Effector mechanisms of IgA antibodies against CD20 include recruitment of myeloid cells for antibody-dependent cell-mediated cytotoxicity (ADCC), and complement dependent cytotoxicity (CDC)

Summary:

ADCC and CDC are recognised as important effector mechanisms for therapeutic IgG1 antibodies. Here, we compared IgG1 with IgA2 isotype variants of the type I CD20 antibody 1F5. Interestingly, IgA2 and IgG1 molecules were similarly effective in depleting syngeneic B cells *in vivo*, and did not differ in their induction of direct cell death *in vitro*. Importantly, IgA2 but not IgG1 effectively triggered ADCC by human PMN. Furthermore, IgA antibodies against CD20 mediated unexpectedly effective CDC. These results suggest that immunotherapy with CD20 antibodies of IgA isotype may represent a promising approach for B cell lymphoma patients.

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The CD20 antibody rituximab has significantly improved the prognosis of lymphoma patients - stimulating intensive research into follow-up antibodies (Maloney 2012). Single nucleotide polymorphism (SNP) analyses of Fcγ receptors and studies in genetically modified mice suggested that ADCC by myeloid effector cells significantly contributes to rituximab's therapeutic efficacy, while CDC was more important in other models (Weiner 2010). While Fc-mediated effector mechanisms of monoclonal CD20 antibodies were long known to be critically affected by their isotype, recent studies demonstrated that this can also occur for F(ab)-mediated direct cell death induction (Könitzer, *et al* 2015). All currently approved and developed CD20 antibodies contain human IgG1 constant regions, which are able to induce CDC by classical C1q binding, and ADCC by recruiting NK cells via FcγRIIIa. However, the IgG1 Fc part of CD20 antibodies is also involved in trogocytosis (membrane exchange

between tumour target and immune effector cells), and in FcγRIIb-mediated CD20 internalisation – two potential mechanisms which may reduce CD20 surface expression and thereby limit both ADCC and CDC efficacy of CD20 antibodies (Taylor and Lindorfer 2015). Bispecific antibodies simultaneously targeting CD20 and the myeloid receptor for IgA (FcαRI; CD89) demonstrated that PMN can effectively kill lymphoma cells by targeting CD20 (Stockmeyer, *et al* 2000). Subsequently, a CD20 antibody of human IgA isotype demonstrated *in vitro* and *in vivo* efficacy against lymphoma cells, but the mode of action of this antibody has not been fully characterised (Pascal, *et al* 2012). IgA antibodies constitute an important part of the mucosal immune system and differ from IgG in structure and function. Meanwhile, IgA antibodies against solid tumour target antigens were shown to effectively recruit myeloid effector cells for antibody-based tumour immunotherapy (Boross, *et al* 2013, Lohse, *et al* 2016). Thus, the aim of the present study was to investigate the MoA of a CD20-directed IgA2 antibody in more detail and to compare it with its respective IgG1 variant.

Human IgA2 and IgG1 variants of the CD20 antibody 1F5 were produced in stably transfected BHK cells. Antibodies were purified using affinity and size exclusion chromatography – resulting in monomeric IgA antibodies which were biochemically analysed (Fig S1A+B). Functional characterisation of the purified proteins demonstrated the expected FcαRI and FcγRIII binding (Figure S1C). Similar target antigen binding of 1F5-IgA2 and 1F5-IgG1 was then confirmed by indirect immunofluorescence analyses on human CD20-transfected CHO cells (Fig 1A). Next, the ability of both isotypes to deplete human CD20 transgenic (hCD20 tg), CFSE-labelled B cells from the spleen of Balb/c mice was assessed in a previously described model (Beers, *et al* 2008). Interestingly, both 1F5-IgA2 and 1F5-IgG1 were similarly effective *in vivo* (Fig 1B), although mice do not express a functional orthologue of human FcαRI. Analysing Fab-mediated effector functions revealed no relevant differences between both isotypes in direct cell death induction measured by Annexin V or 7-

AAD staining, or in homotypic aggregation (Fig S2). Next, effector cell recruitment by CD20-directed IgG1 and IgA2 antibodies was investigated in ^{51}chromium-release assays using either Ramos or freshly isolated primary B-CLL cells as targets, and stimulated PMN or MNC effector cells (Fig 1C-F). Interestingly, with PMN 1F5-IgA2 was more effective than 1F5-IgG1 against both types of target cells, while IgG1 demonstrated the expected efficacy with MNC, but was ineffective with PMN. With isolated monocytes or macrophages both isotypes mediated similar ADCC activity (Fig S3A+B). The activity of 1F5-IgA2 to recruit PMN for ADCC was confirmed against a panel of malignant B cell lines expressing different levels of CD20 (Fig S3C+D).

