Human IgG2 Antibodies against Epidermal Growth Factor Receptor Effectively Trigger Antibody-Dependent Cellular Cytotoxicity but, in Contrast to IgG1, Only by Cells of Myeloid Lineage

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Ab-dependent cellular cytotoxicity (ADCC) is usually considered an important mechanism of action for immunotherapy with human IgG1 but not IgG2 Abs. The epidermal growth factor receptor (EGF-R) Ab panitumumab represents the only human IgG2 Ab approved for immunotherapy and inhibition of EGF-R signaling has been described as its principal mechanism of action. In this study, we investigated effector mechanisms of panitumumab compared with zalutumumab, an EGF-R Ab of the human IgG1 isotype. Notably, panitumumab was as effective as zalutumumab in recruiting ADCC by myeloid effector cells (i.e., neutrophils and monocytes) in contrast to NK cell-mediated ADCC, which was only induced by the IgG1 Ab. Neutrophil-mediated tumor cell killing could be stimulated by myeloid growth factors and was triggered via FcγRIIa. Panitumumab-mediated ADCC was significantly affected by the functional FcγRIIa-R131H polymorphism and was induced more effectively by neutrophils from FcγRIIa-R131H homozygous donors than from -131R individuals. This polymorphism did not affect neutrophil ADCC induced by the IgG1 Ab zalutumumab. The in vivo activity of both Abs was assessed in two animal models: a high-dose model, in which signaling inhibition is a dominant mechanism of action, and a low-dose model, in which effector cell recruitment plays a prominent role. Zalutumumab was more effective than panitumumab in the high-dose model, reflecting its stronger ability to induce EGF-R downmodulation and growth inhibition. In the low-dose model, zalutumumab and panitumumab similarly prevented tumor growth. Thus, our results identify myeloid cell-mediated ADCC as a potent and additional mechanism of action for EGF-R–directed immunotherapy. The Journal of Immunology, 2010, 184: 512–520.

Targeted therapy with tyrosine kinase inhibitors or mAbs offers the potential to improve the selectivity of cancer therapy. The epidermal growth factor receptor (EGF-R) constitutes an attractive molecule for both approaches, because it constitutes a membrane-expressed tyrosine kinase, which is critically involved in the malignant transformation of many solid tumors (1). Indeed, both classes of EGF-R–directed therapeutics have obtained regulatory approval (2). Compared with tyrosine kinase inhibitors, which selectively block tumor cell signaling, mAbs possess the potential advantage of recruiting immune effector mechanisms, such as complement-dependent cytotoxicity (CDC) and Ab-dependent cellular cytotoxicity (ADCC), as additional mechanisms of tumor cell killing (3, 4). Among the mechanisms of action for cancer Abs, ADCC seems to be particularly relevant for rituximab (5), but its contribution for the efficacy of EGF-R Abs is less established (6). Recruitment of immune effector mechanisms by mAbs, such as ADCC, is critically dependent on Ab isotypes (7, 8). Within the IgG subclass, human IgG1 is particularly effective in triggering CDC and ADCC by NK cells and is therefore predominantly used for antitumor immunotherapy (4). Most IgG1 Abs proved to be weak activators of ADCC by polymorphonuclear cells (PMNs), which could effectively kill tumor cells when triggered by IgA Abs (9, 10). Human IgG2 Abs have been selected for immunotherapy in indications for which the recruitment of immune effector mechanisms was not desired (11). However, human IgG2 binds to FcγRIIa, which is widely expressed by myeloid cells, including monocytes/macrophages and PMNs (12, 13). This interaction between IgG2 and FcγRIIa is affected by a common R/H polymorphism at position 131 of the receptor (14–16).

Two EGF-R Abs have Food and Drug Administration approval: cetuximab, a mouse/human chimeric IgG1 Ab, and panitumumab, a human IgG2 Ab (17, 18). Novel EGF-R Abs, such as the human IgG1 Ab zalutumumab, for which mononuclear cell (MNC)-induced ADCC has been described as an important mechanism of action, are being actively investigated (19–21). These observations prompted us to investigate the induction of MNC- and PMN-induced...
ADCC by IgG1 and IgG2 EGF-R–directed Abs in detail. We found that panitumumab did not trigger ADCC by NK cells, in contrast to Abs of the IgG1 isotype. Interestingly, zalutumumab and panitumumab induced ADCC with monocytes and PMN effectors. Neutrophil-mediated killing proved to be triggered via FcγRIIa, was affected by the functional FcγRIIa polymorphism for the IgG2 Ab, and could be enhanced by myeloid growth factors.

Materials and Methods

Experiments reported in this paper were approved by the Ethical Committee of the Christian-Albrechts-University in accordance with the Declaration of Helsinki.

Blood donors and isolation of effector cells

For effector cell isolation, 100 ml peripheral blood were drawn from healthy volunteers after written informed consent was obtained. Briefly, citrate-anticoagulated blood was layered over a discontinuous Percoll (Strom, Berlin, Germany) gradient consisting of 70% and 62% Percoll. After centrifugation, MNCs (mainly consisting of NK cells, B and T lymphocytes, and monocytes) were collected from the plasma–Percoll interface, and neutrophils were collected from the interface between the two Percoll layers. Remaining erythrocytes were removed by hypotonic lysis. The purity of neutrophils was determined by forward/light scatter analysis (Coulter EPICS XL-MCL, Beckman Coulter, Fullerton, CA), and the viability of cells was tested by trypan blue exclusion; both were >95%. NK cells and monocytes were further isolated from MNCs by negative or positive (CD14) selection, respectively, using magnetic beads according to the manufacturer’s instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated monocytes, tested by CD14-immunofluorescence, and NK cells, tested by CD56-immunofluorescence, was >95%. Donor blood was analyzed for the FcγRIIIa119H and FcγRIIa-V158F genotypes. Extracted genomic DNA was analyzed using commercial available Taqman SNP genotyping assays (Applied Biosystems, Foster City, CA), according to the instructions of the manufacturer.

