Immune Effector Functions of Human IgG2 Antibodies against EGFR

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Abstract

Three FDA-approved epidermal growth factor receptor (EGFR) antibodies (cetuximab, panitumumab, and cetrizumab) are clinically available to treat patients with different types of cancers. Interestingly, panitumumab is of human IgG2 isotype, which is considered to have limited immune effector functions. Unexpectedly, our studies unraveled that human IgG2 antibodies against EGFR mediated effective CDC when combined with another noncross-blocking EGFR antibody. This second antibody could be of human IgG1 or IgG2 isotype. Furthermore, EGFR antibodies of human IgG2 isotype were highly potent in recruiting myeloid effector cells such as M1 macrophages and PMN for tumor cell killing by ADCC. Tumor cell killing by PMN was more effective with IgG2 than with IgG1 antibodies if tumor cells expressed lower levels of EGFR. Additionally, lower expression levels of the "don't eat me" molecule CD47 on tumor cells enabled ADCC also by M2 macrophages, and improved PMN and macrophage-mediated ADCC. A TCGA enquiry revealed broadly varying CD47 expression levels across different solid tumor types. Together, these results demonstrate that human IgG2 antibodies against EGFR can promote significant Fc-mediated effector functions, which may contribute to their clinical efficacy. The future challenge will be to identify clinical situations in which myeloid effector cells can optimally contribute to antibody efficacy.

Introduction

Monoclonal antibodies against checkpoint controlling molecules of the adaptive immune system—such as CTLA-4, PD-1, PD-L1, and others—currently revolutionize cancer therapy (1). The scientific and economic interest to identify biomarkers to predict response or resistance to these novel therapies has encouraged studies to investigate closely the mutational status of different tumor types and their immune cell infiltrate (2). The latter studies benefit from recently developed technologies, which enable more detailed analyses of the different tumor-infiltrating cell types as well as their functional states (3). Across many different cancer types, myeloid cells—including monocytes/macrophages, granulocytes, and dendritic cells—were found to make a numerically significant contribution to the tumor cell infiltrate (3). Depending on their state of differentiation, these infiltrating myeloid cells have been divided into antitumorogenic macrophages and neutrophils (M1 and N1, respectively), which can be differentiated from tumor-promoting M2/N2 phenotypes (4, 5). Similar to, but less well studied than in T cells, the tumoricidal activity of innate myeloid cells is also controlled by activating and inhibitory receptors (6, 7). Among the identified inhibitory receptors, SIRPα appears to have a prominent function on myeloid cells, which predominantly interacts with CD47 expressed on malignant and nonmalignant cells to provide a "don't eat me signal." In the clinical setting of antibody-based immunotherapy, activating signals to myeloid effector cells are predominantly provided by antibody-binding Fc receptors. Human myeloid cells express a panel of molecularly and functionally distinct receptors for IgG (8, 9). These receptors, e.g., differ in their affinity for different antibody isotypes (10). Peripheral blood monocytes typically express low levels of FcγRI (CD64), which is upregulated upon exposure to interferon γ, as well as FcγRIIA (CD32a) and the inhibitory FcγRIIB (CD32b) isofrm. Monocyte-derived macrophages can additionally express FcγRIIA (CD16a), but usually do not express FcγRI. Human, but not murine, PMN abundantly express the GPI-linked FcγRIIL isofrm, which does not trigger antibody-dependent cell-mediated cytotoxicity (ADCC), but rather functions as a "decoy" receptor for antibodies with increased FcγRIIIa-binding affinity (11). For FcγRIIA and FcγRIIIA functionally relevant polymorphisms have been identified (12, 13), which were correlated to clinical benefit from tumor-directed antibodies of human IgG1 isotype such as rituximab, herceptin, or cetuximab (14). Although controversial, these studies support the concept that these approved antibodies can mediate their therapeutic efficacy, at least in part, by effector cell recruitment. Studies in mice have
suggested that myeloid cells are particularly relevant for the therapeutic efficacy of several antibodies (15–17), but at present, it is unclear how these results translate to clinical situations in patients. IgG2 is the second most prevalent antibody isotype in human serum, which is predominantly produced in response to bacterial polysaccharide antigens (18). Differences in the antibody isotype repertoire are increasingly recognized to contribute to the protective immune response in infectious diseases (19). At present, human IgG1 is by far the most commonly used isotype in tumor immunotherapy, but also four human IgG2 antibodies are FDA approved, and many more are in clinical development (20). Because human IgG2 only weakly binds to FcγRIIIa (10), this isotype does not effectively recruit NK cells for ADCC (21, 22). These results led to the conclusion that human IgG2 is an isotype with limited immune effector functions. However, myeloid effector cells such as monocytes and PMN mediated ADCC with human IgG2 antibodies against the epidermal growth factor receptor (EGFR) as effectively as with human IgG1 antibodies (22). In comparison with human IgG1, human IgG2 antibodies poorly activated complement-dependent cytotoxicity (CDC; ref. 21). However, at higher target antigen densities and increased serum and antibody concentrations, IgG2 induced CDC by the classic and the alternative complement pathways (23, 24). Nevertheless, the approved IgG2 antibody against EGFR (panitumumab) did not trigger CDC as a single agent, even under optimal experimental conditions (25).

EGFR is a tyrosine kinase receptor, which is overexpressed on many solid tumor types and constitutes a promising target antigen for small molecule and antibody-based approaches (26). Thus, three generations of tyrosine kinase inhibitors are currently available for the treatment of patients with lung cancer, whose tumors harbor characteristic activating mutations in the ATP-binding domain (27). Additionally, EGFR antibodies have been approved for patients with colorectal cancer lacking KRAS mutations (cetuximab and panitumumab), head and neck cancer (cetuximab), and recently also for lung cancer (necitumumab). However, several other EGFR antibodies have also failed during clinical development (e.g., matuzumab, zalutumumab), and many more clinical indications could be envisioned with respect to EGFR’s broad expression profile. A better understanding of the required mechanisms of action in particular tumor indications and respective biomarker identification may increase the chances of successful drug development (27).

