

# Interactions between Ibrutinib and Anti-CD20 Antibodies: Competing Effects on the Outcome of Combination Therapy

Martin Skarzynski<sup>1</sup>, Carsten U. Niemann<sup>1</sup>, Yuh Shan Lee<sup>1</sup>, Sabrina Martyr<sup>1</sup>, Irina Maric<sup>2</sup>, Dalia Salem<sup>1</sup>, Maryalice Stetler-Stevenson<sup>3</sup>, Gerald E. Marti<sup>1</sup>, Katherine R. Calvo<sup>2</sup>, Constance Yuan<sup>3</sup>, Janet Valdez<sup>1</sup>, Susan Soto<sup>1</sup>, Mohammed Z.H. Farooqui<sup>1</sup>, Sarah E.M. Herman<sup>1</sup>, and Adrian Wiestner<sup>1</sup>

## Abstract

**Purpose:** Clinical trials of ibrutinib combined with anti-CD20 monoclonal antibodies (mAb) for chronic lymphocytic leukemia (CLL) report encouraging results. Paradoxically, in preclinical studies, *in vitro* ibrutinib was reported to decrease CD20 expression and inhibit cellular effector mechanisms. We therefore set out to investigate effects of *in vivo* ibrutinib treatment that could explain this paradox.

**Experimental Design:** Patients received single-agent ibrutinib (420 mg daily) on an investigator-initiated phase II trial. Serial blood samples were collected pretreatment and during treatment for *ex vivo* functional assays to examine the effects on CLL cell susceptibility to anti-CD20 mAbs.

**Results:** We demonstrate that CD20 expression on ibrutinib was rapidly and persistently downregulated (median reduction 74%, day 28,  $P < 0.001$ ) compared with baseline. Concomitantly, CD20 mRNA was decreased concurrent with reduced NF- $\kappa$ B signaling. An NF- $\kappa$ B binding site in the promoter of *MS4A1*

(encoding CD20) and downregulation of CD20 by NF- $\kappa$ B inhibitors support a direct transcriptional effect. *Ex vivo*, tumor cells from patients on ibrutinib were less susceptible to anti-CD20 mAb-mediated complement-dependent cytotoxicity than pretreatment cells (median reduction 75%,  $P < 0.001$ ); however, opsonization by the complement protein C3d, which targets cells for phagocytosis, was relatively maintained. Expression of decay-accelerating factor (CD55) decreased on ibrutinib, providing a likely mechanism for the preserved C3d opsonization. In addition, ibrutinib significantly inhibited trogocytosis, a major contributor to antigen loss and tumor escape during mAb therapy.

**Conclusions:** Our data indicate that ibrutinib promotes both positive and negative interactions with anti-CD20 mAbs, suggesting that successfully harnessing maximal antitumor effects of such combinations requires further investigation. *Clin Cancer Res*; 22(1); 86–95. ©2015 AACR.

## Introduction

Ibrutinib is a small-molecule Bruton tyrosine kinase (BTK) inhibitor that has demonstrated exceptional safety and efficacy as a monotherapy for various B-cell malignancies (1), most notably chronic lymphocytic leukemia (CLL; ref. 2), mantle cell lymphoma (MCL; ref. 3), Waldenström's macroglobulinemia (4), and activated B-cell–like diffuse large B-cell lymphoma (ABC-DLBCL; ref. 5). While durable responses to single-agent ibrutinib

are common in CLL (2) and MCL (3), most patients do not experience a complete response. In an effort to improve upon the already impressive activity of ibrutinib monotherapy, many clinical trials are currently exploring the value of combining ibrutinib with monoclonal antibodies (mAb) targeting CD20 (6–10). Anti-CD20 mAbs are foremost among promising therapeutic partners for ibrutinib because they are widely applicable, well-tolerated targeted agents that add clinical benefit to frontline chemotherapy (11). Furthermore, ibrutinib may enhance the effect of CD20-targeting mAbs because it releases CLL cells into the blood from tissue sites (lymph nodes and bone marrow; ref. 12) and thus potentially facilitates the clearing of CLL cells through mAb effector mechanisms (13).

Preliminary data from ibrutinib and CD20-targeting mAb combination trials are promising (6–10); however, there is emerging evidence that ibrutinib may interfere with anti-CD20 mAb activity. Previous work by Bojarczuk and colleagues has shown that BCR pathway inhibitors, including ibrutinib, decrease CD20 expression on target cells *in vitro* (14). This may reduce the benefit of combination therapy because *in vitro* anti-CD20 mAb-mediated complement-dependent cytotoxicity (CDC) is tightly linked to CD20 expression levels (15–17). Indeed, Bojarczuk and colleagues showed that CD20-targeting mAb-mediated CDC against ibrutinib-treated B-cell lymphoma cell lines is diminished (14).

<sup>1</sup>Hematology Branch, National Heart, Lung, and Blood Institute, NIH, Bethesda, Maryland. <sup>2</sup>Department of Laboratory Medicine, Clinical Center, NIH, Bethesda, Maryland. <sup>3</sup>Flow Cytometry Unit, Laboratory of Pathology, Center for Cancer Research, NCI, NIH, Bethesda, Maryland.

**Note:** Supplementary data for this article are available at Clinical Cancer Research Online (<http://clincancerres.aacrjournals.org/>).

S.E.M. Herman and A. Wiestner share senior authorship.

**Corresponding Author:** Adrian Wiestner, Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Building 10, CRC 3-5140, 10 Center Drive, 20892-1202 Bethesda, MD 20814. Phone: 301-496-5093; Fax: 301-496-8396; E-mail: [wiestnera@mail.nih.gov](mailto:wiestnera@mail.nih.gov)

**doi:** 10.1158/1078-0432.CCR-15-1304

©2015 American Association for Cancer Research.

### Translational Relevance

Ongoing clinical trials combine anti-CD20 monoclonal antibodies (mAb) and the Bruton tyrosine kinase (BTK) inhibitor ibrutinib with promising preliminary results. However, preclinical studies have identified a number of negative interactions between ibrutinib and anti-CD20 mAbs that may reduce the efficacy of combination therapy. Given discordant preclinical and clinical observations, we analyzed tumor cells of patients with chronic lymphocytic leukemia (CLL) treated with ibrutinib for changes in CD20 expression and susceptibility to mAb-dependent killing. Ibrutinib substantially reduced CD20 expression on CLL cells *in vivo* and consequently diminished complement-mediated cell killing *in vitro*. Conversely, ibrutinib downregulated CD55, an inhibitor of complement activation, on CLL cells and reduced trogocytosis of anti-CD20 by macrophages, a major mechanism of antigen loss during mAb therapy. Ibrutinib therefore mediates both positive and negative effects on anti-CD20 mAb activity; harnessing these interactions could maximize the clinical benefit of ibrutinib and anti-CD20 mAb combination therapy.

In addition to mediating CDC, mAbs also recruit NK cells and phagocytes to eliminate target cells (18–20). Interestingly, anti-CD20 mAbs have been shown to mediate potent antibody-dependent cellular cytotoxicity (ADCC) even at low CD20 expression levels (16). Although ADCC may help to eliminate B-cell cancers with decreased CD20 expression, Kohrt and colleagues showed that ibrutinib decreases ADCC by directly inhibiting Fc receptor-dependent NK cell activation and cytotoxicity *in vitro*. Furthermore, ibrutinib diminished the *in vivo* antitumor efficacy of the HER2-specific mAb trastuzumab and the CD20-specific mAb rituximab when administered concurrently in a murine xenograft model of breast cancer and lymphoma, respectively (21). Similar to its effect on NK cells, ibrutinib has also been shown to decrease antibody-mediated phagocytosis of target cells by macrophages and neutrophils (22, 23). Taken together, these findings strongly suggest that ibrutinib antagonizes anti-CD20 mAb activity, but are difficult to reconcile with growing clinical evidence that combination therapy can be more effective than either agent alone (6–10).

Here we investigated the effects of *in vivo* single-agent ibrutinib therapy on CLL cells of patients on an investigator-initiated phase II study (NCT01500733). Specifically, we sought to determine how ibrutinib-mediated CD20 downregulation compares with other mechanisms of antigen loss and what role the complement system might play in the context of ibrutinib and anti-CD20 mAb combination therapy. Our findings suggest complex and diverging interactions between ibrutinib and CD20-specific mAbs and may inform how these two agents may best be combined in ongoing and future clinical trials.