Abs

Panitumumab (E7.6.3, Vectibix), a human IgG2 Ab against EGF-R, was purchased from Amgen (Thousand Oaks, CA). A human IgG1 EGF-R Ab (zalutumumab, ZA) (human IgG2 Ab with variable Cγ2 Fc domain) and human IgG1 control Ab against keyhole limpet hemocyanin (HuMab-KLH) were produced at Genmab (Utrecht, The Netherlands). Alexa-647–labeled HuMab-KLH Ab was prepared using a standard Alexa-647 Fluor labeling procedure (Invitrogen Life Technologies, Carlsbad, CA).

N-linked glycosylation of panitumumab and zalutumumab was analyzed by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), as described previously (22). Briefly, IgG was digested with N-glycosidase F (Roche Diagnostics, Mannheim, Germany) to release the N-linked glycans. Samples were centrifuged at 13,000 × g for 10 s before analysis by HPAEC-PAD. HPAEC-PAD analysis was performed on a 2-mm–internal diameter CarboPac PAI column (Dionex, Sunnyvale, CA) with a CarboPac PA100 guard, using a linear elution gradient of 0 to 175 mM sodium acetate in 150 mM sodium hydroxide at a flow rate of 1 ml/min. The PAD occurred with the quadrupole, and peaks were integrated with ChromelSoftware (Dionex). Seventy micrograms digest were loaded for each profile. The N-linked glycosylation profiles of panitumumab and zalutumumab were similar and had the following composition: panitumumab, 93.9% fucose, 39.7% galactose, and 0.7% sialic acid, and zalutumumab, 89.7% fucose, 24.9% galactose, and 0.7% sialic acid (data not shown).

Culture of eukaryotic cells

Human glioblastoma cell line A1207 (originally established by Dr. Aaronson, National Cancer Institute, National Institutes of Health, Bethesda, MD) and human epidermoid carcinoma cell line A431 (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) were cultured in DMEM or RPMI 1640 (Invitrogen Life Technologies), respectively. Media were supplemented with 10% heat-inactivated FCS, 100 U/ml penicillin, 100 U/ml streptomycin, and 4 mM L-glutamine (all from Invitrogen Life Technologies). Cell viability was tested by trypan blue exclusion.

Flow cytometric analyses

For indirect immunofluorescence, 2 × 10⁵ target cells were washed in PBS supplemented with 1% BSA (Sigma-Aldrich, St Louis, MO) and 0.1% sodium azide (FACS buffer). Cells were then incubated with the respective Abs for 30 min on ice. After washing with 500 µl PBS buffer, cells were stained with FITC-labeled polyclonal goat anti-human Ab (Dako Cyto- mation, Glostrup, Denmark). After a final wash, cells were analyzed on a flow cytometer (Coulter EPICS XL-MCL).

FcyR expression on unstimulated PMNs was determined by indirect immunofluorescence with murine Abs 22 (FcγRI), 13 (FcγRIIa), and 3G8 (FcγRIIIa; all from Medarex, Princeton, NJ) after blocking of FcyR with polyclonal human IgG (Bayer Schering Pharma, Berlin, Germany). Surface Abs per cell were calculated using the Qubit Kit (Dako Cytomation) according to the manufacturer’s instructions, and were 757 ± 192 for FcγRI, 14,739 ± 1,685 for FcγRIIa, and 133,826 ± 36,269 for FcγRIII (n = 7; data not shown).

FcyRIIb expression on PMNs was measured after blocking FcγR with rabbit serum by indirect immunofluorescence using human FcγRIIb–specific Ab 2B6 (23). Cells were stained with FITC-labeled polyclonal goat anti- human Ab (Dako Cytomation) and analyzed on a flow cytometer (Coulter EPICS XL-MCL). No FcγRIIb expression was detectable on the surface of this effector cell population (n = 2; data not shown).

EGF-R phosphorylation inhibition assay

EGF-R phosphorylation inhibition was measured in a two-step assay. Cells were cultured overnight in 96-well plates in serum-free medium containing 0.5% human albumin (human albumin 20%; Sanquin, Amsterdam, The Netherlands). Next, mAbs were added in serial dilution ranging from 0–2.5 µg/ml. After a 60-min incubation at 37°C, 50 ng/ml recombinant human EGF (Invitrogen Life Technologies) were added to induce activation of nonblocked EGF-R. Following an additional 30-min incubation, cells were solubilized with lysis buffer (Cell Signaling Technology, Beverly, MA), and the lysates were transferred to ELISA plates coated with 1 µg/ml mouse anti-EGF-R Abs (mAb EGFRI1; BD Pharmingen, San Diego, CA). After a 2-h incubation at room temperature, the plates were washed and binding of phosphorylated EGF-R was detected by using Alexa-647–labeled mouse mAb specific for phosphorylated tyrosines (mAb Eu-N1-P-Tyr-100; PerkinElmer, Waltham, MA). Finally, DELFIA enhancement solution was added, and time-resolved fluorescence (TRF) was measured by exciting at 315 nm and measuring emission at 615 nm on an EnVision plate reader (PerkinElmer).