Classical complement activation was not expected for IgA antibodies, since IgA does not contain a C1q binding site and did not bind C1q in sandwich-ELISA, in contrast to IgG1 (Fig 2A). However, when 1F5-IgG1 and 1F5-IgA2 were compared in CDC assays against a panel of malignant B cell lines or against freshly isolated B-CLL cells, both isotypes were similarly effective (Fig 2B-D). When CDC was analysed under different conditions, IgG1 mediated CDC was significantly faster, required lower serum concentrations, was less sensitive to EGTA inhibition, and was more resistant to heat inactivation than CDC triggered by IgA2 (Fig 2E – H). Interestingly, the *in vivo* activity of both 1F5-IgA2 and -IgG1 in depleting hCD20 tg B cells from the spleen was not abrogated in C1q- or C3- deficient mice (Fig 2I, J) – supporting that CDC is not the predominant mechanism of action for CD20 antibodies in this model (Beers, *et al* 2008).

Together, our results demonstrate that both IgG1 and IgA2 isotypes of the type I CD20 antibody 1F5 were effective in killing B cells *in vitro* and in depleting syngeneic B cells *in vivo*. Interestingly, both isotypes were similarly effective in triggering direct cell death, but differed in Fc-mediated killing mechanisms. Thus, IgA2 and IgG1 differed in the types of effector cells they recruited (MNC vs. PMN), and in the conditions under which they activated the complement pathway. Recent studies demonstrated that different CD20 antibody

constructs, which cannot bind C1q themselves, recruited the surface immunoglobulin of B cells for C1q binding (Engelberts, *et al* 2016) - a mechanism which could also explain CDC by IgA antibodies.

The limited knowledge about the relevance of individual effector mechanisms of CD20 antibodies in humans (Weiner 2010) relates to the most important limitation of our study: we currently cannot propose clinical situations in which IgA2 is expected to be more effective than IgG1. Furthermore, we have excluded complement as the predominant effector mechanism in our particular *in vivo* model, but have not yet elucidated which mechanism led to the excellent therapeutic efficacy *in vivo.* These issues are subject of ongoing studies, which may pave the way for particular applications of IgA antibodies.

In summary, the presented data demonstrates the efficacy of an IgA2 antibody against CD20 in mediating killing of CD20-positive lymphoma cells *in vitro* and *in vivo*. Thus, CD20 antibodies of IgA isotype may further broaden the immunotherapeutic armamentarium for lymphoma patients.

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Conflict of interest: none
Running title: Effector mechanisms of a CD20-directed IgA2 antibody
Keywords: CD20, antibody therapy, ADCC, myeloid effector cells, IgA antibody

Authors' contribution:

Stefan Lohse, Sebastian Loew, Anna Kretschmer, J. H. Marco Jansen, Saskia Meyer and Toine ten Broeke performed *in vitro* experiments, collected and provided data. Mark S. Cragg, Ruth R. French and Thomas R. W. Tipton conducted the animal experiments and provided the data. Thies Rösner, Stefanie Derer, Katja Klausz, Ralf Schwanbeck, Denis M. Schewe, Christian Kellner and Matthias Peipp helped analysing and interpreting data. Stefan Lohse, Thomas Valerius, Jeanette H. W. Leusen, Matthias Peipp and Michael Dechant developed the concept of the project. Stefan Lohse, Anna Kretschmer and Thomas Valerius wrote the paper, which was approved by all authors.

Supporting information:

Fig S1. Production and biochemical characterisation of CD20 antibodies.
Fig S2. Fab-mediated effector mechanisms by CD20 antibodies.
Fig S3. Monocytes and macrophages as effector cells.

