Complement-mediated tumor-specific cell lysis by antibody combinations targeting epidermal growth factor receptor (EGFR) and its variant III (EGFRvIII)

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Monoclonal antibodies (mAb) against variant III of epidermal growth factor receptor (EGFRvIII) hold promise for improving tumor selectivity of EGFR-targeted therapy. Here, we compared Fc-mediated effector functions of three mAb against EGFRvIII (MR1-1, ch806, 13.1.2) with those of zalutumumab, a high affinity EGFR mAb in advanced clinical trials. MR1-1 and ch806 demonstrated preferential and 13.1.2 exclusive binding to EGFRvIII, in contrast to zalutumumab, which bound both wild-type and EGFRvIII. All four human IgG1 mAb mediated antibody-dependent cytotoxicity (ADCC) of EGFRvIII-expressing cells with mononuclear cells and isolated monocytes, while only zalutumumab in addition triggered ADCC by polymorphonuclear cells. Interestingly, combinations of zalutumumab and EGFRvIII mAb specifically mediated complement-dependent cytotoxicity (CDC) of EGFRvIII-transfected but not wild-type cells. Moreover, EGFRvIII-specific CDC was significantly enhanced when zalutumumab was combined with a Fc-engineered variant of MR1-1 (K326A/E333A). These observations confirm the immunotherapeutic potential of antibody combinations against EGFR, and demonstrate that tumor selectivity can be improved by combining therapeutic EGFR mAb with an antibody against EGFRvIII. (Cancer Sci 2011; 102: 1761–1768)

A aberrant expression of epidermal growth factor receptor (EGFR) has been observed in different epithelial tumors and gliomas, and often correlates with a worse clinical outcome for patients. These features make EGFR an attractive target for cancer therapeutics.1,2,3 Like monoclonal antibodies (mAb) and tyrosine kinase inhibitors (TKI),4,5,6,7,8,9,10,11 At present, two EGFR mAb are FDA approved for the treatment of cancer: cetuximab (C225, Erbitux) – a chimeric antibody of IgG1 isotype – and panitumumab (E7.6.3, Vectibix) – a human IgG2 antibody. Zalutumumab (2F8, HuMax-EGFR) is another human IgG1 EGFR antibody in advanced clinical trials.12,13 By binding to domain III of EGFR, these antibodies mediate direct effector mechanisms, resulting in blockade of ligand binding, receptor downmodulation, cell cycle arrest and induction of apoptosis, but also recruitment of immunological activity against cancer cells, such as ADCC and CDC, which have been documented.14 Their clinical activity was associated with certain Fcγ receptor polymorphisms, which are known to affect IgG binding and functional activity.15,16 Furthermore, EGFR antibody combinations were demonstrated to mediate improved CDC and receptor downmodulation.9

Although EGFR is overexpressed to various degrees on cancer cells, EGFR antibodies also target EGFR on normal tissue, such as the liver, skin and gastrointestinal tract. This normal tissue expression can cause dose-limiting side-effects and characteristic toxicities.17,18 Attempts to improve the effector functions of EGFR antibodies might even increase the severity of these reactions.10 Therefore, EGFR antibodies recognizing tumor-specific EGFR epitopes, such as the common EGFR variant III (EGFRvIII), offer the potential to enhance therapeutic specificity. EGFRvIII is frequently expressed in gliomas and carcinomas of the lung, head and neck, breast and prostate, but has not been observed in normal tissues.13,19 It is associated with egr gene amplification in tumor cells and results from an exon 2–7 deletion (thus also named de2–7 EGFR) or ΔEGFR. This in-frame deletion of 801 bp corresponds to 267 truncated amino acids in the extracellular part of the receptor with an additional glycine residue at the fusion junction, which creates a neoepitope. In contrast to wild-type (wt) EGFR, EGFRvIII does not bind EGFR ligands, but is nevertheless constitutively active at low levels. Additionally, its expression correlates with enhanced tumorigenicity, an increase in the invasive phenotype and a worse clinical prognosis. The oncogenic potential and the tumor-restricted expression make EGFRvIII an interesting target for immunotherapeutic approaches. Recently, an EGFRvIII-directed peptide vaccination showed clinical activity in glioma patients.13 In addition, several EGFRvIII-directed antibodies have been generated. Among those, mAb 806 was described to bind both EGFRvIII and approximately 10% of overexpressed wt EGFR.14 Its characterization revealed a binding epitope located between EGFR residues 287–302, accessible in a transitional form of the receptor.13 MR1-1 and 13.1.2 are two other mAb developed against the EGFRvIII neoepitope, with MR1-1 originally created as a single chain Fv (scFv) and immunotoxin. 13.1.2 is a completely human IgG1 mAb with specific binding to EGFRvIII-positive glioblastoma cells and tumor sections.15