EGF-R downmodulation assay

Cells were cultured overnight in 96-well white culture plates (Greiner Bio- One, Frickenhausen, Germany). The next day, Ab solutions were added in triplicate and incubated for 4 h at 37°C. Cells were placed on ice, and cell surface EGF-R was labeled with mouse anti–EGF-R Abs (mAb EGFRI1) in FACS buffer for 30 min at 4°C. Subsequently, cells were washed twice with FACS buffer and stained with europium-conjugated goat anti-mouse Abs (PerkinElmer). Finally, DELFIA enhancement solution was added, and TRF was measured by exciting at 315 nm and measuring emission at 615 nm on an EnVision plate reader. The percentage of EGF-R downmodulation was calculated with the following formula: percentage downmodulation = 1 − (TRF Ab treatment/TRF culture medium control treatment) × 100.

Cell growth inhibition assay

Tumor cell growth in vitro was evaluated, as described previously (24), by measuring vital cell mass using an AlamarBlue assay. Briefly, EGF-R mAb dilutions were added to cell cultures in 96-well flat-bottom tissue culture plates. Plates were incubated at 37°C, and AlamarBlue-labelling solution (Invitrogen Life Technologies) was added directly (time = 0 d) or after 96 h of incubation (time = 4 d). After 4 h of incubation at 37°C, the fluorescence was measured at 530 nm excitation wavelength and 590 nm emission wavelength in a plate reader (Bio-Tek Synergy HT Multidetection mi- croplate reader, Beun de Ronde, Abcoude, The Netherlands).

ADCC assays

ADCC assays against ⁵¹Cr-labeled target cells were performed as described previously (9). Briefly, whole blood or isolated effector cells (MNCs or PMNs), sensitizing Abs at various concentrations, and medium were added to 96-well flat-bottom microtiter plates (Nunc, Rochester, NY). Assays were started by adding effector and target cells at an E:T ratio of 80:1. After 3 h at 37°C, ⁵¹Cr release from triplicate experiments was measured as cpm. The percentage of cellular cytotoxicity was calculated using the following formula: percentage of specific lysis = (experimental cpm − basal cpm)/(maximal cpm − basal cpm) × 100; maximal ⁵¹Cr release was determined by adding perchloric acid (3% final concentration) to target cells, and basal release was measured in the absence of Abs and effector cells. Ab-independent cytotoxicity (effectors without target Abs) was observed in whole blood assays and with MNCs but not with PMNs. ADCC assays with isolated monocytes were performed at an E:T ratio of 40:1 and an...
incubation time of 12 h; those with isolated NK cells were performed at an E:T ratio of 10:1 and an incubation time of 3 h. For analysis of FeR

volvement, F(ab')2 fragments of the FcyRII inhibiting Ab AT10 (Serotec, Düsseldorf, Germany) or control Ab Ox55 (CD2) were added at 10 μg/ml before adding target cells. Ox55 F(ab')2 fragments were kindly provided by Prof. Martin Glennie (Tennis Research Laboratories, Southampton, U.K.). Percent inhibition was calculated with the following formula: percentage of inhibition = (percentage of lysis without blocking Ab) - (percentage of lysis with blocking Ab)/(% lysis without blocking Ab) × 100.

TRF resonance energy transfer

Binding of mAb to FcRs was measured using a competitive TRF resonance energy transfer (TR-FRET) assay. FcγRla, FcγRIla-131R/H, FcγRIIb, FcγRIIa-158VF, and FcγRIIb-Na1/Na2 were cloned, expressed, and purified, as described previously (22). Soluble FcγR proteins comprised the extracellular domains with a 6 × histidine-tag at their C termini. Briefly, 10 μl 2 μg/ml soluble FcγR protein were mixed with 10 μl 0.6 μg/ml Eu-W1024–labeled mouse (IgG1) anti-6 × histidine Abs for LANCE assays (PerkinElmer). Next, 10 μl a serial dilution of test mAb diluted in LANCE detection buffer (PerkinElmer), 0.2% BSA, and 0.03% Tween-20 was added. Finally, 10 μl Alexa-647–conjugated HuMab-KLH Ab diluted in LANCE detection buffer was added (final concentration FcγRla 1.25 μg/ml, FcγRIla-131R/H, FcγRIIb, and FcγRIIa-158VF/6 μg/ml and FcγRIIb-Na1/Na2 15 μg/ml), resulting in a 40 μl final volume. The reaction mix was incubated in a polystyrene 96-well plate (Greiner Bio-One) on a plate shaker overnight at 4°C. The next day, 10 μl reaction mix was transferred to a 384-well (Greiner Bio-One) white plate, and TRF was measured by exciting at 315 nm and measuring emission at 665 nm on an EnVision plate reader.

