Obinutuzumab activates Fc\γRI more potently than other anti-CD20 antibodies in chronic lymphocytic leukemia (CLL)

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\textbf{ABSTRACT}

Treatment with monoclonal antibodies has revolutionized clinical medicine, especially in the fields of cancer and immunology. One of the oldest antibodies, which is widely used for the treatment of lymphomas and autoimmune diseases, is the anti-CD20 antibody rituximab. In recent years, new antibodies against CD20 have been developed including ofatumumab and obinutuzumab. An important mechanism of action of therapeutic monoclonal antibodies is activation of immune cells via Fc receptors (Fc\γRs). However, surprisingly, little is known about triggering of Fc\γRs by different therapeutic antibodies in general and anti-CD20 antibodies in particular. Here we establish a reporter assay to assess whether a particular antibody activates a certain Fc receptor. Using this assay we corroborated previous reports demonstrating obinutuzumab’s ability to highly activate Fc\γRIIa (CD16a). Importantly, we discovered that obinutuzumab also activates Fc\γRI (CD64) significantly more than rituximab and ofatumumab in response to chronic lymphocytic leukemia (CLL) cells obtained from patients. Mechanistically we show that this is due to the lack of Fc\γRIIb-mediated internalization of obinutuzumab following binding to CD20. Moreover, we show that obinutuzumab induces increased phagocytosis by primary macrophages in an Fc\γRI-dependent manner. Beyond the discovery of a new mechanism of obinutuzumab activity, the reporter assay can be applied to other therapeutic antibodies and may assist in developing antibodies with improved immunological properties.

\textbf{KEYWORDS}

anti-CD20; CLL; Fc receptors; obinutuzumab; rituximab

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\textbf{INTRODUCTION}

The anti-CD20 monoclonal antibody rituximab has significantly improved the treatment of B-cell lymphoproliferative diseases\textsuperscript{1} as well as that of autoimmune diseases.\textsuperscript{2} Rituximab is currently part of the backbone treatment regimen of many B-cell lymphoproliferative diseases including diffuse large B-cell lymphoma (DLBCL) and chronic lymphocytic leukemia (CLL).\textsuperscript{3} Rituximab also constitutes an important component in the treatment of several autoimmune diseases, especially autoimmune hemolytic anemia and immune thrombocytopenic purpura (ITP).\textsuperscript{4,5}

In recent years, new antibodies against CD20 have been developed, notably ofatumumab and obinutuzumab.\textsuperscript{6} Both antibodies have been used for the treatment of CLL with possible clinical advantage of obinutuzumab over rituximab.\textsuperscript{7,8} Obinutuzumab was also recently approved for the treatment of relapsed/refractory follicular lymphoma.\textsuperscript{9} Two other anti-CD20 antibodies, ibritumomab and tositumomab, were conjugated to a radioactive substance to enhance their cytotoxic activity, and additional anti-CD20 antibodies are currently being studied in clinical trials.\textsuperscript{8} In this study, we focused on the three anti-CD20 antibodies which are in clinical use in CLL: rituximab, ofatumumab and obinutuzumab.

Understanding how the various anti-CD20 antibodies exert their clinical outcome is of major importance.\textsuperscript{10} Two main structural properties can explain the clinically observed differences of the various anti-CD20 antibodies. The first is the specific recognition of the antigen by the antibody, i.e. different CD20 epitopes are recognized by the different antibodies. In addition, previous studies demonstrated that some anti-CD20 antibodies (e.g., rituximab and ofatumumab) are able to localize CD20 into lipid rafts, while others (e.g., obinutuzumab) are not, referred as “type I” and “type II” antibodies, respectively.\textsuperscript{11} This separation is not merely limited to CD20 localization but it is also associated with several other features, such as reduced recruitment of the complement system\textsuperscript{12} and less internalization by type II antibodies.\textsuperscript{13,14}

The second property is related to differences in the Fc portion of the various antibodies, which could affect complement recruitment and activation of Fc receptors on immune cells. Recognition of the antibody by various Fc receptors may be determined by the specific Fc isotype, specific amino acid sequences or specific glycosylations of the Fc segment. For example, the Fc segment of obinutuzumab has been glycoengineered so that the oligosaccharides attached to asparagine 297 (Asn\textsuperscript{297}) in the Fc region are non-
fucosylated in order to enhance the binding of the antibody to Fcγ receptor IIIa (FcγRIIIa, CD16a). Surprisingly, the interactions of various therapeutic antibodies with various Fc receptors have not been fully elucidated. Moreover, the available data on the interaction of different anti-CD20 antibodies and human Fc receptors is based on affinity tests, mainly surface plasmon resonance (SPR), and not on assays which measure the activation of Fc receptors.

