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## CD20 as target for immunotherapy

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## Ibrutinib interferes with the cell-mediated anti-tumour activities of therapeutic CD20 antibodies: implications for combination therapy

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## ABSTRACT

The novel Bruton tyrosine kinase inhibitor ibrutinib and phosphatidylinositol-4-phosphate 3-kinase- $\delta$  inhibitor idelalisib are promising drugs for the treatment of chronic lymphocytic leukemia and B-cell non-Hodgkin lymphoma, either alone or in combination with anti-CD20 antibodies. We investigated the possible positive or negative impact of these drugs on all known mechanisms of action of both type I and type II anti-CD20 antibodies. Pretreatment with ibrutinib for 1 hour did not increase direct cell death of cell lines or chronic lymphocytic leukemia samples mediated by anti-CD20 antibodies. Pre-treatment with ibrutinib did not inhibit complement activation or complement-mediated lysis. In contrast, ibrutinib strongly inhibited all cell-mediated mechanisms induced by anti-CD20 antibodies rituximab, ofatumumab or obinutuzumab, either in purified systems or whole blood assays. Activation of natural killer cells, and antibody-dependent cellular cytotoxicity by these cells, as well as phagocytosis by macrophages or neutrophils were inhibited by ibrutinib with a half maximal effective concentration of 0.3-3  $\mu$ M. Analysis of anti-CD20 mediated activation of natural killer cells isolated from patients on continued oral ibrutinib treatment suggested that repeated drug dosing inhibits these cells *in vivo*. Finally we show that the phosphatidylinositol-4-phosphate 3-kinase- $\delta$  inhibitor idelalisib similarly inhibited the immune cell-mediated mechanisms induced by anti-CD20 antibodies, although the effects of this drug at 10  $\mu$ M were weaker than those observed with ibrutinib at the same concentration. We conclude that the design of

combined treatment schedules of anti-CD20 antibodies with these kinase inhibitors should consider the multiple negative interactions between these two classes of drugs

## INTRODUCTION

The anti-CD20 antibody rituximab is approved for the treatment of B-cell non-Hodgkin lymphoma (B-NHL) and chronic lymphocytic leukemia (CLL) in combination with chemotherapy. The next generation anti-CD20 monoclonal antibodies, ofatumumab [1] and obinutuzumab (GA101) [2], were approved more recently and are in clinical trials for the treatment of CLL and BNHL, combined with either standard or novel chemotherapeutic agents. Anti-CD20 antibodies are thought to act through immune mediated mechanisms, in particular complement-dependent cytotoxicity (CDC), antibody-dependent cellular cytotoxicity (ADCC) by natural killer cells (NK) and antibody-dependent phagocytosis by macrophages [3]; recent evidence indicates that these monoclonal antibodies can also promote activation of polymorphonuclear neutrophils (PMN) and phagocytosis [4]. Ofatumumab shows enhanced CDC compared to rituximab whereas obinutuzumab, a glycoengineered anti-CD20, shows more potent ADCC [5,6] and PMN-mediated phagocytosis [4]. Obinutuzumab also induces significant direct cell death, at least for some cellular targets [6,7]. Anti-CD20 monoclonal antibodies that mediate substantial CDC, such as rituximab and ofatumumab, are classified as type I, whereas those that induce high homotypic adhesion and direct cell death, such as obinutuzumab, are classified as type II [3]. Despite the advent

of next generation anti-CD20 monoclonal antibodies, these drugs are likely to perform best when combined with chemotherapeutic agents that employ different or synergistic mechanisms of action. Rituximab is most often combined with CHOP (cyclophosphamide, hydroxydaunorubicin, oncovin and prednisone) and with fludarabine and/or cyclophosphamide. The known role of B-cell receptor (BCR) signaling in the pathogenesis of B-cell neoplasms has led to the investigation of several specific kinase inhibitors as potential novel drugs for these diseases [8-10]. The most studied are the Bruton tyrosine kinase (Btk) inhibitor ibrutinib, the phosphatidylinositol 3-kinase- $\delta$  inhibitor idelalisib (CAL-101), the Syk inhibitors fostamatinib and GS-9973 and the pan-kinase inhibitor dasatinib [8, 10, 11]. Ibrutinib has recently been approved as a single agent for the treatment of refractory and relapsed CLL and mantle cell lymphoma, and combinations of ibrutinib with other drugs, including anti-CD20 antibodies, are currently being tested in phase 2-3 clinical trials in the same diseases ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)) [12-17]. The molecular target of ibrutinib, Btk, is a Tec family tyrosine kinase that regulates signaling downstream of the BCR and other immuno-receptors, including Toll-like and chemokine receptors. BCR plays a major role in B-cell development and differentiation and is deleted or mutated in X-linked agammaglobulinemia [8, 18]. Idelalisib is another kinase inhibitor in advanced clinical development [19, 20]. Results of a phase 3 study in relapsed CLL patients has shown a significant therapeutic advantage of rituximab in combination with idelalisib, compared to rituximab alone [19]. In view

of the use of CD20 monoclonal antibodies in combination with novel kinase inhibitors in the clinic, we investigated the effect of ibrutinib and idelalisib on all known mechanisms of action of type I and II anti-CD20 monoclonal antibodies, including direct cell death, CDC, ADCC and phagocytosis by macrophages and PMN.

## MATERIAL AND METHODS

### Target cells and patients

Peripheral blood was obtained from normal donors or patients with CLL or B-NHL, in accordance with the Declaration of Helsinki of 1975, as revised in 2008. All subjects gave written informed consent for their blood products to be used for research under an institutional review board-approved protocol. The MEC-1 (CLL), BJAB (Burkitt lymphoma) and DOHH-2 (B-NHL) cell lines were grown in RPMI1640 medium containing 10% fetal bovine serum (Euroclone, Wetherby, UK) and 110  $\mu$ M gentamycin (PHT Pharma, Milan, Italy).

### Drugs

Rituximab (Mabthera<sup>®</sup>) and obinutuzumab (Gazyva<sup>®</sup>) were obtained from the pharmacies, ofatumumab (Arzerra<sup>®</sup>) from GlaxoSmithKline (Barnard Castle, UK). IgG1k anti-HER2 trastuzumab (Herceptin, Roche) was used as a negative control. Ibrutinib and idelalisib were from Selleckchem (Houston, TX, USA). Lepirudin anti-coagulant (Refludan, Celgene Corporation, Summit, NJ, USA) was a kind gift from Dr. J. Lambris (University of Pennsylvania School of Medicine, PA, USA).

