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

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Running title: Ibrutinib inhibits CD20 antibody activities

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**Abstract**

Novel Bruton Tyrosine Kinase inhibitor ibrutinib and Phosphatidyl-4-5-biphosphate 3-Kinase-δ inhibitor idelalisib are promising drugs for chronic lymphocytic leukemia and B-non Hodgkin's 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. Pre-treatment with ibrutinib for one 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 0.3-3μM. 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 Phosphatidyl-4-5-biphosphate 3-Kinase-δ inhibitor idelalisib similarly inhibited the immune cell-mediated mechanisms induced by anti-CD20 antibodies, although the effects of this drug at 10μM 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

Anti-CD20 antibody rituximab (RTX) is approved for the treatment of B-Non Hodgkin's lymphoma (B-NHL) and chronic lymphocytic leukemia (CLL) in combination with chemotherapy. The next generation anti-CD20 monoclonal antibodies (mAbs), ofatumumab (OFA)[1] and obinutuzumab (OBZ, GA101)[2], have more recently been approved and are in clinical trials for the treatment of CLL and B-NHL, 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 (ADCP) by macrophages[3]; more recent evidence indicates the mAbs can also promote activation of polymorphonuclear neutrophils (PMN) and phagocytosis[4]. OFA shows enhanced CDC compared to RTX whereas OBZ, a glycoengineered anti-CD20, shows more potent ADCC[5,6] and PMN-mediated phagocytosis[4]. OBZ also induces significant direct cell death, at least for some cellular targets[6,7]. Anti-CD20 mAbs that mediate substantial CDC, like RTX and OFA, are classified as Type I, whereas those that induce high homotypic adhesion and direct cell death, like OBZ, are classified as Type II[3].

Despite the advent of next generation anti-CD20 mAbs, these drugs are likely to perform best when combined with chemotherapeutic agents that employ different or synergistic mechanisms of action. RTX 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 PI3kinase-δ 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 (MCL), 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)[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 (XLA)(reviewed in[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 RTX in combination with idelalisib, compared to RTX alone[19].

In view of the use of CD20 mAbs 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 mAbs, including direct cell death, CDC, ADCC and phagocytosis by macrophages and PMN.

## 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's lymphoma) and DOHH-2 (B-NHL) cell lines were grown in RPMI1640 medium containing 10% fetal bovine serum (Euroclone, Wetherby, UK) and 110µM gentamycin (PHT Pharma, Milano, Italy).

*Drugs*

Rituximab (Mabthera®) and obinutuzumab (OBZ, GA101, Gazyva®) were obtained from the pharmacies, Ofatumumab (Arzerra®) from GlaxoSmithKline (Barnard Castle, UK). IgG1κ anti-HER2 trastuzumab (TRZ, Herceptin, Roche) was used as negative control.

Ibrutinib and idelalisib were from Selleckchem (Houston, TX). Lepirudin anti-coagulant (Refludan, Celgene Corporation, Summit, NJ) was a kind gift from Dr. J. Lambris (University of Pennsylvania School of Medicine, PA).

*Cell growth and cytotoxicity assays*

For cytotoxicity assays with the alamar blue dye, $5x10^3$ lymphoma cell lines were incubated for 48 hours with ibrutinib and/or 10 µg/ml anti-CD20 antibodies. 1/10th volume alamar blue was then added and 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 $3x10^4$/well. Percentage and absolute counts of live and dead cells were measured by 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 hour with kinase inhibitors. Anti-CD20 or control mAbs were then added (no wash) and 10 minutes later 20% pooled human serum (HS). After 4 hours incubation at 37°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 of normal donors or CLL was drawn in 50 µg/mL lepirudin[23]. Kinase inhibitors were added, followed one hour later by anti-CD20 opsonized CLL. Complement activation was measured after one hour by staining with FITC-labeled anti-C3b/iC3b/C3d mAb 1H8[24].

Cytotoxicity was assessed after 24 hours by flow cytometry after labelling with anti-CD19-APC and 7-AAD (both from BD Biosciences).

*NK cell activation and ADCC*

To assess NK cell degranulation, PBMC from normal donors were pre-treated for one hour with kinase inhibitors and then CLL cells added at a 1:1 ratio in the presence or absence of 1µg/mL anti-CD20 or control mAbs. After 4 hour incubation at 37°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. A sample was taken either when patients had been at least 1 week off treatment, either before (PRE) or 4 hours after drug administration (POST 4hr). In one case we were able to collect samples before and after first treatment (PRE and POST 4 hours) and on day 21 of continuous treatment (PRE and POST 4 hours). PBMCs were incubated with antibody-opsonized BJAB cells at a 1:1 ratio. After 2 hours, NK degranulation was analyzed by flow cytometry as above.

For ADCC, cell lines were labeled with 100µCi $^{51}Cr$ (Amersham Biosciences, Uppsala, Sweden) and 4-hour ADCC assays were performed according to standard procedures, using PBMC from healthy donors as effector cells at a 100:1 effector:target (E:T) ratio.

*Phagocytosis by macrophages*

Monocyte-derived macrophages were generated as described[25] and pre-treated for one hour with kinase inhibitors before adding targets. CLL cells were stained with 0.1µM Carboxyfluorescein succinimidyl ester (CFSE, Molecular Probes, Thermo Scientific Inc., USA) and incubated with the macrophages in the presence or absence of anti-CD20 or control mAbs. After 2 hour incubation at 37°C, cells were harvested and stained with anti-CD19-APC and anti-CD11b-PE (both BD Biosciences) and analyzed by flow cytometry[7].

*PMN activation and phagocytosis*

PMN were used in whole blood in lepirudin (Refludan), or purified from peripheral blood as described[4], and pre-treated for one hour with kinase inhibitors before adding targets. CLL cells were stained with 2µM PKH26 (for phagocytosis), opsonized with anti-CD20 mAbs and mixed with purified PMN or whole blood at a 3:1 ratio (CLL:PMN). After 6 hours incubation at 37°C, cells were stained with anti-CD11b-PE to measure PMN activation or with anti-CD15-FITC and CD19-APC (BD Biosciences) for phagocytosis and analyzed by flow cytometry[4].

*Statistical analysis*

The data were analyzed using paired or unpaired Student's t-test, or a one-way ANOVA as appropriate. *: $p<0.05$; **: $p< 0.01$; ***: $p< 0.001$.

## 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 the alamar blue vital dye. Treatment for 72 hours with 1-10μM ibrutinib showed that the BJAB cell line was more sensitive than MEC-1, with about 10% versus 40-50% viable cells at 3-10μM ibrutinib, respectively (Figure 1A). $IC_{50}$ was about 1μM for BJAB and 3μM for MEC-1. Experiments where we washed away the kinase inhibitor after different exposure times showed that a 2-hour 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 1-10μM (Supplementary Figure 1). However the drug is significantly cytotoxic only for BJAB at the same drug doses (Figure 1B).

Anti-CD20 type II antibody OBZ 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 OBZ with a 30% decreased number of live cells at 48 hours compared to control (Figure 1C). Pre-treatment for one hour with 0.1-10μM ibrutinib before adding OBZ did not further increase, and if anything reduced, the effect of OBZ (Figure 1C). BJAB or CLL samples did not respond to OBZ or the other anti-CD20 antibodies alone in terms of reduced live cells after 48-72 hours 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 show additive or synergistic effects with anti-CD20 mAbs in terms of inhibition of proliferation and/or direct cell death induction.

*CDC activity 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 hour with 0.1-10μM ibrutinib, after which OFA was added at suboptimal concentrations (1-3 μg/mL) together with 20% HS as complement source. As shown in Figure 2A, ibrutinib pretreatment did not significantly affect OFA-mediated CDC. Similar results were obtained with MEC-1 cells (data not shown).

