IgG Fc domains that bind C1q but not effector Fcγ receptors delineate the importance of complement-mediated effector functions

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Engineered crystallizable fragment (Fc) regions of antibody domains, which assume a unique and unprecedented asymmetric structure within the homodimeric Fc polypeptide, enable completely selective binding to the complement component C1q and activation of complement via the classical pathway without any concomitant engagement of the Fcγ receptor (FcγR). We used the engineered Fc domains to demonstrate in vitro and in mouse models that for therapeutic antibodies, complement-dependent cell-mediated cytotoxicity (CDCC) and complement-dependent cell-mediated phagocytosis (CDCP) by immunological effector molecules mediated the clearance of target cells with kinetics and efficacy comparable to those of the FcγR-dependent effector functions that are much better studied, while they circumvented certain adverse reactions associated with FcγR engagement. Collectively, our data highlight the importance of CDCC and CDCP in monoclonal-antibody function and provide an experimental approach for delineating the effect of complement-dependent effector-cell engagement in various therapeutic settings.

Therapeutic monoclonal antibodies (mAbs) ameliorate disease by two mechanisms that involve the binding and resultant modulation of the function of proteins associated with pathophysiology and the recruitment of effector mechanisms dependent on the crystallizable fragment (Fc) regions of antibody domains; these functions mediate, either directly or indirectly, the neutralization and clearance of targeted substrates, as well as the programming of adaptive immunity1,2. Effector functions arise from the binding of the Fc domain of immunoglobulin G (IgG) to Fcγ receptors (FcγRs) expressed on various leukocyte subsets and also from recruitment of the complement component C1q and the ensuing activation of the classical complement pathway. Human effector FcγRs include, in addition to the well-characterized ‘classical’ (type I) receptors (in humans, FcγRI, FcγRII, FcγRIII and their isoforms), the lectin-like type II receptors (CD23 and CD209), TRIM21 and members of the FCRL family of receptors3,4. The recruitment and signaling of type I receptors via immunocomplexes (ICs) are responsible for antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent cell-mediated phagocytosis (ADCP), reactions that have been established clinically to contribute to the mechanism of action of many therapeutic antibodies5. Alternatively, activation of the classical complement pathway leads to target-cell clearance by two distinct processes6: first, direct cell lysis that results from insertion of the membrane attack complex into the cell membrane (complement-dependent cytotoxicity (CDC)); and second, the deposition of opsonins, such as C3b, that are covalently bound onto the cell surface and in turn are recognized by complement receptors (CRs) on effector cells. The CRs activated by the deposited opsonins trigger complement-dependent cell-mediated cytotoxicity (CDC) and complement-dependent cell-mediated phagocytosis (CDCP)6,7. Additionally, activation of the classical pathway has been established to stimulate B cell and T cell adaptive immune responses8.

Determining in a quantitative way the relative roles of complement-dependent and FcγR-dependent effector mechanisms in mAb function is critical for the development of improved therapeutics9,10. However, this has proven to be a very difficult problem to address.

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exponentially, as evinced by the longstanding debate about the relative importance of complement in the clearance of CD20+ B cells by mAbs (such as rituximab (Rituxan)) to the B cell–specific surface antigen CD20 (refs. 11,12). IgG isotypes capable of activating complement also bind to FcγRs to varying degrees, especially after the formation of highly aggregated ICs on target cells or viruses13,14. As a result, it is not possible to distinguish, in the presence of serum, whether target-cell lysis by antibodies is dominated by ADCC or CDC and, similarly, whether phagocytosis is due to ADCP or CDCP. While ADCP and ADCP can be readily studied by well-established in vitro assays15, there is no straightforward manner with which to quantify the effect of CDCP and CDCP on target-cell clearance by mAbs. Because the C1q- and FcγR-binding sites on the Fc domain are proximal and partially overlap, amino-acid substitutions engineered to diminish the binding of FcγRs also eliminate the recruitment of C1q and vice versa16,17.

Among the cell-elimination pathways triggered by the classical complement pathway, CDC activity is by far the easiest to measure and has been studied in great detail11,15. In contrast, apart from the results of some very early, qualitative studies from more than 40 years ago, with polyclonal antibodies15, practically nothing is known about the kinetics and magnitude of target-cell elimination by CDC and CDCP or their importance in mAb function. In the presence of serum, C3 fragments become deposited onto target cells as a result of release of cytotoxic proteins by effector cells or through phagocytosis. While synergy in the elimination of substrates when both CRs and FcγRs are activated has been inferred from some studies15, other reports have suggested antagonistic or opposing effects19, and the precise role of CDCC and CDCP in the absence of confound effects due to FcγR engagement is not known.

RESULTS

Engineering of aglycosylated C1q-selective IgG1 Fc domains

To delineate in detail the role of CDCC and CDCP in target-cell clearance, among diverse effector functions (Fig. 1a), we focused on engineering C1q-selective, aglycosylated antibodies that lacked the ubiquitous Asn297 glycan in the Fc domain. In aglycosylated mAbs, glycan-mediated effects such as signaling via type II receptors are ubiquitous Asn297 glycan in the Fc domain. In aglycosylated mAbs, the aglycosylated form (the Fc domain of aglycosylated clone 801 (A801) constructed with the Fab arms of Rituxan (RA801)) and the glycosylated form (the Fc domain of glycosylated clone 801 (G801) constructed with the Fab arms of Rituxan (RG801)). As analyzed by surface plasmon resonance, RA801 had no detectable binding to any FcγR, whereas RG801 bound very weakly only to the high-affinity receptor FcγRI (Table 1 and Supplementary Fig. 3a,b). Rituxan clone 802 (with the amino-acid substitutions L245K, G246M, G247R and L351Q) showed selective binding to C1q only when expressed as an aglycosylated protein (RA802) (Table 1 and Supplementary Fig. 3a,b). Its glycosylated form, RG802, showed complete loss of binding to C1q and to effector FcγRs (Table 1 and Supplementary Fig. 3a,b). Both RA801 and RA802 displayed pH-dependent binding to the human neonatal Fc receptor (FcRn), with an affinity at pH 6.0 comparable to that of wild-type glycosylated IgG1 (Supplementary Fig. 3c); this suggested that the substitutions in these antibodies were unlikely to have a negative effect on recycling by FcRn or pharmacokinetics in vivo.

