Engineered Fc variant antibodies with enhanced ability to recruit complement and mediate effector functions

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Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ADCP, antibody-dependent cellular phagocytosis; CDC, complement-dependent cytotoxicity; CR, complement receptor; CRP, complement regulatory protein; FcγR, Fc gamma receptor; mAb, monoclonal antibody; MAC, membrane-attack complex; NK, natural killer; PBMC, peripheral blood mononuclear cell; RSV, respiratory syncytial virus; SPR, surface plasmon resonance

Introduction

Monoclonal antibodies (mAbs) are successful as therapeutics due in part to their ability to bring to bear the destructive capabilities of the immune system against specific target cells. In a variety of in vivo and in vitro settings, antibody coating of targets has been shown to mediate potent killing mechanisms such as complement-dependent cytotoxicity (CDC), antibody-dependent cellular phagocytosis (ADCP), and antibody-dependent cellular cytotoxicity (ADCC). All of these effector functions are mediated by the antibody Fc region and over the past decade engineering of this region to enhance the cytotoxic activity of therapeutic antibodies has been a subject of intense investigation.1,2

Modification of antibodies to boost the response of the complement system is appealing due to its contribution to the mechanism of action of one of the most successful anti-cancer mAbs, anti-CD20 rituximab,3 as well as its important role in destroying invading pathogens. For antibody therapeutics, the relevant arm of the complement system is the classical (antibody-dependent) complement cascade, which consists of over twenty tightly-regulated proteins, C1 through C9. The trigger for classical complement activation is the initial binding to antibody-coated target by complement protein C1q, a bundle of six heterotrimeric subunits composed of globular heads and collagen-like tails. For human IgG1, CH2 domain residues D270, K322, P329 and P331 have been implicated as essential to the interaction between human IgG antibodies and C1q.4,5 Efforts have been made to increase the affinity of this interaction, with the goal of concomitantly increasing progression through the classical complement cascade to enhance lysis of the target cells. Several groups have taken advantage of the differences in CDC activity among human IgG isotypes (IgG3 > IgG1 >> IgG2 ≈ IgG46) by swapping segments between isotypes to generate various chimeric IgG molecules with enhanced complement recruitment.7-10 Others have engineered specific amino acid substitutions into either the hinge region11-13 or the CH2 domain.14

The most widely recognized mechanism of complement-mediated target destruction is lysis by the membrane-attack...
complex (MAC), a transmembrane channel created by complexation of C5b, C6, C7, C8 and C9 proteins. This non-cellular process, commonly referred to as CDC, is thought to be relevant to the clinical activity of some anti-tumor antibodies. Less established for antibody drugs, although potentially no less relevant, are cellular-based complement mechanisms that are mediated by interaction between opsonic C3 and C4 components and complement receptors (CR1, CR3 and CR4) expressed on effector cells. Particularly intriguing from the standpoint of viabilities was determined from Alamar Blue fluorescence and half-maximal effective concentration (EC<sub>50</sub>) values of the antibody-dependent cell lysis were calculated (Table 1). The three single substitutions resulted in potency increases of 1.9- to 3.0-fold relative to native IgG1 ocrelizumab. When the single substitutions were combined, potencies further increased, ranging from 3.3-fold to 5.4-fold for double substitution variants and 6.9-fold for the triple. Several variants surpassed the potency of 113F, an IgG1/IgG3 chimera included as a positive control for CDC enhancement. Similar results were observed when targeting the Burkitt’s lymphoma Raji cell line (data not shown).