In vivo mouse models

SCID mice (C.B.-17/lcrCr/scid/scid) were purchased from Charles River (Maastricht, The Netherlands), and all experiments were performed with 8–12-wk-old female mice. Mice were housed in a barrier unit of the Central Laboratory Animal Facility (Utrecht, The Netherlands) and kept in filter-top cages with sterile water and food provided ad libitum. Mice participating in the experiments were checked thrice weekly for clinical signs of disease and discomfort. All experiments were approved by the Utrecht University animal ethics committee. Subcutaneous tumors were induced by s.c. inoculation of 5 × 10^6 A431 cells in the right flank of mice. Tumor volumes were calculated from digital caliper measurements as 0.52 × length × width^2 (in mm^3). Experimental lung metastases were induced by injecting 1 × 10^7 luciferase-transfected A431 cells (A431-luc, clone L18) into the tail vein. Tumor growth was assessed at weekly intervals using bioluminescence imaging of the back view of the mice. Before imaging, mice were anesthetized by i.p. injection of a mix of ketamine/xylazine/atropine. Synthetic δ-luciferin (Biothera, Handen, Sweden) was given i.p. at a dose of 125 mg/kg. Mice were then placed backside up in the imager; 10 min after luciferin administration, imaging was started using a VersArray 1300B CCCD camera (Roper Scientific, Vianen, The Netherlands). Light was detected in a photon-counting manner over an exposure period of 5 min. Black and white images were taken under illumination for anatomical reference. Metavue software (Universal Imaging, Downingtown, PA) was used for data collection and image analysis. The total photon count from the lung area was taken as the measure of tumor burden. MAbs were injected i.p. as indicated in the Results. During the study, two heparinized blood samples were taken for determination of human mAb plasma concentrations, which were in the expected range (data not shown).

Data processing and statistical analyses

Data are displayed graphically and were analyzed statistically using GraphPad Prism 4.0 (GraphPad, San Diego, CA). Experimental curves were fitted using a four-parameter nonlinear regression model with a sigmoidal dose response (variable slope). Group data are reported as mean ± SEM, unless otherwise indicated. Differences between groups were analyzed by unpaired (or, when appropriate, paired) Student t tests or one-way ANOVA. Significance was accepted when p values were < 0.05.

Results

Mechanisms mediated by Ab binding to EGF-R

Zalutumumab and panitumumab have distinct, but overlapping epitopes on the extracellular domain of EGF-R (24, 25). To compare mechanisms mediated by the binding of these Abs to EGF-R (Fab-mediated mechanisms), we analyzed binding to A431 tumor cells that overexpress human EGF-R [1.8 × 10^6 EGF-R molecules/cell (26)], Growth inhibition of A431 cells by zalutumumab and panitumumab was determined by flow cytometry. Data represent means ± SEM of four independent experiments. B, Zalutumumab and panitumumab mediated inhibition of phosphorylation of EGF-R expressing A431 cells was measured using an EGF-R phosphorylation inhibition assay. Results represent means ± SE of four independent experiments. C, Downmodulation of EGF-R cell surface expression by zalutumumab and panitumumab was determined in A431 cells using a TRF assay. D, Growth inhibition of A431 cells by zalutumumab and panitumumab was determined by measuring conversion of AlamarBlue into a fluorescent product. The fluorescence was determined with a microplate reader at 530 nm excitation wavelength and 590 nm emission wavelength and represented as mean fluorescence intensity (MFI). Results represent means ± SEM of three independent experiments. *, Significant difference (p < 0.05).
By indirect immunofluorescence, the binding characteristics of both Abs proved to be similar, with EC$_{50}$ of 1.3 μg/ml (95% confidence interval [CI], 0.8–2.0 μg/ml) and 0.8 μg/ml (95% CI, 0.5–1.4 μg/ml; n = 4) for zalutumumab and panitumumab, respectively (Fig. 1A).

Because panitumumab and zalutumumab are known to inhibit ligand-induced receptor phosphorylation, we measured inhibition of the phosphorylation status of A431 cells in a TRF assay. In this model, both Abs proved to be equally effective (zalutumumab: EC$_{50}$: 0.72 μg/ml; 95% CI, 0.67–0.77 μg/ml; panitumumab: EC$_{50}$: 0.62 μg/ml; 95% CI, 0.56–0.68 μg/ml; n = 4) (Fig. 1B).