Here we implemented a reporter assay to study whether certain anti-CD20 antibodies activate a particular Fc receptor. Using this system, we were able to demonstrate that obinutuzumab leads to increased activation of FcγRI (CD64) as compared to rituximab or ofatumumab.

Results

A reporter assay to study interactions of anti-CD20 antibodies with human Fcγ receptors (FcγRs)

To study which of the FcγRs can be activated, individually, by various anti-CD20 antibodies we stably transfected mouse BW5147 cells (BW cells) with chimeric FcγRs. These chimeric FcγRs included the extracellular part of a given FcγR fused to the transmembrane and cytoplasmic segments of the mouse CD3ε chain. Activation of a specific FcγR results in secretion of mouse interleukin-2 (mIL-2) which can be detected by ELISA (Fig. 1A).

We transduced BW cells with either of the following human FcyR-CD3ε chimeras: FcγRIIIa (CD16a, low and high affinity variants 158F and 158V respectively), FcγRIIa (CD32 a, low and high affinity variants, 131R and 131H respectively), and FcγRI (CD64). We verified the expression of the extracellular part of the FcγRs in the transfected BW cells by flow cytometry (Fig. 1B).

Obinutuzumab leads to increased activation of FcγRIIIa and FcγRI in response to Raji cells

We subsequently used this BW cell reporter system to study the differential activation of FcγRs by three anti-CD20 antibodies which are in clinical use: rituximab, ofatumumab and obinutuzumab. Obinutuzumab has been glycoengineered to have enhanced affinity to the two variants of FcγRIIIa. To test whether our reporter system is able to recapitulate these previous observations, we first studied the BW cells expressing the two variants of FcγRIIIa (158F and 158V, low and high-affinity respectively). We pre-incubated Raji cells, which express CD20, with rituximab, ofatumumab or obinutuzumab. The mIL-2 level with each antibody was normalized to the mIL-2 level of the control cells (transfected BW cells incubated with Raji cells without any antibody, defined as one). The pre-incubation of each of the three anti-CD20 antibodies with Raji cells resulted in secretion of mIL-2 significantly above the background level (Fig. 2A, the background level is marked with a horizontal line). More importantly, obinutuzumab activated the two variants of FcγRIIIa more efficiently than rituximab and ofatumumab (Fig. 2A). As a negative control, we repeated this experiment with BW cells expressing an empty vector and, as expected in this case, there was no activation by any of the anti-CD20 antibodies (supplemental Figure 1A).

Figure 1. The FcyR-CD3ε reporter system. (A) Schematic representation of the BW reporter system. BW5147 cells were stably transfected with the extracellular portion of different human Fcγ receptors fused to the transmembrane and cytoplasmic domains of mouse CD3ε chain. Activation of a specific FcγR-CD3ε results in secretion of mIL-2. (B) Each of the transfected BW cells was stained with a specific mAb against the particular FcγR it expresses (black histograms). Gray shaded histograms represent the background staining with an isotype-matched control antibody. One representative experiment is shown out of three performed.
Next, we tested two additional human Fcγ receptors. No major changes were noted in the activation of FcγRIIA (Fig. 2B, both low and high-affinity variants). However, we found that obinutuzumab leads to enhanced activation of FcγRI compared to rituximab and ofatumumab (Fig. 2C). To the best of our knowledge, the observation that obinutuzumab activates FcγRI more potently has not been previously reported.

As additional controls to these experiments we incubated the various BW reporter cells with the T cell line Jurkat, which does not express CD20, and, as expected, none of the anti-CD20 antibodies activated any of the FcγRs (supplemental Figure 1B).

