HuMab-7D8, a monoclonal antibody directed against the membrane-proximal small loop epitope of CD20 can effectively eliminate CD20^{low} expressing tumor cells that resist rituximab- mediated lysis

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ABSTRACT

Background
Incorporation of the chimeric CD20 monoclonal antibody rituximab in the treatment schedule of patients with non-Hodgkin's lymphoma has significantly improved outcome. Despite this success, about half of the patients do not respond to treatment or suffer from a relapse and additional therapy is required. A low CD20-expression level may in part be responsible for resistance against rituximab. We therefore investigated whether the CD20-expression level related resistance to rituximab could be overcome by a new group of CD20 mAbs (HuMab-7D8 and ofatumumab) targeting a unique membrane-proximal epitope on the CD20 molecule.

Design and Methods
By retroviral transduction of the CD20 gene into CD20-negative cells and clonal selection of transduced cells a system was developed in which the CD20-expression level is the only variable. These CD20 transduced cells were used to study the impact of rituximab and HuMab-7D8 mediated complement-dependent cytotoxicity. To study the *in vivo* efficacy of these mAbs an *in vivo* imaging system was generated by retroviral expression of the luciferase gene in the CD20-positive cells.

Results
We show that HuMab-7D8 efficiently killed CD20^{low} cells that are not susceptible to rituximab-induced killing *in vitro*. In a mouse xenograft model, we observed a comparable increase in survival time between HuMab-7D8 and rituximab-treated mice. Most significantly, however, HuMab-7D8 eradicated all CD20-expressing cells both in the periphery as well as in the bone marrow whereas after rituximab treatment CD20^{low} cells survived.

Conclusions
Cells that are insensitive to *in vitro* and *in vivo* killing by rituximab as the result of their low CD20-expression profile may be efficiently killed by an antibody against the membrane-proximal epitope on CD20. Such antibodies should, therefore, be explored to overcome rituximab resistance in the clinic.

Key words: CD20, *in vivo* model, antibody therapy, ofatumumab, rituximab.

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Introduction

The non-Hodgkin's lymphomas (NHL) represents a heterogeneous group of lymphoid neoplasms. Their prevalence has been increasing over the years and NHL are now fifth for cancer incidence and mortality.[1,2] Diffuse large B-cell lymphoma (DLBCL) is the most common NHL, followed by follicular lymphoma (FL).[2-4] Since the 1970s, the best treatment option for patients with B-cell NHL consisted of various combinations of chemotherapy with or without radiotherapy.[5-7] During the last decade, inclusion of the monoclonal CD20 antibody rituximab (Mabthera, Rituxan, IDEC-C2B8) in the chemotherapy regimens has significantly improved patient outcome with or without pre-treatment[8-17] and is now accepted as a standard therapy for CD20-positive lymphomas. Furthermore, if patients with low-grade lymphoma respond to a single-agent rituximab treatment, scheduled maintenance therapy with rituximab substantially prolongs the progression free survival and overall survival.[2,18] In addition, if patients achieve complete or partial remission after the combination of chemotherapy and rituximab, maintenance with rituximab also increased the overall and progression free survival.[2,17-19]

Next to its application in hematologic cancers, depletion of B cells by rituximab has also shown promise for the treatment of autoimmune diseases such as rheumatoid arthritis (RA).[20,21]

Despite the success of rituximab, resistance to treatment by this therapeutic antibody develops in patients who, therefore, do not respond or relapse. The mechanisms of rituximab resistance may be host and/or tumor-related, but are still poorly understood.[22-25] Therefore, the need to study rituximab-resistance as well as the development of more potent CD20-directed immunotherapy is imperative.

Rituximab is a chimeric human-mouse CD20 monoclonal antibody (mAb) which activates different effector mechanisms among which complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC) are considered the most important.[25-29] In addition, growth arrest and the induction of apoptosis have been observed, especially after hyper-crosslinking of CD20.[25,26,30] In previous experiments, we demonstrated that the CDC activity of rituximab significantly correlates with the number of CD20 molecules on the cell surface, and that CDC and ADCC show an additive effect. Importantly, we also showed that low CD20 ($CD20^{low}$) expressing cells could not be killed by rituximab.[29,31,32] This may explain the poor response to rituximab of B-cell malignancies expressing low CD20 levels such as B-cell chronic lymphocytic leukemia B-CLL. The number of CD20 molecules on B-CLL was reported to be in the order 22,000 molecules per cell,[33] which is 300 to 600-fold lower than observed in lymphoma.[29,33-35]

Recently, a panel of fully human antibodies including ofatumumab (HuMax-CD20), HuMab-2C6 and HuMab-7D8, were generated in human Ig transgenic mice. This group of human antibodies represents a panel of CD20 mAbs that bind to a unique membrane-proximal CD20 epitope, including the small and large extracellular loop. It has been proposed that the recognition of this epitope leads to exceptionally potent complement-mediated tumor cell lysis.[32] HuMab-7D8 and ofatumumab in addition have a much slower off-rate than rituximab.[31] Ofatumumab is currently under clinical development for B-CLL, NHL and RA.[36,37]

Here, we investigated whether a human antibody directed against the distinct membrane-proximal epitope on CD20 (HuMab-7D8) could overcome the rituximab CD20-expression level-related resistance by comparing the activity of rituximab and HuMab-7D8 *in vitro* and *in vivo* using CD20-transduced T cells. In a xenograft mouse model, we demonstrated that, although the differential effect of rituximab and HuMab-7D8 were not reflected in differences in the increase of survival time, it was evident, however, that rituximab eradication of $CD20^{low}$-expressing cells was poor, while HuMab-7D8 eradicated all CD20-expressing cells from the peripheral compartment as well as from the bone marrow. *In vitro* results demonstrate that HuMab-7D8 showed a higher capacity to kill low CD20-expressing cells than rituximab. We further show that cells that resist killing through rituximab exposure are still sensitive to HuMab-7D8.

