Epidermal Growth Factor Receptor (EGFR) Antibody-Induced Antibody-Dependent Cellular Cytotoxicity Plays a Prominent Role in Inhibiting Tumorigenesis, Even of Tumor Cells Insensitive to EGFR Signaling Inhibition

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Ab-dependent cellular cytotoxicity (ADCC) is recognized as a prominent cytotoxic mechanism for therapeutic mAbs in vitro. However, the contribution of ADCC to in vivo efficacy, particularly for treatment of solid tumors, is still poorly understood. For zalutumumab, a therapeutic epidermal growth factor receptor (EGFR)-specific mAb currently in clinical development, previous studies have indicated signaling inhibition and ADCC induction as important therapeutic mechanisms of action. To investigate the in vivo role of ADCC, a panel of EGFR-specific mAbs lacking specific functionalities was generated. By comparing zalutumumab with mAb 018, an EGFR-specific mAb that induced ADCC with similar potency, but did not inhibit signaling, we observed that ADCC alone was insufficient for efficacy against established A431 xenografts. Interestingly, however, both zalutumumab and mAb 018 prevented tumor formation upon early treatment in this model. Zalutumumab and mAb 018 also completely prevented outgrowth of lung metastases, in A431 and MDA-MB-231-luc-D3H2LN experimental metastasis models, already when given at nonsaturating doses. Finally, tumor growth of mutant KRAS-expressing A431 tumor cells, which were resistant to EGFR signaling inhibition, was completely prevented by early treatment with zalutumumab and mAb 018, whereas ADCC-crippled N297Q-mutated variants of both mAbs did not show any inhibitory effects. In conclusion, ADCC induction by EGFR-specific mAbs represents an important mechanism of action in preventing tumor outgrowth or metastasis in vivo, even of cancers insensitive to EGFR signaling inhibition.  The Journal of Immunology, 2011, 187: 3383–3390.

Monoclonal Abs targeting cancer cells represent an important class of drugs whose action depends on the engagement of multiple mechanisms of action (MoA). First, mAbs may block target functionality via, for example, receptor downmodulation or ligand blockade. Second, mAbs can induce immune effector functions such as complement-mediated cytotoxicity (CDC) or Ab-dependent cellular cytotoxicity (ADCC). Many studies have shown in vitro that therapeutic mAbs can effectively induce ADCC (1–5). The in vivo relevance of in vitro observed ADCC activity is difficult to delineate because more than one MoA can be involved in the in vivo anti-tumor effects of therapeutic mAbs.

Evidence for the role of ADCC was gained from studies on FcγR polymorphisms, which influence the affinity of FcγR for IgG, in patients, in relation to mAb efficacy. In vitro studies showed that effector cells expressing the high-affinity FcγRIIIa-158V/V genotype induce higher levels of target cell killing with rituximab (MabThera, anti-CD20) compared with effector cells expressing the low-affinity FcγRIIIa-158F/F genotype (6). Clinical observations in non-Hodgkin’s lymphoma patients showed rituximab to be most efficient in patients with the high-affinity FcγRIIIa-158V/V genotype (7, 8). Additionally, metastatic breast cancer patients treated with trastuzumab (Herceptin, anti-HER2) showed that patients with the FcγRIIa-158V/V genotype responded better to therapy (9). These clinical studies indicate that ADCC represents a relevant MoA for rituximab and trastuzumab in vivo. For cetuximab (Erbitux, anti-epidermal growth factor receptor [EGFR]) contradictory results have been published on the importance of ADCC as a MoA in metastatic colorectal cancer patients. Zhang et al. (10) showed that cetuximab-treated patients with low-affinity FcγRIIa genotypes had longer progression-free survival than did high-affinity carriers, whereas Bibeau et al. (11) showed the opposite, that is, patients with high-affinity FcγRIIa genotypes had longer progression-free survival. The reason for the discrepancy between these two studies regarding FcγRIIa polymorphism and progression-free survival remains unclear.

Murine xenograft models have been crucial for establishing ADCC as a MoA. Clynes et al. (12) demonstrated the importance of ADCC for efficacy of rituximab and trastuzumab using FcR γ-chain−/− mice, which have no functional activating FcγR. No anti-tumor effect of the mAbs was observed in these mice, demonstrating the importance of IgG–FcγR interactions in inhibition of tumor growth.