### **Cell growth and cytotoxicity assays**

For cytotoxicity assays with the Alamar blue dye,  $5 \times 10^3$  cells from lymphoma cell lines were incubated for 48 h with ibrutinib and/or 10  $\mu\text{g}/\text{mL}$  anti-CD20 antibodies. A one-tenth volume of Alamar blue was then added and the incubation continued overnight. Fluorescence was read in a plate reader (FluoroStar Optima, BMG) [21]. Cell growth and death induction were measured by flow cytometry after plating lymphoma cell lines at  $3 \times 10^4$  cells/well. Percentages and absolute counts of live and dead cells were measured by 7-aminoactinomycin (7-AAD) staining (BD Biosciences) and flow cytometry using calibration beads (Bright Count Microspheres, IQ Products, Groningen, the Netherlands).

### **Complement activation and cytotoxicity**

Cell lines were pre-treated for 1 h with kinase inhibitors. Anti-CD20 or control monoclonal antibodies were then added (no wash) and, 10 min later, 20% pooled human serum. After incubation for 4 h at  $37^\circ\text{C}$ , cells were stained with 7-AAD and cell death was analyzed by flow cytometry (FACSCanto II, BD Biosciences). For measurement of CDC in CLL cells, whole blood assays were performed [7, 22]. Briefly peripheral blood from normal donors or patients with CLL was drawn into 50  $\mu\text{g}/\text{mL}$  lepirudin [23]. Kinase inhibitors were added, followed 1 h later by anti-CD20-opsonized CLL. Complement activation was measured after 1 h by staining with FITC-labeled anti-C3b/iC3b/C3d monoclonal antibody 1H8 [24]. Cytotoxicity was assessed after 24 h by flow cytometry after labeling with

anti-CD19-APC and 7-AAD (both from BD Biosciences).

### **Natural killer cell activation and antibody-dependent cellular cytotoxicity**

To assess NK-cell degranulation, peripheral blood mononuclear cells from normal donors were pre-treated for 1 h with kinase inhibitors and then CLL cells were added at a 1:1 ratio in the presence or absence of 1  $\mu\text{g}/\text{mL}$  anti-CD20 or control monoclonal antibodies. After incubation for 4 h at  $37^\circ\text{C}$ , cells were stained with anti-CD56-APC and anti-CD107a-PE and degranulation measured by flow cytometry as increased percentage of CD107a+ cells in the CD56+ population. For *ex vivo* analysis of NK-cell activation, peripheral blood was collected after informed consent from patients with low grade B-NHL receiving 560 mg oral ibrutinib daily. Samples were taken when patients had been at least 1 week off treatment, either before or 4 h after drug administration. In one case we were also able to collect samples before and after first treatment and before and 4 h after treatment on day 21 of continuous treatment. The peripheral blood mononuclear cells were incubated with antibody-opsonized BJAB cells at a 1:1 ratio. After 2 h, NK-cell degranulation was analyzed by flow cytometry as above. For ADCC, cell lines were labeled with 100  $\mu\text{Ci}$   $^{51}\text{Cr}$  (Amersham Biosciences, Uppsala, Sweden) and 4-h ADCC assays were performed according to standard procedures, using peripheral blood mononuclear cells from healthy donors as effector cells at a 100:1 effector:target ratio.

### Phagocytosis by macrophages

Monocyte-derived macrophages were generated as described elsewhere [25] and pre-treated for 1 h with kinase inhibitors before adding targets. CLL cells were stained with 0.1  $\mu\text{M}$  carboxyfluorescein succinimidyl ester (Molecular Probes, Thermo Scientific Inc., USA) and incubated with the macrophages in the presence or absence of anti-CD20 or control monoclonal antibodies. After 2 h of incubation at 37°C, cells were harvested and stained with anti-CD19-APC and anti-CD11b-PE (both from BD Biosciences) and analyzed by flow cytometry [7].

### Statistical analysis

The data were analyzed using paired or unpaired Student's t-test, or a one-way ANOVA as appropriate.

## RESULTS

### *Ibrutinib does not enhance direct cell death induced by anti-CD20 antibodies*

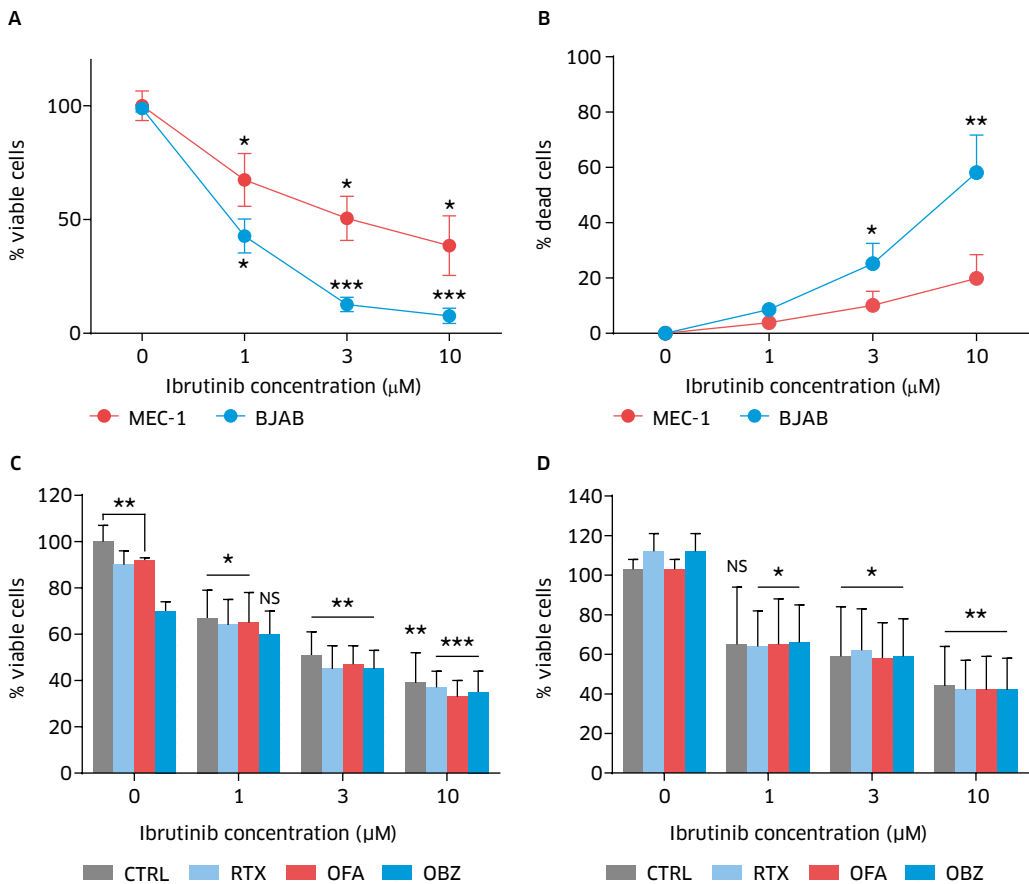
We first investigated the effect of ibrutinib alone on B-cell lymphoma and CLL cell lines using Alamar blue vital dye. Treatment for 72 h with 1-10  $\mu\text{M}$  ibrutinib showed that the BJAB cell line was more sensitive than MEC-1, with about 10% versus 40-50% viable cells, respectively, at concentrations of 3-10  $\mu\text{M}$  ibrutinib (Figure 1A). The IC<sub>50</sub> was about 1  $\mu\text{M}$  for BJAB and 3  $\mu\text{M}$  for MEC-1. Experiments in which we washed away the kinase inhibitor after different periods of exposure showed that a 2 h exposure is sufficient to obtain a full inhibitory effect (data not shown). Since a decrease in viable cells in growing cell lines may be due to either inhibition of proliferation or induction