Design and Methods

Generation of $CD20^{pos}$ CEM cells and $CD20^{pos}$ + lucR-IRES-eGFP-^{pos} CEM cells

CEM T cells were transduced with the CD20-encoding retroviral vector as previously described.[29] Briefly, the Moloney Murine-Leukemia virus based vector (pMX), containing the click beetle luciferase (LucR) and internal ribosomal entry site-enhanced Green Fluorescent Protein (IRES-eGFP) genes, was constructed by digesting the pCBR-Control vector (Promega Corporation, Madison Wi, USA) with *Bgl* II and *Xba* I endonuclease restriction enzymes, releasing the click beetle luciferase fragment. Subsequently, the pMX-IRES-eGFP vector was digested with *BamH* I and *Not* I to create the insertion space for the LucR fragment. Next, the *Xba* I site of the LucR fragment and the *Not* I site of the viral backbone were blunted and subsequently the LucR fragment was ligated into the retroviral backbone.

Transduction of CEM-CD20 cells with the pMX-LucR-IRES-eGFP retroviral vector and generation of viral supernatant was performed as previously described.[29] Transduced CEM-CD20-LucR-IRES-eGFP cells were purified with a fluorescence-activated cell sorter (FACS) (FACSAria, Becton Dickinson, Mountain View, CA, USA) based on eGFP expression. *In vitro* luciferase expression was determined with a luminometer (EG&G Berthold, Lumat LB 5507) by lysing 0.1×10^6 cells with 100 µL lysing solution and adding 100 µL of luciferase substrate according to the manufacturer's protocol (Promega Corporation.)

Cell culture

The CEM T cells were cultured in culture medium consisting of RPMI (Gibco-BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS, Integro, Zaandam, the Netherlands), penicillin (100 U/mL), streptomycin (100 µg/mL) (Gibco-BRL), and 5×10^{-5} M 2-mercaptoethanol (Merck, Darmstadt, Germany). All cells were cultured at 37°C in a humidified 5% CO_2 atmosphere.

Flow cytometric analysis

Expression of CD20 and eGFP was determined by flow cytometry (FACS Calibur, Becton Dickinson, Mountain View, CA, USA). Antibodies used for staining were CD20-phycoerythrin (PE) mAb/allophycocyanin (APC), CD7-fluorescein isothiocyanate (FITC) mAb (BD Biosciences, San Jose, CA, USA). Rituximab was obtained from Roche (Basel, Switzerland). HuMab-7D8 was

described previously.[32] F(ab')2 fragments of goat-anti-human-IgG1-FITC/PE were obtained from Southern Biotech (Birmingham, AL, USA). The absolute numbers of CD20 molecules *per* cell were determined with the QuantiBRITE CD20-PE kit, on a FACS Calibur (both from Becton Dickinson, San Jose, CA, USA) according to the manufacturer's instructions. The antibodies bound *per* cell (ABC) represent the absolute number of CD20 molecules per cell.

Anti-CD20 mediated cytotoxicity assays

All CD20 mAb mediated cytotoxicity assays were performed as previously described.[29,38] Based on optimization assays, we used 10 μg/mL of anti-CD20 mAb, 20% normal human serum as source of complement, and incubated for 30 min at 37°C.

To study complement-dependent-cytotoxicity (CDC) mediated antibody resistance, CD20-positive cells were treated with rituximab or HuMab-7D8 in the presence of human complement for one day at 37°C. The next day normal human serum was washed away and the cells were cultured for 7-14 days. The presence of bound antibody was checked every two days by staining the cells with goat-anti-human-IgG-FITC. After 14 days, no antibody could be detected on the cells.

At this time point, cells were subjected to a second round of antibody-induced cell kill, either by rituximab or by HuMab-7D8 (Figure 1). Cell kill was analyzed by propidium iodide staining as previously described.[29,38] HuMab-7D8 and rituximab alone did not induce lysis of CEM-CD20 cells after prolonged incubation in the absence of complement (up to 72 h), indicating that none of the antibodies induced apoptosis under the conditions used.

