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Lydie Cassard, ... , Wolf H. Fridman, Catherine Sautès-Fridman

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Article

Development

The efficacy of anti-tumor IgG reflects the balance between opposing signals mediated by activating and inhibitory Fcγ receptors (FcγRs) expressed by effector cells. Here, we show that human malignant melanoma cells express the inhibitory low-affinity Fcγ receptor FcγRIIB1 in 40% of tested metastases. When melanoma cells were grafted in nude mice, a profound inhibition of FcγRIIB1 tumor growth that required the intracytoplasmic region of the receptor was observed. IgG immune complexes (ICs) may be required for this inhibition, since sera from nude mice bearing tumors contained IgG that decreased the proliferation of FcγRIIB1-positive cells in vitro, and tumor development of FcγRIIB1-positive melanoma lines was not inhibited in antibody-defective severe combined immunodeficiency (SCID) mice. Passive immunization of SCID mice with anti-ganglioside G<sub>D2</sub> antibody resulted in significant inhibition of growth of FcγRIIB1-positive tumors in an intracytoplasmic-dependent manner. Altogether, these data suggest that human melanoma cells express biologically active inhibitory FcγRIIB1, which regulates their development upon direct interaction with anti-tumor antibodies. Therefore, FcγR expression on human tumors may be one component of the efficacy of antibody-mediated therapies, and FcγR-positive tumors could be the most sensitive candidates for such treatments.

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# Modulation of tumor growth by inhibitory Fc $\gamma$ receptor expressed by human melanoma cells

Lydie Cassard,<sup>1</sup> Joël F.G. Cohen-Solal,<sup>1</sup> Annie Galinha,<sup>1</sup> Xavier Sastre-Garau,<sup>2</sup> Claire Mathiot,<sup>3</sup> Jérôme Galon,<sup>1</sup> Thierry Dorval,<sup>4</sup> Alain Bernheim,<sup>5</sup> Wolf H. Fridman,<sup>1</sup> and Catherine Sautès-Fridman<sup>1</sup>

<sup>1</sup>Unité d'Immunologie Cellulaire et Clinique, Institut National de la Santé et de la Recherche Médicale (INSERM) U 255 and Université Pierre et Marie Curie Paris VI, Centre de Recherches Biomédicales des Cordeliers, Paris, France

<sup>2</sup>Département de Anatomopathologie,

<sup>3</sup>Laboratoire d'Hématologie, and

<sup>4</sup>Service de Médecine Oncologique, Institut Curie, Paris, France

<sup>5</sup>Centre National de la Recherche Scientifique Unité Mixte de Recherche 1599, Laboratoire de Cytogénétique, Institut Gustave Roussy, Villejuif, France

The efficacy of anti-tumor IgG reflects the balance between opposing signals mediated by activating and inhibitory Fc $\gamma$  receptors (Fc $\gamma$ Rs) expressed by effector cells. Here, we show that human malignant melanoma cells express the inhibitory low-affinity Fc $\gamma$  receptor Fc $\gamma$ RIIB1 in 40% of tested metastases. When melanoma cells were grafted in nude mice, a profound inhibition of Fc $\gamma$ RIIB1 tumor growth that required the intracytoplasmic region of the receptor was observed. IgG immune complexes (ICs) may be required for this inhibition, since sera from nude mice bearing tumors contained IgG that decreased the proliferation of Fc $\gamma$ RIIB1-positive cells *in vitro*, and tumor development of Fc $\gamma$ RIIB1-positive melanoma lines was not inhibited in antibody-defective severe combined immunodeficiency (SCID) mice. Passive immunization of SCID mice with anti-ganglioside G<sub>D2</sub> antibody resulted in significant inhibition of growth of Fc $\gamma$ RIIB1-positive tumors in an intracytoplasmic-dependent manner. Altogether, these data suggest that human melanoma cells express biologically active inhibitory Fc $\gamma$ RIIB1, which regulates their development upon direct interaction with anti-tumor antibodies. Therefore, Fc $\gamma$ R expression on human tumors may be one component of the efficacy of antibody-mediated therapies, and Fc $\gamma$ R-positive tumors could be the most sensitive candidates for such treatments.

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## Introduction

The Fc $\gamma$  receptors (Fc $\gamma$ Rs) expressed on hematopoietic cells play a key role in immune defenses by linking humoral and cellular immunity (1). Fc $\gamma$ Rs display coordinate and opposing roles in immune responses depending on their cytoplasmic region and/or their associated chains. Indeed, the activating receptors contain an immunoreceptor tyrosine-based activation motif (ITAM) and initiate inflammatory, cytolytic, and phagocytic activities of immune effector cells. In contrast, the inhibitory receptors that downmodulate the immune responses contain an immunoreceptor tyrosine-based inhibitory motif (ITIM) (2, 3). Three

categories of Fc $\gamma$ R exist: Fc $\gamma$ RI has high affinity for monomeric IgG, whereas Fc $\gamma$ RII and Fc $\gamma$ RIII exhibit low affinity for monomeric IgG but avidly bind IgG-containing immune complexes (ICs). Both in mice and in humans (4), two isoforms of the inhibitory Fc $\gamma$ RIIB (Fc $\gamma$ RIIB1 and Fc $\gamma$ RIIB2) are produced by an alternative splicing, which generates a 47-amino acid insert in the intracellular domain of Fc $\gamma$ RIIB1.

Antibodies directed against neoplastic cells provide new therapeutic approaches against various malignancies, including lymphoma, leukemia, melanoma, and breast and colorectal carcinoma (5, 6). There is increasing evidence that the Fc portion of the anti-tumor IgG is a major component of their therapeutic activity, along with other mechanisms such as activation of apoptosis, blockade of signaling pathways, or masking of tumor antigens. Thus, by binding to activating Fc $\gamma$ Rs expressed by immune effector cells, such as macrophages, monocytes, neutrophils, or NK cells, tumor-specific antibodies trigger the destruction of malignant cells via antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis (7, 8). Recent experiments have shown that inhibitory Fc $\gamma$ R decreases the *in vivo* efficacy of antibodies against tumors. Indeed, the use of mAb's to eradicate a variety of tumors in mice reveals that the Fc $\gamma$ RIIB inhibitory receptor expressed on effector cells

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**Address correspondence to:** Catherine Sautès-Fridman, INSERM U255, Centre de Recherches Biomédicales des Cordeliers, 15 rue de l'École de Médecine, 75006 Paris, France. Phone: 33-1-53-10-04-03; Fax: 33-1-40-51-04-20; E-mail: catherine.fridman@u255.bhdc.jussieu.fr.

