B cell depletion in vitro and in vivo with an afucosylated anti-CD19 antibody

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MEDI-551: an afucosylated anti-CD19 antibody

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Non-standard abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ALL, acute lymphoblastic leukemia; BM, bone marrow; CDC, complement-dependent cytotoxicity; CLL, chronic lymphocytic leukemia; DLBCL, diffuse large b cell lymphoma; FL, follicular lymphoma; mAb, monoclonal antibody; PBS, phosphate buffered saline; PBMC, peripheral blood mononuclear cell; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; Tg, transgenic.

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Abstract

The pan B cell surface antigen CD19 is an attractive target for therapeutic monoclonal antibody (mAb) approaches. We have generated a new afucosylated anti-human CD19 mAb, MEDI-551, with increased affinity to human FcγRIIIA and mouse FcγRIV and enhanced antibody-dependent cellular cytotoxicity (ADCC). During in vitro ADCC assays with B cell lines, MEDI-551 is effective at much lower mAb concentrations than the fucosylated parental mAb anti-CD19-2. Further, the afucosylated CD19 mAb MEDI-551 depleted B cells from normal donor peripheral blood mononuclear cell (PBMC) samples in an autologous ADCC assay, as well as blood and tissue B cells in human CD19/CD20 double transgenic (Tg) mice at lower concentrations than that of the positive control mAb, rituximab. In huCD19/CD20 Tg mice, both macrophage mediated phagocytosis and complement-dependent cytotoxicity (CDC) contribute to depletion with rituximab; MEDI-551 did not require complement for maximal B cell depletion. Further, extended B cell depletion from the blood and spleen was achieved with MEDI-551, which is likely explained by bone marrow B cell depletion in huCD19/CD20 Tg mice relative to the control mAb rituximab. In summary, MEDI-551 has potent B cell depleting activity in vitro and in vivo and may be a promising new approach for the treatment of B cell malignancies and autoimmune diseases.
Introduction

B lymphocytes are an important component of the immune system and are the source of humoral immunity. However, B cells can also be pathogenic and the origin of disease. Deregulated B cell function has been implicated in several autoimmune diseases, including systemic sclerosis, systemic lupus erythematosus (SLE), and rheumatoid arthritis (RA) (Edwards and Cambridge, 2006; Gabrielli et al, 2009). B cells contribute to pathological immune responses through the secretion of cytokines, co-stimulation of T cells, antigen presentation, and the production of autoantibodies (Browning, 2006; Walker and Fritzler, 2007; Yanaba et al, 2008). Furthermore, the majority of human leukemias and lymphomas are of B-cell origin (Kuppers, 2005). Despite recent advances in the clinic, B cell malignancies, such as acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), follicular lymphoma (FL), and diffuse large B cell lymphoma (DLBCL), still present a significant challenge and curative treatment is not possible in most cases (Coffier et al, 2005a; Coffier et al, 2005b).

B cell depletion by targeting B cell restricted cell surface antigens with monoclonal antibodies (mAbs) has gained significant attention in recent years. In particular, the CD20 mAb rituximab, which is already approved for the treatment of NHL and DLBCL, has shown promising results in the treatment of B cell malignancies. While the combination of rituximab with chemotherapy has led to significant improvements in patient survival in FL and other malignancies, many patients relapse after treatment (Maloney, 2005). In addition, low CD20 expression has been associated with poor response to rituximab in DLBCL, and rituximab treatment can lead to down-regulation of CD20 or the selection of CD20-deficient tumor clones (Johnson et al, 2009; Davis et al,
Rituximab has also shown activity in autoimmune diseases, in particular in RA, but reductions in the levels of circulating autoantibodies, which may play an important role in SLE and other indications, are not consistent (Levesque and St Clair, 2008; Tedder, 2009).

CD19 is a B cell-specific surface antigen that is expressed by early pre-B cells from the time of heavy chain rearrangement until the molecule is eventually down-regulated during terminal differentiation into plasma cells (Nadler et al, 1983). CD19 belongs to the immunoglobulin domain-containing superfamily of transmembrane receptors. As a component of the B-cell receptor complex, CD19 regulates the threshold for B-cell activation (Engel et al, 1995). Relatively small changes in CD19 surface expression can lead to loss of tolerance and autoantibody production (Sato et al, 2004).

In hematopoietic malignancies of B cell origin, expression of CD19 is maintained following malignant transformation of B cells and the majority of B cell-derived leukemias and lymphomas consistently express CD19 (D’Arena et al, 2000). Importantly, malignant B cells that have lost CD20 expression during the course of rituximab therapy maintain expression of CD19 (Johnson et al, 2009). Thus, CD19 is an attractive target for antibody-mediated B cell depletion therapy in multiple hematologic malignancies. Due to the expression of CD19 on plasmablasts, which are CD20 negative (Levesque and St. Clair 2008), a depleting CD19 mAb may also have a more significant impact than CD20 targeted therapies on the levels of pathogenic autoantibodies in autoimmune diseases (Tedder, 2009).
Here we report the generation of a new anti-human-CD19 mAb in development for the therapeutic depletion of B cells. The humanized and affinity optimized MEDI-551 mAb has binding characteristics which are favorable for an ADCC-dependent mechanism. MEDI-551 was generated by removal of the fucose carbohydrate modification from the Fc portion of the humanized CD19 mAb anti-CD19-2, which resulted in a selective increase in the affinity for human FcγRIIIA and mouse FcγRIV. The anti-CD19-2 mAb was developed by humanization and affinity optimization of the HB12b mAb, which has potent B cell depleting activity in transgenic (Tg) mice expressing human CD19 (huCD19) (Kansas and Tedder, 1991; Yazawa et al, 2005). During in vitro ADCC assays, MEDI-551 depleted primary human B cells at lower mAb concentrations than the CD20 mAb rituximab. Furthermore, at low mAb doses, MEDI-551 is more effective than rituximab in depleting blood and tissue B cells in huCD19/CD20 double Tg mice, despite the absence of complement-dependent cytotoxicity (CDC), which contributes to in vivo depletion with rituximab. In the Tg mouse model, macrophages are important for in vivo B cell depletion with the afucosylated human IgG1, consistent with the increased binding to mouse FcγRIV. In summary, MEDI-551 is a new anti-CD19 mAb optimized for enhanced ADCC effector function and may be found to be a useful new reagent for the treatment of B cell malignancies and B cell-dependent autoimmune disease.
Material and Methods