Measurement of raft-associated antigen by Triton X-100 insolubility

To study the presence of CD20 in cholesterol-rich microdomains before and after antibody ligation, a rapid flow cytometry method based on Triton X-100 insolubility was performed at low temperature, as described previously.[31] Briefly, cells were washed in phosphate buffered saline (PBS) and resuspended at 2.5×10^6 cells/mL. Cells were incubated with 10 μg/mL of CD20 antibody (rituximab or HuMab-7D8) for 15 min at 37°C. Next, the samples were washed in cold PBS and then divided in half. One half was kept on ice and was used to determine the surface CD20 expression (set at 100%). The other half was treated with 0.5% Triton X-100 (Riedel-de Haen, Seelze, Germany) for 15 min on ice to determine the proportion of CD20 remaining in the Triton X-100-insoluble fraction. Both the treated and non-treated control cell fraction were centrifuged and stained with anti-human IgG-FITC. As a control, CD7-FITC mAb was used as described previously.[29] The mean fluorescence intensity (MFI) was determined by flow cytometry.

Mice, conditioning regimen and transplantation

RAG2$^{-/-}\gamma c^{-/-}$ mice were obtained from the Netherlands Cancer Institute (Amsterdam, the Netherlands). Mice were bred and housed in the specified pathogen-free (SPF) breeding unit of the Central Animal Facility of the University of Utrecht. The animals were supplied with autoclaved sterilized food pellets and distilled water *ad libitum*. All animal experiments were conducted according to Institutional Guidelines after acquiring permission from the local Ethical Committee for Animal Experimentation and in accordance with current Dutch laws on Animal Experimentation.

Mice were used at 8-20 weeks of age. On day 0, all mice received total body irradiation with a single dose of 300 centiGray (cGy). On day 1, cell suspensions containing 4.0×10^6 CEM-CD20-LucR-IRES-eGFP cells in 0.25 mL of PBS/0.1%BSA (GIBCO-BRL) were intravenously (i.v.) injected into the lateral tail vein. Within one hour, PBS (control) or different doses of anti-CD20 antibody were administrated intraperitoneally (i.p). After one week, human or chimeric immunoglobuline subtype G1 (IgG1) levels were determined in serum samples of the mice. Mice lacking human or chimeric IgG1 in the serum were excluded from the analyses.

Bioluminescent imaging (BLI)

Mice were monitored for luciferase expression twice a week using a cooled charge-coupled device (CCCD) camera (Roper Scientific, Princeton instrument, Trenton, NJ, USA). Mice were anesthetized by intramuscular injection of Ketamine/Xylazin/Atropine (ratio 8:7:1, 35 μL). Subsequently, 100 μL of D-luciferin (7.5 mM) (Synchem, Kessel, Germany) was injected intraperitonealy (i.p.) and the ventral side of the mice was imaged for 10 min inside a light-tight chamber. Light emission was quantified by using MetaVue and MetaMorph software (Universal Imaging Corporation, Downingtown, PA, USA). Blood was collected once a week to determine the serum levels of human IgG1. Diseased and paralyzed mice were sacrificed and bone marrow (BM) was collected. Cells were harvested from the BM and the CD20-expression level was detected by flow cytometry after culture for at least 14 days.

Determination of human or chimeric IgG1 concentration

Human or chimeric IgG1 concentrations in mouse serum were determined using a sandwich ELISA. Mouse mAb anti-human IgG MH16-1 (#M1268, CLB Sanquin, the Netherlands) was coated onto 96-well Microlon ELISA plates (Greiner, Germany) at a density of 200 ng/well. After blocking plates with PBS supplemented with 2% chicken serum (Invitrogen, Groningen, the Netherlands) and subsequent washing, samples serially diluted in ELISA buffer (PBS supplemented with 0.05% Tween 20 (Sigma Aldrich, Zwijndrecht, the Netherlands) and 2% chicken serum) were added, and incubated on a plate shaker for 1 h at room temperature. After washing, the plates were incubated with peroxidase-labeled F(ab')2 fragments of goat anti-human IgG immunoglobulin, Fcγ fragment specific (#109-035-098, Jackson, West Grace, PA, USA), 1:5000 diluted in PBS. Plates were developed with 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Roche, Mannheim, Germany), and absorbance measured in a microplate reader (Biotek, Winooski, VT, USA) at 405 nm.

Statistical analysis

Where indicated, the mean values and standard deviation (SD) were calculated. Differences between rituximab and HuMab-7D8 were determined by non-linear regression curve fitting, by use of GraphPad Prism version 5.01 (GraphPad Software, Inc., San Diego, CA, USA). A P value of < 0.05 was considered significant.

Results

Rituximab and HuMab-7D8 binding to CEM-CD20 cells and induction of CDC

Recently, fully human CD20 mAbs (ofatumumab, HuMab-7D8 and HuMab-2C6) were generated in human Ig-transgenic mice. These mAbs bind to a unique membrane-proximal CD20 epitope encompassing the small and large loop of CD20 and induce ADCC and exceptionally potent CDC.[31,32] In this study, we compared the *in vitro* and *vivo* efficacy of rituximab and HuMab-7D8. We first tested the binding of both antibodies to the transduced CEM-CD20 cells at saturating concentrations. Figure 1A

shows that both antibodies bound with similar high specificity. Next, CDC experiments were performed with CEM-CD20 cells in which the cells were incubated with antibody in the presence of normal human serum as a source of complement. For comparison, CDC was performed with both antibodies at a saturating concentration of 10 μg/mL, which was required to achieve maximal lysis with rituximab. It should be noted that maximal lysis with HuMab-7D8 was already observed at a concentration of 500 ng/mL (*data not shown*). Both antibodies induced CDC very rapidly, with maximal levels occurring within 5 min at 37°C in the presence of 20% normal human serum (*data not shown*).