We next examined the binding of the Fc variants to human C1q using surface plasmon resonance (SPR). Sensorgrams (Fig. 2C) were fit with a two-state binding model (Table 1). Although the fitted K<sub>d</sub> values do not represent the actual K<sub>d</sub> between C1q globular head and Fc, they nonetheless reflect the relative affinity of the C1q multimeric bundle for an opsonized surface. The C1q affinities of the variants showed similar rank order to their CDC potencies, with a correlation coefficient of r<sup>2</sup> = 0.90 (p < 0.0005) (Fig. 2D). Consistent with its 6.9-fold increase in CDC potency, the S267E/H268F/S324T (EFT) variant had the tightest C1q binding affinity as well, an increase of 47-fold over native IgG1 ocrelizumab. When the S267E/H268F/S324T (EFT) variant had the tightest C1q binding affinity as well, an increase of 47-fold over native IgG1 ocrelizumab. When the S267E/H268F/S324T (EFT) variant had the tightest C1q binding affinity as well, an increase of 47-fold over native IgG1 ocrelizumab. When the S267E/H268F/S324T (EFT) variant had the tightest C1q binding affinity as well, an increase of 47-fold over native IgG1 ocrelizumab. When the S267E/H268F/S324T (EFT) variant had the tightest C1q binding affinity as well, an increase of 47-fold over native IgG1 ocrelizumab. When the S267E/H268F/S324T (EFT) variant had the tightest C1q binding affinity as well, an increase of 47-fold over native IgG1 ocrelizumab. When the S267E/H268F/S324T (EFT) variant had the tightest C1q binding affinity as well, an increase of 47-fold over native IgG1 ocrelizumab. When the S267E/H268F/S324T (EFT) variant had the tightest C1q binding affinity as well, an increase of 47-fold over native IgG1 ocrelizumab.
EFT + AE variant was the most potent complement mediator, providing enhancement to CDC EC50 by 23-fold in the context of the anti-CD20 (Table 2). A transform of the data based on cell-surface binding indicated that the variants lowered the CD20 set of four variants, each with four or five substitutions. The EFT triple was chosen as the variant with the greatest CDC activity, while the FT double was of interest as the most potent CDC variant lacking S267E, which was expected from previous observations to impair FcγR-mediated effector function due to decreased FcγRIIIa and increased FcγRIIb affinity. For simplicity we refer to these variant combinations by adding the FcγR substitutions as a suffix to the CDC variants, i.e., FT + DE, FT + AE, EFT + DE and EFT + AE. Combination variants were constructed and expressed in the context of anti-CD20 IgG1 ocrelizumab.

CDC assays of the combination variants confirmed that the enhanced potency conferred by the initial engineering remained upon adding the substitutions (Fig. 3A and B). These results were supported by correlated increases in C1q affinity (data not shown). Unexpectedly, we found that the EFT + AE variant gained an additional 3.3-fold in CDC activity (and, similarly, C1q affinity, data not shown) relative to the EFT triple variant (Table 2). However, this synergy was not observed with the EFT + DE combination (Fig. 3B). Subsequent work identified I332E and S267E as the synergistic pair, but that their synergy was absent in the presence of S239D (data not shown). The five-substitution EFT + AE variant was the most potent complement mediator, providing enhancement to CDC EC50 by 23-fold in the context of the anti-CD20 (Table 2). A transform of the data based on cell-surface binding indicated that the variants lowered the CD20

| ID | Variant | EC50 (nM) | Fold | Kd (nM) | Fold |
|----|---------|-----------|------|---------|------|
| IgG1 | Native IgG1 | 0.33 | 1 | 48 | 1 |
| - | S324T | 0.17 | 1.9 | 19 | 2.9 |
| - | S267E | 0.11 | 3.0 | 26 | 1.8 |
| FT | H268F/S324T | 0.098 | 3.3 | 4.6 | 11 |
| EF | S267E/H268F | 0.092 | 3.6 | 2.8 | 17 |
| ET | S267E/S324T | 0.061 | 5.4 | 1.2 | 41 |
| EFT | S267E/H268F/S324T | 0.048 | 6.9 | 1.0 | 47 |
| - | I332F | 0.089 | 3.7 | 6.9 | 7.1 |

*EC50s were from four-parameter sigmoidal dose-response fits (n = 2). Fold = EC50 (Native IgG1)/EC50 (variant). Kd = Kd1/(1 + 1/Kd2) from a global two-state binding fit of SPR data. Fold = Kd (Native IgG1)/Kd (variant). Antibodies had ocrelizumab variable regions.

Figure 2. Fc engineering generates variant anti-CD20 antibodies with enhanced binding affinity for C1q and enhanced cytotoxicity of CD20 Ramos cells. (A and B) CDC activity of Fc variant anti-CD20 mAbs against opsonized Ramos cells using human complement. Antibody-dependent % lysis was measured at multiple antibody concentrations by Alamar Blue-based detection (mean ± SE of duplicate wells). EC50s are listed in Table 1. (C) SPR sensorgrams for native IgG1 (black) and variant EFT (green) are shown. C1q concentrations range from 100 nM to 6.25 nM by 2-fold serial dilution. (D) Correlation between the fold improvements in C1q affinity as determined by SPR (Table 1) and CDC activity (Table 1, A and B).
The EFT CDC-enhancing variant displayed reduced FcγRIIIa affinity and sharply increased binding to FcγRIIa R131 and FcγRIIb. This binding profile is similar to that observed in previous work with the S267E substitution. Combination with the AE substitutions produced a variant (EFT + AE) with substantially higher FcγRIIa affinity, a greater H131 FcγRIIa:FcγRIIb ratio yet high FcγRIIb affinity, and FcγRIIIa binding slightly better than native IgG1. The clearly superior CDC activity of this combination variant made the EFT + AE variant our sole EFT combination choice for further characterization.