## Materials and Methods

### Patients

The analyses presented here are based on samples from 48 patients with CLL enrolled in our ongoing, investigator-initiated phase II study of ibrutinib (NCT01500733). The study was approved by the local ethics committee; informed consent was

obtained from all patients in accordance with the Declaration of Helsinki. Briefly, both treatment naïve and relapsed/refractory patients with either del(17p) or TP53 mutations or patients over the age of 65 years were eligible and treated with ibrutinib 420 mg orally once daily until disease progression or the occurrence of intolerable side effects. Mutation status of the immunoglobulin heavy chain variable (*IGHV*) gene was assessed as described and CLL patients with <2% mutations were classified as unmutated (12, 24). Patient characteristics are summarized in Supplementary Table S1.

### Bone marrow immunohistochemistry

Bone marrow trephine biopsies were fixed in B-Plus fixative, embedded in paraffin, and processed for morphologic evaluation using standard procedures. Immunohistochemical studies with anti-CD20 and anti-CD79a antibodies (Ventana Medical System) were performed using immunoperoxidase staining procedures on an automated immunostainer (Ventana Medical System) according to the manufacturer's instructions. Results were assessed semiquantitatively by two trained hematopathologists on a scale from 0% to 100%. Images were obtained via digital microscopy using an Olympus BX-51 microscope (Olympus America) equipped with a DPlan 40/0.65 numeric aperture objective and captured using an Olympus DP70 digital camera system.

### Evaluation of CD20 antigen expression

CD20 mean fluorescence intensity (MFI) values were obtained before and on ibrutinib using peripheral blood mononuclear cells (PBMC) prepared by density-gradient centrifugation (Lymphocyte Separation Media; ICN Biomedicals) and viably frozen in 90% FBS, 10% dimethyl sulfoxide (Sigma) in liquid nitrogen. Enumeration of anti-CD20-binding sites, antibody-binding capacity (ABC), was performed on fresh PBMCs, and fresh bone marrow cell suspensions in the clinical laboratory using the Quantibrite system (BD Biosciences) as previously described (25). Cells were analyzed on a FACSCanto II flow cytometer using FACS-DIVA 6.1.1, FCSEXPRESS 4 (BD Biosciences) and FlowJo 10 software (TreeStar). In select experiments, the role of NF- $\kappa$ B on CD20 expression was measured using the NF- $\kappa$ B inhibitor Bay 11-7028 (Santa Cruz Biotechnology).

### Gene expression

Total RNA was extracted from CD19+ PBMCs or lymph node core biopsies using RNeasy kits (Qiagen) and cDNA was prepared using the High Capacity cDNA RT Kit (Applied Biosystems). Expression of *MS4A1* (CD20) mRNA was quantified by RT-PCR using inventoried TaqMan primers on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). For lymph node core biopsies, CD20 expression was normalized to CD19 mRNA expression. Expression of six previously validated NF- $\kappa$ B target genes were quantified by RT-PCR on TaqMan Custom Arrays (microfluidic cards; Applied Biosystems) and averaged into a NF- $\kappa$ B signature score as previously described (26).

### CDC, complement deposition, and complement regulatory protein expression

For analysis of CDC, cells were incubated with or without 10  $\mu$ g/mL ofatumumab (commercial supply from GlaxoSmithKline) for 2 hours in the presence of 33% normal human serum (Innovative Research) as a source of complement. The proportion of

lysed CD19<sup>+</sup> cells was assessed by TO-PRO3 (Life Technologies) staining. Complement deposition was measured using the FITC labeled 1H8 mAb (Cedarlane) that binds all forms of C3 (27). Cell surface expression levels were measured on CD19<sup>+</sup> cells using PE-labeled mAbs against CD55, CD46, and CD59 (BD Biosciences). Samples were analyzed using FACSCanto II and Fortessa LSR flow cytometers (BD Biosciences) and FlowJo 10 software (TreeStar). For all *ex vivo* experiments, CLL cells were cultured in AIM V media (Life Technologies).

**FACS and *in vitro* analysis of CD20 subpopulations**

CLL PBMC samples were stained with CD19-FITC and CD20-PE mAbs (BD Biosciences), triple washed, and resuspended in PBS containing 1% FBS and penicillin–streptomycin (Life Technologies) before being sorted on a FACS Aria II cell sorter (BD Biosciences). CD19<sup>+</sup> cells that fell in the top or bottom <15% of the CD20<sup>+</sup> cell distribution were collected, acid washed as previously described (28) to remove staining mAbs, and then cultured in AIM-V media (Life Technologies). Cells were kept at 4°C at all times before being put into culture at 37°C. Cultured cells were treated with vehicle or 1 μmol/L ibrutinib for 48 hours as described previously (14) and finally assessed for CD20 expression changes and cell lysis resulting from ibrutinib treatment.

**Trogocytosis assays**

CLL cells from patients were stained with Vybrant CFDA SE Cell Tracer (Life Technologies), bound with Alexa-Fluor 594 (Life Technologies) labeled ofatumumab, coincubated with THP1 cells (ATCC), and then assessed for Cell Tracer and Alexa-Fluor 594 staining as previously described (29, 30). THP1 cells were incubated in RPMI1640 media (Life Technologies) containing various concentrations of ibrutinib (Selleckchem) or vehicle for 1 hour before coincubation with CLL cells. To abrogate the effect of ibrutinib, THP1 cells were pretreated overnight with 1 μmol/L phorbol 12-myristate 13-acetate (PMA; Sigma) or vehicle (DMSO; Sigma).

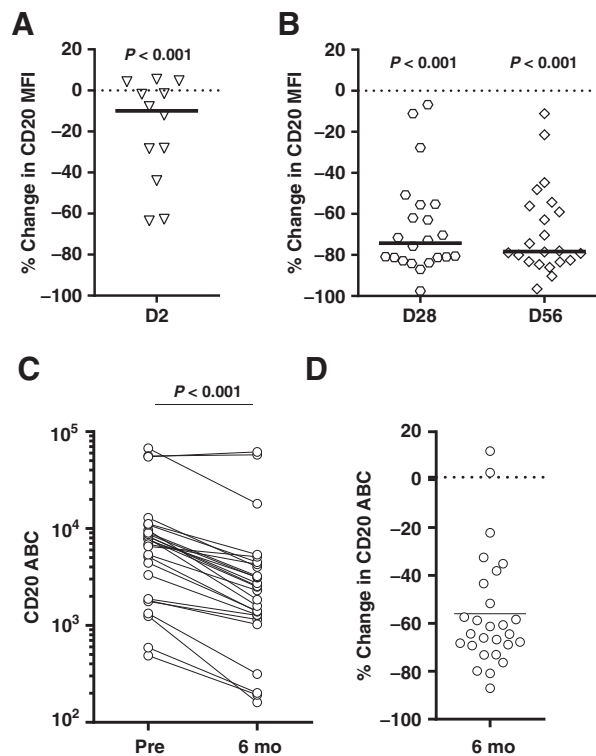
**Statistical analyses**

To determine whether data were normally distributed, the D’Agostino and Pearson (omnibus K<sup>2</sup>) normality test was used. To compare measurements from patients either across time or treatment, two-tailed Student paired *t* tests were applied to normally distributed (Gaussian) datasets, otherwise matched-pairs Wilcoxon signed rank tests were used. Correlations were evaluated using Pearson test (Prism 5, GraphPad).

**Results**

**Ibrutinib decreases CD20 expression on CLL cells *in vivo***

First, we confirmed the *in vitro* observations of CD20 down-regulation on CLL cells by ibrutinib reported by Bojarczuk and colleagues, in clinical samples of patients treated with single-agent ibrutinib in our phase II clinical trial (NCT01500733). We measured CD20 expression on CLL cells in the peripheral blood at baseline and during treatment by flow cytometry. On day 2, after only one dose of ibrutinib, CD20 MFI on CLL cells was already decreased in half of the patients tested, albeit with wide inter-patient variability (*P* = 0.02; Fig. 1A). However, after one (day 28) and two cycles (day 56) of ibrutinib, CD20 MFI on CLL cells was decreased in all patients evaluated (*n* = 22, *P* < 0.0001; Fig. 1B). At day 28, the median decrease compared with baseline was 74%



