A Complement-Optimized EGFR Antibody Improves Cytotoxic Functions of Polymorphonuclear Cells against Tumor Cells

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Complement-dependent cytotoxicity (CDC) has been suggested to be an important mechanism of action of tumor-targeting Abs. However, single unmodified epidermal growth factor receptor (EGFR)–targeting IgG1 Abs fail to trigger efficient CDC. For the current study, we generated a CDC-optimized variant of the EGFR Ab matuzumab (H425 wt) by introducing amino acid substitutions K326A/E333A (H425 mt). This Ab was then used to elucidate the impact of complement activation on the capacity of effector cells such as mononuclear cells (MNC) and polymorphonuclear cells (PMN) to exert Ab-dependent cell-mediated cytotoxicity (ADCC). H425 mt, but not H425 wt, significantly induced complement deposition, release of anaphylatoxins, and CDC against distinct tumor cell lines, whereas no differences in ADCC by MNC or PMN were detected. Notably, stronger cytotoxicity was induced by H425 mt than by H425 wt in whole blood assays and in experiments in which MNC or PMN were combined with serum. Although MNC-ADCC was not affected by C5 cleavage, the cytotoxic activity of PMN in the presence of serum strongly depended on C5 cleavage, pointing to a direct interaction between complement and PMN. Strong cell surface expression of C5a receptors was detected on PMN, whereas NK cells completely lacked expression. Stimulation of PMN with C5a led to upregulation of activated complement receptor 3, resulting in enhanced complement receptor 3–dependent PMN-ADCC against tumor cells. In conclusion, complement-optimized EGFR Abs may constitute a promising strategy to improve tumor cell killing by enhancing the interaction between humoral and cellular effector functions in Ab-based tumor therapy. The Journal of Immunology, 2015, 195: 5077–5087.

Abbreviations used in this article: ADCC, Ab-dependent cell-mediated cytotoxicity; CDC, complement-dependent cytotoxicity; CR, complement receptor; EGFR, epidermal growth factor receptor; MAC, membrane-attack complex; MFI, mean fluorescence intensity; MNC, mononuclear cell; mt, mutated; PMN, polymorphonuclear cell; wt, wild-type.

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patients with metastatic colorectal cancer and acquired resistance to approved anti-EGFR Ab therapy (11). Activation of the complement system constitutes a pivotal defense mechanism in innate immunity against invading pathogens or malignant cells and contributes to removal of apoptotic cells (12). The importance of efficient complement activation during immune responses is highlighted by the fact that activation of the complement cascade comprises three main effector functions: opsonization of target cells with opsonins (e.g., C1q, C3b, or C4b) for phagocytosis by myeloid cells, generation of anaphylatoxins C3a and C5a to trigger chemotaxis of effector cells, and formation of the membrane-attack complex (MAC; C5b-9) for target cell lysis (13). However, in the case of CD20-targeting Abs, complement activation on target cells has been demonstrated to inhibit macrophage-, neutrophil-, and NK cell–mediated cytotoxicity against tumor cells in vitro (14–16). Furthermore, inhibition of rituximab-triggered NK cell–mediated cytotoxicity in the presence of human serum has been mainly ascribed to C3b deposition on target cells (17, 18). In contrast, other studies demonstrated a beneficial cytotoxic activity of rituximab against tumor cells in the presence of mononuclear cells and serum (19). Hence, the impact of complement activation on effector cell activity during Ab-based tumor therapy still remains unclear.

IgG1 Abs have been described as mainly interacting with the globular heads of C1q via their CH2 domains (20–23) after forming IgG hexamers assembled at the cell surface (24). Referring to these findings, Idusogie et al. (25) substituted surface-exposed amino acids in the CH2 domain of the therapeutic CD20 IgG1 Ab rituximab and unreeved, in particular, residues K326 and E333 as suitable candidate amino acids for improvement of C1q binding. Although an increase in C1q binding by IgG1 and C1q activity was already achieved by single amino acid substitutions such as K326A (∼1.5-fold) or E333A (∼2.0-fold) within the CH2 domain, introduction of both amino acid exchanges further enhanced C1q binding and CDC capacity (∼2.5-fold) without altering ADCC activity.

Hence, previously described Fe engineering approaches (i.e., K326A/E333A mutations) were used to boost complement-activating capacities of the EGFR-targeting Ab matuzumab, with the aim of improving its antitumoral immune responses.

Materials and Methods
Study population and consent
Experiments reported in this article 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.