of cell death, flow cytometry experiments were also performed using 7-AAD staining and calibration beads. Analysis of absolute number of live cells at different time points showed that ibrutinib inhibits proliferation of both BJAB and MEC-1 at a concentration of 1-10  $\mu\text{M}$  (Online Supplementary Figure S1). However the drug is significantly cytotoxic only for BJAB at the same drug doses (Figure 1B). The type II anti-CD20 antibody obinutuzumab has been reported to induce direct cell death of some cell lines [26,27]. We therefore investigated whether ibrutinib may synergize with anti-CD20 antibodies. We observed in Alamar blue assays that the MEC-1 cell line responded to obinutuzumab with a 30% decreased number of live cells at 48 h compared to control (Figure 1C). Pre-treatment for 1 h with 0.1-10  $\mu\text{M}$  ibrutinib before adding obinutuzumab did not further increase, and if anything reduced, the effect of obinutuzumab (Figure 1C). BJAB or CLL samples did not respond to obinutuzumab or the other anti-CD20 antibodies alone, as investigated by a reduction of live cells after 48-72 h of treatment, as previously reported [7], and addition of ibrutinib did not modify this (Figure 1D and data not shown). We conclude that ibrutinib does not have additive or synergistic effects with anti-CD20 monoclonal antibodies in terms of inhibition of proliferation and/or direct cell death induction.

### **The complement-dependent cytotoxicity of anti-CD20 antibodies is not affected by ibrutinib treatment**

We next investigated whether ibrutinib affects complement activation or CDC. BJAB cells were pretreated for 1 h with 0.1-10  $\mu\text{M}$  ibrutinib, after which ofatumumab was





**FIGURE 1** Ibrutinib does not synergize with anti-CD20 antibodies in inducing cell death.

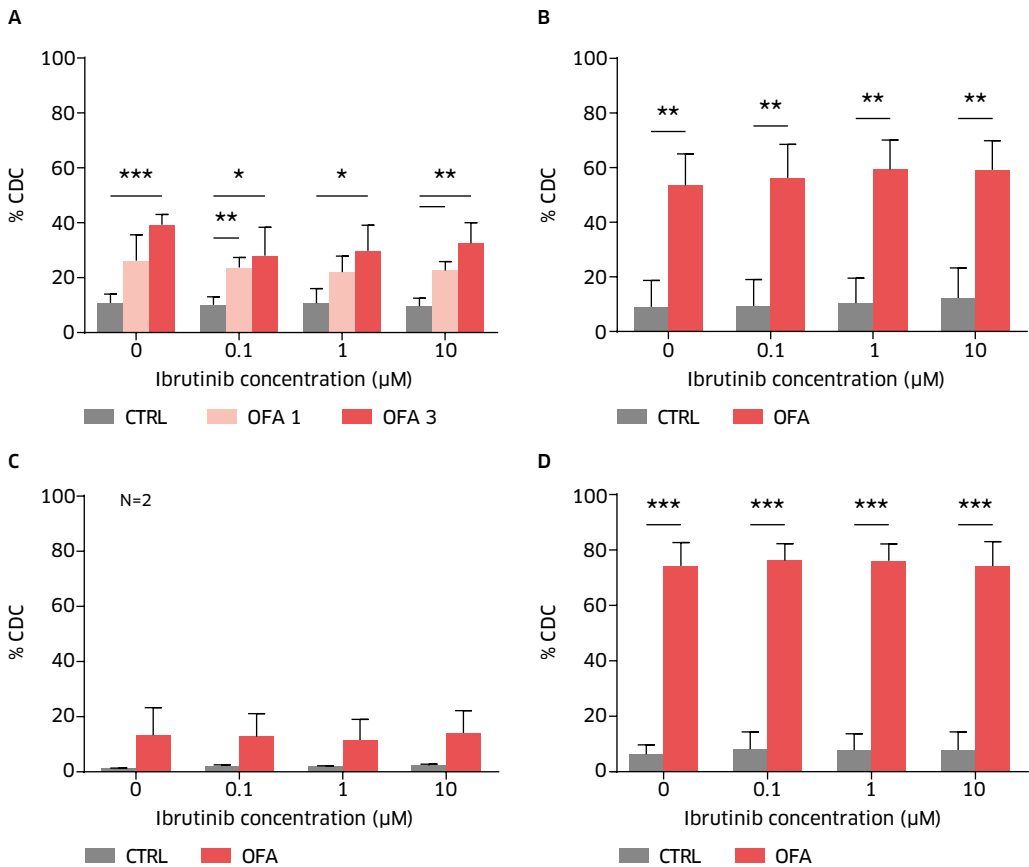
(A) The MEC-1 and BJAB cell lines were treated with 1, 3 or 10 μM ibrutinib. The percentage viable cells was analyzed after 72 h using Alamar vital dye. (B) The MEC-1 and BJAB cell lines were treated with 0.1, 1 or 10 μM ibrutinib. The percentage of dead cells was analyzed by 7-AAD staining and flow cytometry at 72 h. (C, D) The MEC-1 cell line (C) or peripheral blood mononuclear cells from CLL patients (D) were treated for 48 h with the indicated concentrations of ibrutinib, in the presence or absence of 10 μg/mL rituximab (RTX), ofatumumab (OFA) or obinutuzumab (OBZ). The Alamar blue vital dye was then added and the percentage of viable cells was measured after overnight incubation. The results are the mean percentages and standard deviations of viable cells compared to untreated control, from three independent experiments. For all conditions in the presence of ibrutinib, the statistical significance indicated refers to ibrutinib treated versus equivalent samples in the absence of ibrutinib. For samples not treated with ibrutinib (0, panels C and D), statistical significance is shown for samples treated with anti-CD20 monoclonal antibodies versus untreated samples. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001.

