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Immature Neutrophils Mediate Tumor Cell Killing via IgA but Not IgG Fc Receptors[1]

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Antitumor Abs are promising therapeutics for cancer. Currently, most Ab-based therapies focus on IgG Ab, which interact with IgG FcR (FcγR) on effector cells. In this study, we examined human and mouse neutrophil-mediated tumor cell lysis via targeting the IgA FcR, FcαRI (CD89), in more detail. FcαRI was the most effective FcR in triggering tumor cell killing, and initiated enhanced migration of neutrophils into tumor colonies. Importantly, immature neutrophils that are mobilized from the bone marrow upon G-CSF treatment efficiently triggered tumor cell lysis via FcαRI, but proved incapable of initiating tumor cell killing via FcγR. This may provide a rationale for the disappointing results observed in some earlier clinical trials in which patients were treated with G-CSF and antitumor Ab-targeting FcγR. *The Journal of Immunology,* **2005, 174: 5472–5480.**

Over the last few years, therapeutic mAb have been acknowledged as promising drugs for cancer treatment (1). In this approach, tumor cells are linked via antitumor mAb to FcR on immune cells, which leads to tumor cell killing. Clinical studies demonstrated encouraging results in the treatment of malignancies, provided mAb were directed at appropriate tumor Ags, and a number of antitumor mAb have now been approved for cancer therapy by the Food and Drug Administration (2).

As yet, it remains unclear how mAb exert their antitumor properties. Therapeutic mAb may cross-link Ags on tumor cells, leading to proapoptotic or antiproliferative effects (1). Additionally, FcR-mediated effector functions by immune cells, like phagocytosis, enhanced presentation of tumor Ags, and Ab-dependent cellular cytotoxicity (ADCC)[3] may contribute to therapeutic efficacy of mAb, as protection against tumor growth was abrogated in FcR γ-chain-deficient mice (3, 4). ADCC has been well documented for monocytes/macrophages, as well as for NK cells (5, 6). Furthermore, neutrophilic granulocytes (neutrophils) have also been shown to exert ADCC (7).

Until now, neutrophils received relatively little attention as effector cells for Ab therapy, despite their well-documented antitumor properties (8). In vitro, neutrophils have been shown to exert potent cytolytic capacity against a variety of tumor cells in the presence of antitumor mAb, and in vivo studies support a role for neutrophils in tumor rejection (7, 9, 10). It has been furthermore demonstrated that neutrophils can induce Ab-dependent apoptosis in human breast cancer cells (11). Additionally, neutrophils represent the most populous FcγR-expressing leukocyte subset within the blood, and their numbers can be increased by treatment with G-CSF (12). Moreover, activated neutrophils can secrete a plethora of inflammatory mediators and chemokines, such as MIPs, MCPs, and IL-8, hereby attracting other immune cells such as monocytes, dendritic cells, and T cells, which may lead to generalized antitumor immune responses (13, 14). Neutrophils may thus represent an attractive effector cell population for Ab therapy.

All approved therapeutic mAb are of the IgG isotype, which can interact with IgG FcR (FcγR) (2). FcγR are widely expressed on a number of cells, including noncytotoxic cells such as platelets, B cells, and endothelial cells. Binding of IgG to such cells may act as an Ab "sink." In addition, binding of IgG to the inhibitory FcR, FcγRIIb, might lead to down-regulation of immune responses (3, 15). To overcome some of these problems, attempts have been made to selectively target activatory FcR via bispecific Abs (BsAb), recognizing both the FcR and tumor Ag of interest. Neutrophils constitutively express the low affinity FcγRIIa (CD32) and FcγRIIIb (CD16) subclasses (15). Additionally, stimulation of neutrophils with G-CSF (or IFN-γ) induces expression of the high affinity FcγRI (CD64), which represents the predominant cytotoxic FcγR on neutrophils (16). These data stimulated the evaluation of a combined therapy of G-CSF and FcγRI-specific BsAb in a number of clinical trials (17–19). These trials, however, only showed limited therapeutic effects, indicating that improvement of neutrophil-mediated Ab therapy is required.

Recently, the IgA FcR (FcαRI, CD89) has been identified as candidate target for tumor therapy (20, 21). FcαRI is constitutively expressed on myeloid effector cells, including neutrophils, monocytes, macrophages, eosinophils, and dendritic cells, but not on noncytolytic cell populations. Furthermore, FcαRI can potently trigger effector functions such as oxidative burst, cytokine release, and phagocytosis, and has been documented as a potent trigger molecule on neutrophils for tumor cell lysis (22, 23). Notably, targeting FcαRI was able to overcome the Ag restriction observed in neutrophil-mediated ADCC with IgG mAb against the B cell lymphoma tumor Ag CD20 (24). FcαRI may thus represent an

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[3] Abbreviations used in this paper: ADCC, Ab-dependent cellular cytotoxicity; BsAb, bispecific Ab; 3D, three-dimensional; EpCAM, epithelial cell adhesion molecule; mIg, mouse immunoglobin; Tg, transgenic.

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attractive alternative for neutrophil-mediated Ab therapy. Its potential as target molecule for the initiation of tumor cell killing was therefore addressed in detail in the present study.

Materials and Methods

Blood and bone marrow donors

Studies were approved by the Medical Ethical Committee of Utrecht University, in accordance with the Declaration of Helsinki. A peripheral blood sample (30 ml) was drawn from healthy untreated volunteers or healthy donors receiving human rG-CSF (Neupogen, 5 μg/kg of body weight, twice daily for 5 days; Amgen), respectively. Bone marrow samples were obtained from cardiac patients undergoing surgery. All donors gave informed consent.

Transgenic (Tg) mice

Generation of FcαRI $\times$ FcγRI double-Tg mice was described earlier (25). To induce FcγRI expression on neutrophils and increase neutrophil counts in blood, mice were injected s.c. with 15 μg of pegylated G-CSF (kindly provided by Amgen) in 150 μl of PBS 3 days before blood collection. Mice were bred and maintained at the Central Animal Facility of the Utrecht University. All experiments were performed according to institutional and national guidelines.

Cell lines

The breast carcinoma cell line SK-BR-3, which overexpresses the proto-oncogene product HER-2/neu, and the malignant B cell lymphoma RAJI (Burkitt's lymphoma) were obtained from the American Type Culture Collection. Cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FCS and antibiotics (RPMI 1640/10%). SK-BR-3 cells were harvested using trypsin-EDTA (Invitrogen Life Technologies).

Abs and flow cytometry

Abs A77 (mouse immunoglobin G1 (mIgG1) anti-FcαRI), m22 (mIgG1 anti-FcγRI), and 520C9 (mIgG1 anti-Her-2/neu) were produced from hybridomas (Medarex). F3.3 (mIgG1 anti-HLA class II)-producing hybridomas were obtained from the Tenovus Research Laboratory (University of Southampton). Chimeric human/mouse Abs were generated, as previously described (26).