Further, Ab induced downmodulation of cell surface-expressed EGF-R was determined using a plate-based TRF assay. Zalutumumab proved to be more potent than panitumumab with respect to downmodulation of EGF-R (downmodulation at 1 and 10 μg/ml, respectively: zalutumumab: 35.3%; 95% CI, 20.4–50.3% and 34.7%; 95% CI, 27.1–42.3%; panitumumab: 9.0%; 95% CI, 6.5–11.5% and 8.7%; 95% CI, –3.1–20.4%; n = 3) (Fig. 1C). Additionally, A431 cell proliferation was inhibited more effectively by zalutumumab (IC$_{50}$: 0.27 μg/ml; 95% CI, 0.16–0.46 μg/ml; top plateau 41.7% ± 3.1%), whereas panitumumab activated MNCs only weakly (EC$_{50}$: 0.042 μg/ml; 95% CI, 0.006–0.277 μg/ml; top plateau 13.7% ± 1.7%; n = 5) (Fig. 2A). Interestingly, however, zalutumumab and panitumumab induced similar tumor cell killing by PMNs (Fig. 2B; zalutumumab: EC$_{50}$: 0.299 μg/ml; 95% CI, 0.121–0.740 μg/ml; top plateau 20.8% ± 2.7%; panitumumab: EC$_{50}$: 0.122 μg/ml; 95% CI, 0.013–1.212 μg/ml; top plateau 18.3% ± 2.2%; n = 4). Thus, panitumumab triggered effective killing by PMNs but not by MNCs, whereas zalutumumab recruited both effector cell populations. To further analyze effector mechanisms for low levels of panitumumab-mediated ADCC with MNCs, we also performed ADCC experiments with isolated NK cells and monocytes. NK cells were significantly activated by zalutumumab (EC$_{50}$: 0.007 μg/ml; 95% CI, 0.001–0.05 μg/ml; top plateau 15.0% ± 1.8%; n = 3), whereas killing by panitumumab was not different from that with a nonbinding control Ab (Fig. 2C). Interestingly, panitumumab and zalutumumab effectively recruited isolated monocytes for cell killing (Fig. 2D; zalutumumab: EC$_{50}$: 0.06 μg/ml; 95% CI, 0.006–0.56 μg/ml; top plateau 33.4% ± 4.4%; panitumumab: EC$_{50}$: 0.04 μg/ml; 95% CI, 0.004–0.31 μg/ml; top plateau 37.7% ± 4.5%; n = 3). To assess the contribution of the various effector cell populations, we then investigated tumor cell killing in whole blood assays. With unfractionated whole blood as effector source, both Abs induced the capacity of panitumumab and zalutumumab to trigger ADCC against A431 cells. Isolated MNCs and PMNs served as effector cells. As expected, the IgG1 Ab zalutumumab triggered ADCC with MNCs effectively (EC$_{50}$: 0.006 μg/ml; 95% CI, 0.001–0.023 μg/ml; top plateau 41.7% ± 3.1%), whereas panitumumab activated MNCs only weakly (EC$_{50}$: 0.042 μg/ml; 95% CI, 0.006–0.277 μg/ml; top plateau 13.7% ± 1.7%; n = 5) (Fig. 2A). ADCC is considered an important mechanism of action for EGF-R Abs, which is supported by clinical data demonstrating the influence of FcγR polymorphisms on the outcome of cetuximab therapy (26, 27). Because panitumumab is of human IgG2 isotype, this Ab is considered inactive in ADCC. Therefore, we compared the capacity of panitumumab and zalutumumab to trigger ADCC against A431 cells. Isolated MNCs and PMNs served as effector cells. As expected, the IgG1 Ab zalutumumab triggered ADCC with MNCs effectively (EC$_{50}$: 0.006 μg/ml; 95% CI, 0.001–0.023 μg/ml; top plateau 41.7% ± 3.1%), whereas panitumumab activated MNCs only weakly (EC$_{50}$: 0.042 μg/ml; 95% CI, 0.006–0.277 μg/ml; top plateau 13.7% ± 1.7%; n = 5) (Fig. 2A). Interestingly, however, zalutumumab and panitumumab induced similar tumor cell killing by PMNs (Fig. 2B; zalutumumab: EC$_{50}$: 0.299 μg/ml; 95% CI, 0.121–0.740 μg/ml; top plateau 20.8% ± 2.7%; panitumumab: EC$_{50}$: 0.122 μg/ml; 95% CI, 0.013–1.212 μg/ml; top plateau 18.3% ± 2.2%; n = 4). Thus, panitumumab triggered effective killing by PMNs but not by MNCs, whereas zalutumumab recruited both effector cell populations. To further analyze effector mechanisms for low levels of panitumumab-mediated ADCC with MNCs, we also performed ADCC experiments with isolated NK cells and monocytes. NK cells were significantly activated by zalutumumab (EC$_{50}$: 0.007 μg/ml; 95% CI, 0.001–0.05 μg/ml; top plateau 15.0% ± 1.8%; n = 3), whereas killing by panitumumab was not different from that with a nonbinding control Ab (Fig. 2C). Interestingly, panitumumab and zalutumumab effectively recruited isolated monocytes for cell killing (Fig. 2D; zalutumumab: EC$_{50}$: 0.06 μg/ml; 95% CI, 0.006–0.56 μg/ml; top plateau 33.4% ± 4.4%; panitumumab: EC$_{50}$: 0.04 μg/ml; 95% CI, 0.004–0.31 μg/ml; top plateau 37.7% ± 4.5%; n = 3). To assess the contribution of the various effector cell populations, we then investigated tumor cell killing in whole blood assays. With unfractionated whole blood as effector source, both Abs induced

**FIGURE 2.** Panitumumab and zalutumumab differ in effector cell recruitment for ADCC. ADCC by panitumumab or zalutumumab was analyzed in chromium-release assays against A431 cells using isolated MNCs (A), PMNs (B), NK cells (C), monocytes (D), or unfractionated whole blood (E). Efficient lysis by panitumumab was obtained with unfractionated whole blood, PMNs, and monocytes but not with unfractionated MNCs and isolated NK cells. Data presented are means ± SEM of five experiments for A and E, four experiments for B, and three experiments for C and D. Significance was accepted when $p < 0.05$. *, significant lysis compared with control mAb HuMab-KLH; #, significant difference between panitumumab and zalutumumab.
similar killing levels (Fig. 2E; zalutumumab: EC\textsubscript{50}: 0.064 mg/ml; 95% CI, 0.006–0.745 mg/ml; top plateau 38.1% ± 8.3%; panitumumab: EC\textsubscript{50}: 0.068 mg/ml; 95% CI, 0.020–0.226 mg/ml; top plateau 26.8% ± 3.3%; n = 5), demonstrating that MNCs and PMNs contribute to the ADCC activity in human blood.

Then, we investigated whether panitumumab-mediated ADCC by PMNs was restricted by the cell line. As demonstrated in Fig. 3A, killing with panitumumab by PMNs proved to be even greater against A1207 (top plateau 39.2% ± 5.0%; n = 9) than against A431 cells; the former is a glioblastoma cell line with high EGF-R expression (1.7 \times 10^6 EGF-R molecules/cell) (25). To exclude that PMN recruitment was specific for panitumumab, we next compared a human IgG2 switch variant of 2F8 IgG2 with panitumumab. As shown in Fig. 3B, both human IgG2 EGF-R Abs mediated significant ADCC via PMNs, indicating that IgG2-mediated killing via EGF-R is not restricted to panitumumab. To further evaluate the impact of panitumumab-mediated ADCC, we investigated the influence of in vivo G-CSF treatment in whole blood assays (Fig. 3C). G-CSF is routinely administered in certain clinical settings to increase the number and functional activity of PMNs in peripheral blood. With unfractionated whole blood from healthy donors, only low levels of A1207 cell killing by panitumumab were observed (top plateau 14.8% ± 2.4%), whereas killing was markedly enhanced with whole blood from G-CSF primed donors (top plateau 39.2% ± 5.0%; n = 3). At an Ab concentration of 10 µg/ml, the difference in killing between unstimulated and G-CSF–primed blood was significant.