**Obinutuzumab leads to increased activation of FcγRIIa and FcγRI in response to CLL cells**

We next used CLL cells of human patients as target cells for our BW reporter cells. In addition to the wide use of rituximab in this disease, both ofatumumab and obinutuzumab are approved for treatment of CLL.7,8

Staining of CLL cells with the three anti-CD20 antibodies revealed reduced binding of obinutuzumab as compared to rituximab and ofatumumab as previously described (supplemental Figure 2).12

We pre-incubated each of the three anti-CD20 antibodies with CLL cells obtained from patients and tested the activation of the different FcγRs. The mIL-2 level of each patient sample was first normalized to the mIL-2 level of the control cells as above. Afterwards, the normalized results of cells from 15 CLL patients which were tested with a given FcγR/CD3ζ were averaged.

With CLL cells, as with Raji cells, we initially tested the activation of the two variants of FcγRIIIa by anti-CD20 antibodies. As expected, obinutuzumab demonstrated clear and significant advantages over the other two antibodies in activating both FcγRIIIa variants (Fig. 3A). The two variants of FcγRIIIa were similarly activated by the three anti-CD20 antibodies upon interaction with the CLL samples (Fig. 3B).

Next, we tested the activation of FcγRI by the three anti-CD20 antibodies pre-incubated with CLL patient samples. Importantly, obinutuzumab activated FcγRI more potently than the other two antibodies (Fig. 3C). The advantage of obinutuzumab in activating FcγRI was noted in each patient we analyzed separately (supplemental Figure 3A). Although most of our samples included cells from untreated CLL patients, the effect was demonstrated also in patients who were treated in the past or currently being treated for their disease (n = 4). Regardless of the relative advantage of obinutuzumab in activating FcγRI, the variability between the patients in activating this Fc receptor (supplemental Figure 3A) and possibly other Fc receptors by anti-CD20 antibodies should be explored in future studies.

We repeated these experiments under different conditions of incubation time, antibody concentration and effector to target (E:T) ratio. Regardless of the experimental conditions, obinutuzumab was superior in activating FcγRI (supplemental Figure 3B).

**Obinutuzumab induces efficient antibody-dependent cellular cytotoxicity (ADCC) by primary NK cells**

To test the functional relevance of the findings by the BW reporter system, we initially focused on FcγRIIIa. For that we used primary bulk NK cells since NK cells are activated mainly by this FcγR.

We isolated NK cells from two donors which express the low-affinity (158F) or the high-affinity (158V) variants of FcγRIIIa. These variants were identified by genotyping (see supplemental methods) as well as by staining with two different antibodies against FcγRIIIa. The low-affinity variant of FcγRIIIa was stained only with the 3G8 antibody clone (Fig. 4A), whereas the high-affinity variant of FcγRIIIa was stained with both 3G8 and MEM154 antibodies (Fig. 4B).

CLL cells obtained from patients were incubated with each of the three anti-CD20 antibodies or with a control antibody. Then, primary NK cells were added and the extent of NK cell degranulation was assessed by quantifying the percent of CD107a+ NK cells. In agreement with the BW reporter assay, incubation of the CLL cells with obinutuzumab induced more degranulation of NK cells as compared to rituximab and ofatumumab, and the results were similar with both variants of FcγRIIIa (Fig. 4C and 4D).
Stronger activation of FcγRI by obinutuzumab results in enhanced elimination of CLL cells by primary macrophages

To test the functional relevance of FcγRI activation by obinutuzumab, we performed phagocytosis assays with primary macrophages which are known to express this FcγR. Primary macrophages were differentiated from peripheral blood mononuclear cell (PBMCs) by selecting adherent cells and then culturing them with human serum. We examined the purity of the macrophages by analyzing the surface expression of CD14 (Fig. 5A). As expected, these primary macrophages expressed several FcγRs including FcγRI (Fig. 5B).

For the phagocytosis assay, CLL cells were first labeled with CFSE and then incubated with each of the three anti-CD20 antibodies. Primary macrophages were then added and co-incubated for 24 hours. Wright staining of the cells after this incubation period revealed several CLL cells inside macrophages (Fig. 5C).

In order to quantify the effect of each of the antibodies we used flow cytometry to assess the percentages of CFSE<sup>pos</sup> cells in each of the experimental conditions (see methods and<sup>15</sup>). Incubation with obinutuzumab clearly enhanced the elimination of CLL cells as compared to incubation with rituximab (Fig. 5D).