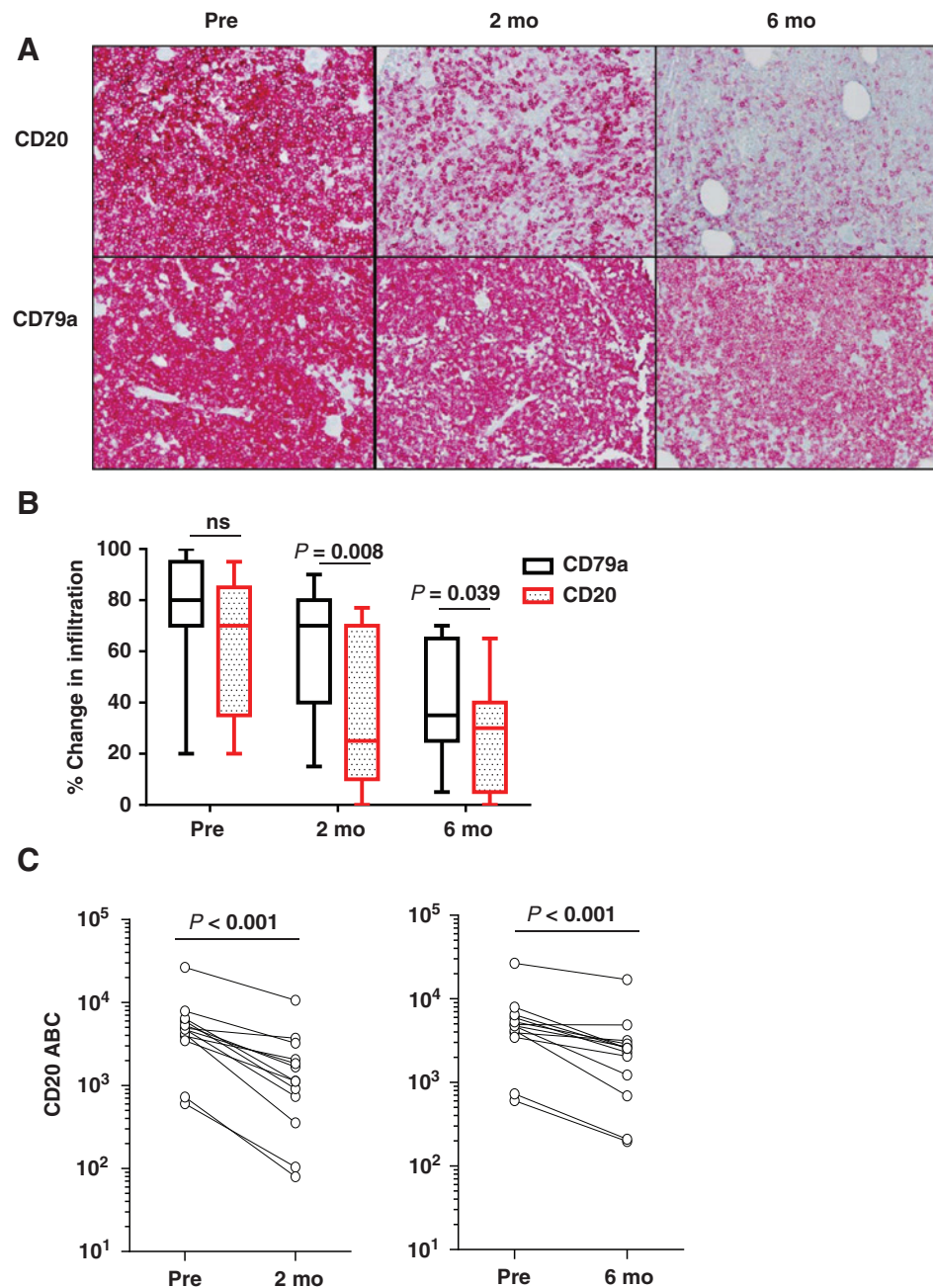
**Figure 1.** *In vivo* ibrutinib treatment decreases CD20 expression on circulating CLL cells. A and B, flow cytometric quantification of the change in CD20 expression on leukemic (CD19<sup>+</sup>, CD5<sup>+</sup>) cells in the peripheral blood of CLL patients (A) after a single dose (day 2) of ibrutinib (*n* = 12) and (B) on days 28 and 56 (*n* = 22) compared with pretreatment. Lines depict median values. C, CD20 ABC values of circulating CLL cells from patients before ibrutinib treatment and at 6 months (*n* = 27). D, percent change in CD20 ABC at 6 months (*n* = 27). ABC measurements were made on fresh samples using Quantibrite beads. *P* values were calculated using the matched-pairs Wilcoxon signed-rank test.

[interquartile range (IQR): 55–82] and at day 56 it was 78% (IQR: 56–83). Supplementary Figure S1 shows the decrease in CD20 expression at day 28 of a representative CLL patient. Reductions in CD20 by ibrutinib were independent of TP53 aberrations (24) and IGVH mutational status (Supplementary Fig. S2A–S2D). In 27 patients, anti-CD20 ABC was quantified using fresh PBMC samples obtained during treatment and after 6 months on ibrutinib using the BD Quantibrite system. In 25 of 27 patients (92.6%), CD20 expression was reduced on treatment compared with baseline. Notably, the two patients that did not show decreased CD20 on ibrutinib had unusually high baseline CD20 expression. The median pretreatment CD20 ABC was 7,572 sites per cell but varied widely from <500 to 67,000 sites per cell (Fig. 1C). The median decrease at 6 months was 64% (IQR, 43–69) compared with the matching pretreatment samples (*P* = 0.001; Fig. 1C).

Bone marrow biopsies were obtained pretreatment, and at 2 months and 6 months on treatment. To assess the degree of tumor infiltration, biopsies were routinely stained with anti-CD20 and anti-CD79a antibodies by immunohistochemistry. Upon review, we noted that in addition to a reduction in tumor infiltration, evidenced by fewer cells staining for CD79a, the intensity of anti-CD20 staining decreased most notably at 2 months on treatment

Downloaded from http://aacrjournals.org/clinccancerres/article-pdf/22/1/86/2030357/86.pdf by guest on 24 August 2022

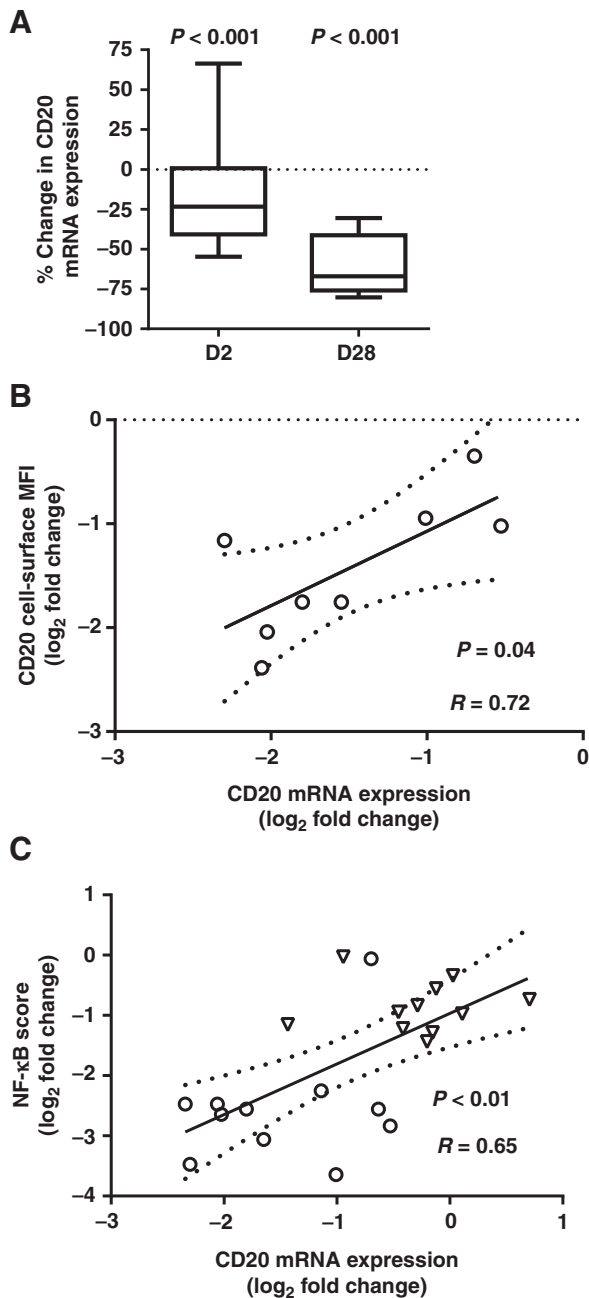




(Fig. 2A for a representative patient; Fig. 2B summarizes data for all patients evaluated). To confirm this decreased expression of CD20 on bone marrow-resident CLL cells, we obtained quantitative measurements of anti-CD20 ABC on CLL cells in fresh bone marrow aspirates. The anti-CD20 ABC on CLL cells in the bone marrow of thirteen patients was significantly decreased after two months on ibrutinib ( $n = 13$ ,  $P = 0.003$ ; Fig. 2C, left) and remained suppressed after 6 months of treatment ( $n = 13$ ,  $P = 0.001$ ; Fig. 2C, right). The median decrease in CD20 expression at 2 months was 69% (IQR, 60–85) and at 6 months it was 60% (IQR, 38–65). Thus, a decrease in the expression of CD20 is equally present in peripheral blood and bone marrow-resident CLL cells throughout at least the first 6 months of therapy.