CDC induced by OFA on CLL cells was also investigated. In this case more physiological whole blood assays in lepirudin were performed, in which OFA is known to act entirely through CDC[22]. Whole blood from healthy normal donors (ND) was pretreated with ibrutinib for one hour,

after which CLL cells opsonized with 3 µg/mL OFA were added and incubation was carried out overnight at 37°C. OFA-mediated CDC was not affected by ibrutinib up to 10µM (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 ibrutinib 1-hour pretreatment (Figure 2D). We also measured whether ibrutinib treatment for 24-72 hours modulated expression of CD20 or the complement inhibitors CD55 and CD59. 1-10µM 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 NK cell activation and ADCC in vitro and in vivo*

We next investigated the effect of ibrutinib on NK cell degranulation and ADCC mediated by anti-CD20 mAbs, a major mechanism of these antibodies[6,28]. Negative control was irrelevant trastuzumab antibody (TRZ). When PBMC were co-cultured with CLL cells opsonized with the different anti-CD20 mAbs, glycoengineered OBZ induced a stronger degranulation of NK cells than RTX or OFA, as expected[7]. One hour pre-treatment of PBMC with ibrutinib before addition of opsonized targets strongly inhibited degranulation in all cases, with an $EC_{50}$ of about 0.1µM (Figure 3A). Similar data were observed using MEC-1 cells (data not shown). Also in CLL whole blood assays, where only OBZ can induce NK cell degranulation, ibrutinib was strongly inhibitory with an $EC_{50}$ of 1µM (Figure 3B and Supplemental Figure 2).

We also analyzed ADCC by PBMC using DOHH-2 and MEC-1 cell lines as targets. Consistent with the degranulation data, ibrutinib inhibited ADCC of both cell lines, with $EC_{50}$ of 0.1-1µM irrespective of the anti-CD20 mAb used (Figure 3C,D). Similar data were obtained when NK cells were pre-activated with IL-2 and then treated with ibrutinib for one hour 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 3 low grade B-NHL patients who had been treated with ibrutinib at a 560 mg oral daily dose. PBMC were purified from blood samples collected when patient were at least one week off treatment, before (PRE) and 4 hours after the first treatment (POST 4h). The 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 8.3-11.6% decrease in the degranulation capacity induced by all anti-CD20 mAbs ($p<0.001$). 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 (about 4%) decrease in degranulation. However, after 21 days of continuous treatment, degranulation induced by OFA and OBZ was down by 33% and 40%, respectively, compared to pre-treatment controls (Figure 4A).

Altogether these data suggest that NK cells are inhibited *in vivo* after ibrutinib administration.

*Ibrutinib inhibits phagocytosis by macrophages and PMN*

We next investigated the effect of ibrutinib on ADCP. Phagocytosis of CLL targets opsonized with 1 µg/mL RTX, OFA or OBZ by *in vitro* differentiated macrophages was measured by flow cytometry[22]. Both no antibody (CTRL) and trastuzumab (TRZ) were used as negative controls. Phagocytosis mediated by all three anti-CD20 mAbs was significantly inhibited by pre-treatment of macrophages for one hour with ibrutinib. $EC_{50}$ was about 1-3µM and phagocytosis was reduced to background levels at 10µM 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 OBZ-opsonized CLL targets and phagocytosis of the same targets in whole blood was inhibited by one hour pre-treatment with ibrutinib, with an $EC_{50}$ of about 1µM (Figure 5B,C).

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-δ 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 observed with idelalisib. All the above anti-CD20 antibody effector mechanisms were investigated, using 10µM ibrutinib or idelalisib. One hour pretreatment with idelalisib did not affect CDC in CLL whole blood assays. Indeed idelalisib alone at 10µM induced about 20% cell death at 24 hours, CDC mediated by OFA by about 50% and both drugs together induced an 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 mAbs, albeit to a lower extent than ibrutinib. Thus NK degranulation induced by OBZ-opsonized CLL cells in whole blood was inhibited by 48% and 92% with 10µM idelalisib and ibrutinib, respectively (Figure 6B, $p<0.01$). ADCC of MEC-1 cells induced by either OFA or OBZ was inhibited only by 18-28% by idelalisib and >90% by ibrutinib (Figure 6C, $p<0.001$, and supplemental Figure 3). Also, OBZ-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 OFA-induced macrophage-mediated phagocytosis, although the difference in inhibition between the two agents was not significant (50% versus 63% inhibition, respectively, Figure 6E).

## Discussion

In this report we have extensively investigated the effect of Btk inhibitor ibrutinib on immune-mediated or direct mechanisms of cell death induced by Type I and II anti-CD20 mAbs and compared it to that of the PI3K-δ inhibitor idelalisib.

Ibrutinib alone inhibited proliferation of BJAB and MEC-1 cell lines at 1-10µM, but was cytotoxic only for BJAB. A two-hour exposure to ibrutinib was sufficient to induce cytotoxicity, in agreement with the fact that ibrutinib is an irreversible inhibitor or Btk (data not shown). Btk plays a central role in BCR signaling. It regulates $Ca^{++}$ flux, phospholipase C-γ2 (PLC-γ2), IkB kinase and subsequent NF-kB activation[8]. *In vitro* and *in vivo*, ibrutinib has been reported to inhibit PLC-γ2 and downstream Akt, Erk and NF-kB activation, CLL/MCL 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 anergy, may determine sensitivity to the cytotoxic effect of ibrutinib[13,30,31], but this was not further investigated here. Rather, we examined the effect of ibrutinib on direct cell death induction by anti-CD20 antibodies. Type II CD20 mAb OBZ alone induced a measurable decrease in viable MEC-1 cells after 48-72 hours, but not had no effect on the BJAB cell line or on CLL cells, as previously reported[7]. More interestingly ibrutinib did not synergize, but if anything diminished, direct cell death induction by anti-CD20 mAbs. Cell death induction by type II CD20 mAbs appears to be independent of caspase activation and to go through a lysosomal pathway instead[27]. Our data suggest therefore that this cell death pathway is not sensitized by Btk inhibition.

Complement activation has been reported to be an important mechanism of action of RTX and OFA *in vitro* and *in vivo*[21,22,32,33]. Complement activation and CDC were not significantly affected by ibrutinib after 1-hour pre-exposure to the kinase inhibitor. Longer exposure times to ibrutinib (72 hours) 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 this report. 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 mAbs, since complement is abundant and fully active in the periphery[22]. CDC efficacy

may however be reduced by the potential down-modulation of CD20 and upregulation 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 mAbs[28], especially for glycoengineered OBZ which binds FcγRIIIA more efficiently[6]. We showed here that ibrutinib strongly inhibited NK cell degranulation and ADCC mediated by all anti-CD20 mAbs. This effect was observed with an $EC_{50}$ of about 0.1-0.3μM, 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 were pre-activated by rhIL-2 (data not shown). Our data are in agreement with and extend the recently published data, that showed inhibition of antibody-induced NK degranulation, NK mediated cytokine production and ADCC by 0.1-1μM 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 XLA patients[39]. In the latter study however, TLR3 rather than FcγR mediated signaling was investigated. Interestingly ibrutinib has been shown recently to also inhibit Itk, another Tec family kinase like Btk[40]. Itk is predominantly expressed by T cells and signals downstream of the TCR[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 report using Itk specific inhibitor CGI-1746 suggests that Btk 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 10μM 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 B-NHL patients treated with 560 mg daily oral ibrutinib. We could show a significant decreased capacity of NK cells to degranulate after a single oral ibrutinib dose in three patients. Furthermore in one patient that could be analyzed, NK cells were even more markedly inhibited with respect to pre-treatment cells after 3 weeks of continuous treatment. Published pharmacokinetics data suggest that peak concentrations of ibrutinib of approximately 0.25μM (100 ng/mL), an effective concentration to inhibit ADCC *in vitro*, may be reached 2 hours after a single 560 mg administration *in vivo*[12]. Furthermore Dubosvky *et al.* also demonstrated ibrutinib binding to Itk *in vivo* after 8 daily oral administrations of the drug[40]. Altogether these data suggest that repeated treatment with ibrutinib may lead to inhibition of NK cell-mediated mechanisms induced by anti-CD20 mAbs *in vivo*.

Analysis of anti-CD20 mAb-mediated phagocytosis by macrophages and PMN showed that ibrutinib also strongly inhibited these reactions. These data are in agreement with reports 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 $EC_{50}$ for this inhibition was in the range of 0.3 to 3µM. 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 for the therapeutic activity of anti-CD20 and other mAbs *in vivo*[4,47-50].