To examine the role of FcγRI, we blocked FcγRI on the macrophages and repeated the phagocytosis assay. Blocking of FcγRI significantly reduced the elimination of CLL cells by obinutuzumab (Fig. 5E). In parallel, we tested a defucosylated variant of rituximab which has enhanced ability to activate FcγRIIIa but not FcγRI compared to WT-rituximab (see below). This enabled us to scrutinize the additive effect of the improved activation of FcγRI by obinutuzumab, and indeed obinutuzumab outperformed this rituximab variant (Fig. 5E). We therefore concluded that activation of FcγRI by obinutuzumab is functional.

Mechanism of action

To investigate why obinutuzumab can efficiently activate FcγRI, we initially studied the glycosylation properties of this antibody. Obinutuzumab has been glycoengineered so that its...
Fc segment has been defucosylated in order to increase the affinity of this antibody to FcγRIIIa. Therefore we first tested whether our BW reporter system is able to corroborate this finding by comparing the activation of FcγRIIIa between rituximab and a non-fucosylated variant of rituximab (rit-hIgG1fut). As expected, the non-fucosylated form of rituximab activated more potently the two variants of FcγRIIIa compared to WT-rituximab (Fig. 6A). Next, we tested the importance of fucosylation of the Fc segment in activating FcγRI. As shown before, obinutuzumab activated FcγRI more potently compared to ofatumumab and rituximab (Fig. 6B). Importantly, however, and in contrast to the results obtained with FcγRIIIa (Fig. 6A), the defucosylated form of rituximab (rit-hIgG1fut) did not enhance the activation of FcγRI as compared to WT-rituximab (Fig. 6B).

We also used our reporter system to study the effect of the isotype on the activation of FcγRI by testing a panel of different variants of rituximab which includes human and mouse isotypes (supplemental Figure 4). The only isotype which activated FcγRI in a significant manner was mIgG2a (rit-mIgG2a). However, since the three anti-CD20 antibodies studied here have the same isotype, this cannot account for the observed differences. We also examined the possibility that a difference in the Fc sequences of rituximab and obinutuzumab might affect the FcγRI activation and found them to be identical (supplemental Figure 5).

Next, we tested the possibility that the increased activation of FcγRI is related to internalization of the anti-CD20 antibodies. It has been reported that obinutuzumab (a type II antibody), is comparatively less internalized upon binding to CD20. We tested the internalization of anti-CD20 antibodies by comparing the CD20 staining on CLL cells using the various anti-CD20 antibodies, at 4°C (where internalization is not expected) and 37°C (where internalization can occur), along different time points (Fig. 6C). This experiment revealed significant internalization of rituximab and ofatumumab, but not obinutuzumab (Fig. 6C).

It was previously reported that the internalization of anti-CD20 antibodies is mediated through bridging of FcγRIIb and CD20. To investigate the role played by FcγRIIb, we initially confirmed that FcγRIIb is expressed on CLL cells (Fig. 6D, boxed) and then we blocked it. Blocking of FcγRIIb on CLL cells significantly reduced the internalization of rituximab and ofatumumab (Fig. 6D).

Next, we examined the role played by FcγRIIb in FcγRI activation. To this aim, FcγRIIb was pre-blocked on CLL cells and then the CLL cells were incubated, as before, with the different anti-CD20 antibodies and subsequently with the BW-FcγRI on the macrophages prior to their incubation with CLL cells. Error bars represent standard deviation of triplicates. * P < 0.05; ** P < 0.01; *** P < 0.001; Student’s t test. One representative experiment is shown out of two performed.

Figure 5. Obinutuzumab induces increased elimination of CLL cells by primary macrophages. (A) CD14 expression on primary macrophages, black histogram. Gray filled histogram represents background staining with an isotype-matched control antibody. (B) Expression of the indicated FcγRs on primary macrophages, black histograms. Gray filled histograms represent the background staining with an isotype-matched control antibody. (C) Wright staining of macrophages which were incubated with CLL cells and obinutuzumab (ET 1:1). White arrow heads: CLL cells inside macrophages. Original magnification, x40, Scale bar 100 μM. (D-E) Quantification of the elimination of CLL cells by primary macrophages. CLL cells were stained with CFSE, pre-incubated with the indicated anti-CD20 antibodies and then co-incubated with primary macrophages. The concentration of CFSE+ cells which remained in the supernatant was assessed by flow cytometry, normalized to control cells and its complement value to 100% was defined as the elimination ratio. (E) CLL cells were incubated with different anti-CD20 antibodies: rituximab, defucosylated rituximab and obinutuzumab, and then with primary macrophages. The phagocytosis assay with obinutuzumab was performed in parallel also with pre-blocking of FcγRI on the macrophages prior to their incubation with CLL cells. Error bars represent standard deviation of triplicates. * P < 0.05; ** P < 0.01; *** P < 0.001; Student’s t test. One representative experiment is shown out of two performed.
Pre-blocking of FcγRIIb on CLL cells significantly increased the activation of FcγRI by rituximab and ofatumumab, but not by obinutuzumab (Fig. 6E).