The influence of the CD20 expression level on CDC was assessed by employing a panel of stably transduced CEM-CD20 clones for which we determined the absolute number of CD20 molecules expressed *per* cell. The CD20 antibodies-bound-per cell (CD20-ABC) ranged from 7,000 to 135,000 and is comparable to CLL samples and low expressing lymphoma samples.[29,33-35] The only variable parameter between these clones is the CD20 expression level and interpretation of results is, therefore, not complicated by differences in expression levels of complement regulatory proteins (CD46, CD55, CD59).[29] The clones were subjected to rituximab- and HuMab-7D8-induced CDC, which demonstrated that rituximab required an approximately 5–6 times greater CD20 expression to induce maximum cell lysis (Figure 1B). By use of non-linear curve fitting a comparison was made between both treatments, evaluating differences in B_{max} and K_D using F-test. This test demonstrated that the increased CDC activity of HuMab-7D8 at low CD20 expression levels compared to rituximab was highly significant (F-test, $P<0.0001$).

In addition, neither antibody was able to induce apoptosis, as defined by propidium iodide staining in CD20 transduced CEM T cells (*data not shown*).

Of note, HuMab-7D8 and rituximab were both effective in ADCC assays (*data not shown*). The combination of CDC and ADCC resulted in an additive killing effect for both antibodies as previously shown (*data not shown*).[29]

Resistance to rituximab-mediated killing of CD20^{low} cells cannot be explained by ineffective CD20 translocation into lipid rafts

Both rituximab and HuMab-7D8 are able to translocate the CD20 antigen into cholesterol rich microdomains or so-called lipid rafts.[31] Efficient lipid raft formation is required for induction of CDC.[39] We addressed the question whether the higher capacity to mediate CDC of CD20^{low} cells was related to a superior ability of HuMab-7D8 to induce lipid raft formation compared to rituximab. To this end, we determined the induction of lipid raft formation by both antibodies using clones with different CD20 expression levels (clones A-F). Figure 1C shows that both antibodies are capable of efficiently translocating CD20 molecules into lipid microdomains, independent of the CD20 expression level. At the same time, HuMab-7D8 is more effective than rituximab in mediating CDC of CEM-CD20 clones with a low surface density of CD20 (Figure 1D). These data suggest that factors other than efficient translocation of CD20 molecules into lipid rich microdomains are responsible for the poor lysis of CD20^{low} cells by rituximab.

In vivo eradication of CD20^{low} cells by HuMab-7D8 but not rituximab

To study the *in vivo* efficacy of both antibodies, we developed a mouse model in which we can trace intravenously (i.v.) injected luciferase-tagged CEM-CD20 cells using bioluminescence imaging. For this we used the

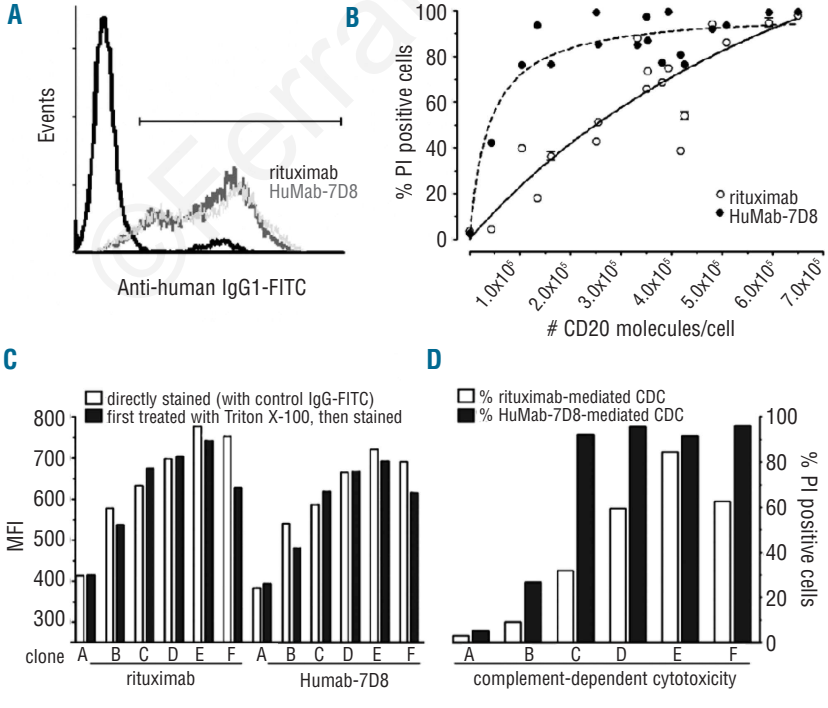

Figure 1. Resistance to rituximab-mediated killing is not explained by ineffective translocation into lipid rafts. (A) FACS-analysis of HuMab-7D8 (light gray) and rituximab (dark gray) binding to CEM-CD20 cells at saturating concentrations. The black line displays transduced cells that were stained with FITC conjugated human IgG. (B) Individual CEM-CD20 clones were incubated with 10 μg/ml rituximab or HuMab-7D8 and CDC was induced by adding 20% normal human serum as source of complement. The expression of CD20 in number of molecules *per* cell was plotted against the extent of CDC (expressed as % PI positive cells). The open dots represent rituximab-mediated CDC and the filled dots HuMab-7D8. (C) CEM-CD20 clones (A-F) with increasing CD20 expression (see Y-axis) were incubated with 10 μg/mL of CD20 mAb. One half of the cells were directly stained with anti-IgG1-FITC (open bars). The other half was first treated with Triton X-100 prior to staining with anti-IgG1-FITC (filled bars). (D) The open bars show the percentage CDC of the clones in the presence of rituximab and normal human serum, the filled bars show the HuMab-7D8-induced CDC. All experiments were performed in duplicate and repeated once with similar results.