Here, we investigated and compared Fc-mediated effector mechanisms of three mAb against EGFRvIII (MR1-1, ch806 and 13.1.2) with those of zalutumumab, an EGFR mAb. Moreover, we aimed to increase the therapeutic activity and tumor selectivity by investigating non-blocking combinations of these antibodies. Interestingly, the combination of zalutumumab and a Fc-engineered variant of MR1-1 proved highly efficacious in triggering CDC selectively against EGFRvIII-expressing tumor cells.

Materials and Methods

The experiments reported here were approved by the Ethics Committee of the Christian-Albrechts-University, Kiel, Germany, in accordance with the Declaration of Helsinki.

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Isolation of human effector cells. Blood donors were randomly selected from healthy volunteers, who gave written informed consent. Mononuclear cells (MNC) and polymorphonuclear cells (PMN) were isolated from peripheral blood as previously described. Monocytes were obtained from MNC by centrifugation (20 min at 1000g, 4°C) and used directly or stored at −80°C.

Culture of eukaryotic cells. Human epidermoid carcinoma cell line A431 (DSMZ, Braunschweig, Germany) and human embryonic kidney HEK293T cells (ATCC, Manassas, VA, USA) were kept in RPMI 1640- or DMEM-Glutamax-I medium (Invitrogen, Carlsbad, CA, USA) containing 10% FCS (Invitrogen), penicillin, streptomycin and 50 μg/mL Geneticin (Invitrogen) or 0.5 mg/mL Hygromycin B (PAA). For production of MR1-1E3, stably transfected CHO-K1 cells were cultured in CD-CHO medium (Invitrogen) containing 1% sodium hypoxanthine and thymidine mixture (HT)-supplement (Invitrogen, Waltham, MA, USA) harboring wt EGFR as a template. Briefly, pSec-EGFRvIII was digested using HindIII/EcoNI and ligated to HindII/EcoNI-digested pUSE-EGFR, resulting in pUSE-EGFRvIII. The coding sequence was confirmed by Sanger sequencing, and thereafter subcloned into pSec Tag2/Hygro C (Invitrogen) to obtain pSec-EGFRvIII. A431 and HEK293T cells were transfected with either pUSE-EGFRvIII or pSec-EGFRvIII using Lipofectamine (Invitrogen) according to the manufacturer’s instructions. Stable clones were generated by limiting dilution and put under selection with the appropriate antibiotic 48 h after transfection.

Antibodies. The EGFR-directed antibodies cetuximab (Erbitux, chimeric IgG1κ) and panitumumab (Vectibix, human IgG2κ) were purchased from Merck (Darmstadt, Germany) and Amgen (Thousand Oaks, CA, USA), respectively. Zalutumumab (2F8) and HuMab-005 (both human IgG1κ) were produced at Genmab (Utrecht, the Netherlands). Variable light and heavy chain sequences of matuzumab and the EGFRvIII antibodies 13.1.2, ch806 and MR1-1 were synthesized according to published sequences. All were cloned into mammalian expression plasmids containing human κ or γ1 constant regions. A human IgG1κ control antibody against keyhole limpet hemocyanin (HuMab-KLH; Genmab) served as an irrelevant control. MR1-1 with enhanced affinity for C1q (named MR1-1E3) was expressed in the pEE14.4 vector (Lonza, Basel, Switzerland) and subcloned into pSec Tag2/Hygro. HEK293T-vIII cells with the lowest EC50 value and a concentration-dependent antibody binding to wt EGFR and EGFRvIII. An irrelevant IgG1 antibody (KLH) served as a control (grey fill).