Finally, we compared the inhibitory effects *in vitro* of the PKI3-δ inhibitor idelalisib with those of ibrutinib. We observed that idelalisib had similar inhibitory effects as ibrutinib on CDC, NK degranulation, ADCC, PMN activation and macrophage phagocytosis, but at lower levels when used at an equal 10µM 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 mAbs and have important implications for the design of schedules for combined therapy. Indeed concurrent RTX plus ibrutinib therapy in mouse models appear 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-mediated, as previously reported, but also macrophage and/or PMN mediated mechanisms triggered by anti-CD20 mAbs. Thus, although initial clinical data suggest a favorable response of patients to ibrutinib or idelalisib in combination with RTX[17,19], modification of dosing schedules may be designed to more efficiently exploit the effector mechanisms of both anti-CD20 mAbs and kinase inhibitors, leading to even further improvements in response. Thus anti-CD20 mAb 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 mAbs with strong CDC capacity. Finally, anti-CD20 mAbs 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 better design appropriate and optimal drug schedules.

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**Authorship and Disclosures**

PWHIP, JG, ECWB and FJB designed and supervised the experiments. FDR and PJE performed the experiments and analyzed data. PP, MI and AR provided funding and critically reviewed the data. GG and AR provided patients material and supervised the clinical aspects of the study. RPT provided reagents. JG wrote and all authors have critically revised the manuscript.

FJB, PJE, ECWB and PWHIP are employees of Genmab, the company developing OFA and own Genmab warrants and/or stocks. JG has received research grants from Roche Glycart AG, the company developing OBZ, and Genmab BV. RPT has received grants and honoraria from Genmab, the company developing OFA. AR and JG have received honoraria from Roche Italia, the firm developing OBZ. There are no other conflicts of interest to declare.

## References


1. Wierda WG, Kipps TJ, Mayer J, Stilgenbauer S, Williams CD, Hellmann A, et al. Ofatumumab as single-agent CD20 immunotherapy in fludarabine-refractory chronic lymphocytic leukemia. J Clin Oncol. 2010;28(10):1749-55.
2. Goede V, Fischer K, Busch R, Engelke A, Eichhorst B, Wendtner CM, et al. Obinutuzumab plus Chlorambucil in Patients with CLL and Coexisting Conditions. N Engl J Med. 2014;370(12):1101-10.
3. Weiner GJ. Rituximab: mechanism of action. Semin Hematol. 2010;47(2):115-123.
4. Golay J, Da Roit F, Bologna L, Ferrara C, Leusen JH, Rambaldi A, et al. Glycoengineered CD20 antibody obinutuzumab activates neutrophils and mediates phagocytosis through CD16B more efficiently than rituximab. Blood 2013;122(20):3482-91.
5. Teeling JL, Mackus WJ, Wiegman LJ, van den Brakel JH, Beers SA, French RR, et al. The biological activity of human CD20 monoclonal antibodies is linked to unique epitopes on CD20. J Immunol. 2006;177(1):362-71.
6. Moessner E, Bruenker P, Moser S, Puentener U, Schmidt C, Herter S, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. Blood 2010;115(22):4393-402.
7. Bologna L, Gotti E, Manganini M, Rambaldi A, Intermesoli T, Introna M, et al. Mechanism of action of type II, glycoengineered, anti-CD20 monoclonal antibody GA101 in B-chronic lymphocytic leukemia whole blood assays in comparison with rituximab and alemtuzumab. J Immunol. 2011;186(6):3762-69.
8. Woyach JA, Johnson AJ, Byrd JC. The B-cell receptor signaling pathway as a therapeutic target in CLL. Blood 2012;120(6):1175-84.
9. Herman SE, Gordon AL, Hertlein E, Ramanunni A, Zhang X, Jaglowski S, et al. Bruton tyrosine kinase represents a promising therapeutic target for treatment of chronic lymphocytic leukemia and is effectively targeted by PCI-32765. Blood 2011;117(23):6287-96.
10. Burger JA, Chiorazzi N. B cell receptor signaling in chronic lymphocytic leukemia. Trends Immunol. 2013;34(12):592-601.
11. Buggy JJ, Elias L. Bruton tyrosine kinase (BTK) and its role in B-cell malignancy. Int Rev Immunol. 2012;31(2):119-32.
12. 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(1):32-42.
13. Vargas L, Hamasy A, Nore BF, Smith CI. Inhibitors of BTK and ITK: state of the new drugs for cancer, autoimmunity and inflammatory diseases. Scand J Immunol. 2013;78(2):130-9.
14. 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(1):88-94.
15. 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(6):507-16.
16. Blum KA, Christian B, Flynn JM, Jaglowski SM, Jones JA, Maddocks K, et al. A Phase I Trial of the Bruton's Tyrosine Kinase (BTK) Inhibitor, Ibrutinib (PCI-32765), in combination with Rituximab (R) and Bendamustine in Patients with relapsed/refractory Non Hodgkin's Lymphoma (NHL). Blood 2012;120(21):a1643.
17. Burger JA, Keating MJ, Wierda W, Hoellenriegel J, Jeyakumar G, Ferrajoli A, et al. Ibrutinib in combination with Rituximab (iR) is well Tolerated and induces high rate of durable remissions in patients with high-risk chronic lymphocytic leukemia (CLL) : New, updated results of a phase II trial in 40 patients. Blood 2013;122(21):a675.
18. Hallek M. Signaling the end of chronic lymphocytic leukemia: new frontline treatment strategies. Blood 2013;122(23):3723-34.

19. Furman RR, Sharman JP, Coutre SE, Cheson BD, Pagel JM, Hillmen P, et al. Idelalisib and Rituximab in Relapsed Chronic Lymphocytic Leukemia. N Engl J Med. 2014;370(11):997-1007.
20. Gopal AK, Kahl BS, de Vos S, Wagner-Johnston ND, Schuster SJ, Jurczak WJ, et al. PI3Kdelta Inhibition by Idelalisib in Patients with Relapsed Indolent Lymphoma. N Engl J Med. 2014;370(11):1008-18.
21. 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(12):3383-89.
22. Bologna L, Gotti E, Da Roit F, Intermesoli T, Rambaldi A, Introna M, et al. Ofatumumab is more efficient than rituximab in lysing B chronic lymphocytic leukemia cells in whole blood and in combination with chemotherapy. J Immunol. 2013;190(1):231-9.
23. Mollnes TE, Brekke OL, Fung M, Fure H, Christiansen D, Bergseth G, et al. Essential role of the C5a receptor in E coli-induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation. Blood 2002;100(5):1869-77.
24. Lindorfer MA, Jinivizian HB, Foley PL, Kennedy AD, Solga MD, Taylor RP. B cell complement receptor 2 transfer reaction. J Immunol. 2003;170(7):3671-8.
25. Leidi M, Gotti E, Bologna L, Miranda E, Rimoldi M, Sica A, et al. M2 macrophages phagocytose rituximab-opsonized leukemic targets more efficiently than m1 cells in vitro. J Immunol. 2009;18(7)2:4415-22.
26. Herter S, Herting F, Mundigl O, Waldhauer I, Weinzierl T, Fauti T, 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):2031-42.
27. Ivanov A, Beers SA, Walshe CA, Honeychurch J, Alduaij W, Cox KL, 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.
28. Cartron G, Dacheux L, Salles G, Solal-Celigny P, Bardos P, Colombat P, et al. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor Fc gammaIIIa gene. Blood 2002;99(3):754-8.
29. Cheng S, Ma J, Guo A, Lu P, Leonard JP, Coleman M, et al. BTK inhibition targets in vivo CLL proliferation through its effects on B-cell receptor signaling activity. Leukemia 2013;28(3):649-57.
30. Rahal R, Frick M, Romero R, Korn JM, Kridel R, Chun Chan F, et al. Pharmacological and genomic profiling identifies NF-kappaB-targeted treatment strategies for mantle cell lymphoma. Nat Med. 2014;20(1):87-92.
31. Yang Y, Shaffer AL, 3rd, Emre NC, Ceribelli M, Zhang M, Wright G, et al. Exploiting synthetic lethality for the therapy of ABC diffuse large B cell lymphoma. Cancer Cell 2012;21(6):723-37.
32. Teeling JL, French RR, Cragg MS, van den Brakel J, Pluyter M, Huang H, et al. Characterization of new human CD20 monoclonal antibodies with potent cytolytic activity against non-Hodgkin lymphomas. Blood 2004;104(6):1793-800.
33. 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(7):3532-41.
34. 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(5):1163-7.
35. Chang BY, Francesco M, De Rooij MF, Magadala P, Steggerda SM, Huang MM, et al. Egress of CD19(+)CD5(+) cells into peripheral blood following treatment with the Bruton tyrosine kinase inhibitor ibrutinib in mantle cell lymphoma patients. Blood 2013;122(14):2412-24.

