Fc-engineered EGF-R antibodies mediate improved antibody-dependent cellular cytotoxicity (ADCC) against KRAS-mutated tumor cells

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Oncogenic mutations of the KRAS gene have emerged as a common mechanism of resistance against epidermal growth factor receptor (EGF-R)-directed tumor therapy. Mutated KRAS leads to ligand-independent activation of signaling pathways downstream of EGF-R. Thereby, direct effector mechanisms of EGF-R antibodies, such as blockade of ligand binding and inhibition of signaling, are bypassed. Thus, a humanized variant of the approved EGF-R antibody Cetuximab inhibited growth of wild-type KRAS-expressing A431 cells, but did not inhibit KRAS-mutated A549 tumor cells. We then investigated whether killing of tumor cells harboring mutated KRAS can be improved by enhancing antibody-dependent cellular cytotoxicity (ADCC). Protein- and glyco-engineering of antibodies’ Fc region are established technologies to enhance ADCC by increasing antibodies’ affinity to activating Fcγ receptors. Thus, EGF-R antibody variants with increased affinity for the natural killer (NK) cell-expressed FcγRIIIa (CD16) were generated and analyzed. These variants triggered significantly enhanced mononuclear cell (MNC)-mediated killing of KRAS-mutated tumor cells compared to wild-type antibodies. Additionally, cells transfected with mutated KRAS were killed as effectively by ADCC as vector-transfected control cells. Together, these data demonstrate that KRAS mutations are not sufficient to render tumor cells resistant to ADCC. Consequently Fc-engineered EGF-R antibodies may prove effective against KRAS-mutated tumors, which are not susceptible to signaling inhibition by EGF-R antibodies. (Cancer Sci 2010; 101: 1080–1088)

After approximately 30 years of translational research, the epidermal growth factor receptor (EGF-R), ErbB1, has emerged as a validated target antigen for molecular therapies.¹ Today, both EGF-R-directed tyrosine kinase inhibitors (TKI) and EGF-R antibodies have obtained approval for tumor therapy.²,³ As expected from clinical experience with TKI against other target antigens,⁴ mutations in the EGF-R kinase domain critically affect sensitivity and resistance against EGF-R directed TKI.⁵ These EGF-R mutations proved less relevant for tumor cell killing by EGF-R antibodies in vitro⁶ and in animal models,⁷ and early clinical data support these observations.⁸ On the other hand, resistance against EGF-R antibodies in colorectal cancer (CRC) patients has been associated with activating mutations of KRAS⁹–¹² and other mediators of downstream signaling such as v-raf murine sarcoma viral oncogene homolog B1 (B-RAF),¹³ phosphoinositide-3-kinase (PI3K), phosphatase and tensin homolog (PTEN), and others.¹⁴ This clinical resistance of KRAS-mutated CRC led to the development of guidelines that recommend to limit the application of EGF-R-directed antibodies to CRC patients bearing wild-type KRAS tumors.¹⁵

KRAS belongs to the family of RAS proto-oncogenes that activate intracellular signaling downstream of receptor kinases.¹⁶ Thereby, oncogenic point mutations in the KRAS gene promote cellular growth and apoptosis resistance – leading to more aggressive tumor phenotypes with increased resistance to chemo- and radiotherapy. Oncogenic RAS proteins display impaired intrinsic GTPase activity preventing their inactivation by GTPase activating proteins (GAPs).¹⁷ This makes the development of small molecule inhibitors inherently difficult.¹⁸ Although promising approaches are emerging,¹⁹ furthermore, inhibitors of RAS processing, such as farnesyltransferase-inhibitors, often do not inhibit KRAS4b – the most common RAS isoform in solid tumors.²⁰ Therefore, substances blocking molecules downstream of KRAS, such as PI3K and MEK are actively investigated²¹ but other strategies to overcome RAS-mediated resistance to tumor therapy are required.