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Figure 1

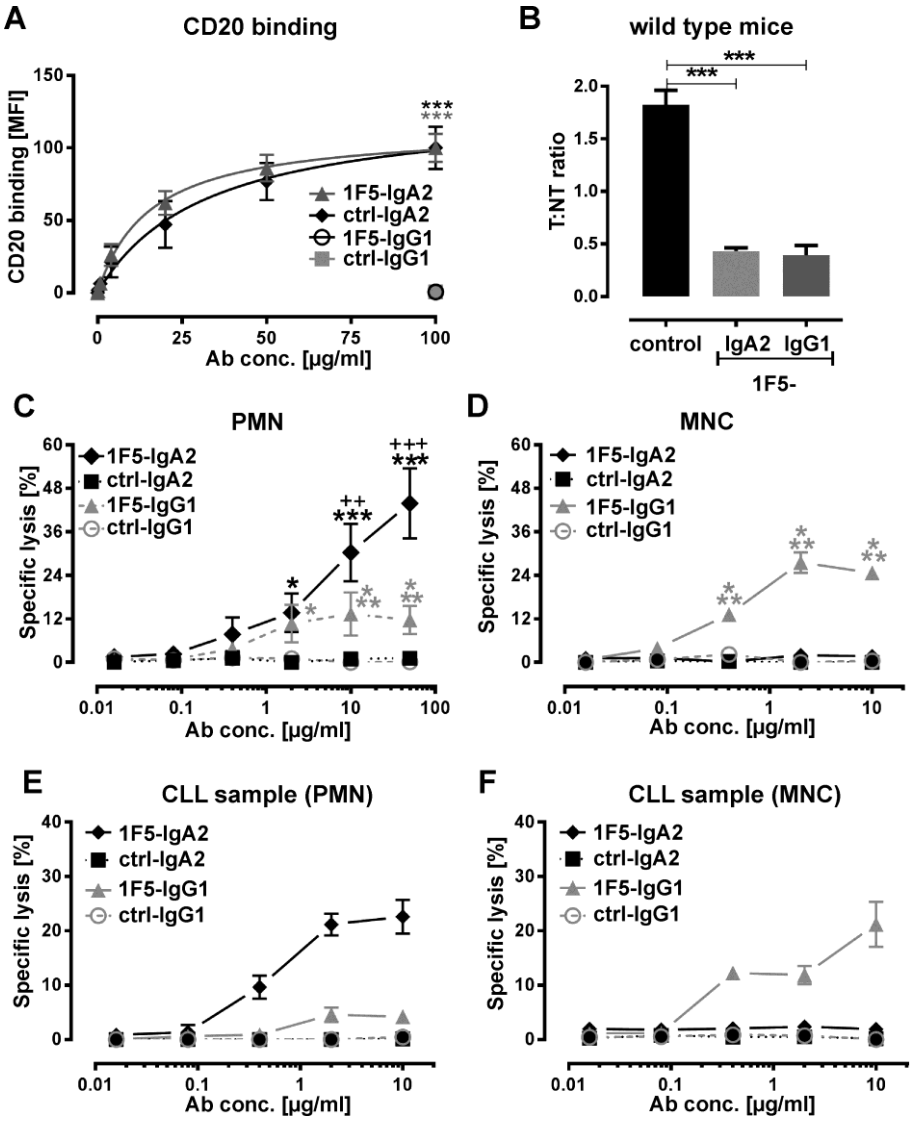

Fig 1. (A) Binding of 1F5-IgG1 or -IgA2 and respective control antibodies to human CD20 transfected CHO-K1 cells was analysed by indirect immunofluorescence using a FITC-labelled human κ-light chain antibody. (B) *In vivo* efficacy of 1F5-IgA2 and 1F5–IgG1 was analysed as described in Beers, *et al* 2008. Spleen suspensions were stained with APC-labelled CD19 antibody, and samples were analysed by flow cytometry to determine the ratio of target to non-target CFSE-labelled cells. Results are presented as mean ± SEM of "T:NT ratio". The capacity of increasing concentrations of 1F5-IgA2 or 1F5-IgG1 to mediate killing of Ramos (C, D), or freshly isolated B-CLL cells (E, F) by isolated PMN (C, E), or MNC (D, F) was analysed in ^{51}chromium-release assays. Results of at least three independent experiments are displayed as mean ± SEM of "mean fluorescence intensity" (A), or "specific lysis [%]" (C, D), or from triplicates (E, F). Significant differences between specific and