Cells and reagents

The Burkitt’s lymphoma cell lines Raji, Daudi and Ramos, and the human monocyte line THP-1 were obtained from the American Type Culture Collection (Manassas, VA.). The KC133 NK cell line expressing human CD16 was obtained from BioWa Inc. (Princeton, NJ). All cell lines were maintained in RPMI medium, supplemented with 10% fetal bovine serum. Blood samples (PBMC and serum) were obtained from healthy donors after obtaining informed consent. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation using Histopaque-1077 according to the manufacturer’s instructions and B cells were purified by negative selection using the Naive B Cell Isolation Kit II according to the manufacturer’s instructions (Miltenyi Biotec; Auburn, CA). The mAbs used were anti-CD22 clone S-HCL-1 (BD Immunocytometry Systems, San Jose, CA) and rituximab (Biogen Idec, Inc; Cambridge, MA) (Anderson et al, 1997). The human IgG1 mAb R347 (MedImmune, Inc) was used as isotype control. Mouse specific mAbs used were B220 (CD45R; Invitrogen, Carlsbad, CA) and 1D3 (CD19; Becton Dickinson Pharmingen, San Diego, CA).

Antibody engineering

To generate a CD19 mAb with enhanced ADCC effector function, the mouse IgG1 mAb HB12b (Kansas and Tedder, 1991), which recognizes human CD19, was humanized and affinity optimized, resulting in mAb anti-CD19-2. To generate a homogenously afucosylated antibody, the humanized IgG1 mAb anti-CD19-2 was expressed in a
fucosyltransferase-deficient producer CHO cell line (BioWa Potelligent® Technology, BioWa Inc.; Princeton, NJ) to generate MEDI-551.

**Cell proliferation assay**

To test the effect of immobilized (cross-linked) mAb on B cell function, 100 ng of anti-CD19-2 mAb or isotype control mAb in phosphate buffered saline (PBS) was used to coat triplicate wells of a flat bottom high-binding 96 well plate (Costar 3361) overnight at room temperature. Next, the wells were washed and the cells were seeded at a density of 10,000 cells per well. Cells were assayed on day three using the CellTiter-Glo® Luminescent Cell Viability Assay according to the manufacturer’s instructions (Promega; Madison, WI).

**BIAcore affinity measurements**

The affinity (K_D) for the binding of human and murine FcγRs to IgG mAbs was measured on a BIAcore 3000 instrument (Uppsala, Sweden). Briefly, the fucosylated and afucosylated forms of the IgG1 humanized CD19 MAb were immobilized onto separate flow cells on a CM5 sensor chips using a standard amino coupling chemistry as outlined by the instrument’s manufacturer. A reference flow cell without mAb was also prepared on each sensor chip. Stock solutions of FcγRs (Oganesyan et al, 2008) were serially diluted using instrument buffer (HBS-EP buffer containing 0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA and 0.005% P-20 detergent). FcγR were injected over both the IgG and reference cell surfaces at a flow rate of 5 μL/min. Binding data were collected for 50 min, followed by a 60 sec pulse of 5 mM HCl between injections to remove bound
FcγR from the IgG surfaces. After all binding data were collected, individual data sets were averaged for binding (Response at equilibrium, or “Req”) at each concentration (C), and then fit to a 1:1 binding isotherm (“Req vs. C”) plot. From this, the equilibrium binding constants, K_D, were derived. Such calculations were performed with BIAevaluation software, v. 4.1 (BIACore, Inc, Uppsala, Sweden).

**ADCC assays**

ADCC assays were performed with a set effector-target cell ratio of 2.5:1. Briefly, Daudi target cells were washed with PBS, resuspended in RPMI-5 Phenol-Free media at a cell density of 0.4x10⁶/mL. KC1333 NK cells were washed once in PBS and resuspended in RPMI-5 Phenol Free media at a cell density 1x10⁶/mL. Assays were performed in U bottom 96 well plates. Experimental wells were set up by combining 50 μL of the appropriate mAb dilution, 50 μL of target cell suspension and 50 μL of effector cell suspension. Reactions were set up in triplicates. Following set up, plates were spun at 120 x g for 3 minutes to pellet the cells. Plates were incubated at 37° C/ 5% CO₂ for 4 hours. Target cell lysis was measured by detecting the release of lactate dehydrogenase (LDH) from the cytoplasm of lysed cells using the CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, WI) performed according to the manufacturer’s directions. Forty five minutes prior to the end of incubation 15 μL of manufacturer provided Lysis Buffer was added to the Target Cell Maximum Release Control wells and Detergent Background wells. After incubation the plate was centrifuged at 120 x g for 3 minutes. 50 μL of the supernatant from each well was transferred to a new flat bottom 96 well plate. 50 μL of reconstituted substrate mix (assembled from manufacturer provided
components) was added and the plate was incubated at room temperature 10-20 minutes protected from light. 50 μL of manufacturer provided stop buffer was added and absorbance at 490 nM was measured in a plate reader.

\[
\text{% Cytotoxicity} = \frac{\text{experimental LDH release} - \text{spontaneous LDH release}}{\text{maximum LDH release} - \text{detergent background}} \times 100.
\]

Prior to calculating the % cytotoxicity all values were reduced by the media background.

Multi-parameter flow cytometry was used to quantify in vitro ADCC activity using peripheral blood mononuclear cells (PBMC) from healthy adult volunteer donors. In this assay, the PBMC are the source of both the natural killer effector cells and the B cell targets. The PBMC were re-suspended at 10^6 cells per mL in culture media (RPMI 1640, 10 % heat in-activated FBS, 2 mM L-glutamine), supplemented with 200 ng/mL recombinant human IL-2 (PeproTech, Inc; Rocky Hill, NJ). A total of 2x10^5 PBMC were added to each well in Nunc U96-well round bottom microwell plates. Serial dilutions of MEDI-551 or rituximab were added in 10 μL to wells in triplicate and incubated for 20 hours at 37°C with 5% CO₂. Cells were then washed once in stain buffer (PBS containing 0.5% BSA) and re-suspended in 100 μL of a cocktail of fluorescently labeled antibodies in stain buffer containing anti-CD19 Phycoerythrin-Cy7 (PE-Cy7), anti-CD20 Pacific Blue, anti-CD22 allophycocyanin (APC) or anti-CD22 phycoerythrin (PE), and anti-FceRIα fluorescein isothiocyanate (FITC). The cells were stained for 30 minutes on ice, washed once in stain buffer then re-suspended in a final volume of 100 μL of stain buffer containing 5 μL of the viability solution, 7-amino-actinomycin D (7-AAD). As a counting standard, 20 μL per well of CountBright Absolute Counting Beads was added to determine cell concentration of cell subsets. Samples were acquired on an LSR II flow
cytometer equipped with a high throughput capability for running 96-well microwell plates.