The binding of ICs onto cells represents an exquisitely sensitive assay for the detection of physiologically relevant IgG–FcγR interactions13,14. To form ICs with defined stoichiometries, we used antibodies comprising the Fc domain of A801 or A802 fused to α-2, 4,6-trinitrophenyl (TNP) Fab arms (TNP-A801 and TNP-A802, respectively) and incubated them with bovine serum albumin (BSA) conjugated to an average of either 4 TNP molecules (TNP4:BSA; low-avidity IC) or 32 TNP molecules (TNP32:BSA; high-avidity ICs). ICs formed with wild-type antibody to TNP (anti-TNP) readily bound to Chinese hamster ovary (CHO) cells expressing each FcγR (Fig. 1b). In contrast, ICs of TNP-A801 and TNP-A802 of higher avidity (TNP4:BSA or TNP32:BSA) showed no binding to FcγR-expressing CHO cells (Fig. 1b). We note that because of the higher surface hydrophobicity of TNP32-BSA, when mixed with IgG, TNP32-BSA ICs show greater binding to CHO cells expressing no FcγRs than that of TNP4-BSA ICs.

RA801 and RA802 mediated potent CDC activity with three widely used CD20+ human Burkitt’s lymphoma cell lines (Raji, Ramos and Daudi), as well as with cells derived from patients with acute lymphocytic leukemia (Fig. 2a,b and Table 2). Complement activation of the classical pathway and opsonization of CD20+ cells by C3b was comparable to that achieved with Rituxan (Fig. 2c and Supplementary Fig. 4a, b). The lysis of Raji cells and Ramos cells via RA801 was characterized by EC50 values (effector concentration for a half-maximum response) 2.3-fold lower and 4.5-fold lower, respectively, than those obtained with Rituxan (Fig. 2a, b and Table 2). We also assessed ofatumumab, an approved second-generation mAb to CD20 whose enhanced clinical activity in chronic lymphocytic leukemia has been established to result from improved CDC24–26, and found that the greater CDC potency of RA801 compared favorably with that of ofatumumab: the EC50 values for Raji cells and Ramos cells obtained with
ofatumumab were 1.9-fold lower and 4.1-fold lower, respectively, than those obtained with Rituxan (Fig. 2d).

The enhanced CDC killing potency of ofatumumab is due to its binding to a membrane-proximal epitope in CD20 that more optimally engages C1q27. Antibodies with the ofatumumab Fab fragments in combination with the clone 801 aglycosylated Fc domain (OA801) or clone 802 aglycosylated Fc domain (OA802) conferred on Raji cells and Ramos cells CDC of even greater potency than that conferred by ofatumumab (Fig. 2d and Supplementary Table 2).

After binding to antigens on the cell surface, IgG1 antibodies self-associate via the Fc domain to form hexamers that, in turn, optimally engage C1q27. Antibodies with the ofatumumab Fab fragments in combination with the clone 801 aglycosylated Fc domain (OA801) or clone 802 aglycosylated Fc domain (OA802) conferred on Raji cells and Ramos cells CDC of even greater potency than that conferred by ofatumumab (Fig. 2d and Supplementary Table 2).

In standard ADCC assays performed with culture medium in the absence of serum, RA801 and RA802 were completely unable to lyse CD20+ cells (Raji or Ramos) with either peripheral blood mononuclear cells (PBMCs) or polymorphonuclear cells (PMNs) as effector cells (Fig. 3a, Table 2 and Supplementary Fig. 4c). In contrast, in the presence of serum (25% of the total volume) that had been depleted of complement component C9 (to abolish the formation of membrane attack complexes and thus cell killing via CDC), RA801 and RA802 were very efficient in mediating target-cell lysis, with EC50 values slightly higher than those obtained with Rituxan under these conditions29 (Fig. 3b).

Table 1 Binding properties of the engineered antibody variants

<table>
<thead>
<tr>
<th>IgG</th>
<th>Gly</th>
<th>C1q</th>
<th>FcγRI</th>
<th>FcγRIIa131</th>
<th>FcγRIIb</th>
<th>FcγRIlaV158</th>
<th>FcγRIlaV158</th>
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<tbody>
<tr>
<td>IgG 801</td>
<td>No (RA801)</td>
<td>0.108 (213-fold)</td>
<td>120</td>
<td>310</td>
<td>1300</td>
<td>195</td>
<td>390</td>
</tr>
<tr>
<td>IgG 802</td>
<td>No (RA802)</td>
<td>0.145 (158-fold)</td>
<td>648 (0.002 fold)</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
<td>NB</td>
</tr>
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</table>

Apparent dissociation constant (Kd) values (nM) for the binding of RA801 and RA802 (far left) to human C1q, human FcγRs (two natural variants of FcγRIla (His131 and Arg131) and FcγRIla (Phe158 and Val158), with variant amino acids subscripted) or human FcRn (assessed at a pH of 6.0) (top row), estimated with a global two-state binding model for C1q binding, the langmuir 1:1 binding model for the binding of the high-affinity receptor FcγRI or a bivalent binding model for dimeric forms of low-affinity FcγRs, as described29; results were calculated by the following equation: Kd (glycosylated IgG)/Kd (igG 801 or 802): NB, no binding detected. Data are representative of three experiments.

Analysis of RA801- and RA802-mediated CDC

In contrast, in the presence of serum (25% of the total volume) that had been depleted of complement component C9 (to abolish the formation of membrane attack complexes and thus cell killing via CDC), RA801 and RA802 were very efficient in mediating target-cell lysis, with EC50 values slightly higher than those obtained with Rituxan under these conditions29 (Fig. 3b).

In these experiments, cell lysis was strictly dependent on the presence of effector cells (PBMCs or PMNs) and was solely the outcome of CDC, given that RA801 and RA802 were unable to bind to FcγRs.
and did not exhibit any CDC activity in serum depleted of C9 or any CDCC activity in in serum depleted of C1q (Fig. 3c,d). Thus, in serum, the efficacy of the lysis of CD20+ cells by RA801 and RA802, which perform only CDC, was at least comparable to or greater than that of Rituxan, even though Rituxan executed target-cell killing via both FcγR-mediated mechanisms (ADCC) and CR-dependent mechanisms (CDCC). CR3 is a key receptor for the lysis of complement-opsonized target cells by effector cells.26,30 The addition of blocking antibody to CR3 inhibited the lysis, by PBMCs and PMNs (as effector cells), of RA801-opsonized Raji cells in serum depleted of C9 (Fig. 3e and Supplementary Fig. 4e). Antibody blockade of CR4 also inhibited the lysis of RA801-opsonized cells by PBMCs and PMNs26,30 (Fig. 3e and Supplementary Fig. 4e). Thus, CR3 and CR4, which are expressed by a variety of myeloid cells, have an important role in CDCC. Finally, in assays performed in complete blood, a condition more relevant to in vivo settings, RA801 and RA802 were slightly more effective than Rituxan at achieving lysis of Raji cells and Ramos cells (Fig. 3f,g, Table 2 and Supplementary Fig. 4f). We concluded that at least within the time frame and conditions used in these experiments, complement activation made a major, if not dominant, contribution to the elimination of antibody-opsonized CD20+ cells.