Fig. 1. Epidermal growth factor receptor variant III (EGFRvIII) recognition by different antibodies. (A) Lysates of EGFRvIII-transfected and parental A431 and HEK293T cells were analyzed by immunoblot using the stated antibodies for detection and β-actin as a loading control. Arrows indicate the expected molecular weights of wild-type (wt) EGFR and EGFRvIII, which appears as a double band. 13.1.2 exclusively detected EGFRvIII, whereas ch806 and MR1-1 also bound to wt EGFR on non-transfected cells. (B) Indirect immunofluorescence revealed a similar binding of EGFRvIII antibodies to EGFRvIII-expressing A431 (A431-vIII) and HEK293T (HEK293T-vIII) cells (lower panel). Additional binding to A431 cells was obtained with MR1-1 (grey line) and ch806 (black line). MR1-1 also displayed reactivity with HEK293T cells, whereas 13.1.2 (dotted line) only bound to EGFRvIII-transfected cells. Zalutumumab (black fill) recognized wt EGFR and EGFRvIII. An irrelevant IgG1 antibody (KLH) served as a control (grey fill). (C) Flow cytometric analyses relative to KLH binding (relative fluorescence intensity [RFI]; n = 3) yielded concentration-dependent antibody binding to HEK293T-vIII cells with the lowest EC50 value and a 95% confidence interval (CI) for zalutumumab ( ), similar values for ch806 ( ) and 13.1.2 ( ), and highest values for MR1-1 ( ).
site-directed mutagenesis kit (Agilent, La Jolla, CA, USA) according to the manufacturer’s instructions. For direct immunofluorescence, EGFRvIII antibodies were FITC-conjugated with the EZ-label-Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer’s protocol.

**Immunoblotting.** Cells were grown overnight and lysed with cell lysis buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). Then, 20 μg total protein per sample were run on 3–8% BisTris acetate gels (Invitrogen) and transferred onto PVDF membranes (GE Healthcare, Munich, Germany). A mixture of polyclonal rabbit antibody against total and phosphorylated EGFR (both Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used at a dilution of 1:1000. EGFRvIII antibodies were used in a final concentration of 2 μg/mL. A mouse antibody against β-actin (Cell Signaling) served as a control (1:1000). HRP-conjugated anti-rabbit or anti-mouse IgG (DakoCytomation, Glostrup, Denmark) or anti-human κ secondary antibody (Biozol, Eching, Germany) were diluted 1:2000. Detection was performed with ECL substrate (Thermo) and ChemiDoc XRS+ (Bio-Rad, Hercules, CA, USA).

**Flow cytometric analyses.** For indirect immunofluorescence, cells were washed with PBS containing 1% BSA and 0.1% NaN₃ (both Sigma-Aldrich), and incubated with the respective antibody at the indicated concentration. After washing, cells were stained with rabbit anti-human IgG FITC (Dako) and analyzed with Epics Profile flow cytometer (Beckman Coulter, Fullerton, CA, USA). Relative fluorescence intensity (RFI) = mean fluorescence specific mAb/mean fluorescence KLH.

**Crossblocking studies.** For competitive binding studies, stably transfected HEK293T cells were incubated with 200-fold excess of unconjugated antibody and the indicated FITC-labeled EGFRvIII antibody at non-saturating concentrations. Samples were analyzed by flow cytometry and competition was calculated: % of maximal mean fluorescence intensity (MFI) = (experimental MFI - background MFI)/(maximal MFI - background MFI) × 100, with maximal MFI defined by a combination of FITC-conjugated EGFRvIII antibody with KLH.