The fluorescence activated cell sorting (FACS) data were analyzed with FlowJo software, version 7.2.2 for PC. The gating strategy used to quantify B cell depletion was as follows. Initially, dead 7-AAD positive cells were excluded, and then live lymphocytes were gated using their forward and light scatter characteristics. Basophils, eosinophils and mast cells within the lymphocyte gate were excluded on the basis of their expression of FcεR1 alpha. With rituximab, it was evident that the antibody treatment reduced CD19 expression. Consequently, for this antibody treatment, the total number of surviving B cells was defined by a single gate of all CD22+ cells. For MEDI-551 treatment, the number of surviving B cells in the CD20+CD22+ gate was determined. The IgG1 afucosylated antibody, R347-aFuc, was used as a non-depleting treatment control and was used to define these gates. The number of absolute counting beads in each sample was quantified in a forward scatter gate. The numbers of surviving B cells in the total CD22+ or CD20+CD22+ gates were converted to cell concentrations using the standard counting beads according to the manufacturer’s instructions.

The B cell depletion (percentage cytotoxicity) was then calculated according to the following formulae. For rituximab:

\[
\% \text{ cytotoxicity} = \left\{ 1 - \frac{\text{rituximab CD22+ (cells/mL)}}{\text{R347-afuc CD19+CD22+ (cells/mL)}} \right\} \times 100.
\]

For MEDI-551:

\[
\% \text{ cytotoxicity} = \left\{ 1 - \frac{\text{MEDI-551 CD20+CD22+ (cells/mL)}}{\text{R347-aFuc CD20+CD22+ (cells/mL)}} \right\} \times 100.
\]
The half-maximal effective concentration (EC₅₀) of B-cell depletion was calculated using a four-variable curve fit equation in GraphPad Prism v5.01 software (GraphPad Software, La Jolla, CA). The results are shown in Table II and include the 95% confidence interval as well as the R² value (goodness of fit).

Complement-dependent cytotoxicity assay

The CDC assay was performed using the same LDH read-out assay as the ADCC assay. Briefly, human donor serum was collected and divided into two separate aliquots. One was treated at 56°C for 30 min to inactivate complement followed by centrifugation for 10 min at 3000 x g while the other remained untreated. Media containing Phenol-free RPMI and 10% of either heat-inactivated or non-heat inactivated serum was prepared. Target cells were washed with PBS, resuspended in either RPMI-1640 containing heat-inactivated or non-heat-inactivated serum, at a cell density of 0.4x10⁶/mL. Experimental wells were set up by combining 50 μL of the appropriate antibody dilution (in either heat inactivated or non-heat inactivated media), 50 μL of target cell suspension (in either heat inactivated or non-heat inactivated media) and 50 μL media (either heat inactivated or non-heat inactivated). Reactions were set up in triplicates and plates were incubated at 37°C/5% CO₂ for 4 hours. Forty five minutes prior to the end of incubation 15 μL of manufacturer provided Lysis Buffer was added to the Target Cell Maximum Release Control well and Detergent Background wells. After incubation the plate was centrifuged at 120 x g for 3 min. 50 μL of the supernatant from each well was transferred to a new flat bottom 96 well plate. 50 μL of reconstituted substrate mix (assembled from manufacturer provided components) was added and the plate was incubated at room
temperature for 10-20 min protected from light. 50 μL of manufacturer provided stop buffer was added and absorbance at 490 or 492 nm was measured in a plate reader. % Cytotoxicity was calculated as described above for ADCC.

B cell depletion in huCD19/CD20 transgenic mice

All mouse experiments were carried out in a pathogen-free environment at the MedImmune animal facility in accordance with IACUC approved protocols. In vivo depletion of B cells by CD19 and CD20 mAbs was evaluated in huCD19/CD20 double Tg mice, which were generated by crossing huCD19 Tg with huCD20 Tg animals (Zhou et al, 1994; Ahuja et al, 2007). Tg mice (10-15 weeks old; 5-8 animals per group) were randomized and treated with MEDI-551 or rituximab by tail vein injection. PBS-treated animals were used as controls (3-5 animals per group). Blood, spleen and bone marrow samples were collected for further analysis using flow cytometry. B cell numbers were determined in each sample by staining with APC-Cy5.5 conjugated B220 (CD45R) and PerCP-Cy5.5 conjugated muCD19 antibodies. Single cell bone marrow and spleen cell suspensions were loaded at 1.5 million cells/well for flow cytometry analysis.

Mechanism of in vivo B cell depletion

Tg mice were treated with MEDI-551 or rituximab in the absence of complement or of one of the potential effector cell populations.

To study the contribution of complement for B cell depletion, hCD19 Tg mice (three mice per group; 8-12 weeks old) were treated with 30 μg/mouse cobra venom factor (CoVF, Sigma; St Lewis, MO) or PBS every other day for five days (Minard-
Colin, et al., 2008). Twenty-four hours following the first i.p. dose of CoVF, mice were treated with either MEDI-551 or rituximab (10 mg/kg, i.v.) or PBS. Serum C3 levels were detected before mAb injection and at the study endpoint by ELISA (Immunology Consultants Laboratory, Newberg, OR) (data not shown).

To deplete NK cells and neutrophils, hCD19 Tg mice were treated with i.p. injections of 0.25 mg/mouse anti-NK1.1 (clone PK136) or anti-GR1 (clone RB6-8C5) (Biolegend; San Diego, CA), respectively (Gong et al., 2005). Anti-NK1.1 treatment occurred every other day for five days (days -1, 1, 3). Anti-GR1 was given as a single injection (day -1). Treatment with MEDI-551 or rituximab (10 mg/kg mAb) followed 24 hours after the first injection (day 0). The depletion of NK cells and neutrophils was verified with FACS analysis prior to treatment with MEDI-551 or rituximab and throughout the duration of B cell depletion. NK cells were detected as CD49b+CD335+ in the blood and CD49b+Ly49+ in the spleen and bone marrow. Neutrophils were detected as CD11b+SSC^{high} (data not shown).