FcγRI $\times$ HER-2/neu BsAb (22 $\times$ 520C9; MDX-H210) was obtained from Medarex. FcαRI $\times$ HER-2/neu BsAb (A77 $\times$ 520C9) and FcαRI $\times$ HLA class II BsAb (A77 $\times$ F3.3) were produced by chemically cross-linking $F(ab')_2$ of 520C9 or F3.3 mAb with $F(ab')_2$ of FcαRI-specific mAb A77, as described (27).

Surface expression of FcαRI and FcγRI on neutrophils (2×10^5 cells or 25 μl of blood) was determined with mAb A77 (FcαRI) or m22 (FcγRI), respectively (10 μg/ml), followed by incubation with FITC-conjugated $F(ab')_2$ of goat anti-mouse IgG Ab (Southern Biotechnology Associates). Percentage of neutrophils in blood and bone marrow was determined with PE-conjugated anti-mouse GR-1 mAb (BD Biosciences) or FITC-conjugated anti-human CD66b mAb (Serotec). Maturation status of isolated human bone marrow cells was measured with FITC-conjugated anti-CD11b mAb (Beckman Coulter) and PE-conjugated anti-CD16 mAb (BD Biosciences) as described previously (28). Cells were analyzed on a FACScan (BD Biosciences).

Isolation of neutrophil effector cells

Neutrophils were isolated from heparin anticoagulated peripheral blood samples by standard Ficoll-Histopaque (Sigma-Aldrich) density gradient centrifugation. Neutrophils isolated from G-CSF-treated donors were used directly after isolation, whereas neutrophils from healthy untreated volunteers were cultured overnight at 37°C with IFN-γ (300 U/ml; Boehringer Ingelheim) to induce FcγRI expression.

Bone marrow neutrophils were isolated, as described previously (29). Bone marrow samples were incubated on ice with a lysis solution of pH 7.4 (0.16 M ammonium chloride, 0.01 M potassium bicarbonate, and 0.1 mM sodium-edetate) for 5 min to remove erythrocytes, after which cells were incubated for 1 h in RPMI 1640/10% at 37°C. Nonadherent cells were harvested, and neutrophils were separated by discontinuous Percoll gradient centrifugation (successively 81, 62, 55, 50, and 45% of Percoll). Percoll layers 1 and 5 in the gradient contained nonmyeloid cells, lipids, cellular debris, and erythrocytes, respectively. Percoll layers 2, 3, and 4 (hereafter labeled as P2, P3, and P4) comprised different neutrophil maturation stages. Neutrophils from bone marrow samples were used directly after isolation.

Chromium release assay

^{51}Cr release assays were performed, as described earlier (30). Briefly, 1×10^6 target cells were incubated with 100 μCi of ^{51}Cr (Amersham) for 2 h at 37°C and washed three times. ^{51}Cr-labeled target cells were plated in 96-well round-bottom microtiter plates (5×10^3 cells/well) in the absence or presence of different concentrations of BsAb or mAb and RPMI 1640/10% containing 4×10^5 or 2×10^5 neutrophils (E:T ratio of 80:1 or 40:1) per well, respectively. After 4 h at 37°C, ^{51}Cr release in the supernatant was measured as cpm. Percentage of lysis of tumor cells was calculated as follows: (experimental cpm $-$ basal cpm)/(maximal cpm $-$ basal cpm) $\times$ 100%.

Real-time video-recording assay

SK-BR-3 cells (5×10^4) were cultured for 2 days in RPMI 1640/10% in a 24-well plate. Next, neutrophils (5×10^5) were added together with BsAb (1 μg/ml), and video recording was performed for 2 h with an inverted phase-contrast microscope (Nikon Eclipse TE300) in a humidified, 7% CO_2 gassed, temperature-controlled (37°C) chamber. A randomly selected field of 220 $\times$ 200 μm was recorded at a speed of 168 images per second using a color video camera (Sony; including a CMAD2 adapter) coupled to a time-lapse video recorder (Sony; SVT S3050P). Percentage of SK-BR-3 cells that had detached after 2 h (indicative of cell death) was determined.

Collagen culture assay

Collagen was isolated from rat tails and dissolved in 96% acetic acid (2 mg/ml). MilliQ, 0.34M NaOH, and DMEM (10×) (Sigma-Aldrich) were mixed (1:1:1), after which 2.3 ml was added to 10 ml of collagen and 1.3 ml of SK-BR-3 cells (5×10^5/ml) on ice. This final mixture was plated in 24-well plates (1 ml/well) and allowed to coagulate, after which 1 ml of RPMI 1640/10% was added. Cultures were grown for 2 wk to allow tumor colony formation, followed by addition of neutrophils in absence or presence of BsAb (0.5 or 1 μg/ml). After 24 h, collagen gels were washed with 150 mM NaAc, pH 5.0, containing india ink (1:100) (30 min), fixed overnight at room temperature with zinc salts-based fixative (0.5 g/L calcium acetate, 5.0 g/L zinc acetate, 5.0 g/L zinc chloride in 0.1 M Tris buffer) (31), and embedded in paraffin.

Immunohistochemistry

Paraffin slides were deparaffinized in ethanol, and endogenous peroxidase was blocked with 0.3% H_2O_2 in methanol (30 min, room temperature). Nonspecific binding was blocked by incubation with 10% normal rabbit serum (15 min, room temperature). Neutrophils were stained with a mouse anti-human CD66b mAb (BD Pharmingen), followed by biotinylated rabbit anti-mouse IgM Ab (Zymed Laboratories) and HRP-labeled streptavidin (Zymed Laboratories). The 3.3-diaminobenzidine was used as substrate (Sigma-Aldrich), resulting in a brown staining. Slides were counterstained with Mayer's hematoxylin (Klinipath), after which they were embedded in Entallan (Merck). The number of neutrophils that migrated into tumor colonies and the percentage of tumor colonies that contained more than one neutrophil were quantified.

Cytospins were stained with Diff-Quick, according to manufacturer's instructions (Dade Behring).

Binding assay

Neutrophils and SK-BR-3 cells were labeled with PKH-67, a FITC-fluorescent membrane marker, or PKH-26, a PE-fluorescent membrane marker, respectively, according to manufacturer's instructions (Sigma-Aldrich). Labeled neutrophils and SK-BR-3 tumor cells were incubated with or without BsAb (1 μg/ml) for 30 min at 4°C in RPMI 1640/10% at different E:T ratios. Binding was analyzed on a FACScan (BD Biosciences).

Calcium mobilization assay

Neutrophils were labeled with 1,5-(and-6)-carboxy seminaphtorhodafluor-1-acetoxymethyl ester (SNARF-1) (2.8 μM) and Fluo-3 (1.4 μM) (Molecular Probes) for 30 min at 37°C, after which cells were washed and incubated with anti-FcαRI (A77) or anti-FcγRI (m22) mAb (10 μg/ml) for 30 min at 4°C. Cells were washed twice and resuspended in calcium mobilizing buffer. Intracellular free calcium levels after cross-linking FcR

with $F(ab')_2$ of goat anti-mouse IgG1 Ab (Southern Biotechnology Associates) were analyzed on a FACScan. The first 20 s of each run, before cross-linking, were used to establish baseline intracellular calcium levels.