To further investigate whether the advantage of obinutuzumab in activating FcγRI is specific to cells expressing FcγRIIb, we over-expressed CD20 in Jurkat cells, which do not express FcγRIIb (Fig. 6F). We examined the activation of BW-FcγRI in response to the CD20-expressing Jurkat cells which were incubated with the different anti-CD20 antibodies and observed similar levels of activation by all three anti-CD20 antibodies (Fig. 6G).

Finally, to demonstrate that rituximab and ofatumumab, but not obinutuzumab can bind to FcγRIIb we used BW-FcγRIIb reporter cells, and found that obinutuzumab activates FcγRIIb significantly less than the other two antibodies (Fig. 6H).

**Discussion**

Here we employed a reporter assay to study the activation of human FcγRs by therapeutic anti-CD20 antibodies. Using this method we were able to corroborate previous reports regarding the enhanced activation of FcγRIIIa by obinutuzumab. More importantly, we discovered that obinutuzumab activates FcγRI more potently than other anti-CD20 antibodies.

The assay we used is based on transfected BW cells, which express human FcγRs fused to CD3ζ. The main advantage of this assay is that it reports to what extent an FcγR is activated. Moreover, the activity of the antibodies is tested when they are bound to their natural ligand (in this case, CD20). Additional advantages of this system are that it is specific to a particular FcγR, sensitive and easy to use. The common methods which are currently available to investigate interactions between antibodies and FcγRs have several limitations. SPR measurements determine binding affinities rather than activation, and the two do not necessarily correlate. In addition, functional tests with immune cells are complicated and primary immune cells usually express several Fc receptors which might confound the results.

Rituximab has dramatically improved the treatment of B-cell lymphoproliferative diseases and several autoimmune diseases. Even better results have been attained in CLL with the
novel anti-CD20 antibody obinutuzumab, yet this has yet to be confirmed in clinical trials using the same dose of both antibodies.8,10 The reasons accounting for the apparent improved efficacy of obinutuzumab were not completely understood. Obinutuzumab is considered to be a type II anti-CD20 antibody as it does not accumulate CD20 in lipid rafts,12 and this property has been associated with several other features, such as reduced recruitment of the complement system. In addition, obinutuzumab has been glycoengineered by defucosylation of asparagine 297 (Asn162)26 in the Fc region,12 in order to enhance the binding of this antibody to FcγRIIIa.17,18 Indeed, affinity tests based on SPR demonstrated that obinutuzumab has increased affinity to both variants of FcγRIIIa. We verified these findings with our reporter assay both in a CD20 expressing cell line (Raji) and, more importantly, in patient-derived CLL cells. We also showed that this advantage of obinutuzumab results in enhanced ADCC of CLL cells by primary NK cells, in line with previous studies which were largely performed with cell lines as targets and sometimes with PBMC’s as effectors.15,19,20,21,22

Whether obinutuzumab binds to FcγRs other than FcγRIIIa has not been clearly elucidated. It was recently reported that obinutuzumab binds to FcγRIIib with higher affinity than does rituximab leading to enhanced activation of polymorphonuclear neutrophils (PMNs).23 We have not assessed FcγRIIib using our system; however, its extracellular domain is highly homologous to that of FcγRIIIa.24