To assess the role of macrophages, hCD19 Tg mice were treated with injections of liposome-encapsulated clodronate (Encapsula; Nashville, TN) (5-7 animals per group). PBS-loaded liposomes were used as control (3-4 animals per group) (Van Rooijen and Sanders, 1994). Loaded liposomes were i.p. injected at 0.2 mL/mouse every other day for five days prior to treatment with 10 mg/kg MEDI-551 or rituximab. An additional 0.2 mL/mouse was injected two days following mAb treatment. The depletion of macrophages was verified by FACS and immunofluorescence histology. The number of macrophages in spleen was determined by FACS staining of F4/80+ cells prior to the treatment with MEDI-551 and again at the endpoint. The spleens of mice were also
harvested for histology. Slides of spleen sections, prepared from OCT blocks, were stained for B cells (Alexa555 or Pacific Blue conjugated B220), T cells (Alexa647 conjugated CD90), red pulp macrophages (Alexa488 conjugated F4/80), marginal zone metaphilic macrophages (biotinylated MOMA-1 followed by pacific blue conjugated strepavidin) and/or marginal zone macrophages (Alexa488 conjugated ERTR9) (data not shown).

**Statistical Analysis**

The data were analyzed and graphed using GraphPad Prism software (GraphPad Software, La Jolla, CA). Mean values and standard deviation (SD) was calculated for experiments as indicated in the figure legends. Statistics were performed using a two-tailed, two sample t test in GraphPad Prism and are determined from triplicate wells of each donor tested. Significant differences are noted by p value: *, p<0.05; **, p<0.01; ***, p<0.005.
Results

Removal of fucose enhances the ADCC effector function of the anti-CD19-2 mAb

ADCC is an important mechanism by which therapeutic mAbs mediate target cell depletion (Glennie et al, 2007). Engagement of effector cells requires the interaction of the mAb Fc with Fcγ receptors on the surface of NK cells, monocytes/macrophages, or neutrophils. It has been demonstrated that the removal of fucose from the carbohydrate modification of the mAb Fc preferentially increases the affinity to FcγRIIIA, resulting in increased ADCC effector function (Shields et al, 2002; Nimmerjahn and Ravetch, 2008). To generate a CD19 mAb with enhanced effector function, the humanized and affinity optimized CD19 mAb anti-CD19-2 was expressed in a fucosyltransferase deficient CHO cell line, which generates homogenously afucosylated mAbs (BioWa Potelligent Technology). The afucosylated form of anti-CD19-2 is termed MEDI-551. We first tested the effect of fucosylation on the binding of the humanized IgG1 anti-CD19 mAb to various human and mouse Fcγ receptors by measuring the equilibrium binding constant (K_D) on a BIAcore instrument. The removal of fucose had no significant effect on the binding to human FcγRI and FcγRIIa (Table I). The afucosylated mAb, however, has ~9-fold increased affinity to the activating FcγRIIIA. Similarly, removal of fucose resulted in a 10-fold increase in binding affinity to mouse FcγRIV, the mouse orthologue of human FcγRIIIA. The binding to mouse FcγRIIb and FcγRIII were unaffected by the removal of fucose. These results are consistent with previously published data and further demonstrate a selective increase in the binding of afucosylated IgG1 mAbs to human FcγRIIIA and mouse FcγRIV (Shields et al, 2002; Nimmerjahn and Ravetch, 2008).
Although the relative contribution of different effector cell populations in various tissues is still not fully established for human subjects, NK cells likely play an important role. In ADCC assays with human PBMC as the source of the effector cells, almost all activity is provided by the NK cells (Desjarlais et al, 2007). Therefore we tested the effector function of MEDI-551 and the fucosylated version the mAb (anti-CD19-2) in assays with NK cells as the effector cell population. The increased affinity of MEDI-551 for FcγRIIIA resulted in a substantial enhancement of its ADCC activity in vitro (Figure 1A). This increase in ADCC effector function was reflected in a lower EC50 value for cytotoxicity (3.1 ng/ml for MEDI-551 versus 57.6 ng/ml for the fucosylated mAb) as well as an increase in overall cytotoxicity (50% for MEDI-551 versus 17% for mAb anti-CD19-2). In repeated assays with Daudi cells as targets, MEDI-551 was also more effective at low mAb concentrations than the rituximab CD20 mAb (EC50 = 41.0 ng/ml), which was included as a positive control (Figure 1A).

To further characterize the anti-CD19 mAb, we tested mAb anti-CD19-2 for additional Fc-dependent and independent effects on B cells. In addition to ADCC, CDC is an Fc-dependent mechanism by which mAbs against cell surface antigens can mediate target cell killing and is well characterized for rituximab (Glennie et al, 2007). When tested in vitro with Daudi cells and human serum as a source of complement, the anti-CD19-2 mAb was not active in a CDC assay, while the rituximab CD20 mAb showed significant cytotoxicity (Figure 1B).

Antibodies against cell surface antigens can also affect proliferation and/or viability of the target cell. Previous reports demonstrated that some, but not all, mAbs against CD19 can inhibit proliferation of B leukemia or lymphoma cells (Ghetie et al, 1994). Therefore
we tested the effect of anti-CD19-2 mAb on cell proliferation of Raji, Daudi, and Ramos B lymphoma cell lines, using a luminescent viability assay. As a control, we also included the monocytic cell line THP-1. As shown in figure 1C, incubation with anti-CD19-2 mAb resulted in a significant decrease in the number of viable B cells as compared to cells treated with the isotype control mAb. Proliferation of THP-1 cells, which do not express CD19, was not affected by anti-CD19-2 mAb. When added in solution, anti-CD19-2 mAb also inhibited the proliferation of primary human B cells stimulated with anti-IgM and CpG-B oligonucleotide (data not shown).

Depletion of primary human B cells ex vivo with MEDI-551

Having demonstrated potent activity in an ADCC assay with a B cell line and NK cells, we asked if MEDI-551 was also effective in depleting primary human B cell in an autologous assay in which donor PBMC are the source of both the target and effector cell populations. Figure 2 shows the results from three representative assays (out of six individual PBMC samples) in which the activities of MEDI-551 and rituximab were compared. The calculated EC50 values for B cell depletion with MEDI-551 mAb and rituximab for all PBMC samples are summarized in Table II. In all assays MEDI-551 demonstrated potent ADCC activity against primary human B cells. The EC50 values determined ranged from 3 to 29 pM for MEDI-551, and from 18 to 431 pM for rituximab. The results demonstrate that MEDI-551 is active against primary human B cells in autologous ADCC assays.