Here, we investigated immune effector functions of human IgG2 antibodies against EGFR in more detail. With the exception of NK cell-mediated ADCC, the human IgG2 isotype proved at least as effective in triggering antibody-mediated effector functions as human IgG1. Thus, tumor cell types or stages of tumor development with a myeloid rather than NK cell predominated immune cell infiltrate may particularly benefit from the application of human IgG2 antibodies against EGFR. Because myeloid cell-mediated tumor cell killing is effectively regulated by CD47/SIRPα interactions, CD47 low-expressing tumor types could be promising candidates for this approach.

Materials and Methods

Experiments with human material were approved by the Ethical Committees of the participating institutions in accordance with the Declaration of Helsinki.

Antibodies

The approved EGFR antibodies panitumumab (human IgG2, E7.6.3, Vectibix) and cetuximab (chimeric human IgG1; 225, Erbitux) were from Amgen and Merck, respectively. Human IgG1 and IgG2 variants of the EGFR antibodies zalutumumab (sequence in patent WO 20020405684772A2). 003, 005, 018 (sequences in patent WO 2009/030239A1) and matuzumab (sequence in patent WO 2009/034940A1) were produced at Genmab and in Southampton (Cancer and Vaccine Group, Cancer Sciences, University of Southampton, UK), respectively. Antibodies were purified by protein A affinity chromatography (rProtein A FF; GE Healthcare), dialyzed overnight to PBS, and filter sterilized over 0.22 µm dead-end filters. The concentration of purified IgGs was determined by absorbance at 280 nm.

Quality assessment of purified antibodies was performed by SDS/PAGE (>90% intact IgG, >95% HC + LC under reducing conditions), ESI-TOF mass spectrometry (identity confirmation), and HP-SEC (aggregate level < 5%), as described previously (28).

Cell lines

Cell lines were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures), ECACC (European Collection of Authenticated Cell Culture) or kindly provided by Dr. M. Binder (University Hospital Eppendorf, Hamburg, Germany). All cells were grown in media as recommended by the suppliers and were obtained between 2011 and 2016.

C1q ELISA

C1q binding was measured by ELISA. Microtiter stripes (Thermo Fisher Scientific) were coated with serial dilutions (0–50 µg/mL) of IgG1 or IgG2 at 4 °C before 25% NHS was added as source of human C1q. Bound C1q was quantified by 10 µg/mL monoclonal mouse-anti-human C1q antibody (Quidel) visualized by 1:000 diluted HRP-conjugated goat-anti-mouse antibody (West Grove). Absorbance was measured by a SunRise microplate reader (TECAN).

Generation of CD47 knockout A431 cells

gRNA directed against the coding sequence of CD47 were designed using the Zhang laboratory online tool (http://crispr.mit.edu/). As a control oligo, 5′-GCACTACCAAGGCTAACTCA3′ from pCas-Scramble CrispR vector (Origene) was used. The control oligo or CD47 coding sequences were cloned into pLentiCrispR-v2, a second-generation SIN lentiviral plasmid. Lentiviral particles were produced by transient transfection of pLentiCrispR-v2 –CD47ko or -control and co-constructs psPAX2 and pCMV-VSVG in 293T cells. On days 2 and 3 after transfection, supernatants of transfected cells were filtered through a 0.45 µm filter and added to A431 cells. Transduced cells were selected by 1 µg/mL puromycin. Single-cell clones were obtained by limiting dilution.

Growth inhibition

Growth inhibition of DiFi cells was analyzed using the 3-(3,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium inner salt (MTS) assay (Promega). DiFi cells were seeded at a density of 5 × 10^4 cells/well and treated with antibodies at the indicated concentrations. After incubation for 72 hours at 37 °C, MTS was added and absorption at 490 nm/670 nm was measured. Percentage of growth inhibition was
Flow-cytometric analyses

EGFR and CD47 expressions were quantified using the murine antibodies m245 (EGFR) and B6H12 (CD47, Bio XCell). Binding of murine antibodies was determined by FITC-conjugated goat anti-mouse F(ab)_2, staining. A murine CD20 antibody served as a control.

PE-labeled antibodies against CD16 (FcγRIII), CD32 (FcγRII), CD64 (FcγRI, Beckman Coulter) or against CD80, CD163, CD47, or SIRPα (Milltenyi Biotec) were used for direct immunofluorescence analyses. Fcγ receptor blockade was achieved by IVIG (Privigen). Immunofluorescence was analyzed on flow cytometers (Epics Profile or Navios; Beckman Coulter).

Complement deposition

Deposition of complement components on target cells was determined by incubating 2.5 × 10^5 cells with control or single EGFR antibodies (all at 10 μg/mL) alone or in combination with a noncross-blocking EGFR antibody (anti-EGFR-IgG1 003 at 5 μg/mL) for 15 minutes at 4°C. Subsequently, 25% v/v human serum was added in the presence of a C5-blocking antibody (eculizumab, Alexion Pharma; 100 μg/mL) to avoid CDC. Samples were either stained with polyclonal FITC-conjugated C1q or C4b/c antibodies (both from Agilent) or polyclonal FITC-conjugated anti-iC3c serum (Abcam) and analyzed on flow cytometers (Epics Profile or Navios; Beckman Coulter).

CD47 siRNA-induced knockdown experiments

Target cells were seeded at a density of 2.5 × 10^5/well in 6-well plates. Four hours later, transfection of siRNA was performed using lipofectamine 2000 (Invitrogen). Synthetic siRNA-targeting CD47 (ID HS.4446414) or a control siRNA (Low GC Duplex #1 from Invitrogen) were used. Efficacy of siRNA-induced knockdown of CD47 was analyzed using a PE-conjugated mouse anti-human CD47 antibody and an irrelevant PE-conjugated antibody (both Miltenyi Biotec).

CDC assays

CDC assays were performed as described (25). Briefly, target cells were preincubated with 200 μg/mL [51Cr] for 2 hours. As the source of complement, 25% v/v freshly drawn human serum was used in the presence of the indicated antibodies. For combination assays, noncross-blocking EGFR antibodies of IgG1 isotype (003, 005, 018 all from Genmab) were used at a final concentration of 5 μg/mL. Percentage of [51Cr] release was calculated using the formula: % growth inhibition = absorption with antibody/absorption without (w/o) antibody × 100.

