Clustering: a rational design principle for potentiated antibody therapeutics

Oostindie, S.C.

4

POTENT PRECLINICAL EFFICACY OF DUOHEXABODY-CD37 IN B-CELL MALIGNANCIES

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TO THE EDITOR,

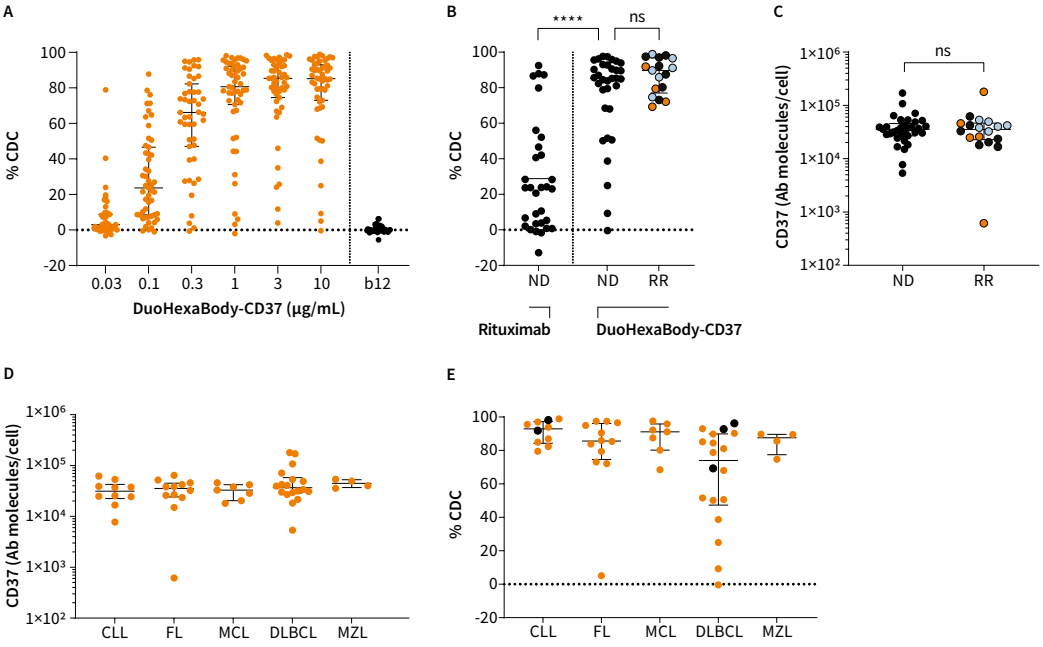
Over the last decades, several surface antigens have been identified and validated for treatment of B-cell malignancies. CD20-targeting antibodies have emerged as an effective therapy for patients with B-cell malignancies and are now broadly used in clinical practice. Nonetheless, many patients develop resistance against CD20-targeting therapies, with little or no further treatment options.[1] Tetraspanin CD37 is expressed almost exclusively on hematopoietic cells with high expression on mature B-cells, including their malignant counterparts,[2,3] and is a well described and validated target for B-cell malignancies. A number of CD37 targeting agents are in (pre)clinical development, including antibody-drug conjugates (IMGN529 and AGS67E), an Fc-engineered antibody (BI836826), a homodimeric therapeutic protein (otlertuzumab/TRU-016), a radioimmunoconjugate (^{177}Lu-lilotomab), and chimeric antigen receptor T-cells.[4-6] We recently reported on the development of DuoHexaBody-CD37, a novel biparatopic CD37-bispecific immunoglobulin G1 (IgG1) antibody with an E430G hexamerization-enhancing single point mutation in the Fc-domain.[7] In contrast to all other newly developed CD37 targeting agents, DuoHexaBody-CD37 mediates potent complement-dependent cytotoxicity (CDC), a powerful anti-tumor effector mechanism,[8,9] in addition to its Fc gamma receptor (FcγR)-mediated tumor cell kill mechanisms including antibody-dependent cellular cytotoxicity and cellular phagocytosis. DuoHexaBody-CD37 was designed based on recent discoveries that (1) initiation of CDC is dependent on Fc-mediated hexamer-formation of IgG1 antibodies after target engagement on the cell surface,[10] (2) antibody Fc–Fc interactions and hexamer formation can significantly be improved by introducing an E430G single point mutation in the Fc domain,[10,11] and (3) dual-epitope targeting of CD37 can potentiate or further enhance CDC.[7]