EGF-R antibodies can recruit a broad panel of tumor cell killing mechanisms.²² These may be divided into those mediated by the F(ab’) region – called direct mechanisms – and indirect mechanisms that are triggered by antibodies’ constant Fc region.²³ For EGF-R antibodies, direct mechanisms such as blockade of ligand binding, inhibition of signaling, and receptor down-regulation are considered to be particularly important.²⁴ However, in vitro EGF-R antibodies can also effectively recruit indirect mechanisms such as complement-dependent cytotoxicity (CDC)²⁵ and antibody-dependent cellular cytotoxicity (ADCC).²⁶ In mice, the efficacy of ErbB2 (HER-2/neu)-directed antibodies has been demonstrated to critically depend on the expression of activating Fcγ receptors,²⁷ and – against other target antigens – on antibodies’ relative binding affinities to activation compared to inhibitory Fc receptors (A:I ratio)²⁸ importantly, ADCC operated at lower antibody concentrations compared to direct effector mechanisms.²⁹ The contribution of ADCC to ErbB antibodies’ efficacy in patients is supported by studies demonstrating associations between therapeutic efficacy and the expression of certain Fcγ receptor allotopes,²⁹–³¹ which display enhanced antibody binding and increased ADCC activity compared to their less active allotforms.³²,³³ Together, these observations stimulate research into antibodies with enhanced affinity to activating Fcγ receptors.³⁴,³⁵

So far, two approaches of engineering antibodies’ Fc regions have proved to be particularly powerful for enhancing Fcγ receptor binding and for improving Fcγ receptor-mediated effector functions: reducing the amount of fucose in the α2H2 attached glycosylation³⁶–³⁷ (glyco-engineering), and site-directed mutagenesis of amino acids in the hinge or CH2 regions of antibodies (protein-engineering).³⁸ Here, we demonstrate that EGF-R antibodies of IgG1 isotype can kill KRAS-mutated tumor cells via ADCC. Tumor cell killing was significantly enhanced
when Fc-engineered EGF-R antibodies were compared to their wild-type counterpart. These results support approaches to enhance the ADCC activity of EGF-R antibodies, particularly in patients with KRAS-mutated tumors, who otherwise have a low probability of responding to currently available EGF-R-directed therapies.\cite{15}

**Material and Methods**

**Study population and consent.** Experiments reported here were approved by the Ethics Committee of the Christian-Albrechts-University, Kiel, Germany, in accordance with the Declaration of Helsinki. Blood donors were randomly selected from healthy volunteers, who gave written informed consent before analyses. Distribution of FcγRIIb genotypes among analyzed individuals was 25% (V/V), 33.3% (F/F), and 41.7% (V/F).

**Culture of human EGF-R-expressing cancer cell lines.** Epidermoid carcinoma cell line A431, non-small cell-lung cancer (NSCLC) cell line H2030 (both ATCC, Manassas, VA, USA), and colorectal carcinoma (CRC) cell line SW-480 (kindly provided by Gennab, Utrecht, The Netherlands) were kept in RPMI-1640-Glutamax-I medium (Invitrogen, Carlsbad, CA, USA). NSCLC cells A549 and CRC cells HCT-116 (both ATCC) were kept in DMEM (Invitrogen, Carlsbad, CA, USA). All media were supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The growth medium for the H2030 cell line was additionally supplemented with 2 mM l-glutamine, 10 mM sodium pyruvate, 4.5 g/L glucose, and 1.5 g/L sodium bicarbonate (all Sigma-Aldrich, St. Louis, MO, USA).

**Construction of a KRAS expression vector and stable transfection of A431 cells.** Full-length wild-type KRAS4b was amplified by PCR from cDNA of H1975 cells. The G12V mutation was inserted by PCR, and the resulting PCR product was cloned between the HindIII and NotI sites of the expression vector pSeph (Invitrogen). An N-terminal myc-tag was added into the Nhel and HindIII restriction sites with the following oligonucleotides 5′-CTAGCATGAGGACGAAGCTGAGCCAG-3′ and 5′-AGCTATATTTCAAGTCACAGGTCGCTCCAGGATGATATATA-3′. The control vector expression pSec (Invitrogen) following the manufacturer's protocol. Transfectants were sorted for GFP expression with a FACSAria cytofluorometer (BD Biosciences, Erembodegem, Belgium).