Isolation of human effector cells

Human effector cells (MNC, PMN) were isolated from peripheral blood of healthy volunteers, as previously described (11) using polymorphprep (Progen). Macrophages were generated by adherence of MNC using monocyte-attachment medium (PromoCell). After attachment, cells were incubated for 24 hours in serum-free X-Vivo medium (Lonza). Subsequently, cytokines for macrophage generation and polarization were added. M1-like macrophages were generated by incubation with 10 ng/mL GM-CSF for 6 days followed by 10 ng/mL interferon γ and 100 ng/mL LPS for 2 additional days. To generate M2-like macrophages, attached MNC were incubated with 25 ng/mL M-CSF for 6 days. Subsequently, IL4 at 10 ng/mL was added for 2 additional days to complete M2-like polarization. All cytokines were from PeproTech, LPS from Sigma-Aldrich.

ADCC assays

ADCC was analyzed in [51Cr] release assays as described (11). Briefly, isolated effector cells (MNC, PMN, monocytes or macrophages), antibodies at various concentrations, and medium were added to round-bottom microtiter plates (Nunc). Assays were started by adding effector and target cells at indicated E:T ratios. After 3 hours (MNC and PMN) or 16 hours (macrophages) at 37°C, [51Cr] release from triplicate samples was measured. The percentage of cellular cytotoxicity was calculated using the formula: percentage of specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) × 100; maximal [51Cr] release was determined by adding Triton-X (2% final concentration) to target cells, and basal release measured in the absence of sensitizing antibodies and effector cells.

Trogocytosis assay

The transfer of membrane from tumor cells to neutrophils was measured by flow cytometry as described (29). Briefly, lamin-B-TRQ–transfected A431 cells were labeled at 37°C with the lipophilic membrane dye DiO (5 μmol/L, Invitrogen). Labeled target cells were then incubated with GM-CSF–stimulated neutrophils at an effectortarget cell ratio of 5:1 in the absence or presence of either 5 μg/mL cetuximab (IgG1 [225]) or panitumumab [IgG2 (E7.6.3)]. After 60 minutes, samples were fixed and measured on a flow cytometer. After gating for the TRQ-negative neutrophil population, the percentage of DiO-positive cells and their mean fluorescent intensity (MFI) were evaluated.

TCGA data analysis

RNA-sequencing data sets (RNAseqV2) from 17 different human cancer types (ovary, thyroid, lung, cervix, head and neck, prostate, glioma, pancreas, colon, stomach, breast, endometrium, urothelium, melanoma, testis, kidney, liver) were obtained from The Cancer Genome Atlas (TCGA; https://portal.gdc.cancer.gov/) via The Human Protein Atlas (https://www.proteinatlas.org/ENSG00000196776-CD47/pathology). Expression in fragments per kilobase per million (FPKM values) was compared with box-whisker plots (whiskers indicating the 10th and 90th percentiles).

Data processing and statistical analyses

Data were generated from at least three independent experiments with blood from different healthy donors. Graphical and statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software). Group data are reported as mean ± SEM. Significance was determined by two-way ANOVA-repeated measures test with Bonferroni post hoc correction. EC50 values were calculated from dose–response curves, reported as means ± SEM.

Results

EGFR-directed IgG1 and IgG2 antibodies do not differ in Fab-mediated effector functions

Although IgG1 and IgG2 share approximately 90% sequence homology, both isotypes differ in structural aspects, especially in their heavy and light chain disulfide linkage (Fig. 1A). Although
IgG1 antibodies contain disulfide bonds between the C terminus of the light chains and the upper hinge region of the heavy chains, IgG2 antibodies are linked between the light chains’ C-terminus and the N-terminal regions of the heavy chains’ CH1 domains. Thus, we analyzed whether these structural differences influenced Fab-mediated effector functions induced by EGFR-directed IgG1 and IgG2 antibodies. First, the ability of EGFR-directed IgG1 as well as IgG2 antibodies for target antigen binding was compared. Both clinically approved EGFR antibodies cetuximab (IgG1, 225) and panitumumab (IgG2, E7.6.3) displayed similar binding to EGFR-expressing A431 cells, as indicated by similar EC50 values (1.6 ± 1.3 μg/mL and 1.5 ± 1.3 μg/mL for cetuximab and panitumumab, respectively). Cetuximab and panitumumab contain different variable regions and bind to different, although overlapping EGFR epitopes (25). Thus, we next compared IgG1 and IgG2 isotype switch variants of zalutumumab (2F8) in binding to EGFR-expressing cells (Fig. 1B). Again, the binding characteristics of both IgG variants were similar (EC50 = 3.2 ± 1.4 μg/mL and 2.0 ± 1.4 μg/mL for IgG1 and IgG2, respectively), and no significant differences in the number of EGFR-binding sites were observed between IgG1 and IgG2 antibodies (Fig. 1B; Supplementary Fig. S1A). Next, we used MTS assays to investigate growth inhibition of DiFi cells in the presence of increasing concentrations of EGFR antibodies of IgG1 or IgG2 isotypes. All three EGFR antibodies (225, E7.6.3, 2F8) mediated similar maximal growth inhibition of DiFi cells (Fig. 1C). No differences between IgG1 (225) and IgG2 (E7.6.3) were observed, while the IgG2 variant of 2F8 was less efficient at lower antibody concentrations than 2F8-IgG1. These results demonstrated that Fab-mediated effector functions of EGFR antibodies were marginally affected by selecting IgG1 or IgG2 isotypes.