Here, we investigated the ex vivo therapeutic potential of DuoHexaBody-CD37 by evaluating its unique CDC-inducing capacity in primary tumor cell samples from a large cohort of newly diagnosed (ND) and relapsed/refractory (RR) patients with a broad range of B-cell malignancies, including chronic lymphocytic leukemia (CLL) and B-cell non–Hodgkin lymphoma, including diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), mantle cell lymphoma, and marginal zone lymphoma (Supplementary Material and Methods). DuoHexaBody-CD37 induced potent, dose-dependent CDC of malignant primary B-cells in 45-minute complement assays with a median maximal lysis of 86% (n = 51, range: 0%-99%) and a median half maximal effective concentration (EC_{50}) of 0.10 µg/mL (range: 0.004-15.21 µg/mL) (Figure 1A). Strikingly, sensitivity of the patient samples to DuoHexaBodyCD37-induced

CDC was highly homogeneous. Only in 8 of 51 patient samples maximal lysis was lower than 60%, of which 3 samples demonstrated responses below 20% lysis. The CDC activity of DuoHexaBody-CD37 was comparable in samples from ND patients (n = 33; median maximal lysis of 85%; range CDC 0%-98%) and RR patients (n = 18; median maximal lysis of 89%; range CDC 5%-99%). Furthermore, DuoHexaBody-CD37 was significantly more potent than the CD20-targeting antibody rituximab in samples from ND patients, that is, CD20 antibody treatment naive patients (Figure 1B), even though CD37 expression was generally lower than expression of CD20 (Supplementary Figure 1).

Potent cytotoxicity was also observed in samples from patients who relapsed from (Figure 1B, blue symbols; n = 7) or were refractory to treatment regimens containing CD20- targeted antibodies (defined by progression of the disease within 6 months post treatment; Figure 1B, red symbols; n = 5), with the exception of one CD20-refractory FL patient. CD37 expression analysis (Figure 1C) revealed that this patient lacked CD37 expression on tumor B-cells. Since it has been reported that anti-CD20 therapy may induce CD20 antigen loss,[12] and CD20 colocalizes with CD37 on the cell surface,[13] we evaluated the possibility that CD37 expression levels were reduced on tumor B-cells derived from patients who had been exposed to anti-CD20. However, all anti-CD20–treated patient samples in our cohort showed high CD37 expression levels and no significant differences were observed in CD37 expression levels between samples from ND and RR patients (Figure 1C). In addition, CD37 was expressed homogeneously in all B-cell malignancy subtypes (Figure 1D), which is in alignment with several other reports2,6 except for a recent study that suggested variable CD37 expression in DLBCL.[14] This study-related discrepancy could be due to differences in assay-dependent detection limits in our study (flow cytometry) versus the other (immunohistochemistry) study. DuoHexaBody-CD37 induced an effective and homogeneous CDC response in all samples from CLL (n = 10; median max lysis 93%; range, 80%-99%), mantle cell lymphoma (n = 7; 91%; 69%-98%) and marginal zone lymphoma (n=4; 88%; 75%-90%), and 11 of 12 FL patient samples (n=12; 86%; 5%-98%), while a more heterogeneous response was observed in DLBCL patient samples (n = 18; 74%; 0%-96%) (Figure 1E) (Kruskal-Wallis one-way analysis of variance (ANOVA), $*P = 0.0321$). There were no differences in sensitivity between activated B-cell and germinal center B-cell subtypes of DLBCL (Supplementary Figure 2). Of note, also samples from ibrutinib-refractory CLL (n = 2) and double-hit DLBCL (n = 3) patients with poor prognosis were highly susceptible to DuoHexaBodyCD37–induced CDC (maximum lysis >60%) (Figure 1E).