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**Nonstandard abbreviations used:** Fc $\gamma$  receptor (Fc $\gamma$ R); immunoreceptor tyrosine-based activation motif (ITAM); immunoreceptor tyrosine-based inhibitory motif (ITIM); immune complex (IC); antibody-dependent cellular cytotoxicity (ADCC); severe combined immunodeficiency (SCID).

is a potent regulator of ADCC *in vivo*, downmodulating the activation of monocytes and macrophages via ITAM-containing Fc $\gamma$ R (9).

It has previously been suspected that human and mouse tumors from nonhematopoietic tissues may express low-affinity Fc $\gamma$ R (10). However, whether the Fc $\gamma$ R-expressing cells originated as tumor or inflammatory cells was debated. It was difficult to answer this question because tumor cells lose Fc $\gamma$ R expression in culture (11). Interestingly, the expression of Fc $\gamma$ R can be recovered after a single passage of tumor cells *in vivo*, and some tumor cell lines expressed Fc $\gamma$ R (12). We report here, for the first time to our knowledge, that human metastatic melanoma cells express inhibitory Fc $\gamma$ RIIB1 *in vivo* and *ex vivo*. This expression is associated with an inhibition of development of melanoma tumors grafted subcutaneously in nude mice, but not in severe combined immunodeficiency (SCID) mice. The anti-G<sub>D2</sub> mAb IgG3, whose Fc portion binds to human tumor Fc $\gamma$ RIIB1 but not to host-cell mouse Fc $\gamma$ Rs, reduced growth of Fc $\gamma$ RIIB1-positive tumors when it was inoculated in SCID mice. Altogether, our data suggest a novel mechanism by which anti-tumor IgG might directly regulate tumor development.

## Methods

**Human melanoma tumors and cell lines.** The human malignant melanoma lines A375 and HT144 (American Type Culture Collection, Manassas, Virginia, USA) were maintained in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin. A375 cells, which do not express Fc $\gamma$ RII mRNA, were stably transfected (13) with pBR322-zeo vector containing human Fc $\gamma$ RIIB1 (A375IIB1), Fc $\gamma$ RIIB2 (A375IIB2), or Fc $\gamma$ RIIA (A375IIA) cDNAs. HT144 cells were stably transfected with the same vector containing human Fc $\gamma$ RIIB1 cDNA (HT144IIB1) or with Fc $\gamma$ RIIB(Cyto<sup>-</sup>) cDNA, which contains no cytoplasmic residues beyond the juxtamembrane region [HT144IIB(Cyto<sup>-</sup>)]. Transfectants were cloned by limiting dilution and tested for Fc $\gamma$ RII expression by flow cytometry. The human lymphoma ST486 (American Type Culture Collection) was cultured in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 5% sodium pyruvate, 20  $\mu$ M 2-mercaptoethanol, and 1% penicillin-streptomycin.

The cutaneous (patient 201), liver (patients 212, 721, and 926), or lymph node (patient 193) metastasis from patients with melanoma was analyzed. The 721 cell line was derived by culturing liver metastasis in DMEM supplemented with 10% FCS, 2 mM L-glutamine, 1% sodium pyruvate, and 1% penicillin-streptomycin. After 1 week of culture, Fc $\gamma$ RII-positive and -negative clones were selected using anti-Fc $\gamma$ RIIA+B mAb (AT10) (14) coupled to magnetic beads (Immunotech, Marseille, France).

***In vivo tumor growth.*** Melanoma cells were injected subcutaneously into Swiss nude mice or into CB17 SCID mice. Tumor development at the site of injection

was evaluated twice a week. Tumor volume (in mm<sup>3</sup>) was estimated from the length, *a*, and width, *b*, of the tumor: volume =  $ab^2/2$ . The mice were bled 1 week before and 14 days after tumor cell injection, and IgGs were purified by protein A-Sepharose column (Pharmacia Biotech AB, Uppsala, Sweden). In some experiments, SCID mice were inoculated subcutaneously with melanoma cells on day 0 and intravenously with 50  $\mu$ g anti-G<sub>D2</sub> mAb 7A4 (15) or PBS on days 0, 3, 6, 9, 13, and 16.

**Flow cytometry analysis.** Cells were incubated with AT10, anti-Fc $\gamma$ RIIA mAb IV.3 (16), anti-G<sub>D2</sub> mAb 7A4, Mel/14 mAb (17), a 1:10 dilution of sera from mice or with medium alone followed by FITC-conjugated goat anti-mouse IgG Ab (FITC-GAM) (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA). In some experiments, cells were incubated with FITC-coupled anti-HLADR mAb or FITC-coupled isotypic control IgG2a (Immunotech). Flow cytometry analysis was performed with a FACScan (Becton Dickinson, Mountain View, California, USA).

**Cytogenetics.** Cell lines were incubated with colcemid (GIBCO BRL; Life Technologies Inc., Cergy Pontoise, France) for 2 hours, then with buffer containing 0.075 M KCl, and fixed with methanol/acetic acid (3 vol/1 vol). After spreading, R banding was performed and analyzed using a digital imaging system.

**Western blot analysis.** The Fc $\gamma$ RIIs were immunoprecipitated with AT10 as described previously (13). In some experiments, HT144IIB1 cells (10<sup>7</sup>/ml) were incubated with 100  $\mu$ M pervanadate for 10 minutes at 37°C. Cells were centrifuged and lysed, and Fc $\gamma$ RIIB1 was immunoprecipitated as above. Immunoprecipitated material was fractionated by SDS-PAGE (Bio-Rad Laboratories Inc., Hercules, California, USA), transferred and Western blotted with anti-SHIP1, anti-SHIP2 (Upstate Biotechnology Inc., Lake Placid, New York, USA), anti-SHP1, anti-SHP2, or anti-phosphotyrosine antibodies (Transduction Laboratories, Lexington, Kentucky, USA), or with Fc $\gamma$ RIIA (260) or Fc $\gamma$ RIIB/IC antisera followed by horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA). Peroxidase-labeled antibodies were detected using an ECL kit (Amersham Biosciences Europe GmbH, Orsay, France). The anti-Fc $\gamma$ RIIA, 260 or anti-Fc $\gamma$ RIIB/IC sera were prepared by hyperimmunization of rabbits with Fc $\gamma$ RIIA2 (18) or with a GST fusion protein of residues from the first to the third intracytoplasmic domains of Fc $\gamma$ RIIB1, respectively (13).

**Immunohistochemistry.** Staining of Fc $\gamma$ RII was performed on unfixed and unsaturated tissue sections from frozen tumor samples using KB61 supernatant (19) or mouse IgG1 (DAKO SA, Trappes, France) followed by alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc.). Signal was developed using an alkaline phosphatase substrate (Fast Red TR/Naphthol AS-MX; Sigma Chemical Co., St. Louis, Missouri, USA) and counterstained with Mayer's hematoxylin.