control antibodies ($p < 0.01$) are indicated by *** in (A). (B-F) Significant differences ($p \leq 0.001$) between CD20-directed and control antibodies are indicated by *, between 1F5-IgA2 and 1F5-IgG1 by +.

Figure 2

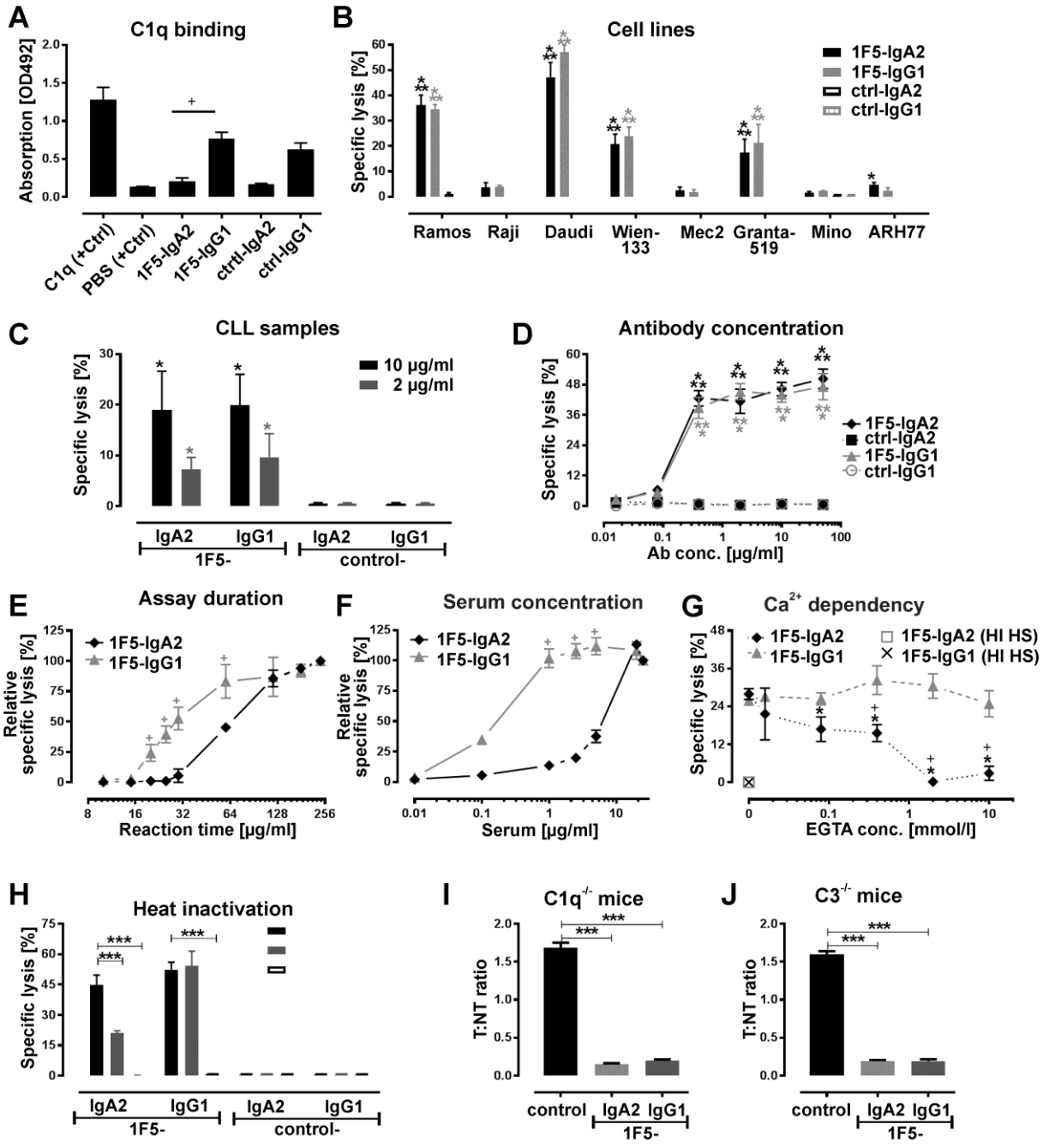

Fig 2. (A) C1q binding to 1F5-IgG1 or 1F5-IgA2 antibodies was determined by ELISA with peroxidase-labelled polyclonal mouse anti-human C1q antibody. The capacity of CD20 or control antibodies (10 µg/ml) to mediate CDC of different lymphoma cell lines (B), or B-CLL cells from patients (C) was evaluated in ^{51}chromium-release assays using 25% v/v healthy donor serum or heat inactivated healthy donor serum (HI HS). 1F5-IgA2 and 1F5-IgG1 were compared in CDC of Ramos cells using increasing concentrations of respective antibodies (D). (E)-(H) CDC assays were performed using a fixed concentration of respective antibodies (10 µg/ml) and differing assay conditions: time dependency (E), serum concentration-

dependency (F), (G) Ca^{2+-} dependency and (H) heat sensitivity of CDC. Results of at least three independent experiments are presented as mean ± SEM of "absorption [OD492]" in (A), "specific lysis [%]" in (B, C, F+G), or "relative specific lysis [%]" in (D, E). Significant differences ($p \leq 0.001$) between CD20-directed and control antibodies are indicated by *, between 1F5-IgA2 and 1F5-IgG1 by +. (I) and (J) *In vivo* efficacy of 1F5-IgG and –IgA2 in depleting syngeneic B cells from the spleen of mice was determined as described (Beers, *et al* 2008). Results are presented as mean ± SEM of "T: NT ratio". Significant differences ($p \leq 0.001$) between CD20-directed and control antibodies are indicated by ***.