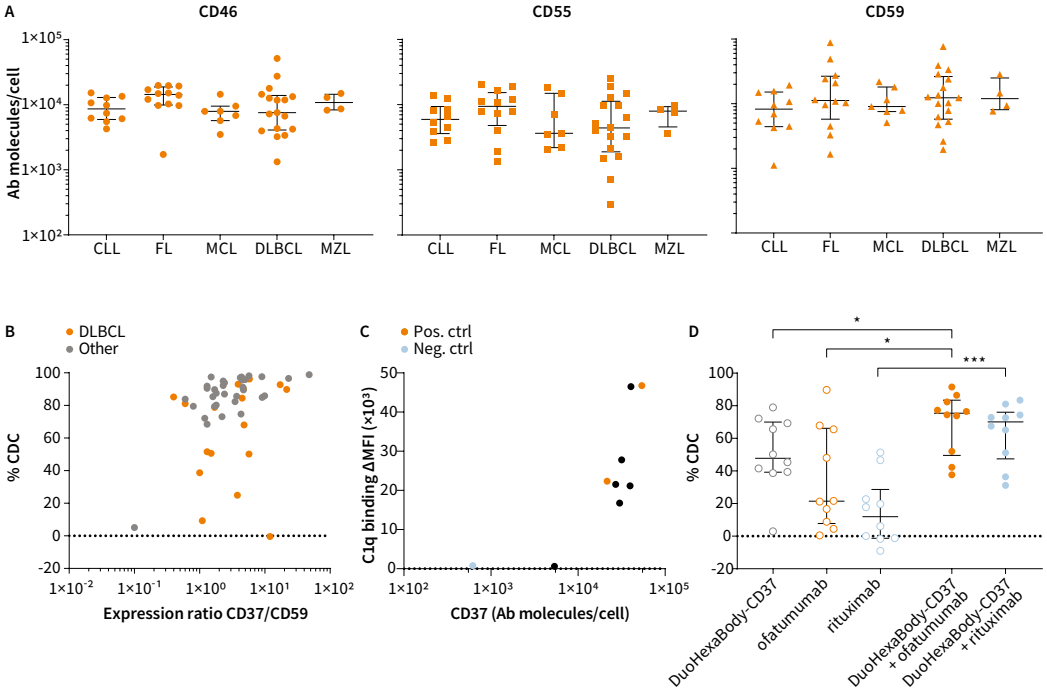
Complement activation and CDC-mediated tumor cell lysis is not only dependent on antigen density but also on expression levels of complement regulatory proteins (CRPs) CD46 and CD55 that inhibit complement convertases, and



▲ **Figure 1**

DuoHexaBody-CD37 induced potent CDC in samples obtained from ND and RR patients with various B-cell malignancies. (**A**) Dose-dependent CDC induced by DuoHexaBody-CD37 in tumor B-cells (n = 51) derived from patients with various B-cell malignancy subtypes grouped together, in the presence of 20% NHS and in comparison to IgG1-ctrl (10 µg/mL). Levels of CDC-mediated tumor cell kill (% CDC) were determined by 7AAD-positive tumor cell staining, relative to a no antibody control sample. (**B**) Comparison of CDC activity of 10 µg/mL DuoHexaBody-CD37 in samples from ND (n = 33) and RR (n = 18) patients (ns; Mann-Whitney U test), and comparison of rituximab (10 µg/mL) and DuoHexaBody-CD37 in ND patients (****P < 0.0001; Wilcoxon matched-pairs signed rank test). RR patients include CD20 therapy-refractory patients (n=5; orange symbols), defined by progression of the disease within 6 months post therapy, and CD20 therapy-relapse patients (n=6; blue symbols). (**C**) CD37 expression levels (defined as the number of CD37 molecules on the cell surface, assessed by quantitative flow cytometry), on tumor B-cells in ND and RR patient samples (ns; Mann-Whitney U test). (**D**) Quantified CD37 expression levels (antibody molecules per cell) on tumor B-cells stratified according to B-cell malignancy subtype (ns, nonparametric Kruskal-Wallis test). All data are shown as the median and interquartile range. (**E**) CDC activity of DuoHexaBody-CD37 (10 µg/mL) stratified according to B-cell malignancy subtype, including CLL (n = 10), FL (n = 12), MCL (n = 7), DLBCL (n = 18), and MZL (n = 4) (*P = 0.0321, nonparametric Kruskal-Wallis test with Dunn's multiple comparisons [n]). Black symbols indicate patient samples with poor prognosis: ibrutinib-refractory CLL (n = 2) and double-hit DLBCL (n =3).

CD59 that inhibits the formation of the membrane attack complex.[15] Expression of CRPs was not associated with B-cell malignancy subtype (Figure 2A) or treatment status (Supplementary Figure 3). Furthermore, no correlation was observed between DuoHexaBody-CD37–induced CDC and expression of CD37 or CRPs (Supplementary Figure 4). A weak, but statistically significant correlation was observed between sensitivity to DuoHexaBody-CD37 and the ratio of CD37 and CD59 expression levels (Figure 2B). However, in DLBCL patient samples, differences in (the ratio of) CD37 and CD59 expression could not explain the heterogeneous response to DuoHexaBody-CD37 ex vivo (Figure 2B,



orange symbols). We therefore conclude that there is a limited impact of CRP expression on DuoHexaBody-CD37–mediated CDC. This was in contrast to rituximab-induced CDC, which already showed a strong correlation with CD20 expression but an even stronger correlation with the ratio of CD20 and CD55 (Supplementary Figure 5).