RAG2$^{-/-}\gamma c^{-/-}$ mouse model, in which human cells can efficiently engraft.[40-43] To determine whether CEM T cells would successfully engraft in these mice, they were subjected to total body irradiation with a sublethal dose of 350 cGy, and cells were titrated (1.0×10^6 – 15.0×10^6 cells) by i.v. injection into the tail vein. A dose of 4.0×10^6 cells was found to result in a reproducible engraftment and outgrowth of the CEM cells in the bone marrow compartment and soft tissues such as the liver whereas towards the terminal stage of tumor development paralysis of the hind legs by compression of the cervical spine occurs after approximately three weeks. The latter was an endpoint to euthanize the animals. Collection of blood samples revealed the presence of CEM T cells (*data not shown*).

For *in vivo* imaging, CEM cells were transduced with a CD20 expression vector (CD20-WPRE-INS) and a retroviral vector expressing luciferase and eGFP (LucR-IRES-eGFP)[38] (Figure 2A). The CD20-WPRE-INS retroviral vector was selected because of its highly stable CD20 expression *in vitro*.[38] To mimic the *in vivo* situation in patients, we chose not to use a clonal population of cells for this experiment. Instead, CD20 and eGFP double positive cells were purified by FACS sorting. We selected a cell population predominantly made up of CD20low cells, but also containing cells with higher or even lower CD20 expression varying in a 3-log range for CD20 expression with a mean of ~100 MFI (Figure 2B). The 90% of cells positive for eGFP and CD20, as indicated in Figure 2B, were shown to also express high levels of luciferase (Figure 2C). The *in vitro* sensitivity for rituximab- and HuMab-7D8-induced CDC of the selected pool was determined before injection into mice using a concentration of 10 μg/mL. Consistent with the low CD20 expression, rituximab induced poor CDC (10% cell lysis) compared to HuMab-7D8 (50% cell lysis) in this cell population (Figure 2D).

At day 1, mice were injected i.v. with the FACS-sorted CEM-CD20-LucR cell pool, after which 50 μg of antibody (rituximab or HuMab-7D8 in PBS) or an equivalent volume of matrix alone (PBS; control group) was injected i.p. Bioluminescent imaging was performed twice a week. The first luciferase signal above background (10^5: relative light units (RLU)) was detected in the femurs of the mice in the control group at day 7. No bioluminescence signal was detected for both the rituximab- or HuMab-7D8-treated mice at that time point (Figure 3B). Over the next ten days the signal increased rapidly in the control group and became visible in the abdomen and the cervical spine. At day 20, the mice of the control group suffered from hind limb paralysis and were euthanized (Figure 3A and B). On that same day, the first signal became visible in the femurs of the treatment groups and, in time, also in the abdomen (soft tissues, e.g. liver and spleen) and cervical spine. Rituximab- and HuMab-7D8-treated mice survived longer, until at day 40 all mice had been euthanized because of hind limb paralysis. Thus, treatment of the mice with rituximab and HuMab-7D8 significantly prolonged the median survival of the mice from 20 to 40 days (Figure 3C) compared to the control group. No significant difference in survival of the mice between rituximab and HuMab-7D8 treatment was observed. Specific luciferase expression analyses on different parts of the mice revealed a trend that both antibodies were more effective at the site of the abdomen than at the site of the femurs. Yet, the relatively low luciferase signal in the femurs (maximally 10 times the background of 10^5) hindered statistical confirmation (*data not shown*). Mice treated with a lower amount of 5 μg of antibody gave similar results, and, antibody treatment at day 3 instead of day 1 also delayed the outgrowth of the cells compared to non-treated mice (*data not shown*).

After euthanizing the mice, cells were harvested from

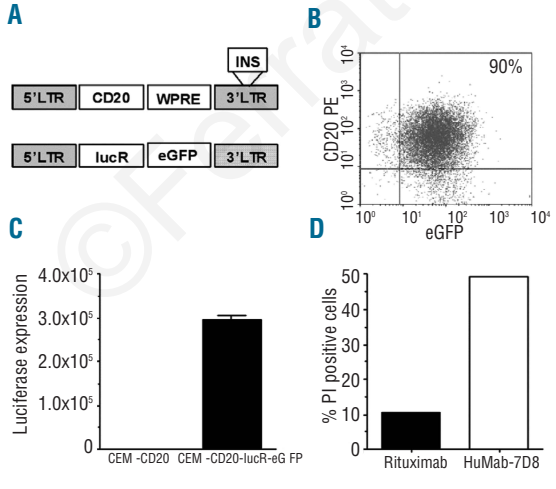

Figure 2. Preparation of luciferase expressing CEM-CD20 cells. (A) Schematic representation of CD20- and LucR-encoding retroviral vectors. (B) FACS analysis of transduced and FACS-sorted LucR-tagged CEM-CD20 cell pool. (C) *In vitro* luciferase activity of LucR-tagged CEM-CD20 cells and control CEM-CD20 cells. (D) *In vitro* CDC assay of the LucR-CEM-CD20 cell pool prior to *in vivo* use; cell kill is expressed as % PI positive cells.