Here we show that obinutuzumab activates FcγRI more potently than rituximab and ofatumumab using CLL cells as targets which leads to enhanced elimination of the CLL cells by macrophages. Contradictory results were previously reported regarding the level of antibody dependent cellular phagocytosis (ADCP) induced by these antibodies. This might be explained by different experimental conditions, especially incubation times and effector cells (mouse vs. human cells, which express different FcγRs) as well as the different methods used to quantify the level of phagocytosis. Some studies, including in xenograft models, reported comparable levels of ADCP between obinutuzumab and rituximab.19,25 However, other studies imply that obinutuzumab induces stronger ADCP as compared to rituximab, especially with longer incubation periods.13,14,22

We also studied the mechanism which explains the advantage of obinutuzumab in activating FcγRI. Antibody defucosylation per se is expected to affect the antibody binding solely to FcγRIIa and FcγRIIib since these FcγRs are the only ones which are glycosylated at a particular position of asparagine 162 (Asn162).26 Indeed, antibody defucosylation did not affect the binding to FcγRI17 and glycoengineered obinutuzumab showed a comparable level of ADCP to that of the non-glycoengineered form of this antibody.27 The results of our BW reporter system support these observations, since defucosylation of rituximab increased the activation of FcγRIIIa but not of FcγRI.

We also tested the possibility that different levels of antibody internalization would explain the advantage of obinutuzumab. In recent years it has been reported that obinutuzumab undergoes less internalization as compared to rituximab and the internalization of rituximab is mediated through binding to FcγRIIb on the surface of CLL cells.13,14,22 We confirmed these observations and, more importantly, found that blocking of FcγRIib increased the activation of FcγRI by rituximab. In addition, we also showed that when CD20 is expressed in cells which do not express FcγRIib, the advantage of obinutuzumab in activating FcγRI is abolished. Finally, using our reporter system, we showed that obinutuzumab does not activate FcγRIIib, in contrast to rituximab.

Whether increased activation of FcγRI is a unique property of obinutuzumab or a general feature of type II anti-CD20 antibodies should be better elucidated in the future. A critical obstacle in addressing this issue is that the isotype of other type II antibodies is murine. Despite this, and in agreement with our results, type II anti-CD20 antibodies have been reported to undergo less internalization compared to type I antibodies.13,22 In addition, the type II antibody tositumomab has been shown to phosphorylate less FcγRIIib as compared to rituximab,13 and this was hypothesized to be a general property of type II antibodies.6

In summary, we established a novel reporter system to compare the activation of human FcγRs by anti-CD20 antibodies. Based on this method, we found that obinutuzumab leads to enhanced activation of FcγRI compared to both rituximab and ofatumumab. We suggest that this assay will enable better evaluation of new therapeutic antibodies against CD20 or against other targets in general.

Materials and methods

Cells and antibodies

The cell lines used in this study were Raji, Jurkat (both human) and the mouse cell line BW5147.

The following anti-CD20 antibodies were used: rituximab and obinutuzumab (Roche), ofatumumab (GSK) and rituximab isotypes (anti-hCD20 isotype collection, InvivoGen). Details of the antibodies used are included in the supplemental methods.

Generation of transfected BW cells and the BW reporter assay

The extracellular domain of human FcγRIIa (CD16a), FcγRIIa (CD32a), or FcγRI (CD64) was fused to the transmembrane and intracellular domains of the mouse CD3ζ chain and cloned into the pcDNA3 vector. Details regarding the generation of the transfected BW cells are included in the supplemental methods.

For the BW reporter assay, we first incubated target cells with different anti-CD20 antibodies for one hour on ice. As target cells, we used mostly human CLL cells obtained from patients and also Raji or Jurkat cells. Raji and Jurkat cells were irradiated prior to their incubation with the antibodies (6000 rad). Then the various BW cells were added, and incubated at 37°C and 5% CO₂ for 48 hours in a final volume of 200 μl. In each well we incubated 50,000 BW cells and 50,000 target cells (in the case of Raji or Jurkat) or 200,000 target cells (in the case of CLL cells). In most of the experiments we used 0.5μg of the anti-CD20 antibodies per well (5μg/ml), except when using Raji cells and the two variants of FcγRIIa, where we used 0.01 μg of the anti-CD20 antibodies per well (0.1 μg/ml). All cells were suspended in RPMI medium (Sigma-
Aldrich) supplemented with 1% glutamine, pyruvate, non-essential amino acids, penicillin-streptomycin (Biological industries) and 10% fetal bovine serum (FBS, Sigma-Aldrich), hereafter referred to as complete medium. The assay was performed in triplicates. After 48 hours, supernatants were collected, and the level of mouse interleukin-2 (mIL-2) was quantified, and the level of mouse interleukin-2 (mIL-2) was quantified by using anti-IL-2 mAbs and standard enzyme-linked immunosorbent assay (ELISA) (Powerwave XS Plate Reader, Biotek). As control antibodies for the anti-CD20 antibodies in the reporter assays we used trastuzumab (Roche) or human IgG1 isotype control. We performed the BW reporter assay under different experimental conditions including various incubation times, antibody doses and effector to target ratios.