Currently, due to the discrepancy in clinical results for cetuximab, it remains to be established whether ADCC induction contributes to the in vivo efficacy of EGFR-specific mAbs. The present...
study addresses the in vivo MoA of zalutumumab, a human IgG1 mAb specific for human EGFR, previously demonstrated to have a dual MoA, namely signaling inhibition and ADCC (13). To distinguish ADCC induction from signaling inhibition we generated a matched set of EGFR-specific mAbs, including one mAb with ADCC induction and one mAb with signaling inhibition as the sole MoA. We studied the effects of this mAb panel in several mouse tumor xenograft models, mimicking either metastatic colonization or solid tumor growth. Using these tools, we obtained a better insight into when, where, and at what mAb dose ADCC induction plays a role in the treatment of solid tumors with EGFR-specific mAbs.

Materials and Methods

Cell lines

A431 cells (epidermoid cell line) were obtained from DSMZ (Braunschweig, Germany; cell line no. ACC 91). The A431-luciferase clone L18 cell line (further referred to as A431-luc) was generated by stable transfection of the A431 cells with pGwLuciferase (construct from Gene Therapy Systems, San Diego, CA). MDA-MB-231-luc-D3H2LN cells (adenocarcinoma, mammary gland, further referred to as MDA-MB-231-luc) were obtained from Caliper Life Sciences (Hopkinton, MA). A431-KRAS4bG12V cells were provided by Thomas Valerius [University Hospital Schleswig-Holstein and Christian-Albrechts-University Kiel, Kiel, Germany (14)]. A431 cells were cultured in RPMI 1640 medium (Lonza, Verviers, Belgium), supplemented with 10% heat-inactivated Cosmic Calf Serum (HyClone, Logan, UT), 50 μg/ml penicillin, and 50 μg/ml streptomycin (Lonza). The growth medium for the A431-KRAS4bG12V cells was supplemented with 0.7 mg/ml hygromycin B (Invitrogen, Carlsbad, CA). MDA-MB-231-luc cells were cultured in DMEM medium (Lonza), supplemented with 10% heat-inactivated Cosmic Calf Serum, 50 IU/ml penicillin, 50 μg/ml streptomycin, 1 mM sodium pyruvate (Lonza), and 0.1 mM nonessential amino acids (Invitrogen). Cells were treated with 0.05% trypsin-EDTA (Invitrogen) in PBS (B. Braun, Melsungen, Germany). For in vivo tumor studies, cells were harvested in log phase and tested for EGFR expression and potential mycoplasm contamination.

Abs

The human IgG1, κ, EGFR-specific mAb zalutumumab (HuMax-EGFR, clone 2F8) and mAb 018 be generated by immuHuMab mice (Medarex, Milpitas, CA) and produced as recombinant proteins as described previously (13). The N297Q mutation in the Fc part of the Fc fragment of zalutumumab and mAb 018, referred to as zalu-N297Q and mAb 018-N297Q, and the K322A mutation in zalutumumab, referred to as zalu-K322A, were introduced using the QuikChange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA). Mutagenesis was confirmed by sequencing (LGGenomics, Berlin, Germany). IgG concentrations were determined by A280 measurements. Human IgG1, κ mAb specific for keyhole limpet hemocyanin (HuMab-KLH), also generated in HuMab mice, was included in all experiments as isotype control mAb.

Flow cytometry

EGFR binding was assessed by incubating EGFR-expressing cells with serial dilutions of mAb in PBS, 0.05% BSA (Roche, Meylan, France), and 0.012% sodium azide (Sigma-Aldrich, St. Louis, MO) at 4°C for 30 min. Cells were washed and incubated with FITC-conjugated F(ab′)2 fragments of goat anti-human κ L chain (BD Biosciences, Aalst, Belgium) at 4°C for 30 min in the dark. Samples were analyzed by FACS analysis (FACSCanto II; BD Biosciences).

EGFR phosphorylation inhibition assay

Inhibition of EGFR autophosphorylation was evaluated in a two-step assay using the A431 cell line as described previously (15). In short, cells starved overnight were incubated with serially diluted EGFR-specific mAbs. After 60 min incubation, 50 ng/ml recombinant human EGF (Invitrogen) was added and incubated for 30 min. Cells lysates were transferred to ELISA plates and EGFR was captured with mouse EGFR-specific mAb (mAb EGFR1; BD Pharmingen, San Diego, CA). Phosphorylated EGFR was detected using europium-labeled mouse mAb, specific for phosphorylated tyrosines (mAb Eu-N1 P-Tyr-100; PerkinElmer, Boston, MA).