**Immunoprecipitation.** Cells were seeded in 10-cm dishes at 5×10⁶ cells/dish and grown overnight. The next day, cells were washed with ice-cold PBS and pelleted by centrifugation. Cells were lysed according to the manufacturer's instructions with native lysis buffer (NLB; Cell Signaling Technology, Danvers, MA, USA). Five-hundred µg of native protein were incubated with 10 µL of Raf-1 RBD agarose beads (Millipore, Billerica, MA, USA) for 1 h at 4°C. After washing, beads were stained with FITC-conjugated Fab′ fragments rabbit anti-human IgG (Dako, Glostrup, Denmark), respectively. Quantitative surface EGF-R expression was determined using murine EGF-R and control antibodies, and the QIFIKIT (Dako), according to the manufacturer’s instructions. Immunofluorescence was analyzed on a flow cytometer (Epics Profile; Beckman Coulter, Fullerton, CA, USA).

**Data processing and statistical analyses.** Data are displayed graphically and were statistically analyzed using GraphPad Prism 4.0. Curves were fitted using a nonlinear regression model with a sigmoidal dose response (variable slope). Statistical significance was determined by the Student’s t-test (paired, two-tailed) and the respective results are displayed as mean ± SEM. P-values were calculated and null hypothesis was rejected when P ≤ 0.05.

**Results**

**Characterization of used cell lines.** Cell lines were selected according to their KRAS status from the Cancer Genome Project online database at the Sanger Institute and from literature.\cite{42} While the A431 cell line harbors wild-type KRAS\cite{43} A549, H2030, HCT-116, and SW-480 cells carry activating mutations of the KRAS gene. As measured by using the QIFIKIT, all of these cell lines differ in the expression of EGF-R molecules per cell, with A431 cells showing the highest and HCT-116 cells the
lowest EGF-R expression. The characteristics of the aforementioned cell lines are summarized in Table 1. To analyze whether point mutations of the KRAS gene, described for A549, H2030, HCT-116, and SW-480 cells, influence the activation status of RAS, all five cell lines were assayed by immunoprecipitation against activated RAS\(^{\text{GTP}}\). As expected, enhanced activation of RAS was detected for the cell lines harboring oncogenic KRAS (A549, H2030, HCT-116, and SW480) when compared to A431 cells (Fig. 1).

**Table 1.** Cell lines used in this study

| Cell line | Origin | EGF-R molecules/cell† | EGF-R | KRAS |
|-----------|--------|-----------------------|-------|------|
| A431      | Epidermoid carcinoma | 2 056 108 ± 399 361 | Wild-type, amplified | Wild-type |
| A549      | NSCLC  | 255 890 ± 21 063      | Wild-type | G12S homozygous |
| H2030*   | NSCLC  | 166 400 ± 31 407      | Wild-type | G12V homozygous |
| HCT-116† | CRC    | 40 056 ± 6538         | Wild-type | G12D heterozygous |
| SW-480   | CRC    | 48 059 ± 6478         | Wild-type | G12V homozygous |

† Determined by indirect immunofluorescence using the QIFIKIT; ‡additional homozygous p53 mutation (G262V); §additional heterozygous PIK3CA mutation (H1047R). CRC, colorectal cancer; EGF-R, epidermal growth factor receptor; NSCLC, non-small-cell lung cancer. Source: Cancer Genome Project online database at Sanger Institute (http://www.sanger.ac.uk) and reference.

Figure 1. Oncogenic mutations of the KRAS gene are accompanied by constitutive activation of KRAS. Cells were grown overnight in 10-cm dishes at 5 × 10^4 cells/dish. The next day, immunoprecipitation against activated RAS\(^{\text{GTP}}\) was performed using Raf-1 RBD agarose beads and 500 ng native protein extracts. Precipitated, activated RAS was separated by SDS-PAGE and immunoblotted against RAS. The amount of total RAS protein in the analyzed cell lines was controlled by separation of 15 μg of whole protein extracts per sample by SDS-PAGE and immunoblotting against RAS.
to directly inhibit cell proliferation could be detected – demonstrating F(ab’)-mediated effector functions not to be affected by Fc protein-engineering.