### Quantitative analysis of NK cell–mediated CDCC

Natural killer (NK) cells are by far the most effective cytolytic leukocyte population among PBMCs in vitro. NK cells can form stable conjugates with antibody-opsonized target cells via either FcγRIIa (CD16a) or CRs26,30–32; this results in the release of cytotoxic granules, which leads to apoptosis and target-cell lysis. We used high-throughput single-cell killing assays to evaluate, at high resolution, details of the kinetics of the killing of Raji cells by human NK cells. We used single-cell assays because the time constants for processes such as the formation of synapses between effector and target cells, time required for cell lysis and the efficiency with which effector cells perform multiple killings cannot be determined by conventional macroscopic
assays. In brief, fluorescence-labeled NK cells from two healthy donors were incubated in nanowells together with an average of one to three Raji (target) cells that had been opsonized with either Rituxan or RA801. Cell motility and lysis were monitored by time-lapse imaging microscopy in nanowell grids (TIMING)\(^3^3\) (Supplementary Fig. 5). The time to establish stable conjugation, the duration of conjugation before tumor-cell apoptosis and the time to tumor-cell apoptosis were determined for >150 single-cell killing events per sample (Fig. 4a,b). We observed no significant difference, following opsonization with Rituxan or RA801, in the frequency of one- or two-target-cell killing events (Fig. 4b). Mechanistically, killing via RA801 involves the formation of synapses between CRs and opsonins deposited on target cells, whereas with Rituxan, synapses can form both as a result of opsonin deposition and via the binding of antibody to FcRIIIa on NK cells. The similar overall NK cell–killing activity or serial killing of cells on antibody-opsonized target cells. The finding that phagocytosis with RA801 or RA802 occurred only in the presence of serum unequivocally demonstrated that CDCC could be a major mechanism in the cytotoxic effect of NK cells on antibody-opsonized target cells.

**Figure 3** Killing of CD20\(^{+}\) cells by CDCC. (a,b) Lysis of Raji tumor cells (opsonized by various concentrations (horizontal axes) of isotype-matched control antibody (as in Fig. 1d), Rituxan, RA801 or RA802 (key)) by PBMCs (as effector cells), at an effector cell/tumor cell ratio of 10:1, in RPMI-1640 medium without pooled human serum (No PHS) (a) or supplemented with 25% pooled human serum depleted of C9 (PHS – C9) (b); results are presented relative to those obtained by incubation in SDS lysis buffer. (c) Lysis (by CDC) of Raji cells opsonized by various concentrations (horizontal axes) of IgG antibodies as in a (key), assessed in the presence of undepleted serum (+C9) or serum depleted of C9 (–C9). (d) Lysis of Raji cells (opsonized by 20 µg/ml, 4 µg/ml or 0.8 µg/ml (dark to light bar shading) of isotype-matched control antibody, Rituxan or RA801 (key)) by PBMCs or PMNs in RPMI-1640 medium supplemented with 25% serum depleted of C9. (e) Lysis of Raji cells (opsonized as in a) by PBMCs, in RPMI-1640 medium supplemented with 25% pooled human serum. (f) Lysis of Raji cells (opsonized with 10 µg/ml, 2.5 µg/ml or 0.6 µg/ml (shading as in d) of antibodies as in a (key)) by PBMCs, in RPMI-1640 medium supplemented with 25% pooled whole blood. Data are from one experiment representative of three experiments (a-d,f,g) or two experiments (e) (error bars, s.d.).

Analysis of CDCP by RA801 and RA802

In the absence of serum, RA801- or RA802-opsonized CD20\(^{+}\) cells were not phagocytosed by classically activated (M1) macrophages, even at the highest concentration of antibody tested (400 nM) (Fig. 5a). However, RA801 and RA802 were effective in performing phagocytosis by macrophages via complement when human serum depleted of C9 was added to the assay (Fig. 5b,c). The finding that phagocytosis with RA801 or RA802 occurred only in the presence of serum unequivocally demonstrated that it was mediated by complement deposition; i.e., it was due solely to CDCP. In the presence of serum, phagocytosis of RA801- or RA802-opsonized cells was slightly more effective than that of Rituxan-opsonized cells, with EC\(_{50}\) values of 3.7 ± 0.04 nM, 4.6 ± 0.1 nM and 17.4 ± 0.2 nM (mean ± s.d.), respectively (Fig. 5b,c). Notably, at saturating concentrations of antibody, the presence of serum markedly increased the magnitude of phagocytosis and resulted in >80% clearance of Raji cells with either RA801 or Rituxan, compared with 30% cell clearance observed with ADCP (Fig. 5b,c). However, consistent with published reports\(^3^4\), serum increased the EC\(_{50}\) for phagocytosis via Rituxan (17.4 ± 0.2 nM with serum, compared with 37 ± 5 pM for medium only (mean ± s.d.); Fig. 5b,c).
Anti-tumor effects by complement in an in vivo model

We first established that RA801 did not bind to mouse FcγRs but was able to activate mouse complement and mediate CDC (Supplementary Fig. 6a–c). We evaluated the anti-tumor effect of RA801 in the treatment of established subcutaneous tumors formed by Ramos cells in outbred nude mice, which lack a thymus and mature T cells but produce macrophages and NK cells. When the tumors reached a diameter of 40–45 mm², the mice were treated three times with RA801 or Rituxan (5 mg per kg body weight), administered intravenously. No significant difference between the Rituxan-treated mice and RA801-treated mice was observed in terms of tumor growth (Fig. 6a). In contrast, administration of RA801 had a significant effect in slowing tumor growth in FcγR-null mice (Fig. 6c,d).

The presence of micro-aggregates in antibody preparations can trigger systemic, FcγR-mediated anaphylactic responses. It has been reported that intravenous administration of heat-aggregated Rituxan results in a rapid reduction in body temperature38. In contrast, we observed no change in body temperature in mice given injection of heat-aggregated RA801 (Fig. 6e). Separate from that, the engagement of FcγRs by anti-CD20 has been shown to lead to decreased surface expression of CD20 on B cells, a process that is mediated in part by the co-ligation of FcγRIIb and internalization of antibody–antigen complexes in cis39,40. Consistent with those observations, Raji cells incubated with Rituxan showed a significant decrease in surface expression of CD20, with a reduction of >40% by 6 h (Fig. 6f and Supplementary Fig. 6d). In contrast, cells incubated with RA801 showed no decreasing expression of surface CD20 (Fig. 6f and Supplementary Fig. 6d).

