

IMMUNOBIOLOGY

Antigenic modulation limits the effector cell mechanisms employed by type I anti-CD20 monoclonal antibodies

Thomas R. W. Tipton,¹ Ali Roghanian,¹ Robert J. Oldham,¹ Matthew J. Carter,¹ Kerry L. Cox,¹ C. Ian Mockridge,¹ Ruth R. French,¹ Lekh N. Dahal,¹ Patrick J. Duriez,² Philip G. Hargreaves,³ Mark S. Cragg,¹ and Stephen A. Beers¹

¹Antibody and Vaccine Group, Cancer Sciences Unit, University of Southampton Faculty of Medicine, and ²Southampton Experimental Cancer Medicine Centre/Cancer Research UK, Protein Core Facility, General Hospital, Southampton, United Kingdom; and ³Promega UK Ltd., Southampton Science Park, Southampton, United Kingdom

Key Points

- Antigenic modulation significantly impacts natural killer cell and macrophage ability to mediate Fc γ receptor-dependent killing.
- hIgG1 mAbs are unable to elicit natural killer-mediated ADCC in the mouse, supporting ADCP as the dominant effector mechanism.

Following the success of rituximab, 2 other anti-CD20 monoclonal antibodies (mAbs), ofatumumab and obinutuzumab, have entered clinical use. Ofatumumab has enhanced capacity for complement-dependent cytotoxicity, whereas obinutuzumab, a type II mAb, lacks the ability to redistribute into lipid rafts and is glycoengineered for augmented antibody-dependent cellular cytotoxicity (ADCC). We previously showed that type I mAbs such as rituximab have a propensity to undergo enhanced antigenic modulation compared with type II. Here we assessed the key effector mechanisms affected, comparing type I and II antibodies of various isotypes in ADCC and antibody-dependent cellular-phagocytosis (ADCP) assays. Rituximab and ofatumumab depleted both normal and leukemic human CD20-expressing B cells in the mouse less effectively than glycoengineered and wild-type forms of obinutuzumab, particularly when human immunoglobulin G1 (hIgG1) mAbs were compared. In contrast to mouse IgG2a, hIgG1 mAbs were ineffective in ADCC assays with murine natural killer cells as effectors, whereas ADCP was equivalent for mouse IgG2a and hIgG1. However, rituximab's ability to elicit both ADCC and ADCP was reduced by antigenic modulation, whereas type II antibodies remained unaffected. These data demonstrate that ADCP and ADCC are impaired by antigenic modulation and that ADCP is the main effector function employed *in vivo*. (*Blood*. 2015;125(12):1901-1909)

Introduction

Rituximab is the archetypal anti-CD20 monoclonal antibody (mAb), licensed in 1997 and now used alongside chemotherapy to treat non-Hodgkin lymphoma.¹ It is proposed to operate through 4 effector functions: programmed cell death, complement-dependent cytotoxicity (CDC), and the 2 Fc γ receptor (Fc γ R)-dependent mechanisms antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP).¹⁻³

Following on from the success of rituximab are the next-generation anti-CD20 mAbs ofatumumab and obinutuzumab. Ofatumumab was approved for chronic lymphocytic leukemia (CLL) treatment in 2009 and shows enhanced CDC, likely through its low off-rate and cell-surface proximal epitope.^{4,5} Obinutuzumab, licensed in 2013 for first-line CLL treatment (in combination with chlorambucil), has a glycoengineered Fc region, resulting in higher affinity binding to human Fc γ RIIIa (hFc γ RIIIa) and b, thus enhancing hFc γ RIII-dependent effector functions.⁶⁻⁹

Despite the success of rituximab, patients often become resistant to therapy and relapse. Acute resistance can be associated with loss of CD20 from the cell surface, particularly in the case of CLL.¹⁰ We have previously demonstrated that antigenic modulation, whereby CD20

antibody:antigen complexes are internalized after type I mAb binding, will contribute to CD20 loss and that this process is accelerated by hFc γ RIIb.¹¹⁻¹³

Anti-CD20 mAbs are categorized as type I (rituximab, ofatumumab) or II (tositumomab, obinutuzumab) depending upon their propensity to elicit CD20 redistribution into lipid rafts and trigger efficient CDC (type I) or homotypic adhesion and lysosomal nonapoptotic programmed cell death (type II).¹⁴⁻¹⁶ Type II antibodies are also resistant to antigenic modulation and display an enhanced B-cell depletion in hCD20 mice.^{11,17}

Using a combination of mouse Fc γ R^{-/-} (mFc γ R^{-/-}) mice¹⁸ and intravital imaging,^{19,20} it has been clearly shown that anti-CD20 mAbs require Fc γ R-mediated effector mechanisms for successful therapy. Further evidence for Fc γ R effector mechanisms came from the observations that hFc γ RIIIa and hFc γ RIIa polymorphisms correlate with clinical efficacy,^{21,22} although roles for complement activation and apoptosis induction have also been proposed.^{1,23}

Multiple cell types express Fc γ R with variable expression patterns: With the exception of a minor proportion of the human population who express an open reading frame for hFc γ RIIc,²⁴ B cells express only the

Submitted July 17, 2014; accepted January 17, 2015. Prepublished online as *Blood* First Edition paper, January 28, 2015; DOI 10.1182/blood-2014-07-588376.

M.S.C. and S.A.B. are the senior authors and contributed equally to this study.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

© 2015 by The American Society of Hematology

inhibitory hFcγRIIb (mFcγRII), natural killer (NK) cells express only hFcγRIIIa (mFcγRIII), and macrophages variably express the full repertoire of FcγR.²⁵

Here we assessed the potential effector functions employed by type I and II mAbs, how they are affected by the internalization process, and, through the use of isotype variants, identified the key effector mechanisms involved in B-cell depletion. We demonstrate that antigenic modulation affects ADCP and ADCC in both the mouse and human systems *in vitro* and that ADCP is the dominant effector mechanism of B-cell depletion in the mouse. These data explain the greater efficacy of type II antibodies *in vivo* in mice and have implications for future antibody selection and development in humans.

Methods

Animals, clinical samples, and antibodies

Details relating to animal experiments, clinical sample preparation, and antibody generation/preparation can be found in the supplemental Methods, available on the *Blood* Web site.

Flow cytometry

Flow cytometry was as described previously²⁶ with samples assessed on a FACScan, FACSCalibur, or FACSCanto II (BD Biosciences) and data analyzed with FCS Express V3 (De Novo Software). To determine cell-surface expression of CD20, cells were incubated with anti-CD20 mAbs, washed twice, and then stained using phycoerythrin-F(ab')₂ goat-anti-mouse or anti-human Fcγ-specific reagents (Jackson ImmunoResearch). The mean number of antibodies/cell was determined using BD QuantiBRITE beads (BD Biosciences).

ADCP

ADCP assays were performed as described previously with mouse^{11,17} or human macrophages.¹² Further details are shown in the supplemental Methods.

ADCC

Target cells were loaded with calcein-AM (10 μg/mL), opsonized with anti-CD20 antibody (10 μg/mL), and cultured with murine NK (mNK) cells for 2 hours at an effector to target (E:T) ratio of 10:1 or peripheral blood mononuclear cells for 4 hours at an E:T of 50:1. Subsequently, 75 μL of supernatant was collected and analyzed using a Varioskan Flashplate reader at 495 nm (Thermo Scientific). Results are reported as percentage maximum lysis obtained when incubating targets alone with 4% triton X-100. For human targets, ADCC was also measured using the ADCC Reporter Bioassay (Promega) according to the manufacturer's instructions.