**FcγR involvement and influence of the FcγRIIA-R131H polymorphism**

To determine FcγR involvement in PMN-mediated ADCC, killing of A1207 tumor cells was analyzed in the presence of F(ab\textsuperscript{9})\textsubscript{2}-fragments against FcγRII (AT10) or against a control Ag (Ox55, both at 10 µg/ml). A1207 cells served as targets, and “% of maximum lysis” was calculated as described in Materials and Methods. Data presented are means ± SEM of four experiments. * Significant (p < 0.05) inhibition of ADCC. The influence of the FcγRII-R131H polymorphism on ADCC of A1207 cells via panitumumab (B) or zalutumumab (C) was investigated by comparing -131H/H or -131R/R homozygous donors in PMN-mediated ADCC assays. Data are presented as means ± SEM of five (panitumumab) or three (zalutumumab) independent experiments. * Significant (p < 0.05) difference between both allotypes.
the control Ab did not inhibit ADCC (Fig. 4A), indicating that panitumumab and zalutumumab kill via FcγRIa. Because binding of human IgG2 Abs to FcγRIIa is known to be influenced by the FcγRIIa-R131H polymorphism, we compared FcγRIIa-131H/H and -131R/R homozygous donors in panitumumab-mediated ADCC by PMNs (Fig. 4B). Killing levels obtained with PMNs from 131-H/H homozygous donors (top plateau 58.5% ± 2.6%) were significantly higher than those from -131-R/R homozygous donors (top plateau 48.8% ± 3.1%; n = 5). As expected, we did not observe differences in killing levels between these FcγRIIa allotypes with zalutumumab (Fig. 4C; n = 3).

Next, we analyzed binding characteristics of panitumumab and zalutumumab to soluble FcγR by TR-FRET binding assays (Fig. 5). The IgG2 Ab panitumumab demonstrated stronger binding to FcγRIIa-131H (IC₅₀: 22.3 μg/ml; 95% CI, 8.2–36.4 μg/ml; n = 3) than to FcγRIIa-131R (IC₅₀: 126 μg/ml; 95% CI, 108–160 μg/ml; n = 3), whereas the IgG1 Ab zalutumumab bound to both allotypes similarly. In contrast, panitumumab required very high concentrations for binding to FcγRIIIa-158V and -158F, whereas zalutumumab bound slightly more strongly to FcγRIIIa-158V than to -158F at therapeutic Ab concentrations. Further, panitumumab did not bind to FcγRIIb or both allotypes of FcγRIIIb, whereas zalutumumab demonstrated strong binding to FcγRIIb (IC₅₀: 0.3 μg/ml; 95% CI, 0.03–2.6 μg/ml; n = 3) and weak binding to FcγRIIIb (FcγRIIIb-NA1, IC₅₀: 64 μg/ml; 95% CI, 10–416 μg/ml; FcγRIIIb-NA2, IC₅₀: 72 μg/ml; 95% CI, 15–362 μg/ml; n = 3). Finally, an affinity difference for FcγRIIb was observed between zalutumumab (IC₅₀: 55 μg/ml; 95% CI, 14–225 μg/ml; n = 3) and panitumumab (IC₅₀: 362 μg/ml; 95% CI, 33–3989 μg/ml; n = 3).

Low-dose antitumor activity of EGF-R Abs

To further elucidate the in vivo relevance of our observations for zalutumumab and panitumumab, we tested both Abs in two different A431 xenograft models in mice (Fig. 6). Previous studies showed that blockade of signaling is required for maximum antitumor effects of zalutumumab, which only occurred at high Ab doses, giving complete receptor saturation (19). In small tumor xenografts, we also observed antitumor effects at low Ab doses, which correspondingly were most likely mediated by immune effector functions rather than blockade of EGF-R. First, we compared the effects of zalutumumab and panitumumab at a high dose level in a model with established s.c. A431 tumors. In contrast to zalutumumab, panitumumab had a limited effect on tumor growth, suggesting that blocking of signaling by panitumumab was less effective (Fig. 6A). Next, we made a comparison in an experimental metastasis model whereby a single dose was applied shortly after the i.v. inoculation of A431-luc cells. Zalutumumab and panitumumab were protective at very low doses in this model (i.e., 1 μg/mouse s.c., corresponding
or 10

KLH was used as control mAb. Data are means by panitumumab. Interestingly, however, zalutumumab and pan-

previous studies, MNCs were effectively recruited by zalutumumab which often requires longer-duration assays. As expected from

Fc

g
killing target cells after activation. Monocytes express Fc

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IgG2 was demonstrated to bind to Fc

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RIIa and clear their targets predominantly by phagocytosis, which often requires longer-duration assays. As expected from previous studies, MNCs were effectively recruited by zalutumumab in classic 3-h chromium-release assays, but only to a minor degree by panitumumab. Interestingly, however, zalutumumab and pan-

Discussion
In this study, we investigated effector mechanisms of the two EGF-R Abs panitumumab and zalutumumab, which are of human IgG2 or IgG1 isotypes, respectively. Binding studies on A431 cells demon-

on day 15, 17, 23, and 30 at a dose of 1 mg/mouse, i.p. B. Experimental metastasis model in which tumors were

used s.c. inoculation of 5 3 10^6 A431 cells in three groups of nine mice. For treatment, Abs were given

in vivo effect at dose levels, which are expected to provide only partial receptor blockade and partial inhibition of phosphorylation (Fig. 1), suggests that induction of immune ef-

to ~50 µg/kg and subsaturating peak serum levels <0.5 µg/ml). The strong in vivo effect at dose levels, which are expected to provide only partial receptor blockade and partial inhibition of phosphorylation (Fig. 1), suggests that induction of immune effector functions plays a role in the antitumor effects in this model.