**C1q deposition.** C1q deposition was determined by incubating cells with an individual antibody or antibody combinations (10 μg/mL final), followed by the addition of 1% human serum and incubation at 37°C for 10 min. After washing, samples were stained with polyclonal FITC-conjugated C1q antibody (Dako) and analyzed by flow cytometry.

**ADCC and CDC assays.** The ADCC assays with MNC, monocytes and PMN with an E : T ratio of 40:1 (MNC and PMN) or 80:1 (monocytes) were performed as previously described.(17) The CDC assays were performed with 25% human serum instead of effector cells. The PMN were additionally stimulated with 10 U/well GM-CSF (Cellgenix, Antioch, IL, USA) during 3 h incubation time. Cytotoxicity was calculated as follows: % specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) × 100, with maximal 51Cr-release determined by the addition of 5% TritonX-100 to target cells, and basal release measured in the absence of antibodies and effector cells or serum.

**Data processing and statistical analyses.** Data are displayed graphically and were statistically analyzed using GraphPad Prism 4.0 (GraphPad Software, La Jolla, CA, USA). Curves were fitted using a non-linear regression model with a sigmoidal dose response (variable slope). Group data are reported as mean ± SEM from triplicates of at least four independent experiments with different donors.
mean ± SEM, and differences between groups were analyzed by unpaired (or, when appropriate, paired) Student’s t-tests. Significance was accepted with P < 0.05.

Results

Binding characteristics of EGFRvIII antibodies. Because tumor cell lines for unknown reasons usually do not express EGFRvIII, we established two cellular models by stably transfecting A431 and HEK293T cells. Thus, cell lines expressing EGFRvIII with either high (A431-vIII) or low (HEK293T-vIII) endogenous wt EGFR co-expression were generated. The deletion mutant revealed the expected lower molecular mass (145 kDa) compared with wt EGFR (170 kDa) and appeared as a characteristic double band in western blot experiments (Fig. 1A). As previously published, 13.1.2 demonstrated selective reactivity with lysates from EGFRvIII-transfected cells (16) while MR1-1 and ch806 gave additional signals with non-transfected cells. Thus, ch806 stained a subset of wt EGFR from EGFR-overexpressing A431 cells, as previously described (14) and MR1-1 bound wt EGFR not only to A431, but also on HEK293T cells.

To verify surface expression of EGFRvIII, we performed indirect immunofluorescence experiments (Fig. 1B). Zalutumumab, a high affinity EGFR mAb directed against ligand-binding domain III, served as a positive control and bound to EGFR-positive A431 and HEK293T cells, as expected (24). Importantly, zalutumumab demonstrated stronger binding to EGFRvIII-expressing cells, which was explained by the higher antigen density. Reactivity of EGFR mAb (i.e. zalutumumab and cetuximab) with EGFRvIII has previously been described, and confirms that EGFR antibodies against domain III can also recognize EGFRvIII (25,26). As expected, the EGFRvIII antibodies bound to EGFRvIII-transfected cells (Fig. 1B, lower panel). MR1-1 reacted with both non-transfected A431 and HEK293T cells, while ch806 only bound to parental A431, but not HEK293T cells (upper panel). 13.1.2 proved its EGFRvIII specificity and did not bind to cells expressing wt EGFR. In order to semi-quantitatively assess the apparent avidity of the EGFRvIII antibodies, concentration-dependent binding to HEK293T-vIII cells was analyzed (Fig. 1C). The experiments revealed the lowest EC50 for zalutumumab (EC50, 0.6; confidence interval [CI], 0.3–1.6 μg/mL) and highest for MR1-1 (2.9; 1.3–6.2). Similar values were obtained for ch806 (1.9; 0.7–5.4) and 13.1.2 (1.6; 0.6–4.3). In conclusion, the immunofluorescence results confirmed the data from the western blot analyses, and demonstrated the successful generation of two different cellular models expressing EGFRvIII for further investigation.