**Fc engineering enhanced ADCC against KRAS-mutated tumor cells.** Furthermore, wild-type antibody and its protein-engineered variants were compared in ADCC assays against the KRAS-mutated tumor cell lines HCT-116, SW-480 (Fig. 4A,B), A549, and H2030 (Fig. 4C,D). Significantly enhanced killing (up to 2.5-fold) of all cell lines was observed when protein-engineered antibody variants were compared with the respective wild-type EGF-R antibody. The wild-type antibody demonstrated significantly lower ADCC activity with all cell lines tested. Moreover, MNC mediated ADCC against KRAS-mutated tumor cells with both protein- and glycoengineered antibodies triggered significant lysis (Fig. 4D).

To assess whether glyco-engineering also results in enhanced ADCC against KRAS-mutated tumor cells, similar experiments were performed comparing a non-fucosylated and the wild-type antibody (Fig. 5). Interestingly, all four cell lines proved susceptible to ADCC by the glyco-engineered EGF-R antibody. The wild-type antibody demonstrated significantly lower ADCC activity with all cell lines tested. Moreover, MNC mediated ADCC against KRAS-mutated tumor cells with both protein- and glycoengineered variants is not only distinguished by lower EC50 concentrations compared to wild-type, but also by remarkably increased killing at saturating antibody concentrations.

In conclusion, data presented in Figures 5 and 6 demonstrated that KRAS-mutated cells were susceptible to ADCC, and that ADCC activity could be enhanced by protein- or glyco-engineering of EGF-R antibodies.

**Fc engineering improved ADCC activity independently of the FcγRIIa allotype.** To analyze whether ADCC activity triggered by Fc-engineered EGF-R antibodies was influenced by different FcγRIIa-158F/V genotypes, ADCC experiments were performed with KRAS-mutated SW-480 cells and MNC effector cells from healthy donors, genotyped for the FcγRIIa-158F/V polymorphism. Irrespective of the individual FcγRIIa allotype, stronger ADCC activity was observed for protein- and glycoengineered EGF-R antibodies.

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**Table 2. Binding affinities of EGF-R antibody variants to human Fc receptors**

| Antibody variant | $K_d$ (nM) (fold increase to IgG1wt) |
|------------------|------------------------------------|
|                  | FcγRIIa R131 | FcγRIIa V158 | FcγRIIa F158 |
| Wild-type        | 640 (1.0)    | 870 (1.0)    | 250 (1.0)    | 920 (1.0)    |
| Afucosylated IgG1| 680 (0.94)   | 610 (1.4)    | 14 (18)      | 84 (11)      |
| I332E            | 400 (1.6)    | 530 (1.6)    | 34 (7.4)     | 200 (4.6)    |
| S239D/I332E      | 210 (3.0)    | 160 (5.4)    | 5.0 (50)     | 24 (38)      |

*EGF-R, epidermal growth factor receptor.*

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**Fig. 2.** Humanized Cetuximab did not induce growth inhibition, but triggered antibody-dependent cellular cytotoxicity (ADCC) against the KRAS-mutated tumor cell line A549. Wild-type KRAS-expressing A431 cells and KRAS-mutated A549 cells served as targets in MTS growth inhibition and 51chromium-release ADCC assays. (A,C) Cells were incubated in the presence or absence of wild-type or control antibodies (0.013 to 200 μg/mL). After 72 h of incubation, cells were analyzed by MTS assay. (B,D) For ADCC assays, cells were incubated with human mononuclear cells (MNCs) at an E:T ratio of 80:1 in the presence or absence of wild-type or isotype control antibodies (final concentration 2 μg/mL). After 3 h, lysis of tumor cells was determined by measurement of 51Cr release. Data are presented as mean ± SEM of multiple independent experiments (MTS A431 n = 9; MTS A549 n = 3; ADCC A431/A549 n = 5) with different donors. Asterisks (*) indicate significant lysis or growth inhibition ($P < 0.05$).
glyco-engineered antibodies compared to their wild-type counterpart (Fig. 6).