36. Ponader S, Chen SS, Buggy JJ, Balakrishnan K, Gandhi V, Wierda WG, 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(5):1182-89.
37. Fiorcari S, Brown WS, McIntyre BW, Estrov Z, Maffei R, O'Brien S, et al. The PI3-Kinase Delta Inhibitor Idelalisib (GS-1101) Targets Integrin-Mediated Adhesion of Chronic Lymphocytic Leukemia (CLL) Cell to Endothelial and Marrow Stromal Cells. PLoS One 2013;8(12):e83830.
38. 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(12):1957-60.
39. Bao Y, Zheng J, Han C, Jin J, Han H, Liu Y, et al. Tyrosine kinase Btk is required for NK cell activation. J Biol Chem. 2012;287(28):23769-78.
40. Dubovsky JA, Beckwith KA, Natarajan G, Woyach JA, Jaglowski S, Zhong Y, et al. Ibrutinib is an irreversible molecular inhibitor of ITK driving a Th1-selective pressure in T lymphocytes. Blood 2013;122(15):2539-49.
41. Berg LJ, Finkelstein LD, Lucas JA, Schwartzberg PL. Tec family kinases in T lymphocyte development and function. Annu Rev Immunol. 2005;23(1):549-600.
42. Khurana D, Arneson LN, Schoon RA, Dick CJ, Leibson PJ. Differential regulation of human NK cell-mediated cytotoxicity by the tyrosine kinase Itk. J Immunol. 2007;178(6):3575-3582.
43. Burger JA, Buggy JJ. Bruton tyrosine kinase inhibitor ibrutinib (PCI-32765). Leuk Lymphoma. 2013;54(11):2385-91.
44. Lindvall JM, Blomberg KE, Valiaho J, Vargas L, Heinonen JE, Berglof A, et al. Bruton's tyrosine kinase: cell biology, sequence conservation, mutation spectrum, siRNA modifications, and expression profiling. Immunol Rev. 2005;203(1):200-15.
45. Jongstra-Bilen J, Puig Cano A, Hasija M, Xiao H, Smith CI, Cybulsky MI. Dual functions of Bruton's tyrosine kinase and Tec kinase during Fcgamma receptor-induced signaling and phagocytosis. J Immunol. 2008;181(1):288-98.
46. Ormsby T, Schlecker E, Ferdin J, Tessarz AS, Angelisova P, Koprulu AD, et al. Btk is a positive regulator in the TREM-1/DAP12 signaling pathway. Blood. 2011;118(4):936-945.
47. Uchida J, Hamaguchi Y, Oliver JA, Ravetch JV, Poe JC, Haas KM et al. The innate mononuclear network depletes B lymphocytes through Fc receptor-dependent mechanisms during anti-CD20 antibody immunotherapy. J Exp Med. 2004;199(12):1659-69.
48. Beers SA, Chan CH, James S, French RR, Attfield KE, Brennan CM, et al. Type II (tositumomab) anti-CD20 monoclonal antibody out performs type I (rituximab-like) reagents in B-cell depletion regardless of complement activation. Blood 2008;112(10):4170-7.
49. Albanesi M, Mancardi DA, Macdonald LE, Iannascoli B, Zitvogel L, Murphy AJ, et al. Cutting edge: FcgammaRIII (CD16) and FcgammaRI (CD64) are responsible for anti-glycoprotein 75 monoclonal antibody TA99 therapy for experimental metastatic B16 melanoma. J Immunol. 2012;189(12):5513-17.
50. Montalvao F, Garcia Z, Celli S, Breart B, Deguine J, Van Rooijen N, et al. The mechanism of anti-CD20-mediated B cell depletion revealed by intravital imaging. J Clin Invest. 2013;123(12):5098-103.

## Figure Legends

**Figure 1. Ibrutinib does not synergize with anti-CD20 antibodies in cell death induction**

(A): The MEC-1 and BJAB cell lines were treated with 1, 3 or 10μM ibrutinib. The percentage viable cells was analyzed after 72 hours using the alamar vital dye. (B): The MEC-1 and BJAB cell lines were treated with 0.1, 1 or 10μM ibrutinib. Percentage of dead cells was analyzed by 7-AAD staining and flow cytometry at 72 hours. (C, D): The MEC-1 cell line (C) or PBMC from CLL patients (D) were treated for 48 hours with the indicated concentrations of ibrutinib, in presence or absence of 10 μg/mL RTX, OFA or 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 3 independent experiments. For all conditions in presence of ibrutinib, the statistical significance indicated refers to ibrutinib treated versus equivalent samples in absence of ibrutinib. For samples not treated with ibrutinib (0, panels C/D), statistical significance is shown for anti-CD20 mAb-treated versus untreated samples. *:$p<0.05$; **: $p<0.01$; ***:$p<0.001$.

**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 hour, after which 1 or 3 μg/mL OFA and 20% HS were added. 7-AAD was added after 1 hour and cell death (%7-AAD$^+$) measured by flow cytometry. (B): Ibrutinib was added to whole blood from normal donors and after 1 hour at 37°C, CLL cells opsonized with 3μg/ml OFA were added. The percentage of CD19$^+$/7-AAD$^-$ viable CLL cells was measured by flow cytometry after 24 hours. (C). Ibrutinib was added to whole blood from CLL patients and after 1 hour 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 hours. (D) Ibrutinib was added to whole blood from normal donors and after 1 hour at 37°C, CLL cells opsonized with 3 μg/mL OFA were added; C3 deposition was measured after 1 hour. Unless otherwise indicated all results are the means and standard deviations for 3 independent experiments. Statistical significance is indicated and refers to presence versus absence of OFA. Differences between ibrutinib treated versus untreated samples were not statistically significant.

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

(A,B): PBMC (A) or whole blood (B) from CLL patients were treated with 0.1 to 10µM ibrutinib for 1 hour, after which the indicated anti-CD20 mAbs were added at 1µg/mL. NK degranulation was analysed after further 4 hours incubation, by double staining with anti-CD56-APC and anti-CD107a-PE and flow cytometry. (C,D): ADCC assays were performed by $^{51}$Cr release assays, using the DOHH-2 (C) or MEC-1 (D) cell lines as targets, in absence or presence of the indicated anti-CD20 mAbs at 1µg/mL and/or increasing concentrations of ibrutinib. The results are the means and standard deviations for 3 independent experiments. The statistical significance indicated above each bar refers to CD20 mAb-treated versus untreated control sample, for values obtained in absence of ibrutinib (0). Statistical significance in presence of different doses of ibrutinib (1-10µM) was calculated with respect to the equivalent controls in absence of the drug. In absence of ibrutinib, statistical significance is with compared to without anti-CD20 antibodies.

**Figure 4. Antibody-mediated NK cell activation *ex vivo* is inhibited following *in vivo* ibrutinib treatment**

(A): Blood samples from 3 patients with B-NHL were collected before the initiation of treatment (PRE), or 4 hours after administration of the first 560 mg ibrutinib tablet (POST 4h). PBMCs were purified from all samples and co-cultured with the BJAB cell line opsonized with 1 µg/mL anti-CD20 mAbs and degranulation analyzed 2 hours later by double staining with anti-CD56-APC and anti-CD107a-PE 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 PBMC from this patient was then measured as in (A).

**Figure 5. Ibrutinib inhibits antibody-dependent phagocytosis mediated by both macrophages and PMN**

(A): *In vitro* differentiated macrophages were pretreated with ibrutinib for 1 hour, after which CFSE-labelled CLL cells either untreated (CTRL), or opsonized with 10 µg/mL anti-CD20 or control TRZ were added. After 2 hours 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 hour, after which CLL cells, either untreated (CTRL), or opsonized with 10 µg/mL OBZ or control TRZ mAb were added. PMN activation after 6 hours was measured by staining with CD11b-PE and flow cytometry. (C): Whole blood from normal donors

was incubated with ibrutinib for 1 hour, after which PKH26-labelled-CLL cells opsonized with 10 µg/mL OBZ or control TRZ mAb were added. Phagocytosis was measured after 20 hours by staining with anti-CD15-FITC and anti-CD19-APC and flow cytometry. All results are the means and standard deviations for 3 independent experiments. The statistical significance was calculated as defined in Figure 1 legend.