Detection of hIgG

The level of human immunoglobulin G (hIgG) in mouse serum was assessed by standard enzyme-linked immunosorbent assay (supplemental Methods).

SPR

Surface plasmon resonance (SPR) analysis of FcγR:mAb binding was performed as described previously.²⁷ Further details are in the supplemental Methods.

In vivo B-cell depletions

Systemic B-cell depletion assays were performed as previously described.¹¹ Mice were given a single intravenous dose of anti-CD20 mAb (250 μg) and the proportion of B cells remaining in the blood assessed by flow cytometry over time using dual staining with anti-B220-PerCP and anti-CD19-APC (BD Biosciences).

Εμ-Tcl-1 × hCD20 Tg leukemia studies

Εμ-Tcl-1 × hCD20 transgenic (Tg) tumors were generated by intercrossing Εμ-Tcl-1 and hCD20 Tg mice and harvesting the resulting tumors. Splenic tumors were confirmed as expressing hCD20 by flow cytometry and stored in liquid nitrogen. For therapy experiments, cells were thawed and 1×10^7 given intraperitoneally to congenic female hCD20 Tg C57BL/6 mice. Leukemic burden was assessed by monitoring the percentage of CD5⁺B220^{lo} cells by flow cytometry. When tumor cells could be clearly observed in the blood (after approximately 35–42 days), mice were randomized to receive different mAbs (250 μg/mouse) intravenously and the effect on circulating tumor and normal B cells was monitored.

Statistical analysis

Statistical analysis comparing treatment groups was performed using 1- or 2-way analysis of variance (ANOVA) where appropriate. Statistical analysis was performed using GraphPad Prism, version 6, for Windows (GraphPad Software).

Results

hIgG1 type II but not type I mAbs effectively deplete B cells in hCD20 transgenic mice

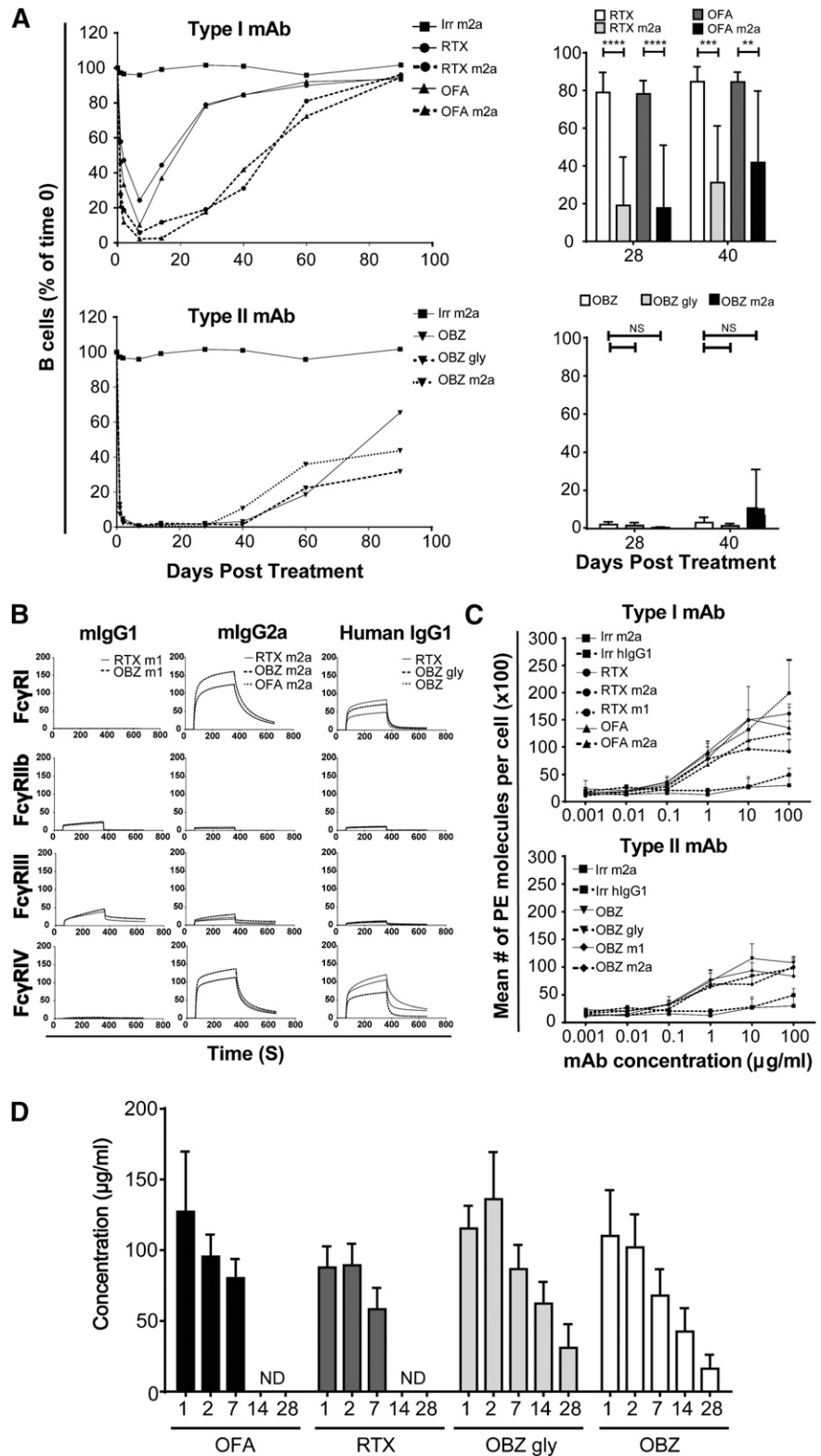
We previously showed that type II anti-CD20 mAbs are superior to type I in depleting hCD20 transgenic B cells *in vivo* and that this superior efficacy correlates with the propensity of type I mAbs to undergo antigenic modulation.^{11,17} Previous work has also demonstrated a hierarchical role for IgG isotype in FcγR interaction, effector cell function, and target cell deletion capacity *in vivo*.²⁸ Therefore, to extend our previous observations, we investigated the impact that alternate antibody isotypes had on B-cell depletion with type I and II mAbs. We treated hCD20 Tg mice with either type I or type II anti-CD20 antibodies engineered with mIgG2a or hIgG1 isotypes.

For type I antibodies, the mIgG2a isotype provided significantly prolonged B-cell depletion compared with hIgG1 (Figure 1A). However, there was no difference in B-cell depletion when comparing hIgG1 (glycoengineered obinutuzumab and wild-type non-glycoengineered obinutuzumab [OBZ gly]) and wild-type non-glycoengineered mIgG2a versions of the type II reagents. As anticipated from previous work with mCD20 as a target,²⁸ the mIgG2a isotype was superior to mouse IgG1 (mIgG1), independent of whether type I or II anti-hCD20 reagents were examined (supplemental Figure 1).

To better understand why type II reagents are superior and why mIgG2a and hIgG1 isotypes are equally efficacious, we assessed the ability of these mAbs to engage mFcγR using SPR. As seen in Figure 1B and supplemental Table 1, mIgG2a isotypes possess similar mFcγR binding profiles in accordance with those previously reported,²⁷ with strong affinity for mFcγRI and IV, and we report similar binding profiles for hIgG1. Although the mIgG2a had an intermediate affinity for mFcγRIII, hIgG1 had a lower affinity. In contrast, mIgG1 mAbs had no measurable binding to mFcγRI or IV, but did have an intermediate affinity for mFcγRIII. Importantly, these binding patterns were independent of antibody type and equivalent for both types I and II and therefore do not explain the propensity of type II reagents to provide prolonged B-cell depletion *in vivo*.