Cell lines and Abs
Human epidermoid carcinoma cell line A431 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was kept in RPMI 1640 medium, whereas the glioblastoma cell line A1207 (originally established by Dr. Aaronson, National Cancer Institute, National Institutes of Health, Bethesda, MD) and the colorectal carcinoma cell line DiFi (European Collection of Cell Cultures, Salisbury, U.K.) were cultivated in DMEM medium. Both cell culture mediums were supplemented with 10% (v/v) heat-inactivated FCS, 100 U/ml penicillin, and 100 µg/ml streptomycin.

VH and VL regions of the humanized EGFR-directed IgG1 Ab matuzumab (H425; patent WO 92/15683) were cloned into an expression vector (Refludan; Pharmion, Hamburg, Germany), sensitizing Abs (10 µg/ml), followed by staining for 1.5 h at 4˚C with H425 wt (0–50 µg/ml), H425 mt (0–50 µg/ml), anti-human C1qR (20 µg/ml; Abcam, Cambridge, U.K.), mouse anti-human C11b or activated CD11b (clone 2L.PM10c; 0–10 µg/ml; Thermo Fisher Scientific, Rockford, IL; or clone CBRM1/5; 10 µg/ml; OriGene Technologies, Rockville, MD, respectively), or irrelevant control Abs. Afterward, cells were stained with FITC-conjugated goat anti-mouse Fcy F(ab)2 (Jackson ImmunoResearch Laboratories, West Grove, PA). For direct immunofluorescence, 1 × 10⁵ cells were stained for 30 min at 4˚C in the presence of human IV IgG (Intratect; Biostest AG, Dreieich, Germany) with CD93-P3E (BioLog, San Diego, CA), FITC-conjugated mouse anti-human gC1qR (Abcam), CD35-FITC (Beckman Coulter, Krefeld, Germany), CD11b-FITC (Beckman Coulter), CD11c-PC7 (Beckman Coulter), CD21-PE (Beckman Coulter), PE-conjugated mouse anti-human C5L2 (BioLog), CD88-FITC (Bio-Legend), PE-conjugated mouse anti-human C3R (BioLegend), CD16-PE (Beckman Coulter), CD64-PE (Beckman Coulter), or respective irrelevant control Abs. Alternations in expression patterns of complement receptors (CRs) or FcγRs on PMN were elucidated by time-dependent (0–80 min) stimulation of PMN with GM-CSF (50 U/ml) or a combination of C5a (100 ng/ml; Complement Technology, Tyler, TX) and GM-CSF.

Relative fluorescence intensity was calculated by the following formula: mean fluorescence intensity (MFI)–specific Ab/MFI control Ab. Immunofluorescence was analyzed on a flow cytometer (Navios; Beckman Coulter, Fullerton, CA).

Complement deposition
Binding of the initial complement component C1q by indicated Abs on the cell surface of A431 cells was determined by incubation of 1.5 × 10⁵ cells with H425 wt, H425 mt, or control Ab (all at 10 µg/ml) for 15 min at 4˚C. Subsequently, 25% v/v human serum was added supplemented with complement components (50 µg/ml; Alexion Pharmaceuticals, Cheshire, CT) or left untreated, followed by incubation for 10 min at 37˚C. After washing, samples were stained with polyclonal FITC-conjugated anti-human C1q or C4b(c) (both DAKO, Eching, Germany), with mouse anti-human (i) C3b mAb (Thermo Fisher Scientific, Waltham, MA), as well as with mouse anti-human Factor Bb or C5b-9 mAbs (both from Quidel, San Diego, CA), followed by staining with FITC-conjugated goat anti-mouse Fcy fragment-specific F(ab′)2 (Jackson ImmunoResearch Laboratories). Samples were analyzed on a flow cytometer (Epics Profile; Beckman Coulter).

Cell death analysis
To analyze complement-mediated lysis of target cells, 1 × 10⁵ A431 cells were incubated with H425 mt or control Ab (both at 10 µg/ml), 25% v/v human serum, 25% v/v heat-inactivated (30 min at 60˚C) human serum, or without serum for 3 h at 37˚C and 5% CO2. Afterward, cells were stained using the FITC Annexin V Apoptosis Detection Kit I (BD Pharmingen, Heidelberg, Germany) according to the manufacturer’s instructions and analyzed by flow cytometry.