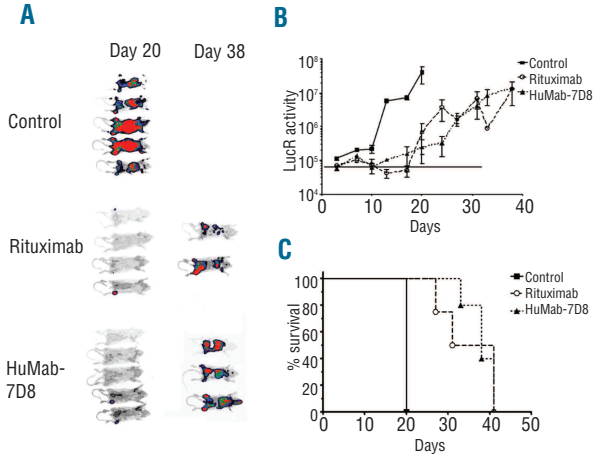

Figure 3. *In vivo* bioluminescence imaging. (A) *In vivo* imaging at day 20 and day 38 of mice inoculated with LucR-CEM-CD20 cells as described in the *Design and Methods* section. Mice were treated either with PBS, rituximab or HuMab-7D8. (B) After the last bioluminescence (on day 38) the mice were euthanized. Luciferase activity in relative light units was plotted against time. (C) Survival curve of mice inoculated with LucR-CEM-CD20 cells treated with PBS (control), rituximab or HuMab-7D8.

the bone marrow and cultured for at least two weeks in normal culture medium to eliminate mouse cells. Next, the CD20 expression of these cells, that survived *in vivo* antibody treatment, was analyzed. As a control, we confirmed that no remaining rituximab or HuMab-7D8 was present on the cell surface at that time (*data not shown*). Cells harvested from the control mice showed similar CD20 expression levels as the original cells that were injected into the mice (Figure 4A and B). Cells harvested from mice treated with rituximab still expressed CD20, but at a lower level compared to the original cells (Figure 4C). Remarkably, cells from mice injected with HuMab-7D8 did not exhibit any CD20 expression (Figure 4D). Together, these data suggest that CD20^{low} cells escaped CDC-mediated lysis by rituximab *in vivo*, while CD20^{low}-expressing cells were eliminated by HuMab-7D8.

CD20^{low} cells which escape rituximab can be killed by HuMab-7D8

We next addressed the question whether rituximab-resistant CEM-CD20 cells could be killed by HuMab-7D8 and *vice versa*. In parallel experiments, CD20^{low} CEM-CD20 cells were subjected to either rituximab- or HuMab-7D8-induced CDC using an mAb concentration of 10 μg/mL, respectively, and the extent of cell death was determined using propidium iodine FACS staining (10% for rituximab *vs.* 50% for HuMab-7D8; Figure 5A). As a control experiment, no CDC was observed in the presence of human serum alone (Figure 5A) or with heat-inactivated serum (*data not shown*). The cells surviving rituximab or HuMab-7D8 treatment were kept in culture for 14 days and subjected to a second round of CDC by incubating the cells with either one of the CD20 antibodies in the presence of normal human serum as source of complement (Figure 5B). A second incubation with rituximab resulted in only 5% cell lysis of the cells previously treated with rituximab. Notably, incubation of these rituximab-resistant CEM-CD20 cells with HuMab-7D8 resulted in 40% cell death, indicating that rituximab-resistant CEM-CD20 cells remained sensitive to lysis by the human antibody. A second incubation of HuMab-7D8 treated CEM-CD20 cells with HuMab-7D8 resulted in 25% cell death, whereas this cell population was completely resistant to killing by rituximab.

Discussion

In this report, we compared the human CD20 antibody HuMab-7D8 with rituximab for its ability to overcome resistance to CD20 antibody therapy in relation to the