**Figure 6. Idelalisib also inhibits cell-mediated activities of CD20 mAbs, but more weakly than ibrutinib**

The following immune-mediated mechanisms of anti-CD20 mAbs were studied in absence or presence of 10µM ibrutinib or 10µM idelalisib: (A) OFA-dependent CDC of CLL cells in normal donor whole blood, (B) NK cell degranulation in PBMC induced by OBZ-opsonized CLL cells, (C) OFA- and OBZ-dependent ADCC of 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 3 independent experiments.

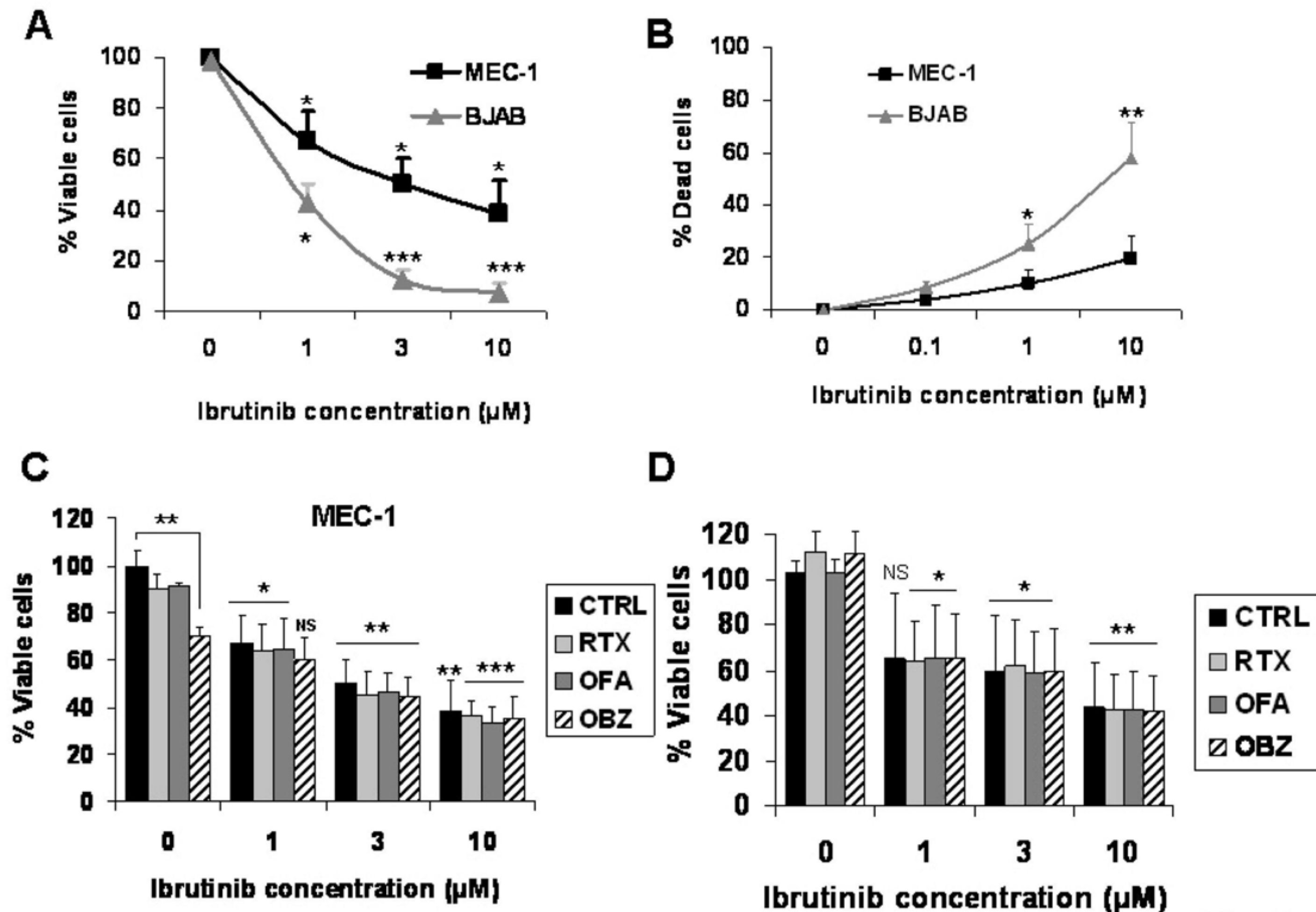
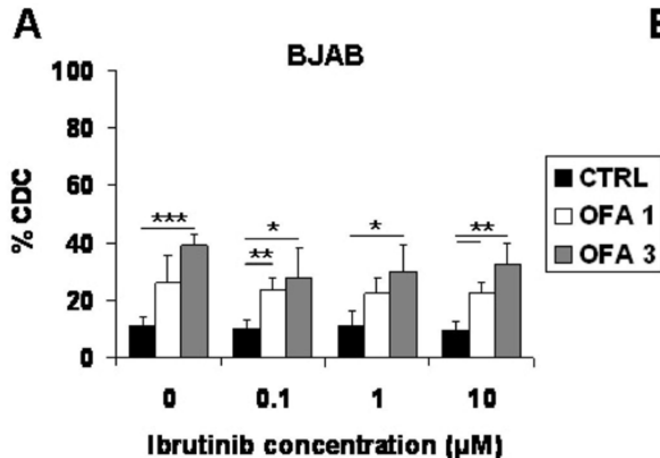
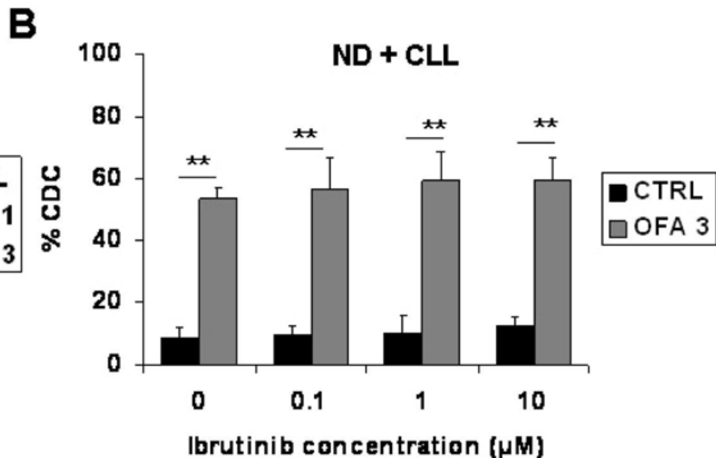
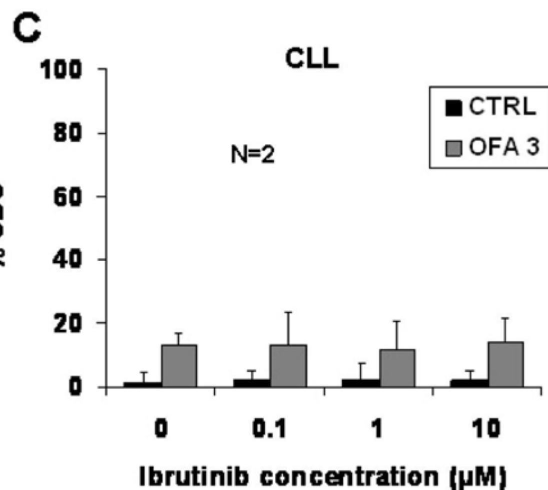