MAPK phosphorylation assay

Neutrophils were labeled with anti-FcαRI (A77) or anti-FcγRI (m22) mAb (10 μg/ml) for 30 min at 4°C. After washing, FcR were cross-linked with $F(ab')_2$ of goat anti-mouse IgG1 Ab (Southern Biotechnology Associates) at 37°C for different time points (varying between 0 and 60 s). Ice-cold PBS was added to stop reactions, after which samples were boiled in reducing sample buffer, run on 10% SDS-PAGE gels, and electrotransferred to nitrocellulose membranes (0.45 μm; Millipore). Membranes were blocked with 5% BSA (Roche Diagnostic Systems) and probed with anti-phospho-p44/42 MAPK or anti-total MAPK Ab for 2 h (Cell Signaling Technology). Following washing, membranes were further incubated for 1 h with peroxidase-conjugated goat anti-rabbit Ab (Pierce). Staining was visualized using the ECL detection system (Amersham).

Statistical analysis

Data are shown as mean ± SD. Group data are shown as mean ± SEM. Statistical differences were determined using two-tailed unpaired Student's *t* test or ANOVA. Significance was accepted when $p < 0.05$.

Results

Neutrophil ADCC

Mature neutrophils express FcαRI, but only low levels of FcγRI (16) (Fig. 1*A*). To compare FcαRI- and FcγRI-mediated Ab therapy, neutrophils were therefore stimulated with IFN-γ (IFN-γ neutrophils), which enhanced FcγRI expression (Fig. 1*B*). Additionally, neutrophils from G-CSF-stimulated healthy donors (G-CSF neutrophils) were collected, and FcαRI and FcγRI expression was assessed (Fig. 1*C*). IFN-γ neutrophils, as well as G-CSF neutrophils from 64% of

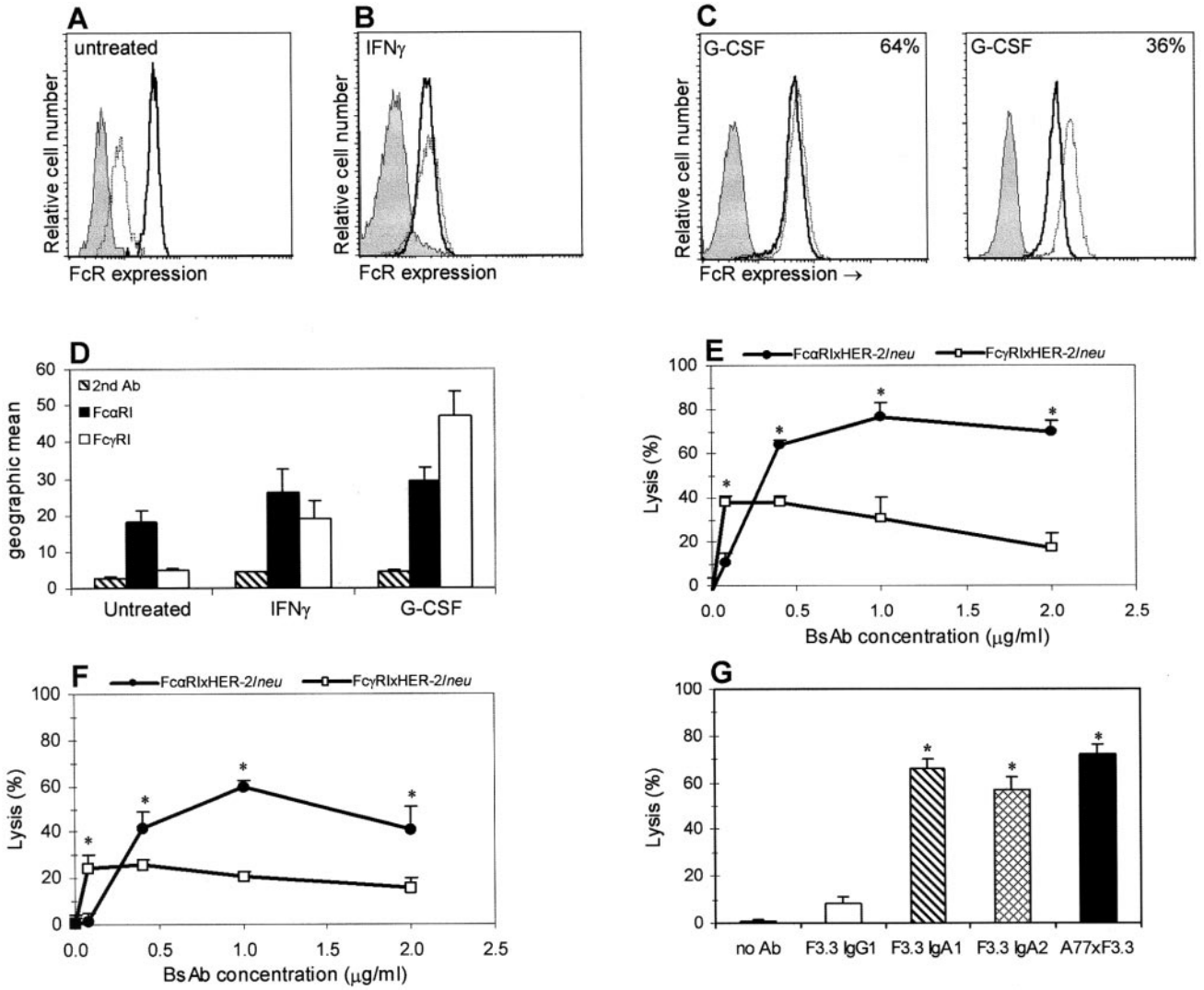

FIGURE 1. Neutrophil-mediated ADCC of SK-BR-3 and RAJI cells. Surface expression of FcαRI (bold line) and FcγRI (thin line) determined by flow cytometry on isolated untreated (*A*), IFN-γ (*B*), and G-CSF neutrophils (*C*). FcαRI and FcγRI expression level on G-CSF neutrophils was similar in 64% of donors (*C*, *left plot*). Neutrophils from 36% of G-CSF-treated donors had slightly higher FcγRI expression compared with FcαRI expression (*C*, *right plot*). Neutrophils were stained with A77 (FcαRI) or m22 (FcγRI), followed by incubation with FITC-labeled goat anti-mouse IgG. The filled area represents secondary Ab only. *D*, Mean fluorescent indexes (geographic mean ± SEM) of secondary Ab (▨), FcαRI (■), and FcγRI (□) expression on untreated, IFN-γ, and G-CSF neutrophils. Lysis of SK-BR-3 tumor cells by IFN-γ (*E*) or G-CSF neutrophils (*F*) in the presence of increasing concentrations of FcαRI × HER2/neu (A77 × 520C9; ●) or FcγRI × HER2/neu (22 × 520C9; □) BsAb. Chromium release from triplicates was measured, and data are expressed as mean ± SD. One representative experiment of 4 or of 11 is shown, respectively. *G*, Lysis of RAJI cells by G-CSF neutrophils in the presence of anti-HLA class II (F3.3) IgG1 (□), IgA1 (▨), IgA2 (▩) mAb, or FcαRI × HLA class II BsAb (A77 × F3.3; ■) (2 μg/ml). Data are presented as mean percentage lysis ± SEM from six individual experiments. ∗, $p < 0.05$ compared with FcγR.