Proliferation inhibition assay

The ability of EGFR-specific mAbs to inhibit tumor cell proliferation was tested in a proliferation assay as described previously (15). A431 cells were seeded at a density of 500 cells per well in 96-well culture plates. EGFR-specific mAbs were added in serial dilutions in culture medium and cultured for 5 d. Alamar Blue (20 μl; Invitrogen) was used for measuring vital cell mass.

Isolation of PBMCs from human blood

PBMCs were isolated from buffy coats obtained from regular blood bank donations (after informed consent; Sanquin Blood Bank, Utrecht, The Netherlands) using density separation with lymphocyte separation medium (Lymphoprep; Lonza), followed by washing with PBS to remove platelets.

Culture of bone marrow-derived mouse macrophages

Bone marrow was isolated from the hind legs of SCID mice by flushing the femurs. Bone marrow was passed through a cell strainer and seeded in petri dishes (58 cm²) with 10 ml/petri dish at 1.25 × 10⁵ cells/ml in DMEM medium containing 10% fetal calf serum, 2 mM glutamine, 50 IU/ml penicillin, and 50 μg/ml streptomycin. Cells were cultured with 50 μM M-CSF (Prospec, Rehovot, Israel) at 37°C and 5% CO₂ for 7 d. Cultured macrophages were stimulated with 250 U/ml IFN-γ (BD Biosciences) and 25 ng/ml LPS (Sigma-Aldrich) 24 h prior to use. Macrophages were detached with Versene (Invitrogen) and characterized by FACS analysis for staining with F4/80-A488 (AbD Serotec, Oxford, U.K.) and CD80-PE (eBioscience, San Diego, CA).

Ab-dependent cellular cytotoxicity

ADCC was evaluated in a 51Cr-release assay in which A431 target cells (5 × 10⁵ cells) were labeled with 100 μCi Na⁵¹CrO₄ (Amersham Biosciences, Uppsala, Sweden) at 37°C for 1 h. Cells were washed twice with PBS and resuspended in culture medium at 1 × 10⁵ cells/ml. Labeled cells (5 × 10⁵) were added in 96-well plates and incubated with mAb (room temperature, 15 min). In the ADCC assay with human PBMCs, mAbs were added in 5-fold serial dilutions in culture medium (triplicate wells) and 5 × 10⁵ PBMCs/well. In the ADCC assay with mouse macrophages, a fixed mAb concentration of 10 μg/ml was used (six replicates) and 1 × 10⁶ mouse macrophages per well. Instead of mAb, culture medium was added to determine the background 51Cr release (negative control), and Triton X-100 (Sigma-Aldrich) (1.6% final concentration) was added to determine the maximal 51Cr release (positive control). After 24 h incubation at 37°C supernatants were collected and 51Cr release was measured in a β-counter (cpm). Percentage of cellular cytotoxicity was calculated using the following formula: percentage specific lysis = [experimental release (cpm) – negative control (cpm)]/[positive control (cpm) – negative control (cpm)] × 100%.

Mouse tumor xenograft models

SCID mice (C.B.-17/IcrCrl-scid/scid) were purchased from Charles River (Maastricht, The Netherlands). All experiments were performed with 8- to 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 water and food provided ad libitum. Mice were checked at least twice a week for clinical signs of disease and discomfort. All experiments were approved by the Utrecht University Animal Ethics Committee. Subcutaneous tumors were induced by inoculation of 5 × 10³ A431 cells or 1 × 10⁶ A431-KRAS4bG12V cells in the right flank of mice. Tumor volumes were calculated from digital caliper measurements as 0.52 × length × width² (mm³). Experimental lung metastases were induced by injecting 1 × 10⁶ A431-luc cells or 0.25 × 10⁵ MDA-MB-231-luc cells into the tail vein. At weekly intervals, tumor growth was assessed using bioluminescence imaging. Before imaging, mice were anesthetized by i.p. injection of a mix of ketamine (Ve´toquinol, Lure, France), xylazine (Tryptar, Bioanalysis, Amsterdam, The Netherlands), and midazolam (Médiavet, Paris, France; MDA-MB-231-luc model). During imaging, black-and-white images were made for anatomical reference. Orthogonal views were reconstructed using Metavue software (Universal Imaging, Downingtown, PA) and imported into M3 Vision software (BioSpace Lab, Paris, France; MDA-MB-231-luc model). During illumination, black-and-white images were made for anatomical reference. Metavue software (Universal Imaging, Downingtown, PA) was used for data collection and image analysis of the A431-luc model. M3 Vision software (BioSpace Lab) was used for image analysis of the MDA-MB-231-luc model. mAbs were injected i.p. at indicated time points at four different dosing levels: 2 μg/mouse (0.1 mg/kg), 10 μg/mouse (0.5 mg/kg), 100 μg/mouse (5 mg/kg), and 1 mg/mouse (50 mg/kg). For in vivo study, heparinized blood samples were taken for determination of human IgG levels in plasma using a Behring Nephelometer II (Siemens Health care Diagnostics, Erlangen, Germany).
Statistical analysis