EGFR-directed IgG2 antibodies induced efficient CDC in combination with nonepitope-overlapping IgG1 or IgG2 antibodies

Combinations of IgG1 antibodies against nonoverlapping EGFR epitopes were shown to efficiently induce complement deposition and CDC (25). Results from ELISA experiments confirmed the previously reported low C1q-binding capacity of human IgG2 compared with IgG1 antibodies under these experimental conditions (Fig. 2A). Next, we analyzed whether IgG2 antibodies will induce complement deposition on tumor cells and CDC in combination with IgG1 antibodies to nonoverlapping EGFR epitopes. As a combination partner the EGFR antibody 003 was chosen, which reacts with a nonligand-binding EGFR epitope and does not interfere with cetuximab or panitumumab binding (25). As expected, all individual EGFR antibodies did not induce complement binding or deposition on A431 cells as single molecules, as previously shown (25). Interestingly, however, the IgG1/IgG2 combination of antibody 003 and panitumumab was as efficient in inducing C1q binding (Fig. 2B), C4b deposition (Fig. 2C) and C3b deposition (Fig. 2D) as the IgG1/IgG1 combination of antibody 003 and cetuximab. Subsequently, the CDC activity of the respective IgG1/IgG2 (mAb 003 and panitumumab) and IgG1/IgG1 (mAb 003 and cetuximab) combinations was analyzed in 51Cr release assays against A431 cells. Importantly, the EGFR-directed IgG1/IgG2 antibody combination was as effective as the IgG1/IgG1 combination in triggering CDC, while the individual antibodies were ineffective (Fig. 2E). Control experiments with heat-inactivated human serum or addition of the C5-blocking antibody eculizumab confirmed that the
Figure 2.
Complement activation by EGFR antibodies of human IgG1 and IgG2 isotypes. **A**, IgG1 and IgG2 antibodies significantly differed in C1q binding in ELISA. Combinations of EGFR-IgG1 and -IgG2 antibodies (5 μg/mL each) induced C1q binding (**B**), C4b/c deposition (**C**), and C3b/c deposition (**D**) on tumor cells, while single agents did not result in complement activation. Complement binding as well as deposition was analyzed by indirect immunofluorescence analyses. **E**, CDC by EGFR-directed IgG1/IgG2 and IgG1/IgG1 combinations was analyzed in 51Cr release assays. Fresh or heat-inactivated human serum (NHS and HI-NHS, respectively) served as complement source. To inhibit MAC formation, NHS was supplemented with 100 μg/mL of the C5-blocking antibody eculizumab (ECU). **F**, CDC induced by EGFR-directed IgG1/IgG1 and IgG1/IgG2 combinations was similarly dependent on antibody concentrations (left) or time course (right). **G**, EGFR-directed IgG1/IgG1 and IgG1/IgG2 combinations (5 μg/mL each) triggered CDC against different cell lines (left: DiFi, right: A1207). **H**, IgG2 antibodies (5 μg/mL) against different EGFR epitopes (E7.6.3, 425, 2F8) induced CDC in combination with a nonepitope-overlapping IgG1 antibody (003, 5 μg/mL). **I**, EGFR-directed IgG1/IgG1 and IgG1/IgG2 combinations induced similar CDC using different IgG1 combination partners (antibodies 003, 005, or 018). **J**, EGFR antibody combinations of IgG1/IgG1, IgG1/IgG2, or IgG2/IgG2 isotypes (5 μg/mL each) triggered efficient CDC. CDC was analyzed by 51Cr-release assays using 25% NHS as a source of complement. Mean values ± SEM of at least three independent experiments are displayed. Data were analyzed by ANOVA, and significant differences (P < 0.05) are depicted by *. 

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observed tumor cell killing was indeed complement mediated (Fig. 2E). In addition, the CDC activity of IgG1/IgG2 and IgG1/ IgG1 combinations was also determined using a fixed 003 anti- body concentration and increasing concentrations of panitumumab or cetuximab. Both EGFR-directed IgG combinations displayed similar dose-dependent CDC efficacy (Fig. 2F, left). Next, the complement activation kinetics of IgG1/IgG2 or IgG1/IgG1 combinations were analyzed by stopping the assays at different time points. Interestingly, both IgG1/IgG2 and IgG1/IgG1 combinations showed similarly fast kinetics in complement activation (Fig. 2F, right). Together, these results indicate that both EGFR-directed IgG1/IgG2 and IgG1/IgG1 combinations triggered CDC predominantly via the classic complement pathway. Furthermore, the ability of the IgG1/IgG2 combination to trigger CDC of other target cell lines was tested. Also against A1207 and DiFi cells, both IgG1/IgG2 and IgG1/IgG1 combinations lead to similar CDC activity (Fig. 2G). In addition, IgG2 variants of other EGFR antibodies against different target antigen epitopes were tested. In combination with 003-IgG1, 2F8-IgG2 triggered similar CDC (max. lysis = 30.5 ± 4.7%) as panitumumab (max. lysis = 28.7 ± 8.4%), while 425-IgG2 was less potent in combination with 003-IgG1 (max. lysis = 7.8 ± 2.3%) (Fig. 2H). In addition, cetuximab and panitumumab were combined with either nonepitope-overlapping EGFR-directed IgG1 combinatorial partners (003, 005, or 018). Although IgG1/IgG2 and IgG1/IgG1 combinations utilizing 005 (225/005 max. lysis = 30.9 ± 8.3%; E7.6.3/ 005 max. lysis = 25.8 ± 7.4%) led to similar CDC as 003 combinations (225/003 max. lysis = 30.1 ± 7.7%; E7.6.3/003 = 25.8 ± 6.9%), 018 was a more potent combination partner (225/018 max. lysis = 45.9 ± 3.4%; E7.6.3/018 max. lysis = 40.3 ± 6.3%; Fig. 2I). Next, we analyzed the capacity of human IgG2 antibodies against nonoverlapping EGFR epitopes to mediate ADCC (max. lysis = 25.8/003 max. lysis = 45.9/003 max. lysis = 40.3 ± 6.3%; Fig. 2J). Together, these results indicate that both EGFR-directed IgG1/IgG2 and IgG1/IgG1 combinations were analyzed by stopping the assays at different time points. Interestingly, both IgG1/IgG2 and IgG1/IgG1 combinations showed similarly fast kinetics in complement activation (Fig. 2F, right). Together, these results indicate that both EGFR-directed IgG1/IgG2 and IgG1/IgG1 combinations triggered CDC predominantly via the classic complement pathway. Furthermore, the ability of the IgG1/IgG2 combination to trigger CDC of other target cell lines was tested. Also against A1207 and DiFi cells, both IgG1/IgG2 and IgG1/IgG1 combinations lead to similar CDC activity (Fig. 2G). In addition, IgG2 variants of other EGFR antibodies against different target antigen epitopes were tested. In combination with 003-IgG1, 2F8-IgG2 triggered similar CDC (max. lysis = 30.5 ± 4.7%) as panitumumab (max. lysis = 28.7 ± 8.4%), while 425-IgG2 was less potent in combination with 003-IgG1 (max. lysis = 7.8 ± 2.3%) (Fig. 2H). In addition, cetuximab and panitumumab were combined with either nonepitope-overlapping EGFR-directed IgG1 combinatorial partners (003, 005, or 018). Although IgG1/IgG2 and IgG1/IgG1 combinations utilizing 005 (225/005 max. lysis = 30.9 ± 8.3%; E7.6.3/ 005 max. lysis = 25.8 ± 7.4%) led to similar CDC as 003 combinations (225/003 max. lysis = 30.1 ± 7.7%; E7.6.3/003 = 25.8 ± 6.9%), 018 was a more potent combination partner (225/018 max. lysis = 45.9 ± 3.4%; E7.6.3/018 max. lysis = 40.3 ± 6.3%; Fig. 2I). Next, we analyzed the capacity of two human IgG2 antibodies against nonoverlapping EGFR epitopes to mediate complement activation. Thus, IgG1 and IgG2 isotype variants of EGFR antibody 018 were combined with either cetuximab (IgG1) or panitumumab (IgG2), resulting in IgG1/IgG1, IgG1/ IgG2, and IgG2/IgG2 combinations, respectively. Interestingly, the IgG2/IgG2 combination displayed similar efficiency in C1q binding, C4b/c and C3b deposition (Supplementary Fig. S1B) and triggered similar levels of CDC (Fig. 2J) as the respective IgG1/ IgG1 and IgG1/IgG2 combinations. These results clearly show that human IgG2 EGFR antibodies can induce efficient CDC in combination with another EGFR antibody of either human IgG1 or IgG2 isotype.