First, we examined MNCs, which mainly contain NK cells and monocytes as cytotoxic FcγR-bearing effector cell populations. NK cells constitutively express FcγRIIa and are capable of rapidly killing target cells after activation. Monocytes express FcγRI and FcγRIIa and clear their targets predominantly by phagocytosis, which often requires longer-duration assays. As expected from previous studies, MNCs were effectively recruited by zalutumumab in classic 3-h chromium-release assays, but only to a minor degree by panitumumab. Interestingly, however, zalutumumab and pan-

FIGURE 6. Antitumor effects in mouse A431 xenograft models. A. Subcutaneous model in which tumors were induced by s.c. inoculation of 5 3 10^6 A431 cells in three groups of nine mice. For treatment, Abs were given

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50 µg/kg and subsaturating peak serum levels <0.5 µg/ml). The strong in vivo effect at dose levels, which are expected to provide only partial receptor blockade and partial inhibition of phosphorylation (Fig. 1), suggests that induction of immune effector functions plays a role in the antitumor effects in this model.

Importantly, we could not detect FcγRI on PMNs for ADCC to be a general characteristic of this Ab isotype. A contribution of PMNs to the clinical activity of therapeutic Abs has been postulated but not proven (28, 29). So far, combination ther-

518 ADCC BY HUMAN IgG2 Abs

A

Tumor size (mm^3)

0 250 500 750

Panitumumab

2

Zalutumumab

control mAb

Dosing 50 mg/kg

B

Bioluminescence (AU)

control mAb

Panitumumab 0.05 mg/kg

Zalutumumab 0.05 mg/kg

Panitumumab 0.5 mg/kg

Zalutumumab 0.5 mg/kg

Dosing

Days after inoculation

5

10

15

20

25

30

35

40

45

50

55

60

65

70

Panitumumab triggered effective ADCC by PMN effector cells (Fig. 2). Panitumumab triggered ADCC by PMNs against different target cell lines, and PMNs were also effective with an IgG2 isotype switch variant of zalutumumab (Fig. 3). Previously, we demonstrated that chimeric IgG2 Abs against HLA class II triggered ADCC by PMNs against lymphoma cells (9), indicating that IgG2 Abs can trigger PMN-mediated tumor cell killing via different target Ags. Together, these findings suggest the capacity of human IgG2 Abs to recruit PMNs for ADCC to be a general characteristic of this Ab isotype. A contribution of PMNs to the clinical activity of therapeutic Abs has been postulated but not proven (28, 29). So far, combination ther-

panitumumab bound more effectively to FcγRIIa-131H than to -131R, but it required very high concentrations for binding to both FcγRIIa-V158F allotypes. In contrast, the FcγRIIa-R131H polymorphism did not influence zalutumumab’s binding characteristics. Because both Abs proved to also bind to FcγRIIb, we

were interested in the potential role of ADCC by PMNs as an ef-

these findings suggest the capacity of human IgG2 Abs to recruit PMNs for ADCC to be a general characteristic of this Ab isotype. A contribution of PMNs to the clinical activity of therapeutic Abs has been postulated but not proven (28, 29). So far, combination ther-

because EGF-R Abs are particularly potent in recruiting PMN ef-

Blocking studies revealed FcγRIIa to be the FcγR on PMNs responsible for ADCC by panitumumab and zalutumumab. The interaction between human IgG2 and FcγRIIa is affected by a common R/H polymorphism at position 131 of the receptor, whereas binding of human IgG1 was not affected by this poly-

were presented addressing this issue experimentally. In contrast, human IgG2 Abs were repeatedly demonstrated to trigger effective CDC and ADCC, whereas human IgG2 Abs are often regarded as relatively inert in their Fc functions (7, 11). Therefore, this latter isotype has been chosen by some investigators, when Fc-

mediated effector functions were not desired. Accordingly, nu-

were interested in the potential role of ADCC by PMNs as an ef-

First, we examined MNCs, which mainly contain NK cells and monocytes as cytotoxic FcγR-bearing effector cell populations. NK cells constitutively express FcγRIIa and are capable of rapidly killing target cells after activation. Monocytes express FcγRI and FcγRIIa and clear their targets predominantly by phagocytosis, which often requires longer-duration assays. As expected from previous studies, MNCs were effectively recruited by zalutumumab in classic 3-h chromium-release assays, but only to a minor degree by panitumumab. Interestingly, however, zalutumumab and pan-

Effectively, we observed that the binding differences for pan-

Because panitumumab-mediated ADCC proved to be triggered via FcγRIIa and because we consistently observed panitumumab
to mediate low but significant levels of ADCC with MNCs, we investigated the contribution of NK cells and monocytes individually. We used 12-h chromium-release assays to analyze killing by isolated monocytes, the FcγRIIa-expressing effector cell fraction of MNCs, which is regarded as an important effector cell population for Ab immunotherapy in vivo (5, 33, 34). Interestingly, panitumumab and zalutumumab recruited monocytes effectively for tumor cell killing (Fig. 2), expanding their therapeutic potential. In contrast, isolated NK cells were activated by zalutumumab but not by panitumumab.