**MTT assay.** Cells ( $2 \times 10^3$  per well) were plated in triplicate into 96-well plates and incubated at 37°C. After the indicated periods of time, cell numbers were assessed by the MTT method (20).

**In vitro growth inhibition assays.** Cells were seeded into 96-well plates in medium supplemented with 1% insulin-transferrin-sodium selenite (ITS) (Sigma Chemical Co.) for 24 hours at 37°C. In the culture medium, the FCS was replaced by ITS to avoid potential interference of xenoreactive serum IgG in the assay. Then, purified IgG from sera of nude mice, the same volume of material eluted from sera of SCID mice processed similarly, anti- $G_{D2}$  mAb 7A4, or control mouse IgG3 P51.1 (anti-phosphorylcholine) were added to cells for 24 hours. This was followed by a 24-hour pulse with  $^3H$ -thymidine (1  $\mu$ Ci/well; Amersham Biosciences Europe GmbH).

**Statistical analysis.** The in vivo comparison of tumor size between the various groups of mice was done by Student's *t* test with ANOVA. *P* values less than 0.05 were considered significant.

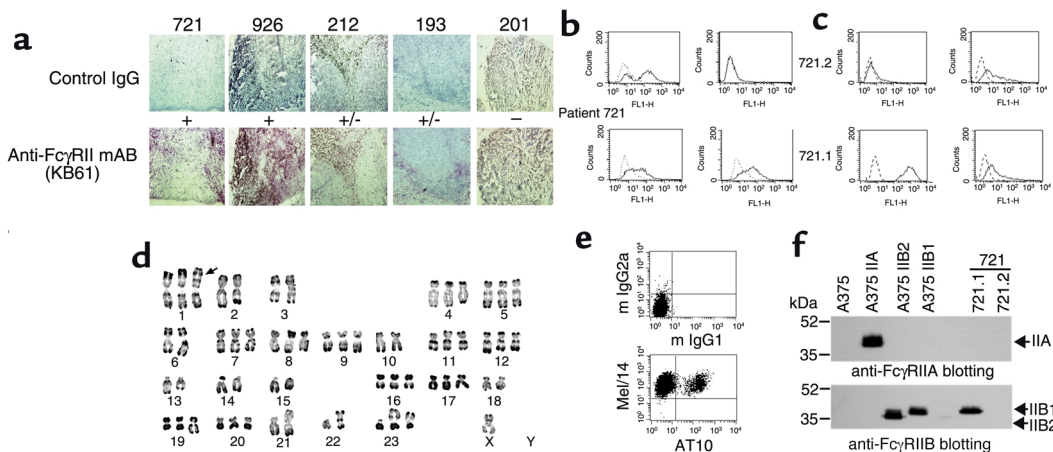
## Results

**Expression of Fc $\gamma$ RIIB1 by human metastatic melanoma.** The expression of Fc $\gamma$ RIIs was investigated by immunohistochemistry in metastasis from patients with melanoma. Figure 1a illustrates representative experiments, suggesting that human metastatic melanomas express Fc $\gamma$ RIIs. This expression was heterogeneous. We found that 20–50% of melanoma cells reacted with anti-Fc $\gamma$ RIIA+B mAb KB61 (patients 721 and 926) whereas others were either faintly positive

(patients 212 and 193) or negative (patient 201). Out of 19 metastases tested, eight showed clear positivity by immunohistochemistry.

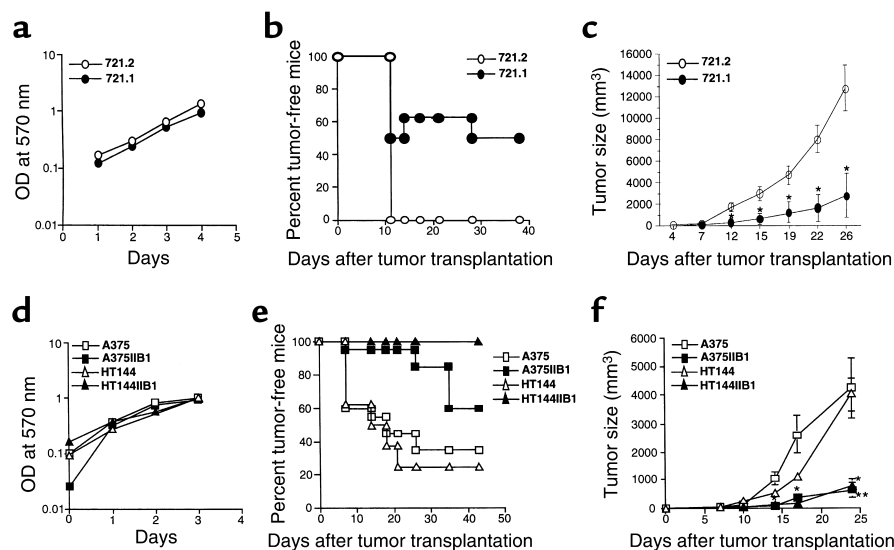
A cell line, 721, was derived from liver metastasis of patient 721. The cells expressed the melanoma antigen ganglioside  $G_{D2}$  and MHC class II molecules (Figure 1, b and c) but no detectable hematopoietic markers such as CD45, CD14, CD1a, CD4, CD15, and CD20 (data not shown). They contained between 50 and 60 human chromosomes with multiple chromosomal abnormalities, including abnormalities of 1p, but did not contain tetrasomic chromosome (Figure 1d). About 40% of cells reacted with the pan-anti-Fc $\gamma$ RII mAb but not with the anti-Fc $\gamma$ RIIA specific mAb (Figure 1b). Double-labeling experiments showed that Fc $\gamma$ RII-positive and -negative 721 cells expressed the melanoma marker Mel/14 (Figure 1e). These results demonstrate that the 721 line is a melanoma line and not a cell line derived from inflammatory cells or from a fusion between an inflammatory cell and a melanoma cell. Several clones were derived from the 721 line, among which 721.1 and 721.2 expressed high or undetectable levels of Fc $\gamma$ RII, respectively, but remained  $G_{D2}$ -positive (Figure 1c).