EGFRvIII antibodies effectively triggered ADCC. To investigate Fc-mediated effector functions, we first analyzed ADCC against parental and EGFRvIII-transfected A431 cells (Fig. 2). The

![Fig. 3. Crossblocking experiments and C1q deposition. (A) Cross-competition studies were performed with epidermal growth factor receptor variant III (EGFRvIII)-expressing HEK293T cells stained with the FITC-labeled EGFRvIII antibody of interest in the presence of 200-fold excess of the indicated unconjugated antibodies. The percentage of maximal mean fluorescence intensity (MFI) was calculated as described in the Materials and Methods. None of the EGFRvIII antibodies demonstrated overlapping epitopes with any of the EGFR antibodies used. MR1-1 and 13.1.2 crossblocked each other, indicating an overlapping epitope, while ch806 did not compete with any other antibody. (B) C1q deposition on A431 and EGFRvIII-transfected A431 (A431-vIII) cells was analyzed in the presence of individual antibodies or combinations (10 μg/mL final mAb concentration). While zalutumumab plus HuMab-005 triggered efficient C1q deposition, none of the individual antibodies or the combination of zalutumumab and KLH (isotype control) was effective on either cell line. Zalutumumab induced significant C1q deposition in combination with each of the EGFRvIII antibodies selectively on A431-vIII (right panel), but not on A431 cells (left panel). Data are presented as mean ± SEM of at least three independent experiments. In (A), (x) indicates significant (P < 0.05) binding differences of the FITC-labeled antibody in the presence of the unconjugated antibody vs KLH. In (B), (x) marks significant differences in C1q binding after incubation with zalutumumab vs zalutumumab combinations. RFI, relative fluorescence intensity.](1764)
MNC, isolated monocytes or GM-CSF-stimulated PMN served as effector cells in $^{3}$Cr-release assays. Zalutumumab demonstrated significant ADCC with all effector cell populations against both target cell lines. Notably, killing of A431-vIII cells was slightly more effective than lysis of non-transfected cells (Fig. 2, respectively). The three EGFRvIII antibodies mediated effective lysis of A431-vIII cells using MNC and monocytes as effector cell populations. Both MR1-1 and ch806 also triggered MNC-mediated ADCC against non-transfected A431 cells. Interestingly, only zalutumumab recruited PMN as effector cells against both target cell lines.

**Epitope mapping and C1q deposition.** Combinations of antibodies binding to non-overlapping EGFR epitopes were shown to trigger effective CDC against EGFR-expressing tumor cells. To identify EGFRvIII antibody candidates for combination therapy with EGFR antibodies, crossblocking experiments with fluorescence-labeled MR1-1, ch806 and 13.1.2 were performed on EGFRvIII-transfected HEK293T cells (Fig. 3A). EGFRvIII antibody binding was not inhibited by zalutumumab, indicating that MR1-1, ch806 and 13.1.2 bind to epitopes distinct from the EGFR ligand-binding domain III. To exclude that this was restricted to zalutumumab, we also tested EGFR mAb cetuximab, panitumumab and matuzumab, which are known to bind different domain III epitopes. None of these antibodies inhibited EGFRvIII antibody binding. However, MR1-1 and 13.1.2, both generated against the EGFRvIII neoepitope displayed no overlap with any other antibody tested.

Based on these results, we analyzed C1q deposition triggered by MR1-1, ch806 or 13.1.2 on A431 and A431-vIII cells (Fig. 3B). Neither any individual antibody nor the combination of zalutumumab with the isotype control antibody KLH induced C1q deposition. However, significant C1q deposition on both cell lines was observed in the presence of zalutumumab and HuMab-005, two non-overlapping EGFR antibodies with CDC capacity, as previously shown. Importantly, combinations of zalutumumab with MR1-1, ch806 or 13.1.2 triggered significant C1q deposition only on EGFRvIII-transfected A431 cells, but not on parental A431 cells, although MR1-1 and ch806 demonstrated binding to these cells (Fig. 1).