#### Inhibition of CD20 mRNA expression by ibrutinib correlates with reduced NF- $\kappa$ B activity

To assess whether decreased CD20 cell surface expression was correlated with a reduction in CD20 mRNA (encoded by the *MS4A1* gene), we performed quantitative RT-PCR on RNA from purified CLL cells. Already within 24 hours of initiating ibrutinib (day 2), a trend toward decreased CD20 mRNA expression was seen in most patients, although it did not reach statistical significance (Fig. 3A). After one cycle (day 28), CD20 mRNA expression in the CLL cells was decreased in all patients assessed, with a median reduction of 67% ( $n = 12$ ,  $P < 0.001$ ; Fig. 3A). Reduction in CD20 mRNA and CD20 cell surface expression correlated well ( $n = 8$ ,  $R = 0.72$ ,  $P = 0.04$ ; Fig. 3B).



**Figure 3.** Inhibition of CD20 mRNA expression by ibrutinib correlates with reduced NF- $\kappa$ B activity. A, expression of CD20 mRNA was quantified by RT-PCR in CD19<sup>+</sup> PBMCs on day 2 ( $n = 8$ ) and after 1 month (day 28,  $n = 12$ ) on ibrutinib. The percent change on treatment compared with baseline is shown. B, correlation between CD20 mRNA (*MS4A1* gene) expression and CD20 cell surface expression on CLL cells on day 28,  $n = 8$ . C, correlation between NF- $\kappa$ B signature score and CD20 gene expression. LN core biopsies (day 2,  $n = 11$ ) are shown as triangles, whereas day 28 peripheral blood samples are represented as circles ( $n = 12$ ). Panels B and C are shown on a  $\log_2$  scale.  $P$  values for all comparisons were calculated using the matched-pairs Wilcoxon signed-rank test. Correlations were determined using the Pearson test. The whiskers on all box plots depict the range (minimum and maximum values).

We previously demonstrated that ibrutinib decreases the expression of NF- $\kappa$ B target genes in CLL cells (26). To investigate the possibility that NF- $\kappa$ B acts as a mechanistic link between BTK inhibition and CD20 downregulation, we scanned the *MS4A1* gene (NCBI Entrez Gene ID: 931) for the canonical NF- $\kappa$ B binding consensus sequence (31, 32) and found one (GGGACTTTCC, 6594-6603) downstream of the transcriptional start site (6567) and another (GGGGTCCCCC, 6957-6966) between exons 1 and 2. We next compared the effect of ibrutinib on the averaged expression of six NF- $\kappa$ B target genes, referred to as NF- $\kappa$ B signature score (26), with its effect on CD20 expression by quantitative RT-PCR. There was a strong positive correlation ( $R = 0.65$ ,  $P < 0.01$ ) between the treatment-induced downregulation of CD20 mRNA and the NF- $\kappa$ B signature score ( $n = 22$ ; Fig. 3C).

To further examine the role of NF- $\kappa$ B in regulating CD20 expression, we tested the *in vitro* effect of the NF- $\kappa$ B inhibitor Bay11-7028 on primary CLL cells collected from patients prior to the start of ibrutinib. CD20 surface staining was decreased in a dose-dependent manner by *in vitro* treatment with the NF- $\kappa$ B inhibitor ( $n = 8$ ,  $P < 0.001$ ; Supplementary Fig. S3). Together, these data suggest that inhibition of NF- $\kappa$ B signaling by ibrutinib decreases the expression of CD20 through a transcriptional mechanism.

#### Ibrutinib decreases ofatumumab-mediated CDC

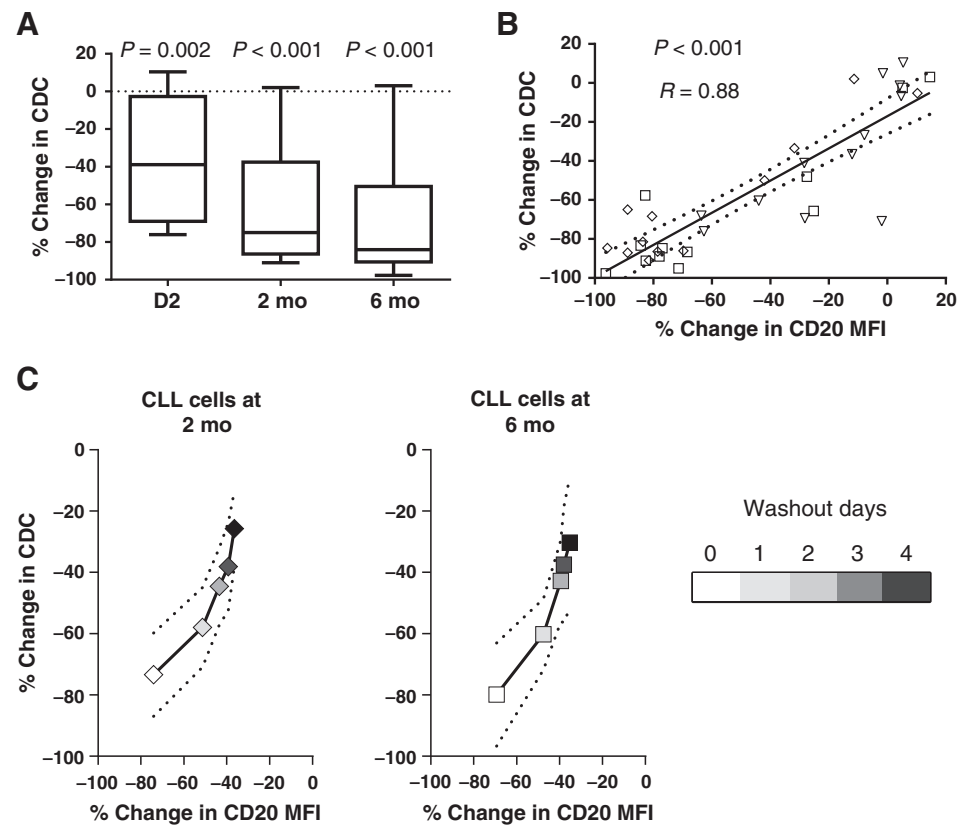
To characterize the functional consequences of reduced CD20 expression on CLL cells during treatment with ibrutinib, we measured ofatumumab induced CDC *ex vivo*. Compared with the matched pretreatment samples, CDC was already significantly reduced in CLL cells obtained on day 2 of ibrutinib and decreased further with continued therapy ( $n = 12$ ,  $P \leq 0.02$ ; Fig. 4A). After 2 and 6 months of therapy, the median reduction in ofatumumab-induced CDC was 75% (IQR 38–86) and 84% (IQR 50–91), respectively. Across all patients and time points, there was a strong correlation between the decrease in CD20 expression and the ability of ofatumumab to induce CDC ( $R = 0.88$ ;  $P < 0.001$ ; Fig. 4B).

To assess the possibility that the observed loss of CD20 was due to selective elimination of CLL cells with the highest CD20 expression, we sorted PBMCs obtained from CLL patients into CD20-bright and -dim fractions and measured their CD20 expression and viability after 48 hours of *in vitro* treatment with ibrutinib or vehicle. Subpopulations from the same CLL patient responded similarly to treatment in terms of the change in percent viability and CD20 expression ( $n = 4$ , data not shown). These results argue against a major role for preferential killing of CD20-bright CLL cells in the observed downregulation of CD20 during ibrutinib therapy.

To investigate whether ibrutinib-mediated downregulation of CD20 and the resultant loss of CDC are permanent, we cultured CLL cells from patients on ibrutinib that demonstrated a treatment-induced decrease in CD20 expression for up to 96 hours *in vitro* and reassessed CD20 expression and CDC at 24-hour intervals. The recovery in CD20 expression was rapid, already apparent after 24 hours of culture, and reached 60% to 70% of pretreatment levels by 96 hours (Fig. 4C). In parallel, CDC also recovered reaching 70% to 80% of pretreatment values by 96 hours (Fig. 4C). These results indicate that the effects of ibrutinib on CDC and CD20 expression are rapidly reversible.