The Fc $\gamma$ RII isoform expressed by 721.1 cells was determined by immunoprecipitation of Fc $\gamma$ RII followed by isoform-specific Western blot analysis (13, 21). Western blot with anti-Fc $\gamma$ RIIB/IC antibody revealed 40-kDa polypeptides in 721.1 and A375IIB1 lysates, and 36-kDa polypeptides in A375IIB2 lysate. In the same assay, the anti-Fc $\gamma$ RIIA antibody revealed 40-kDa polypeptides only in A375IIA lysate (Figure 1f). In



**Figure 1**

Human metastatic melanomas express inhibitory Fc $\gamma$ RII. (a) Immunohistochemical analysis using anti-Fc $\gamma$ RII mAb KB61 in liver (patients 721, 926, and 212), lymph node (patient 193), or subcutaneous (patient 201) metastases from patients with cutaneous (patients 926, 193, and 201) or uveal (patients 212 and 721) melanoma (bottom). No immunostaining was detected when primary antibody was replaced with isotype control (top).  $\times 30$ . (b and c) Cell line derived from patient 721 expresses Fc $\gamma$ RII. Cells from this melanoma line (721 and its derivative clones, 721.1 and 721.2) were incubated with anti-Fc $\gamma$ RIIA+B (AT10) or anti-Fc $\gamma$ RIIA (IV.3) mAb's, anti-melanoma mAb's ( $G_{D2}$  mAb's), anti-HLA-DR mAb, or control isotype (dotted histograms). (d and e) Cell line derived from patient 721 is a melanoma cell line. (d) R-banded karyotype of 721 cell line. Numerous structural and numerical chromosomal aberrations are present. See the abnormality of 1p (arrow), probably involving 3p. (e) Double staining was performed by incubating cells with AT10 and anti-melanoma marker (Mel/14) mAb's (bottom) or with control isotypes (top). (f) Analysis of the Fc $\gamma$ RII isoforms expressed by the 721 cell line, by immunoprecipitation of Fc $\gamma$ RII with AT10 in cell lysates from 721.1, 721.2, A375, A375IIB1, A375IIB2, and A375IIA cell lines, and by immunoblotting with anti-Fc $\gamma$ RIIA (260) (top panel) or anti-Fc $\gamma$ RIIB/IC (bottom panel) polyclonal antibodies. Molecular weight standards (kDa) are shown in the left lane. mIgG, mouse IgG.



**Figure 2**

FcγRIIB1 expression is associated with inhibition of development of human melanoma tumors in nude mice. (a and d) In vitro proliferation of FcγRIIB1-negative (721.2, A375, or HT144) and FcγRIIB1-positive (721.1, A375IIB1, or HT144IIB1) melanoma lines, determined by an MTT assay on days 1–4. (b and e) Tumor uptake in nude mice, determined by subcutaneous inoculation of  $2 \times 10^6$  721.1 and 721.2 cells (b) or  $1 \times 10^6$  A375, HT144, A375IIB1, or HT144IIB1 cells (e). Results are expressed as percentage of mice ( $n = 30$ ) that remained tumor-free after challenge in three experiments. (c and f) Tumor growth, measured by volume after injection of  $4 \times 10^6$  721.1 and 721.2 cells (c) or  $2 \times 10^6$  A375, HT144, A375IIB1, or HT144IIB1 cells (f). Ten mice were used per experiment in each group, and mean tumor volume values, with SD, of mice bearing tumor are shown. Data are representative of three independent experiments. \* $P < 0.03$ , \*\* $P < 0.002$  between FcγRIIB1-positive and FcγRIIB1-negative tumors.

addition, we did not detect 36- or 40-kDa polypeptides in 721.2 lysate. Altogether, these results demonstrate that FcγRIIB1 is expressed by human melanoma line 721 and its FcγRII-positive clone derivative 721.1.

*FcγRIIB1 inhibits growth and uptake of human melanoma in nude mice.* To investigate the putative role of FcγRIIB1 on melanoma growth in vivo, 721.1 and 721.2 lines were injected subcutaneously into nude mice. As shown in Figure 2b, 100% of mice injected with FcγRIIB1-negative melanoma 721.2 developed a tumor at day 11, whereas 50% of mice inoculated with FcγRIIB1-positive melanoma 721.1 remained tumor-free and viable at the end of the observation period (day 40). To determine the effect of FcγRIIB1 on melanoma growth, cells were grafted into nude mice at a cellular density at which no effect of FcγRIIB1 was observed on tumor uptake (data not shown). Whereas both clones expanded similarly in vitro (Figure 2a), the growth of FcγRIIB1-positive melanoma cells was strongly inhibited in vivo as compared with that growth of the FcγRIIB1-negative clone, since up to 75% inhibition of tumor mass from day 12 to day 26 was observed ( $P < 0.01$  at day 26) (Figure 2c). Similar results were observed using other FcγRIIB1-positive and -negative clones from patient 721 (data not shown).

To demonstrate the role of FcγRIIB1, we used human metastatic melanoma transfected with cDNA encoding FcγRIIB1. Although in vitro growth kinetics of cell lines had not significantly changed after transfection (Figure 2d), a profound effect of FcγRIIB1 was seen on tumorigenicity and tumor growth, reminis-

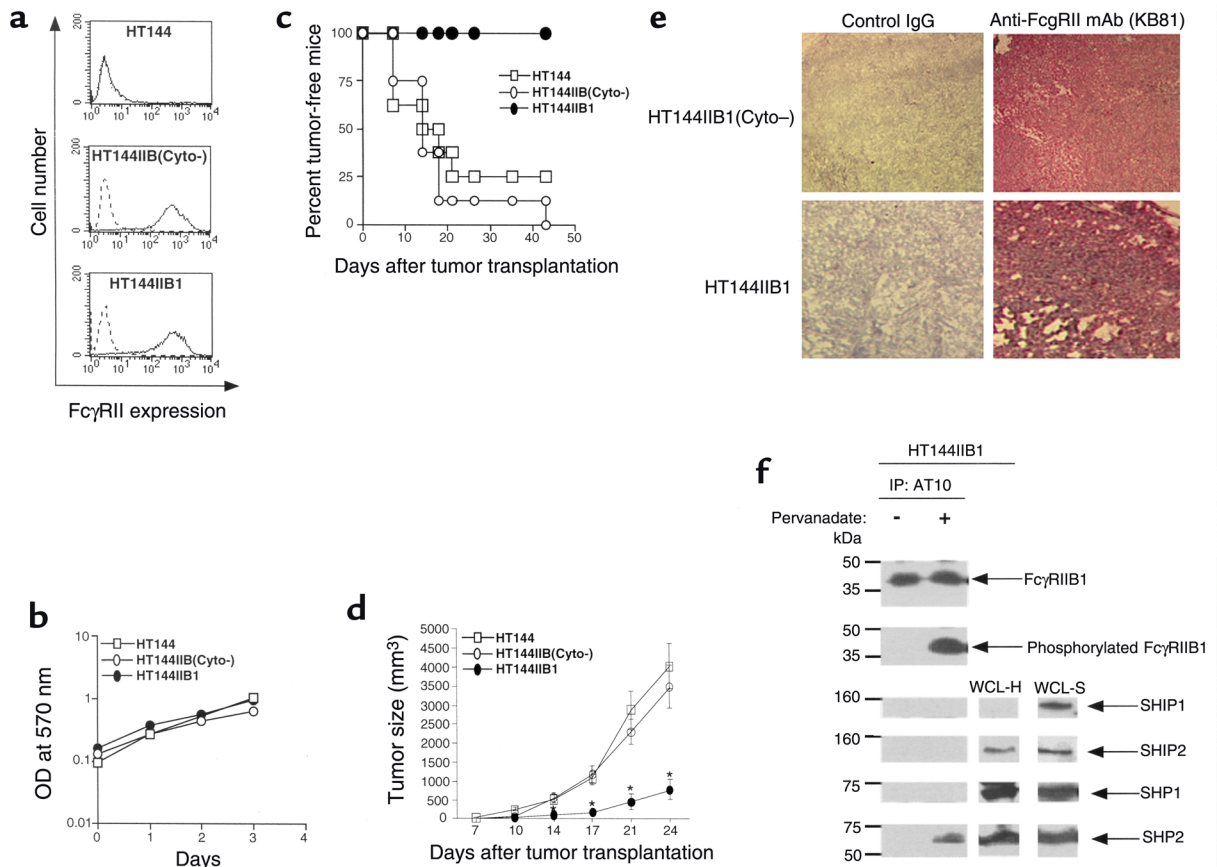
cent of our observation in cell lines from patient 721. For instance, 100% of mice inoculated with HT144IIB1 remained tumor-free and viable at the end of the observation period (day 42), whereas only 25% of mice injected with HT144 remained tumor-free (Figure 2e). To determine the effect of FcγRIIB1 on growth of melanoma tumors, we inoculated into nude mice a cellular dose at which no significant effect of FcγRIIB1 on tumor uptake was observed (80% and 100% uptake of FcγRIIB1-positive and FcγRIIB1-negative tumors, respectively). As shown in Figure 2f, expression of FcγRIIB1 led to a pronounced inhibition of growth of both A375 and HT144 melanomas starting from day 14, up to 80% inhibition of tumor volume at day 24 ( $P < 0.001$ ). Altogether, these results suggest that FcγRIIB1 inhibits the uptake and growth of human melanoma cells in nude mice.