**EGFRvIII-specific CDC mediated by EGFR/EGFRvIII antibody combinations.** To analyze the efficacy of EGFRvIII-targeting antibodies in CDC, wt and stably transfected A431 and HEK293T cells were incubated with different antibody combinations and human serum (Fig. 4). As expected, no killing by any individual antibody was observed, whereas the combination of zalutumumab and HuMab-005, again used as the control, exhibited significant lysis of both A431 and A431-vIII cells. Non-transfected HEK293T cells were complement resistant under these conditions. Importantly, none of the EGFRvIII antibodies (MR1-1, ch806 or 13.1.2) in combination with zalutumumab triggered CDC against A431 or HEK293T cells, but all of these combinations were capable of inducing significant CDC against EGFRvIII-transfected A431 or HEK293T cells. Thus, combinations of EGFRvIII antibodies and zalutumumab triggered CDC exclusively against EGFRvIII-bearing cells, suggesting a tumor-specific, complement-mediated therapeutic approach.

**Fc engineering of MR1-1 further improved EGFRvIII-specific CDC.** Among the EGFRvIII antibodies compared here, MR1-1 proved to be the most potent partner for zalutumumab. Thus, MR1-1 was selected to introduce K326A and E333A mutations, previously demonstrated to enhance CDC activity of IgG1 antibodies. This variant, named MR1-1E3, displayed similar binding as the parental antibody, but triggered higher C1q deposition (data not shown). Correspondingly, MR1-1E3 alone was capable of mediating CDC against HEK293T-vIII (Fig. 5A).

Next we investigated whether combinations of EGFRvIII antibodies might trigger CDC against EGFRvIII-expressing tumor cells. Because ch806 did not compete with MR1-1 or 13.1.2 (Fig. 3A), it was an appropriate partner. Interestingly, combinations of ch806 and MR1-1 or 13.1.2 triggered efficient CDC against HEK293T-vIII (Fig. 5B, right panel), while the combination of ch806 and MR1-1E3 was even more effective and additionally killed EGFRvIII-expressing A431 cells (Fig. 5B, left panel). If used in combination with zalutumumab (10 μg/mL final concentration), MR1-1E3 demonstrated similar...
CDC as the MR1-1 combination against A431-vIII and HEK293T-vIII cells (Fig. 5A, left panel). However, at lower antibody concentrations, combinations of zalutumumab and MR1-1E3 were significantly more effective against both cell lines (Fig. 5A, right panel).

Discussion

In the present study, we demonstrated EGFRvIII-specific CDC when EGFRvIII antibodies were used in combination with EGFR antibodies, suggesting that these combinations might enhance the tumor specificity and effectiveness of EGFR-directed therapy. So far, two EGFR antibodies have obtained FDA approval, and several EGFRvIII antibodies have been developed, with ch806 being the only one in clinical trials (clinicaltrial.gov/NCT00291447). Due to its tumor specificity, EGFRvIII is currently also being investigated as a target antigen in vaccination trials, and for other therapeutic approaches. The EGFR mAb act via Fab- and Fc-mediated effector mechanisms. For example, zalutumumab blocks EGFR signaling by inhibition of ligand binding and receptor downmodulation and induces efficient ADCC, a Fc-mediated mechanism. For EGFRvIII mAb, several studies reported Fab-mediated effects, while evidence for Fc-mediated killing is more limited. In accordance with the "Weinstein hypothesis", EGFR or EGFRvIII must not only be present on the cell surface, but must also contribute to growth and survival of cancer cells to make them responsive to direct effector mechanisms of EGFR/EGFRvIII therapeutics. For Fc-mediated mechanisms, like ADCC and CDC, only the presence of the target antigen, not dependence, might be sufficient. Thus, we focused on Fc-mediated effector functions of the EGFRvIII mAb MR1-1, ch806 and 13.1.2, all of the IgG1 isotype.