Structural analysis of the clone 801 Fc

To understand the molecular basis of the unique selectivity of the clone 801 Fc fragment for C1q, we solved the crystal structures of the
Figure 5 CDCP of CD20⁺ cells. (a,b) Phagocytosis of antibody-opsonized Raji cells (antibodies in key; concentration, horizontal axes) by monocyte-macrophages (top left) or macrophages without Raji cells incubated for 0, 2, 4 or 6 h (horizontal axis) with 100 nM IgG (antibodies in key; concentration, horizontal axes) by monocyte-macrophages (top left) or macrophages without Raji cells (bottom left), with opsonization by isotype-matched control antibody, Rituxan, RA801 or RA802 in the presence of serum depleted of C9 (top row, low magnification; bottom row, high magnification); top and right ‘strips’ show different views (xz and yz) of the same cells at a confocal plane. Scale bars, 20 µm. Data are from one experiment representative of three experiments (error bars a, b, s.d. of technical triplicates).

Fc domain of both the aglycosylated clone 801 (A801-Fc) and glycosylated clone 801 (G801-Fc) at 2.3 Å and 3.2 Å, respectively (Table 3). The most striking feature of the A801-Fc structure was the extreme asymmetric orientation of C2γ in chain B (C2γA), relative to that of C2γ in chain A (Fig. 7a). Topologically, the orientation of C2γ relative to C2γ is described by the C2γ–C2γ dihedral angle, which is defined from the Cα atoms of Tyr300 and Tyr319 in C2γ, and Gln362 and Met428 in C2γ (ref. 41). Chain A of A801-Fc had a dihedral angle of −20.5°, which was slightly smaller than yet comparable to the dihedral angles of chain A observed in the ten glycosylated Fc (G-Fc) structures (−27° ± 4.1° (mean ± s.d.)) or the comparable angle (−25° ± 9.4°) in the four aglycosylated or deglycosylated human Fc proteins (A-Fc) in the Protein Data Bank (PDB) (Fig. 7a and Supplementary Table 3). However, chain B of A801-Fc showed an extreme twist and packed against C2γA, which resulted in a dihedral angle of 136° (Fig. 7a). As a comparison, in all other Fc domains in the PDB, chains A and B have similar dihedral angles (−23° ± 3.8° for ten G-Fc structures, and −25° ± 1.9° for four A-Fc structures). This extreme twist was observed only for A801-Fc, but G801-Fc showed similar dihedral angles for both chain A and chain B, comparable to those in other Fc structures (Supplementary Table 3). Although it is well established that the two homodimeric Fc chains crystallize with distinct temperature factors, dynamics and geometries for each chain(41), the different orientation of C2γA and C2γA relative to that of C2γ in A801-Fc is unprecedented, to our knowledge. No direct physical contact of C2γ with other molecules in the asymmetric unit was observed (Supplementary Fig. 7a), which suggested that the dihedral angle in chain B of A801-Fc was probably not the result of crystal-packing artifacts. We used cross-linking with the carbodiimide EDC and liquid chromatography–tandem mass spectrometry to determine whether the observed crystal structure reflected the conformation of A801-Fc in solution. We observed a cross-linked product in A801-Fc between a peptide with Lys334 in chain A and Glu269 in chain B (Fig. 7a). In the highly asymmetric A801-Fc structure, the side chains of Lys334 in chain A and Glu269 in chain B were only 3.6 Å apart (Fig. 7b), a distance compatible with the formation of cross-linkage via EDC (−4 Å). In contrast, Lys334 in chain A and Glu269 in chain B were 24.1 Å apart in G-Fc (PDB accession code 3AVE) and were 24.1 Å apart in A-Fc (PDB accession code 3SGG) (Fig. 7b). Thus, the results of the crosslinking experiments (Fig. 7c) were fully consistent with, and provided independent support for, the proposal that the crystal structure of A801-Fc reflected the conformation of the molecule in solution.

In the A801-Fc structure, loops BC and FG in C2γ are involved in the asymmetric interactions between the two chains (Figs 6 and 7). The C2γ chain A, which packs against C2γ chain B, has a dihedral angle of −20.5° relative to C2γ chain A, which packs against C2γ chain B, of 136° (Fig. 7a). In the asymmetric unit was observed (Supplementary Fig. 7a), which suggested that the dihedral angle in chain B of A801-Fc was probably not the result of crystal-packing artifacts. We used cross-linking with the carbodiimide EDC and liquid chromatography–tandem mass spectrometry to determine whether the observed crystal structure reflected the conformation of A801-Fc in solution. We observed a cross-linked product in A801-Fc between a peptide with Lys334 in chain A and a second peptide with Gln362 and Met428 in chain B (Fig. 7a). As a comparison, in all other Fc domains in the PDB, chains A and B have similar dihedral angles (−23° ± 3.8° for ten G-Fc structures, and −25° ± 1.9° for four A-Fc structures). This extreme twist was observed only for A801-Fc, but G801-Fc showed similar dihedral angles for both chain A and chain B, comparable to those in other Fc structures (Supplementary Table 3). Although it is well established that the two homodimeric Fc chains crystallize with distinct temperature factors, dynamics and geometries for each chain(41), the different orientation of C2γA and C2γA relative to that of C2γ in A801-Fc is unprecedented, to our knowledge. No direct physical contact of C2γ with other molecules in the asymmetric unit was observed (Supplementary Fig. 7a), which suggested that the dihedral angle in chain B of A801-Fc was probably not the result of crystal-packing artifacts. We used cross-linking with the carbodiimide EDC and liquid chromatography–tandem mass spectrometry to determine whether the observed crystal structure reflected the conformation of A801-Fc in solution. We observed a cross-linked product in A801-Fc between a peptide with Lys334 in chain A and a second peptide with Gln362 and Met428 in chain B (Fig. 7a). As a comparison, in all other Fc domains in the PDB, chains A and B have similar dihedral angles (−23° ± 3.8° for ten G-Fc structures, and −25° ± 1.9° for four A-Fc structures). This extreme twist was observed only for A801-Fc, but G801-Fc showed similar dihedral angles for both chain A and chain B, comparable to those in other Fc structures (Supplementary Table 3). Although it is well established that the two homodimeric Fc chains crystallize with distinct temperature factors, dynamics and geometries for each chain(41), the different orientation of C2γA and C2γA relative to that of C2γ in A801-Fc is unprecedented, to our knowledge. No direct physical contact of C2γ with other molecules in the asymmetric unit was observed (Supplementary Fig. 7a), which suggested that the dihedral angle in chain B of A801-Fc was probably not the result of crystal-packing artifacts. We used cross-linking with the carbodiimide EDC and liquid chromatography–tandem mass spectrometry to determine whether the observed crystal structure reflected the conformation of A801-Fc in solution. We observed a cross-linked product in A801-Fc between a peptide with Lys334 in chain A and a second peptide with Gln362 and Met428 in chain B (Fig. 7a). As a comparison, in all other Fc domains in the PDB, chains A and B have similar dihedral angles (−23° ± 3.8° for ten G-Fc structures, and −25° ± 1.9° for four A-Fc structures). This extreme twist was observed only for A801-Fc, but G801-Fc showed similar dihedral angles for both chain A and chain B, comparable to those in other Fc structures (Supplementary Table 3). Although it is well established that the two homodimeric Fc chains crystallize with distinct temperature factors, dynamics and geometries for each chain(41), the different orientation of C2γA and C2γA relative to that of C2γ in A801-Fc is unprecedented, to our knowledge. No direct physical contact of C2γ with other molecules in the asymmetric unit was observed (Supplementary Fig. 7a), which suggested that the dihedral angle in chain B of A801-Fc was probably not the result of crystal-packing artifacts. We used cross-linking with the carbodiimide EDC and liquid chromatography–tandem mass spectrometry to determine whether the observed crystal structure reflected the conformation of A801-Fc in solution.
the interaction interface\(^2\) (Fig. 7d). The principal contacts at this surface were dominated by hydrophobic interactions between residues Phe241, Phe243 and Val264 of chain A with backbone atoms of residues Glu269, Asp270, Lys326 and Pro329 in chain B. Furthermore, a salt bridge was formed between Asp265 of chain A and Lys326 residues Glu269, Asp270, Lys326 and Pro329 in chain B. Furthermore, a salt bridge was formed between Asp265 of chain A and Lys326 of chain B.