Affinities of the variant anti-CD20 antibodies to activating and inhibitory human FcγRs were examined by SPR (Table 3). The FT double substitution marginally affected FcγR binding if at all, with the most significant perturbation being a slightly lower affinity for FcγRIIb. Addition of the DE and AE substitutions to this variant dramatically improved FcγR binding, resulting in variants with broad enhancement to FcγRs, particularly the isoforms of FcγRIIIa (FT + DE), or selective enhancement for FcγRIIa and FcγRIIIa relative to FcγRIIb (FT + AE). The EFT CDC-enhancing variant displayed reduced FcγRIIa affinity and sharply increased binding to FcγRIIa R131 and FcγRIIb. This binding profile is similar to that observed in previous work with the S267E substitution. Combination with the AE substitutions produced a variant (EFT + AE) with substantially higher FcγRIIa affinity, a greater H131 FcγRIIa:FcγRIIb ratio yet high FcγRIIb affinity, and FcγRIIIa binding slightly better than native IgG1. The clearly superior CDC activity of this combination variant made the EFT + AE variant our sole EFT combination choice for further characterization.
To examine the effect of these FcγR binding profiles on effector recruitment, the anti-CD20 variant antibodies were studied in cell-based ADCC (Fig. 3C and D) and ADCP assays (Fig. 3E and F). These experiments used the Ramos cell line as target cells, and either purified peripheral blood mononuclear cells (PBMCs) or monocyte-derived macrophages as effectors for ADCC and ADCP respectively. For both sets of experiments donor allotypes were determined to be heterozygous for both FcγRIIa (H131/R131) and FcγRIIIa (V158/F158). For ADCC, we and others have previously shown that FcγRIIIa-expressing natural killer cells are the primary effectors.19 For macrophage phagocytosis, we have demonstrated that FcγRIIa is the dominant receptor, with less prominent but still significant contributions from FcγRI and FcγRIIIa. The H268F/S324T variant had similar ADCC activity to native IgG1 and slightly improved ADCP (Fig. 3C and E). Combination with the AE and DE variants resulted in moderate (5.3-fold) and dramatic (22-fold) enhancements in ADCC activity, respectively (Fig. 3C, Table 2) as a consequence of their increased binding to FcγRIIIa (Table 3). The FT + AE and FT + DE variants also showed 4- to 5-fold improvements in macrophage ADCP (Fig. 3E, Table 2), consistent with their greater binding to the activating receptors, particularly FcγRIIa (Table 3). The EFT variant, which has 70% reduced FcγRIIIa affinity (Table 3), mediated lower ADCC activity, both in terms of its potency and efficacy (Fig. 3D, Table 2), and ADCP comparable to native IgG1 (Fig. 3F, Table 2). Addition of the AE substitutions restored ADCC to IgG1 level (Fig. 3D, Table 2). Interestingly, the EFT + AE combination did not enhance phagocytosis (Fig. 3F, Table 2), despite its improved affinity for the activating receptors and particularly strong binding to FcγRIIa. This result may reflect a role of the inhibitory receptor FcγRIIB, which binds tightly to this variant, distinguishing this outcome from observations in previous work.19 Regardless, together the variants provide a range of effector function activities, including dramatically improved complement-mediated yet preserved FcγR-mediated activities (EFT + AE), and simultaneously enhanced CDC, ADCC and ADCP (FT + AE, FT + DE).