Data analysis was performed using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA) and PASW Statistics 18.0 (SPSS, Chicago, IL). Data were reported as means ± SEM. Differences between groups were analyzed using one-way ANOVA followed by a Tukey posttest (GraphPad Prism 5.0). Selected data were also analyzed using log-rank test (PASW Statistics 18.0).

Results

In vitro characterization of EGFR-specific Abs

To study the differential roles of signaling inhibition and ADCC induction by EGFR-specific mAbs, four mAbs with different functionalities were developed. Next to zalutumumab, the EGFR-specific mAb 018, which binds EGFR at a different epitope, was used (16). mAb 018 induces ADCC but does not inhibit EGFR phosphorylation and proliferation of A431 cells (see below). From both zalutumumab and mAb 018, mutants were generated in which the site for N-linked glycosylation in the Fc domain was eliminated by mutating the asparagine at position 297 to glutamine, referred to as zalu-N297Q and mAb 018-N297Q. This mutation leads to loss of Fc glycosylation, which results in abrogation of IgG Fc receptor interactions and Clq binding, and thereby of ADCC and CDC functions as previously described (17).

By flow cytometric analyses on A431 cells, the binding characteristics of zalutumumab, mAb 018, and their N297Q mutants were demonstrated to be similar. Half-maximal binding concentrations (EC_{50}) for zalutumumab and zalu-N297Q were 0.36 μg/ml (95% confidence interval [CI], 0.22–0.59 μg/ml) and 0.37 μg/ml (95% CI, 0.22–0.64 μg/ml) and for mAb 018 and mAb 018-N297Q, 1.4 μg/ml (95% CI, 0.84–2.34 μg/ml) and 1.9 μg/ml (95% CI, 1.27–2.88 μg/ml) (Fig. 1A).

The capacity to induce ADCC in A431 cells was comparable for mAb 018 (EC_{50}, 0.03 μg/ml; 95% CI, 0.02–0.05 μg/ml) and zalutumumab (EC_{50}, 0.04 μg/ml; 95% CI, 0.01–0.14 μg/ml). Both N297Q mutants were unable to induce ADCC, consistent with their loss of FcγR binding (Fig. 1B). As expected, the N297Q mutation did not affect zalutumumab’s ability to inhibit signaling, as both Abs demonstrated a similar inhibition of EGFR phosphorylation. mAb 018 and mAb 018-N297Q did not inhibit EGF-induced phosphorylation (Fig. 1C). Finally, we demonstrated that zalutumumab and zalu-N297Q inhibited proliferation, whereas mAb 018 and mAb 018-N297Q did not (Fig. 1D). Previously, it had been observed that neither zalutumumab alone nor mAb 018 alone was able to induce CDC with human complement (16).

Taken together, we generated a well-assorted panel of EGFR-specific mAbs in which zalutumumab inhibits signaling and induces ADCC; mAb 018 solely induces ADCC, zalu-N297Q only inhibits EGFR signaling, and mAb 018-N297Q is inert for both MoA. This matched set of mAbs with a unique combination of MoA allowed us to focus on the differential role of ADCC in xenograft models.