Antibody-binding levels of type I and II reagents may provide a trivial explanation for their differing efficacy *in vivo*; therefore, we stained hCD20 Tg B cells with type I or type II reagents and looked for the amount of cell surface-bound antibody. Figure 1C shows that 10 μg/mL is sufficient to saturate hCD20 Tg B cells with both types of mAbs and that, as previously reported,¹⁷ at 10 μg/mL, type I

Figure 1. In vivo depletion using human and mouse type I and II antibodies. (A) Left panels show that transgenic hCD20 mice were administered 250 μ g anti-CD20 by tail-vein injection and the percentage of circulating B220, CD19⁺ B cells measured over 90 days (n = 4). Right panels show statistical comparison of human vs mouse isotype mAbs at days 28 and 40. Statistical analyses were carried out using 2-way ANOVA with multiple comparisons and significance was accepted at *****P* < .0001, ****P* < .001, and ***P* < .01. (B) SPR analysis of anti-human CD20 mAbs (hIgG1, mIgG1, and mIgG2a) binding to mouse Fc γ R1, IIb, III, and IV. Recombinant, soluble Fc γ R proteins were passed over mAbs immobilized at 2000 RU. Sensorgrams are shown. (C) Binding comparison of type I and II anti-CD20 mAbs to transgenic hCD20 B cells. Cells were opsonized with 0.001 to 100 μ g/mL anti-CD20 mAbs and analyzed by flow cytometry. The mean number of phycoerythrin (PE) molecules per cell was quantified by indirect staining with anti-mouse or anti-human Fc PE-conjugated F(ab')₂ and comparison of the geometric mean fluorescent index with BD QuantiBRITE beads. (D) The concentration of anti-CD20 mAbs in the sera of mice administered 250 μ g of human IgG1 mAbs was determined by enzyme-linked immunosorbent assay; n = 4 mice per group. ND, not detectable. Bars represent mean \pm standard deviation. Irr, WR17; OFA, ofatumumab; RTX, rituximab.



antibodies bind ~2-fold compared with type II antibodies, although some variation in binding was seen, presumably from differences in affinity.

After ruling out differences in Fc:Fc γ R binding affinities and antibody binding levels as explanations for our earlier observations, we next determined the serum half-life of hIgG1 mAbs during our in vivo depletions (Figure 1A). Figure 1D demonstrates that type I antibodies

have a shorter serum half-life compared with type II reagents, likely because of B cell-dependent internalization of antibody.¹¹⁻¹³ The reduced quantity of type I antibody available may therefore affect effector cell mechanisms.

Previously, we showed that complement and apoptosis were redundant effector mechanisms in depleting hCD20⁺ B cells with

mIgG2a anti-CD20 mAbs, whereas depletion of macrophages with clodronate or treatment of γ chain^{-/-} mice arrested B-cell depletion¹¹; we carried out similar experiments here confirming these observations for hIgG1 mAbs (supplemental Figures 2 and 3). Given these confirmatory findings, we decided to investigate the differential effects of Fc γ R-mediated ADCC and ADCP on B-cell depletion using clinical CLL samples and in the hCD20 mouse.

Type II reagents show superior ADCC activity, but ADCC is not the dominant mechanism of action in vivo

To correlate our in vitro data with the observed in vivo results, we began by assessing the efficacy of our antibodies in assays with murine effectors and then confirmed these results with human cells. We started by expanding mNK cells ex vivo in the presence of interleukin-2 (200 μ g/mL). The resulting NK cells possessed a highly activated phenotype, displaying increased CD69 and Fc γ RIII expression (supplemental Figure 4). At all concentrations tested, the type II mAbs were able to elicit ADCC to a greater extent than were the type I mAbs (Figure 2A). This increased ADCC activity was not due to altered glycosylation because both obinutuzumab mIgG2a and mIgG1 are wild-type mAbs and not defucosylated. This observation is perhaps surprising given the relative binding levels determined in Figure 1C. Also, when we directly compared type I or II mIgG2a and mIgG1 mAbs, we observed equivalent levels of ADCC with mNK effectors (Figure 2B) in contrast to our in vivo findings, in which the mIgG1 antibodies (even type II) performed relatively poorly (supplemental Figure 1), suggesting ADCC is not the dominant mechanism of action in vivo. Furthermore, although type II hIgG1 mAbs were highly effective at depleting B cells in vivo, they demonstrated negligible ADCC activity (Figure 2B). The Fc γ R binding affinities indicate that in contrast to mIgG2a and mIgG1 isotypes, hIgG1 has a low affinity for mFc γ RIII, but a high affinity for mFc γ RI and IV (Figure 1B). Because mNK cells only express mFc γ RIII,²⁹ these data explain the inability of hIgG1 mAbs to elicit substantial ADCC with mouse effectors. Moreover, coupled with the depletions observed in hCD20 Tg mice (Figure 1A), they demonstrate that ADCC is not the dominant effector cell mechanism depleting B cells in the mouse.

We next repeated these experiments using human effector cells and primary human CLL cells as targets. Type II antibodies again showed higher levels of ADCC than type I (Figure 2C). Although obinutuzumab displayed the greatest levels of ADCC, by virtue of its glycomodification and increased affinity for hFc γ RIIIa, the wild-type non-glycoengineered version also provided good levels of ADCC compared with type I antibodies, suggesting an inherent ability of type II to outperform type I antibodies in ADCC. These results were further confirmed using an hFc γ RIIIa ADCC bio-reporter assay, which measures the extent of hFc γ RIIIa engagement (supplemental Figure 5).

Types I and II mIgG2a and hlgG1 reagents show a similar propensity to elicit ADCP

Macrophages were recently implicated as the key effector in antibody-mediated depletion.^{11,19,20} Because ADCC failed to adequately explain our in vivo results, we assessed the abilities of type I and II antibodies to mediate ADCP. First, we compared type I and II mIgG2a antibodies and demonstrated that all antibodies elicit phagocytosis optimally at 10 μ g/mL with murine effectors (Figure 3A). In addition, type I reagents displayed a trend toward inducing greater levels of phagocytosis than type II, perhaps reflecting the higher levels of bound antibody (Figure 1C).

We next examined the impact of antibody isotype. In the case of murine targets and effectors, with type I and II reagents, the mIgG2a