The A801-Fc structure suggested a molecular basis for the selective binding to C1q and lack of binding to FcγRs. The K320E amino-acid substitution lay within the predicted C1q-binding region\(^43–46\) and probably mediated binding to C1q by electrostatic interactions. The binding of A801-Fc to C1q was probably the result of more subtle effects probably related to differences in the conformational flexibility of C2. Within the A801-Fc structure, the apex of C\(γ\)2 \(γ\)1 folded over to support and stabilize C\(γ\)2 of this ‘soft’ conformation observed only in A-Fc (PDB accession code 3S7G).\(^3\) Other biochemical data has also shown that removal of the Asn297 glycan increases the conformational flexibility of C\(γ\)2 (ref. 23). We postulate that the enthalpic contribution from the K320E substitution, probably coupled with the lower flexibility of C\(γ\)2, was a key reason for the efficient binding of A801-Fc to C1q.

X-ray structures also provided an explanation for the complete loss of binding of FcγR to A801-Fc.\(^3\) With IgG1, FcγRs dock on both C\(γ\)2 \(γ\)A and C\(γ\)2 \(γ\)B and interact with two subsites centered near the C′E loop of one chain and the hinge LLPP motif of the other.\(^47\) Interactions with both subsites are needed for FcγR binding, which results in an interaction surface of ~1,000 Å\(^2\), of which about 600 Å\(^2\) of interface is formed with the LLPP motif.\(^48\) However, the change in the orientation of C\(γ\)2 \(γ\)B in A801-Fc precluded the simultaneous interaction of FcγRs with both subsites (Supplementary Fig. 7c).

The two FcγR-binding subsites of G801-Fc were sterically accessible; however, they seemed to be highly flexible and/or disordered. Specifically, in three of the four molecules within each asymmetric unit, either the LLPP motif or the C′E loop was disordered (Fig. 7e and Supplementary Fig. 7d,e). In the only molecule within the asymmetric unit in which these regions were sufficiently ordered to be traced, the C′E loop assumed a ‘soft’ conformation observed only in A-Fc, which does not bind to FcγRs.\(^34\) The high flexibility of the C′E loop of this ‘soft’ conformation would probably ‘penalize’ FcγR binding with a high entropic cost. Collectively, the X-ray crystal structures of A801-Fc and G801-Fc provided a structural explanation for the enhanced C1q binding and the lack of recognition by FcγRs.

**DISCUSSION**

Effector cells express both FcγRs and CRs; therefore, in the presence of serum, ADCC (or ADCP) and CDC (or CDCP) occur at the same time.\(^13–15\) Whereas ADCC and ADCP can be assayed readily without CDC or CDCP taking place, simply through the use of medium depleted of serum and C1q, the reverse is not the case. For analysis of CDC or CDCP alone, without ADCC or ADCP also taking place, it is necessary to block the binding of ICs to the multitude

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**Table 3 Data collection and refinement statistics for G801-Fc and A801-Fc**

<table>
<thead>
<tr>
<th>Data collection</th>
<th>G801-Fc</th>
<th>A801-Fc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Space group</td>
<td>P 1 2 1</td>
<td>P 6(^5)</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td>a, b, c (Å)</td>
<td>93.6, 141.0, 98.9</td>
</tr>
<tr>
<td></td>
<td>α, β, γ (°)</td>
<td>90.0, 117.7, 90.0</td>
</tr>
<tr>
<td>Resolution (Å)</td>
<td>40.0–3.22 (3.28–3.22)</td>
<td>50.0–2.25 (2.29–2.25)</td>
</tr>
<tr>
<td>R(_{free})</td>
<td>0.150 (0.808)</td>
<td>0.089 (0.826)</td>
</tr>
<tr>
<td>R(_{free})</td>
<td>9.69 (1.55)</td>
<td>19.27 (1.24)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.90 (99.80)</td>
<td>99.30 (96.40)</td>
</tr>
<tr>
<td>Redundancy</td>
<td>3.6 (3.6)</td>
<td>5.1 (3.7)</td>
</tr>
<tr>
<td>Refinement</td>
<td>Resolution (Å)</td>
<td>40.0–3.22 (3.28–3.22)</td>
</tr>
<tr>
<td>No. reflections</td>
<td>36958</td>
<td>81432</td>
</tr>
<tr>
<td>No. atoms</td>
<td>11344</td>
<td>6713</td>
</tr>
<tr>
<td>Sugar atoms</td>
<td>493</td>
<td>0</td>
</tr>
<tr>
<td>Water</td>
<td>0</td>
<td>125</td>
</tr>
<tr>
<td>B-factors (Å(^2))</td>
<td>84</td>
<td>64</td>
</tr>
<tr>
<td>Protein</td>
<td>84</td>
<td>64</td>
</tr>
<tr>
<td>Sugar atoms</td>
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<td>NA</td>
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<tr>
<td>Water</td>
<td>0</td>
<td>51</td>
</tr>
<tr>
<td>r.m.s. deviations</td>
<td>Bond lengths (Å)</td>
<td>0.002</td>
</tr>
<tr>
<td>Bond angles (°)</td>
<td>0.61</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Shell of highest resolution is in parenthesis. NA, not applicable.
of FcRs expressed by effector cells. We reasoned that a much more experimentally tractable approach for the study of CDCC and CDCP would be to engineer human IgG1 Fc domains that bound only to C1q and activated complement without engaging FcRs. For this purpose, it was critical to silence FcγR binding, since even very low-affinity interactions between IgGs and FcγRs can trigger effector phenotypes. The engineering of Fc domains with absolute or nearly absolute C1q selectivity involved starting with aglycosylated antibodies in which binding to both C1q and FcγRs was substantially, albeit not completely, attenuated, followed by screening of very large mutant libraries for both binding to fluorescence-labeled C1q and the simultaneous absence of binding to high-avidity FcγRs. Using this approach, we isolated A801-Fc; of its two amino-acid substitutions, K320E in Cγ2 conferred substantial affinity for C1q, and Q386R in Cγ3 resulted in a radical conformational change that abolished any binding to FcγRs.