*The intracytoplasmic region of FcγRIIB1 is required for inhibition of growth and uptake of human melanoma in nude mice.* The FcγRIIB1-mediated inhibition of cellular activation has been shown to require the receptor's cytoplasmic tail. To determine whether the intracytoplasmic region of FcγRIIB1 is involved in the inhibition of melanoma development in nude mice, we compared the growth of HT144 transfected with vector containing FcγRIIB(Cyto<sup>-</sup>) cDNA [HT144IIB(Cyto<sup>-</sup>)] with that of HT144 and HT144IIB1. HT144IIB1 and HT144IIB(Cyto<sup>-</sup>) cell lines had similar expression levels of FcγRII (Figure 3a) and similar growth rates in vitro (Figure 3b). As shown in Figure 3c, 100% and 75% of mice inoculated with HT144IIB(Cyto<sup>-</sup>) or HT144,

respectively, developed a tumor at day 42. In contrast, 100% of mice grafted with HT144IIB1 remained tumor-free and viable over a 42-day observation period. The effect of the intracytoplasmic region of FcγRIIB1 on tumor growth was then evaluated. The results showed that, from day 14 to the sacrifice of the animals at day 24, the growth of HT144IIB1 was highly and significantly inhibited (up to 80% inhibition of tumor volume) in comparison with that of HT144 or HT144IIB(Cyto<sup>-</sup>) ( $P < 0.008$ ), the growth of HT144IIB(Cyto<sup>-</sup>) being no different from that of HT144 (Figure 3d). Expression levels of FcγRIIB were similar in the HT144IIB(Cyto<sup>-</sup>) and HT144IIB1 tumors at day 21 (Figure 3e). These results indicate that the intracytoplasmic region of FcγRIIB1 plays a prominent role in the inhibition of development of human melanoma tumors in nude mice. In hematopoietic

cells, FcγRIIB-dependent negative regulation of cell proliferation requires recruitment of phosphatases by phosphorylated ITIM present in the cytoplasmic tail of FcγRIIB1. Western blot analysis of whole cell lysates showed that HT144 expressed tyrosine phosphatases SHP2 and SHP1, and the inositol phosphatase SHIP2, but not the hematopoietic lineage-specific SHIP1 (Figure 3f). Pervanadate treatment of HT144IIB1 led to recruitment of SHP2 but not of SHP1 or SHIP2 by phosphorylated FcγRIIB1 (Figure 3f), suggesting that FcγRIIB can be functional in human melanoma cells.

*The FcγRIIB1-dependent inhibition of tumor development does not occur in SCID mice.* The FcγRIIB1 expressed by human melanoma binds mouse IgG. Although nude mice lack a thymus, they have significant amounts of circulating IgG produced against T cell-independent antigens such as gangliosides expressed by human



**Figure 3**

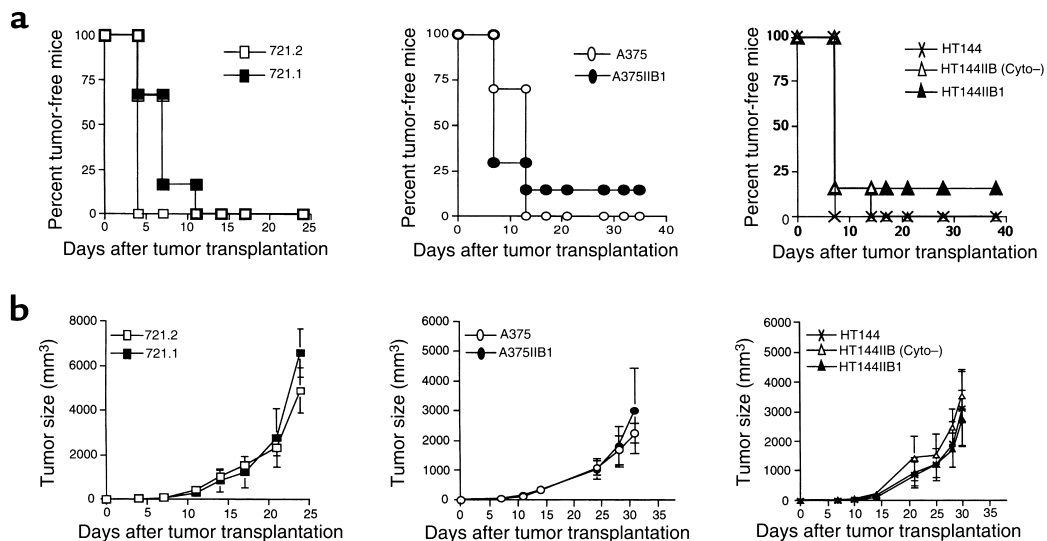
The FcγRIIB1-mediated inhibition of melanoma development in nude mice requires the receptor's cytoplasmic tail. HT144IIB1 and HT144IIB(Cyto<sup>-</sup>) cell lines were used. (a) Expression of FcγRIIB1 as assessed the day before injection by flow cytometry using AT10 or control IgG1 (dotted histograms). (b) In vitro proliferations of melanoma lines were determined by an MTT assay on days 1–4. (c) Tumor uptake incidence as determined by subcutaneous inoculation of nude mice with  $1 \times 10^6$  cells. Results are expressed as percentage of mice ( $n = 20$ ) that remained tumor-free after challenge in two experiments. (d) Tumor growth as measured by volume after inoculation of  $2 \times 10^6$  cells. Ten mice were used per experiment in each group, and mean tumor volume values of mice bearing tumor with SDs are shown. Data are representative of two independent experiments. \* $P < 0.01$  between HT144IIB1 tumors and HT144 or HT144IIB(Cyto<sup>-</sup>) tumors. (e) Immunohistochemical analysis of FcγRIIB expression using KB61 mAb (right panels), on tumor tissues at day 21 after injection in nude mice of HT144IIB1 (bottom) or HT144IIB(Cyto<sup>-</sup>) (top) cells.  $\times 30$ . (f) Recruitment of SHP2 phosphatase to phosphorylated FcγRIIB1. HT144IIB1 cells ( $6 \times 10^7$ ) were treated or not treated with 100  $\mu$ M pervanadate for 10 minutes. Cells were lysed and FcγRIIB was immunoprecipitated using AT10 mAb. Immunoprecipitated materials were electrophoresed and Western blotted with anti-FcγRIIB/IC, anti-phosphotyrosine, anti-SHIP1, anti-SHIP2, anti-SHP1, and anti-SHP2 Ab's. Whole cell lysates of HT144IIB1 (WCL-H) or ST486 (WCL-S) were used as positive controls.