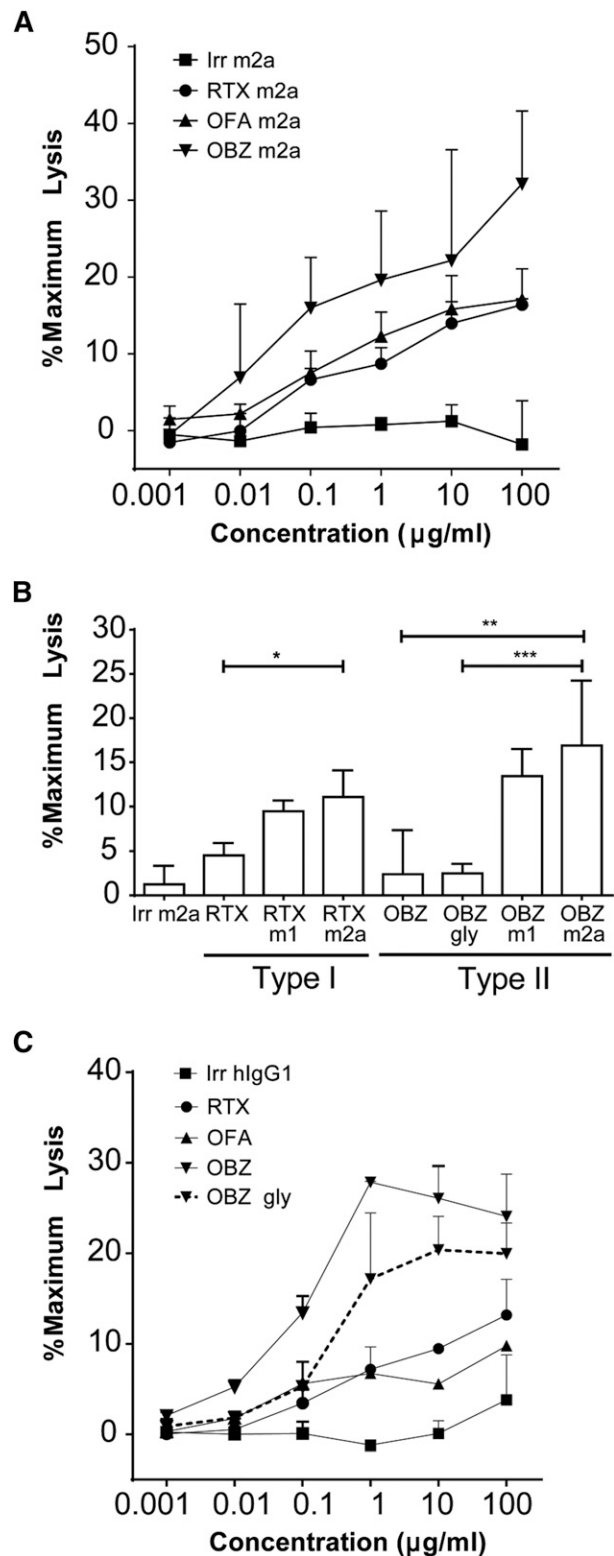


Figure 2. ADCC of type I and II antibodies. (A) Transgenic hCD20 murine B cells were loaded with Calcein-AM and opsonized with anti-CD20 mAbs ($N = 3$). B cells were then cocultured with mNK cells for 2 hours at an E:T ratio of 10:1; supernatant was assayed for calcein release at 490 nm. (B) Levels of ADCC when target hCD20 murine B cells were opsonized with 10 μ g/mL mAbs and cocultured with mNK cells. Statistical analyses were carried out using 1-way ANOVA with multiple comparisons and significance was accepted at $*P < .05$, $**P < .01$, and $***P < .001$. (C) Primary human CLL cells were loaded with Calcein-AM and opsonized with 0.001 to 10 μ g/mL anti-CD20 mAbs ($N = 2$). CLL cells were then cocultured with human peripheral blood mononuclear cells for 4 hours at an E:T ratio of 50:1; supernatant was assayed for calcein release at 490 nm.

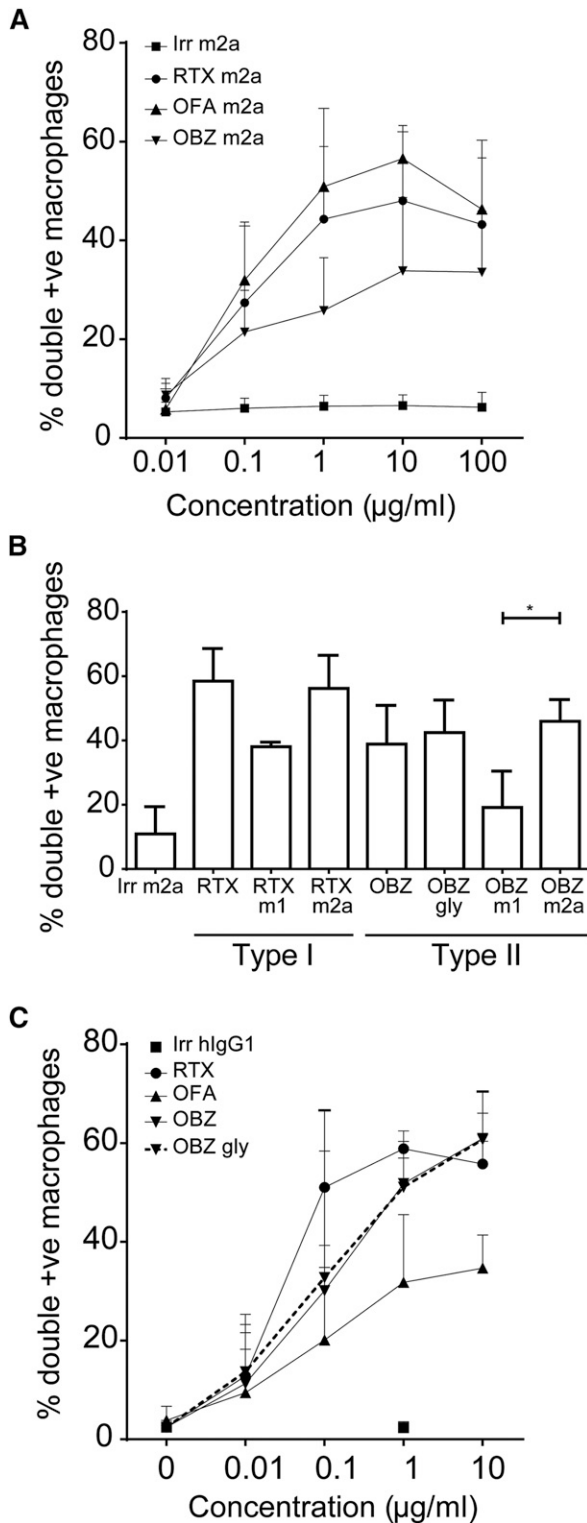


Figure 3. ADCC of type I and II antibodies. (A) Transgenic hCD20 murine B cells were carboxyfluorescein diacetate succinimidyl ester-labeled and opsonized with 0.01 to 100 µg/mL anti-CD20 mAbs (N = 5). Opsonized B cells were cocultured with murine bone marrow-derived macrophages (BMDMs) for 30 minutes to permit phagocytosis and then analyzed by flow cytometry. (B) Levels of ADCC when target hCD20 murine B cells were opsonized with 10 µg/mL mAbs and cocultured with murine BMDMs. Statistical analyses were carried out using 1-way ANOVA with multiple comparisons and significance was accepted at **P* < .05. (C) Primary human CLL samples were carboxyfluorescein diacetate succinimidyl ester-labeled and opsonized with 0.01 to 10 µg/mL anti-CD20 mAbs (N = 3). Opsonized CLL B cells were cocultured with human marrow-derived macrophages (MDMs) for 60 minutes to permit phagocytosis and then analyzed by flow cytometry.

was superior to the mIgG1 isotype at eliciting phagocytosis (Figure 3B), correlating with the superior in vivo depletion observed (supplemental Figure 1). Furthermore, and in contrast to our ADCC data, hIgG1 antibodies showed equivalent levels of phagocytosis compared with mIgG2a antibodies for both type I and II reagents, in keeping with their similar FcγR binding profiles. When we repeated these experiments using primary human CLL cells and human macrophages (Figure 3C), type I reagents again displayed a propensity to outperform type II at suboptimal doses. Both the glycoengineered obinutuzumab and wild-type OBZ Gly displayed equivalent levels of ADCC.