added at suboptimal concentrations (1-3  $\mu\text{g}/\text{mL}$ ) together with 20% human serum as a source of complement. As shown in Figure 2A, ibrutinib pre-treatment did not significantly affect ofatumumab-mediated CDC. Similar results were obtained with MEC-1 cells (data not shown). CDC induced by ofatumumab on CLL cells was also investigated. In this case more physiological whole blood assays in lepirudin were performed, in which ofatumumab is known to act entirely through CDC [22]. Whole blood from healthy normal donors was pretreated with ibrutinib for 1 h, after which CLL cells opsonized with 3  $\mu\text{g}/\text{mL}$  ofatumumab were added and the mixture incubated overnight at 37°C. Ofatumumab-mediated CDC was not affected by ibrutinib at a concentration up to 10  $\mu\text{M}$  (Figure 2B). Similar results were obtained using whole blood from CLL patients (Figure 2C). In agreement with these results, C3 complement fragment deposition on CLL cells in whole blood was not affected by 1 h pretreatment with ibrutinib (Figure 2D). We also measured whether exposure to ibrutinib for 24-72 h modulated expression of CD20 or the complement inhibitors CD55 and CD59 and found that 1-10  $\mu\text{M}$  ibrutinib did not significantly modulate expression of any of these molecules in either DOHH-2 or MEC-1 cell lines (data not shown). We conclude that ibrutinib has no significant effect on CDC induced by anti-CD20 antibodies.

**Ibrutinib inhibits natural kill cell activation and antibody dependent cellular cytotoxicity *in vitro* and *in vivo***

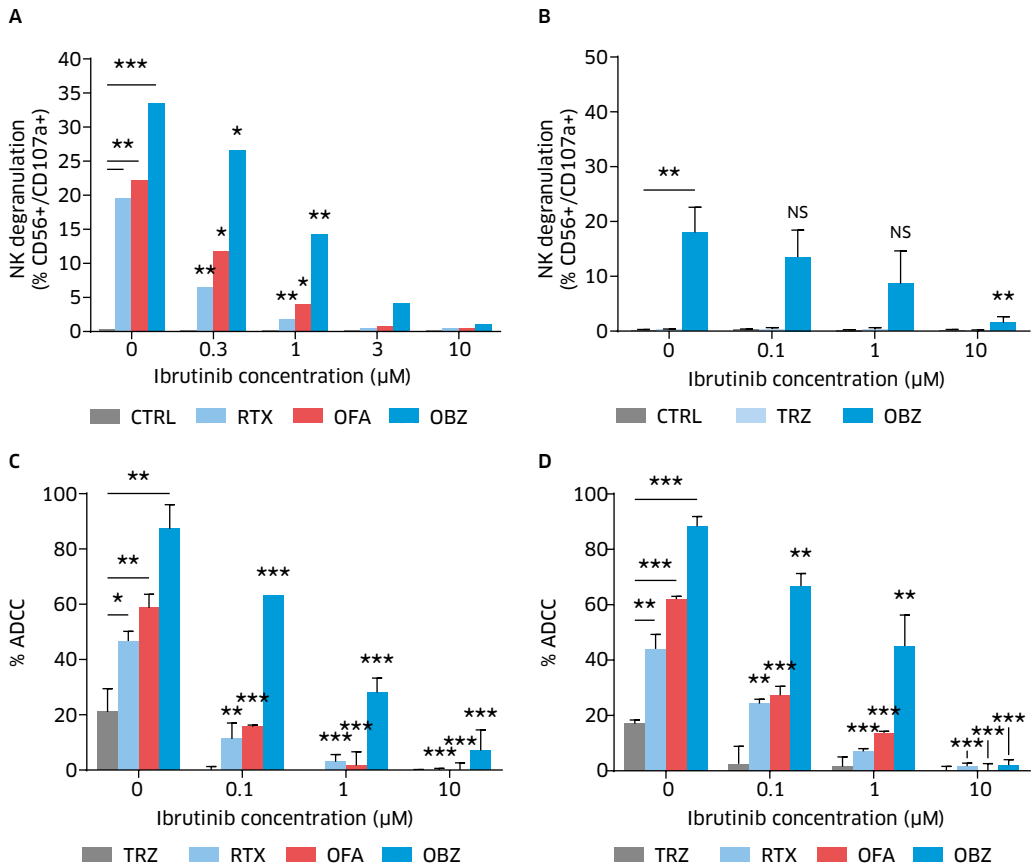
We next investigated the effect of ibrutinib on NK-cell degranulation and ADCC mediated by anti-CD20 monoclonal antibodies,

a major mechanism of these antibodies [6,28]. The negative control was irrelevant trastuzumab antibody. When peripheral blood mononuclear cells were co-cultured with CLL cells opsonized with the different anti-CD20 monoclonal antibodies, glycoengineered obinutuzumab induced a stronger degranulation of NK cells than did rituximab or ofatumumab, as expected [7]. One hour pre-treatment of peripheral blood mononuclear cells with ibrutinib before addition of opsonized targets strongly inhibited degranulation in all cases, with an EC50 of about 0.1  $\mu\text{M}$  (Figure 3A). Similar findings were made using MEC-1 cells (data not shown). Also in CLL whole blood assays, in which only obinutuzumab can induce NK-cell degranulation, ibrutinib was strongly inhibitory with an EC50 of 1  $\mu\text{M}$  (Figure 3B and Online Supplementary Figure S2). We also analyzed ADCC by peripheral blood mononuclear cells using DOHH-2 and MEC-1 cell lines as targets. Consistent with the degranulation data, ibrutinib inhibited ADCC of both cell lines, with EC50 of 0.1-1  $\mu\text{M}$  irrespectively of the anti-CD20 monoclonal antibody used (Figure 3C, 3D). Similar results were obtained when NK cells were pre-activated with interleukin-2 and then treated with ibrutinib for 1 h before performing degranulation and ADCC assays (data not shown). To determine whether NK cell inhibition may take place *in vivo*, we analyzed the degranulation capacity of NK cells isolated from three patients with low-grade BNHL who had been treated with oral ibrutinib 560 mg/die. Peripheral blood mononuclear cells were purified from blood samples collected before and 4 h after the first treatment when the patients had been off treatment for at least 1 week. The