MEDI-551 depletes blood and tissue B cells in vivo
The HB12b and anti-CD19-2 mAbs are specific for human CD19 and do not bind rodent CD19 or CD19 from non-human primates (Yazawa et al., PNAS 2005 and data not shown). Also, rituximab does not cross-react with rodent CD19 (Gong et al, 2005). Therefore, we made use of a Tg mouse model to compare the in vivo B cell depletion activity of the two mAbs. Double Tg animals were generated by crossing huCD19 Tg mice with huCD20 Tg mice. Both strains have been well characterized previously, express the transgene in a B cell restricted fashion, and have been used successfully to study B cell depletion with CD19 and CD20 mAbs, respectively. (Zhou et al, 1994; Ahuja et al, 2007; Yazawa et al, PNAS 2005). As shown in Figure 3A, the B cell restricted expression of both transgenes is also maintained in the huCD19/CD20 double Tg animals. To further characterize the expression of huCD19 and huCD20 in this model, purified spleen B cells were incubated with serial dilutions of anti-CD19-2 mAb, rituximab, or the R347 isotype control mAb and mAb binding was determined by FACS. The results demonstrate that maximal surface binding to huCD19/CD20 Tg B cells is comparable for the anti-CD19-2 and rituximab mAbs. The anti-CD19 mAb, however, appears to have stronger binding at low mAb concentrations, suggesting a higher affinity than rituximab (Figure 3B).

To compare in vivo B cell depletion with MEDI-551 and rituximab, huCD19/CD20 Tg mice received a single dose of each mAb at 0.5, 2, or 10 mg/kg, and remaining blood and spleen B cell numbers were determined four days later by flow cytometry. At the highest dose of 10 mg/kg both mAbs were very effective in depleting B cells from blood and spleen (Figures 4 A and B). At the lower doses, however, B cell depletion with rituximab was incomplete, while depletion with MEDI-551 was as effective at 0.5 mg/kg as at 10
mg/kg of mAb. For example, a single dose of 0.5 mg/kg rituximab reduced splenic B cells on average by 42%, while MEDI-551 mAb used at this dose eliminated 85% of B cells from treated mice (Figure 4B).

Further analysis showed that B cell depletion in blood and spleen was maintained for more than two weeks following a single 10 mg/kg administration of MEDI-551 (Figure 4C and D). In mice treated with rituximab, significant recovery of blood and spleen B cell numbers was observed by day 14, despite almost complete depletion of blood and spleen B cells during the first days after dosing. In clinical studies with rituximab, immature B cells are often the first B cells detectable in peripheral blood during B cell recovery, suggesting repopulation from the bone marrow (BM) (Roll et al., 2008). To test if differences in the depletion of BM B cells between CD19 and CD20 mAbs could account for the differences in the kinetics of B cell recovery in the periphery and secondary lymphoid organs, we analyzed the extent of B cell depletion in this compartment with the two mAbs. As shown in Figure 4E, a single dose of MEDI-551 mAb resulted in a substantial reduction (on average by 91.4% by day three) in BM B220+muCD19+ B cells, while rituximab depleted fewer than 50% of the cells. Thus, the shorter time to B cell recovery in mice treated with rituximab is likely caused by the partial depletion of BM B cells.

**Macrophages are required for efficient in vivo B cell depletion with MEDI-551**

Several cell types, including NK cells, neutrophils, and macrophages, can potentially contribute to antibody-mediated target cell depletion in vivo (Glennie et al., 2007; Nimmerjahn and Ravetch, 2008). Previous studies have emphasized the importance of
monocytes/macrophages for B cell depletion in mice with mouse IgG2a mAbs (Uchida et al, 2004; Gong et al, 2005; Yazawa et al, 2005). However, the mechanism by which an afucosylated human IgG1 mediates its effector function has not yet been determined in a mouse model. To explore the mechanism of in vivo B cell depletion with MEDI-551, huCD19/CD20 Tg mice were treated with the CD19 mAb in the absence of one of the potential effector cell populations or following complement neutralization.

Elimination of NK cells or neutrophils with anti-NK1.1 or anti-GR-1 mAbs, respectively (Gong et al, 2005), did not affect the efficiency of B cell depletion (data not shown). However, elimination of monocytes/macrophages, by treatment of mice with liposome-encapsulated clodronate (Uchida et al, 2004), almost completely prevented depletion of blood and spleen B cells with MEDI-551 (Figure 5A and B). Elimination of monocytes/macrophages had a similar effect on blood B cell depletion with rituximab. Depletion of splenic B cells with rituximab, however, was less affected. Therefore, we also tested for a potential contribution of complement to the depletion of spleen B cells with CD19 and CD20 mAbs. Treatment of Tg mice with cobra venom factor (CoVF), which almost completely eliminated serum C3 levels as determined by ELISA (data not shown) (Minard-Colin et al, 2008), significantly reduced spleen B cell depletion by rituximab (Figure 5C). Depletion of spleen B cells by MEDI-551 was much less affected by the elimination of complement with CoVF. To further confirm the contribution of macrophages for depletion of mouse B cells we tested MEDI-551 and rituximab in a phagocytosis assay with mouse peritoneal macrophages. As negative control we also included a Fc-mutated version of mAb anti-CD19-2, mAb anti-CD19-2-TM, which is devoid of effector function (Oganesyan et al, 2008). As shown in Figure 5D, the MEDI-
551 mAb efficiently mediated phagocytosis of mouse B cells, while the effector-less anti-CD19-2-TM mAb was inactive. Although rituximab was also active in the phagocytosis assay as expected, the overall activity was less than with MEDI-551.

In summary, macrophages play an important role for in vivo B cell depletion in mice with the afucosylated human IgG1 mAb MEDI-551. While the results do not rule out some contribution of NK cells and neutrophils, especially in the absence of macrophages, these cells appear to play a minor role and are not required for efficient depletion when functional macrophages are present. Further, consistent with the in vitro data, the anti-CD19 mAb does not mediate CDC in vivo. For the depletion of blood B cells, rituximab also largely depends on macrophages. For the depletion of splenic B cells with rituximab, however, CDC appears to also play a role, in accordance with the results from Gong et al (2005).
Discussion

B cell depletion with targeted mAbs has proven successful for the treatment of hematologic malignancies of B cell origin as well as for autoimmune diseases. Given the success of rituximab, a variety of new agents targeting CD20 are now in pre-clinical and clinical development (Cheson and Leonard, 2008). Targeting CD20, however, also has limitations and the antigen can be lost from malignant cells through down-regulation and/or selection (Kennedy et al, 2004; Takei et al, 2006; Davis et al, 1999). Recent data from clinical trials of autoimmune patients treated with rituximab suggest that the depletion of memory B cells from lymphoid tissues is not always complete. Importantly, the presence of memory cells, rather than naive immature and mature B cells, at the time of repopulation was generally associated with a poor response to rituximab therapy (Leandro et al, 2006; Roll et al, 2008; Pers et al, 2008). The broader expression along the B cell lineage and presence on most malignant B cells make CD19 an attractive alternative to CD20 for therapeutic mAb approaches.

