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Effectors mechanisms of IgA antibodies against CD20 include recruitment of myeloid cells for antibody-dependent cell-mediated cytotoxicity and complement-dependent cytotoxicity

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 antibody-dependent cell-mediated cytotoxicity (ADCC) by myeloid effector cells significantly contributes to rituximab’s therapeutic efficacy, while complement-dependent cytotoxicity (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 natural killer (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 that may reduce CD20 surface expression and thereby limit both ADCC and CDC efficacy of CD20 antibodies (Taylor & Lindorfer, 2015). Bispecific antibodies simultaneously targeting CD20 and the myeloid receptor for IgA (FcεRI; CD89) demonstrated that polymophonuclear cells (PMN) could
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 mechanism of action (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 FcγRI and FcγRIII binding (Fig 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 isoforms to deplete human CD20 transgenic (hCD20 tg) cells was analysed by indirect immunofluorescence using a fluorescein isothiocyanate (FITC)-labelled human κ-light chain antibody. (B) In vivo efficacy of 1F5-IgA2 and 1F5-IgG1 was analysed as described by Beers et al. (2008). Spleen suspensions were stained with allophycocyanin (APC)-labelled CD19 antibody, and samples were analysed by flow cytometry to determine the ratio of target (T) to non-target (NT) carboxyfluorescein succinimidyl ester (CFSE)-labelled cells. Results are presented as mean ± SEM of the T:NT ratio. The capacity of increasing concentrations of 1F5-IgA2 or 1F5-IgG1 to mediate killing of Ramos (C, D), and freshly isolated B-cell chronic lymphocytic leukaemia (CLL) cells (E, F) by isolated polymorphonuclear cells (PMN) (C, E), or mononuclear cells (MNC) (D, F) was analysed in 51chromium-release assays. Results of at least three independent experiments are displayed as mean ± SEM of “mean fluorescence intensity” (A), “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 ≤ 0.001) between CD20-directed and control antibodies are indicated by *, between 1F5-IgA2 and 1F5-IgG1 by +. Ab, antibody.
Fig 2. (A) C1q binding to 1F5-IgG1 or 1F5-IgA2 antibodies was determined by enzyme-linked immunosorbent assay with peroxidase-labelled polyclonal mouse anti-human C1q antibody. The capacity of CD20 or control antibodies (10 μg/ml) to mediate complement-dependent cytotoxicity (CDC) of different lymphoma cell lines (B), or B-cell chronic lymphocytic leukaemia (CLL) cells from patients (C) was evaluated in 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), Ca²⁺ dependency (G) and heat sensitivity of CDC (H). Results of at least three independent experiments are presented as mean ± SEM of “absorption [OD492]” in (A), “specific lysis [%]” in (B, C, D, G, H), or “relative specific lysis [%]” in (E, F). Significant differences (P ≤ 0.001) between CD20-directed and control antibodies are indicated by *, between 1F5-IgA2 and 1F5-IgG1 by †, between IgA2 and IgG1 by ‡, and between IgA2 and IgG1 by ††. (I) and (J) In vivo efficacy of 1F5-IgG1, and 1F5-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 the Target:non-target (T:NT) ratio. Significant differences (P ≤ 0.001) between CD20-directed and control antibodies are indicated by ***.
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, because IgA does not contain a C1q binding site and did not bind C1q in sandwich-enzyme-linked immunosorbent assay, 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 (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid) 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 MoA 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 versus 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 that 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 the 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.

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 Rössner, Stefanie Derer, Katja Klausz, Ralf Schwanbeck, Denis M. Schewe, Christian Kellner and Matthias Peipp helped to analyse and interpret the 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.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**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.