Fig. 1



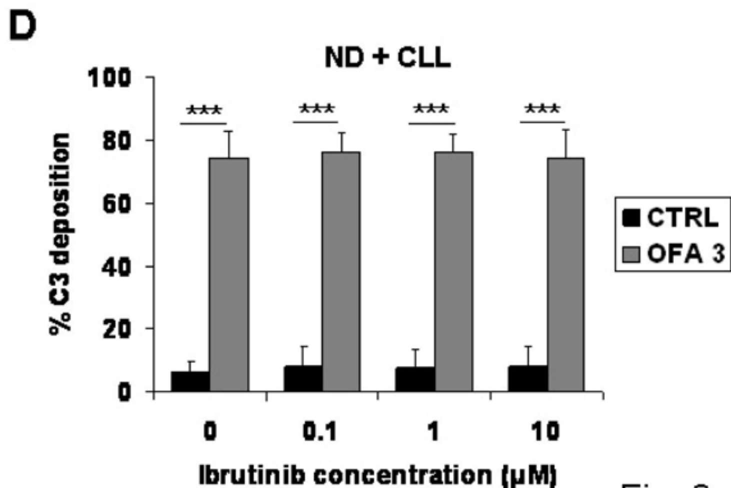
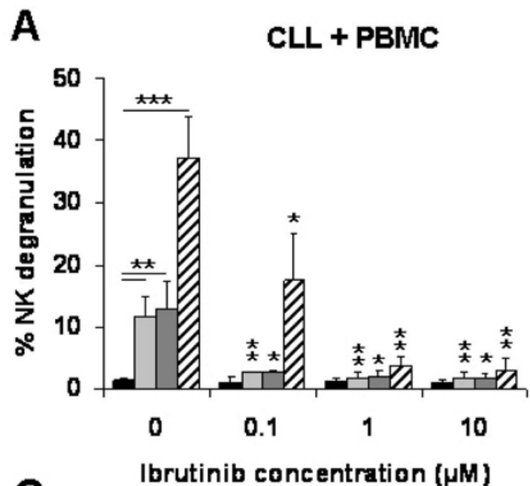
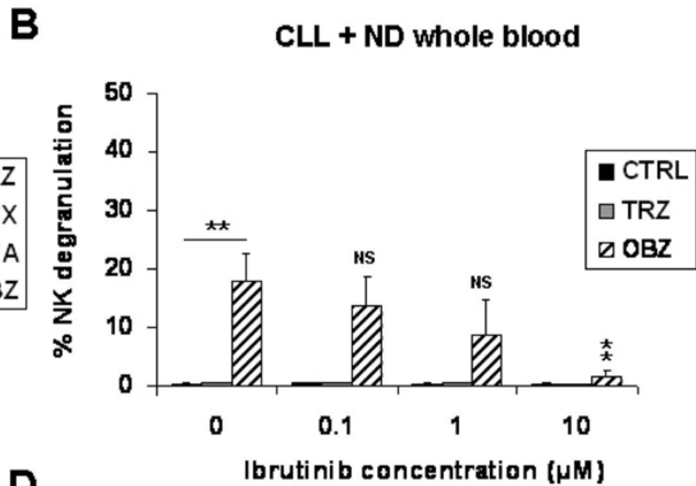
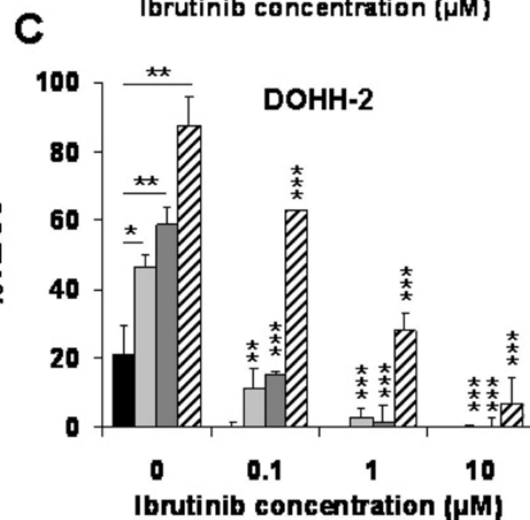




Fig. 2



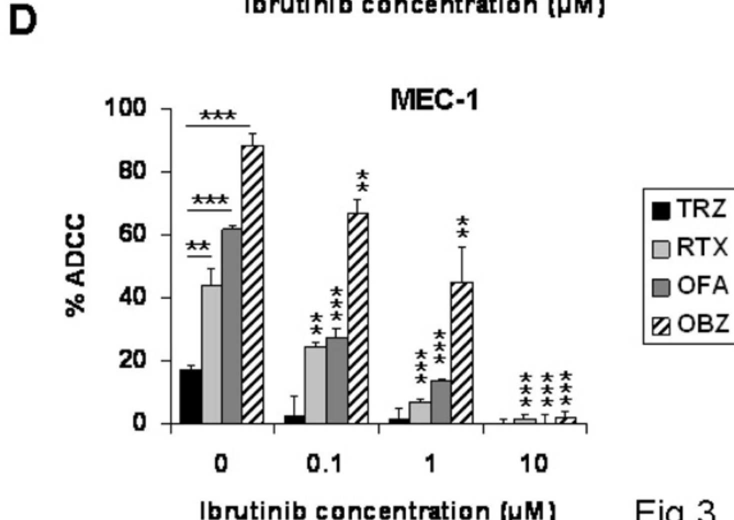
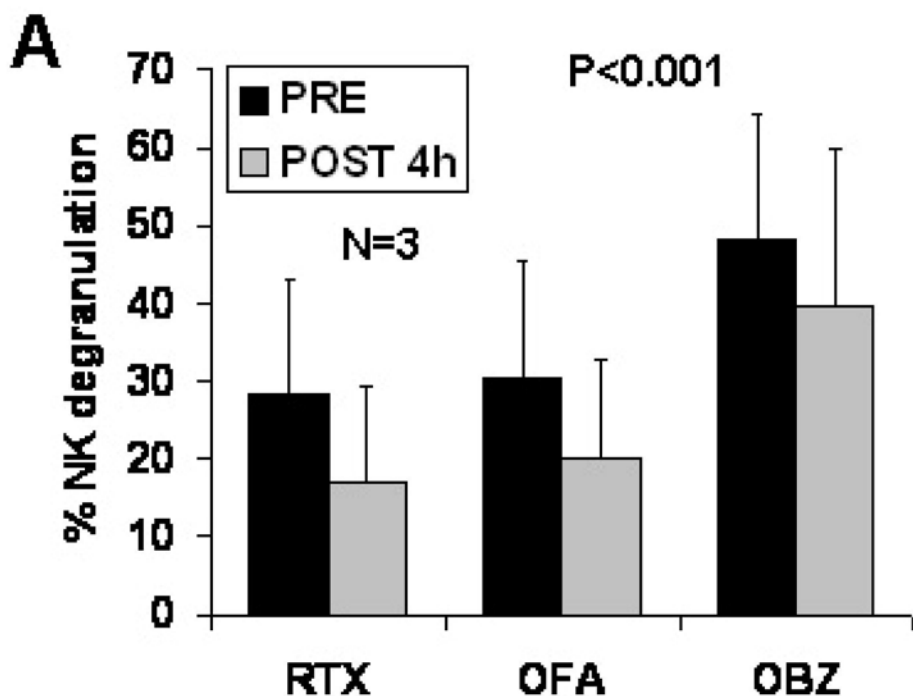