Zalutumumab and mAb 018 are functional in ADCC with murine effector cells

Because we employed a mouse model to study the in vivo impact of ADCC, we first checked the interaction of our mAbs with mouse effector cells in vitro. Bone marrow-derived macrophages were used, since they are important effector cells expressing all FcγRs. The cultured macrophages were positive for F4/80-A488 and CD80-PE, and they therefore represent mature and activated macrophages (data not shown). To study ADCC, mouse macrophages were incubated with ^{51}Cr-labeled A431 cells in an E:T ratio of 20:1 in the presence of saturating mAb concentrations (10 μg/ml). As expected, zalutumumab and mAb 018 induced a comparable percentage of specific lysis of A431 cells with mouse macrophages (mean ± SEM, 23.8 ± 2.5 and 22.0 ± 1.8%), whereas the N297Q mutants were inactive in ADCC (Fig. 2). These results were confirmed by using mouse NK cells and mouse neutrophils as effector cells (data not shown).
Having established the different functionalities of our EGFR-mAbs to induce ADCC was analyzed in a 51Cr-release assay using mouse bone marrow-derived macrophages as effector cells, and A431 cells as target cells. mAbs were tested at a concentration of 10 μg/ml with six replicates. Data represent mean ± SEM of three independent experiments.

Inhibition of signaling or induction of ADCC is sufficient to prevent tumor growth upon early treatment in A431 s.c. xenografts

Knowing that both zalutumumab and mAb 018 can induce ADCC with mouse effector cells, we tested these mAbs and their N297Q mutants in an A431 xenograft model. Mice were treated i.p. with 5 mg/kg mAbs within 2 h after tumor induction (early treatment). Zalutumumab and zalu-N297Q were both found capable of inhibiting tumor growth (p < 0.001; one-way ANOVA, day 26), indicating signaling inhibition alone to be sufficient to inhibit tumor growth upon early treatment (Fig. 3A). mAb 018 was also capable of inhibiting tumor growth (p < 0.001; one-way ANOVA, day 26), indicating that also ADCC alone suffices for inhibition of tumor growth in this experimental setting (Fig. 3B). The N297Q mutation completely removed the anti-tumor effect of mAb 018, confirming mAb 018 indeed to be inactive in vivo in the absence of its capability to induce ADCC.

Impact of dosing and timing on zalutumumab and mAb 018 efficacy in A431 s.c. xenografts

Having established the different functionalities of our EGFR-specific Ab panel in vivo, the influence of dosing and timing on efficacy was examined. First, 0.5 and 5 mg/kg mAb doses were tested in early treatment (Fig. 4A). Dosing of 0.5 mg/kg zalutumumab or mAb 018, leading to expected maximum plasma concentrations of 5 μg/ml (18), resulted in a delay in tumor growth (p < 0.001; one-way ANOVA, day 21). This suggests that ADCC is already effective at mAb doses that are not expected to fully saturate EGFR with mAbs. At a dose of 5 mg/kg, both zalutumumab and mAb 018 completely inhibited tumor growth (p < 0.001; one-way ANOVA, day 21).

Additionally, the impact of timing on the role of ADCC was studied in established tumor models in which we examined efficacy of zalutumumab and mAb 018 on variable tumor volumes. A delay in tumor growth was observed when mice with tumor volumes of 80–100 mm3 (days 2–4) were treated with 0.5 or 5 mg/kg mAb (p < 0.05 and p < 0.001; one-way ANOVA, days 24 and 18, respectively). Complete inhibition of tumor growth, however, was no longer observed (Fig. 4B). At a tumor volume of 200 mm3 (day 15) mice were treated with a repeated dose of 50 mg/kg mAb, which is expected to give full target saturation in established tumors (13). Treatment with mAb 018 did not inhibit tumor growth in this setting, whereas complete abrogation of tumor growth with zalutumumab (p < 0.01; one-way ANOVA, day 29) was observed (Fig. 4C).

FIGURE 2. Zalutumumab and mAb 018 are functional in an ADCC assay with murine effector cells. The capacity of the different mAbs to induce ADCC was analyzed in a 51Cr-release assay using mouse bone marrow-derived macrophages as effector cells, and A431 cells as target cells. mAbs were tested at a concentration of 10 μg/ml with six replicates. Data represent mean ± SEM of three independent experiments.

FIGURE 3. Early treatment with mAb 018-N297Q does not prevent tumor growth. Eight mice per group were injected s.c. with 5 × 106 A431 cells. mAb (5 mg/kg) was administered within 2 h after tumor inoculation. Tumor volumes were calculated as described in Materials and Methods. A. Efficacy of zalutumumab and zalu-N297Q. B. Efficacy of mAb 018 and mAb 018-N297Q. Data shown are mean tumor volumes ± SEM.