Figure S1

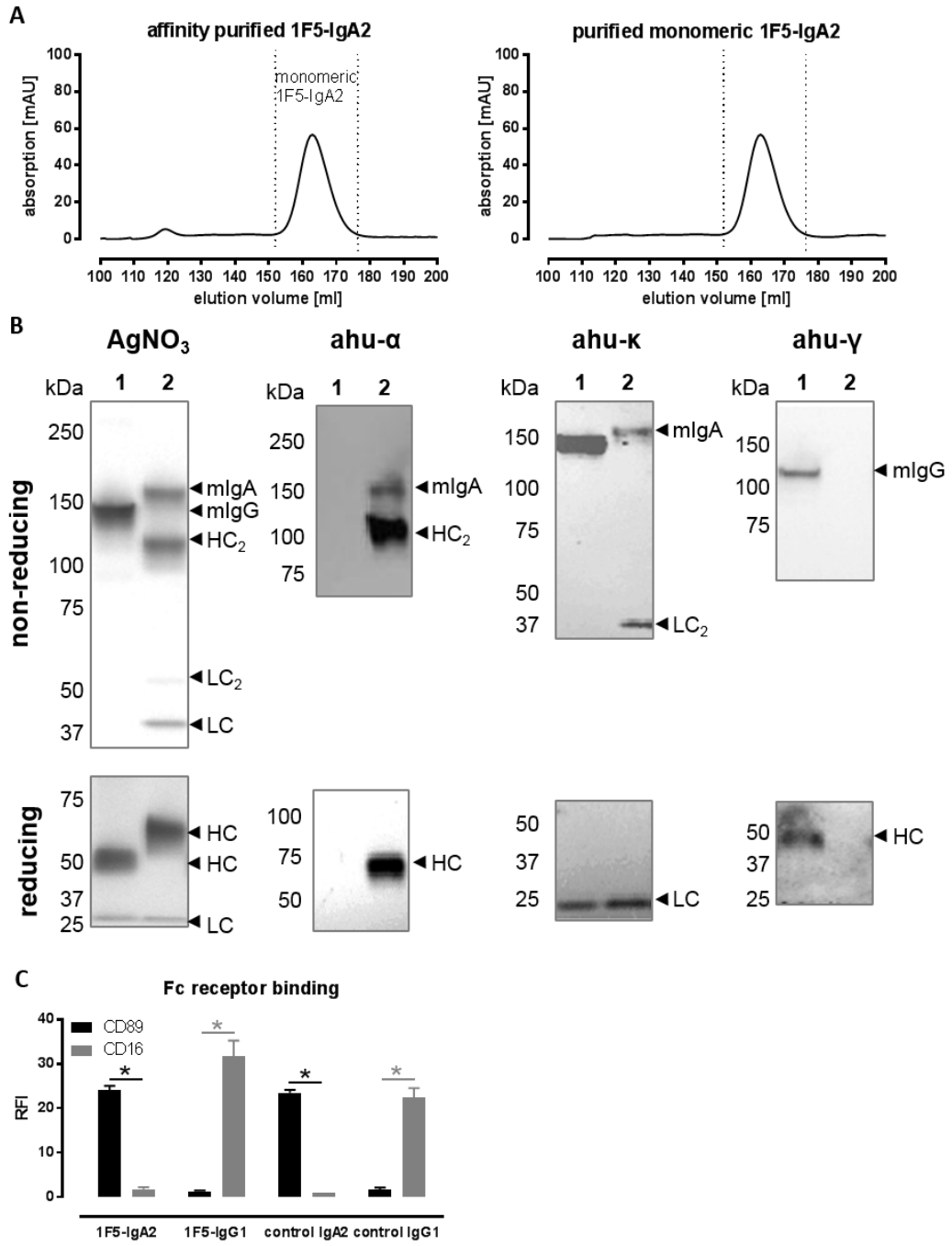

Fig S1. Production and biochemical characterisation of CD20 antibodies: (A) 1F5-IgA2 was purified after anti-human kappa-directed bead affinity chromatography using size exclusion chromatography to specifically isolate monomeric IgA. (B) Antibodies were separated on denaturing 3-8 % tris-acetate gels under non-reducing (top row) and reducing (bottom row) conditions. Purity was analysed using denaturing SDS-PAGE and silver staining. Proteins were transferred onto PVDF membranes and stained with peroxidase-labelled antibodies

directed against human α, κ or γ-chains as indicated, respectively. Lanes: 1. 1F5-IgG1, 2. 1F5-IgA2. Symbols: monomeric IgA/G: mIgA/G; monomers and dimers of heavy and light chains are indicated with HC/LC and HL2/LC2, respectively. (C) Binding of 1F5-IgG1, 1F5-IgA2 and respective control antibodies to FcαRI/FcRγ (CD89) or FcγRIII/FcRγ (CD16) co-transfected cells was analysed using a FITC-labelled human κ-light chain-directed antibody in indirect immunofluorescence analyses. Results of at least three independent experiments are displayed as "relative mean fluorescence intensity". Significant differences between IgG1 and IgA2 antibodies in (B) ($p < 0.05$) are indicated by *.