Overwhelmingly, the effector function of therapeutic antibodies is thought to be dominated by FcγR-dependent processes and/or by CDC. We demonstrated in various assays that effector-cell-mediated substrate-clearance mechanisms triggered by activation of the classical complement pathway resulted in very extensive and rapid target-cell lysis and phagocytosis. Our results suggest that the extent and kinetics of the clearance of CD20+ cells via CDCC or CDCP are at least comparable to those due to ADCC or ADCP, respectively. Consequently, CDCC and CDCP deserve careful consideration and optimization in studies of the mechanism of action of mAbs.

The relative importance of CDCC and CDCP probably depends heavily on the target and disease setting. First, optimal activation of complement is critically dependent on the density and organization of antigens on the surface of target cells. Second, many pathogenic cells have high expression of complement-inhibitory proteins, and clearly, in these cases, activation of the classical pathway is compromised. Third, it is well established that in vivo extensive activation of complement due to a considerable burden of target cells and antibody can rapidly lead to the depletion of either C1q or downstream complement components, such as C2 (ref. 49). Clearly, in such circumstances, ADCC and ADCP are probably the dominant antibody effector mechanisms. In conclusion, mAbs with absolute C1q-binding selectivity, as described here, might be useful for the eradication of pathogenic cells in certain disease states while circumventing FcγR-dependent adverse effects, as well as the lower responsiveness to mAbs in patients with low-affinity FcγR polymorphisms.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS


COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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24. Pawluczkowicz, A.W. et al. Binding of submaximal C1q promotes complement-dependent cytotoxicity (CDC) of B cells opsonized with anti-CD20 mAbs ofatumumab (OFA) or rituximab (RTX): considerably higher levels of CDC are induced by OFA than by RTX. J. Immunol. 183, 749–758 (2009).
ONLINE METHODS
Cells and reagents. All cancer cell lines were tested for mycoplasma contamination before cytotoxicity assays. Burkitt's lymphoma Raji (ATCC CCL-86), Ramos (ATCC CRL-1596), Daudi (ATCC CCL-213) and DB can -

Surface-plasmon-resonance analysis. To determine the affinities of antibody variants with C1q, FcRs and FcRn, a Biacore 3000 instrument (GE Healthcare) was used with HBS-EP running buffer (GE Healthcare). Bovine serum albumin (BSA) was immobilized in the reference channel of the CM5 sensor chip, and each antibody was immobilized on the CM5 sensor chip by amine coupling at a pH of 5.0. All FcRs (400 nM) or the serially diluted C1q proteins (1–40 nM) were injected into the CM5 chip at 30 µl/min for 2 min, followed by 10 min of dissociation. The chip was regenerated after each binding event with 10 mM glycine, pH 3.0, with a contact time of 1 min. Monomeric FcRn–β2-microglobulin–glutathione S-transferase (730 nM) was injected in the CM5 chip at 30 µl/min in HBS-EP, pH 7.4, for 90 s, and the chip was regenerated after each binding event with 10 mM glycine, pH 3.0, with a contact time of 1 min. The resulting sensorgrams were fit with a global two-state binding model for C1q, and with a 1:1 Langmuir isotherm model for monomeric FcRn and FcRn using Biaevaluation 3.0 software. Because C1q is hexameric protein, the measured K_D values do not correspond to the true equilibrium dissociation constants for the interaction of the C1q globular head with IgG and are used here only as a measure of the relative affinity of the C1q for each antibody.

C3b deposition assays. Cells cultured in complete RPMI-1640 medium were mixed with an equal volume of normal human serum (NHS), and then mAbs were added to a final concentration of 10 µg/ml. After incubation for 30 min at 37 °C, the cells were washed twice with 1% BSA in PBS and developed with FITC-conjugated mouse mAb 3E7 to C4d (BioLegend; 1:50 dilution). Flow cytometry was performed on a FACScalibur flow cytometer (BD Biosciences), and mean fluorescence intensity was converted into molecules of equivalent soluble fluorochrome with calibrated beads (Spherotech).

Solution-phase complement-activation assay. Complement activation in the absence of tumor cells was determined by measurement of the concentration of C4d, a product of the classical complement activation pathway, after incubation of 100 µg antibody in 1 ml 90% NHS for 1 h at 37 °C. C4d concentrations were measured by enzyme-linked immunosorbent assay (MicroVue C4d EIA kit, Quidel Corporation) according to the manufacturer’s instructions.

Size-exclusion chromatography (SEC). SEC was performed on Agilent 1100 high-performance liquid chromatography system using a SuperdexTM200 10/300GC, (GE Healthcare), with a mobile phase of PBS, pH 7.4, at a flow rate of 0.5 ml/min. Proteins were detected by monitoring the absorbance at 280 nm. The injection amount was 100 µg protein in a volume of 100 µl.

Enzyme-linked immunosorbent assay. To measure binding antibodies to FcRs and C1q, 96-well plates (Qiagen) were coated with 1 µg/well of each antibody at 4 °C for 16 h and washed three times with PBS containing 0.05% Tween20 (PBST). The plates were blocked for 1 h at 25 °C with 3% skim milk in PBS and were washed three times with PBST. FcRs (50 nM or 500 nM) was added in plates. After 1 h of incubation at 25 °C, the plates were washed with PBST and incubated with 50 µl of PBS containing 1:5,000 goat anti-histidine (Abcam; Cat# ab1269) or antibody to glutathione S-transferase IgG conjugated to HRP (Rockland; Cat# 600-103-200) for 1 h. After washing three times with PBST, 50 µl TMB substrate was added per well (Thermo Scientific), 50 µl of 1 M H_2SO_4 was added for neutralization, and the absorbance at 450 nm was recorded.

Crystalization and data collection. Crystallization conditions for the 801-Fc domain were screened initially by sparse-matrix solutions using sitting drop-diffusion on Phenix instrument (Art Robins Instruments). Crystals for A801-Fc were obtained by mixing protein at a concentration of 20 mg/ml with buffer containing 50 mM sodium citrate, pH 5.0, as a pH buffer, and 0.44 M NaCl with 24% wt/vol PEG 4000 as precipitant at 1:1 ratio at 25 °C. G801-Fc at
a concentration of 10 mg/ml crystallized in 40 mM potassium phosphate, 16% wt/vol PEG 8000 and 20% vol/vol glycerol. The crystals were cryo-protected in 20% glycerol before vitrified in liquid nitrogen for data collection54.