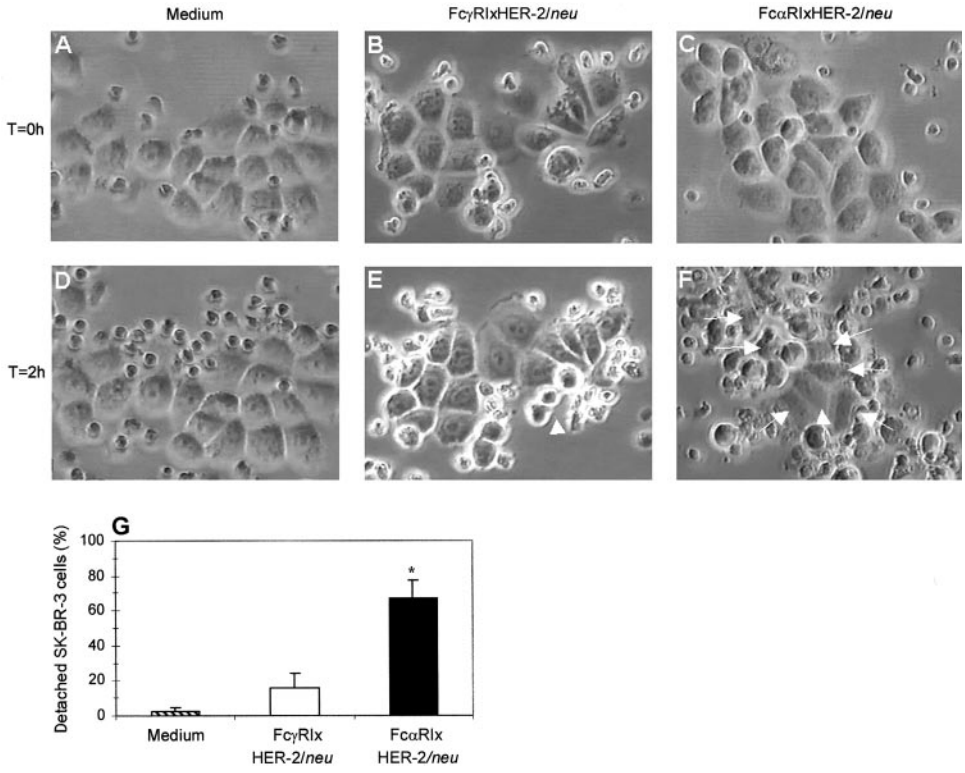

FIGURE 2. Real-time video recording of BsAb-induced SK-BR-3 killing by neutrophils. G-CSF neutrophils (smaller cells) were added to adherently growing SK-BR-3 cells (larger cells) in the absence (*A* and *D*) or presence of 1 μg/ml FcγRI $\times$ HER-2/neu (*B* and *E*) or FcαRI $\times$ HER-2/neu (*C* and *F*) BsAb. Interactions between cells were recorded for 2 h. Time points 0 (*A–C*) and 2 h (*D–F*) are shown. *E*, SK-BR-3 cells that were detached in presence of FcγRI $\times$ HER-2/neu BsAb are indicated by arrowheads. *F*, In the presence of FcαRI $\times$ HER-2/neu BsAb, high numbers of SK-BR-3 cells were detached. SK-BR-3 cells that were unaffected by neutrophils are indicated by arrows. *G*, Percentage of SK-BR-3 cells that were detached by neutrophils in the absence (▨) or presence of FcγRI $\times$ HER-2/neu (□) or FcαRI $\times$ HER-2/neu (■) BsAb after 2 h. A representative experiment of three is shown. Data represent mean $\pm$ SD. $*$, $p < 0.05$ compared with FcγRI $\times$ HER-2/neu BsAb.

the donors (Fig. 1*C*, *left plot*), showed similar FcαRI and FcγRI expression levels. Neutrophils from 36% of G-CSF-treated donors had higher FcγRI expression levels (Fig. 1*C*, *right plot*), resulting in a slight increase in overall FcγRI expression compared with FcαRI (Fig. 1*D*).

Levels of neutrophil-mediated tumor cell lysis varied greatly between donors. However, triggering of FcαRI on IFN-γ neutrophils via FcαRI $\times$ HER-2/neu (A77 $\times$ 520C9) BsAb consistently resulted in higher SK-BR-3 tumor cell lysis, compared with FcγRI targeting (MDX-H210) (Fig. 1*E*). Mean tumor cell lysis in the presence of 1 μg/ml FcαRI or FcγRI BsAb was 94 $\pm$ 16% and 38 $\pm$ 11%, respectively ($n = 4$). In ~75% of G-CSF donors, ADCC of SK-BR-3 tumor cells, mediated by G-CSF neutrophils, was higher via triggering FcαRI compared with FcγRI (Fig. 1*F*). Mean tumor cell lysis in the presence of 1 μg/ml BsAb was 52 $\pm$ 13% and 26 $\pm$ 11% for targeting FcαRI and FcγRI, respectively ($n = 8$). No difference in tumor cell lysis was observed using neutrophils from ~25% of G-CSF donors (targeting FcαRI or FcγRI resulted in 42 $\pm$ 17% or 46 $\pm$ 28% tumor cell lysis at 1 μg/ml BsAb, respectively; $n = 3$). In the presence of low BsAb concentrations (0.08 μg/ml), neutrophil-mediated tumor cell killing was more efficient via FcγRI BsAb. Maximal FcγRI-mediated tumor cell lysis, however, never reached the levels that were obtained upon engagement of FcαRI. A further increase of BsAb concentration did not lead to higher levels of tumor cell killing, which is most likely due to saturation of both neutrophils and tumor cells at high BsAb concentrations, hereby interfering with efficient neutrophil-tumor cell interactions. At low E:T ratios, neutrophils from all donors were less efficient in initiating tumor cell killing via FcγRI BsAb compared with FcαRI BsAb, including at low BsAb concentrations (data not shown). Cross-linking of neither FcαRI nor FcγRI by anti-FcR mAb resulted in tumor cell killing, hereby ruling out any bystander killing of tumor cells as a result of neutrophil degranulation (data not shown).

We next examined whether the observed differences in tumor cell killing could be reproduced when other tumor Ags were targeted. We therefore examined FcαRI- and FcγR-mediated tumor cell killing of RAJI B cell lymphoma cells that express HLA class II. Both anti-HLA class II (F3.3) IgA1 and IgA2 mAb triggered lysis of RAJI cells more efficiently than anti-HLA class II (F3.3) IgG1 mAb (Fig. 1*G*). Furthermore, FcαRI $\times$ HLA class II BsAb (A77 $\times$ F3.3) was equally effective in mediating tumor cell killing as IgA mAb.