**FIGURE 2** Exposure to ibrutinib does not significantly affect CDC induced by anti-CD20 antibodies.

(A) BJAB cells were incubated with increasing concentrations of ibrutinib for 1 h, after which 1 or 3 µg/mL ofatumumab (OFA) and 20% human serum were added. 7-AAD was added after 1 h and cell death (%7-AAD+) was measured by flow cytometry. (B) Ibrutinib was added to whole blood from normal donors (ND) and after 1 h at 37°C, CLL cells opsonized with 3 µg/mL OFA were added. The percentage of CD19+ /7-AAD- viable CLL cells was measured by flow cytometry after 24 h. (C) Ibrutinib was added to whole blood from CLL patients and after 1 h at 37°C, 3 µg/mL OFA were added. The percentage of CD19+ /7-AAD- viable CLL cells was measured by flow cytometry after 24 h. (D) Ibrutinib was added to whole blood from normal donors and after 1 h at 37°C, CLL cells opsonized with 3 µg/mL OFA were added; C3 deposition was measured after 1 h. Unless otherwise indicated all results are the means and standard deviations for three independent experiments. Statistical significance is indicated (\*P<0.05; \*\*P<0.01; \*\*\*P<0.001) and refers to the presence versus absence of OFA. Differences between ibrutinib-treated versus untreated samples were not statistically significant. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001.



**FIGURE 3** Ibrutinib strongly inhibits antibody-mediated NK-cell degranulation and ADCC.

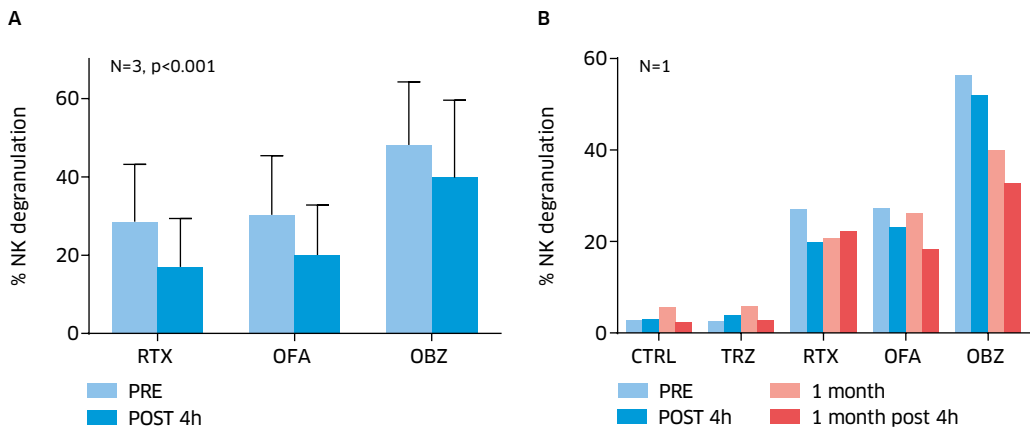
(A,B): Peripheral blood mononuclear cells (PBMC) (A) or whole blood (B) from CLL patients were treated with 0.1 to 10 μM ibrutinib for 1 h, after which the indicated anti-CD20 monoclonal antibodies were added at 1 μg/mL. NK-cell degranulation was analyzed after a further 4 h of incubation, by double staining with anti-CD56-APC and anti-CD107a-PE and flow cytometry. (C,D) ADCC assays were performed by <sup>51</sup>Cr release assays, using the DOHH-2 (C) or MEC-1 (D) cell lines as targets, in the absence or presence of the indicated anti-CD20 monoclonal antibodies at 1 μg/mL and/or increasing concentrations of ibrutinib. The results are the means and standard deviations for three independent experiments. The statistical significance (\*P<0.05; \*\*P<0.01; \*\*\*P<0.001) indicated above each bar refers to CD20 monoclonal antibody-treated versus untreated control sample, for values obtained in the absence of ibrutinib (0). Statistical significance in the presence of different doses of ibrutinib (1-10 μM) was calculated with respect to the equivalent controls in the absence of the drug. In the absence of ibrutinib, statistical significance refers to the comparison with versus without anti-CD20 antibodies. TRZ: trastuzumab; RTX: rituximab; OFA: ofatumumab; OBZ: obinutuzumab.

capacity of the NK cells from these samples to degranulate in response to anti-CD20 opsonized BJAB cells was then measured. As shown in Figure 4A, a single ibrutinib administration resulted in an 8.3-11.6% decrease in the degranulation capacity induced by all anti-CD20 monoclonal antibodies ( $P < 0.001$ ). In one case, we were able to collect samples pre- and post-ibrutinib administration on both day 1 and day 21 of continuous treatment. The results obtained suggest that daily ibrutinib administration leads to accumulation of inhibition with repeated dosing (Figure 4B). Indeed the first administration of ibrutinib led in this case to a rather small decrease (about 4%) in degranulation. However, after 21 days

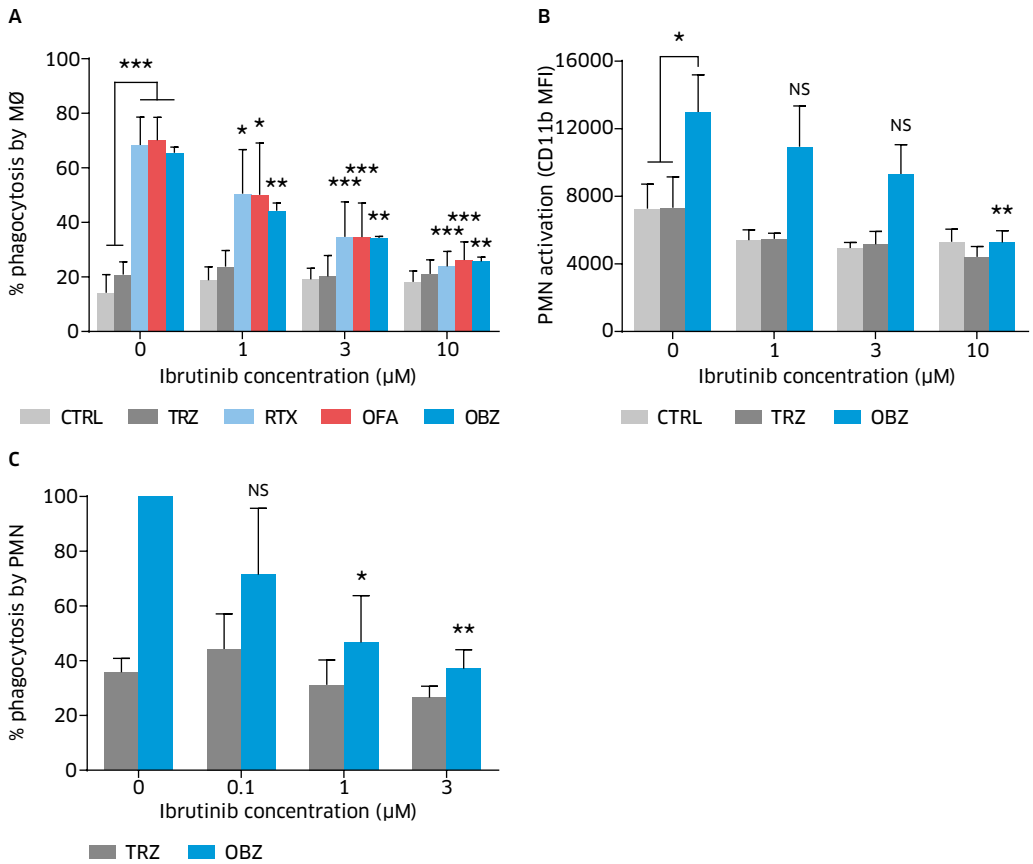
of continuous treatment, degranulation induced by ofatumumab and obinutuzumab was down by 33% and 40%, respectively, compared to levels in pretreatment controls (Figure 4B). Collectively, these data suggest that NK cells are inhibited *in vivo* after ibrutinib administration.