Our earlier data showed that although ADCC was not elicited by hIgG1 type II mAbs with murine cells, they were efficacious in depleting target B cells in vivo. In contrast, these data demonstrate that hIgG1 mAbs were effective in ADCC assays, unlike mIgG1 equivalents. Taking into account their respective in vivo activities, these data indicate ADCC is the key effector mechanism in the mouse. However, we have also shown here that type I reagents performed better than type II reagents in ADCC assays, suggesting that type I antibodies should perform better than type II in vivo—and this is evidently not the case. Based upon our earlier observations in vitro and previous literature,^{11,17} we speculated that antigenic modulation may limit the effector functions of type I mAbs. To address this, we next examined the impact antigenic modulation had on both ADCC and ADCCP.

Antigenic modulation limits both ADCC and ADCCP with type I anti-CD20 reagents

To investigate the kinetics of antigenic modulation, we stained for antibody on opsonized cells 0 to 6 hours after mAb ligation. Antibody was rapidly lost from the cell surface of hCD20 Tg B cells and primary human CLL cells with type I mAbs (Figure 4A-B), as previously reported.¹¹

To determine which effector mechanisms were critically affected by modulation, we opsonized hCD20 Tg B cells with antibody for 0 to 6 hours and then examined them in our ADCC and ADCCP assays. Murine ADCC levels were significantly (*P* = .0001) impacted by antigenic modulation, with a 6-hour preincubation clearly detrimental for type I but not type II reagents (Figure 4C). We then repeated these experiments with hNK effectors and primary human CLL target cells (Figure 4D). Again, a 6-hour preincubation period led to significant antigenic modulation and demonstrated a trend toward a detrimental effect on the ADCC capacity of rituximab, whereas there was no difference with type II reagents examined at the start and end of the 6-hour culture period. The differences observed with rituximab are likely not significant because the long (4 hour) target and effector incubation period used for the human ADCC assay, which would allow modulation to occur even in the 0-hour samples. We next looked into the impact of antigenic modulation on phagocytosis. Type I reagents were significantly (*P* < .05) affected by modulation, whereas type II reagents remained unaffected in both murine (Figure 4E) and human systems (Figure 4F).

These findings clearly demonstrate that modulation severely reduces the efficacy of key FcγR-dependent effector systems employed by directly targeting mAbs such as rituximab in both human and mouse systems against both normal and malignant B-cell targets.

Prolonged depletion of hCD20 Tg Eµ-TCL-1 B cells by type II anti-CD20 mAbs

To confirm the clinical relevance of these results for hematological immunotherapy, we performed experiments in hCD20 Tg mice bearing hCD20 Tg Eµ-TCL-1 B-cell tumors, produced recently (manuscript

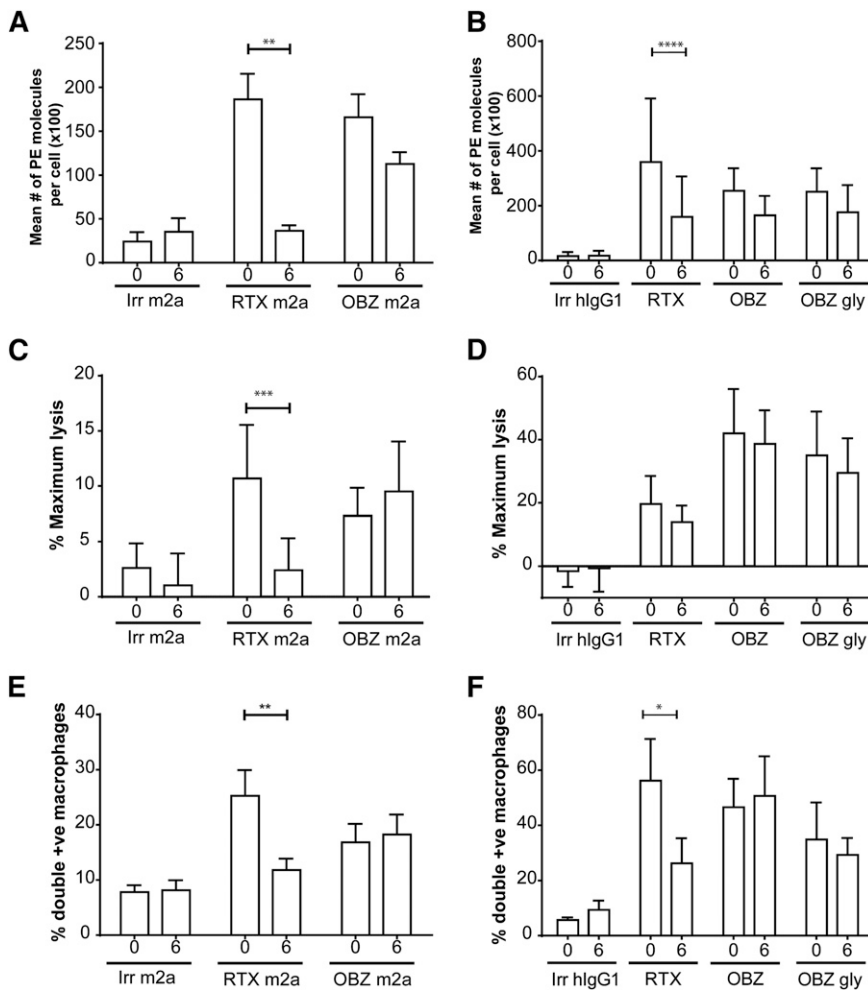


Figure 4. Impact of modulation on ADCC and ADCP effector mechanisms. (A-B) Surface levels of CD20 after incubation with type I and II mAbs. Target murine hCD20 (A) or primary human CLL (B) B cells were opsonized with 10 μ g/mL anti-CD20 mAbs for 30 minutes or 6 hours; samples were then stained for 30 minutes with anti-mouse (A) or anti-human (B) Fc PE and assessed by a flow cytometer, using BD QuantiBRITE beads to calculate mean number of PE molecules/cell ($N = 3$). (C-D) Impact on ADCC, murine hCD20 (C), or primary human CLL (D) B cells were loaded with calcein-AM and incubated with anti-CD20 mAbs for either 30 minutes or 6 hours with 10 μ g/mL anti-CD20 mAbs. Samples were then cocultured for either 2 hours with mNK cells (C) or 4 hours with human peripheral blood mononuclear cells (D) at an E:T ratio 10:1 and 50:1, respectively. Sample supernatant was assessed for fluorescence at 495 nm. (E-F) Impact on ADCP, murine hCD20 (E), or primary human CLL (F) B cells were stained with CFSE and incubated with 10 μ g/mL anti-CD20 mAbs for either 30 minutes or 6 hours. Samples were then cocultured for 30 minutes with murine BMDMs (E) or 1 hour with human MDMs (F) and then assessed by flow cytometer. Statistical analyses were carried out using 2-way ANOVA with multiple comparisons and significance was accepted at * $P < .05$, ** $P < .01$, *** $P < .001$, and **** $P < .001$.

in preparation). The E μ -TCL-1 mouse model of CLL³⁰ coupled to expression of hCD20 allows us to assess the ability of the clinically relevant mAbs to delete malignant cells in a fully syngeneic, immune-competent context. The data demonstrate that, as with normal hCD20 Tg B cells, hCD20 Tg E μ -TCL-1 tumor B cells displayed significantly ($P < .05$) prolonged depletion when treated with type II (OBZ gly) compared with type I (rituximab) mAbs, correlating with a longer serum half-life (Figure 5). These results confirm our findings with normal B cells and demonstrate the superior effects in vivo of the type II mAbs in a clinically relevant mouse model of CLL.