Taken together, these data indicate that at an early time point in tumor development, ADCC as the only MoA is capable of reducing tumor growth and that low mAb concentrations are sufficient. In contrast, inhibition of EGFR signaling is required for inhibiting established tumors with a large tumor volume, where ADCC induction alone is ineffective.

Both zalutumumab and mAb 018 are effective at low doses in experimental metastasis models

Because ADCC has been an effective MoA upon early treatment, we hypothesized that ADCC might be very effective in killing metastasizing tumor cells. Therefore, the efficacy of ADCC as a MoA was studied in two different experimental metastasis models in which tumor cells colonize the lungs. Development was assessed weekly by optical imaging. First, early treatment with 0.5 mg/kg mAb was tested in the i.v. A431-luc model. Tumor development of A431-luc cells in the lungs became visible at day 23, and starting from day 43 tumor volume enhanced exponentially in the control group. The light intensities of A431-luc cells at day 49 are shown in Fig. 5A. Tumor growth was completely inhibited upon treatment with zalutumumab or mAb 018 (p < 0.01 and p < 0.05; log-rank test, progression-free set at <200,000 counts). These results were confirmed in a second model in which mice were injected i.v. with MDA-MB-231-luc cells. To evaluate the dose requirements, mAb 018 was tested in this model at different dose levels. Tumor development became visible at day 21 and tumor volume enhanced exponentially in the control group, whereas in the mAb 018-treated mice a delay in tumor growth was observed. The light intensity of the MDA-MB-231-luc cells in the lungs at day 33 in the mAb 018-treated groups was ∼10- to 18-fold lower than in the group treated with control mAb (Fig. 5B). Even a low dose of 0.1
mg/kg mAb 018 was able to significantly delay tumor progression ($p < 0.05$; log-rank test, progression-free set at $< 5000$ cpm). Both zalutumumab and mAb 018 are effective on tumors resistant to inhibition of EGFR signaling. The data so far indicate that ADCC occurs independently of effects on signaling. This is further supported by the fact that proliferation of the MDA-MB-231-luc cell line was found to be insensitive to EGFR signaling inhibition in vitro (Supplemental Fig. 1), but in vivo growth of this cell line can be delayed with EGFR-specific mAb. To further evaluate ADCC efficacy in tumors insensitive to EGFR signaling inhibition we used A431-KRAS4bG12V cells. Schlaeth et al. (14) demonstrated previously that an A431 cell line stably transfected with oncogenic KRAS4bG12V remained sensitive to ADCC, but not to EGFR signaling inhibition, in vitro. To study this in vivo, we treated mice with a single dose of 5 mg/kg mAb after s.c. A431-KRAS4bG12V tumor inoculation. Indeed, both zalutumumab and mAb 018 were able to inhibit tumor growth completely ($p < 0.001$; one-way ANOVA, day 21; Fig. 6A). The N297Q mutation completely removed the anti-tumor effects from zalutumumab and mAb 018, confirming that ADCC is indeed an effective MoA against A431-KRAS4bG12V tumor cells in vivo, which are insensitive to EGFR signaling inhibition. **No major role for CDC as MoA of zalutumumab in mice** So far, we assumed that CDC does not play a role in the anti-tumor effects of zalutumumab or mAb 018 in mouse models, because no CDC induction was observed in assays with human serum (16). To confirm that this also holds true in a mouse model we used a zalutumumab Fc mutant in which the lysine at position 322 was mutated to alanine, referred to as zalu-K322A. Duncan and Winter (19) suggested that the K322 position in mouse IgG2b is located in the binding site for C1q. Furthermore, Idusogie et al. (20) showed that the K322 position is also in human IgG1 the epicenter for human, rabbit, and guinea pig C1q binding. To extend this to mice, we confirmed that the K322A mutation also leads to strongly reduced binding of mouse C1q (Supplemental Fig. 2A) (29). Furthermore, we showed that this mutation leads to loss of CDC of human cells induced by human IgG1 mAb in mouse serum (Supplemental Fig. 2B). Having established loss of CDC by the K322A mutation with mouse complement, we compared zalutumumab and zalu-K322A in an in vivo model in which we treated A431-KRAS4bG12V s.c. xenografts within 2 h after tumor inoculation with 0.5 or 5 mg/kg mAb. If CDC was an additional MoA of zalutumumab in vivo, a reduced effect on tumor growth inhibition by zalu-K322A would be expected. However, zalu-K322A was as effective in tumor.
inhibition as zalutumumab, and therefore we can exclude CDC as a major MoA of zalutumumab (Fig. 6).