**Figure 4.**

Ibrutinib decreases ofatumumab-mediated complement-dependent cytotoxicity. A, flow cytometric analysis of CDC mediated by ofatumumab in 33% normal human serum (NHS) against CLL cells obtained from 12 patients before treatment (pre), the day after treatment began (day 2), and at 2 and 6 months. B, correlation of ibrutinib effect on CD20 expression (x-axis) and CDC (y-axis) in the patients shown in A,  $n = 12$ . C, CLL samples from the 10 patients with  $>20\%$  decrease in CD20 expression and CDC were reassessed for CD20 expression and susceptibility to CDC after 1 to 4 days of culture in AIM V media. Symbols represent mean values.  $P$  values for all comparisons were calculated using the two-tailed Student paired  $t$  test. Correlations were determined using the Pearson test. The whiskers on all box plots depict the range (minimum and maximum values).



### Downregulation of CD55 by ibrutinib rescues ofatumumab-mediated complement deposition

While ofatumumab-mediated CDC was clearly compromised concurrent with CD20 downregulation, the biologic effects of the complement system are not limited to direct cytotoxicity. In particular, the complement component C3 is deposited, and remains covalently attached, to cells targeted by a complement-activating antibody such as ofatumumab; priming them for phagocytosis. We therefore measured the various forms of deposited C3 (denoted simply as C3d) on CLL cells after ofatumumab treatment *ex vivo*. The C3d opsonization of CLL cells correlated well to the degree of CDC achieved, both pretreatment ( $n = 16$ ,  $R = 0.81$ ,  $P < 0.001$ ; Fig. 5A, left) and after 2 months on ibrutinib ( $n = 16$ ,  $R = 0.73$ ,  $P < 0.001$ ; Fig. 5A, right). In parallel to CDC, C3d opsonization was also diminished on ibrutinib; however, the reduction in CDC in ibrutinib-treated samples was significantly more pronounced (median reduction 67%; IQR 53–79) than the decrease in C3d (median reduction 30%; IQR 11–54;  $n = 16$ ,  $P < 0.001$ ; Fig. 5B). Thus, complement deposition was relatively preserved despite decreased CD20 expression, prompting us to investigate the expression of regulators of complement activation on CLL cells.

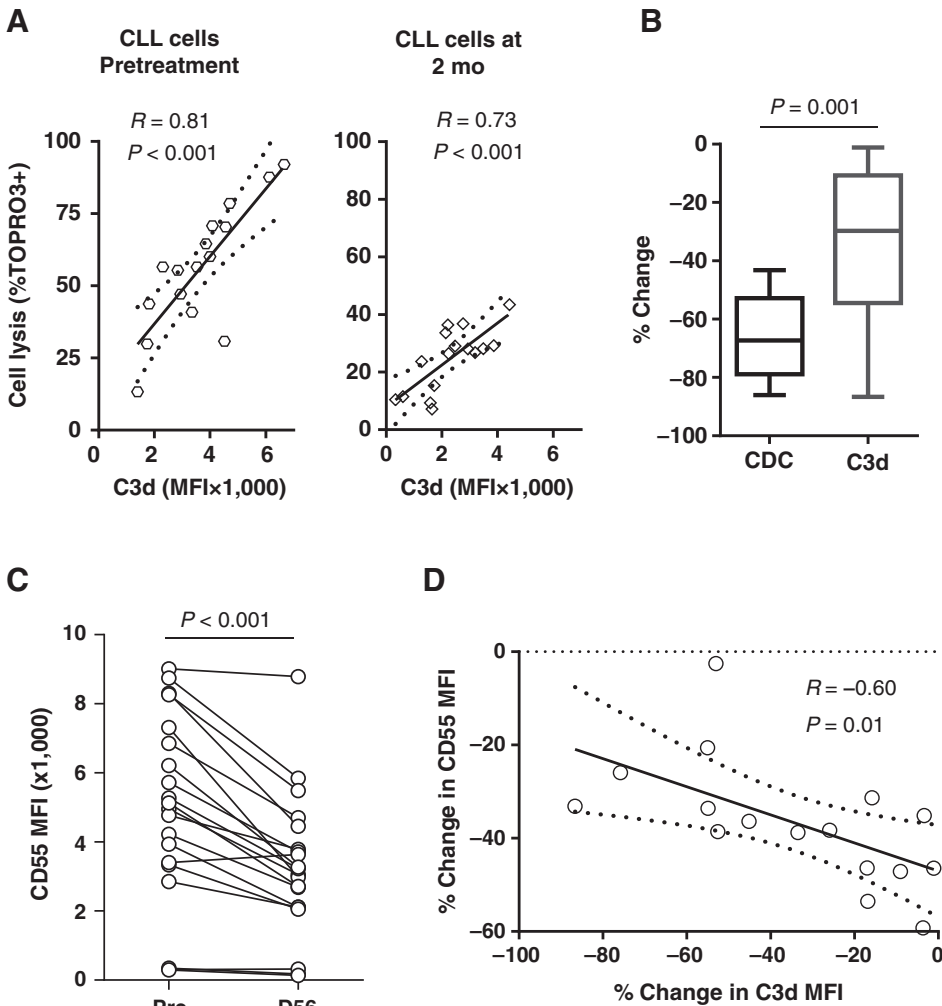
We compared expression of complement regulatory proteins (CRP) on CLL cells at 2 months compared with baseline. Notably, expression of CD55 (decay accelerating factor) was significantly diminished in patients on ibrutinib (median decrease 37%, IQR 32%–46%,  $P < 0.001$ ; Fig. 5C). CD55 promotes the decay of proteases that amplify C3d opsonization (15, 33). The reduction in CD55 expression on ibrutinib was inversely correlated to the decrease in C3d on CLL cells treated with ofatumumab *ex*

*vivo* ( $n = 16$ ,  $R = -0.60$ ,  $P = 0.01$ ; Fig. 5D). Downregulation of CD55 by ibrutinib could explain the minimal reduction in C3d opsonization compared with CDC (Fig. 5B). In contrast, CD46 (membrane cofactor protein) and CD59 (protectin) levels were not consistently affected (median change  $-5.4\%$  and  $4.2\%$ , respectively; Supplementary Fig. S4A and S4B). These results implicate CD55 as an important inhibitor of CLL cell surface opsonization by C3d. Furthermore, diminished expression of CD55 may play a significant role in augmenting the complement activation mediated by ofatumumab in the face of downregulated CD20 expression.

### Ibrutinib reduces ofatumumab-induced loss of CD20 by inhibiting trogocytosis

CLL cells have relatively lower CD20 expression than other B-cell malignancies. In addition, it is well established that anti-CD20 antibody therapy promotes the rapid loss of the antigen through trogocytosis (34). We used the methods of Beum and colleagues to study whether ibrutinib affects trogocytosis mediated by ofatumumab (28–30).

Figure 6A shows a representative experiment in which CFSE-labeled CLL cells were coincubated with THP-1 cells (monocyte cell line). At the beginning of coincubation, Alexa Fluor 594 labeled ofatumumab-stained CLL cells but did not bind to the monocyte cell line (Fig. 6A, left). After 2 hours of coculturing THP-1 cells with ofatumumab-bound CLL cells, CD20-labeled antibody was transferred to THP-1 cells, while a CD20-negative CLL population emerged (Fig. 6A, middle). In contrast, THP-1 cells treated with ibrutinib for 1 hour showed a dose-dependent decrease in trogocytosis (Fig. 6A, right; Fig. 6B;  $n = 9$ ,  $P <$



**Figure 5.** Inhibition of CD55 expression by ibrutinib rescues ofatumumab-mediated complement opsonization. A, correlation between cell lysis and the degree of C3d opsonization of CLL cells exposed to ofatumumab and normal human serum *ex vivo*. CLL cells were obtained from patients ( $n = 16$ ) pretreatment and at 2 months on ibrutinib. B, the percent change in ofatumumab-mediated CDC and C3d opsonization on ibrutinib compared with baseline ( $n = 16$ ). C, the mean fluorescence intensity of CD55 on the surface of CLL cells obtained from patients before ibrutinib (pre) and on day 56 ( $n = 20$ ). D, correlation between the percent change in CD55 expression and C3d opsonization ( $n = 16$ ).  $P$  values for all comparisons were calculated using the two-tailed Student paired  $t$  test. Correlations were determined using the Pearson test. The whiskers on all box plots depict the range (minimum and maximum values).

0.01). This appears to be a direct inhibitory effect of ibrutinib as trogocytosis mediated by THP-1 cells is restored when BTK blockade by ibrutinib is bypassed by pretreatment with the PKC activator phorbol myristate acetate (Fig. 6B, far right).