Engineering of CDC-enhanced anti-CD19 and -CD40 antibodies. In a final experiment, we examined the transferability of the variants to other antibodies. We tested the substitution combination with the most potent CDC enhancement, EFT + AE, in the context of humanized anti-human CD19 and anti-human CD40 antibodies. Both native IgG1 and CDC-enhanced variants were examined in the CDC assay against Ramos cells as described above. The Fc variant antibodies exhibited improved CDC activity, both in terms of potency and efficacy (Fig. 4), consistent with the anti-CD20 results. Strikingly, the variant anti-CD19 antibody mediated complement activity even when the native IgG1 version was completely lacking, reaching approximately 60% lysis with an EC50 of 3.2 nM. The variant anti-CD40 showed remarkable gains in efficacy (2.5-fold) and potency (620-fold) relative to the native IgG1 version. These results demonstrated that the identified substitutions are not only broadly useful for anti-cancer antibodies, but can confer potent CDC activity even when it is absent or weak in a native IgG1.

Discussion

Over the past decade substantial progress has been made towards improving the cytotoxic potency of mAb drugs. The strongest
motivation for the consideration of complement activity as an optimization goal is its contribution to the mechanism of action of one of the most successful antibody drugs—rituximab anti-CD20. Support includes the dependence of rituximab activity on complement in mouse models, the association between expression of complement regulatory proteins (CRPs) and resistance to rituximab therapy and the consumption of complement upon rituximab treatment in chronic lymphocytic leukemia (CLL) patients. Yet inconsistencies remain, including the absence of observed complement-mediated cytotoxicity in vitro using tumor cells from different response groups and the uncompromised activity of other anti-CD20 mAbs in complement-deficient mice. Complicating the picture further is the recent observation that complement protein C3 can inhibit rituximab-mediated natural killer (NK) cell activation and ADCC, impacting antibody activity in vivo. Yet strong complement-mediated activity is an argued benefit of the next generation anti-CD20 ofatumumab and the relevance of complement to the in vivo activity of mAbs targeting other tumor antigens suggests a broader role for complement in anti-cancer immunotherapy. Beyond oncology, complement intuitively plays a prominent mechanistic role for antibodies that target pathogens, particularly given the activation of complement pathways by, and the absence of CRPs on, microbial surfaces. The capacity of mAbs to destroy microbes using complement mechanisms is well-established by both in vitro and in vivo data. Unfortunately, little clinical data is available concerning the mechanisms of action of anti-pathogenic mAbs, due principally to the low number of such drugs that have progressed through clinical trials. Regardless, although antibodies modified for improved complement have not progressed into clinical development at the same rate as those enhanced for FcγR-mediated effector functions, optimization for complement has enormous potential for improving the next generation of mAbs.

Our objective was to engineer variants that improve Fc engagement to optimize the entire repertoire of cytotoxic effector functions. Our CDC-enhancing modifications, consisting of various combinations of substitutions S267E, H268F and S324T, improve CDC up to 6.9-fold and C1q affinity up to 47-fold in the context of an anti-CD20 antibody. The direct relationship we observed between effector function and the effector ligand, an essential foundation for virtually all Fc engineering efforts, is consistent with previous work by others. The most potent single substitution we identified, S267E, modulates the charge of the C1q domain, similar to other CDC-altering variants such as D270A, K322A, E333A and 113F which includes K274Q and N276K. The Fc-C1q interaction has been shown to have a strong ionic component, potentially mediated through several exposed arginines on C1q subunit B. It is possible that S267E interacts with one of these arginines, a hypothesis that requires further studies to confirm.

Combination of CDC-enhancing substitutions with previously characterized substitutions generated a set of variants that improved affinity to both complement and FcγRs. The binding sites on the Fc region for C1q and FcγR are overlapping, illustrated by the different properties of the FT and EFT combinations. The FT variant possesses not only improved complement activity, but FcγR affinities favorable for ADCC and ADCP. The FcγR-binding properties of the AE and DE variants stacked additionally on top of the complement improvements provided by the FT variant, generating variants (FT + AE and FT + DE) with simultaneously enhanced ADCC (up to 22-fold), ADCP (up to 4.7-fold), and CDC (up to 3.3-fold). In contrast, the S267E substitution acts as a trade-off between CDC activity and FcγR-mediated effector function. The poor ADCC activity of the EFT variant was not only rescued by the AE mutations, but the synergy between S267E and I332E provided an additional boost to complement-mediated effector function, thereby resulting in a variant (EFT + AE) with a native IgG1 level of ADCC activity and 23-fold enhancement in CDC.