In conclusion, we have demonstrated that ADCC induction by EGFR-specific mAbs alone is sufficient for preventing tumor outgrowth, even of tumor cells that are insensitive to EGFR signaling inhibition.

Discussion

To evaluate the in vivo role of ADCC in the anti-tumor effects of zalutumumab, a matched set of EGFR-specific mAbs was generated. Each EGFR-specific mAb displayed a different MoA, permitting us to distinguish between ADCC and signaling inhibition. We evaluated the efficacy of these EGFR-specific mAbs in several murine xenograft models to answer the questions when, where, and at what mAb dose levels ADCC induction may play a role in the treatment of solid tumors with therapeutic mAbs.

Previous work already demonstrated a role for ADCC in preventing tumor growth. The data from Clynes et al. (12) was confirmed by de Haij et al. (1) in a recent study in mice deficient in FcR \( \gamma \)-chain signaling (NOTAM mice). In these mice, tumor growth was no longer inhibited by rituximab. These studies established ADCC as MoA, but did not address the questions under which conditions it is effective and how it relates to other potential MoA. To clarify these questions, we treated mice at different time points of tumor development. Early treatment (at day 0) in a s.c. A431 xenograft model completely inhibited tumor growth with ADCC (mAb 018) and/or signaling inhibition (zalutumumab N297Q) as MoA (zalutumumab has both MoA). Treatment at a time point on which the tumors were established but still had a small tumor volume was less effective, but still resulted in a delay of tumor growth by ADCC induction or inhibition of signaling. However, growth of established tumor with a large tumor volume (day 15) was only inhibited by a signaling blocking mAb (zalutumumab) at high dose (50 mg/kg). mAb 018, a mAb with only ADCC as a MoA, did not reduce growth of established tumors. These results demonstrate ADCC to be especially effective in early treatment, suggesting that nonestablished tumors, in particular, are susceptible to eradication via ADCC. This is consistent with and explains the previous finding of Clynes et al. (12) and de Haij et al. (1) who employed nonestablished tumor models for their experiments.

To investigate what EGFR-specific mAb dose level is required to induce efficient ADCC, mice were treated with three different mAb doses. A dose of 0.5 mg/kg is expected to yield a maximum plasma concentration of 5 mg/ml, which gives incomplete receptor saturation according to in vitro binding data. Doses of 5 and 50 mg/kg are expected to yield maximum plasma concentrations of 50 and 500 mg/ml, which both give full receptor saturation in vitro. In the current study, a single dose of 0.5 mg/kg mAb at day 0 was sufficient to strongly inhibit tumor development via ADCC as the only MoA. We conclude that EGFR saturation is not required for effective ADCC induction in vivo. This conclusion is supported by a study in Fc\(\gamma\)RIIB knockout mice, in which it was shown that mice deficient in the inhibitory receptor, Fc\(\gamma\)RIIB, had complete inhibition of tumor growth already at submaximal trastuzumab doses (12).

The importance of ADCC in the treatment of hematological tumors with mAb has been shown in several studies (21). A role for
ADCC in the treatment of solid tumors with mAb is less clear. An exception is trastuzumab, which is used for the treatment of solid tumors and for which ADCC has been described as an important MoA (22). In this study, we demonstrate that s.c. growth of a solid tumor can only be inhibited via ADCC when mAb treatment is started shortly after tumor induction. These results suggest that ADCC by EGFR-specific mAbs has no or limited impact on an established solid tumor mass. In experimental metastasis models, in which A431-luc cells or MDA-MB-231-luc cells were inoculated i.v., complete inhibition or a delay, respectively, of tumor growth by ADCC as single MoA was observed, even at a single low dose of 0.1 mg/kg mAb. These data suggest that tumor cells must be easily accessible for the therapeutic mAb and/or effector cells to be effectively depleted via ADCC. Ineffectiveness of the immune system to access xenografts was shown by Yu et al. (23) who observed, upon i.v. injection of light-emitting microorganisms in mice bearing MCF-7 human metastatic mammary carcinoma tumors, that the primary breast tumor and metastasis in the left breast were colonized by bacteria 2 d after injection. Eight days after injection the bacteria were no longer detectable in the metastasis, but they were present in the primary tumor for over 45 d. These results indicate that the tumor microenvironment is an immune-privileged site. Comparable findings have been obtained by Gong et al. (24) for B cell clearance by CD20 targeting Ab. Using a human CD20 transgenic mouse model they observed that upon administration of CD20-specific mAb, circulating B cells were rapidly cleared through the reticuloendothelial system, but B cells residing within the marginal zone compartment were only partially cleared. The partial clearance of B cells from the marginal zone was mediated by CDC induction, indicating that the mAb did reach this compartment. Clearance of the remaining resistant B cells required trafficking of these cells through the circulation. Our results, showing that zalutumumab can inhibit tumor growth of an established solid tumor via signaling inhibition, demonstrate that indeed the therapeutic mAb does reach the tumor. These data imply that an established solid tumor protects itself from ADCC, not by excluding the therapeutic mAb, but by excluding the effector cells from the tumor microenvironment. Protection from the tumor against the immune system can be overcome by enhancing the affinity of therapeutic mAb for activating FcyR. Junttila et al. (25) showed that enhanced affinity of trastuzumab for FcyR resulted in enhanced anti-tumor effects in established solid tumors in vivo. Enhancing the affinity of mAb for activating FcyR may well lead to enhanced capture of effector cells in the tumor microenvironment.