melanoma. To further investigate the mechanism by which FcγRIIB1 inhibits tumor development, the melanoma lines were grafted into SCID mice, which do not mount any adaptive immune response, including antibody production. As shown in Figure 4a, 100% of SCID mice inoculated with FcγRIIB1-negative melanoma (721.2, A375, HT144) and 85% of their FcγRIIB1-positive counterparts expressing wild-type (721.1) or recombinant FcγRIIB1 (A375IIB1, HT144IIB1) developed a tumor within 13 days. No significant difference in tumor uptake was seen between HT144IIB1 and HT144IIB(Cyto<sup>-</sup>) cells (Figure 4a). In addition, the growth rates of all three FcγRIIB1-positive tumors were similar to that of their FcγRIIB1-negative counterparts, and no significant difference was found between growth of HT144IIB1 and growth of HT144IIB1(Cyto<sup>-</sup>) tumors over a 31-day period (Figure 4b).

*Role of antibodies in the FcγRIIB1-dependent inhibition of melanoma growth in vitro and in vivo.* The fact that FcγRIIB1-positive human melanoma cells behaved differently in nude and SCID mice raised the possibility that IgG ICs or IgG directed against tumor cells play an important role in melanoma development in nude mice. Immunofluorescence assays were performed on FcγR-negative HT144 cells to ensure that sera from nude mice contained anti-tumor IgG. Thus, sera taken at day 14 from nude mice bearing HT144IIB1 or HT144IIB(Cyto<sup>-</sup>) tumors contained IgG binding to HT144, some reactivity being also detected at day 0 (Figure 5a). IgGs isolated from the sera of nude mice bearing day-14 tumors were tested for their effect on melanoma cell proliferation after 2 days of culture.

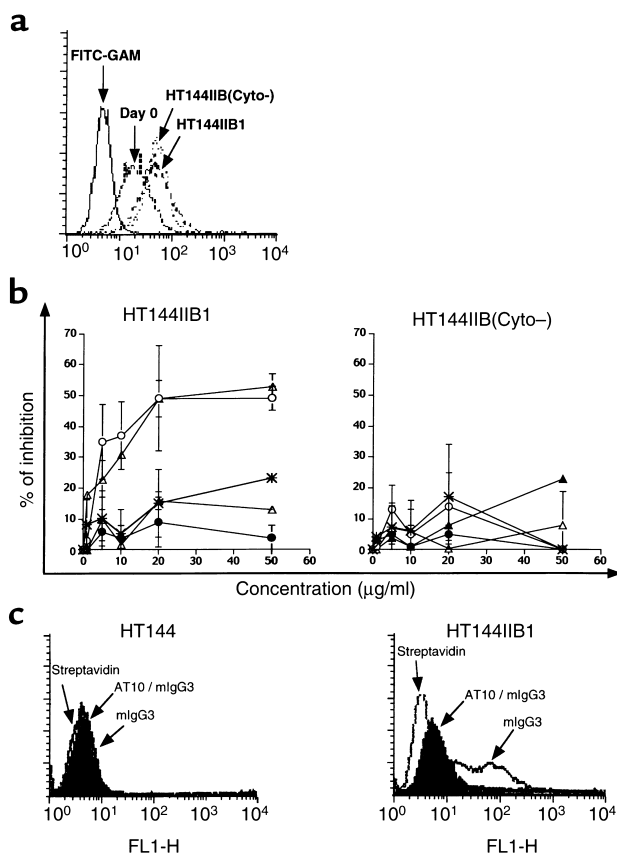
The proliferation of HT144IIB1 but not that of HT144IIB(Cyto<sup>-</sup>) cells was inhibited in a dose-dependent manner by IgG isolated from sera of nude mice bearing HT144IIB1 or HT144IIB(Cyto<sup>-</sup>) tumors (up to 40% inhibition reached at 20 μg/ml IgG) (Figure 5b). A high dose of IgG isolated from day-0 sera of nude mice slightly inhibited cell proliferation (25%). Moreover, eluate from sera of SCID mice bearing tumor, treated under the same conditions, did not contain antibodies (data not shown) and failed to inhibit the proliferation of both cell lines (Figure 5b). Another HT144IIB1 clone and two other HT144IIB(Cyto<sup>-</sup>) clones gave the same results (data not shown), which indicate that anti-melanoma IgGs are produced in nude mice and inhibit proliferation of FcγRIIB1-positive melanoma in an intracytoplasmic-dependent manner. Since these antibodies were mainly of the IgG3 isotype (data not shown), we investigated whether mouse IgG3 bound human FcγRIIB. As shown in Figure 5c, a significant binding of mouse IgG3 was detected in HT144IIB1 and not in HT144. This binding was inhibited by AT10, suggesting that human FcγRIIB1 is responsible for it. Taken with data shown in Figure 4, these results suggest that antibodies could play a direct role in FcγRIIB1-mediated inhibition of uptake and growth of human melanoma in nude mice.