An important result in the present study was the capacity of the variants to function not only in other antibodies, but in one case to impart CDC activity onto an antibody that otherwise did not mediate it. Whereas most mAbs mediate ADCC in vitro, fewer seem capable of mediating complement activity (unpublished results). This is likely a contributing factor to the lower emphasis on complement relative to FcγRs for mAb cancer therapy. One possible reason for the high bar for complement activity is that it requires high antibody opsonization density, which is consistent with the fact that pentameric IgM is the most active isotype for complement. The capacity of the EFT + AE variant to impart CDC onto anti-CD19, and moreover the dramatic (>600-fold) enhancement over the poor CDC activity of anti-CD40 are encouraging for the broad applicability of complement-enhancing approaches to antibody drug optimization.

There are multiple cytotoxic mechanisms accessible by the classical complement pathway. These include not only the non-cellular CDC activity mediated by MAC as described here, but a number of cellular activities. Opsonic C3b, iC3b, and C4b proteins on target cells result in two cellular complement activities, both of which are mediated by interaction with complement receptors CR1 (CD35), CR3 (CD11b-CD18) and CR4 (CD11c-CD18) expressed on NK cells, neutrophils, and monocyte phagocytes. CR-dependent phagocytosis and cytotoxicity, also referred to as complement-dependent cellular cytotoxicity (CDC), are analogs of the FcγR-dependent mechanisms of similar name, except that effector function is mediated directly by binding of CR to opsonin. The other mechanism involves enhancement of FcγR-mediated effector functions by the CR/opsonin interaction. CR-enhancement of cellular effector functions is activated by opsonic complement protein C5a, which is not only chemotactic for effector cells, but also selectively increases macrophage expression of activating FcγRs relative to FcγRIIB, providing additional crosstalk between FcγR and complement effector arms.

Two key issues related to these cellular mechanisms include the dependence of CR activation on binding to pathogen-associated molecular patterns (PAMPs), for example cell wall β-glucan, and the negative regulation of complement activation by CRPs, which include both soluble and cell surface...
proteins. While these regulatory mechanisms do not hinder antibodies that target pathogens, they pose barriers for anti-cancer antibodies. Addressing these issues will undoubtedly require a greater understanding of complement biology as it relates to immunotherapy. Yet our hope is that enhanced recruitment of complement, possibly synergistically with improved FcγR engagement, may help overcome these obstacles, analogous to the capacity of high FcγRIIIa affinity to induce activation of NK cells.60,61 Indeed, encouraging the immune system to deal with tumor cells as it would an invading microorganism is in essence the basis of the present strategy, and that of many others, for improving the therapeutic activity of anti-cancer antibodies.

Materials and Methods

Cells and reagents. Burkitt lymphoma Ramos cell line was obtained from DSMZ (German Collection of Microorganisms and Cell Lines). Burkitt’s lymphoma Raji cell line was obtained from American Type Culture Collection. The extracellular domains of these were subcloned into the vector pcDNA3.1Zeo (Invitrogen) with a C-terminal 6xHis tag, transfected into HEK293T cells and purified using nickel affinity chromatography (Qiagen, Valencia, CA). Human Fc gamma receptor protein FcγRI, FcγRIIIa receptor proteins were produced at Xencor. FcγRIIa, FcγRIIb and FcγRIIIa genes were obtained from the Mammalian Gene Collection (American Type Culture Collection). The extracellular domains of these were subcloned into the vector pCNA3.1Zeo (Invitrogen) with a C-terminal 6xHis tag, transfected into HEK293T cells and purified using nickel affinity chromatography (Qiagen, Valencia, CA).

Construction, expression and purification of antibody variants. The variable region VH and VL domains of ocrelizumab (also known as PRO70769 or rhuMab 2H7) anti-human CD20 antibody20 were generated by gene synthesis (Blue Heron Biotechnology, Bothell, WA) and subcloned into the vector pIIT5 (National Research Council, Canada)58 encoding human heavy IgG1 and light Ck constant regions. Substitutions in the Fc domain were introduced using site-directed mutagenesis (QuikChange, Stratagene, Cedar Creek, TX). Positions are numbered according to the EU index.59 The 116F variant positive control for enhanced CDC activity was also constructed,21,63 To serve as an Fc isotype control, engineered as described. 21,63 To serve as an Fc isotype control, the anti-CD19, CD40 and RSV antibodies were constructed by subcloning into the appropriate IgG1 and Cκ pIIT5 vectors from ocrelizumab Fc variants.