Real-time video recording of neutrophil-mediated tumor cell killing

To visualize differences in neutrophil-mediated ADCC between FcαRI and FcγRI in time, a real-time video-recording assay was established. In the absence of BsAb, G-CSF neutrophils accumulated around SK-BR-3 cells, but were not activated (characterized by round-shaped neutrophils). Furthermore, no tumor cell killing was observed (Fig. 2, *A*, *D*, and *G*). In the presence of FcγRI $\times$ HER-2/neu BsAb, as well as FcαRI $\times$ HER-2/neu BsAb, irregularly shaped neutrophils (reflecting activation) bound to SK-BR-3 cells. Minimal detachment of SK-BR-3 cells, indicative of cell death (32), was observed in the presence of FcγRI $\times$ HER-2/neu BsAb (Fig. 2, *B*, *E*, and *G*, and supplemental movie 1).[4] Addition of FcαRI $\times$ HER-2/neu BsAb, however, resulted in significantly higher numbers of detached SK-BR-3 cells (Fig. 2, *C*, *F*, and *G*, and supplemental movie 2).

Neutrophil migration toward tumor colonies

A three-dimensional (3D) collagen culture assay was set up to study migration of G-CSF neutrophils in the presence of BsAb toward tumor cell colonies. Random neutrophil migration into collagen was present in the absence of BsAb, but no noticeable interactions with tumor cells were found (Fig. 3, *A* and *D*). Although migration into tumor colonies was observed in presence of different concentrations of FcγRI $\times$ HER-2/neu BsAb (Fig. 3, *B* and *D*,

[4] The online version of this article contains supplemental material.

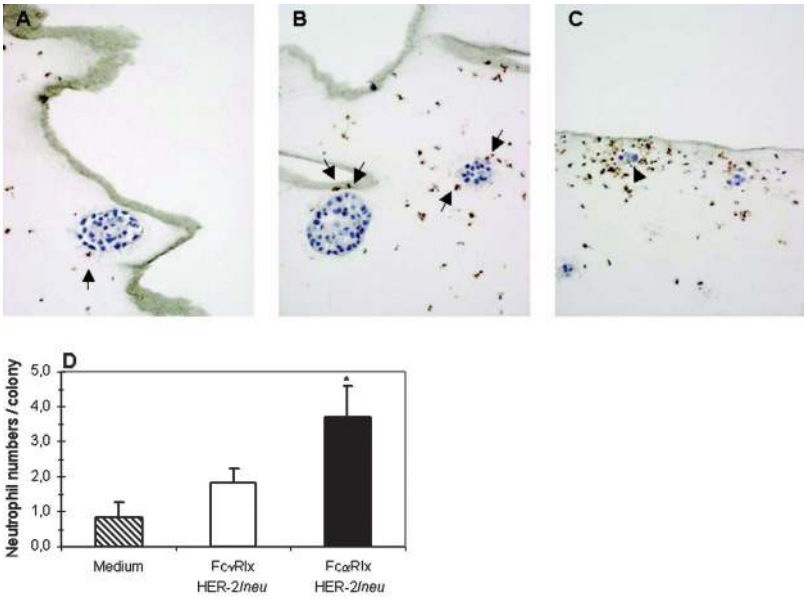

FIGURE 3. BsAb-induced neutrophil migration toward tumor colonies. G-CSF neutrophils were added to SK-BR-3 tumor colonies in collagen, either in the absence (*A*) or presence of 0.5 μg/ml FcγRI × HER-2/neu (*B*) or FcαRI × HER-2/neu (*C*) BsAb. Collagen was fixed and slides were stained for CD66b (neutrophils, brown). Neutrophils attached to tumor colonies are indicated in *A* and *B* by arrows. In *C*, remnants of a SK-BR-3 tumor colony are marked by an arrowhead. *D*, Numbers of neutrophils per colony in the absence (▨) or presence of FcγRI × HER-2/neu (□) or FcαRI × HER2/neu (■) BsAb. Results represent mean ± SEM from three individual experiments. ∗, $p < 0.05$, compared with FcγRI × HER-2/neu BsAb.

and data not shown), addition of FcαRI × HER-2/neu BsAb resulted in significantly higher neutrophil numbers that accumulated in and around SK-BR-3 tumor colonies (Fig. 3, *C* and *D*). In addition, the percentage of positive tumor colonies (containing more than one neutrophil) was also higher in presence of FcαRI × HER-2/neu BsAb, compared with FcγRI × HER-2/neu BsAb (89 ± 10%, compared with 45 ± 7%, respectively; $n = 3$). Furthermore, only FcαRI × HER-2/neu, but not FcγRI × HER-2/neu BsAb induced tumor colony destruction (Fig. 3*C*).

FcαRI- and FcγRI-mediated signaling in neutrophils

We studied binding of neutrophils to tumor cells in the presence of BsAb to exclude poorer binding via FcγRI as a factor of importance in the observed difference in tumor cell killing. Therefore, fluorescent cells were incubated at 4°C, and binding of neutrophils to SK-BR-3 tumor cells in the presence of BsAb was studied. However, higher levels of neutrophil-tumor cell interactions were observed at varying E:T ratios in the presence of FcγRI × HER-2/neu BsAb, compared with FcαRI × HER-2/neu BsAb (Fig. 4, *A* and *B*). Similar results were obtained when longer times of incubation (up to 3 h) or other BsAb concentrations were used (0.5–2 μg/ml) (data not shown).

Next, signaling via both FcR was studied in calcium mobilization and MAPK phosphorylation assays. After cross-linking either FcαRI or FcγRI, levels of intracellular calcium started rising after 10 s. However, cross-linking of FcαRI resulted in maximal intracellular calcium mobilization after 25 s, whereas calcium mobilization peaked 55 s after FcγRI cross-linking. Furthermore, cross-linking of FcαRI or FcγRI led to rapid MAPK phosphorylation, which was detected within 15 s after cross-linking of either FcαRI or FcγRI (Fig. 4*D*). The quantity of phosphorylation, however, was consistently higher upon triggering FcαRI, compared with FcγRI. Thus, triggering of FcαRI resulted in faster and more robust signaling compared with FcγRI.

Cytolytic capacity of bone marrow neutrophils

G-CSF is frequently used in cancer patients to enhance blood neutrophil numbers as it mobilizes neutrophils from the bone marrow (12). Therefore, we next assessed the capacity of immature bone marrow neutrophils to initiate ADCC. Neutrophils were isolated from human bone marrow using a Percoll discontinuous density gradient, resulting in separation of neutrophil precursors into three distinct populations (Fig. 5, *A–I*). Neutrophils from the second Percoll layer (P2 neutrophils) contained early neutrophil precursors, which are defined by intermediate CD11b and low CD16 expression (Fig. 5*B*), as well as round- to kidney-shaped nuclei (Fig. 5*C*). P3 neutrophils were immature band neutrophils, with intermediate CD11b and heterogeneous CD16 expression (Fig. 5*E*). Nuclei were horseshoe shaped (Fig. 5*F*). Percoll layer P4 was mainly composed of mature neutrophils (P4 neutrophils), which had high CD16 expression levels (Fig. 5*H*) and a segmented nucleus (Fig. 5*I*). FcαRI expression was low on P2 neutrophils, but expression levels increased during neutrophil maturation, whereas P2 neutrophils had high FcγRI expression, which decreased during development (Fig. 5, *A*, *D*, and *G*).