Discussion

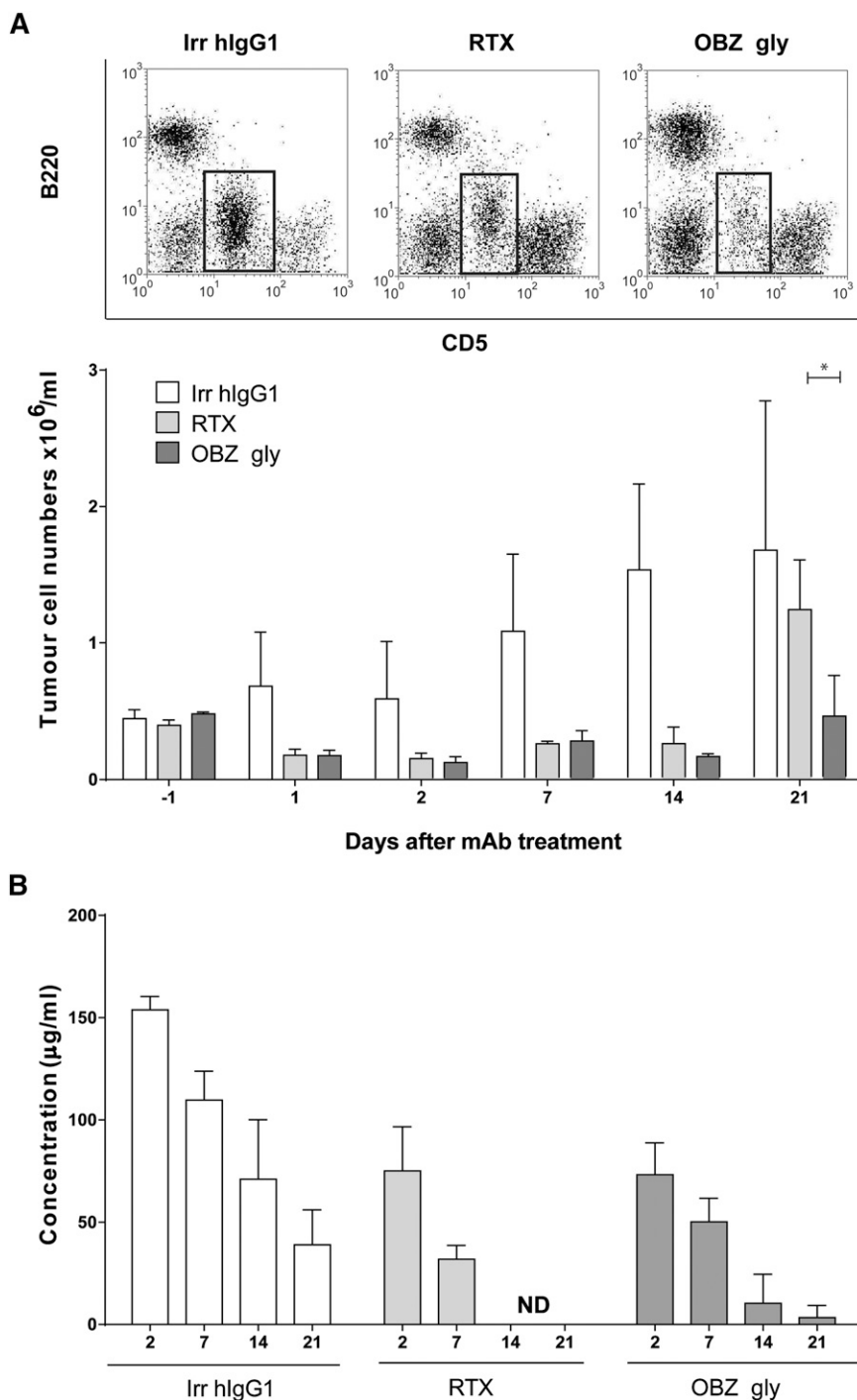
Previously, we demonstrated that mIgG2a versions of type II anti-CD20 mAbs outperform type I and that antigenic modulation takes place more rapidly with type I antibodies.^{11,17} Here we show that modulation affects both Fc γ R-dependent effector mechanisms engaged by mAbs ADCC and ADCP, thus explaining the propensity for type II antibodies to outperform type I in vivo in both normal B-cell depletion and a model of CLL. These same effector mechanisms are similarly impacted with clinically relevant reagents targeting primary human CLL cells. Finally, based on the observation that hlgG1 was efficacious in depleting hCD20 B cells in vivo but showed little ADCC activity because of its minimal binding to mFc γ RIII, we demonstrate that ADCP is the dominant effector mechanism responsible for target cell depletion in the mouse.

Previous work comparing clinically relevant antibodies have demonstrated that ofatumumab and obinutuzumab are superior to rituximab in different ways: ofatumumab displays enhanced CDC and obinutuzumab enhanced ADCC and direct cell death.^{31,32} Although insightful, these experiments did not take into account the potentially detrimental effect of antigenic modulation, which we show here to have a significant impact on Fc γ R-dependent effector mechanisms. However, work by van Meerten et al demonstrated a strong correlation between CDC and CD20 expression, whereas they found no such association with regard to ADCC.³³ This suggests that antigenic modulation would perhaps have a larger impact on CDC, although we have been unable to demonstrate a significant role for CDC in mice (supplemental Figure 2).¹¹ We and others have, however, been able to demonstrate a critical need for macrophages and ADCP (supplemental Figure 3)^{11,19,20} and would suggest that perhaps the levels of CD20 compared by van Meerten et al did not reach a sufficiently low level to affect Fc γ R mechanisms—a threshold that is crossed by significant loss of antibody from modulation.

We found that glycoengineered obinutuzumab elicited greater potency in our human ADCC assays than its wild-type counterpart, thus highlighting the benefit of defucosylation. However, we also found that, in vivo, both obinutuzumab and OBZ Gly displayed equivalent levels of B-cell depletion akin to that seen with mIgG2a. Examination of binding affinity data revealed that both hlgG1 and mIgG2a antibodies have similar affinities for mFc γ RI and IV, although interestingly, obinutuzumab did have a slower dissociation rate for mFc γ RIV. hFc γ RIIIa is glycosylated at residue Asn162,³⁴ with mFc γ RIV glycosylated at a similar location, likely explaining this

Figure 5. In vivo depletion of E μ -TCL-1 \times hCD20 Tg leukemic B cells using type I and II antibodies.

(A) E μ -TCL-1 \times hCD20 Tg splenic tumors were administered intraperitoneally to hCD20 Tg mice and treated intravenously (250 μ g) with anti-CD20 mAbs when CD5⁺ B220^{low} tumor B cells were clearly detectable by flow cytometry 35 to 42 days later. The percentage of circulating tumor was then measured for the following 21 days (n = 3). Example dot-plots showing tumor cell populations on day 21 (tumor indicated in the boxed area) above, with mean numbers of tumor cells/mL below. Statistical analyses were carried out using 2-way ANOVA with multiple comparisons and significance was accepted at *P < .05. (B) The concentration of anti-CD20 mAbs in the sera of mice in panel A were determined by enzyme-linked immunosorbent assay. ND, not detectable. The results clearly show that RTX is completely lost from the sera by day 14, whereas OBZ gly remains detectable out to day 21, coincident with more prolonged tumor depletion. n = 3 mice per group. Bars represent mean \pm standard deviation.