Anaphylatoxin release assay
Fresh human plasma (25% v/v), supplemented with 25 µ/ml lepirudin (Relfudan; Pharmion, Hamburg, Germany), sensitizing Abs (10 µg/ml), and RPMI 1640 (10% FCS) were added to a 96-round-well plate. As a control, target cells were incubated only with RPMI 1640 (10% FCS). The assay was started by adding 5 × 10⁵ A431 cells in 50 µl culture medium, resulting in a final volume of 200 µl per well. After 3 h at 37˚C, supernatants were collected by centrifugation, and anaphylatoxin release was determined by calibrated flow cytometry using the Human Anaphylatoxin Kit (BD Biosciences, San Diego, CA) according to the manufacturer’s instructions.
Isolation of human effector cells

Human effector cells such as peripheral mononuclear cells (MNC), or polymorphonuclear cells (PMN) were isolated from peripheral blood drawn from healthy volunteers, as previously described (26). NK cells were isolated from whole MNC fraction by indirect MACS cell separation (NK Cell Isolation Kit, Order no. 130-092-657; Miltenyi Biotec, Auburn, CA).

Cytotoxicity assays

ADCC assays

ADCC assays were performed as described (26). The E:T ratio was 40:1 unless otherwise indicated. ADCC experiments with PMN were performed in the presence of GM-CSF. In respective experiments, PMN were preincubated with 100 ng/ml human C5a, GM-CSF (50 U/ml), or a combination of C5a (100 ng/ml; Complement Technology, TY) and GM-CSF (50 U/ml) for 30 min prior to the addition of target cells and analyzed Abs. For blockade of CD11b, FcyRIII (CD16), or FcyRII (CD32), on PMN during ADCC experiments, PMN were preincubated with saturating concentrations of a CD11b Ab (clone 2LP19c; 200 g/ml), whose Fc domain had been modified to abolish Fc receptor binding (CD16-IgG1-Ko) (27), or a CD32-F(ab’2), fragment (1 mg/ml; AT10) for 15 min at 37°C before addition of Abs and target cells. Percentage of cytotoxicity was calculated using the following formula: % specific lysis = experimental cpm – basal cpm)/(maximal cpm – basal cpm) × 100.

CDC assays

CDC assays were performed as described in Ref. 8. In short, target cells (5 × 10⁴), labeled with 200 μCi [³⁵Cr], freshly drawn human serum (25% v/v), and indicated Abs at various concentrations were added to microtiter plates and incubated for 3 h at 37°C and 5% CO₂. Percentage of cytotoxicity was calculated using following formula: % specific lysis = (experimental cpm – basal cpm)/(maximal cpm – basal cpm) × 100.

Complement-dependent cell-mediated cytotoxicity assays. Complement-dependent cell-mediated cytotoxicity assays were performed as CDC or ADCC assays with A431 cells described above, except that MNC or PMN were coincubated with 25% v/v human serum or 25% v/v human heat-inactivated (30 min at 60°C) serum in the presence or absence of the C5-neutralizing Ab eculizumab (50 μg/ml) or with 25% v/v C6-depleted serum (Complement Technology) reconstituted with C6 (65 μg/ml; Complement Technology).

Complement-dependent cell-mediated cytotoxicity assays were also performed using whole human blood (25% v/v) from normal healthy volunteers or from GS-CSF–primed healthy volunteers as effector source. Whole blood samples were anticoagulated with 25 μg/ml lepirudin (Pharmion), stimulated with GM-CSF (50 U/ml) in the case of whole blood from normal healthy volunteers or from GS-CSF–primed healthy volunteers, or from MNC (Fig. 3A) or PMN (Fig. 3B), no significant differences were detected between H425 wt and H425 mt regarding dose–dependence of CDC triggered by H425 wt more strongly depends on CD55 or CD59 expression levels than that triggered by H425 mt. Complement-mediated lysis of A431 cells evoked by H425 mt was accompanied by a significant generation of the anaphylatoxins C3a, C4a, and C5a, whereas no anaphylatoxin release was detected for H425 wt (Fig. 2D).

Chemotaxis assay

Migration of human neutrophils toward the anaphylatoxin C5a was assayed in 96-well microplate chambers (HTS Transwell-96 Permeable Support with 3.0-μm Pore Polycarbonate Membrane; Corning Costar, Tewksbury MA). The lower part of the Boyden chamber, containing human C5a (0–1 μg/ml) diluted in HBSS (Invitrogen), supplemented with 2% BSA, was separated from the upper plate containing neutrophils (8 × 10⁶ cells per milliliter, resuspended in HBSS + 2% BSA + 50 U/ml human GM-CSF) by a polycarbonate membrane with a 3-μm pore size. After 3 h incubation of the plate at 37°C and 5% CO₂, the upper chamber plate was removed. Migrated PMN were quantified by a PMN standard curve in the bottom chamber plate by MTS assay according to the manufacturer’s instructions (Promega, Madison, CA).

Data processing and statistical analyses

Data are displayed graphically and were statistically analyzed using GraphPad Prism 5.0. Curves were fitted using a nonlinear regression model with a sigmoidal dose response (variable slope if applicable). Statistical significance was determined by the one-way or two-way ANOVA repeated measures test with the Bonferroni post test. The respective results were displayed as mean ± SEM of at least three independent experiments. The p values were calculated and null hypotheses were rejected when p ≤ 0.05.