Overexpression of oncogenic KRAS did not affect MNC-mediated ADCC. Previous experiments demonstrated that KRAS-mutated tumor cell lines were susceptible to ADCC by Fc-engineered EGF-R antibodies, but did not address the question whether KRAS mutations directly affected ADCC. To investigate this interesting issue, A431 cells harboring wild-type KRAS were stably transfected with oncogenic KRAS4bG12V or a control vector (see Materials and Methods). Tumor cells were assayed by immunoprecipitation against activated RASGTP and western blot experiments. Results from these experiments demonstrated that both transfectants stably expressed GFP (data not shown) and basal levels of active, wild-type KRAS, whereas only in KRAS4bG12V-transfected cells a second protein band could be detected – representing active myc-tagged KRAS4bG12V (Fig. 7A).

Next, ADCC experiments with MNC effector cells were performed with A431 KRAS4bG12V cells and A431 cells transfected with the control vector. As presented in Figure 7(B), no differences in Cetuximab-triggered cytotoxicity were observed between both transfectants – demonstrating that oncogenic KRAS did not affect MNC-mediated cytotoxicity.

Discussion

In the present study, a humanized variant of Cetuximab did not inhibit the growth of KRAS-mutated A549 cells, but triggered limited ADCC activity against these cells. Interestingly, established approaches to enhance ADCC activity of antibodies by protein- or glyco-engineering significantly improved killing against a panel of KRAS-mutated tumor cells by MNC effector cells. This difference between wild-type and Fc-engineered antibodies is well explained by the homology between FcRIIa – the predominant Fcγ receptor on human natural killer (NK) cells(38) that constitute the main effector cell population in MNC. Interestingly, protein-engineered antibodies with increased binding affinity for FcγRIIIa (I332E) demonstrated lower ADCC activity with PMN(39) which is not a FcγRIIIb/(40,41) receptor on human natural killer cells(38) that constitute the main effector cell population in MNC. Interestingly, protein-engineered antibodies with increased binding affinity for FcγRIIIa improved ADCC by MNC effector cells, but reduced tumor cell killing by PMN(41). Similar results were observed in the present study with a glyco-variant of the humanized Cetuximab antibody Zalutumumab where low-fucosylated compared to completely-fucosylated Zalutumumab demonstrated higher binding to FcγRIIa and the PMN effector cells than the respective wild-type antibody (Fig. 3D). We have previously reported similar results for glyco-engineered variants of the EGF-R antibody Zalutumumab where low-fucosylated compared to completely-fucosylated Zalutumumab demonstrated higher binding to FcγRIIa, improved ADCC by MNC effector cells, but reduced tumor cell killing by PMN. Similar results were observed in the present study with a glyco-variant of the humanized Cetuximab antibody (data not shown). These unexpected results with PMN effector cells may be explained by the homology between FcγRIIa and the PMN-expressed FcγRIIB. Thus, improving the affinity for FcγRIIa simultaneously increases binding to FcγRIIB(42), which is not a cytotoxic trigger molecule on PMN. Glyco-engineering predominantly modulates binding affinity to FcγRIIIa, while protein-engineering can alter binding characteristics to FcγRIIIa and FcγRIId, as well as FcγRI and FcγRIIB. Whether these differential FcγR binding profiles of protein- and glyco-engineered antibodies will impact their therapeutic effects in vivo will require further studies.