Figure S2

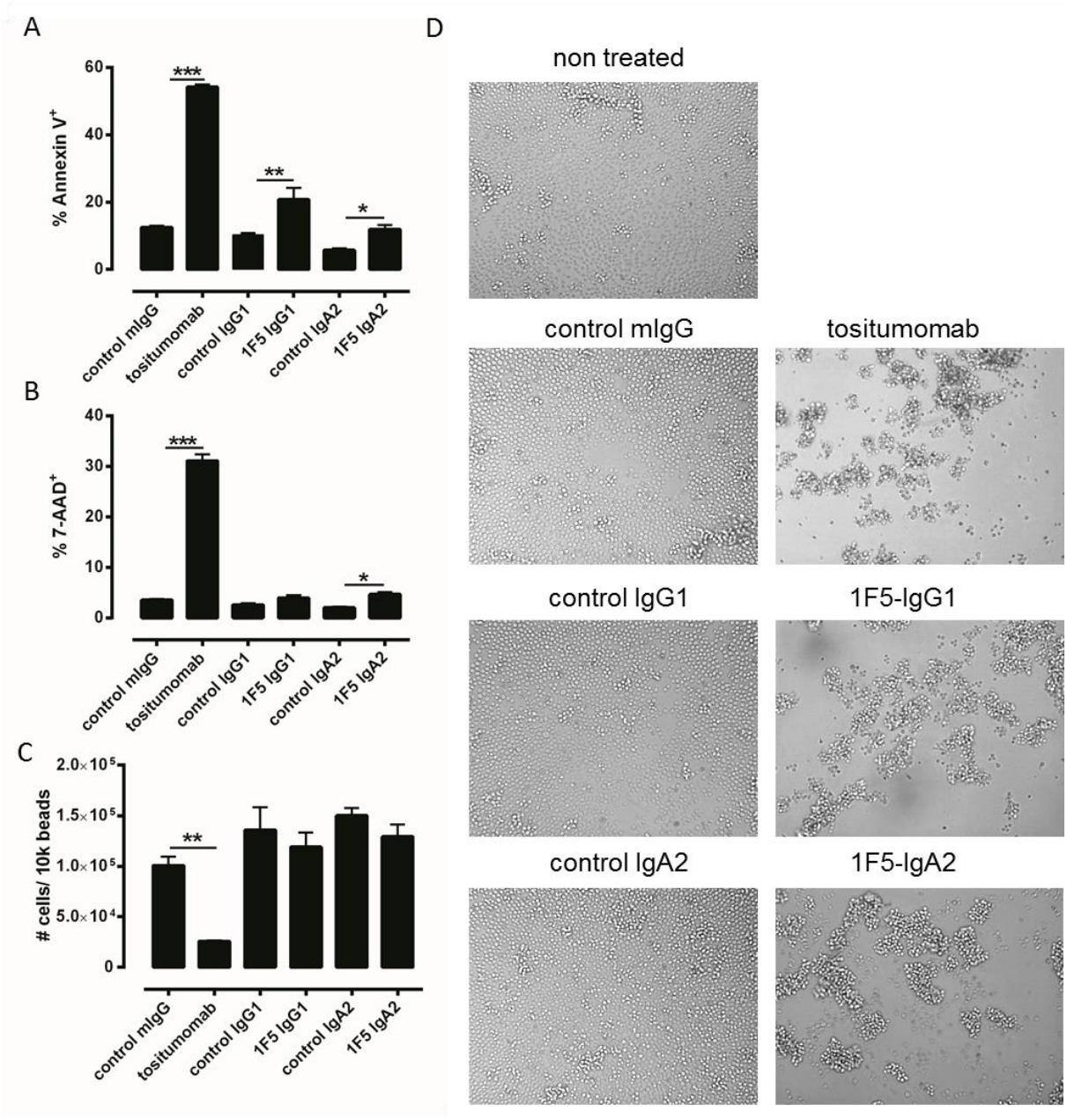

Fig S2. Fab-mediated effector mechanisms of CD20 antibody isotype variants. Induction of cell death was analysed by incubating Ramos cells for 48 h with respective antibodies. Annexin V-FITC (A) and 7-AAD (B) staining was measured, and total number of viable Ramos cells in relation to reference beads (C) was calculated. Results of three independent experiments are presented as mean ± SD of "% Annexin V+" in (A), "% 7-AAD+" in (B), and "#cells/10k beads" in (C). Significant differences are indicated by * for $p \leq 0.05$, ** for $p \leq 0.01$ and *** for $p \leq 0.001$. (D) Representative images of homotypic aggregation of Ramos cells were taken with an EVOS microscope and are representative for three independent experiments.