Results

Generation of a CDC-optimized variant of H425

As unmodified EGFR-targeting Abs do not trigger CDC against tumor target cells (8), amino acids at positions K326 and E333 in the CH2 domain of IgG1 were substituted by alanine (= H425 mt)—a strategy to improve the capacity of IgG1 molecules to recruit complement (28) (Fig. 1A). H425 wt and H425 mt were expressed and purified by affinity chromatography. Potential remaining contaminants or protein aggregates were removed by gel filtration, resulting in homogeneous protein preparations (Fig. 1B). Automated capillary gel electrophoresis was performed under reducing or nonreducing conditions and revealed high purity and correct assembly of the heavy and light chains of Abs (Fig. 1C). Both wt (H425 wt) and mt matuzumab (H425 mt) displayed identical dose-dependent EGFR binding, as determined by flow cytometry analyses (Fig. 1D).

As expected from the CDC-optimized IgG1-CH2 protein backbone, H425 mt significantly bound C1q at the cell surface of A431 cells, whereas H425 wt did not Fig. 2A, leading to cell death induction by the mutated Ab in the presence of normal human serum, but not heat-inactivated serum (Fig. 2B). Furthermore, H425 mt, but neither H425 wt nor a control Ab, triggered significant dose-dependent induction of CDC against A1207 and A431 cells when intact human sera were used (Fig. 2C), whereas no CDC against A431 cells was detected in experimental settings with heat-inactivated sera (data not shown). Notably, low levels of CDC were observed by H425 wt against DiFi cells, with H425 mt displaying significantly superior CDC compared with H425 wt (Fig. 2C). As previously published, DiFi cells display lower CD55 and CD59 expression levels, compared with A431 or A1207 cells (9). Hence, one may hypothesize that the extent of CDC triggered by H425 wt more strongly depends on CD55 or CD59 expression levels than that triggered by H425 mt. Complement-mediated lysis of A431 cells evoked by H425 mt was accompanied by a significant generation of the anaphylatoxins C3a, C4a, and C5a, whereas no anaphylatoxin release was detected for H425 wt (Fig. 2D).

mt and wt H425 similarly activate effector cell–mediated tumor cell lysis

Because Fc receptors on effector cells also bind to IgG1 molecules via the CH2 domain to mediate ADCC against target cells, it was studied whether H425 wt and H425 mt differ in their capacity to trigger ADCC. In [³⁵Cr] release assays using either isolated MNC (Fig. 3A) or PMN (Fig. 3B), no significant differences were detected between H425 wt and H425 mt regarding dose–dependence of ADCC against A431 cells. However, in experiments in which GM-CSF–primed whole blood from healthy donors was used as effector source, solely H425 mt triggered significant cytolysis of A431 cells (Fig. 3C, left panel). Of note, killing was completely blocked by the C5-neutralizing Ab ecuclizumab (Fig. 3C, right panel). In the next step, whole blood from G-CSF–primed donors, which contained higher amounts of PMN with strong cell surface expression of the high-affinity FcγRI (CD64) than did GM-CSF–primed whole blood, was analyzed. In these assays, H425 wt also triggered cytolysis of A431 cells that, however, could not be prevented by ecuclizumab. This finding points to a mere activation of effector cells rather than of the complement system. In comparison with H425 wt, superior cytotoxic activity was detected for H425 mt, which was reduced to the extent of H425 wt triggered cytolysis in the presence of ecuclizumab (Fig. 3D). Together, wt H425 is able to efficiently recruit effector cells for tumor cell destruction, whereas the CDC-optimized H425 can activate both complement and effector cells.

Superior cytotoxicity is induced by CDC-optimized matuzumab in the presence of effector cells and complement