Crystal diffraction data were collected at the Advanced Light Source beamline BL5.0.3 (Berkeley, CA) and Advanced Photo Source BL 23-ID (Chicago, IL). The data were processed using the program HKL2000 (ref. 54). The diffractions were scaled to 2.3 Å for A801-Fc and 3.2 Å for G801-Fc. The statistics for data collection are summarized in Table 3.

Structure determination, refinement and analysis. The structures of A801 was determined by molecular replacement using wild-type Fc as the search model (PDB code: 3AVE). The first round of molecular refinement using program Phaser in CCP4 suite55,56 identified two dimers per asymmetric unit and revealed that the Cγ2-Cγ3 dihedral angle varied considerably from those in all other Fc structures in the database. Therefore, the Cγ2 and Cγ3 were searched separately by Phaser55,56, and the initial solution was identified and then confirmed with electron-density-fitting the model. Rigid-body and CNS refinement were carried out in Phenix.refine57. The model was optimized by iterative cycles of manual model building using COOT 58 followed by refinement with Phenix.refine52 to improve the quality of the model. Prior to the refinement, 5% of the diffraction data were reserved as an unbiased test set for cross-validation (Rfree)59. The final model exhibits an Rwork of 20% and an Rfree of 24%.

The structure of G801-Fc was also determined by molecular replacement using the structure of glycosylated human Fc as the search model (PDB code: 3AVE). A total of four Fc molecules were found per asymmetric unit. The fitted model was rebuilt using Phenix.refine57, and several iterative cycles of optimization were carried out with manual building and refinement. The final model showed an Rwork of 25% and an Rfree of 29%.

Both structures were evaluated by MolProbity60 and Procheck. For A801, the MolProbity Score was 1.71 and placed this at the 99th percentile of all structures for the similar resolution range in database. For G801-Fc, the MolProbity Score was 1.47 and reached 100th percentile. Detailed refinement statistics for A801-Fc and G801-Fc are summarized in Table 3. Figures were prepared using PyMol (DeLano, 2002) 61.

C1q cell-surface-binding assay. Raji cells were resuspended in 5% NHS and then incubated with mAbs at a final concentration of 10 µg/ml at 37 °C for various times (0–30 min). Classical complement activation reactions were then quenched with 20 volumes of ice-cold 1% BSA-PBS. The cells were pellet and washed once and then were probed with FITC anti-C1q (Dako; Cat# F0254; 1:200 dilution) for 30 min at 25 °C. The samples were washed and then analyzed by flow cytometry (FACSCalibur flow cytometer; BD Biosciences), and mean fluorescence intensity was converted to molecules of equivalent soluble fluorochrome with calibrated beads (Spherotech).

Binding of ICs to FcγRs expressed on CHO cells. ICs were generated by co-incubation of 5 µg/ml of variants of anti-TNP IgG (clone 7B4; provided by F.N.) and 2.5 µg/ml of TNP-coupled BSA (TNP25-BSA or TNP32-BSA; Biosearch Technologies) for 3 h at 25 °C (ref. 14). The relative size of TNP25-BSA and TNP32-BSA ICs was analyzed by polyethylene glycol (PEG) precipitation. ICs were then incubated with 1 × 104 CHO cells stably expressing human FcγRs for 1 h with gentle shaking at 4 °C, followed by detection of bound ICs by flow cytometry using a PE-conjugated goat anti-human IgG F(ab′)2 fragment at 0.5 µg/ml (Jackson Laboratories; Cat# 109-096-006). ICs were also generated by mixing 10 µg/ml of antibodies and 5 µg/ml of PE-conjugated F(ab′)2 goat anti-human IgG F(ab′)2 (identified above) for 30 min at 37 °C. CHO cells expressing mouse FcγRs were incubated with these ICs for 1 h on ice and were analyzed by flow cytometry with the method described above. Data were analyzed with Flow Cytometry Analysis Software (FlowJo).

Complement-dependent cytotoxicity (CDC) assays. CDC assays were performed as described previously52. In brief, serially diluted antibodies were incubated with 25% pooled human serum and cancer cells loaded with calcein AM (Life Technologies) at 37 °C for 1 h in 96-well plates. The plates were centrifuged at 1,000 g for 10 min and then the supernatants were collected. The released calcein-AM was detected by spectrophotometry using an excitation wavelength of 485 nm and emission wavelength of 535 nm. The percent of tumor cell lysis was calculated relative to SDS lysis buffer. The detailed equation is provided in Supplementary Figure 4. CDC assays with serum depleted of C9 or pooled mouse serum were performed exactly as described above.

PBMC or PMN purification. All in vitro assays were performed under a protocol approved by the UT Austin Institutional Review Board (IRB). Human peripheral blood mononuclear cells (PBMCs) and polymorphonuclear leukocytes (PMNs) were isolated from human blood by flow cytometry using a PE-conjugated goat anti-human IgG F(ab′)2 (identiﬁed above) for 30 min at 37 °C. PMNs were aspirated in the interphase between Histopaque and medium, and PMNs were collected from the pellet. Both human PBMCs and PMNs were resuspended with red-blood-cell lysis buffer (155 mM NH4Cl, 12 mM NaHCO3 and 0.1 mM EDTA) and were washed twice with PBS.

Complement- or FcγR-mediated cellular cytotoxicity assays. For ADCC assays, 5 × 104 human PBMCs or PMNs were mixed with various concentrations of IgG variants and 5 × 104 of calcein–AM–loaded cancer cells. For complement-mediated cellular cytotoxicity assays, 25% of serum depleted of C9 or pooled human serum was added in wells containing PBMCs (or activated PMNs), antibodies and cancer cells. Before use, PMNs were stimulated by GM-CSF (10 ng/ml) for 24 h. After 4 h at 37 °C, the fraction of lysed tumor cells was determined by the same method as CDC assay. For all assays, an E:T ratio of 10:1 was used.

Complement-receptor-inhibition assays. Calcein–AM–loaded cancer cells were pre-incubated with antibodies for 30 min. Antibody-opsonized Raji cells were incubated with effector cells (PBMC or PMN) in RPMI-1640 medium supplemented with 25% serum depleted of C1q. After 4 h at 37 °C, the fraction of lysed tumor cells was determined as described for CDC assays above. For all assays, an E:T ratio of 10:1 was used.

Blockade assays for CR3 or CR4 were also performed. 10 µg/ml of anti-CR3 or anti-CR4 (BioLegend; Cat# 301302 or Cat# 301616) was pre-incubated with effector cells for 30 min. Calcein–AM–loaded and antibody-opsonized CD20+ cancer cells were then incubated with anti-CR3- or anti-CR4-coated effector cells in RPMI-1640 medium supplemented with 25% serum depleted of C9. After 4 h at 37 °C, the fraction of lysed tumor cells was determined as described for CDC assays above. For all assays, an E:T ratio of 10:1 was used.