Fig. 3. Fc engineering enhanced mononuclear cell (MNC)-mediated but decreased polymorphonuclear cell (PMN)-mediated antibody-dependent cellular cytotoxicity (ADCC). (A) Model structure of human IgG1 highlighting the amino acid substitutions and the fucose residue in the antibodies’ heavy chains. Gray = heavy chain; white = light chain. The picture was generated using Accelrys DS visualizer software. To characterize the antibody variants, indirect immunofluorescence and ADCC assays with wild-type KRAS and epidermal growth factor receptor (EGF-R)-high-expressing A431 cells were performed. (B) For indirect immunofluorescence A431 cells were incubated with increasing concentrations (0.013 to 200 μg/mL) of wild-type, Fc variant (I332E or S239D/I332E), or control antibodies, and stained with F(ab’2) fragments of polyclonal FITC-conjugated rabbit antihuman IgG. Each data point represents the mean fluorescence intensity (MFI) of respective antibody at the indicated concentration. To analyze the cytotoxic activity of Fc-engineered antibodies, ADCC assays (C–E) were performed. Target cells were opsonized with increasing concentrations of wild-type, Fc variant (I332E or S239D/I332E), or control antibodies, respectively. Isolated MNC (C) or PMN (D) at an E:T ratio of 80:1 or whole blood (E) served as effector cells in 51Cr release assays. Data are presented as mean ± SEM of at least three independent experiments. *P < 0.05 control vs wild-type antibody; #P < 0.05 variants vs wild-type antibody.
Notably, Fc-engineered EGF-R antibodies proved more effective than the wild-type antibody in ADCC against both KRAS-unmutated and -mutated cell lines (Figs 3–5). The negative impact of KRAS mutations on the clinical efficacy of EGF-R antibodies in CRC has now been confirmed in numerous clinical trials (reviewed in reference(15)), while more preliminary studies in NSCLC patients suggested that the KRAS status may not be predictive for Cetuximab’s efficacy in these patients. (8,45) Presently, it is not clear whether these differences are explained by statistical considerations (lower response rates and lower incidence of KRAS mutations in NSCLC vs CRC) or whether they reflect biological differences between both tumor types. There is experimental evidence to suggest that the relative contribution of antibodies’ effector mechanisms depends on antibody concentrations(28) and potentially on the tumor location. (46) Further studies are required to address these interesting issues.

Results from in vitro studies with tumor cell lines are often confounded by multiple genetic abnormalities that are commonly observed in these cells. These often poorly defined alterations impair the interpretation of results with respect to specific mutations. Thus, results presented in Figures 4 and 5 demonstrate that KRAS-mutated tumor cells can be killed by ADCC,
and that mutated KRAS is not sufficient to render tumor cells resistant to ADCC, but they do not allow the direct assessment of the impact of KRAS mutations on the susceptibility of tumor cells against ADCC. To address this important issue, wild-type KRAS-expressing A431 cells were transfected with oncogenic KRAS4bG12V or a control vector. Interestingly, both cell lines were similarly susceptible to MNC-mediated ADCC by EGF-R antibodies. Furthermore, HCT-116 cells additionally carry an activating PI3K mutation (http://www.sanger.ac.uk), which is discussed as another mechanism of resistance against EGF-R antibodies. Our preliminary results suggest that also this mechanism of resistance could be overcome by Fc-engineered EGF-R antibodies. Potentially, enhancing Fc-mediated effector functions of EGF-R antibodies – such as ADCC and CDC – may prove as a more general approach to overcome resistance against EGF-R-directed therapy.

An important question is whether our in vitro results have relevance for the understanding of the mechanisms of action for EGF-R antibodies in vivo, and whether they impact potential approaches to enhance the efficacy of EGF-R directed therapy. Recent recommendations suggested to exclude patients with KRAS-mutated CRC from EGF-R directed therapies, as they showed low response rates in clinical trials. These clinical observations supported the concept that inhibition of EGF-R-mediated signaling might be the predominant mechanism of action for EGF-R antibodies. Accordingly, activating mutations of downstream signaling molecules – like KRAS or v-raf murine sarcoma viral oncogene homolog B1 (B-RAF) – would render tumor cells resistant to EGF-R inhibitors. Further support for the contribution of Fc-mediated effector mechanisms for Cetuximab’s clinical efficacy was derived from statistical correlations between Fcγ receptor alloforms and clinical outcomes. These studies suggested that Fc-mediated effector functions were relevant for Cetuximab’s clinical efficacy in CRC patients – as previously demonstrated for other therapeutically approved antibodies. Importantly, individual patients with favorable Fcγ receptor alloforms (FcγRIIa-131H and/or FcγRIIIa-158V) responded to Cetuximab therapy – even when their tumors harbored oncogenic KRAS mutations. As previously reported by others, Fc engineering improved ADCC by FcγRIIIa-158F/F, -158F/V, and 158V/V donors.