Fig.3



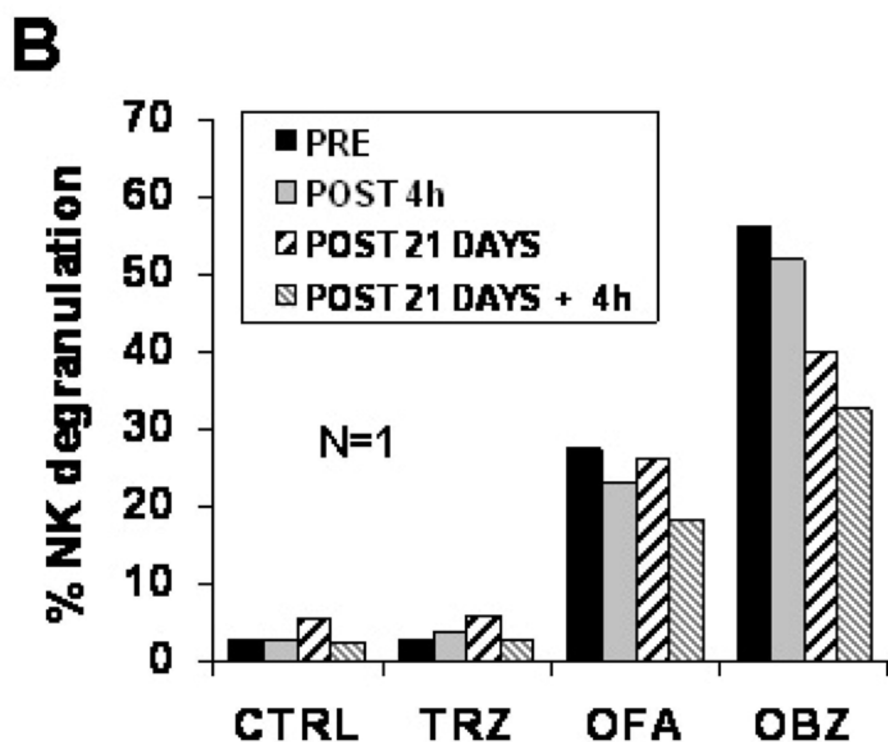
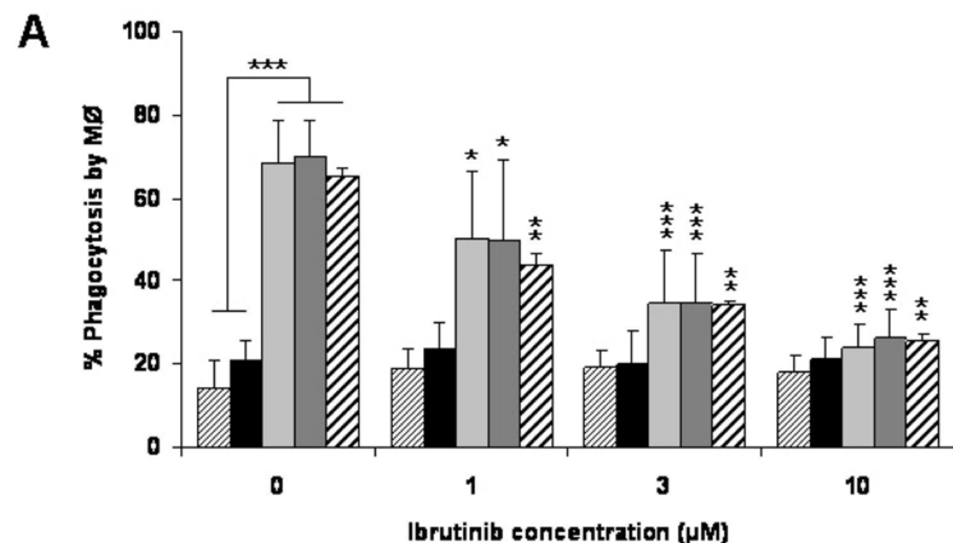
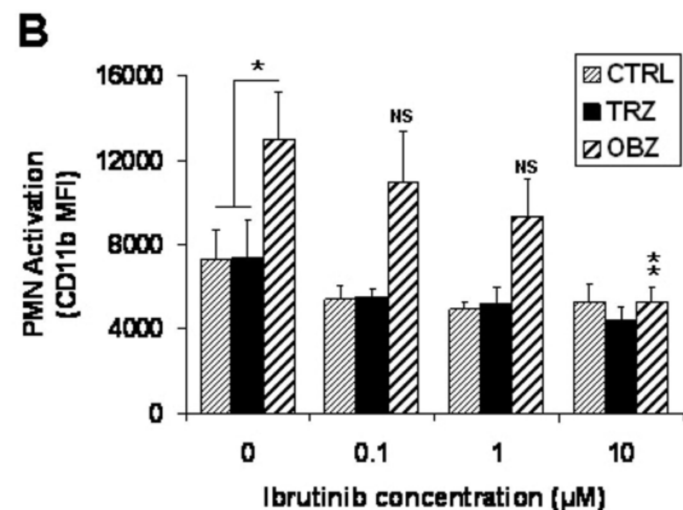