Several different mechanisms, including ADCC, CDC, and induction of apoptosis, can lead to target cell elimination by therapeutic mAbs, such as rituximab (Glennie, 2007). While CDC may play a role in B cell depletion with rituximab, the contribution of direct apoptosis to in vivo B cell depletion is controversial (Glennie et al, 2007).

In contrast to CDC and apoptosis, the relevance of ADCC and antibody-mediated phagocytosis for in vivo activity is now well established (Desjarlais et al, 2007; Glennie, 2007). The results from this study demonstrate that MEDI-551 has potent ADCC activity, but does not mediate CDC. While potentially contributing to overall activity of CDC-competent mAbs, such as rituximab, activation of complement may also be
involved in infusion-related reactions, a side effect often observed with therapeutic mAbs. Using different forms of a mAb against HLA-DR in preclinical animal models, Tawara et al (2008) recently demonstrated that infusion reactions were correlated to the ability of the mAbs to mediate CDC. It will have to be determined in the clinic, if the absence of CDC in MEDI-551 may result in fewer infusion reactions as compared to other, CDC-competent, mAbs.

The ADCC activity of therapeutic mAbs can be enhanced by increasing the affinity of the mAb Fc for activating Fcγ receptors, in particular FcγRIIIA (Desjarlais et al, 2007). This can be achieved by the introduction of point mutations in the Fc or by modification of the Fc carbohydrate. The removal of fucose from the Fc of the anti-CD19 mAb anti-CD19-2 resulted in ~9-fold increased affinity to FcγRIIIA. Interestingly, the increase in ADCC activity was much greater than the fold affinity increase to FcγRIIIA. Not only did the EC50 drop by ~20-fold, also the maximum level of cytotoxicity was significantly greater for the afucosylated mAb MEDI-551 as compared to the fucosylated mAb anti-CD19-2 (Figure 1A and data not shown). Since the activity and potency of the anti-CD20 mAb rituximab have been well characterized, we included this mAb as control in this study. The results from ADCC assays with B cell lines as well as primary B cells, demonstrate that MEDI-551 is more effective at low mAb concentrations than rituximab (Figures 1A and 2; data not shown). Also in vivo, using huCD19/CD20 double Tg mice as a model system, MEDI-551 effectively depleted blood and tissue B cells. At high doses MEDI-551 and rituximab achieved comparable depletion in blood and secondary lymphoid organs. However, MEDI-551 was more effective in eliminating B cells at lower mAb doses. The longer duration of B cell depletion with MEDI-551 is likely a result of the
greater impact MEDI-551 has on BM B cells. CD20 is expressed by immature and mature BM B cells, but, in contrast to CD19, is not yet present on earlier stages of B cell development (Levesque and St Clair, 2008). Given that rituximab was almost as effective as MEDI-551 in depleting B cells from blood and spleen when used at high doses, the relatively incomplete depletion of B cells from the BM of Tg mice, is therefore likely a result of the differential expression of the two antigens along the B cell lineage.

It has been noted that in RA patients treated with rituximab clinical benefit often lasts for the duration of B cell depletion (Dorner et al, 2010). Thus, more complete depletion of early B cells from the BM could provide additional benefit in this indication and possibly other autoimmune diseases.

Consistent with previous reports, the removal of fucose selectively increased the affinity of the IgG1 anti-CD19 mAbs to human FcγRIIIA, which is expressed on NK cells, monocytes/macrophages, and neutrophils (Shields et al, 2002; Nimmerjahn and Ravetch, 2008). Among mouse Fcγ receptors tested, the affinity increase was limited to FcγRIV. In contrast to human FcγRIIIA, however, mouse FcγRIV is not expressed on NK cells. This has potential consequences for the in vivo activity when testing the afucosylated mAb in a murine model. Analysis of the effector mechanisms engaged in the mouse model by MEDI-551 clearly demonstrates an important contribution by macrophages for in vivo B cell depletion. Elimination of neutrophils or NK cells on their own had no effect on the ability of MEDI-551 to deplete B cells from blood and lymphoid tissues (data not shown). MEDI-551 also efficiently depleted splenic B cells in the absence of complement, although a minor, but statistically significant difference was observed between CoVF treated and un-treated animals (Figure 5C). In previous studies carried
out in huCD19 Tg animals, elimination of complement by CoVF had no noticeable effect on depletion of blood or spleen B cells by MEDI-551 (data not shown). Overall, the finding are consistent with the results from the \textit{in vitro} CDC assay (Figure 1B). The absence of CDC activity is likely the result of the inability of the anti-CD19 mAb to mobilize the target antigen into lipid rafts, a phenomenon required for anti-CD20 mAbs to mediate CDC (data not shown) (Craigg et al, 2003). Complement, however, was required to achieve maximal depletion of spleen B cell with rituximab (Figure 5C). Also using a huCD20 Tg mouse model, Gong et al (2005) investigated the mechanisms of B cell depletion with anti-huCD20 mAbs. Consistent with the findings presented here, neutralization of complement reduced the efficiency of B cell depletion in the spleen. Interestingly, the marginal zone B cells were selectively dependent on complement for anti-CD20 mediated depletion (Gong et al, 2005). Using wild type mice and anti-mouse-CD20 mAbs, however, Minard-Colins et al (2008) did not observe a significant contribution of complement to depletion of spleen B cells. It is possible that these different observations reflect differences in potency of the mAbs used. In the huCD19/CD20 Tg model used here, marginal zone and follicular B cells were equally susceptible to depletion with MEDI-551, despite the lack of CDC activity (data not shown). Thus, the combined \textit{in vivo} data demonstrate that the afucosylated human IgG1 mAb behaves most similar to a mouse IgG2a mAb, which has the highest affinity to FcγRIV and largely relies on macrophages for target cell depletion in mouse models (Uchida et al, 2004; Gong et al, 2005; Yazawa et al, 2005).