**NK cell purification and CD107a degranulation assay**

Primary NK cells were isolated from the peripheral blood of healthy human volunteers, activated, and tested for purity as previously described. Low and high-affinity FcyRIIa-expressing donors were identified by genotype analysis as well as by FACS staining as described in the supplemental methods.

Details of the CD107a degranulation assay are included in the supplemental methods.

**Macrophage extraction and phagocytosis assay**

Macrophages were isolated and differentiated from the peripheral blood of healthy human volunteers by plastic adhesion (see for example). Peripheral blood mononuclear cells (PBMCs) were cultured in 10-cm plates with serum free medium for two hours. Then the medium was replaced with complete medium supplemented with 10% human serum (H4522, Sigma-Aldrich). This medium was replaced every few days until receiving a confluent plate of adherent cells.

For the phagocytosis assay, macrophages were harvested with EDTA, and seeded in 8-well chamber slides (SPL) at a concentration of 50,000 cells/chamber. The phagocytosis assay was performed on the following day. CLL cells were stained with CFSE (CellTrace, catalog c34554, Thermofisher scientific) and then incubated with different anti-CD20 antibodies for one hour on ice. The stained CLL cells were added to the macrophages and incubated at 37°C and 5% CO2 for 24 hours. We used 50,000 macrophages together with 50,000 target cells per chamber in a final volume of 200–250 μL. We used 0.5 μg (5 μg/ml) anti-CD20 antibody per chamber. For the blocking experiments, macrophages were pre-incubated for one hour with the anti-CD64 antibody (cat. 305002, BioLegend) at a dose of 1 μg (6.7 μg/ml). After 24 hours of incubation the medium of each chamber was extracted and the concentration of CFSE positive CLL cells in each chamber was assessed by flow cytometry. The reading of the cell concentration was performed in triplicates. For each chamber we calculated an elimination percentage which was defined as:

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\frac{(1 - \text{Concentration of CFSEfluorescent CLL cells in test chamber})}{\text{Concentration of CFSEfluorescent CLL cells in control chamber}} \times 100.
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As controls we used cells incubated with a control antibody or without any antibody. In addition, after extracting the medium the slides were rinsed, fixed and Wright stained (Hematek, Siemens).

**Internalization assays and ectopic expression of CD20**

For the internalization assays, CLL cells were incubated as mentioned above with anti-CD20 antibodies at 37°C and 4°C. For pre-blocking of FcyRIIb on the CLL cells we used anti-CD32 antibody (GTX74628, clone AT10) at a dose of 1 μg (10 μg/ml). A CD20 expressing plasmid was kindly provided by Professor Daniel Davis, the University of Manchester. CD20 was cloned into pHAGE-DsRED(−)-eGFP(+) lentiviral vector using a restriction free (RF) methodology. Lentiviral virions were produced by transient three-plasmid transfection of 293T cells as previously described. These viruses were used to transduce Jurkat cells and surface expression of CD20 was monitored by FACS.

**Human samples**

The collection of patient samples was approved by the institutional Helsinki committee of Hadassah Medical Center. Peripheral blood samples of CLL patients were collected in heparin tubes after obtaining informed consent. The samples were separated using a Ficoll gradient and the mononuclear fraction was aliquoted, cryopreserved in freezing solution containing 90% fetal bovine serum and 10% DMSO and later thawed for use.

**Statistics**

Excel and GraphPad Prism software version 7.02 were used for statistical analysis. For statistical tests we used Student’s t test and ANOVA. A statistical test was considered significant when \( p < 0.05 \).

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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Author contributions

S.E. designed and performed experiments, analyzed results, and wrote the paper; S.K. and R.K. assisted in performing experiments; N.K. generated the transfected BW cells; and O.M. supervised the project.

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