Fig. 4



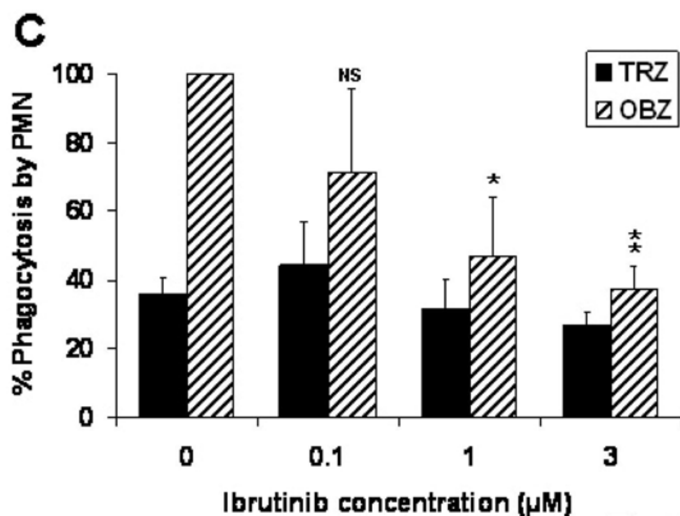




Fig.5

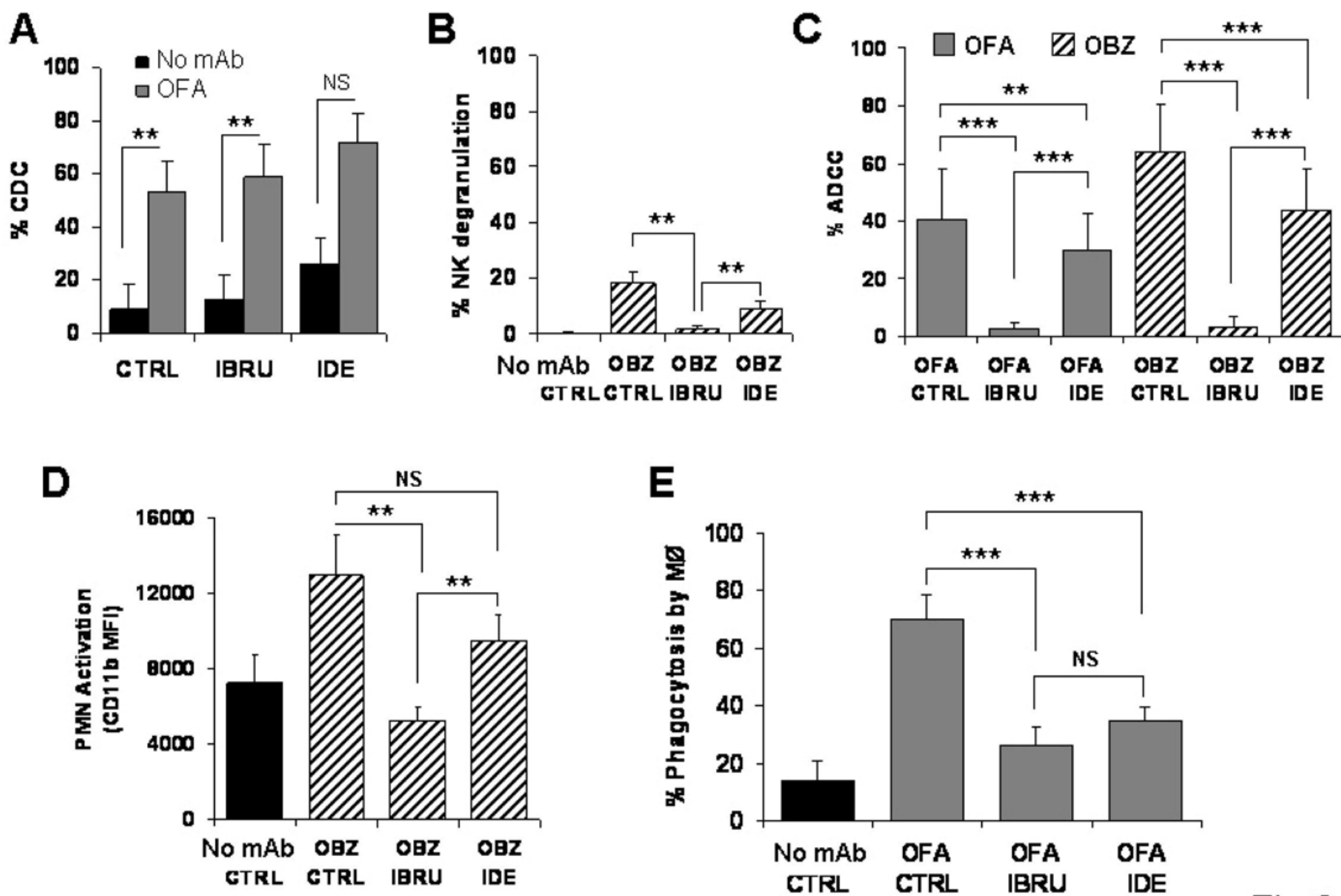
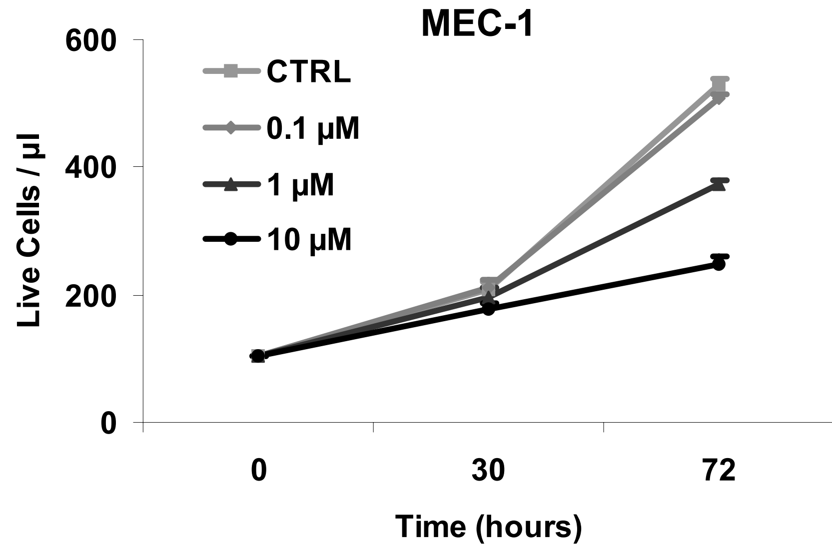
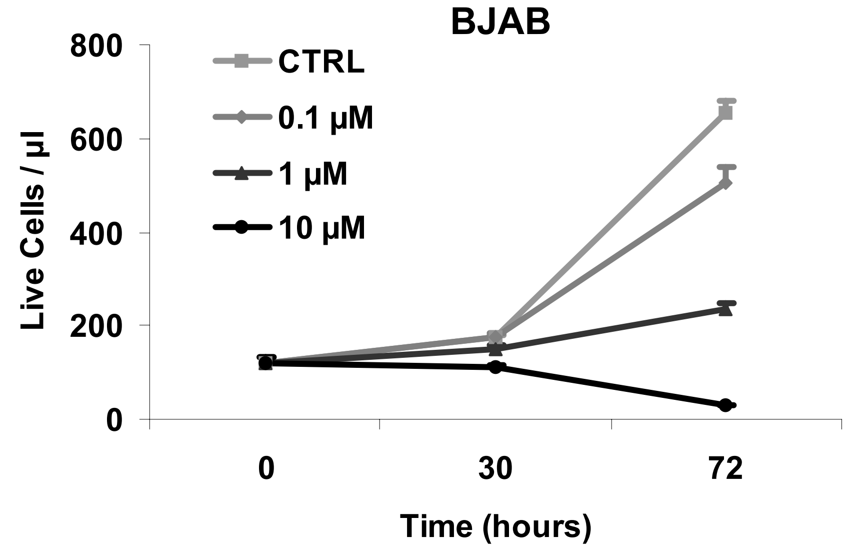


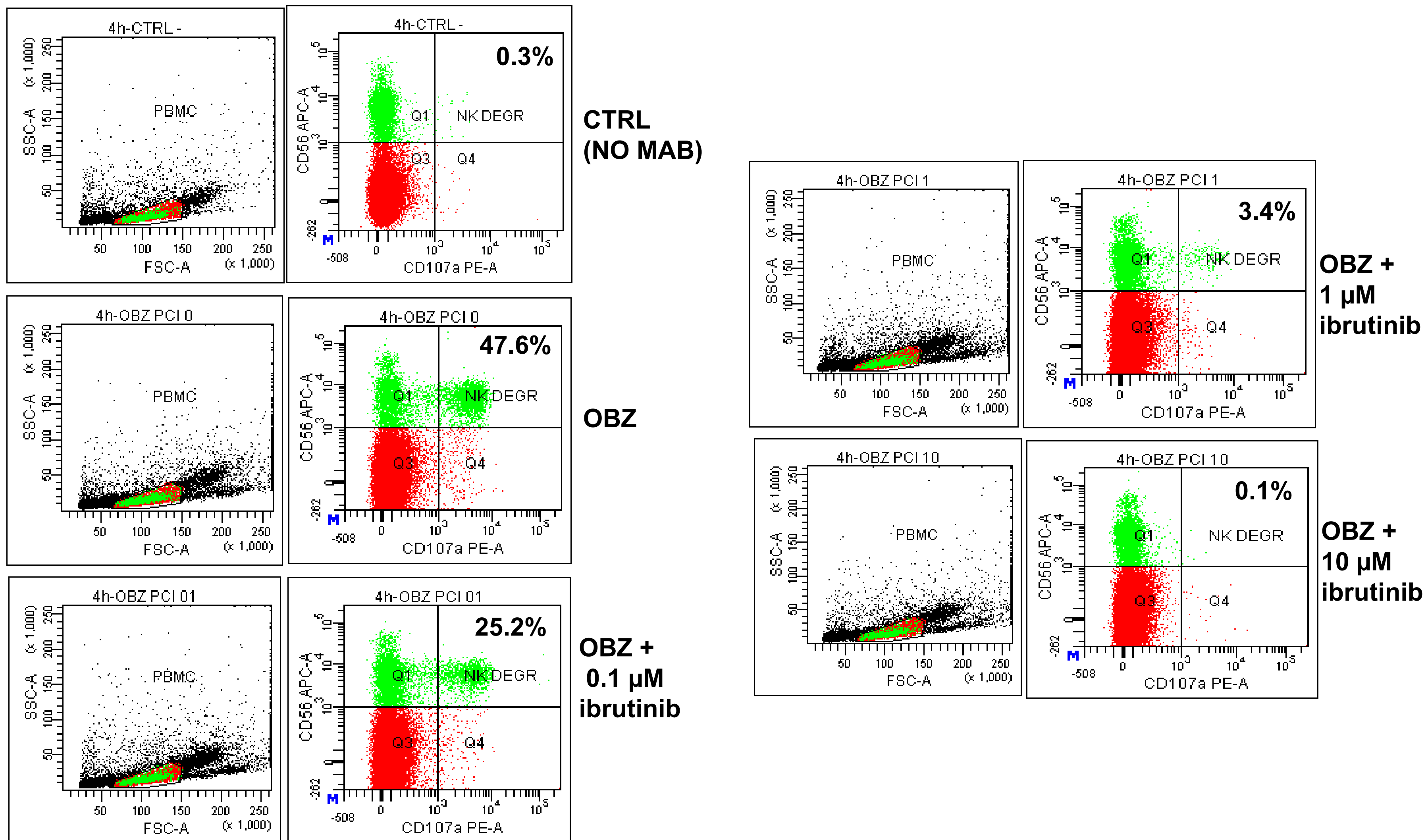

Fig.6

**Supplementary Figure 1. Effect of ibrutinib alone on MEC-1 and BJAB.** Cells were plated in presence or absence of 0.1 to 10 μM ibrutinib. Absolute numbers of live cells were measured at 30 and 72 hours by flow cytometry, using 7-AAD and calibration beads.



**Supplementary Figure 2. Dot plot of degranulation of NK cells by flow cytometry.** PBMC were untreated or treated for 1 hour with 0.1,1 or 10 μM ibrutinib followed by 4 hours incubation with CLL (CTRL) or CLL opsonised with OBZ. Percentages of CD107a⁺ CD56⁺ NK cells are shown in each panel



**Supplementary Fig. 3. Results of ADCC assays of MEC-1 cell line with 10 μM ibrutinib (A,C) or 10 μM idelalisib (B,D) induced by OFA (A,B) or OBZ (C,D)**