As mentioned above, the affinity of mAbs for Fcγ receptors can be increased by point mutations in the Fc (Desjarlais et al, 2007). Although a selective increase in FcγRIIA
binding appears difficult, several mAbs with significantly enhanced ADCC have been reported (Bowles et al, 2006; Lazar et al, 2006). One such mAb is XmAb5574, an anti-CD19 mAb which has been engineered for enhanced ADCC by introducing two point mutations in the Fc (Horton et al, 2008). These mutations result in a significant increase in affinity not only to FcγRIIIA, but also to FcγRIIa and the inhibitory receptor FcγRIIb. It is, however, difficult to compare the in vitro activity of XmAb5574 with the activity of MEDI-551, due to differences in the assay systems used. Using rituximab as a template, Masuda et al (2007) compared the effect of removal of fucose to Fc mutations on Fcγ receptor binding and ADCC activity. In their study the afucosylated mAb had ~10-fold better binding to FcγRIIIA while the affinity of the Fc mutant versions of rituximab was improved up to ~90-fold. When tested in vitro for ADCC activity, however, the afucosylated and Fc mutant mAbs were equipotent over a range of E:T ratios (Masuda et al, 2007). The results suggest the existence of a threshold effect with regard to the affinity of the mAb Fc to FcγRIIIA. Further, point mutations in the Fc can potentially result in undesired effects, such as increased immunogenicity or decreased stability. Removal of fucose, however, does not affect mAb stability and is unlikely to change the immunogenicity in vivo.

While this manuscript was in preparation, Cardarelli et al (2009) reported another afucosylated anti-CD19 mAb, MDX-1342. Although the same approach for ADCC enhancement was used in the generation of MEDI-551 and MDX-1342, it is difficult to compare the activity in vitro and in vivo from the published data. MDX-1342 cross-reacts with CD19 from cynomolgus monkeys and was compared with rituximab for in vivo B cell depletion. When given as a single dose, extent and duration of blood B cell
depletion with the two mAbs was comparable, although the onset of depletion with
rituximab appeared more rapid (Cardarelli et al, 2009). In addition to Fc modifications,
the ADCC potency of mAbs is also determined by other factors. The anti-CD19 mAb
anti-CD19-2 was generated by humanization and affinity maturation, which improved the
binding characteristics with decreased internalization rate, and prolonged residence time
on the cell surface, features favorable for an ADCC-dependent mechanism (Herbst et al,
unpublished). Ghetie et al (1994) have shown that some, but not all, CD19 mAbs can
inhibit B cell proliferation, suggesting that binding properties and/or the particular
epitope recognized play an important role for this effect. Here we show that the CD19
mAb anti-CD19-2 (the fucosylated version of MEDI-551) also reduces proliferation of B
cells; thus, over time, the mAb could also impact B cells in vivo, independent of ADCC effector function. To test this we also generated a Fc-mutated version of anti-CD19-2
(anti-CD19-2-TM) that is unable to bind to Fcγ receptors (Oganesyan et al, 2008) and
compared the effect to MEDI-551 in SCID mouse human lymphoma xenograft models.
Although less potent than MEDI-551, anti-CD19-2-TM resulted in noticeable tumor
growth inhibition, which is likely the result of the anti-proliferative effect of the mAb
(Ward et al, manuscript in preparation).

In summary, MEDI-551 is a new glycoengineered anti-CD19 antibody, optimized for
ADCC effector function. The removal of fucose resulted in a selective increase in
affinity to human FcγRIIIA and mouse FcγRIV and enhanced potency in vitro and in vivo.
Furthermore, the in vitro activity of MEDI-551 with lymphoma cells and primary
human B cells compared favorably to the anti-CD20 mAb rituximab which was included
as positive control in our experiments. Given the broad expression of CD19 in B cell malignancies and continued expression on late stage memory B cells and plasmablasts, B cell depletion with MEDI-551 has therapeutic potential in the treatment of B cell malignancies as well as in autoimmune disease. Clinical studies with MEDI-551 have already been initiated.
References


Figure Legends

Figure 1: Effector function of fucosylated and afucosylated anti-CD19 mAbs

A) Afucosylated anti-CD19 mAb MEDI-551 has increased ADCC effector function against Daudi Burkitt lymphoma cells as compared to the fucosylated mAb anti-CD19-2. ADCC activity was determined using an LDH release assay and KC1333 NK cells as effectors (E:T = 2.5:1). Rituximab was included for comparison. All ADCC assays were done in triplicate. Results are mean +/- SD and representative of three independent experiments.

B) Anti-CD19 mAb anti-CD19-2 is devoid of CDC activity. Daudi cells were incubated with mAb anti-CD19-2 or rituximab as a positive control in the presence of human serum as a source of complement. In contrast to rituximab, mAb anti-CD19-2 has no detectable CDC activity. Cells incubated with mAb in the presence of heat inactivated (hi) serum served as a negative control. All measurements were done in duplicate. The results are representative of two independent experiments.

C) The anti-CD19 mAb anti-CD19-2 inhibits the proliferation of B lymphoma cells. The Burkitt lymphoma lines Raji, Daudi, and Ramos and the monocytic cell line THP-1 were cultured for three days in multi-well plates coated with anti-CD19-2 mAb or the control mAb R347. The effect of mAb treatment on cell proliferation was determined using the CellTiterGlo assay. The results shown are mean +/- SD of triplicate samples.

Figure 2: MEDI-551 has potent ADCC activity in an autologous ADCC assays with normal donor PBMCs. PBMC samples from healthy donors were incubated with MEDI-551 and rituximab as indicated. Cytotoxicity was measured after over night incubation using a FACS based method (see Materials and Methods). Shown are representative
results with PBMCs from three individual donors (out of six donors tested). Donor #374 (middle panel) is heterozygous for the high affinity allotype at amino acid position 158 of FcγRIIIA (158 V/F), while all other samples tested were from donors homozygous for the high affinity allotype (158 V/V). The results shown are mean +/- SD of triplicate samples.

**Figure 3:** Cell surface expression of huCD19 and huCD20 in huCD19/20 double Tg mice. (A) Live blood mononuclear cells and splenocytes from huCD19/CD20 Tg mice were analyzed for expression of human CD19 and CD20, along with murine CD19, a marker for B cells. (B) Purified mouse spleen B cells from huCD19/CD20 Tg mice were incubated with serial diluted anti-huCD19 mAb anti-CD19-2, anti-huCD20 mAb rituximab or isotype control mAb for 1 hour at 37°C. The cell surface bound mAbs were then detected with a mouse anti-human IgG1 secondary antibody and measured by FACS. The graph shows one of two experiments with similar results.