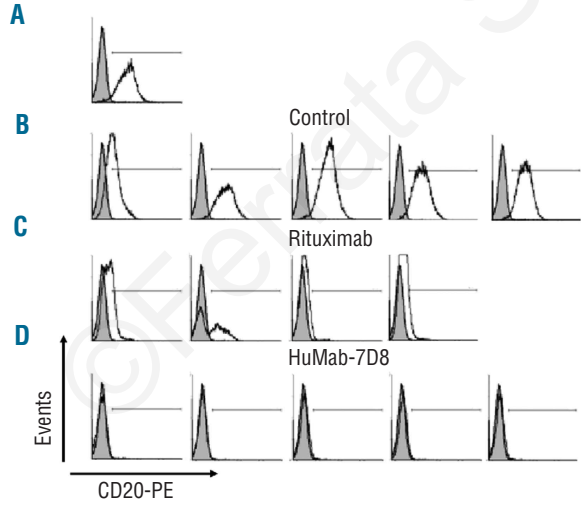

Figure 4. HuMab-7D8 eradicates CD20^{low}-expressing CEM cells *in vivo*. FACS-analyses of CEM-CD20 cells harvested from bone marrow of treated mice and cultured for two weeks. Each graph represents cells from an individual mouse except graph A (see below). The gray plots represent the non-transduced CEM cells. (A) CD20 expression of the pool of retrovirally CD20-transduced CEM cells that was injected into the mice and kept in culture during the experiment. (B) CD20 expression of CEM cells retrieved from the PBS-treated mice. (C) CD20 expression of CEM cells retrieved from rituximab-treated mice and (D) CD20 expression of CEM cells retrieved from the HuMab-7D8-treated mice.

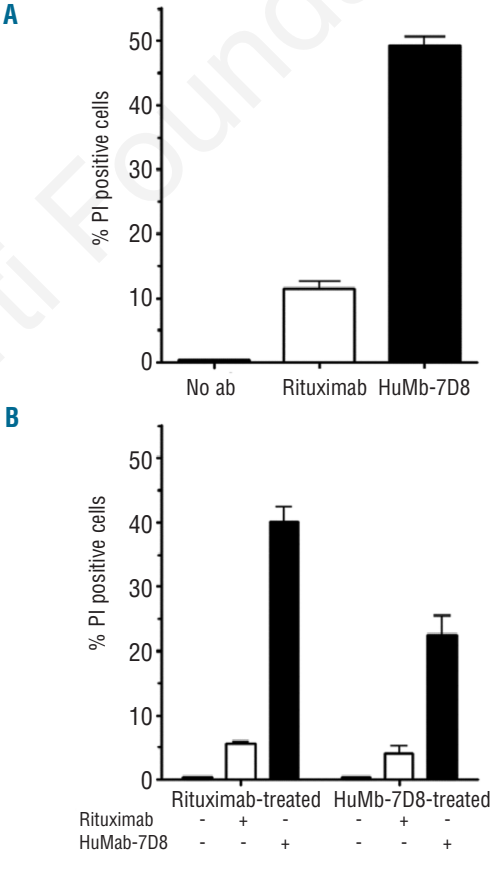

Figure 5. Rituximab-resistant cells are lysed by HuMab-7D8. (A) CD20 mAb-induced CDC of a CEM-CD20 clone with low CD20-expression (MFI of 325) by rituximab and HuMab-7D8. Cells were incubated with human serum only or with either rituximab or HuMab-7D8 and human serum. After 24 h the cells were washed and a sample was taken and analyzed for CDC. Results are expressed as % PI positive cells. The bars indicate the mean ± SD of triplicate measurements. (B) The cells that survived the one day initial rituximab- or Humab-7D8-mediated killing were cultured for an additional two weeks. Rituximab treated cells were subjected to a second exposure of CDC induction using either rituximab or HuMab-7D8. Conversely, the HuMab-7D8-treated cells from the first experiment were subjected to CDC induction using either rituximab or HuMab-7D8. Background lysis was assessed by adding normal human serum only.

CD20-expression level of cells. We demonstrated *in vitro* that compared to rituximab, HuMab-7D8 is significantly more efficient in killing CD20-transfected human CEM T leukemia cells.

HuMab-7D8 and rituximab both contain human IgG1 constant regions and have the same inherent C1q binding capacity when C1q is deposited on a plastic surface.[31] After binding to CD20 on cells, HuMab-7D8, however, activates complement more efficiently than rituximab and induces superior cell lysis.[31,32,44-46] HuMab-7D8, together with HuMab-2C6 and ofatumumab, the latter of which is in advanced clinical development and was recently approved by the FDA and EMA for the treatment of fludarabine and alemtuzumab refractory CLL, belong to a group of human antibodies that bind to a CD20 epitope which is distinct from that recognized by all other CD20 mAbs.[32] These novel antibodies bind to a membrane-proximal epitope, which includes the small 7-mer loop of the CD20 molecule. The A170xP172 motif in the large 44-mer loop that harbors the binding site of rituximab and most other CD20 mAbs, is not relevant for binding by HuMab-7D8 and ofatumumab-type antibodies.[32]

There are three (functional) characteristics of HuMab-7D8 that could potentially contribute to its superiority to rituximab in eliciting CDC-mediated cell killing.[32] First, there is superior binding of HuMab-7D8, resulting in a slower off-rate. However, as CDC occurs in minutes and the antibody off-rate occurs in hours to days, a slower off-rate probably contributes only marginally to the differences in CDC induction in our *in vitro* experiments (Figure 1B). In addition, another human CD20 antibody, HuMab-2C6, identified in the same panel as HuMab-7D8, (IgG1-2C6) has a faster off-rate than rituximab, but retains a much better capacity to activate complement.[32] Second, the movement of CD20 molecules into lipid rafts is crucial for the activation of complement which, therefore, may be affected by differences in the efficiency of CD20 antibodies to induce such translocation.[26,39] Here we demonstrate, however, that raft formation occurs similarly for both HuMab-7D8 and rituximab independent of CD20 expression, and thus the reduced activity of rituximab against CD20low cells is not due to inefficient raft formation (Figure 1C and D). A third characteristic is the proximity of the cognate epitope to the cell membrane. Binding of an antibody to the small loop epitope (HuMab-7D8) probably results in a more membrane-proximal localization as compared to binding to the larger 44-mer loop (rituximab). The localization and orientation of antibody binding, therefore, could give rise to more favorable IgG:IgG interactions and a closer positioning of the antibody Fc region to the membrane. Short lived thioester activated C4b and C3b thus may fixate more efficiently on the cell membrane.[46-49] This might explain why HuMab-7D8 needs less CD20 molecules than rituximab for inducing cell death, and is capable of killing rituximab-resistant cells (Figure 1B).