The impact of complement activation on effector cell–mediated cytotoxicity is controversially discussed (14, 19). Hence, we aimed
to analyze the extent of cytotoxicity against A431 cells triggered by H425 wt or H425 mt during coincubation of MNC or PMN with serum. In the first step, expression patterns of distinct CRs on the cell surface of PMN or NK cells, the main effector cell population in MNC, that mediate ADCC in MNC under these experimental settings were determined by flow cytometry. Although all analyzed CRs (C1qRp, cC1qR, gC1qR, CR1, CR3, CR4, C3aR, C5aR, or C5L2), except for CR2, were detected on the cell surface of PMN, NK cells expressed only CR3, CR4, and low amounts of C3aR (Fig. 4A). In the next step, we performed \[^{51}Cr\] release experiments with A431 cells in the presence of effector cells, serum, or effector cells plus serum. Although no differences in the extent of cytotoxicity against A431 cells were detected for H425 wt between MNC alone or MNC plus serum, H425 mt displayed increased cytotoxicity when MNC and serum were combined in comparison with MNC alone. However, cytotoxicity by H425 mt in the presence of MNC plus serum was similar to that observed with serum alone (Fig. 4B). Notably, compared with the extent of ADCC mediated by PMN alone, cytotoxicity by H425 wt was dramatically reduced when PMN were combined with serum. This reduction may be a result of high Ig levels in serum, competing with H425 wt for FcγR binding on PMN (data not shown). In contrast, H425 mt promoted significantly superior cytotoxicity against A431 cells in the presence of PMN and serum in comparison with serum alone as effector sources (Fig. 4C). To study the impact of H425 mt–triggered complement activation in serum on MNC- or PMN-mediated cytotoxicity against A431 cells, experiments presented in Fig. 4B and 4C were also performed using heat-inactivated human serum (Fig. 4D and 4E). As expected, no target cell lysis was detected for H425 wt and H425 mt in the presence of heat-inactivated serum alone. Combination of heat-inactivated serum with MNC did not affect H425 wt– or H425 mt–triggered MNC-mediated cytotoxicity against A431 cells, whereas heat-inactivated serum significantly reduced PMN-mediated cytotoxicity. During all these experiments, no cytotoxicity was observed in the absence of Abs or in the presence of the control Ab. It should be mentioned that detected differences in the extent of H425 mt–triggered CDC against A431 cells in the presence of serum alone (Fig. 4B, ~60%, and Fig. 4C, ~40%) may be due to the use of different blood donors in independent experiments.

**PMN- but not MNC-mediated cytotoxicity by H425 mt depends on C5 activation in the presence of serum**

To elucidate whether improved cytotoxicity against A431 cells is a result of enhanced effector cell activation by complement activation or of parallel lysis of cells by either complement or effector cells, we performed \[^{51}Cr\] release assays in the presence of eculizumab. Eculizumab blocks the assembly of the MAC of
complements by preventing C5 cleavage (Fig. 5A). Of interest, improvement of H425 mt–triggered cytotoxicity against A431 cells by the combination of MNC and serum was reduced by eculizumab to the level of cytotoxicity mediated by MNC alone—leading to the hypothesis that C5 cleavage is not necessary for MNC-ADCC in the presence of serum. In contrast, superior cytotoxicity against A431 cells was also detected using PMN plus serum, compared with cytotoxicity by PMN alone, but this cytotoxicity was almost completely prevented by addition of eculizumab to experiments in which PMN were combined with serum (Fig. 5B).

These experiments were repeated in the presence of heat-inactivated serum to study non–complement-mediated effects (data not shown). In these circumstances, MNC-mediated ADCC against A431 cells was not affected either by addition of heat-inactivated serum or by eculizumab. As already presented in Fig. 4E and discussed above, PMN-mediated ADCC against A431 cells triggered by H425 mt was significantly reduced in the presence of heat-inactivated serum. However, addition of eculizumab had no effect in these experiments. Altogether, data from experiments with PMN may point to a pivotal role of C5 cleavage during complement activation in promoting PMN-mediated ADCC in the presence of serum. Hence, the impact of C5 cleavage on PMN-ADCC was further

**FIGURE 2.** The CDC-optimized H425 Ab displays enhanced CDC activity against tumor cell lines. (A) Binding of C1q on A431 cells was analyzed by direct immunofluorescence in the presence of 25% v/v human serum and indicated Abs (10 μg/ml). (B) A431 cells were incubated for 3 h at 37˚C with or without normal or heat-inactivated (HI) human serum in the presence of H425 mt or an irrelevant IgG1 Ab (both at 10 μg/ml). Afterward, cell death was analyzed by flow cytometry after staining with Annexin V-FITC and propidium iodide (PI). (C) For [51Cr] release assays, A431 cells, A1207 cells, or DiFi cells were incubated for 3 h at 37˚C in the absence or presence of increasing concentrations of the indicated Abs and 25% v/v serum. (D) Release of anaphylatoxins C3a, C4a, or C5a during CDC assays. A431 cells were incubated with 25% v/v plasma and indicated Abs (10 μg/ml) for 3 h at 37˚C.