In the process of metastasis, tumor cells seem to be better accessible for the immune effector cells, as indicated by the observation that bacteria are cleared from the metastasis after 8 d (23) and the low dose of mAb 018 we needed to strongly diminish the experimental lung metastases. We suggest ADCC to represent a powerful MoA in inhibiting metastasis, and several studies with trastuzumab support this hypothesis. In a mouse xenograft model where the primary tumor does no longer respond to trastuzumab treatment, there was a reduction in circulating tumor cells (26).

Furthermore, a clinical study with trastuzumab showed that therapy-resistant CK-19 mRNA-positive disseminated occult breast cancer cells in the peripheral blood and bone marrow can be effectively targeted by trastuzumab administration (27).

Finally, the question was addressed whether ADCC is effective in tumors insensitive to EGFR signaling inhibition due to mutations downstream of EGFR. An in vitro study from Schlaeth et al. (14) showed that tumor cells expressing mutant KRAS, which results in cells insensitive to EGFR signaling inhibition, can be effectively killed via ADCC. In this study, we studied the role of ADCC induction on tumor growth of MDA-MB-231-luc cells and A431-KRAS\(\text{G12V}\) cells in vivo. The MDA-MB-231-luc tumor cells originated from a metastatic site in a MDA-MB-231 xenograft are described to be insensitive to EGFR stimulation (28). In vitro proliferation demonstrated these cells indeed to be insensitive to EGFR signaling inhibition via zalu-tumumab. Tumor growth of an i.v. MDA-MB-231-luc tumor model could be delayed via mAb 018 treatment, even at a dose of 0.1 mg/kg, indicating again that full receptor saturation is not necessary for ADCC activity. No complete tumor growth inhibition was observed in contrast to the A431-luc experimental metastatic model also not at a dose of 5 mg/kg mAb 018. The fact that MDA-MB-231-luc cells are not fully sensitive to ADCC needs further investigation, but might be due to a lower EGFR density on the MDA-MB-231-luc cells or due to a higher expression of ADCC suppressor proteins. Early treatment (day 0) with 5 mg/kg zalutumumab or mAb 018 in a s.c. A431-KRAS\(\text{G12V}\) tumor model completely prevented tumor growth. Zalu-N297Q and mAb 018-N297Q, which lack ADCC activity, were not able to inhibit tumor growth, and treatment with zalu-K322A did not result in an abrogation of the anti-tumor effect, indicating that CDC has no major role in the EGFR-specific mAb anti-tumor effect of A431-KRAS\(\text{G12V}\) cells. This demonstrates that growth of tumor cells insensitive to EGFR signaling inhibition can be inhibited completely in vivo by induction of solely ADCC.

In conclusion, our study supports ADCC induction by EGFR-specific mAbs, such as zalutumumab, to represent a powerful MoA in metastasis, as well as early stages of tumor development, even in cancers insensitive to EGFR signaling inhibition due to, for example, KRAS mutations. ADCC, therefore, is likely an important MoA for treatment of solid tumors via the prevention of metastasis.
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Disclosures
All authors are current employees of Genmab with warrant and/or stock ownership. T.V. has served on Genmab’s Board of Directors. J.J.L.v.B., T.V., J.G.J.v.d.W., P.W.H.I.P., and W.K.B are zalutumumab patent holders.

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