Cell-based assays. For CDC assays, target Ramos or Raji cells were washed 2x in RHB Buffer (RPMI Medium 1640 containing 20 mM HEPES, 2 mM glutamine, 0.1% BSA, pH 7.2) by centrifugation and resuspension and seeded at 40,000 cells per well. Native IgG1 or variant antibody was added at the indicated final concentrations. Human serum complement (Qiudel, San Diego, CA) was diluted with RHB buffer and added to opsonized target cells. Final complement concentration was one-eighteenth original stock. Plates were incubated for 2 hr at 37°C, Alamar Blue was added, cells were cultured overnight, and fluorescence was measured in relative fluorescence units. Data were normalized to maximal (Triton X-100) and minimal (complement alone) lysis and fit to a four-parameter sigmoidal dose-response curve using GraphPad Prism (La Jolla, CA).

ADCC was determined by lactate dehydrogenase release as described,19 except that Ramos cells were used as targets (seeded at 10,000 per well) and effector cells were added at a 50:1 PBMC/target cell ratio. Macrophage ADCP was determined by flow cytometry as described,19 except that Ramos cells were used as targets and labeled with CFSE (Guava Technologies, Hayward, CA). Purified PBMCs used in these assays were DNA genotyped for FcγRIIa (position 131) and FcγRIIIa (position 158) using methods by and as a commercial service at Gentris Clinical Genetics (Morrisville, NC).

Surface plasmon resonance determination of binding affinities. SPR measurements were performed in HBS-EP running buffer (10 mM HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% v/v surfactant P20, GE Healthcare) using a Biacore 3000 instrument (GE Healthcare). FcγR affinity was determined as described,19 and the results reported are the average obtained from separate Langmuir fittings of the data from the two independent flow cells of the biosensor chip. For determining Clq affinity, a protein A (Pierce Biotechnology) CM5 biosensor chip (GE Healthcare) was generated using a standard primary amine coupling protocol. The chip’s reference channel was coupled to bovine serum albumin (BSA) to minimize nonspecific binding of Clq. Antibodies at 50 nM were immobilized on the protein A surface for 0.5 or 1 min at 10 µL/min. Clq in 2-fold serial dilutions (starting at 100 or 25 nM, 5 concentrations total) was injected over antibody-bound surface for 3 min at 30 µL/min followed by a 4.5 min dissociation phase. Clq molarity was calculated using the molecular weight of the Clq hexameric bundle, 410 kDa. Response units for Clq association and dissociation never dropped below the RU level of protein A-captured antibody for native IgG1 or any of the variants, suggesting that antibody was not displaced from the protein A chip upon binding to Clq and that protein A and Clq can be bound simultaneously. After each cycle, the surface was regenerated by injecting glycine buffer (10 mM, pH 1.5). In order to subtract nonspecific Clq binding to antibody-coated protein A surface, an Fc variant with greatly ablated CDC activity was included. Sensorgrams were processed by zeroing time and response before the injection of Clq and by subtracting appropriate nonspecific signals
Kinetic parameters were determined by global fitting of association and dissociation phase data with a two-state binding model $(A + B \approx AB \approx AB^*). K_c$ was calculated as $K_{d1}/(1 + K_{d2})$.