EGFR-directed IgG2 antibodies activate M1-like macrophages for ADCC

Numerous studies suggested the importance of macrophages in antibody-based immunotherapy [reviewed in (7)]. Thus, we analyzed the ability of human IgG2 antibodies to activate macrophages for tumor cell killing. Monocyte-derived macrophages were generated by adhesion and were either stimulated with GM-CSF/interferon γ/LPS for M1-like polarization, or with M-CSF/IL-4 for M2-like differentiation. As expected, M1-like macrophages displayed strong expression of CD80, while M2-like macrophages expressed lower levels. In contrast, CD163 expres- sion was higher on M2- than on M1-like macrophages (Fig. 3A). Next, Fcγ receptor expression of M1- or M2-like macrophages was determined by direct immunofluorescence. FcγRIIa (CD16) was only weakly expressed by both types of macrophages. Interest- ingly, M2-like macrophages displayed higher CD32 expression, while M1-like macrophages showed a higher expression of CD64 (Fig. 3B). Next, we analyzed the ability of EGFR-directed IgG1 or IgG2 antibodies to mediate ADCC by macrophages of either M1- or M2-like differentiation. A431, SAT and Kyse-150 cells with high, intermediate or low EGFR expression levels, respectively, were used as target cells (Fig. 3C). Although EGFR-directed IgG1 was able to induce efficient ADCC by M1-like macrophages against all tested target cell lines, EGFR-directed IgG2 antibodies only induced ADCC by M1-like macrophages against high and moderate EGFR-expressing cells (A431 and SAT; Fig. 3D, top). Interestingly, neither EGFR-directed IgG1 nor IgG2 antibodies induced significant ADCC by M2-like macrophages (Fig. 3D, bottom).

EGFR antibodies of human IgG2 isotype are more effective in PMN-mediated ADCC than their IgG1 variants

Human IgG2 antibodies effectively activated myeloid cells for ADCC, which was triggered by FcγRIIA (CD32a) and affected by the FcγRIIa-131H/R polymorphism of donors (22). Here, we extended these observations and investigated PMN recruitment by IgG2 antibodies in more detail. First, Fcγ receptor profiling showed GM-CSF–primed granulocytes to express high levels of CD16, moderate levels of CD32, and low levels of FcγRI (CD64; Fig. 4A). Next, we studied to a broader spectrum of target cell lines expressing different levels of EGFR (Fig. 4B), because target antigen expression levels were correlated to PMN-mediated ADCC (30). These experiments confirmed that EGFR antibodies of IgG1 and IgG2 isotypes were similarly effective against A431 and A1207 cells, expressing very high EGFR levels. Interestingly, IgG2 antibodies (003, 005, or 018) were significantly more effective than IgG1 against target cell lines expressing moderate (Kyse-30, SAT) or low (Kyse-150, SCC-25) EGFR levels (Fig. 4B). Correlation analyses between EGFR expression levels and the amount of PMN-mediated killing demonstrated a linear positive correlation for both IgG1 and IgG2 antibodies (Fig. 4C). Next, we used Kyse-30 cells, which display moderate EGFR expression, as target cells for concentration-dependent induction of PMN-mediated ADCC by EGFR-directed IgG1 or IgG2 antibodies. In comparison with IgG1, IgG2 was significantly more effective in triggering PMN-mediated ADCC in a concentration-dependent manner (Fig. 4D, left). Furthermore, IgG2 mediated significantly more efficient ADCC at lower effector to target cell ratios than IgG1 (Fig. 4D, right). Additionally, three IgG2 antibodies targeting different EGFR epi- topes were tested for their capacity to induce PMN-mediated ADCC. All three human IgG2 antibodies were effective in inducing PMN-mediated ADCC against Kyse-30 cells, with panitumumab being the most efficient at lower antibody concentrations (Fig. 4E). Next, we were interested to analyze the mechanism of IgG2-mediated tumor cell killing by PMN, which was recently reported to be induced by trogocytosis (29). Incubation of PMN with DiO-membrane labeled A431 cells resulted in the uptake of tumor cell membranes by PMN in the presence of EGFR antibodies (Fig. 4F/I), but not in the absence of antibodies. EGFR antibodies of both IgG1 (225) or IgG2 (E7.6.) isotypes were similarly effective in triggering trogocytosis—measured as increase in “MFI of all neutrophils” (Fig. 4F/II) or as % of neutrophils staining positive for DiO (Fig. 4F/III). Together, these novel data demonstrated superior activity of human IgG2 antibodies to induce PMN-mediated ADCC, especially against target cells displaying moderate to low EGFR expression.
Inhibition of CD47/SIRPα interactions improves PMN-mediated ADCC by EGFR antibodies