Afterward, supernatants were analyzed by flow cytometry regarding anaphylatoxin release. Data are presented as mean ± SEM from at least three independent experiments with different blood donors. *p ≤ 0.05 H425 mt versus control, #p ≤ 0.05 H425 mt versus H425 wt. RFI, relative fluorescent intensity.
analyzed using C6-depleted serum, which does not allow formation of a functional MAC. In these experiments, addition of C6-reconstituted serum to PMN again increased cytotoxicity against A431 cells in comparison with PMN alone. Notably, when PMN were combined with C6-depleted serum without reconstitution of C6, no complete inhibition of PMN-mediated ADCC was observed (Fig. 5C, right panel), as seen in equivalent experiments with eculizumab (Fig. 5B, right panel). These results support the idea that C5 cleavage during complement activation, rather than constitution of the MAC, plays an important role in activating PMN-mediated ADCC in the presence of serum (Fig. 5C). To test whether eculizumab-altered H425 mt initiated deposition of complement components on A431 cells, flow cytometry analyses were performed. As presented in Fig. 5D, addition of eculizumab to serum samples did not affect H425 mt–promoted binding of C1q or deposition of C4b(c) or (i)C3b on the cell surface of A431 cells. However, as expected, no MAC (C5b-9) was assembled on the cell surface of A431 cells when coincubating H425 mt with eculizumab, whereas MAC was effectively assembled in the absence of eculizumab (Fig. 5D). As presented in Fig. 2D, complement activation by H425 mt promoted generation of C3a by C3 cleavage. Furthermore, PMN as well as NK cells expressed C3aR (Fig. 4A). Hence, the effect of C3a stimulation on PMN- or MNC-mediated ADCC against A431 was studied by [51Cr] release experiments. Notably, C3a did not affect PMN- as well as MNC-mediated ADCC against A431 cells (data not shown), again suggesting a crucial role for C5a in PMN-mediated tumor cell lysis.

C5a induces chemotaxis of PMN and improves CR3-dependent PMN-mediated cytotoxicity

C5a has been demonstrated to potently trigger chemotaxis of PMN (29). Hence, migration of PMN toward distinct concentrations of C5a was analyzed in a Transwell microchamber assay to verify the

FIGURE 3. H425 wt and H425 mt display similar ADCC capacity with isolated effector cells but differ in cytotoxic activity in whole blood. [51Cr] release assays were performed with A431 cells, indicated Abs at increasing concentrations and MNC (A), GM-CSF–stimulated PMN (B), whole blood stimulated with GM-CSF (C), or whole blood drawn from G-CSF–treated healthy individuals (D). For MNC- or PMN-mediated ADCC experiments, an E:T ratio of 40:1 was chosen. The contribution of CDC in whole blood assays was investigated by performing experiments in the absence (left panels) or presence (right panels) of eculizumab (C and D). Data are presented as mean ± SEM from at least three independent experiments with different blood donors. *p ≤ 0.05 H425 mt versus control Ab, #p ≤ 0.05 H425 mt versus H425 wt.
biologic activity of human C5a used in the current study. Notably, significant migration of PMN was detected at 100 ng/ml C5a, whereas nearly none was measured at 10 or 1000 ng/ml C5a (Fig. 6A).

PMN-mediated ADCC triggered by human IgG is mainly driven through engagement of CD32a by the Fc domain of the Abs, whereas CR3 has also been demonstrated to play an important role (30, 31). On the basis of findings that PMN express high amounts of C5aR on their cell surface, we aimed to investigate the impact of C5a, generated by C5 cleavage during complement activation, on the expression of CRs as well as of FcRs CD16, CD32, or CD64 on PMN. For this purpose, PMN were stimulated at different time points with GM-CSF or with GM-CSF plus C5a or were left untreated (Fig. 6B, Supplemental Fig. 1). Stimulation of PMN with GM-CSF or GM-CSF plus C5a resulted in strong upregulation of CR3, CR4, or CD16 and intermediate upregulation of C1qRp, CR1, and CD32. Although no significant differences could be observed between GM-CSF and C5a plus GM-CSF samples in a 30-min incubation period, the combination of C5a and GM-CSF triggered significantly higher cell surface expression of CR3 during long-term incubation (>120 min) in comparison with GM-CSF stimulation. However, no alterations in expression of CD64, cC1qR, or gC1qR (Fig. 6B, Supplemental Fig. 1) as well as in cell morphology (data not shown) could be measured throughout all stimulation experiments.