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References

1. Dejarlas JR, Lazar GA, Zhukovsky EA, Chu SY. Optimizing engagement of the immune system by anti-tumor antibodies: an engineer's perspective. Drug Discov Today 2007; 12:898-910.
2. Presta LG. Molecular engineering and design of therapeutic antibodies. Curr Opin Immunol 2008; 20:640-70.
3. Zhou X, Hu W, Qin X. The role of complement in the mechanism of action of rituximab for B-cell lymphoma: implications for therapy. Oncology 2008; 13:954-66.
4. Idusogie EE, Presta LG, Gazzano-Santoro H, Torp A, Wong P, Ultsch M, et al. Mapping of the Clq binding site on rituximab, a chimeric antibody with a human Fab and murine Fc. J Immunol 2000; 164:1478-84.
5. Thommesen JE, Michaelen TE, Loet G, Sandlie I, Brekke OH. Lysine 322 in the human IgG3 (C(H)2) domain is crucial for antibody dependent complement activation. Mol Immunol 2000; 37:995-1004.
6. Dang JL, Wensel TG, Morrison SL, Stryer L, Herzenberg LA, Ot VT. Seguelal flexibility and complement fixation of genetically engineered chimeric human, rabbit and mouse antibodies. EMBO J 1988; 7:1989-94.
7. Natsume A, In M, Takahama H, Nakagawa T, Shimizu Y, Kitajima K, et al. Engineered antibodies of IgG1/IgG3 mixed isotype with enhanced cytotoxic activities. Cancer Res 2008; 68:3863-72.
8. Natsume A, Shimizu-Yokoyama Y, Satoh M, Shitara K, Niwa R. Engineered anti-CDC20 antibodies with enhanced complement-activating capacity mediate potent anti-lymphoma activity. Cancer Sci 2009.
9. Senell MG, Kane LM, Morrison SL. Amino acid differences in the N-terminus of Cl(H)2 influence the relative abilities of IgG2 and IgG3 to activate complement. Mol Immunol 1997; 34:1019-29.
10. Xu Y, Oomen R, Klein MH. Residue at position 331 in the IgG1 and IgG4 CH2 domains contribute to their differential ability to bind and activate complement. J Biol Chem 1994; 269:3469-74.
11. Dall’Acqua WF, тож Cook KE, Damschroder MM, Woods DE, et al. Engineered antibodies with increased activity to recruit complement. J Immunol 2006; 177:2517-21.
12. Vugmeyer Y, Beyer J, Howell K, Combs D, Fielder P, Yang J, et al. Depletion of B cells by a humanized anti-CDC20 antibody PRO70769 in Macaca fascicularis. J Immunother 2005; 28:212-9.
13. Izaguirre DA, Desjarlais JR, Jacinto J, Sarki S, Hammond PW. A molecular immunology approach to antibody humanization and functional optimization. Mol Immunol 2007; 44:1986-98.
14. Chu SY, Vostar I, Karki S, Moore GL, Lazar GA, Pong E, et al. Inhibition of B cell receptor-mediated activation of primary human B cells by co-engagement of CDC19 and FcgammaRIIB with Fc-engineered antibodies. Mol Immunol 2008; 45:3926-33.
15. Craig MG, Glennie MJ. Antibody specificity controls in vivo effector mechanisms of anti-CDC20 reagents. Blood 2004; 103:2784-93.
16. Di Gaetano N, Cittera E, Nota R, Vecchi A, Greco V, Scianzani E, et al. Combination activation determines the therapeutic activity of rituximab in vivo. J Immunol 2003; 171:1581-7.
17. Golay J, Cittera E, Di Gaetano N, Mangolini M, Mosca M, Nebuloni M, et al. The role of complement in the therapeutic activity of rituximab in a murine B lymphoma model homing in lymph nodes. Haematologica 2006; 91:176-83.
18. Bannerji R, Iradiana S, Flinn IW, Pearson M, Young D, Reed JC, et al. Complement-regulating and complement protecting protein expression in chronic lymphocytic leukemia: relationship to in vivo rituximab resistance. J Clin Oncol 2003; 21:1466-71.
19. Trefon SE, Mitaides G, Mitiaides N, Young G, Doss D, Schlossman R, et al. Tumor cell expression of CD59 is associated with resistance to CD20 serotherapy in patients with B-cell malignancies. J Immunol 2001; 204:1265-71.
20. Kennedy AD, Beam PV, Solga MD, DiLillo DJ, Lindorfer MA, Hess CE, et al. Rituximab infusion promotes rapid complement depletion and acute CD20 loss in chronic lymphocytic leukemia. J Immunol 2004; 172:3280-8.
21. Wang WK, Levy R. Expression of complement inhibitors CD46, CD55 and CD59 on tumor cells does not predict resistance to rituximab in patients with follicular non-Hodgkin lymphoma. Blood 2001; 98:1352-7.
22. Hamaguchi Y, Uchida J, Chiu DW, Venturi GM, Poe JC, Haas KM, et al. The peritumoral cavity provides a protective niche for B1 and conventional B lymphocytes during anti-CD20 immunotherapy in mice. J Immunol 2005; 174:4389-99.
23. Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM, et al. The innate mononuclear phagocyte network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. J Exp Med 2004; 199:1659-69.
24. Wang SY, Raclca E, Taylor RP, Weiner GJ. NK-cell activation and antibody-dependent cellular cytoxicity induced by rituximab-coated target cells is inhibited by the C3b component of complement. Blood 2008; 111:1456-63.
25. Wang SY, Veeranani S, Raclca E, Caglay J, Fritzinger D, Vogel CW, et al. Depletion of the C3 component of complement enhances the ability of rituximab-coated target cells to activate human NK cells and improves the efficacy of monoclonal antibody therapy in an in vivo model. Blood 2009; 114:5322-30.
26. Glennie MJ, French RR, Cragg MS, Taylor RP. Mechanisms of killing by anti-CD20 monoclonal antibodies. Mol Immunol 2007; 44:3832-37.
27. Pawlukowczyk AW, Beurskens FJ, Beum PV, Lindorfer MA, van de Winkel JG, Parren PW, 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 2009; 183:749-58.
28. Imai M, Landen C, Ohira R, Cheung NK, Tomilson S. Complement-mediated mechanisms in anti-GD2 monoclonal antibody therapy of murine metastatic cancer. Cancer Res 2005; 65:10562-8.
29. Zent CS, Secreto CR, LaPlant TG, Beum PV, Lindorfer MA, van de Winkel JG, Parren PW, 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 2009; 183:749-58.
30. Presto MJ, Gericke AA, Reff ME, Pier GB. Production and characterization of a set of mouse human chimeric immunoglobulin G (IgG) subclass and IgA monoclonal antibodies with identical variable region sequences specific for Pseudomonas aeruginosa serogroup O6 lipopolysaccharide. Infect Immun 1998; 66:4137-42.
31. Kelly-Quintos C, Cavacini LA, Posner MR, Goldmann D, Pier GB. Characterization of the opsonic and protective activity against Staphylococcus aureus of fully human monoclonal antibodies specific for the bacterial surface polysaccharide poly-N-acetylglucosamine. Infect Immun 2006; 74:2742-50.
32. Han Y, Kosei TR, Zhang MX, MacGill RS, Carroll MC, Curlet JE. Complement is essential for protection by an IgM and an IgG1 monoclonal antibody against experimental, hematogenously disseminated candidiasis. J Immunol 2001; 167:1590-7.
33. Wells J, Haidaris CG, Wright TW, Gigliotti F. Complement and Fc function are required for optimal antibody prophylaxis against Pneumocystis carinii pneumonia. Infect Immun 2006; 74:390-3.
34. Baker M. Anti-infective antibodies: finding the path forward. Nat Biotechnol 2006; 24:1491-3.
35. Burton DR, Boyd J, Beamont AD, Easterbrook-Smith SB, Emanuel EJ, Novotny J, et al. The C1q receptor site on immunoglobulin G. Nature 1980; 288:338-44.
44. Gaboriaud C, Juanhuix J, Gruez A, Lacroix M, Darnault C, Pignol D, et al. The crystal structure of the globular head of complement protein C1q provides a basis for its versatile recognition properties. J Biol Chem 2003; 278:46974-82.