observation. Importantly, hNK cells express hFc γ RIIIa, which shares higher sequence identity to mFc γ RIV than mFc γ RIII. Mouse Fc γ RIV, however, is not expressed on mNK cells and so these results may underestimate the differences that glycoengineering and also ADCC may elicit in humans.^{35,36} Moreover, because our SPR data only show the binding affinities of monomeric IgG, it would be interesting to determine the interaction of multimeric, complexed hlgG1 with mFc γ RIII using a similar methodology to that described recently.³⁷

As expected from SPR data, when we examined hlgG1 antibodies in assays with mouse effectors, we found that they had limited ADCC activity but high ADCP activity (equivalent to mIgG2a), demonstrating that ADCP is the dominant effector mechanism for normal and

malignant B-cell targets in vivo, at least in mice. Furthermore and in direct contrast to their activity in vivo, both mouse isotypes had equivalent activity in mouse ADCC assays, but mIgG2a outperformed mIgG1 in mouse ADCP assays, correlating with the enhanced depleting activity of the mIgG2a antibodies in vivo. This conclusion is further supported by recent intravital imaging experiments that have shown liver Kupffer cells to be critical for effective antibody therapy.^{19,20}

Intriguingly, when using human CLL cells as targets, obinutuzumab displayed equivalent levels of phagocytosis to OBZ Gly. Although obinutuzumab showed superior ADCC activity as expected, OBZ Gly also displayed substantially higher levels of ADCC than both rituximab and ofatumumab. Therefore, the superior activity of obinutuzumab

over rituximab in vitro may largely reflect the inherent difference between type I and II mAbs. In keeping with these in vitro observations, our in vivo results in the hCD20 Tg E μ -TCL-1 CLL mouse model fit well with a recently reported trial of CLL patients given chlorambucil alone or in combination with either rituximab or obinutuzumab. In this trial, patients receiving obinutuzumab and chlorambucil demonstrated improved progression-free survival and higher rates of complete response compared with those receiving rituximab and chlorambucil, although the obinutuzumab arm did receive a larger dose of antibody.³⁸ In our CLL model, we compared identical doses of normally glycosylated hIgG1 isotype mAbs as monotherapy and still saw a benefit for OBZ Gly over rituximab, suggesting that, at least in part, some of the additional clinical benefit of obinutuzumab may be attributable to its type II character rather than enhanced Fc γ R interactions occurring as a result of its glycoengineered Fc.

With regard to ADCC, it is important to reconcile that there was no difference between murine type I and II antibodies, which could suggest the contribution of other Fc γ R-independent mechanisms. Based on our previous findings¹¹ and those presented here, we contend that the difference between murine types I and II B-cell depletion can be accounted for by ADCP differences alone and that no Fc γ R-independent mechanisms are required. This conclusion is supported by the observation that C3^{-/-} mice show no difference in B-cell depletion compared with wild-type controls (supplemental Figure 2). Recently, it has been shown that in the presence of competing IgG, obinutuzumab is superior to rituximab in ADCC experiments.⁹ In vivo then, in the presence of serum IgG, it may be hypothesized that obinutuzumab would be superior to OBZ Gly by virtue of its enhanced affinity for hFc γ RIIIa. With regard to serum antibody titers following the administration of hIgG1 antibody, we report in Figures 1D and 5B the superior pharmacokinetics of OBZ Gly compared with rituximab and that this correlated with their observed level of normal and malignant B-cell depletion, respectively. We have previously demonstrated a similar relationship in terms of mIgG2a antibodies whereby Ritm2a showed diminished pharmacokinetics compared with tositumomab and that this also correlated with B-cell depletion in hCD20 mice.¹¹

Rituximab ligation results in reorganization of CD20 into polarized caps; this has been proposed to augment ADCC by NK cells.³⁹ However, here we show that type II antibodies, which do not reorganize CD20 to the same extent as rituximab, are superior at ADCC. In their study, Rudnicka et al used established B-cell lines that we have previously shown not to modulate to the same extent as primary B cells. Therefore, although rituximab-induced capping may provide superior ADCC activity, in primary cells loss of antibody from the cell surface through modulation during the 2- to 4-hour ADCC assay may overcome this beneficial effect. This observation provides further impetus for discovering means to inhibit modulation in order to improve type I anti-CD20 efficacy.

We have previously demonstrated that increased levels of Fc γ RIIb augments antigenic modulation by anti-CD20 antibodies.¹² Given our previous findings and the results presented here, we would suggest that cells with increased surface expression of Fc γ RIIb would be even more affected in terms of antibody effector engagement, and so treatment with type I antibodies would be even

worse with cells expressing high levels of Fc γ RIIb when compared with those treated with type II.

In conclusion, we demonstrate that antigenic modulation has a detrimental effect on the known Fc γ R-dependent effector mechanisms, leading to superior activity of nonmodulatory type II reagents in vivo. Importantly, using a variety of mAb isotypes and in vitro assays assessing effector function, we demonstrate that ADCP is the dominant effector mechanism in the mouse in vivo. Therefore, future attempts to augment immunotherapy with this class of direct targeting mAbs should focus on new avenues investigating ways of enhancing ADCP and minimizing modulation.

Acknowledgments

The authors thank Prof Mark Shlomchik for providing hCD20 Tg mice and Dr Christian Klein, Roche Pharmaceutical Research and Early Development, for providing material and advice on the manuscript. The authors also thank Dr Egle, Dr Pekarsky, and Prof Croce for providing the E μ -TCL-1 mouse model and Dr Stefania Gobessi and Dimitar Efremov for advice on its use. The authors also thank the Experimental Cancer Medicine Centre-funded University of Southampton, Faculty of Medicine Human Tissue Bank (Human Tissue Authority licence 12009), for sample storage. The authors also thank Drs Francesco Forconi, Andrew Duncombe, Kathleen N. Potter, Andrew Steele, Ian Tracy, and Isla Wheatley for provision and assistance with clinical material as well as the National Blood Service Blood Transfusion unit at Southampton General Hospital.

This work was funded by Leukaemia and Lymphoma Research (grants 10055 and 12050) and Cancer Research UK (grants C34592/A12343, C1477/A10834, and C34999A/A18087).

Authorship

Contribution: T.R.W.T. helped design the research, performed experiments, analyzed results, produced figures, and wrote the paper with M.S.C. and S.A.B.; A.R., R.J.O., C.I.M., R.R.F., and L.N.D. performed experiments and produced figures; M.J.C. and K.L.C. characterized the hCD20 \times E μ -TCL-1 mouse model; P.G.H. and P.J.D. provided critical reagents for the study; and M.S.C. and S.A.B. designed the research, analyzed results, and wrote the manuscript with T.R.W.T.

Conflict-of-interest disclosure: M.S.C. acts as a consultant for Bio-Invent and has received research funding from them as well as from Roche. P.G.H. is an employee of Promega UK Ltd., Southampton, United Kingdom. The remaining authors declare no competing financial interests.

Correspondence: Mark S. Cragg, Antibody and Vaccine Group, Cancer Sciences Unit, University of Southampton Faculty of Medicine, Southampton General Hospital, Southampton, SO16 6YD, United Kingdom; e-mail: msc@soton.ac.uk.