A431 and HEK293T cells were selected as model cell lines representing targets with either amplified or low levels of wt EGFR, respectively. Stable transfectants expressing EGFRvIII showed enhanced growth (data not shown) and proved to be reactive with the EGFRvIII antibodies. As reported earlier,
zalutumumab displayed strong and concentration-dependent ADCC with different human immune effector cells, with enhanced lysis rates in the presence of EGFRvIII. The EGFRvIII antibodies mediated ADCC against variant III-expressing cells with MNC and monocytes, but were not effective with PMN. The ADCC by PMN has previously been demonstrated to require higher levels of target cell sensitization (target antigen density, antibody concentrations) than killing by MNC. (38) The reasons for these differences are poorly understood, but might be related to the involvement of different Fcγ receptors (FcγRIIa of PMN, FcγRIIIa of NK cells), and differences in killing mechanisms of PMN (lysoenzymes, hydrolases, respiratory burst) and NK cells (release of granzymes, perforin, interferon-γ [IFNγ]). In the present study, zalutumumab exhibited binding to EGFR and EGFRvIII, whereas the EGFRvIII-directed antibodies exclusively bound to EGFRvIII (13.1.2) or EGFRvIII and a subset of wt EGFR (ch806 and MR1-1). Therefore, target cell sensitization was higher with zalutumumab (RFI 229.7 and 398.4 for A431 and A431-vIII cells, respectively) than with ch806 (17.2 and 131.0), MR1-1 (4.9 and 142.1) or 13.1.2 (1.1 and 99.6). Ch806 and MR1-1 induced additional killing of non-transfected A431, which were bound by both antibodies. Due to its binding characteristics, ADCC of 13.1.2 was specific to EGFRvIII-expressing cells. Interestingly, ADCC was not enhanced when EGFR and EGFRvIII antibody combinations were tested (data not shown). This observation is potentially explained by the steep dose-response curve observed in ADCC experiments. (38) Thus, target cell sensitization could be either below or above the threshold for effective ADCC, irrespective of the presence of one or two mAb. Alternatively, antibody combinations lead to larger complexes that increase C1q opsonization and complement activation, while the interaction of antibody-opsonized cells with FcR on effector cells already has a high avidity and might not benefit from additional antigen clustering. Hence, efficient ADCC by MR1-1, ch806 or 13.1.2 was restricted to target cells expressing EGFRvIII and/or amplified wt EGFR.

Complement activation is a tightly regulated, Fc-mediated mechanism of action of antibodies, (38) which is attractive for engagement in the treatment of cancer, (40,41) and was recently described as being part of cetuximab’s antitumor activity against non-small-cell lung cancer in vivo. (42) In previous studies, only combinations of EGFR antibodies (i.e. zalutumumab plus antibody 005, as also shown here), but not individual EGFR antibodies were capable of inducing pot ent CDC. (38) As demonstrated for the murine mAb 806 and 528, EGFRvIII and EGFR mAb can bind simultaneously and act synergistically. (43) To identify suitable combination partners among our antibodies tested, we first performed cross-competition studies, which revealed overlapping binding epitopes for MR1-1 and 13.1.2. In contrast, non-crossblocking combinations of ch806 with MR1-1 or 13.1.2 triggered efficient CDC against EGFRvIII-expressing HEK293T cells. Furthermore, combinations of zalutumumab with any of the EGFRvIII mAb induced EGFRvIII-specific CDC. Even though MR1-1 and ch806 also bound overexpressed wt EGFR on parental A431 cells, complement activation was restricted to EGFRvIII-expressing cells. Additionally, we demonstrated that Fc engineering of MR1-1 (MR1-1E3) improved EGFRvIII-specific CDC, while no increase in killing of EGFR wt-expressing cells was observed. Because EGFRvIII is exclusively found on cancer cells, these observations suggest a more tumor-specific therapeutic approach, which directs complement activity specifically to EGFRvIII-expressing cancer cells and leaves normal cells unaffected.

In conclusion, data presented in the present study provide novel insights into ADCC and CDC as potential effector mechanisms of EGFRvIII antibodies. Thus, EGFR-vIII mAb (MR1-1, ch806 and 13.1.2) proved to be effective in ADCC against cancer cells with amplified wt EGFR and concurrent EGFRvIII expression. Furthermore, combinations of EGFR (i.e. zalutumumab) and EGFRvIII-restricted mAb induced complement killing. These combinations might increase the therapeutic window for EGFR-directed therapy by more effectively and more selectively targeting tumor cells.

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