternative methods and have generated stable transfectants with the anti-apoptotic gene c-FLIP. We are currently screening clones for expression.
In the interim since this grant was written it has been established that chemotherapeutic agent and antitumor antibodies can act synergistically in vivo although the mechanisms are still unclear.

Research Accomplishments:

• We established a general requirement for FcR activation for the in vivo activity of antitumor antibodies including the clinical therapeutic mAbs, Herceptin and Rituxan.
• We established that the inhibitory receptor FcRII dramatically reduces the in vivo acitivity of antitumor antibodies including the Herceptin and Rituxan.
• We have determined that FcRIII engagement is not required for the anti-tumor activity of Herceptin and anti-melanoma antibodies suggesting that NK cells are not involved and that FcRI expressed on myeloid cells may be sufficient to induce fully effective ADCC in vivo.
• We have generated transgenic mice that express activating Fc receptors ONLY in NK cells or myeloid cells. Tissue specific expression and functional reconstitution has been confirmed; NK cell FcR-mediated ADCC in NK-γ tg+ and FcR-mediated macrophage phagocytosis in Mac-γ tg+ mice. These mice have so far demonstrated that neither lineage alone is sufficient for antibody-mediated tumor protection. Further studies with systemic cytokines chosen to enhance the activity and number of the specific effectors involved will be initiated. Further the mice will be mated to each other two generate a strain with activating FcR expression in both myeloid and NK cells.

Reportable Outcomes: Year 1/Year 2

Publications:

Year 1Clynes RA, Towers TL, Presta LG, Ravetch JV, Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nature Medicine* 6:443-446 (2000).
Year 3 Rafiq, K., Bergtold, A., and **R. Clynes,** Immune complex-mediated antigen presentation induces tumor immunity, *JCI* 110:1 (2002)
J. Trcka, Y. Moroi, **R. Clynes**, S. Goldberg, A. Bergtold, M-A Perales, M. Ma, C. Ferrone, M. C. Carroll, J. V. Ravetch, and A. N. Houghton Redundant and Alternative Roles for Activating Fc Receptors and Complement in an Antibody-Dependent Model of Autoimmune Vitiligo *Immunity* 16 861-868 (2002)

Grants/Awards Received

Year 1 Cancer Research Institute Investigator Award 2000
Year 2 Charles Carrington Award in Biomedical Research 2000
Year 2 Kimmel Cancer Investigator Award 2001
Year 2 Speaker Biomedical Research Award 2001
Year 2 R01 NCI CA94037-01 FcR Enhancement of Antigen Presentation;
Implications for Vaccine Development:
Year 2 P01AI50514-01 Project leader (project 3)
"Fc Receptors in Asthma Pathogenesis".

Invited Meetings

Year 1 Invited Speaker Keystone Symposia 2000 (Cellular Immunity and Immunotherapy of Cancer)
Year 2 Stanford University Department of Pathology Invited Lecturer (Charles Carrington Award Recipient)
Year 3 2002 1st Annual Symposium on Anti-Receptor Signaling Human Neoplasia
2002 International Congress on Antitumor Antibodies
2002 Keystone Symposia: Antibody-Based Therapeutics for Cancer

Conclusions

We are pleased that our work has been recognized in Nature Medicine (please see commentary in attached appendix) for its significance in providing a unifying general mechanism for antitumor antibodies. We are excited to pursue this mechanism in further detail beginning with the identification of the required cellular effectors responsible for Fc receptor mediated cellular cytotoxicity in vivo.

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 *FcγRIIB*$^{-/-}$ 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[9,13]. *In vitro* studies with rituximab and its mouse 'parent' antibody 2B8 have indicated a direct pro-apoptotic activity may be associated with this antibody[14].

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 (*FcRγ*$^{-/-}$) (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 *FcRγ*$^{-/-}$/*nu*/*nu* and *FcγRIIB*$^{-/-}$/*nu*/*nu* mice for

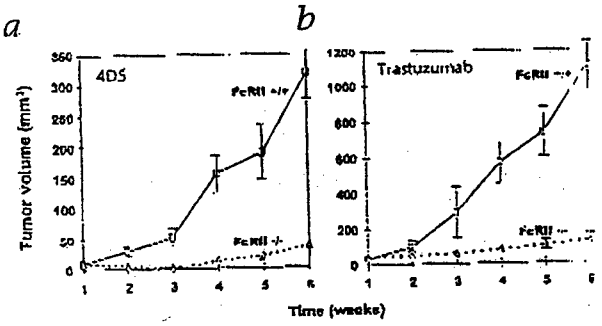

Fig. 3 Anti-breast tumor activity of 4D5 and trastuzumab is enhanced FcγRIIB-deficient mice. Nude mice (n = 8 per group) were injected with BT474M1 cells and treated with a 0.4-μg/g loading dose and 0.2 μg/g weekly (a sub-therapeutic dose for wild-type mice) of 4D5 (*a*) or trastuzumab (*b*). There is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

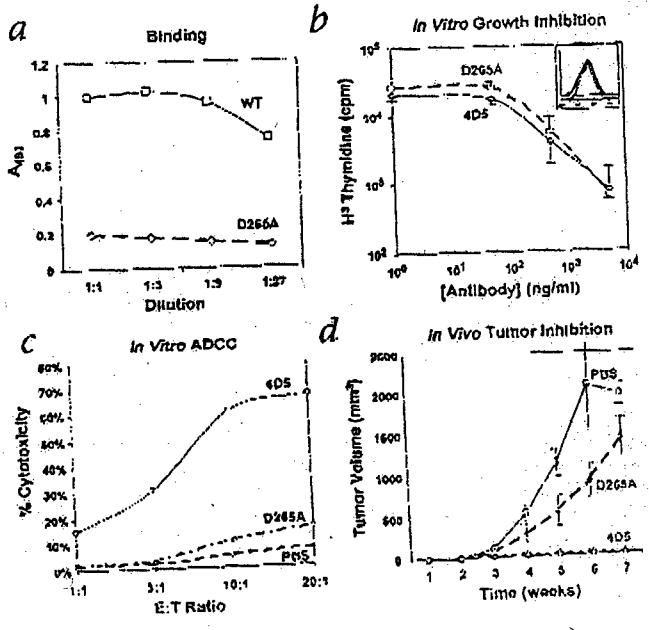

Fig. 4 *In vitro* and *in vivo* properties of the D255A mutant antibody. *a*, FcγRIII binding. Both wild-type and mutant Fc fragments were grafted onto an anti-human IgE Fab fragment. Solid-phase binding assays used hexameric complexes of human IgE and anti-human IgE and plates coated with recombinant 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 NIH/B/c nu/nu mice were implanted with BT474M1 xenografts and their growth in response to treatment with 4D5, D265A or PBS was measured

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 *FcRγ*$^{-/-}$ 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, *FcRγ*$^{+/+}$ BALB/c nude mice or *FcγRII*$^{-/-}$ 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 *FcRγ*$^{-/-}$ 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 *FcRγ*$^{-/-}$ 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 Pharmaceuticals, 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 non-adherent to nylon-wool. Four-hour ADCC reactions used as target cells 5 × 10^6 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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