In addition, we found that rituximab-resistant cells were sensitive to HuMab-7D8-mediated lysis in the presence of complement. Significantly, HuMab-7D8 treated cells that survived an initial treatment remained sensitive to HuMab-7D8 but could not be lysed by rituximab (Figure 5).

Our *in vivo* studies are consistent with the observations *in vitro*. The improved killing of CD20low cells by HuMab-7D8 *in vitro* accordingly translates to their decreased survival *in vivo*. The RAG2$^{-/-}\gamma c^{-/-}$ mice lack B, T and NK cells, but have an active complement system as well as monocytes and macrophages.[41] Administration of either rituximab or HuMab-7D8 resulted in a significantly increased survival of the mice when compared to controls (Figure 3C). A critical mechanism involved in killing of opsonized CD20-positive cell *in vivo* is the complement system. Previously, Golay *et al.* demonstrated that complement is required for elimination of human CD20-expressing murine lymphoma cells in syngeneic mice.[50] In the studies by Golay and co-workers, killing of all human CD20-positive cells by rituximab leads to 100% survival of the mice. In our study, outgrowth of the tumor cells in the mice was not totally prevented although we did observe a significant increase in survival time for both groups of treated compared to untreated mice (Figure 3C). A major difference with the Golay studies is that we employed a polyclonal CD20low human CEM cell population with varying CD20-expression levels (Figure 1A) and also containing CD20-negative cells. In contrast to the Golay studies, which employed a cell line homogenously expressing high levels of CD20 we, therefore, observed CD20low and CD20neg cells to grow (i.e. resulting in increasing luminescence signals over time). Indeed, surviving cells were found in the bone marrow, liver, spleen and lymph nodes, which upon analysis showed some interesting differences between rituximab-treated and HuMab-7D8-treated mice. Cells surviving in rituximab-treated mice still expressed CD20, albeit at a low level, while the surviving cells in HuMab-7D8-treated mice were entirely CD20-negative. Whilst this is a most important observation, it is not reflected in significant differences in tumor load reduction (Figure 3A and B) or in differences in survival times (Figure 3C) between HuMab-7D8 and rituximab-treated mice. Our CDC data showed that the difference between rituximab and HuMab-7D8 only becomes apparent for cells with low CD20 expression, which, in our cell line, is only a minor fraction (Figure 1A). The polyclonal CD20-positive cells that were injected in the mice, however, also contained a very small fraction of CD20-negative cells. Obviously, the latter cannot be eliminated either by rituximab or HuMab-7D8. Hence, the small difference between the fraction of non-expressing plus low expressing cells surviving in the rituximab-treated mice *versus* the fraction of non-expressing cells only surviving after HuMab-7D8 does not leave much room for improvement. We estimate this to be in the order of 2-fold and this will not result in significant differences in survival time or BLI signal (*Online Supplementary Figure S1*). Thus, the most striking finding in this study is that HuMab-7D8 eradicates all CD20-expressing cells, from low to high, while rituximab only eradicates the higher CD20-expressing cells (Figure 4).

Previously, it was described that CD20 can be transiently down-regulated by shaving of the antibody-CD20 complex by monocytes.[51,52] It is unlikely that shaving is responsible for the effects observed here, as extended *in vitro* culture (14 days) of the surviving cells did not result in CD20 re-expression. The pool of CD20/eGFP-transduced CEM cells used in our *in vivo* mouse model contained a very low fraction of cells expressing no CD20 at all. In B-cell leukemic patients, in contrast, a complete loss of CD20 appears extremely rare and has only been reported in a very small number of case reports. This, in combination with our observations in mice, suggests that escape of

HuMab-7D8 therapy by downregulation of CD20 is quite unlikely.

In conclusion, HuMab-7D8 is able to efficiently kill both $CD20^{high}$ as well as $CD20^{low}$ cells. Human CD20 antibodies that bind to the membrane-proximal epitope, such as HuMab-7D8 and ofatumumab, therefore, seem good candidates to overcome the CD20-expression level-related resistance to rituximab, either as a first-line treatment or as a re-treatment/maintenance treatment strategy following rituximab relapse. Consequently, HuMab-7D8 and its clinical counterpart ofatumumab (currently in phase III clinical trials[36,37] and marketed for the treatment of fludarabine and alemtuzumab refractory CLL), are promising novel CD20 mAbs in the fight against B-cell malignancies.

Authorship and Disclosures

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