To test the impact of C5a stimulation on PMN-mediated ADCC, 51Cr release assays were performed with A431 cells and PMN that had been preincubated with C5a for 30 min, followed by further C5a stimulation during the whole assay. Of interest, C5a-improved H425 mt evoked ADCC significantly in a concentration-dependent manner, reaching maximum lysis at 100 ng/ml C5a (Fig. 6C). In addition, ADCC assays with dose–response curves for H425 mt were performed, either in the presence or in the absence of a fixed C5a concentration (100 ng/ml) (Fig. 6D).
In this situation, C5a stimulation of PMN triggered a significant increase of ADCC against A431 cells at saturating H425 mt concentrations (Fig. 6E). To unravel whether H425 mt–triggered tumor cell killing is mediated by CD16 or CD32 ligation and whether this interaction is altered by C5a stimulation, PMN-mediated ADCC experiments were performed in the presence or absence of CD16 or CD32 antagonists. As expected from previous studies, CD16 blockade did not affect the extent of PMN-mediated ADCC, whereas CD32 blockade completely prevented tumor cell killing. Furthermore, blocking of CD16 or CD32 during PMN-mediated ADCC experiments did not unveil differences in the extent of tumor cell killing between C5a-stimulated or C5a-untreated PMN (Fig. 6F).

In the next step, we tested by flow cytometry whether C5a-triggered upregulation of CR3 on PMN was also accompanied by an increase of CR3 activation. Activated CR3 can be measured by the specific Ab CBRM1/5 that exclusively detects neo-epitopes exposed after conformational changes of the receptor (32). As presented in Fig. 6G, expression levels of activated CR3 were significantly more upregulated than whole CR3 expression levels after C5a stimulation of PMN. To further elucidate the role of C5a-induced upregulation of activated CR3 in PMN-mediated ADCC, cytotoxicity experiments were performed in the presence or absence of saturating concentrations of a CR3 (CD11b) blocking Ab. Notably, PMN-mediated ADCC by H425 mt was significantly inhibited (~50%) by CR3 blockade when PMN were preincubated with C5a, but not in experimental settings without C5a (Fig. 6H).

Taken together, these data point to an improvement in the activation status and especially the migratory activity of PMN toward potential tumor target cells by C5a stimulation. As a functional consequence, elevated CR3-dependent cytotoxic activity of PMN against tumor target cells was detected.
The salient conclusion of findings presented in this study is the potential improvement of the interplay between humoral and cellular effector functions in EGFR-targeting Ab-based tumor therapy by improving the complement-activating capacity of the EGFR-targeting Ab matuzumab. CDC optimization was performed by Fc engineering of the CH2 domain (K326A/E333A), resulting in efficient complement activation on tumor cell lines. Improved complement activation was accompanied by augmented tumor cell lysis in experimental settings covering humoral and cellular immunity. In the presence of serum, C5a cleavage was found to be not necessary for MNC-mediated ADCC, whereas it was crucial for PMN-mediated ADCC.

**FIGURE 6.** C5a amplifies CR3-dependent ADCC mediated by PMN. (A) Induction of PMN chemotaxis by C5a at increasing concentrations was analyzed in a 3-h Transwell microchamber plate assay. Data are presented as mean ± SEM from at least three independent experiments with different blood donors. *p ≤ 0.05 C5a versus without C5a, #p ≤ 0.05. (B) PMN were stimulated with 100 ng/ml C5a in a time-dependent manner. Afterward, cell surface expression levels of CR3, CD16, CD32, or CD64 were determined by direct flow cytometry analyses (MFI). Specific Ab, fluorochrome-labeled CR3/CD16/CD32/CD64 Abs; control Ab, respective fluorochrome-labeled control Abs. (C–E) PMN-mediated ADCC against A431 (E:T ratio of 80:1) triggered by H425 mt in the presence or absence of human C5a. 

**Discussion**

The salient conclusion of findings presented in this study is the potential improvement of the interplay between humoral and cellular effector functions in EGFR-targeting Ab-based tumor therapy by improving the complement-activating capacity of the EGFR-targeting Ab matuzumab. CDC optimization was performed by Fc engineering of the CH2 domain (K326A/E333A), resulting in efficient complement activation on tumor cell lines.

Improved complement activation was accomplished by augmented tumor cell lysis in experimental settings covering humoral and cellular immunity. In the presence of serum, C5 cleavage was found to be not necessary for MNC-mediated ADCC, whereas it was crucial for PMN-mediated ADCC. Further analyses revealed that the complement-derived anaphylatoxin C5a directly enhanced CR3-dependent PMN-mediated ADCC against tumor cells, whereas C3a did not affect effector cell-mediated cytotoxicity triggered by H425 (wt or mt) against tumor cells. In line with these findings, previously published data also linked C5a-controlled CR3 expression to effector mechanisms of granulocytes. Although phagocytosis of *E. coli*, oxidative burst, and CR3 expression by granulocytes in human blood were inhibited by a C5aR antagonist or an anti-C5 Ab, CR3 expression on granulocytes was demonstrated to be upregulated by C5a stimulation (33), but not by C3a stimulation (34). Immunologic functions of C3a have been demonstrated to consist of induction of respiratory burst in neutrophils (29) and reduction of IFN-γ production (35), as well as...
cytotoxic activity (36) in the case of NK cells. Furthermore, CR3 that also binds complement-derived opsonins such as C3b and its derivatives has been described as physically interacting with FcγRIIIB via its lectin-like site to facilitate efficient immune complex–triggered calcium release and superoxide production by neutrophils (37–39). These findings supported the Ehlenberger-Nussenzweig hypothesis that complement binding to complement receptors is crucial to increase IgG presentation to phagocyte Fc receptors such as FcγRIIb, thereby increasing IgG-mediated effector functions against pathogens (40).