The first step of complement activation is the binding of C1q to membrane-bound antibodies that together with C1r and C1s forms the C1 complex, the first component of the classical complement pathway. Toward understanding the heterogeneity in CDC responses observed in DLBCL patient samples, we investigated the C1q binding capacity of membrane-bound DuoHexaBody-CD37 in DLBCL patient samples for which DuoHexaBody-CD37–mediated CDC levels were lower than 60% (N = 6). The C1q binding capacity in low CDC responders was comparable to that of 2 patient samples highly susceptible to DuoHexaBody-CD37–induced CDC (Figure 2C), indicating that the first step of complement activation is not impaired. Whether other steps in the complement activation pathway, such as membrane attack complex assembly and stability, or cell intrinsic mechanisms, play a role in resistance to CDC induction remains to be elucidated. Finally, we also evaluated whether combination with rituximab and/or ofatumumab could further enhance the CDC activity of DuoHexaBody-CD37, as we have previously reported enhanced CDC

◀ **Figure 2**

Low sensitivity to DuoHexaBody-CD37–mediated CDC is not associated with expression of CD37 and CRPs and can be improved by combination with CD20 mAbs.

(**A**) Quantified expression levels (antibody molecules per cell) of complement regulatory proteins CD46, CD55, and CD59 on tumor B-cells in specific B-cell malignancy subtypes; CLL (n = 10), FL (n = 12), MCL (n = 7), DLBCL (n = 18), and MZL (n = 4) (ns; nonparametric Kruskal-Wallis test). Data shown are CDC in individual patient samples, in addition to the median and interquartile range. (**B**) CDC activity of DuoHexaBody-CD37 (10 μg/mL) correlated with the ratio of CD37/CD59 expression levels for all B-cell malignancy subtypes grouped together, for DLBCL patient samples specifically and for B-cell malignancy subtypes other than DLBCL (Spearman's correlation r = 0.4423, $^{**}P = 0.0013$; r = 0.2843, $P = 0.2678$; and r = 0.5158, $^{**}P = 0.0025$, respectively). Data are shown relative to a no antibody control sample. (**C**) Expression levels of CD37 (antibody molecules per cell) correlated with C1q binding (ΔMFI) for six samples with low CDC response (CDC < 60%), one CD37-negative sample as negative control (blue symbol) and two high responding samples as positive control (orange symbol) (Spearman's correlation r = 0.7833, $^{*}P = 0.0172$). (**D**) CDC induced by a combination of DuoHexaBody-CD37 and rituximab or ofatumumab (10 μg/mL + 10 μg/mL) versus single antibody (10 μg/mL) (n = 10) ($^{*}P < 0.05$, $^{***}P < 0.001$; Friedman test with Dunn's multiple comparisons test) relative to a no antibody control sample. All data are shown as the median and interquartile range.

in CLL and B-cell non–Hodgkin lymphoma primary patient cells with combinations of CD20 and CD37 antibodies.[13] Indeed, in 10 patient samples including samples with intermediate to low sensitivity to DuoHexabody-CD37-mediated CDC (<80% CDC), the combination of DuoHexaBody-CD37 with rituximab and ofatumumab significantly enhanced the tumor cell kill in an additive manner (Figure 2D). In contrast to ofatumumab, rituximab as single antibody could generally not induce CDC in these samples and the CDC-effects of the combination with DuoHexaBody-CD37 are less striking.

In conclusion, this preclinical study indicates high therapeutic potential for DuoHexaBody-CD37 in a broad spectrum of B-cell malignancies either as single agent or in combination with CD20-targeting antibodies, and supports the recently initiated first-in-human clinical trial of DuoHexaBody-CD37 for patients with relapsed or refractory B-cell NHL (NCT04358458).

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MATERIALS AND METHODS

Therapeutic Antibodies

DuoHexaBody-CD37 and the negative control anti-HIV-1 gp120 antibody IgG1-b12 (mentioned in manuscript as IgG1-ctrl) were generated by Genmab (Utrecht, The Netherlands) as previously described.[7] Rituximab (MabThera®) and ofatumumab (Arzerra®) were obtained from Genmab.