The cytolytic capacity of these neutrophil populations was evaluated with standard chromium release assays. P2 neutrophils were unable to initiate SK-BR-3 killing (data not shown; $n = 3$), which is presumably due to low granule levels. P4 neutrophils exhibited efficient cytolytic capacity, but had low FcγRI expression levels, hereby excluding them from further studies in which FcαRI and FcγRI function was compared. P3 neutrophils showed similar expression levels of FcαRI and FcγRI, and were used as effector cells (Fig. 5*J*). Targeting FcαRI resulted in efficient lysis of SK-BR-3 cells. Targeting of P3 neutrophils via FcγRI × HER-2/neu BsAb, however, did not lead to tumor cell killing (mean tumor cell lysis was 34 ± 25% (1 μg/ml) or 74 ± 39% (2 μg/ml) after targeting to FcαRI, and 0 ± 1% or 0 ± 2% (in the presence of either 1 or 2 μg/ml) on targeting FcγRI; $n = 4$). Moreover, the anti-HER-2/neu mAb Herceptin was not able to initiate P3 neutrophil-mediated SK-BR-3 cell lysis either, indicating that all FcγR, which are expressed on immature neutrophils, were ineffective in mediating ADCC (data not shown; $n = 8$). Similar data were observed in collagen culture assays, in which P3 neutrophils migrated efficiently into SK-BR-3 tumor colonies in the presence of FcαRI × HER-2/neu BsAb, whereas targeting to FcγRI did not result in interactions between neutrophils and tumor colonies (Fig. 5*K*).

Ex vivo triggering of FcαRI and FcγRI on mouse blood and bone marrow cells

Syngeneic animal models provide important tools for unraveling mechanisms of Ab therapy, provided they mirror the human situation. FcαRI × FcγRI double-Tg mice were previously described

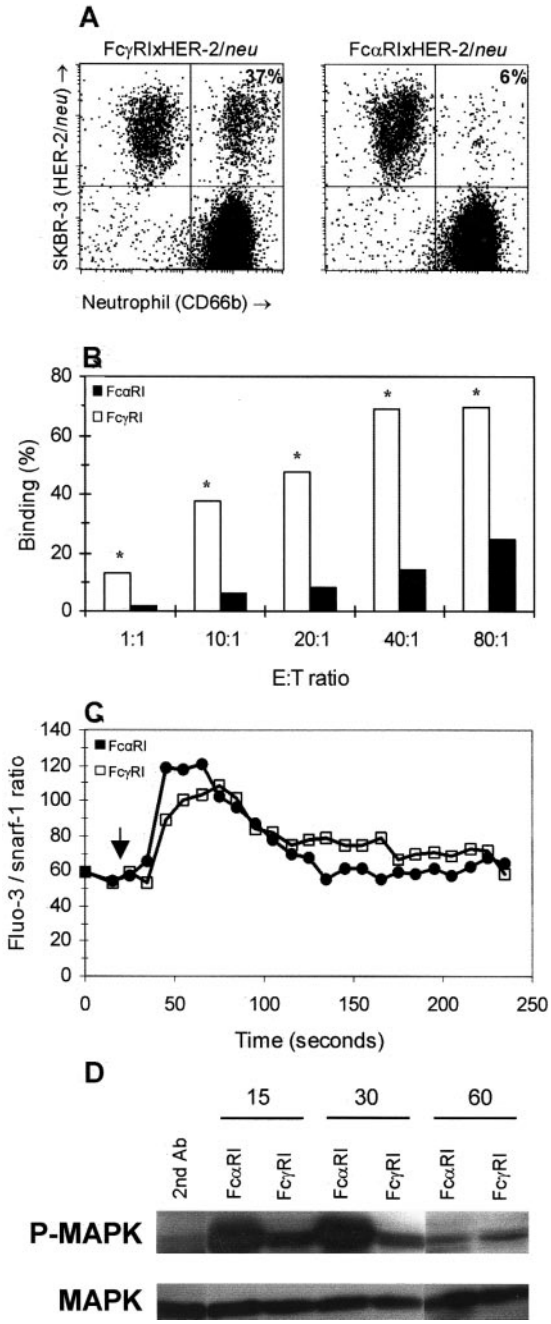

FIGURE 4. FcαRI- and FcγRI-mediated signaling in neutrophils. *A*, SK-BR-3 cells and G-CSF neutrophils were stained with red (PKH26) and green (PKH67) fluorescent labels, respectively, and binding (double-positive cells) was analyzed after incubation at 4°C for 30 min with 1 μg/ml FcγRI × HER-2/neu (*left panel*) or FcαRI × HER-2/neu (*right panel*) BsAb. *B*, Percentage binding in presence of FcγRI × HER-2/neu (□) or FcαRI × HER-2/neu (■) BsAb was determined at varying E:T ratios. Experiments were repeated four times, yielding essentially similar results. *, $p < 0.05$. *C*, Intracellular free calcium levels were measured after cross-linking FcαRI (●) or FcγRI (□). Neutrophils were incubated with anti-FcαRI (A77) or anti-FcγRI (m22) mAb, and baseline calcium levels (Fluo-3/SNARF-1 ratio) were established for 20 s, after which a cross-linking Ab was added (arrow). Calcium mobilization assays were repeated three times, yielding similar results. *D*, After incubation of neutrophils with anti-FcαRI (A77) or anti-FcγRI (m22) mAb, FcR were cross-linked with a secondary Ab for 15, 30, or 60 s. As a negative control, unlabeled neutrophils were incubated with secondary Ab only. Samples were boiled with reducing sample buffer and analyzed on a Western blot with anti-phospho-p44/42 MAPK Ab. The membrane was stripped and reprobed with an anti-total MAPK Ab as an indicator of protein loading. MAPK phosphorylation assays were repeated three times, yielding similar results.

(25) and express human FcαRI constitutively on mature neutrophils, whereas human FcγRI expression can be induced by treatment with G-CSF, which is comparable to humans. To study whether the observed differences between FcαRI- and FcγRI-mediated ADCC could be extrapolated to FcαRI × FcγRI double-Tg mice, mouse blood and bone marrow cells were collected and evaluated in functional studies.