References

- Glennie MJ, French RR, Cragg MS, Taylor RP. Mechanisms of killing by anti-CD20 monoclonal antibodies. *Mol Immunol*. 2007;44(16):3823-3837.
- Lim SH, Beers SA, French RR, Johnson PW, Glennie MJ, Cragg MS. Anti-CD20 monoclonal antibodies: historical and future perspectives. *Haematologica*. 2010;95(1):135-143.
- Boross P, Leusen JH. Mechanisms of action of CD20 antibodies. *Am J Cancer Res*. 2012;2(6):676-690.
- Teeling JL, French RR, Cragg MS, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. *Blood*. 2004;104(6):1793-1800.
- Teeling JL, Mackus WJ, Wiegman LJ, et al. The biological activity of human CD20 monoclonal antibodies is linked to unique

- epitopes on CD20. *J Immunol.* 2006;177(1):362-371.
6. Mössner E, Brünker P, Moser S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. *Blood.* 2010;115(22):4393-4402.
 7. Umaña P, Jean-Mairet J, Moudry R, Amstutz H, Bailey JE. Engineered glycoforms of an antineuroblastoma IgG1 with optimized antibody-dependent cellular cytotoxic activity. *Nat Biotechnol.* 1999;17(2):176-180.
 8. Golay J, Da Roit F, Bologna L, et al. Glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab. *Blood.* 2013;122(20):3482-3491.
 9. Herter S, Birk MC, Klein C, Gerdes C, Umana P, Bacac M. Glycoengineering of therapeutic antibodies enhances monocyte/macrophage-mediated phagocytosis and cytotoxicity. *J Immunol.* 2014;192(5):2252-2260.
 10. Jilani I, O'Brien S, Manshuri T, et al. Transient down-modulation of CD20 by rituximab in patients with chronic lymphocytic leukemia. *Blood.* 2003;102(10):3514-3520.
 11. Beers SA, French RR, Chan HT, et al. Antigenic modulation limits the efficacy of anti-CD20 antibodies: implications for antibody selection. *Blood.* 2010;115(25):5191-5201.
 12. Lim SH, Vaughan AT, Ashton-Key M, et al. Fc gamma receptor IIb on target B cells promotes rituximab internalization and reduces clinical efficacy. *Blood.* 2011;118(9):2530-2540.
 13. Vaughan AT, Iriyama C, Beers SA, et al. Inhibitory FcγRIIb (CD32b) becomes activated by therapeutic mAb in both cis and trans and drives internalization according to antibody specificity. *Blood.* 2014;123(5):669-677.
 14. Alduaij W, Ivanov A, Honeychurch J, et al. Novel type II anti-CD20 monoclonal antibody (GA101) evokes homotypic adhesion and actin-dependent, lysosome-mediated cell death in B-cell malignancies. *Blood.* 2011;117(17):4519-4529.
 15. Ivanov A, Beers SA, Walshe CA, et al. Monoclonal antibodies directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. *J Clin Invest.* 2009;119(8):2143-2159.
 16. Chan HT, Hughes D, French RR, et al. CD20-induced lymphoma cell death is independent of both caspases and its redistribution into triton X-100 insoluble membrane rafts. *Cancer Res.* 2003;63(17):5480-5489.
 17. Beers SA, Chan CH, James S, et al. Type II (tositumomab) anti-CD20 monoclonal antibody outperforms type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. *Blood.* 2008;112(10):4170-4177.
 18. Clynes RA, Towers TL, Presta LG, Ravetch JV. Inhibitory Fc receptors modulate in vivo cytotoxicity against tumor targets. *Nat Med.* 2000;6(4):443-446.
 19. Montalvo F, Garcia Z, Celli S, et al. The mechanism of anti-CD20-mediated B cell depletion revealed by intravital imaging. *J Clin Invest.* 2013;123(12):5098-5103.
 20. Gül N, Babes L, Siegmund K, et al. Macrophages eliminate circulating tumor cells after monoclonal antibody therapy. *J Clin Invest.* 2014;124(2):812-823.
 21. Weng WK, Levy R. Two immunoglobulin G fragment C receptor polymorphisms independently predict response to rituximab in patients with follicular lymphoma. *J Clin Oncol.* 2003;21(21):3940-3947.
 22. Wu J, Edberg JC, Redecha PB, et al. A novel polymorphism of FcγgammaRIIIa (CD16) alters receptor function and predisposes to autoimmune disease. *J Clin Invest.* 1997;100(5):1059-1070.
 23. Golay J, Cittera E, Di Gaetano N, 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(2):176-183.
 24. Li X, Wu J, Ptacek T, et al. Allelic-dependent expression of an activating Fc receptor on B cells enhances humoral immune responses. *Sci Transl Med.* 2013;5(216):216ra175.
 25. Jönsson F, Mancardi DA, Albanesi M, Bruhns P. Neutrophils in local and systemic antibody-dependent inflammatory and anaphylactic reactions. *J Leukoc Biol.* 2013;94(4):643-656.
 26. Tutt AL, French RR, Illidge TM, et al. Monoclonal antibody therapy of B cell lymphoma: signaling activity on tumor cells appears more important than recruitment of effectors. *J Immunol.* 1998;161(6):3176-3185.
 27. White AL, Chan HT, Roghianian A, et al. Interaction with FcγRIIb is critical for the agonistic activity of anti-CD40 monoclonal antibody. *J Immunol.* 2011;187(4):1754-1763.
 28. Hamaguchi Y, Xiu Y, Komura K, Nimmerjahn F, Tedder TF. Antibody isotype-specific engagement of Fcγgamma receptors regulates B lymphocyte depletion during CD20 immunotherapy. *J Exp Med.* 2006;203(3):743-753.
 29. Bruhns P. Properties of mouse and human IgG receptors and their contribution to disease models. *Blood.* 2012;119(24):5640-5649.
 30. Bichi R, Shinton SA, Martin ES, et al. Human chronic lymphocytic leukemia modeled in mouse by targeted TCL1 expression. *Proc Natl Acad Sci USA.* 2002;99(10):6955-6960.
 31. Rafiq S, Butchar JP, Cheney C, et al. Comparative assessment of clinically utilized CD20-directed antibodies in chronic lymphocytic leukemia cells reveals divergent NK cell, monocyte, and macrophage properties. *J Immunol.* 2013;190(6):2702-2711.
 32. Herter S, Herting F, Mundigl O, et al. Preclinical activity of the type II CD20 antibody GA101 (obinutuzumab) compared with rituximab and ofatumumab in vitro and in xenograft models. *Mol Cancer Ther.* 2013;12(10):2031-2042.
 33. van Meerten T, van Rijn RS, Hol S, Hagenbeek A, Ebeling SB. Complement-induced cell death by rituximab depends on CD20 expression level and acts complementary to antibody-dependent cellular cytotoxicity. *Clin Cancer Res.* 2006;12(13):4027-4035.
 34. Ferrara C, Grau S, Jäger C, et al. Unique carbohydrate-carbohydrate interactions are required for high affinity binding between FcγgammaRIII and antibodies lacking core fucose. *Proc Natl Acad Sci USA.* 2011;108(31):12669-12674.
 35. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcγgammaRIV: a novel FcR with distinct IgG subclass specificity. *Immunity.* 2005;23(1):41-51.
 36. Hirano M, Davis RS, Fine WD, et al. IgE immune complexes activate macrophages through FcγgammaRIV binding. *Nat Immunol.* 2007;8(7):762-771.
 37. Lux A, Yu X, Scanlan CN, Nimmerjahn F. Impact of immune complex size and glycosylation on IgG binding to human FcγRs. *J Immunol.* 2013;190(8):4315-4323.
 38. Goede V, Fischer K, Busch R, et al. Obinutuzumab plus chlorambucil in patients with CLL and coexisting conditions. *N Engl J Med.* 2014;370(12):1101-1110.
 39. Rudnicka D, Oszmiana A, Finch DK, et al. Rituximab causes a polarization of B cells that augments its therapeutic function in NK-cell-mediated antibody-dependent cellular cytotoxicity. *Blood.* 2013;121(23):4694-4702.