Patient samples

According to the Dutch Central Committee on Research involving Human Subjects (CCMO), this type of study does not require approval from an ethics committee. Primary patient samples wereh collected at Amsterdam University Medical Center, location VUmc, according to the *code of conduct for medical research*. Tissues from lymph node biopsies were mechanically disrupted, cultured overnight in a fully humidified incubator and the cells in suspension were collected and cryopreserved in liquid nitrogen until further use. For seven CLL samples and one MCL sample tumor cells were isolated from mononuclear cells of the bone marrow (BMNCs) or peripheral blood (PBMCs) by density-gradient centrifugation (Ficoll-Paque PLUS, Cytiva, MA, USA) according to manufacturer's instructions, and were either used directly or cryopreserved in liquid nitrogen until further use.

Tumor-specific surface markers

B cells were identified using flow cytometry by gating for $CD3^{-}CD19^{+}$ cells within the $CD45^{+}$ population and made up 20-80% of the total lymph node cell population. Malignant B cells were identified by kappa/lambda staining to screen for clonality and if possible also by the tumor-specific markers $CD10^{+}$ (FL and DLBCL), $CD5^{+}$ (CLL), $CD5^{+}CD23^{-}$ (MCL). The following antibodies were used: CD45-KO and CD19-PC7 (Beckman Coulter, CA, USA), CD3-V450, CD5-APC, CD10-APC-H7 and kappa-APC-H7 (BD, NJ, USA), CD5-PE, CD10-PE and kappa-PE (DAKO, Agilent Technologies, CA, USA), CD23-FITC (BioLegend, CA, USA) and lambda-FITC (Emelca Bioscience, Clinge, Netherlands).

Quantitated expression assays

Target cells were incubated with purified mouse anti-human IgG1 antibodies against CD37, CD46, CD55, CD59 and CD20 (BioLegend) and expression levels were quantified by flow cytometry using an indirect immunofluorescence assay (QIFIKIT®, Agilent Technologies) according to manufacturers protocol, followed by a staining for tumor specific cell surface markers.

Complement-dependent cytotoxicity assays

Complement-dependent cytotoxicity assays were performed by incubation of single cell suspensions ($1x10^5$ cells/well) with serial dilutions (0.01-10 µg/mL) of DuoHexaBody-CD37 for 45 minutes at 37°C in the presence of 20% normal human serum (NHS; pooled from eight healthy donors) as a source of complement. Cells were analysed by flow cytometry and viability (%) and lysis (%) of malignant B cells was determined according to the following formulas:

$$\%\ \text{viability} = 100 * \left(\frac{\%\ \text{7AAD} - \text{negative cells of test sample}}{\%\ \text{7AAD} - \text{negative cells of control sample}} \right)$$

$$\%\ \text{lysis} = (100 - \%\ \text{viability})$$

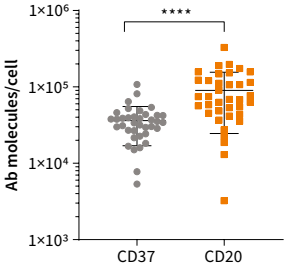
C1q binding assays

Target cells were incubated with DuoHexaBody-CD37 (10 µg/mL) and purified human complement component C1q (2.5 µg/mL; Quidel, San Diego, CA) for 45 min at 37°C. After washing, purified C1q was labeled with a fluorescein isothiocyanate (FITC)-conjugated rabbit anti-human C1q antibody (Dako, Glostrup, Denmark). Binding was detected by flow cytometry and expressed as the median fluorescent intensity (MFI). MFI values of purified C1q labeled with FITC anti-human C1q were subtracted by MFI values of negative control (FITC-anti-human C1q only).

Statistical analysis

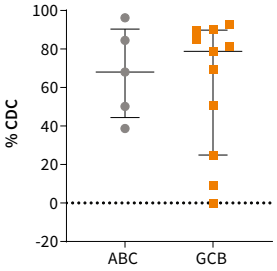
Flow cytometry data were analyzed using FACS DIVA software. Graphs were plotted using GraphPad Prism 8.2. for Windows. Statistical analysis was performed with the appropriate parametric or non-parametric test, as indicated in the figure legends. Two-sided *P* values < .05 were considered statistically significant.