After G-CSF treatment, FcαRI expression level on blood neutrophils was slightly higher compared with FcγRI (Fig. 6*A*). Bone marrow neutrophils from untreated mice showed no difference in expression levels (Fig. 6*B*). Similar to human blood neutrophils, SK-BR-3 tumor cell lysis by mouse blood cells was higher in the presence of FcαRI × HER-2/neu BsAb, compared with FcγRI × HER-2/neu BsAb (Fig. 6*C*). Mean tumor cell lysis in the presence of 1 μg/ml BsAb was 84 ± 16% or 36 ± 4% after targeting to FcαRI or FcγRI, respectively ($n = 3$). Additionally, efficient killing of SK-BR-3 cells was observed upon engagement of FcαRI on mouse bone marrow cells, whereas SK-BR-3 cell lysis was absent in the presence of FcγRI × HER-2/neu BsAb or anti-HER-2/neu mAb, which is identical with results obtained with human cells (Fig. 6*D* and data not shown). Mean tumor cell lysis was 34 ± 11% (1 μg/ml) or 53 ± 18% (2 μg/ml) on targeting FcαRI, and 0 ± 2% or 0 ± 1% (in the presence of either 1 or 2 μg/ml) after targeting to FcγRI ($n = 3$).

Discussion

Neutrophils have previously been proposed as attractive effector cell population for Ab therapy, because they represent the most populous FcR-expressing leukocyte subset in blood and their numbers can be easily increased. It was demonstrated that FcαRI represents the most potent FcR on neutrophils for induction of ADCC, which has been shown for a variety of tumor Ags, including the epidermal growth factor receptor, HLA class II, CD20, CD30, HER-2/neu, and epithelial cell adhesion molecule (EpCAM) (24, 26, 33–37). In our studies, maximal tumor cell killing was higher upon targeting FcαRI, both in ^{51}Cr release assays and real-time video recordings, although FcγRI-mediated tumor cell killing was somewhat higher in the presence of low BsAb concentrations. This is presumably due to the higher number of neutrophil-tumor cell interactions in the presence of FcγRI BsAb compared with FcαRI BsAb. At low BsAb concentrations, the number of FcαRI-mediated neutrophil-tumor cell interactions may be insufficient to reach the threshold necessary for induction of tumor cell killing, as tumor cell lysis was absent at E:T ratios lower than 10:1 (data not shown). The difference in FcR-mediated binding of neutrophils and tumor cells is presently unclear as receptor expression levels, as well as affinities of the used anti-FcR mAb were similar. Differences in FcR distributions within the cell membrane of neutrophils might represent a possible explanation, as FcγRI has recently been observed to constitutively reside in so-called lipid rafts, whereas membrane FcαRI is only partially raft localized (38–40). This dissimilarity in cell membrane distribution may influence the accessibility for BsAb and tumor cells. We furthermore observed that effectiveness of IgG mAb differed greatly between donors (data not shown), which is most likely linked to a polymorphism in the extracellular domain of FcγRIIa, located at aa position 131, as it was shown that neutrophils from FcγRIIa-H/H131 donors were significantly less effective in triggering Ab-dependent apoptosis than neutrophils isolated from FcγRIIa-R/R131 donors (11, 30).

Importantly, FcαRI was the only FcR that consistently induced neutrophil migration toward tumor cells in 3D collagen culture assays, which led to destruction of tumor colonies. This was observed after targeting Her-2/neu on SK-BR-3 mamma-carcinoma

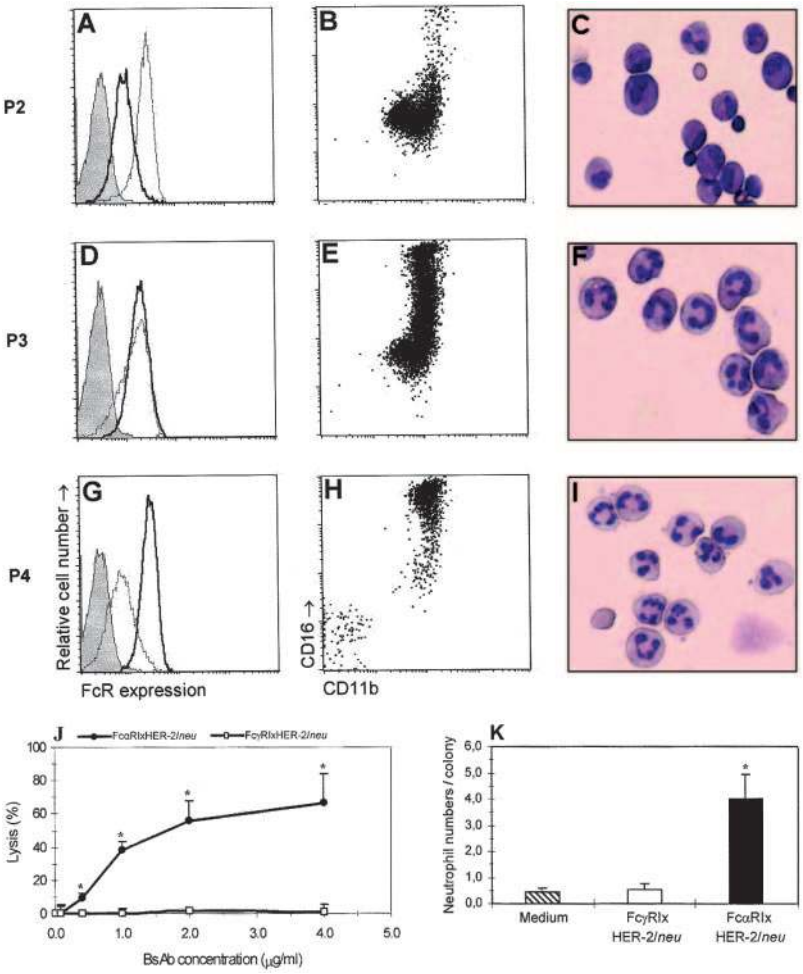

FIGURE 5. Bone marrow neutrophils as effector cells for tumor cell killing. Bone marrow neutrophils were separated into three neutrophil maturation stages, labeled immature P2 (*A–C*), intermediate P3 (*D–F*), and more mature P4 (*G–I*) neutrophils. FcαRI (bold lines) and FcγRI (thin lines) expression levels (*A*, *D*, and *G*) were measured by flow cytometry (filled areas represent secondary Ab only). Expression of CD11b (FITC) and CD16 (PE) (*B*, *E*, and *H*) and cell morphology (*C*, *F*, and *I*) were used to confirm maturation state of bone marrow neutrophils. *J*, Lysis of SK-BR-3 cells by P3 neutrophils (E:T ratio 80:1) in the presence of increasing amounts of FcαRI × HER-2/neu (●) or FcγRI × HER-2/neu (□) BsAb. Data represent mean ± SD of triplicate samples. One representative experiment of four is shown. *K*, Numbers of P3 neutrophils per SK-BR-3 tumor colony in the absence (▨) or presence of FcγRI × HER-2/neu (□) or FcαRI × HER-2/neu (■) (1 μg/ml). Results represent mean ± SEM from three individual experiments. ∗, $p < 0.05$.

cells as well as targeting EpCAM on colon carcinoma SW948 tumor cells with anti-EpCAM mAb (Fig. 3, and data not shown). FcγRI BsAb proved ineffective in inducing neutrophil migration in the 3D collagen. Furthermore, release of IL-8, which is the prototypic neutrophil chemokine, was only observed in the presence of FcαRI BsAb, which may explain the higher migration of neutrophils toward tumor colonies (data not shown).