*Effect of anti-G<sub>D2</sub> mAb on melanoma growth in vitro and in vivo.* The ganglioside G<sub>D2</sub> is a major melanoma-associated glycolipid antigen that induces production of IgG in melanoma patients (22, 23). 7A4 is an IgG3 mAb that reacts with G<sub>D2</sub> expressed by neuroectoderm-derived tumors such as melanoma and has been used for tumor therapy. To extend the role of IgG in



**Figure 4**

The development of human melanomas is independent of FcγRIIB1 expression in SCID mice. (a) Tumor uptake, determined by inoculation of  $2 \times 10^6$  721.1 or 721.2 cells or  $1 \times 10^6$  A375, A375IIB1, HT144, HT144IIB(Cyto<sup>-</sup>), or HT144IIB1 cells in SCID mice. Results are expressed as percentage of mice ( $n = 18$ ) that remained tumor-free after challenge in two experiments. (b) Tumor growth, measured by volume after inoculation of  $4 \times 10^5$  721.1 or 721.2 cells or  $2 \times 10^6$  A375, A375IIB1, HT144, HT144IIB(Cyto<sup>-</sup>), or HT144IIB1 cells. Nine mice were used per experiment in each group, and mean tumor volume values, with SD, of mice bearing tumor are shown. Data are representative of two independent experiments.



**Figure 5**

Sera from nude mice contain IgG antibodies that bind to melanoma cells and inhibit proliferation of FcγRIIB1-positive melanoma cells in an intracytoplasmic-dependent manner. (a) Day-0 and day-14 sera from nude mice bearing HT144IIB(Cyto<sup>-</sup>) or HT144IIB1 tumors, or medium alone (FITC-GAM), were incubated with HT144 cells, and binding of IgG was assessed by flow cytometry. (b) HT144IIB1 (left panel) or HT144IIB(Cyto<sup>-</sup>) (right panel) cells ( $5 \times 10^3$ /well) were incubated with the indicated concentrations of IgG isolated from sera of nude mice at day 0 (cross) or bearing day-14 HT144IIB1 (open circles) or HT144IIB(Cyto<sup>-</sup>) (open triangles) tumors or with the same volume of control eluates from sera of SCID mice bearing day-14 HT144IIB1 (filled circles) or HT144IIB(Cyto<sup>-</sup>) (filled triangles) tumors for 24 hours to FCS-free medium. This was followed by a 24-hour pulse with <sup>3</sup>H-thymidine. The percentages of inhibition were estimated with the formula  $[(1 - \text{cpm of stimulated cells} / \text{cpm of unstimulated cells}) \times 100]$ . Each point represents results from three independent experiments performed in triplicate (mean  $\pm$  SD). (c) HT144 (left panel) or HT144IIB1 (right panel) cells were preincubated with AT10, then with biotin-labeled mouse IgG3, and then with FITC-conjugated streptavidin, or were not preincubated. Binding of mouse IgG3 was compared with background binding of FITC-conjugated streptavidin.

FcγRIIB1-mediated inhibition of cell proliferation, we studied the effect of 7A4 mAb on the proliferation of HT144, HT144IIB1, and HT144IIB(Cyto<sup>-</sup>) melanoma cells. These cell lines express similar levels of G<sub>D2</sub> (Figure 6a). The proliferation of HT144IIB1 cells was significantly inhibited in a dose-dependent manner by 7A4 mAb at day 2 (50% inhibition of <sup>3</sup>H-thymidine incorporation at 100 µg/ml), but not by control mouse IgG3, although the proliferation of HT144 and

HT144IIB(Cyto<sup>-</sup>) cells was not significantly modified (less than 20% inhibition at the same dose) (Figure 6b). Since 7A4 seems to reproduce the FcγRIIB1-dependent negative regulation of cell proliferation with IgG produced in nude mice, we investigated whether this mAb could be used to regulate melanoma growth in SCID mice. For this purpose, HT144IIB1 and HT144IIB(Cyto<sup>-</sup>) cells were injected subcutaneously into SCID mice. The intravenous injections of anti-G<sub>D2</sub> mAb resulted in significant inhibition of growth of HT144IIB1 melanoma from day 6 to day 28 (up to 50% inhibition of tumor mass at day 28,  $P < 0.0001$ ) (Figure 6c). In contrast, the anti-G<sub>D2</sub> mAb failed to inhibit the growth of FcγRIIB(Cyto<sup>-</sup>) melanoma (Figure 6d). Altogether, these results display that anti-G<sub>D2</sub> mAb's are able to inhibit FcγRIIB1-positive melanoma growth in vitro and in vivo and that these inhibitions require the cytoplasmic tail of FcγRIIB1.

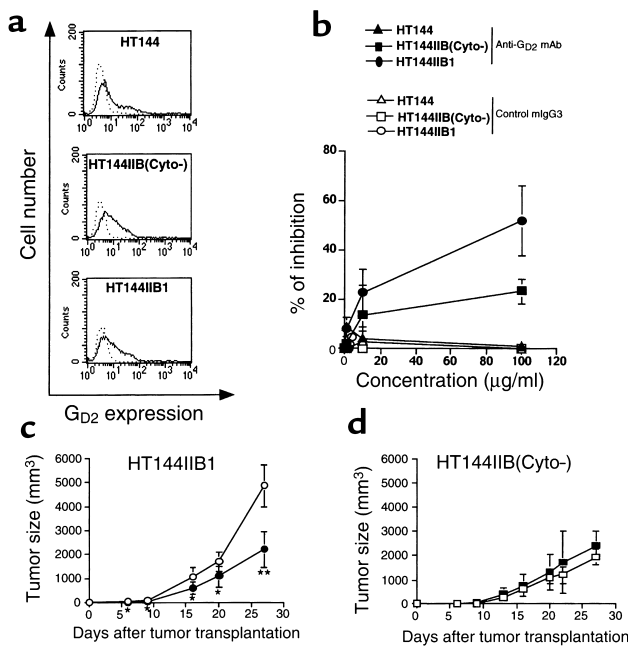
## Discussion

Tumor cells present in metastasis of melanoma patients were found to express inhibitory FcγRIIB as detected in situ and on tumor-derived melanoma lines. In tumor sections, melanin was clearly detected in FcγRII-positive cells, and melanoma marker S100 was coexpressed with FcγRII (data not shown). The cell line derived from patient 721 had the chromosomal characteristics and cell surface marker expression of a melanoma line. Isoform-specific analysis of FcγRII showed that the 721 cell line expressed only FcγRIIB1 isoform. Other cell lines derived from FcγRII-positive biopsies of liver (212 line) or cutaneous metastases (three lines) were also found to react with anti-FcγRIIA+B but not with anti-FcγRIIA mAb (data not shown).