45. Kojouharova MS, Gadiya MG, Tsacheva IG, Zlarinova A, Roumenina JT, Tchorbadjieva MI, et al. Mutational analyses of the recombinant globular regions of human C1q A, B and C chains suggest an essential role for arginine and histidine residues in the C1q-IgG interaction. J Immunol 2004; 172:4351-8.

46. Marques G, Anton LC, Barrio E, Sanchez A, Ruiz S, Gavilanes F, et al. Arginine residues of the globular regions of human C1q involved in the interaction with immunoglobulin G. J Biol Chem 1993; 268:10393-402.

47. Dechant M, Weisner W, Berger S, Peipp M, Beyer T, Schneider-Meck T, et al. Complement-dependent tumor cell lysis triggered by combinations of epidermal growth factor receptor antibodies. Cancer Res 2008; 68:4998-5003.

48. Macor P, Tedesco F. Complement as effector system in cancer immunotherapy. Immunol Lett 2007; 111:6-13.

49. Spittidon CI, Ghiette MA, Uhr J, Marches R, Li J, Shen GL, et al. Targeting multiple Her-2 epitopes with monoclonal antibodies results in improved antitumor activity of a human breast cancer cell line in vitro and in vivo. Clin Cancer Res 2002; 8:1720-30.

50. Konrad S, Baumann U, Schmidt RE, Gesner JE. Intravesicular immunogold Del-goldated―mediated neutralization of C5a: a direct mechanism of IVIG in the maintenance of a high Fc gammaRIII expression ratio on macrophages. Br J Haematol 2006; 134:345-7.

51. Gausch P. Complement: a unique innate immune sensor for danger signals. Mol Immunol 2004; 41:1089-98.