Interestingly, signaling via FcαRI is believed to be mediated via similar signaling routes that are also used by other FcR, and requires association with the common FcR γ-chain. Earlier work showed that effector functions such as ADCC by either FcγRI or FcαRI were dependent on the ITAM signaling motifs within the FcR γ-chain (41, 42). Several phenomena might explain the observed differences between FcαRI- and FcγRI-mediated tumor cell killing. First, FcαRI and FcγRI may initiate different killing mechanisms by neutrophils. Boiling of cytoplasm and membrane blebbing of tumor cells, indicative of apoptosis (32), were observed in our real-time video-recording experiments after addition of neutrophils and FcαRI × Her-2/neu BsAb, which is in concordance with earlier data in which neutrophil-mediated apoptosis of human breast cancer cells was demonstrated after targeting FcαRI (11). Second, FcαRI strongly associates with the FcR γ-chain, based on an additional electrostatic interaction within the transmembrane regions, which may trigger enhanced neutrophil activation (43). Third, FcαRI may initiate additional signaling pathways, as it has been shown that a subpopulation of FcαRI is expressed on neutrophils without associated FcR γ-chain (44). FcαRI might thus interact with an, up until now, unidentified molecule. Our observation that FcαRI cross-linking results in a more rapid rise in intracellular free calcium and higher levels of MAPK phosphorylation (Fig. 4, *C* and *D*) supports the notion that FcαRI initiates more efficient signaling pathways.

Because G-CSF mobilizes immature neutrophils from the bone marrow (12), we also investigated ADCC capacity of bone marrow neutrophils. Only FcαRI BsAb proved capable of triggering tumor cell killing, whereas FcγRI BsAb were ineffective. It was previously shown that maximal simultaneous triggering of FcαRI and FcγRI in IFN-γ neutrophils led to decreased FcγRI-mediated tumor cell killing (25). This suggests that receptors may compete for available FcR γ-chain, a phenomenon that has also been observed for FcεRI and FcγRIII in mast cells (45). It was furthermore demonstrated that FcR γ-chain is required for stable FcγRI expression in IIA1.6 transfectants, as expression was lost over time in the absence of FcR γ-chain (41). The observation that FcγRI expression decreases during neutrophil maturation suggests that FcγRI is not associated with FcR γ-chain. Neutrophils were reported to express relatively low FcR γ-chain levels, compared with monocytes (46). It is therefore possible that limited availability of FcR γ-chain in immature neutrophils results in a favorable association with FcαRI due to a stronger electrostatic interaction, explaining the inability to induce tumor cell killing via FcγRI. Additionally, bone marrow neutrophils proved unable to induce ADCC via IgG Ab. FcγRIIa, which is involved in neutrophil-mediated ADCC, bears an ITAM signaling motif within its cytoplasmic tail, and as such can convey its own signaling irrespective of the FcR γ-chain. It has been demonstrated although that association of FcγRIIa with FcR

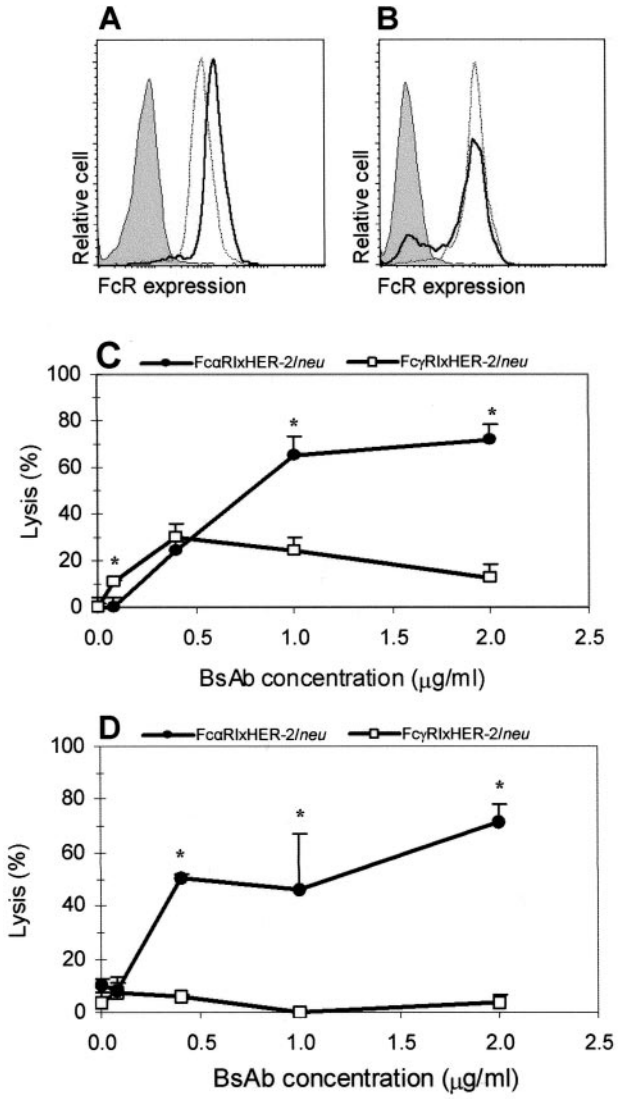

FIGURE 6. Ex vivo triggering of FcαRI and FcγRI on mouse blood and bone marrow cells. Expression levels of FcαRI (bold line) and FcγRI (thin line) were determined on neutrophils from G-CSF-treated mice (*A*) and bone marrow cells from untreated mice (*B*) (filled area represents secondary Ab only). Lysis of SK-BR-3 cells by mouse blood cells (*C*) or mouse bone marrow cells (*D*) in the presence of FcαRI × HER-2/neu (●) or FcγRI × HER-2/neu (□) BsAb. Data are expressed as mean ± SD of triplicates. Experiments were repeated three times, yielding similar results. *, $p < 0.05$.

γ-chain was required for initiation of Ag presentation and cytokine production (47). It is therefore conceivable that FcγRIIa-mediated ADCC depends on interaction with the FcR γ-chain as well.

Another explanation for the differences in neutrophil-mediated tumor cell killing might be due to interactions of FcαRI or FcγRI with other interacting proteins, as Beekman et al. (48) recently described that periplakin was involved in FcγRI-mediated ligand binding and function. Differences in periplakin expression in immature neutrophils might therefore affect FcγRI-mediated ADCC. Alternatively, as FcαRI can be expressed without the FcR γ-chain (44), other proteins might additionally interact with this FcR in immature neutrophils, which may circumvent the FcR γ-chain dependency for its effector functions. Further research on this topic is necessary to clarify differences between FcαRI- and FcγR-mediated tumor cell killing.

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Disclosures

The authors have no financial conflict of interest.

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