Phagocytosis assays (ADCP or CDCP). CD14+ monocytes were first isolated from PBMCs by EasySep (STEMCELL Inc). Monocytes were differentiated into M1 macrophages by culture for 7 d in RPMI-1640 medium containing 10% FBS and 100 ng/ml GM-CSF in 5% CO2 incubator. On the day of the assay, adherent macrophages were detached with HyQtase (GE Healthcare). For ADCP assays, 1 × 105 M1 macrophages were mixed with various concentrations of IgG variants and 1 × 104 of calcein–AM–loaded cancer cells. For CDCP assays, 25% serum depleted of C9 was added to wells containing M1 macrophage, antibodies, and cancer cells. After 2 h, M1 macrophages were labeled with anti-human-CD11b-APC (BioLegend; Cat# 301310) and anti-human-CD14-APC (BioLegend; Cat# 301807). Phagocytosis was evaluated by flow cytometry on an LSRFortessa (BD Bioscience), and results are reported as the ratio of cells positive for both calcein–AM and CD11b–CD14 to the total number of tumor cells in the sample.

Fluorescent images of macrophages phagocytosing Raji cells were obtained by confocal microscopy using calcein–AM–loaded Raji cells opsonized with 5 µg/ml of Rituxan, RA801 and RA802 and incubated with serum depleted of C9 and M1 macrophages at 37 °C for 2 h. Approximately 1 × 106 labeled Raji cells and 1 × 106 macrophages were co-incubated in a total volume of 1 ml. Subsequently, the co-incubated cells were labeled with anti-human-CD11b–APC (BioLegend; Cat# 301310) and anti-human-CD14–APC (BioLegend; Cat# 301807). Phagocytosis was visualized by confocal microscopy using a Zeiss LSM 710/Elyra S.1.

TIMING assays. Thin Nanowell arrays were fabricated following an adaptation of previously described protocol62, and a piece 2-cm × 2-cm in size was

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doi:10.1038/ni.3770
cut and fixed on a #1.5 glass-bottomed, 50-mm diameter Petri dish (Ted Pella). The single-cell cytotoxicity assay was performed as described before. In brief, Raji cells (targets) and freshly isolated blood NK cells (effectors) were labeled for 2 min with 1 µM of red PKH26 and green PKH67 (Sigma-Aldrich), respectively. Except for the control in which Raji cells were not opsonized with antibodies, Raji cells were incubated with 1 µg/ml of RA801 or rituximab for 30 min at 4 °C in complete medium containing serum depleted of C9. NK cells and Raji cells were loaded sequentially onto Nanowell arrays at a concentration of 1 × 10^6 cells/ml, allowed to settle for 3 min, and the entire chip was imaged in phenol-red-free complete medium containing 1/60 (vol/vol) annexin V-AlexaFluor-647 (Life Technologies) and, depending on the condition, the antibodies. Images were acquired on an AxioObserver mounted with a 5% CO2, 37 °C and 100% humidity incubator, piloted with Zen software (Carl Zeiss) and fitted with a Hamamatsu EM-CCD camera and a 20× 0.8 NA objective. TIMING images were taken for 6 h at intervals of 5 min, and data were processed and analyzed as described previously. In brief, a semi-automated pipeline of image analysis takes care of image renaming and timelapse stacking, spectral overlap ‘un-mixing’, background subtraction, cropping of images around the wells and detection of cells by binarization of cell fluorescence, tracking of cell along time, and feature computation.

Animal studies. All animal experiments were performed under a protocol approved by UT Austin institutional Animal Care and Use Committee (IACUC). 1 × 10^6 Ramos cells in 100 µl PBS with 50% Matrigel (BD Bioscience) were injected subcutaneously in the right flank of athymic nude mice (Jackson Laboratories, Foxn1 null mice (IACUC). 1 × 10^6 Ramos cells in 100 µl PBS with 50% Matrigel (BD Bioscience) were injected subcutaneously in the right flank of athymic nude mice (Jackson Laboratory). Administration of antibodies (10 mg/kg) or PBS was begun when the tumor area reached to an average of 30 mm^2 and was repeated a week later. For measurement of tumor growth by bioluminescence (IVIS SpectrumCT, PerkinElmer), the antibodies. Images were acquired on an AxioObserver mounted with a 5% CO2, 37 °C and 100% humidity incubator, piloted with Zen software (Carl Zeiss) and fitted with a Hamamatsu EM-CCD camera and a 20× 0.8 NA objective. TIMING images were taken for 6 h at intervals of 5 min, and data were processed and analyzed as described previously. In brief, a semi-automated pipeline of image analysis takes care of image renaming and timelapse stacking, spectral overlap ‘un-mixing’, background subtraction, cropping of images around the wells and detection of cells by binarization of cell fluorescence, tracking of cell along time, and feature computation.

Anaphylaxis assay. Heat aggregated IgG variants, Fab as negative control, mouse Rituximab as positive control and RA801 were heat aggregated by incubation at 63 °C for 1 h. 600 µg of each heat-aggregated antibodies was injected into C57BL/6j mice (The Jackson Laboratory) intravenously. Mouse core body temperature was measured every 5 min using a rectal thermoprobe.

FcγRIII mediated internalization assay. CD20^+FcγRIII^+ Raji, HBL-1 or TMD8 cells were incubated with 10 µg of antibodies for 0, 2, 4 or 6 h in RPMI-1640 medium supplemented with 10% FBS (Invitrogen). The level of cell-surface bound antibodies was detected by goat anti-human Fc with FITC (Abcam; Cat# ab97224; 1:200 dilution).

Data analysis and statistics. Data were post-processed using Excel and GraphPad Prism. Statistical testing was run using Fisher’s exact test when contingency numbers were compared, log-rank test when survival curves were compared, and elsewhere we used analysis of variance and t-tests.

Data availability. All the other data that support the findings of this study are available from the corresponding author upon request. PDB accession codes for the protein structural data are 5V43 for A801-Fc and 5V4E for G801-Fc.

**Corrigendum:** IgG Fc domains that bind C1q but not effector Fcγ receptors delineate the importance of complement-mediated effector functions


*Nat. Immunol.*; doi:10.1038/ni.3770; corrected online 27 June 2017

In the version of this article initially published online, the labels identifying each plot in Figure 1b were missing. The labels are as follows (left to right): CHO, FcγRI, FcγRIIA_{R131}, FcγRIIA_{H131}, FcγRIIb, FcγRIIIa_{F158} and FcγRIIIa_{V158}. Also, the reference cited in the accompanying legend (ref. 21) is incorrect. The correct reference is ref. 14. The errors have been corrected in the print, PDF and HTML versions of this article.