**Discussion**

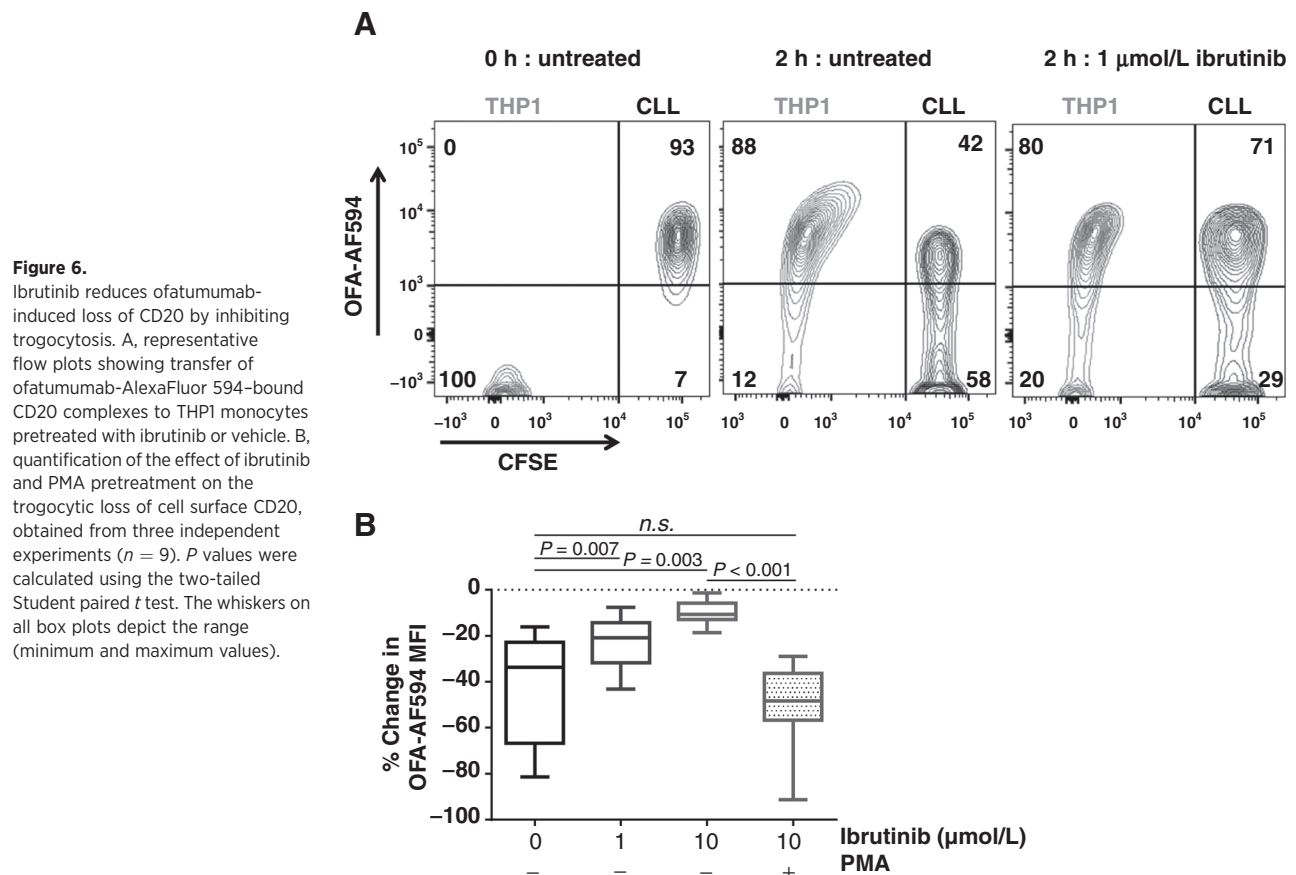
Combinations of kinase inhibitors with monoclonal antibodies (mAb) may improve upon the depth of responses obtained with single-agent therapy and are currently being explored in clinical studies (6–10). However, preclinical reports show multiple negative interactions between these two classes of drugs (14, 21–23). Here, we demonstrate that the BTK inhibitor ibrutinib exerts several effects on CLL cells and mAb effector mechanisms, some of which could reduce, while others could enhance, the efficacy of the combination. We found that CD20 expression was downregulated on tumor cells in peripheral blood and bone marrow of CLL patients treated with ibrutinib. We demonstrate that CD20 downregulation by ibrutinib is transcriptional and tightly correlated to inhibition of NF- $\kappa$ B signaling. In parallel to the decrease in CD20 expression, we observed a striking reduction in CDC mediated by the CD20-specific mAb ofatumumab.

Despite diminished CDC, complement deposition on CLL cells was relatively well maintained, which may be due to the concomitant downregulation of the complement regulatory protein CD55 on CLL cells by ibrutinib. In addition, ibrutinib inhibited antibody-mediated trogocytosis *in vitro*, a major mechanism of CD20 loss during mAb infusion (28–30, 35–38), suggesting that ibrutinib may have positive as well as negative effects on CD20 expression.

We provide the first demonstration of CD20 downregulation on CLL cells by ibrutinib *in vivo*, confirming previous *in vitro* studies by others (14). Previously, Bojarczuk and colleagues have shown that *in vitro* treatment with ibrutinib and other BCR pathway inhibitors decreases CD20 mRNA and protein expression. Our data implicate NF- $\kappa$ B, which is downstream of BTK in the BCR pathway (39, 40), as a link between BCR signaling and CD20 expression. Previous work by our group demonstrated that BCR and NF- $\kappa$ B signaling are both diminished upon ibrutinib treatment in a closely correlated manner (26). Here, we identified an NF- $\kappa$ B binding site at the CD20 gene transcription start site and show a tight correlation between ibrutinib-mediated changes in NF- $\kappa$ B and CD20 mRNA expression. In addition, we found a direct NF- $\kappa$ B inhibitor decreased CD20 surface expression on CLL

Downloaded from <http://aacrjournals.org/clinccancerres/article-pdf/22/1/86/2030537/86.pdf> by guest on 24 August 2022





cells *in vitro*. Taken together, our data suggest inhibition of BCR-mediated NF- $\kappa$ B signaling as a mechanism for the *in vivo* reduction in CD20 surface expression during ibrutinib treatment.

Next, we examined the functional consequences of CD20 downregulation on the efficacy of anti-CD20 mAb therapy. CDC mediated by ofatumumab against CLL cells from ibrutinib-treated patients was severely impaired and the reduction of CDC *ex vivo* was strongly correlated to the *in vivo* downregulation of CD20 expression. These findings are consistent with the well-established connection between CD20 expression levels and the efficacy of anti-CD20 mAb-mediated CDC (15–17). Notably, *in vivo* ibrutinib-treated CLL cells recovered CD20 expression and CDC susceptibility during *in vitro* culture, indicating that ibrutinib-mediated CD20 downregulation is reversible.

Sequential administration of these agents could conceivably mitigate the effects of ibrutinib-mediated antigen loss. Indeed, administering ibrutinib before or after the anti-CD20 mAb rituximab yielded better antitumor activity in a mouse lymphoma model than concurrent administration (21). Furthermore, a clinical trial in CLL showed that starting ibrutinib first, followed by concurrent administration of ibrutinib and ofatumumab resulted in an objective response rate of 100% and was superior to sequential treatment with ofatumumab before ibrutinib or simultaneous administration of ibrutinib and ofatumumab (8). These data suggest that the benefit of combination therapy may depend on the sequence of administration.

Although ofatumumab-mediated CDC was strongly reduced, we noted that *ex vivo* opsonization of CLL cells by the complement

protein C3 upon exposure to the mAb was relatively well-maintained. C3 is the central component of all complement activation pathways and a necessary element of convertases that deposit additional C3 molecules and initiate the formation of the membrane attack complex that is responsible for CDC (41). This prompted us to investigate whether ibrutinib modulated the expression of complement regulatory proteins, of which CD55 (decay accelerating factor) was found to be potently inhibited on ibrutinib. CD55 catalyzes the inactivation of convertases that amplify complement deposition (33).

The decrease in CD55 expression correlated with cell surface deposited C3 (C3d). Thus, the nonlytic functions of complement are partially preserved, despite reduced CD20 expression, when CD55 is concurrently downregulated. This suggests complement-opsonized cells could be eliminated by complement receptor-mediated phagocytosis, a mechanism of cell clearance distinct from Fc-receptor dependent elimination of antibody-opsonized cells (18, 42–44). The biologic implications of complement opsonization warrant further study in combination therapy clinical trials.