Figure S3

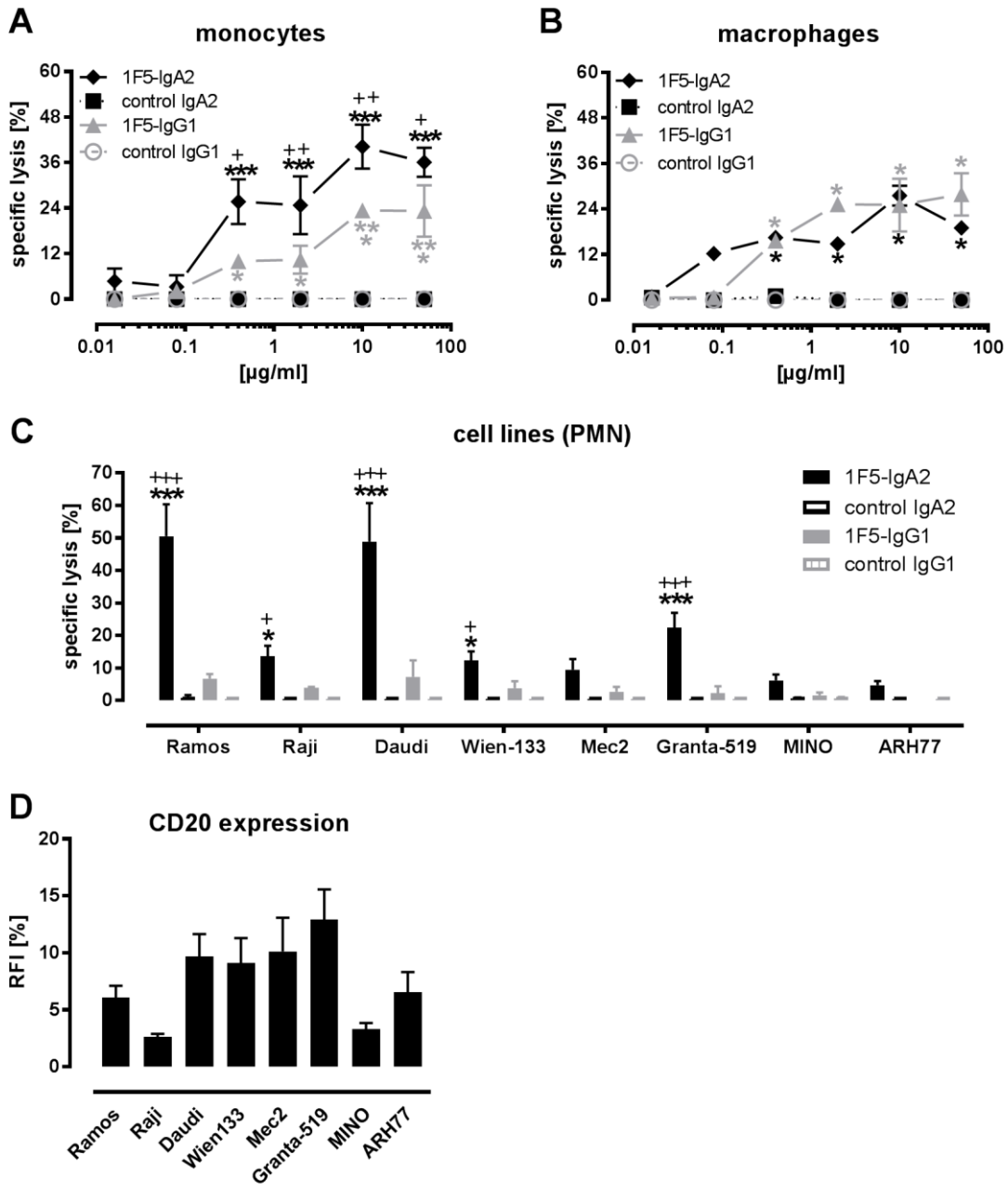

Fig S3. The capacity of increasing concentrations of 1F5-IgA2 or 1F5-IgG1 to mediate killing of Ramos cells by isolated monocytes (A) or monocyte-derived macrophages (B) was analysed in ^{51}chromium-release assays. (C) The capacity of CD20 antibodies (10 µg/ml) to trigger PMN-mediated ADCC of different lymphoma cell lines was evaluated. (D) Expression of CD20 on different lymphoma cell lines was determined using direct immunofluorescence. Results of at least three independent experiments are displayed as mean ± SEM of "specific lysis [%]" (A-C), or "relative mean fluorescence intensity" in (D). Significant differences ($p \leq 0.001$) between CD20-directed and control antibodies are indicated by *, between 1F5-IgA2 and 1F5-IgG1 by +.