### Ibrutinib inhibits phagocytosis by macrophages and polymorphonuclear neutrophils

We next investigated the effect of ibrutinib on antibody dependent phagocytosis. Phagocytosis of CLL targets opsonized with 1  $\mu\text{g}/\text{mL}$  rituximab, ofatumumab or obinutuzumab by macrophages differentiated *in vitro* was measured by flow cytometry [22].



**FIGURE 4** Antibody-mediated NK-cell activation *ex vivo* is inhibited following *in vivo* ibrutinib treatment. (A) Blood samples from three patients with B-NHL were collected before the initiation of treatment (PRE), or 4 h after administration of the first 560 mg ibrutinib tablet (POST 4h). Peripheral blood mononuclear cells were purified from all samples and co-cultured with the BJAB cell line opsonized with 1  $\mu\text{g}/\text{mL}$  anti-CD20 monoclonal antibodies and degranulation was analyzed 2 h later by double staining with anti-CD56-APC and anti-CD107aPE and flow cytometry. (B) In one case, blood samples were also collected at day 21 of continuous treatment, before (POST 21 days) and 4 hour after administration of ibrutinib on day 21 (POST 21 days + 4h). The degranulation capacity of peripheral blood mononuclear cells from this patient was then measured as in (A). CTRL: no antibody; TRZ: trastuzumab; RTX: rituximab; OFA: ofatumumab; OBZ: obinutuzumab.



**FIGURE 5** Ibrutinib inhibits antibody-dependent phagocytosis mediated by both macrophages and PMN. (A) *In vitro* differentiated macrophages were pretreated with ibrutinib for 1 h, after which CFSE-labeled CLL cells either untreated (CTRL), or opsonized with 10 µg/mL anti-CD20 or control trastuzumab (TRZ) were added. After 2 h the cells were stained with CD19-APC and CD11b-PE and phagocytosis was measured by flow cytometry and triple fluorescence analysis. Phagocytosis was defined as the percentage of CD11b+ cells that were CFSE+ /CD19-. (B) Whole blood from normal donors was incubated with ibrutinib for 1 h, after which CLL cells, either untreated (CTRL), or opsonized with 10 µg/mL obinutuzumab (OBZ) or control TRZ monoclonal antibody were added. PMN activation after 6 h was measured by staining with CD11b-PE and flow cytometry. (C) Whole blood from normal donors was incubated with ibrutinib for 1 h, after which PKH26-labeled-CLL cells opsonized with 10 µg/mL OBZ or control TRZ monoclonal antibody were added. Phagocytosis was measured after 20 h by staining with anti-CD15-FITC and anti-CD19-APC and flow cytometry. All results are the means and standard deviations for three independent experiments. The statistical significance was calculated as defined in the legend to Figure 1. \*P<0.05; \*\*P<0.01; \*\*\*P<0.001.

Both no antibody and trastuzumab were used as negative controls. Phagocytosis mediated by all three anti-CD20 monoclonal antibodies was significantly inhibited by pre-treatment of macrophages for 1 h with ibrutinib. The EC50 was about 1-3  $\mu\text{M}$  and phagocytosis was reduced to background levels at 10  $\mu\text{M}$  in all cases (Figure 5A). Anti-CD20 opsonized targets also mediate activation of PMN and phagocytosis [4]. We therefore analyzed the effect of ibrutinib on these mechanisms. Both PMN activation by obinutuzumab-opsonized CLL targets and phagocytosis of the same targets in whole blood was inhibited by 1 h of pre-treatment with ibrutinib, with an EC50 of about 1  $\mu\text{M}$  (Figure 5B, 5C). We conclude that ibrutinib interferes with several cell mediated mechanisms of anti-CD20 antibodies: ADCC, phagocytosis by macrophages, activation and phagocytosis by PMN.