Interactions between CD47 on tumor cells and SIRPα on PMN were shown to affect PMN-mediated ADCC by the HER-2/neu antibody trastuzumab (31). Here, we characterized the impact of CD47/SIRPα interactions on PMN-mediated ADCC by EGFR antibodies of IgG1 or IgG2 isotype. To address this issue, a CD47-negative variant and a control cell line of EGFR-positive A431 cells were generated by CRISPER/Cas9 knockout of the CD47 gene (A431 CD47 Ko) or a control vector (A431 crtl; see Materials and Methods section). Although EGFR expression was not altered (Supplementary Fig. S1C), both cell lines differed in their CD47 expression, as displayed in Fig. 5A. In addition, expression of SIRPα on GM-CSF-stimulated granulocytes as well as M1 and M2 macrophages was measured by indirect immunofluorescence (C). Macrophage-mediated ADCC by EGFR-directed antibodies of human IgG1 and IgG2 isotypes (5 μg/mL) was analyzed in 51Cr-release assays (D). A431, SAT, and Kyse-150 cells with high, moderate, and low EGFR expression, respectively, served as target cells. M1 (GM-CSF/IFNγ/LPS stimulated) or M2 (M-CSF/IL4 stimulated) macrophages were utilized as effector cells at an E:T ratio of 10:1. Mean values ± SEM of at least three independent experiments are displayed. Data were analyzed by ANOVA, and significant differences (P ≤ 0.05) are depicted by *. 

Figure 3. EGFR-directed IgG2 antibodies induced ADCC by M1, but not M2 macrophages. A, M1 (A/I) and M2 macrophages (A/II) were characterized by CD80 and CD163 expression and further analyzed for Fcγ receptor expression (CD16/CD32/CD64). B, EGFR expression of A431, SAT, and Kyse-150 cells was analyzed by indirect immunofluorescence (C). Macrophage-mediated ADCC by EGFR-directed antibodies of human IgG1 and IgG2 isotypes (5 μg/mL) was analyzed in 51Cr-release assays (D). A431, SAT, and Kyse-150 cells with high, moderate, and low EGFR expression, respectively, served as target cells. M1 (GM-CSF/IFNγ/LPS stimulated) or M2 (M-CSF/IL4 stimulated) macrophages were utilized as effector cells at an E:T ratio of 10:1. Mean values ± SEM of at least three independent experiments are displayed. Data were analyzed by ANOVA, and significant differences (P ≤ 0.05) are depicted by *.
Figure 4.
Human IgG2 antibodies against EGFR trigger effective PMN-mediated ADCC. A, Fcγ receptor expression of GM-CSF–stimulated PMN was measured by direct immunofluorescence analyses. B, ADCC by GM-CSF stimulated PMN and EGFR antibodies of human IgG1 or IgG2 isotypes (5 μg/mL) was analyzed by 51Cr-release assays against different solid tumor cell lines (E:T ratio 80:1) expressing different amounts of EGFR (indicated as MFI). IgG2 was significantly more effective than IgG1 against tumor cell lines expressing intermediate or low EGFR levels.

C, EGFR expression levels positively correlated with PMN-mediated ADCC for antibodies of both IgG1 and IgG2 isotypes (r values 0.92 and 0.94, respectively). D, Against Kyse-30 target cells, IgG2 mediated more effective ADCC by GM-CSF–stimulated PMN than IgG1, requiring lower antibody concentrations (left, E:T ratio 80:1) and lower E:T ratios (right, mAb concentration 5 μg/mL).

E, IgG2 antibodies against different EGFR epitopes (E7.6.3, 2F8, 425) mediated significant ADCC by GM-CSF–stimulated PMN against Kyse-30 target cells. Mean values ± SEM of at least three independent experiments are displayed as “% cytotoxicity.” Data were analyzed by ANOVA, and significant differences (P < 0.05) are depicted by *. F, Induction of trogocytosis by IgG1 (225) or IgG2 (E7.6.3) EGFR antibodies was analyzed by flow cytometry. DiO-labeled A431-lamin B-TRQ cells and neutrophils were gated as shown in F/I. After incubation with EGFR antibodies of IgG1 or IgG2 isotype for 60 minutes, PMN became DiO positive. Trogocytosis was quantified as “MFI of the total neutrophil population” (F/II), or as the “% of neutrophils that became DiO positive” (F/III). Results are shown as averages ± SEM of 3 independent experiments with neutrophils from at least 5 individual donors. Data were analyzed by ANOVA with statistical significance as depicted (*, P < 0.05).
cells. In these assays, both EGFR antibodies were unable to induce efficient PMN-mediated ADCC against wild-type A431 cells. Interestingly, in the absence of CD47 only the EGFR-directed IgG2 antibody was capable of inducing significant ADCC by PMN (max. lysis = 25.7 ± 6.8%), while the EGFR-directed IgG1 antibody did not (Fig. 5D/I). Subsequently, the efficacy of EGFR-directed IgG1- and IgG2-mediated ADCC by PMN in the presence or absence of CD47 expression was measured using
GM-CSF–stimulated granulocytes. The ability of EGFR-directed IgG1 (A431 wt: max. lysis = 28.1 ± 6.8% / A431 CD47 ko: max. lysis = 58.6 ± 8.9%) and IgG2 (A431 wt: max. lysis = 24.6 ± 8.8%/A431 CD47 ko: max. lysis = 69.6 ± 10.6%) to mediate ADCC by GM-CSF–stimulated PMN was strongly improved in the absence of CD47 expression (Fig. 5D/II). In addition, EGFR-directed antibodies mediated significantly higher ADCC by PMN at lower effector to target ratios in the absence of CD47 (Fig. 5D/III). Next, siRNA-mediated downregulation of CD47 expression on SAT (moderate EGFR expression levels) and Kyse-150 (low EGFR expression levels) was used to investigate the impact of CD47 expression on other target cell lines. Again, CD47 knockdown did not affect EGFR expression (Supplementary Fig. S1C), while CD47 expression was reduced by approximately 50% (Fig. 5E, left). In ADCC assays, target cells with reduced CD47 expression were significantly more susceptible to IgG1- and IgG2-directed antibodies mediated cytotoxicity by GM-CSF–stimulated PMN compared with control target cells. Although both EGFR antibodies were similarly effective in PMN-mediated ADCC against CD47-siRNA–treated SAT cells, the EGFR-directed IgG2 antibody was more potent than IgG1 against CD47-siRNA–treated Kyse-150 cells (Fig. 5E, right). Next, three IgG2 antibodies, targeting different EGFR epitopes (E.6.3, 425, 2F8), were tested for their ADCC activity against A431 control and CD47 ko cells, respectively, using GM-CSF–stimulated PMN as effector cells. In the presence of CD47, all three antibodies mediated low levels of ADCC, which was improved for all three IgG2 antibodies in the absence of CD47 expression (Fig. 5F). Although 2F8 and panitumumab were similarly effective, ADCC by 425-IgG2 was less potent. These data demonstrate that similar to IgG1–also IgG2-mediated ADCC by PMN is regulated by the interaction of CD47 and SIRPα.