Over the last few years, Ab glycosylation was demonstrated to significantly impact Fc-mediated effector mechanisms of IgG1 Abs (see review in Ref. 35). The presence or absence of fucosylation, in particular, was shown to affect FcγR binding and ADCC. For NK cells, low levels of fucose increased binding to FcγRIIa and, consequently, ADCC activity (see review in Ref. 35). To our knowledge, only two studies have addressed the influence of fucose on PMN activity (22, 36). In both studies, low-fucose IgG1 Abs bound to neutrophils’ FcγRIIIb with increased affinity, resulting in reduced ADCC (22) but increased phagocytic (36) capacity of PMNs. For particular, was shown to affect FcγR.

Relevant information was derived from studies that correlated Ab concentrations (19), these results suggested triggering FcγR-R binding and ADCC. For NK cell-mediated effector functions. Importantly, we found glycosylation profiles of zalutumumab and panitumumab to contain similar levels of fucosylation (see Materials and Methods), indicating that the observed differences in the ADCC efficacy of both Abs were related to their isotypes and not to differences in carbohydrate composition.

Animal studies have provided conflicting results concerning the relative contributions of Fab- and Fc-mediated effector mechanisms to the therapeutic efficacy of EGF-R Abs. One study reported that Ab E7.5.2, which is a nonblocking human IgG2 EGF-R Ab, had no inhibitory effect on A431 xenograft tumor growth (17). Importantly, EGF-R Abs have not been analyzed in FcR γ-chain knockout mice, which provided important insight into the contribution of FcγR-mediated mechanisms of action for other therapeutic Abs (38). However, the relative contribution of different mechanisms of action is also highly dependent on the type of the investigated tumor model, time point of treatment, and the Ab dose applied (19). Further, significant differences were reported between the human and mouse FcR system (38, 39). Mice do not express FcγRIIa. The functional equivalent of this receptor in the mouse is FcγRIV, which is expressed, similarly to the human FcγRIIa, on monocytes, neutrophils, and dendritic cells. Further, affinity differences to human IgG may exist between human and murine FcR. Thus, conclusions regarding the effector cells’ contribution to ADCC induced by human IgG in mouse models should be made with caution. Results from our in vivo studies demonstrated that panitumumab had limited efficacy in established tumors, suggesting that it is not a very effective inhibitor of signaling in vivo. Nevertheless, panitumumab and zalutumumab were able to protect animals at low doses in a metastasis model (Fig. 6). Using doses of 1 μg/mouse i.p. in this model, peak serum levels were predicted to remain <0.5 μg/ml shortly after injection, declining thereafter by monoexponential decay (40). Under these conditions, Ab levels in vivo were presumably too low to achieve effective EGF-R blockade (Fig. 1) (19). In addition, in previous studies, both EGF-R Abs, as single agents, did not induce CDC (25). Because ADCC operates already at lower Ab concentrations (19), these results suggested triggering FcγR-mediated effector functions to be relevant for the therapeutic activity of zalutumumab and panitumumab in vivo.

In the clinical setting, effector mechanisms of therapeutic tumor Abs are probably even more complex than in animal studies (38, 41), and their relative importance for ErbB Abs has been debated (20, 42). Relevant information was derived from studies that correlated therapeutic responses to certain FcγR polymorphisms. Interestingly, Musolino et al. (43) demonstrated that clinical outcomes after trastuzumab therapy correlated with the FcγRIIIa-158V and the FcγRIIa-131H alleles of the receptors. Similar observations were reported for cetuximab, another IgG1 EGF-R Ab (27). However, another study on cetuximab conflicted with the favorable impact of the FcγRIIIa-158V allele on clinical outcomes (26). Although the interpretation of these results is complicated by a potential linkage of FcγRIIa and FcγRIIIa alleles (44, 45), these results suggest ADCC to be a relevant mechanism of action for EGF-R Abs in vivo. Furthermore, the contribution of FcγRIIa alleles suggests myeloid cells as a significant effector cell population. Our in vitro results demonstrate that panitumumab also triggers significant ADCC by myeloid effector cells, but studies on the impact of FcγR polymorphisms for clinical outcomes after panitumumab therapy have not been reported.

In conclusion, important and unexpected differences were observed between ADCC induced by an IgG1 and IgG2 therapeutic Ab against EGF-R. Interestingly, the IgG1 Ab, zalutumumab, as well as the IgG2 Ab, panitumumab, mediated significant ADCC with myeloid effector cells in vitro, which may contribute to panitumumab’s efficacy in vivo. In contrast, only zalutumumab was able to induce potent ADCC through MNCs, most likely by effective triggering of NK cells via FcγRIIa. The functional FcγRIIa-R131H polymorphism described for PMNs was shown to affect killing by panitumumab but not zalutumumab, which acted independently on this polymorphism. In vivo, zalutumumab was more potent than panitumumab in a high-dose treatment in a xenograft model, for which signaling inhibition is a major mechanism of action (19). In contrast, the IgG1 and IgG2 Abs were similarly effective in a low-dose model, suggesting myeloid cell-mediated ADCC as a novel and significant in vivo mechanism of action for EGF-R–directed immunotherapy.

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Disclosures

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