Fc-receptor-mediated phagocytosis is an important mechanism of antibody activity (45, 46). However, Fc-receptor bearing cells can also contribute to antigen loss through trogocytosis, a process in which the effector cells takes up the antibody-antigen complex from a target cell but fails to engulf it entirely (34). It has been shown that trogocytosis causes near complete antigen loss within hours of mAb infusion in CLL patients (35, 36). Different strategies for preventing trogocytosis have been explored,



including low or fractionated dosing of anti-CD20 mAbs (47–49). However, no pharmacologic inhibitors of trogocytosis have been developed for clinical use. Here, we report that ibrutinib potently inhibits trogocytosis of the ofatumumab-CD20 complex from CLL cells *in vitro*. Thus, ibrutinib could potentially avert antigen loss through trogocytosis during treatment with anti-CD20 antibodies. Assessing CD20 expression and C3d deposition on tumor cells before and after infusion of therapeutic mAbs in clinical trials that investigate combinations of antibody therapy with ibrutinib could clarify the relevance of these diverging effects.

A recent clinical trial demonstrated that combination therapy with ibrutinib and ofatumumab can be effective against relapsed/refractory CLL. Specifically, lead-in treatment with single-agent ibrutinib followed by concurrent administration of ofatumumab and ibrutinib achieved a 100% overall response rate and an 88.7% 12-month progression-free survival rate (8). Notably, this schedule appeared to be superior to immediately beginning concurrent administration of ibrutinib and ofatumumab. The results of this clinical trial suggest that the combination can yield clinical benefit and that the outcome of combination therapy depends heavily on the schedule of administration. Further data will be necessary to conclusively determine the optimal administration sequence that can minimize the negative impact of ibrutinib on anti-CD20 mAb activity and make the most of the positive interactions between these therapeutic agents.

Clinical trials of ibrutinib in combination with anti-CD20 antibodies in CLL have reported encouraging results (6, 8, 9). Ibrutinib-mediated egress of tissue-resident CLL cells into the peripheral blood (12) and inhibition of CLL homing to tissue sites (50) could increase exposure of CLL cells to antibodies and serum complement and thus enhance the effect of mAb therapy. In addition, downregulation of CD20 and CD55, inhibition of effector cell function and mitigation of Fc-receptor dependent CD20 loss through trogocytosis likely impact the efficacy of mAb therapy. In extension to previous studies demonstrating a multitude of negative effects of ibrutinib on anti-CD20 mAb activity (14, 21–23), the data presented here suggest that beneficial interactions between ibrutinib and anti-CD20 mAbs could mitigate some of the negative effects. The kinetics and extent of positive and negative interactions and their net effect should be examined further in clinical trials that assess the utility of combining ibrutinib with anti-CD20 mAbs. Our data indicate that maximizing the antitumor activity of combination therapy

requires successful harnessing of the opposing effects of ibrutinib on tumor cells and effector functions of anti-CD20 mAbs.

### Disclosure of Potential Conflicts of Interest

C.U. Niemann is a consultant/advisory board member for Janssen and Roche. G.E. Marti is a consultant/advisory board member for Amerimmune Laboratory. A. Wiestner reports receiving a commercial research grant from and is a consultant/advisory board member for Pharmacyclics. No potential conflicts of interest were disclosed by the other authors.

### Authors' Contributions

**Conception and design:** M. Skarzynski, C.U. Niemann, S.E.M. Herman, A. Wiestner

**Development of methodology:** M. Skarzynski, C.U. Niemann, D. Salem, M. Stetler-Stevenson, S.E.M. Herman

**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** M. Skarzynski, C.U. Niemann, Y.S. Lee, S. Martyr, I. Maric, D. Salem, M. Stetler-Stevenson, C. Yuan, J. Valdez, S. Soto, S.E.M. Herman

**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** M. Skarzynski, C.U. Niemann, I. Maric, D. Salem, K.R. Calvo, C. Yuan, S.E.M. Herman

**Writing, review, and/or revision of the manuscript:** M. Skarzynski, C.U. Niemann, D. Salem, M. Stetler-Stevenson, S.E.M. Herman, A. Wiestner

**Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases):** M. Skarzynski, C. Yuan

**Study supervision:** M.Z.H. Farooqui, A. Wiestner

**Other (saw patients in the clinic and participated in the bone marrow biopsy reviews of this patient population):** G.E. Marti

**Other (clinical care):** M.Z.H. Farooqui

### Acknowledgments

The authors thank the patients who participated in this trial and their families, Lone B. Pedersen and Christian H. Geisler for providing IGHV mutational data, and helpful comments from employees of Pharmacyclics, who reviewed a draft of this article.

### Grant Support

This work was funded by the Intramural Research Program of the NHLBI and NCI. C.U. Niemann was supported by The Danish Cancer Society. Pharmacyclics provided study drug and research support. This work was also supported by the Intramural Research Program of the NHLBI.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received June 3, 2015; revised July 30, 2015; accepted August 4, 2015; published OnlineFirst August 17, 2015.

### References

- Advani RH, Buggy JJ, Sharman JP, Smith SM, Boyd TE, Grant B, et al. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765) has significant activity in patients with relapsed/refractory B-cell malignancies. *J Clin Oncol* 2013; 31:88–94.
- Byrd JC, Furman RR, Coutre SE, Flinn IW, Burger JA, Blum KA, et al. Targeting BTK with ibrutinib in relapsed chronic lymphocytic leukemia. *N Engl J Med* 2013;369:32–42.
- Wang ML, Rule S, Martin P, Goy A, Auer R, Kahl BS, et al. Targeting BTK with ibrutinib in relapsed or refractory mantle-cell lymphoma. *N Engl J Med* 2013;369:507–16.
- Treon SP, Tripsas CK, Meid K, Warren D, Varma G, Green R, et al. Ibrutinib in Previously Treated Waldenström's Macroglobulinemia. *N Engl J Med* 2015;372:1430–40.
- Wilson WH, Young RM, Schmitz R, Yang Y, Pittaluga S, Wright G, et al. Targeting B cell receptor signaling with ibrutinib in diffuse large B cell lymphoma. *Nat Med* 2015;21:922–6.
- Burger JA, Keating MJ, Wierda WG, Hartmann E, Hoellenriegel J, Rosin NY, et al. Safety and activity of ibrutinib plus rituximab for patients with high-risk chronic lymphocytic leukaemia: a single-arm, phase 2 study. *Lancet Oncol* 2014;15:1090–9.
- Wang M, Gordon LI, Rule S, Goy A, Hermine O, Rizo A, et al. A phase III study of ibrutinib in combination with bendamustine and rituximab (BR) in elderly patients with newly diagnosed mantle cell lymphoma (MCL). *J Clin Oncol* 31:15s, 2013 (suppl; abstr TPS8613).
- Jagowski SM, Jones JA, Nagar V, Flynn JM, Andritsos LA, Maddocks KJ, et al. Safety and activity of BTK inhibitor ibrutinib combined with ofatumumab in chronic lymphocytic leukemia: a phase 1b/2 study. *Blood* 2015;126:842–50.
- Brown JR, Barrientos JC, Barr PM, Flinn IW, Burger JA, Tran A, et al. The Bruton's tyrosine kinase (BTK) inhibitor, ibrutinib, with chemoimmunotherapy in patients with chronic lymphocytic leukemia. *Blood* 2015; 125:2915–22.