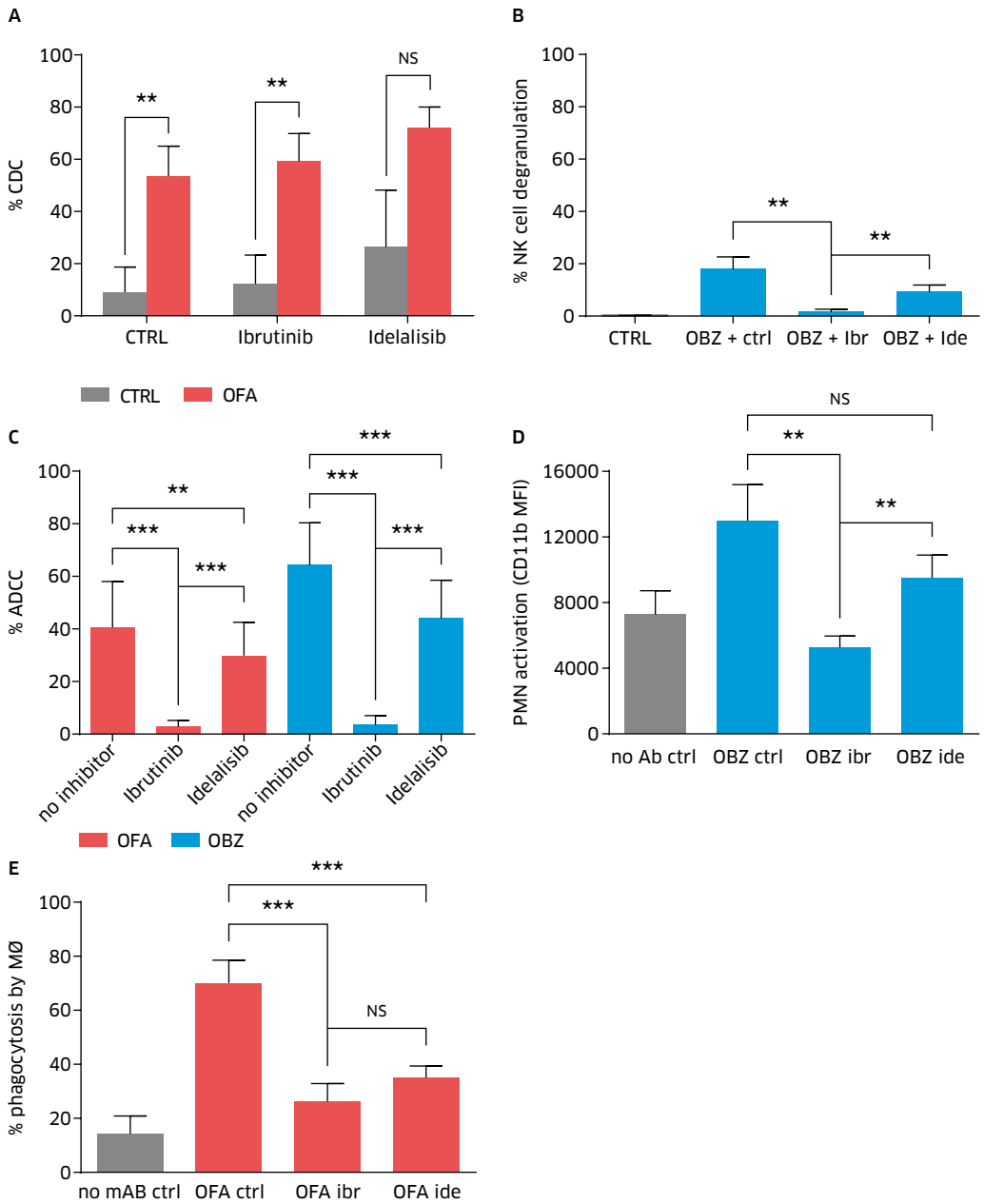
### Comparison with idelalisib

The PI3K- $\delta$  inhibitor idelalisib also acts downstream of the BCR [8, 18-20]. We, therefore, investigated whether the observed inhibition of cell-mediated effector mechanisms could also be seen with idelalisib. All the above anti-CD20 antibody effector mechanisms were investigated, using 10  $\mu\text{M}$  ibrutinib or idelalisib. Pretreatment with idelalisib for 1 h did not affect CDC in CLL whole blood assays. Indeed the cell death at 24 h induced by idelalisib 10  $\mu\text{M}$  alone was about 20%, the CDC mediated by ofatumumab was about 50% and both drugs together induced approximately 70% cell death, an effect which is just about additive (Figure 6A). In contrast idelalisib inhibited all cell-mediated effector mechanisms

induced by CD20 monoclonal antibodies, albeit to a lower extent than ibrutinib. Thus NK-cell degranulation induced by obinutuzumab-opsonized CLL cells in whole blood was inhibited by 48% and 92% with 10  $\mu\text{M}$  idelalisib and ibrutinib, respectively (Figure 6B, >90% by ibrutinib (Figure 6C,  $P < 0.001$ , and Online Supplementary Figure S3). Furthermore, obinutuzumab-dependent PMN activation was less strongly inhibited by idelalisib (27% inhibition) than ibrutinib (60% inhibition) (Figure 6D,  $P < 0.01$ ). The same was true for ofatumumab-induced macrophage-mediated phagocytosis, although the difference in inhibition between the two agents was not statistically significant (50% versus 63% inhibition, respectively, Figure 6E).

## DISCUSSION

In this study we extensively investigated the effect of the Btk inhibitor ibrutinib on immune-mediated or direct mechanisms of cell death induced by type I and II anti-CD20 monoclonal antibodies and compared it to that of the PI3K- $\delta$  inhibitor idelalisib. Ibrutinib alone inhibited proliferation of BJAB and MEC-1 cell lines at concentrations of 1-10  $\mu\text{M}$ , but was cytotoxic only for BJAB. Exposure to ibrutinib for 2 h was sufficient to induce cytotoxicity, concordant with the fact that ibrutinib is an irreversible inhibitor of Btk (data not shown). Btk plays a central role in BCR signaling. It regulates  $\text{Ca}^{++}$  flux, phospholipase C- $\gamma 2$ , I $\kappa$ B kinase and subsequent NF- $\kappa$ B activation [8]. *In vitro* and *in vivo*, ibrutinib has been reported to inhibit phospholipase C- $\gamma 2$  and downstream Akt, Erk and NF- $\kappa$ B activation, CLL/mantle cell





**FIGURE 6** Idelalisib (IDE) also inhibits cell-mediated activities of CD20 monoclonal antibodies (mAb), but more weakly than ibrutinib (IBR).

The following immune-mediated mechanisms of anti-CD20 monoclonal antibodies were studied in the absence or presence of 10  $\mu$ M IBRU or 10  $\mu$ M IDE: (A) Ofatumumab (OFA)-dependent CDC of CLL cells in normal donor whole blood, (B) NK-cell degranulation in peripheral blood mononuclear cells induced by obinutuzumab (OBZ)-opsonized CLL cells, (C) OFA- and OBZ-dependent ADCC of the MEC-1 cell line, (D) PMN activation in whole blood in response to OBZ-opsonized CLL and (E) phagocytosis by macrophages of OFA-opsonized CLL. All results are the means and standard deviations for three independent experiments.