CD47 is differentially expressed across different human solid cancer types

CD47 is highly expressed on many hematologic malignancies, where overexpression of CD47 often correlated with worse clinical outcomes. Here, we showed that EGFR-directed IgG2 antibodies could induce efficient tumor cell killing by PMN, particularly against tumor cells with lower CD47 expression. Therefore, we were interested in analyzing CD47 expression across different solid tumor entities. For this purpose, we made use of RNA-seq data obtained from TCGA. Interestingly, CD47 expression varied considerably between the analyzed cancer types (Fig. 6). Although ovarian cancer displayed the highest CD47 expression, CD47 expression in liver cancer was particularly low. EGFR antibodies are currently approved for colorectal, head and neck, and lung cancer. Among these three entities, lung and head and neck cancers displayed higher CD47 expression levels compared with colorectal cancer. Additional studies would be interesting to investigate the impact of CD47 expression on clinical outcomes during EGFR antibody therapy, particularly for panitumumab.

Discussion

Human IgG2 has traditionally been selected as antibody isotype in immunotherapy when Fc-mediated effector functions were undesired (10, 20). The interest in human IgG2 was recently fostered by studies confirming older observations that human IgG2 can exist in two different isoforms, IgG2A and IgG2B. These two isoforms differ in the conformation of their disulfide bonds in the hinge region and their Fab arm linkage, leading to differences in the rigidity of the hinge (32). The IgG2B isomorph with the more rigid hinge region was found to be particularly effective in mediating immune cell activation via CD40 antibodies of human IgG2 isotype (33). Currently available IgG2 antibodies can switch between the IgG2A and IgG2B isoforms, depending, e.g., on redox conditions. Thus, efforts are in progress to better characterize the IgG2 hinge region and to generate IgG2 variants with well-defined and stable disulfide profiles. Here, we did not observe differences in Fab-mediated effector functions between IgG1 and IgG2 antibodies against EGFR (Fig. 1C). Furthermore, also isoform skewed IgG2A and IgG2B variants of matuzumab did...
not differ in inhibiting growth of EGFR-positive DiFi cells (Supplementary Fig. S1D). Others have reported that human IgG2 variants of CD20 antibodies were associated with more effective direct tumor cell killing than their respective IgG1 variants (34). Together, these results suggest that the impact of the isotype and the rigidity of the hinge on Fab-mediated effector functions may critically depend on the selected target antigen and its epitope, as recently elegantly demonstrated for CD40 antibodies (35).

EGFR epitopes have been identified from crystal structures for cetuximab (C225), matuzumab (H425) and panitumumab (E7 6.3; refs. 36–38) and by mutational studies for zalutumumab (2F8) (39), which were all mapped to the ligand-binding domain III. The epitopes for the nonligand blocking antibodies 003, 005, and 018 were defined by cross-blocking experiments (25). Studies with hexamerization-enhanced variants of EGFR antibodies have demonstrated that matuzumab is particularly efficient in triggering CDC, which may be related to its particular orientation of binding (37). Recently reported results have demonstrated that characteristics of target antigen epitopes—such as their distance to the tumor cell membrane—differently affect the efficacy of particular effector mechanisms of therapeutic antibodies (40, 41).

Fc-mediated effector functions such as ADCC, ADCC, and CDC can contribute to the therapeutic efficacy of EGFR antibodies (27). Frustrated phagocytosis leading to tumor cell death (trogocytosis/trogoptosis) is a documented mechanism of tumor cell killing by macrophages (42) and has recently been described as PMN’s tumor killing mechanism (29). Individual EGFR antibodies do not trigger CDC against human tumor cells, unless they are specifically Fc engineered (43, 44). However, combinations of two noncross-blocking EGFR antibodies of human IgG1 isotype were demonstrated to trigger significant CDC (25). Results from clinical studies with EGFR antibody combinations may reveal whether CDC contributes to their clinical efficacy (45–47). Here, we demonstrate that unexpectedly similar levels of CDC were observed when the second EGFR antibody was of human IgG2 instead of human IgG1 isotype (Fig. 2). Importantly, also two IgG2 antibodies against nonoverlapping EGFR epitopes triggered CDC (Fig. 2f). Although human IgG2 has low affinity for C1q in ELISA, these observations suggest that C1q binding by human IgG2 is sufficient for effective CDC. Recent studies have demonstrated that effective CDC requires hexamerization of antibodies’ Fc parts on the tumor cell surface, which can be modified by Fc engineering (48, 49). Our results suggest that multimerization is also facilitated by EGFR antibody combinations, and that IgG2’s low affinity for C1q sufficiently supports this initial step of complement activation.