Presently, there is a wide gap between results from in vitro models addressing mechanisms of sensitivity and resistance against EGF-R-directed antibody therapy and clinical data that validate them. Unfortunately, studies in small animals are of

Fig. 6. Fc-engineering improved antibody-dependent cellular cytotoxicity (ADCC) activity independently of the FcγRIIIa-158F/V genotype. For ADCC experiments, SW-480 cells were incubated with increasing concentrations of wild-type, variant, or control antibodies and mononuclear cell (MNC) effector cells from healthy donors (E:T ratio 80:1) that were genotyped for the FcγRIIIa-158F/V polymorphism. After 3 h of incubation, 51Cr-release was determined, and relative tumor cell lysis was calculated as described in Materials and Methods. Graphs represent means of triplicates from experiments with MNC from FcγRIIIa-158F/V (A), -158F/F (B), or -158V/V (C) genotyped donors, respectively. Legends are as shown.

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Fig. 7. Oncogenic KRAS did not affect mononuclear cell (MNC)-mediated antibody-dependent cellular cytotoxicity (ADCC) activity. A431 cells were stably transfected with oncogenic myc-tagged KRAS4bG12V or control vector and stable vector expression was controlled by sorting for GFP expression. (A) Functional analysis of transfected KRAS4bG12V was performed by immunoprecipitation against activated RASGTP using Raf-1 RBD agarose beads and 500 μg native protein extracts. Precipitated, activated RAS was separated by SDS-PAGE and immunoblotted against activated RAS. As previously reported by others, Fc engineering improved ADCC by FcγRIIIa-158F/F, -158F/V, and 158V/V donors.

Presently, there is a wide gap between results from in vitro models addressing mechanisms of sensitivity and resistance against EGF-R-directed antibody therapy and clinical data that validate them. Unfortunately, studies in small animals are of
limited value for many of these specific questions due to differences between the murine and the human Fcγ receptor systems. In studies investigating the impact of Fc engineering on antibody efficacy, those differences in species characteristics additionally impede the transfer of in vitro results to in vivo relevance. (12) On the other hand, protein- and glyco-engineered antibodies against other target antigens (e.g., CD30, CD20) have already entered clinical trials, while trials with glyco-engineered EGF-R antibodies are expected to start soon. Another approach to clinically assess the contribution of ADCC versus other antibody effector mechanisms would include studies that prospectively investigate the impact of Fcγ receptor polymorphisms on therapeutic outcomes, for example in FcγRIIa-158V/V donors, as is currently on-going for Trastuzumab (a humanized monoclonal IgG1 antibody directed against human epidermal growth factor receptor 2, ErbB2).

In conclusion, KRAS-mutated tumor cells can be effectively killed by ADCC, indicating that mutated KRAS is not sufficient to confer resistance to antibody-mediated effector cell killing. Consequently, approaches to enhance ADCC may have the potential to increase the clinical activity of EGF-R antibodies – particularly in patients whose tumors harbor activating KRAS mutations.

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Disclosure Statement
Greg A. Lazar is employed by Xencor, Monrovia, CA, USA.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Antibody expression in LEC13 cells resulted in an afucosylated variant of 225-IgG1. Expression constructs harboring the light- and heavy-chain cDNAs of the 225 antibody were expressed in LEC13 and CHO-K1 cells by transient transfection. The purified antibodies were analyzed by SDS-PAGE and immunoblotting with HRP-conjugated antihuman-IgG antibodies and a HRP-conjugated Aleuria Aurantia lectin specific for fucose linked (α-1,6) to N-acetylglucosamine or (α-1,3) to N-acetyllactosamine-related structures. Lane 1 = 225-IgG1 expressed in LEC13 cells; lane 2 = 225-IgG1 expressed in CHO-K1 cells. One representative experiment out of three performed is shown.

Fig. S2. Fc engineering did not influence F(ab')-mediated effector functions. To characterize the antibody variants’ F(ab')-mediated effector functions, MTS assays with wild-type KRAS-expressing A431 cells and oncogenic KRAS-expressing A549 cells were performed. Inhibition of cell growth was analyzed by incubating cells in the presence or absence of increasing concentrations (see above) of the antibodies. After 72 h, vital cell masses were measured by MTS assays. *P < 0.05 control vs wild-type antibody.

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