Although it has been demonstrated in mouse models that C5a promotes activation and recruitment of neutrophils to sites of viral (41), bacterial, or fungal (42) infections, opposing roles of C5a in tumor progression have been described (43). Notably, tumor-bearing mice with C5a-transfected in comparison with control vector–transfected xenografted tumors displayed lower tumor burden. In contrast, tumor-bearing mice with high C5a-producing in comparison with low C5a-producing syngeneic lymphomas showed higher myeloid cell accumulation in the spleen and accelerated tumor progression (43). In line with these findings, tumor growth–promoting activities of complement activation, especially by the C5a signaling cascade, have been described as being evoked by augmented tumor infiltration of myeloid-derived suppressor cells, leading to the suppression of antitumoral T cell responses (44). Hence, depending on the tumor environment, complement is able to exert bifunctional actions such as tumor promotion or tumor suppression.

The affinity of integrins, such as CR3, to their ligands is controlled by G-protein–coupled receptor–initiated inside-out signaling in leukocytes (45). Hence, one may hypothesize that enhanced CR3 activation triggered by C5a may result from the G-protein–coupled receptor C5aR-mediated inside-out signaling in PMN. This inside-out signaling enables further CR3-dependent activation of the tyrosine kinase Src that promotes phosphorylation of ITAM-containing CD32a, resulting in down-stream activation of the tyrosine kinase Syk (46). In the context of EGFR-targeting Abs, it is well known that the ADCC activity of granulocytes against tumor cells is mainly driven through CD32a ligation (30). In line with these findings, enhanced CR3-dependent PMN-mediated ADCC activity against EGFR-positive tumor cells was detected in the current study. The impact of C5a on Fc receptors has also been studied in various mouse models. In these studies, binding of C5a to its receptor C5aR resulted in the upregulation of activating Fc receptors and the parallel down-regulation of the inhibitory FcγRIIB on alveolar or peritoneal macrophages as well as on Kupffer cells. This regulatory function of C5aR signaling led to a relatively higher cell surface expression of activating versus inhibitory Fc receptors that was accompanied by stronger activation of effector cells (47–49).

The importance of the interplay between complement receptors and Fc receptors on effector cells during Ab therapy has been further supported by syngeneic mouse tumor models using soluble yeast β-glucan, a naturally occurring CR3-activating polysaccharide. In this situation, β-glucan treatment improved CR3-dependent antitumoral activities of tumor-targeting Abs (anti-CD2 or anti-MUC1). In this study, granulocytes were supposed to be the main effector population, which was recruited under β-glucan treatment, as no antitumoral effects were detected in granulocyte-depleted mice (50). Furthermore, these data were extended by another study unraveling C5a signaling during β-glucan treatment to be the pivotal mechanism leading to enhanced Ab-based tumor elimination by granulocytes in the same mouse tumor model (51). Two years later, the same group published another study demonstrating similar effects of β-glucan treatment on tumor elimination by anti-Her2/neu (trastuzumab) or anti-EGFR (cetuximab) in mouse tumor models (52), thus revealing the importance of the complex interplay between Fc receptors and complement receptors on granulocytes in Ab-based tumor therapy. Notably, a recent study demonstrated in tumor mouse models the pivotal role of concerted actions between innate and adaptive immune responses involving neutrophils, NK cells, macrophages, and CD8+ T cells in Ab-based tumor therapy (53). Hence, enhanced activation of the effector mechanisms of granulocytes by an efficient recruitment of the complement system should be targeted for successful immunotherapy of EGFR-positive tumors.

In summary, the current study provides novel insights into how therapeutic EGFR-targeting Abs with enhanced complement-activating capacities may trigger improved antitumoral activity. In detail, complement-optimized EGFR Abs are able to use either the complement system or immune effector cells separately to trigger tumor cell destruction. Of interest, they are also able to opsonize tumor cells with complement-derived opsonins or to recruit and activate the cytotoxic activities of granulocytes via C5a. Hypothetically, these effects will be accompanied by an improvement of Ab-based tumor therapy in patients.

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Disclosures

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