IgG1 and IgG2 isotype variants of the 2F8 EGFR antibody have demonstrated similar activity in immunodeficient mice (22). Furthermore, the n vivo activity of the human IgG2 EGFR antibody panitumumab was enhanced, when CD47/SIRPα interactions were abolished (50). However, translation of these results to humans is complicated by the lack of a FcyRIla homologue in mice, which is functionally the predominant Fcy receptor for human IgG2 in man. Furthermore, mice do not express a homologue of human FcyRIIIB, which can inhibit ADCC by human IgG1 antibodies (11), but does not bind human IgG2 with relevant affinity (10). Thus, studies in conventional mice may significantly underestimate the immunotherapeutic potential of human IgG2 antibodies when these antibodies can recruit PMN as effector cells.

Many EGFR-expressing tumor entities demonstrate an infiltration by myeloid cells, which is often associated with a worse clinical prognosis for patients (3). Myeloid cells can directly promote tumor growth by different mechanisms, e.g., by providing growth factors, increasing tumor cell angiogenesis or by mediating immunosuppressive functions on tumor-directed T cells—all leading to increased tumor progression. On the other hand, myeloid cells, and macrophages in particular, were identified as the predominant effector cell population for many tumor-directed monoclonal antibodies (15–17). Additionally, PMN contributed to tumor control in several murine tumor models (51). Thus, optimal activation and recruitment of myeloid cells may improve the efficacy of antibody therapy. Previous studies demonstrated that PMN as the most numerous myeloid cell population were particularly effective in tumor cell killing when activated by tumor-directed antibodies of human IgG1 isotype (52, 53). However, the introduction of human IgA antibodies into clinical studies is still in progress (54), while experience in the clinical development of human IgG2 antibodies is broadly available. Thus, human IgG2 may be selected as antibody isotype for clinical situations where recruitment of myeloid rather than NK effector cells appears promising.

For example, early lung cancer appears to actively exclude Nk-cell infiltration, while myeloid cells are prominently present (55, 56). In addition to the tumor stage also underlying genetic alterations may critically affect the composition of the immune cell infiltrate. For example, Myc activation was demonstrated to actively increase myeloid cell infiltration in lung cancer models, while lymphoid cell numbers were significantly reduced (57). At present, it is unclear why necitumumab was successful in prolonging survival in lung cancer patients (58), while cetuximab demonstrated therapeutic efficacy only in patients with high EGFR-expressing lung cancer (59). Both antibodies are of human IgG1 isotype, recognize similar/overlapping epitopes and thus block EGFR signaling similarly. Interestingly, addition of panitumumab to chemotherapy did not improve therapeutic outcome in two phase II lung cancer studies but was associated with increased toxicity (60, 61).

The impact of Fc-mediated effector functions on clinical outcomes after cetuximab treatment are still controversial, although individual studies have suggested that Fcy receptor polymorphisms are associated with cetuximab’s efficacy in colorectal cancer patients (14). Human IgG2 has moderate affinity for FcyRIla, but very low affinity for FcyRIIa and FcyRIIB (10), which is in line with its ADCC activity by different effector cell populations (11, 22). Preclinical studies using human IgG2 antibodies have demonstrated that the 131-I alloform of the FcyRIla receptor interacts more effectively with human IgG2 than the 131-R alloform (12), leading to increased PMN-mediated ADCC by EGFR antibodies of human IgG2 isotype (22). However, we are not aware of studies addressing the impact of Fcy receptor polymorphisms on the clinical efficacy of human IgG2 antibodies. Furthermore, no direct comparisons between isotype variants of individual antibodies have been performed in patients. Nevertheless, there are results from a head-to-head comparison between the two EGFR antibodies cetuximab (IgG1) and panitumumab (IgG2) in patients with metastatic colorectal cancer. Interestingly, both antibodies demonstrated similar clinical activity (62). Subgroup analyses of this informative study could assist to identify biomarkers to
predict clinical responses to EGFR antibodies of human IgG2 isotype. A similar scenario is developing for CTLA-4 antibodies, where an IgG1 antibody (ipilimumab) is approved, while an IgG2 antibody (temelimubab) is in late-stage clinical development. Myeloid effector cell-mediated depletion of regulatory T cells (Treg) by Fc-mediated mechanisms contributes to the therapeutic efficacy of ipilimumab and was recently also demonstrated for temelimubab (63–65).

The identification of appropriate biomarkers has been demonstrated to increase the chances of patients to respond to novel therapies and to pave the way for accelerated approval of innovative drugs (27). Our in vitro studies suggest the clinical evaluation of several potential biomarkers for antibodies of human IgG2 isotype. For example, the amount of myeloid cells within tumors would be expected to be relevant. Additionally, myeloid cells require rather high EGFR expression levels for effective M-2 MC (59), a biomarker that is actively investigated in NSCLC therapy (59). Furthermore, patients expressing the 131-H allotype of FcRIIa should have higher chances to respond than patients homozygous for the FcRIIa-131R allorm.

Following the therapeutic success of immune-checkpoint blocking antibodies (1), novel approaches aim to improve myeloid cell recruitment for tumor immunotherapy. Prominent examples include antibodies blocking CD47/SIgPz interactions (6) as well as, e.g., antibodies against CD27 (66). To develop their full therapeutic activity, these novel antibodies probably need to be combined with antibodies against classic "tumor antigens" such as CD20 or EGFR. Our studies presented here suggest that the human IgG1 isotype is expected to be suboptimal for these approaches, and that human IgG2 antibodies may improve myeloid effector cell activation. Thus, additional studies are required "to harvest the high hanging fruits" in EGFR-directed antibody therapy (67).

Disclosure of Potential Conflicts of Interest
F. Beuksens is an employee of Gennab. No potential conflicts of interest were disclosed by the other authors.

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