\*P<0.05; \*\*P<0.01; \*\*\*P<0.001.

lymphoma cell survival and interaction within the tumor microenvironment [9,29]. Different mutations in Btk or in downstream signaling effector molecules in neoplastic B cells, as well as B-cell energy, may determine sensitivity *in vitro* and *in vivo* [21,22,32,33]. Complement activation and CDC were not significantly affected by ibrutinib after 1-h pre-exposure to the kinase inhibitor. Longer periods of exposure to ibrutinib (72 h) did not significantly modify expression of CD20 or the complement inhibitors CD55 and CD59 (data not shown), suggesting that altogether ibrutinib has little effect on CDC. A recently published report describes that ibrutinib down-modulates CD20 protein expression and gene transcription in some cell lines (e.g. Raji) and CLL samples, and that this leads to inhibition of CDC [34]. The difference with our data may be due to the different cell lines tested, since these authors showed no modulation of CD20 by ibrutinib in MEC-1, in agreement with our findings. In the context of complement, it is worth noting that ibrutinib and other kinase inhibitors acting downstream of the BCR induce early lymphocytosis *in vivo* due to egress of neoplastic B cells into the peripheral blood [35-37]. This property may be an advantage for subsequent CDC

induction by co-administered anti-CD20 monoclonal antibodies, since complement is abundant and fully active in the periphery [22]. The efficacy of CDC may, however, be reduced by the potential down modulation of CD20 and up-regulation of CD55 after prolonged exposure to ibrutinib, at least in some cases [34]. ADCC is thought to be a major effector mechanism of anti-CD20 monoclonal antibodies [28], especially for glycoengineered obinutuzumab which binds Fc $\gamma$ RIIIA more efficiently [6]. We showed here that ibrutinib strongly inhibited NK-cell degranulation and ADCC mediated by all anti-CD20 monoclonal antibodies. This effect was observed with an EC<sub>50</sub> of about 0.1-0.3  $\mu$ M, both using cell lines and CLL samples, as well as in whole blood assays, i.e. in relatively physiological conditions. Furthermore inhibition of degranulation and ADCC was observed even if NK cells were pre-activated by recombinant human interleukin-2 (data not shown). Our findings are in agreement with and extend recently published data that showed inhibition of antibody-induced NK-cell degranulation, NK-cell-mediated cytokine production and ADCC by 0.1-1  $\mu$ M ibrutinib [34,38]. A direct role of Btk in NK cell activation and cytotoxicity has emerged from work with Btk knock-out mice as well

as in patients with X-linked agammaglobulinemia [39]. In the latter study, however, TLR3 rather than FcγR-mediated signaling was investigated. Interestingly ibrutinib has also been shown recently to inhibit Itk, another Tec family kinase like Btk [40]. Itk is predominantly expressed by T cells and signals downstream of the T-cell receptor [41] and has been reported to be required for the activation of human NK cells through FcγRIII [42]. Thus ibrutinib may inhibit NK cells through either Btk or Itk, or both. A recent study using the Itk-specific inhibitor CGI-1746 suggests that Itk in NK cells is an important signaling molecule to mediate ADCC [38]. Finally ibrutinib is not fully specific for Btk, so other kinases, such as Tec, Blk and Bmx, may be involved in the effects of the drug in B cells as well as immune effector cells described here, especially at the highest 10 μM dose [43]. However, the precise definition of the kinases involved in each case is beyond the scope of this article. We were also able to study the degranulation capacity of NK cells isolated from patients with B-NHL treated with oral ibrutinib 560 mg/die. We found a significantly decreased capacity of NK cells to degranulate after a single oral dose of ibrutinib in three patients. Furthermore in one patient who could be analyzed after 3 weeks of continuous treatment, NK cells were even more markedly inhibited with respect to pre-treatment cells. Published pharmacokinetic data suggest that peak concentrations of ibrutinib of approximately 0.25 μM (100 ng/mL), an effective concentration to inhibit ADCC *in vitro*, may be reached 2 h after a single 560 mg administration *in vivo* [12]. Furthermore Dubosvky et al. also demonstrated ibrutinib

binding to Itk *in vivo* after eight daily oral administrations of the drug [40]. Together these data suggest that repeated treatment with ibrutinib may lead to inhibition of NK-cell-mediated mechanisms induced by antiCD20 monoclonal antibodies *in vivo*. Analysis of anti-CD20 monoclonal antibody-mediated phagocytosis by macrophages and PMN showed that ibrutinib also strongly inhibited these reactions. These data are in agreement with studies showing that Btk is expressed by myeloid lineage cells and is involved in the activation, cytokine production and chemotaxis of macrophages, granulocytes and mast cells [44-46]. The EC50 for this inhibition was in the range of 0.3 to 3 μM. It is possible that these processes may be inhibited by standard ibrutinib dosing *in vivo*, although this was not directly demonstrated here. This is particularly important since phagocytosis and myeloid cells have been implicated as major players in the therapeutic activity of anti-CD20 and other monoclonal antibodies *in vivo* [4, 47-50]. Finally, we compared the inhibitory effects *in vitro* of the PK13-δ inhibitor idelalisib with those of ibrutinib. We observed that idelalisib had similar inhibitory effects as ibrutinib on CDC, NK cell degranulation, ADCC, PMN activation and macrophage phagocytosis, but at lower levels when used at an equal 10 μM dose. These data suggest that these properties may be common to several kinase inhibitors acting downstream of the BCR [8, 18], although the observed differences in potency may lead to different consequences *in vivo* and need to be more fully investigated. The data presented here underline the multiple negative interactions between ibrutinib and

anti-CD20 monoclonal antibodies and have important implications for the design of schedules for combined therapy. Indeed concurrent rituximab plus ibrutinib therapy in mouse models appears to lead to worse outcome compared to sequential drug schedules (whichever the order) [38]. The data presented here suggest that the reason for these observations may be ascribed to inhibition not only of NK cell-mediated mechanisms, as previously reported, but also macrophage- and/or PMN-mediated mechanisms triggered by anti-CD20 monoclonal antibodies. Thus, although initial clinical data suggest a favorable response of patients to ibrutinib or idelalisib in combination with rituximab [17, 19], modification of dosing schedules may be designed to exploit the effector mechanisms of both anti-CD20 monoclonal antibodies and kinase inhibitors more efficiently, leading to even further improvements in response. Thus anti-CD20 monoclonal antibody administration should be timed relative to the initiation of ibrutinib treatment to preserve at least the early cell-mediated mechanisms of action of the antibodies. Although continued treatment seemed to reduce NK-cell activity, CDC activity may not be significantly affected. Timing of ibrutinib dosing might, therefore, be less critical when combined with anti-CD20 monoclonal antibodies with strong CDC capacity. Finally, anti-CD20 monoclonal antibodies may also be usefully given as a maintenance therapy after cessation of the kinase inhibitor. In this case, however, the timing of the recovery of full cell-mediated effector functions will need to be more precisely investigated. Indeed we propose that several of the

markers investigated here (NK-cell degranulation and PMN activation capacity) may be used in the future to better define the immune function of blood cells isolated from patients at various stages of kinase inhibitor treatment and to design appropriate and optimal drug schedules.

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#### Authorship and Disclosures

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at [www.haematologica.org](http://www.haematologica.org).

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