Our observations of ectopic expression of FcγRIIB1 by human melanoma are reminiscent of previous observations suggesting that tumor cells of non-hematopoietic origin may express low-affinity FcγR (10, 12, 24). None of the tumor-derived cell lines expressed FcγRI or FcγRIII (data not shown). Expression of FcγRII on tumor cells was heterogeneous and was found in about 40% of tested metastases. This raises the question of what mechanisms control FcγR expression in tumor cells. Culture of melanoma lines A375, HT144, and 721.2 in the presence of cytokines such as IFN-γ, TNF-α, GM-CSF, and TGF-β did not induce significant expression of FcγRII (data not shown). Moreover, the expression level of FcγR did not decrease during primary cultures of FcγRII-positive melanomas and remained stable after several years in culture. Thus it is likely that the expression of FcγRII is an intrinsic property of tumor cells. It is possible that IgG-containing ICs may upregulate FcγRII expression during the primary stages of tumor formation, as previously observed in vitro on cell lines of lymphoid origin (25).

A prominent and significant inhibitory role of FcγRIIB1 in tumor development was found to occur in nude but not in SCID mice. This inhibition was





**Figure 6** Anti-G<sub>D2</sub> mAb inhibits growth of FcγRIIB1-positive tumor cells in vitro and in vivo, in an intracytoplasmic-dependent manner. (a) Expression of G<sub>D2</sub> on cells as assessed by flow cytometry using 7A4 or a control isotype (dotted histograms). (b) Inhibition of proliferation of melanoma cells in vitro. Cells ( $5 \times 10^3$  cells per well) were incubated with the indicated concentrations of anti-G<sub>D2</sub> mAb 7A4 (filled symbols) or control mouse IgG3 (open symbols). <sup>3</sup>H-thymidine incorporation rate and percentages of inhibition were measured as described in Figure 5. Each point represents results from three independent experiments performed in triplicate (mean  $\pm$  SD). (c and d) Inhibition of growth of HT144IIB1 but not of HT144IIB(Cyto<sup>-</sup>) tumors in SCID mice injected intravenously with anti-G<sub>D2</sub> mAb. SCID mice were inoculated subcutaneously with  $2 \times 10^6$  HT144IIB1 (c) and HT144IIB(Cyto<sup>-</sup>) (d) cells, followed by biweekly intravenous injections of 50  $\mu$ g of mAb 7A4 (filled symbols) or PBS (open symbols). Eight mice were used per experiment in each group, and mean tumor volume values, with SD, of mice bearing tumor are shown. \* $P < 0.05$ , \*\* $P < 0.0001$ .

observed by using two FcγRIIB1-positive and two FcγRIIB1-negative clones independently derived from 721 melanoma. The in vivo growth properties of the noncloned cell lines (A375 and HT144) transfected or not transfected with cDNA encoding FcγRIIB1 showed the same results (data not shown). These results strongly suggest that this phenomenon is not due to cellular variants occurring during cell culture. We found that nude mice develop mostly antibodies of the IgG3 isotype (data not shown), some of which react with melanoma antigens. These antibodies are most likely directed against the carbohydrate antigens expressed by melanoma cells, which may act as T-independent antigens in this system. Addition of IgG, isolated from sera of nude mice bearing tumor, led to a significant inhibition of proliferation of HT144 melanoma cells expressing whole FcγRIIB1 but not FcγRIIB(Cyto<sup>-</sup>). Small amounts of IgG binding to melanoma cells were detected in nude mice before

tumor injection that slightly inhibited HT144IIB1 cell proliferation in vitro. Such IgG may be responsible for inhibition of uptake of FcγRIIB1-positive tumors during the first stages of tumor development.

The fact that growth of HT144IIB(Cyto<sup>-</sup>) tumors was similar to that of HT144 in nude mice and was not inhibited by anti-G<sub>D2</sub> in SCID mice reflects, most probably, the low capacity of mouse IgG3s both to activate complement in vivo (26) and to trigger ADCC, a deficiency that results from their inability to bind mouse FcγR expressed by mouse effector cells such as macrophages (data not shown) and NK cells (27). Several mechanisms might be responsible for the in vitro and in vivo FcγRIIB1 intracytoplasmic-dependent inhibition of melanoma's growth. Mouse IgG3 in the form of complexes indeed binds to human FcγRIIB1. The recruitment of SHP2 tyrosine phosphatase by phosphorylated FcγRIIB1 in HT144 cells suggests that human FcγRIIB1 can be fully active in human malignant melanoma. In a chicken B cell line, FcγRIIB1 mediates an apoptotic response upon homoaggregation, independently of its cytoplasmic region (28). One possibility is that ICs act similarly on melanoma cells. However, aggregation of FcγRIIB1 had no effect on proliferation of FcγRIIB1-positive melanoma in vitro (data not shown). An alternative hypothesis is that antibodies directed against melanoma antigens or growth factor receptors may recruit FcγRIIB1 into cell activation units. It has been reported that G<sub>D2</sub> and ErbB2 localize in membrane microdomains (29, 30) and that activated tyrosine kinase receptors (such as c-kit) are sensitive to FcγRIIB1 inhibitory signals (31). Thus one possible explanation is that cross-linking of FcγRIIB1 to such molecules brings the signaling molecules required for inhibitory activity toward the microdomains, as recently observed when FcγRIIB1 is coaggregated to the B cell receptor (32). We tested this hypothesis in preliminary experiments. Although no significant FcγRIIB1 phosphorylation was detected using anti-G<sub>D2</sub> or nude mouse antibodies, cross-linking of FcγRIIB1 and ErbB2 with anti-ErbB2 mAb induced phosphorylation of FcγRIIB1 and recruitment of SHP2 by phosphorylated FcγRIIB1. Interestingly, anti-ErbB2 mAb also inhibited the in vitro proliferation of FcγRIIB1-positive human metastatic melanoma lines in an Fc-dependent manner (data not shown).

The T-independent anti-tumor IgG produced in nude mice may also be directed against ganglioside G<sub>D2</sub> or other raft-localized antigens. While anti-G<sub>D2</sub> mAb's and antibodies specific for xenoreactive antigens may indeed have an FcγRIIB1-inhibitory role, it remains to be seen whether this role is shared by antibodies with other specificities, particularly those produced in syngeneic hosts.

The anti-CD20 and anti-ErbB2 mAb's which were generated in xenogeneic hosts, currently represent therapeutic options against lymphoma (5) and cancers of nonhematopoietic origin (33), respectively. In vivo

study has indicated that ADCC may be important for antitumor activities of these mAb's (9). The present work raises the possibility that, in addition to their well-documented activity via FcγR-positive host effector cells, antibodies may act directly via Fc-dependent pathway on tumor cells. Since antibody-mediated therapies showed some efficacy only in some groups of treated patients (34), it is likely that unknown mechanisms of action are behind these effects and these variable responses. Our data support an alternative hypothesis for the efficacy of therapeutic antibodies, depending on inhibitory receptor FcγRIIB1 expression on tumor cells.

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