**Figure 4:** Depletion and recovery of B cells following injection of a single dose of MEDI-551 or rituximab. HuCD19/CD20 Tg mice were treated with MEDI-551 or rituximab at 0.5 mg/kg, 2 mg/kg or 10 mg/kg. Control animals were treated with PBS only. Mice (6-7 animals per dose group and time point) were sacrificed for analysis of blood and tissue B cell depletion. The dose-response for B cell depletion in blood (A) and spleen B cells (B) was analyzed on day 3 after mAb administration. To compare the duration of B cell depletion between MEDI-551 and rituximab, additional groups of mice treated with 10 mg/kg mAb were sacrificed and analyzed on
days 7 and 14 after mAb administration. Shown are depletion of blood (C), spleen (D), and BM (cell number per femur) (E) over time. Statistical significance at individual time points between groups treated with MEDI-55 and rituximab is indicated.

**Figure 5:** MEDI-551 leads to depletion of B cell by mouse macrophages *in vivo* and phagocytosis of murine B cells *ex vivo*. (A) HuCD19/CD20 Tg mice were pretreated with clodronate liposome or control liposome and subsequently treated with either MEDI-551 or rituximab at 10 mg/kg. Numbers of remaining B220+muCD19+ blood (A) spleen (B) B cells were determined on day four by FACS. Depletion of spleen macrophages and liver Kupffer cells was confirmed by FACS and histology analysis of F4/80+ cells (not shown). (C) Effect of complement neutralization with cobra venom factor (CoVF; 30 μg/mouse on days -1, 1 and 3) on spleen B cell depletion with MEDI-551 and rituximab. (D) B cells from huCD19/CD20 Tg mice and B6 wild type mice were stained with fluorescence dyes, CFSE and PKH-26, respectively. The cells were mixed at a 1:1 ratio and cultured with mouse peritoneal macrophages, in the presence of serially diluted MEDI-551 or anti-CD19-2-TM mAbs, or with rituximab. After 24 hours, cells were recovered and the numbers of CFSE+ or PKH26+ B cells were determined by FACS. Percentage of B cell depletion was calculated as described under Materials and Methods. The graph shows one of two experiments with similar results.
**Table I**
BLAcore Affinity Measurements of Fucosylated and Afucosylated Anti-CD19 Antibodies to Mouse and Human Fcγ Receptors

<table>
<thead>
<tr>
<th>FcγR</th>
<th>Fucosylated anti-CD19 IgG1 KD [nM]</th>
<th>Afucosylated anti-CD19 IgG1 KD [nM]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mouse</td>
<td>Human</td>
</tr>
<tr>
<td>I</td>
<td>NT b</td>
<td>20</td>
</tr>
<tr>
<td>IIa</td>
<td>NA c</td>
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</tr>
<tr>
<td>IIb</td>
<td>1230</td>
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<tr>
<td>IIIA (V158)</td>
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<td>656</td>
</tr>
<tr>
<td>III</td>
<td>3440</td>
<td>NA</td>
</tr>
<tr>
<td>IV</td>
<td>306</td>
<td>NA</td>
</tr>
</tbody>
</table>

*a* dissociation constant  
*b* not tested  
*c* not applicable
### Table II
Depletion of B cells from normal donor PBMC samples with the afucosylated anti-CD19 mAb MEDI-551 and the anti-CD20 mAb rituximab

<table>
<thead>
<tr>
<th>Donor</th>
<th>MEDI-551 EC(_{50}) [nM] (95% confidence interval)</th>
<th>(R^2)</th>
<th>rituximab EC(_{50}) [nM] (95% confidence interval)</th>
<th>(R^2)</th>
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<tr>
<td>343</td>
<td>0.016 (0.0096-0.0264)</td>
<td>0.991</td>
<td>0.431 (0.2158-0.8625)</td>
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<tr>
<td>310</td>
<td>0.014 (0.0073-0.0251)</td>
<td>0.970</td>
<td>0.252 (0.2166-0.2947)</td>
<td>0.998</td>
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<tr>
<td>374</td>
<td>0.029 (0.0132-0.0645)</td>
<td>0.968</td>
<td>0.063 (0.0500-0.0802)</td>
<td>0.989</td>
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<tr>
<td>421</td>
<td>0.0006 (0.0003-0.0011)</td>
<td>0.896</td>
<td>0.032 (0.0240-0.0416)</td>
<td>0.989</td>
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<tr>
<td>442</td>
<td>0.003 (0.0005-0.0183)</td>
<td>0.989</td>
<td>0.018 (0.0157-0.0208)</td>
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<tr>
<td>467</td>
<td>0.0007 (0.0001-0.0055)</td>
<td>0.957</td>
<td>0.039 (0.0348-0.0442)</td>
<td>0.992</td>
</tr>
</tbody>
</table>
Figure 1

A

% Cytotoxicity

Log antibody concentration [μg/mL]

B

% Cytotoxicity

Log antibody concentration [μg/mL]

C

Relative Light Units

Cell Lines

Raji

Daudi

Ramos

THP1

Control

anti-CD19-2

rituximab

MEDI-551

rituximab-hi

anti-CD19-2-hi

***  

**

***

***

***
Figure 3

A

<table>
<thead>
<tr>
<th></th>
<th>Blood</th>
<th>Spleen</th>
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<tr>
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<td>huCD20</td>
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<tr>
<td>isotype control</td>
<td>muCD19</td>
<td>muCD19</td>
</tr>
</tbody>
</table>

B

- isotype control
- anti-CD19-2
- rituximab

Mean fluorescence intensity vs. Antibody concentration (M)
Figure 4

A

B

C

D

E

This article has not been copyedited and formatted. The final version may differ from this version.
Figure 5

A

B

C

D

PBS MEDI-551 rituximab
PBS MEDI-551 rituximab
PBS MEDI-551 rituximab
PBS MEDI-551 rituximab

PBS Clod. PBS Clod. PBS Clod.
PBS Clod. PBS Clod. PBS Clod.
PBS Clod. PBS Clod. PBS Clod.
PBS Clod. PBS Clod. PBS Clod.

number of spleen B cells

Antibody concentration [M]