SUPPLEMENTARY FIGURES



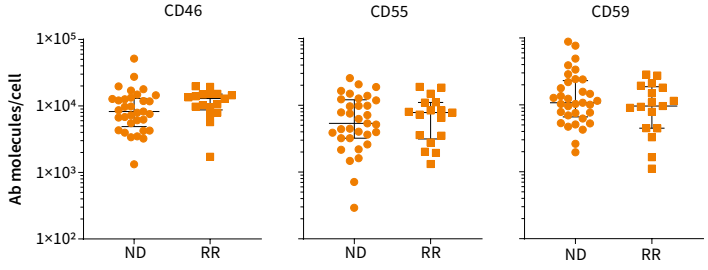
▲ **Supplementary Figure 1**

Expression levels (antibody molecules per cell) of CD37 and CD20 on tumor B cells in samples from ND patients (****$P < 0.0001$; Wilcoxon matched-pairs signed rank test). Data are shown as the median and interquartile range.



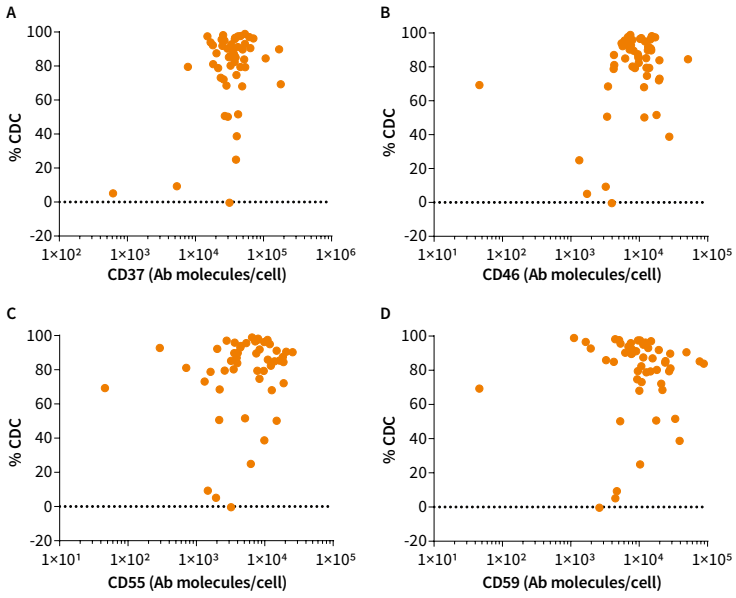
▲ **Supplementary Figure 2**

CDC activity of DuoHexaBody-CD37 (10 µg/mL) in DLBCL patient samples of ABC (n=5) and GCB subtype (n=11) (ns; Mann-Whitney U-test). Data are shown as the median and interquartile range.



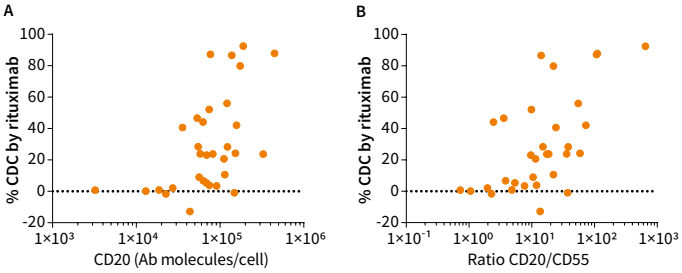
▲ **Supplementary Figure 3**

Expression levels (antibody molecules per cell) of complement regulatory proteins CD46, CD55 and CD59 on tumor B cells, according to treatment status; ND versus RR (ns; Mann-Whitney U test). Data are shown as the median and interquartile range.



▲ **Supplementary Figure 4**

CDC activity of DuoHexaBody-CD37 (10 µg/mL) correlated with quantified expression levels (antibody molecules per cell) of (**A**) CD37 and (**B-D**) complement regulatory proteins CD46, CD55 and CD59 (Spearman's correlation r = 0.1408, *P* = 0.3243; r = 0.1929, *P* = 0.1751; r = 0.1901, *P* = 0.1814; r = -0.2024, *P* = 0.1542, respectively). Data are shown relative to a no antibody control sample.



▲ **Supplementary Figure 5**

CDC activity of rituximab (10 µg/mL) correlated with (**A**) quantified CD20 expression (antibody molecules per cell; Spearman's correlation r=0.5488, **p=0.0011) and (**B**) the ratio of expression levels of CD20 to complement regulatory protein CD55 (Spearman's correlation r=0.5957, ***p=0.0003). Data are shown relative to a no antibody control sample.