10. Maddocks K, Christian B, Jaglowski S, Flynn J, Jones JA, Porcu P, et al. A phase 1/1b study of rituximab, bendamustine, and ibrutinib in patients with untreated and relapsed/refractory non-Hodgkin lymphoma. *Blood* 2015;125:242–8.
11. Hallek M, Fischer K, Fingerle-Rowson G, Fink AM, Busch R, Mayer J, et al. Addition of rituximab to fludarabine and cyclophosphamide in patients with chronic lymphocytic leukaemia: a randomised, open-label, phase 3 trial. *Lancet* 2010;376:1164–74.
12. Herman SE, Niemann CU, Farooqui M, Jones J, Mustafa RZ, Lipsky A, et al. Ibrutinib-induced lymphocytosis in patients with chronic lymphocytic leukemia: correlative analyses from a phase II study. *Leukemia* 2014; 28:2188–96.
13. Herishanu Y, Katz BZ, Lipsky A, Wiestner A. Biology of chronic lymphocytic leukemia in different microenvironments: clinical and therapeutic implications. *Hematol Oncol Clin North Am* 2013;27:173–206.
14. Bojarczuk K, Siernicka M, Dwojak M, Bobrowicz M, Pyrzynska B, Gaj P, et al. B-cell receptor pathway inhibitors affect CD20 levels and impair antitumor activity of anti-CD20 monoclonal antibodies. *Leukemia* 2014; 28:1163–7.
15. Golay J, Lazzari M, Facchinetti V, Bernasconi S, Borleri G, Barbui T, et al. CD20 levels determine the in vitro susceptibility to rituximab and complement of B-cell chronic lymphocytic leukemia: further regulation by CD55 and CD59. *Blood* 2001;98:3383–9.
16. van Meerden 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;12:4027–35.
17. Bellosillo B, Villamor N, López-Guillermo A, Marcé S, Esteve J, Campo E, et al. Complement-mediated cell death induced by rituximab in B-cell lymphoproliferative disorders is mediated in vitro by a caspase-independent mechanism involving the generation of reactive oxygen species. *Blood* 2001;98:2771–7.
18. Taylor RP, Lindorfer MA. The role of complement in mAb-based therapies of cancer. *Methods* 2014;65:18–27.
19. Wang S-Y, Weiner G. Complement and cellular cytotoxicity in antibody therapy of cancer. *Exp Opin Biol Ther* 2008;8:759–68.
20. Weiner GJ. Rituximab: mechanism of action. *Semin Hematol* 2010;47: 115–23.
21. Kohrt HE, Sagiv-Barfi I, Rafiq S, Herman SE, Butchar JP, Cheney C, et al. Ibrutinib antagonizes rituximab-dependent NK cell-mediated cytotoxicity. *Blood* 2014;123:1957–60.
22. Borge M, Almejún MB, Podaza E, Colado A, Grecco HF, Cabrejo M, et al. Ibrutinib impairs the phagocytosis of rituximab-coated leukemic cells from chronic lymphocytic leukemia patients by human macrophages. *Haematologica* 2015;100:e140-e2.
23. Da Roit F, Engelberts PJ, Taylor RP, Breij EC, Gritti G, Rambaldi A, et al. Ibrutinib interferes with the cell-mediated anti-tumor activities of therapeutic CD20 antibodies: implications for combination therapy. *Haematologica* 2015;100:77–86.
24. Farooqui MZ, Valdez J, Martyr S, Aue G, Saba N, Niemann CU, et al. Ibrutinib for previously untreated and relapsed or refractory chronic lymphocytic leukaemia with TP53 aberrations: a phase 2, single-arm trial. *Lancet Oncol* 2015;16:169–76.
25. Jasper GA, Arun I, Venzon D, Kreitman RJ, Wayne AS, Yuan CM, et al. Variables affecting the quantitation of CD22 in neoplastic B cells. *Cytometry B Clin Cytom* 2011;80:83–90.
26. Herman SE, Mustafa RZ, Gyamfi JA, Pittaluga S, Chang S, Chang B, et al. Ibrutinib inhibits BCR and NF- $\kappa$ B signaling and reduces tumor proliferation in tissue-resident cells of patients with CLL. *Blood* 2014;123:3286–95.
27. Lindorfer MA, Jinivizian HB, Foley PL, Kennedy AD, Solga MD, Taylor RP. B Cell Complement Receptor 2 Transfer Reaction. *J Immunol* 2003;170: 3671–8.
28. Beum PV, Peek EM, Lindorfer MA, Beurskens FJ, Engelberts PJ, Parren PW, et al. Loss of CD20 and bound CD20 antibody from opsonized B cells occurs more rapidly because of trogocytosis mediated by Fc receptor-expressing effector cells than direct internalization by the B cells. *J Immunol* 2011;187:3438–47.
29. Beum PV, Kennedy AD, Williams ME, Lindorfer MA, Taylor RP. The shaving reaction: rituximab/CD20 complexes are removed from mantle cell lymphoma and chronic lymphocytic leukemia cells by THP-1 monocytes. *J Immunol* 2006;176:2600–9.
30. Beum PV, Mack DA, Pawluczkoysz AW, Lindorfer MA, Taylor RP. Binding of rituximab, trastuzumab, cetuximab, or mAb T101 to cancer cells promotes trogocytosis mediated by THP-1 cells and monocytes. *J Immunol* 2008;181:8120–32.
31. Chen FE, Huang D-B, Chen Y-Q, Ghosh G. Crystal structure of p50/p65 heterodimer of transcription factor NF- $\kappa$ B bound to DNA. *Nature* 1998;391:410–3.
32. Linnell J, Mott R, Field S, Kwiatkowski DP, Ragoussis J, UdaloVA IA. Quantitative high-throughput analysis of transcription factor binding specificities. *Nucleic Acids Res* 2004;32:e44.
33. Morgan HP, Schmidt CQ, Guariento M, Blaum BS, Gillespie D, Herbert AP, et al. Structural basis for engagement by complement factor H of C3b on a self surface. *Nat Struct Mol Biol* 2011;18:463–70.
34. Taylor RP, Lindorfer MA. Fc $\gamma$ -receptor-mediated trogocytosis impacts mAb-based therapies: historical precedence and recent developments. *Blood* 2015;125:762–6.
35. Beurskens FJ, Lindorfer MA, Farooqui M, Beum PV, Engelberts P, Mackus WJ, et al. Exhaustion of cytotoxic effector systems may limit monoclonal antibody-based immunotherapy in cancer patients. *J Immunol* 2012;188: 3532–41.
36. Kennedy AD, Beum 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.
37. Baig NA, Taylor RP, Lindorfer MA, Church AK, LaPlant BR, Pettinger AM, et al. Induced resistance to ofatumumab mediated cell clearance mechanisms, including complement dependent cytotoxicity, in chronic lymphocytic leukemia. *J Immunol* 2014;192:1620–9.
38. Lindorfer MA, Wiestner A, Zent CS, Taylor RP. Monoclonal antibody (mAb)-based cancer therapy: Is it time to reevaluate dosing strategies? *Oncoimmunology* 2012;1:959–61.
39. Woyach JA, Johnson AJ, Byrd JC. The B-cell receptor signaling pathway as a therapeutic target in CLL. *Blood* 2012;120:1175–84.
40. Burger JA, Chiorazzi N. B cell receptor signaling in chronic lymphocytic leukemia. *Trends Immunol* 2013;34:592–601.
41. Gros P, Milder FJ, Janssen BJC. Complement driven by conformational changes. *Nat Rev Immunol* 2008;8:48–58.
42. Carter D, Lieber A. Protein engineering to target complement evasion in cancer. *FEBS Lett* 2014;588:334–40.
43. Derer S, Beurskens FJ, Rosner T, Peipp M, Valerius T. Complement in antibody-based tumor therapy. *Crit Rev Immunol* 2014;34:199–214.
44. Melis JP, Strumane K, Ruuls SR, Beurskens FJ, Schuurman J, Parren PW. Complement in therapy and disease: Regulating the complement system with antibody-based therapeutics. *Mol Immunol* 2015;67:117–30.
45. Gul N, Babes L, Siegmund K, Korthouwer R, Bogels M, Braster R, et al. Macrophages eliminate circulating tumor cells after monoclonal antibody therapy. *The J Clin Invest* 2014;124:812–23.
46. Montalvo F, Garcia Z, Celli S, Breart B, Deguine J, VanRooijen N, et al. The mechanism of anti-CD20-mediated B cell depletion revealed by intravital imaging. *J Clin Invest* 2013;123:5098–103.
47. Williams ME, Densmore JJ, Pawluczkoysz AW, Beum PV, Kennedy AD, Lindorfer MA, et al. Thrice-weekly low-dose rituximab decreases CD20 loss via shaving and promotes enhanced targeting in chronic lymphocytic leukemia. *J Immunol* 2006;177:7435–43.
48. Aue G, Lindorfer MA, Beum PV, Pawluczkoysz AW, Vire B, Hughes T, et al. Fractionated subcutaneous rituximab is well-tolerated and preserves CD20 expression on tumor cells in patients with chronic lymphocytic leukemia. *Haematologica* 2010;95:329–32.
49. Zent CS, Taylor RP, Lindorfer MA, Beum PV, LaPlant B, Wu W, et al. Chemoimmunotherapy for Relapsed/Refractory and Progressive 17p13 Deleted Chronic Lymphocytic Leukemia (CLL) Combining Pentostatin, Alemtuzumab, and Low Dose Rituximab is Effective and Tolerable and Limits Loss of CD20 Expression by Circulating CLL Cells. *Am J Hematol* 2014;89:757–65.
50. Ponader S, Chen SS, Buggy JJ, Balakrishnan K, Gandhi V, Wierda WC, et al. The Bruton tyrosine kinase inhibitor PCI-32765 thwarts chronic lymphocytic leukemia cell survival and tissue homing *in vitro* and *in vivo*. *Blood* 2012;119:1182–9.