Clinical Cancer Research

Novel BAFF-Receptor Antibody to Natively Folded Recombinant Protein Eliminates Drug-Resistant Human B-cell Malignancies *In Vivo*

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Abstract

Purpose: mAbs such as anti-CD20 rituximab are proven therapies in B-cell malignancies, yet many patients develop resistance. Novel therapies against alternative targets are needed to circumvent resistance mechanisms. We sought to generate mAbs against human B-cell–activating factor receptor (BAFF-R/TNFRSF13C), which has not yet been targeted successfully for cancer therapy.

Experimental Design: Novel mAbs were generated against BAFF-R, expressed as a natively folded cell surface immunogen on mouse fibroblast cells. Chimeric BAFF-R mAbs were developed and assessed for *in vitro* and *in vivo* monotherapy cytotoxicity. The chimeric mAbs were tested against human B-cell tumor lines, primary patient samples, and drug-resistant tumors.

Results: Chimeric antibodies bound with high affinity to multiple human malignant B-cell lines and induced potent antibody-dependent cellular cytotoxicity (ADCC) against multiple subtypes of human lymphoma and leukemia, including primary tumors from patients who had relapsed after anti-CD20 therapy. Chimeric antibodies also induced ADCC against ibrutinib-resistant and rituximab-insensitive CD20-deficient variant lymphomas, respectively. Importantly, they demonstrated remarkable *in vivo* growth inhibition of drug-resistant tumor models in immunodeficient mice.

Conclusions: Our method generated novel anti–BAFF-R antibody therapeutics with remarkable single-agent antitumor effects. We propose that these antibodies represent an effective new strategy for targeting and treating drug-resistant B-cell malignancies and warrant further development. *Clin Cancer Res; 24(5); 1114–23. ©2017 AACR.*

Introduction

mAb immunotherapy is highly successful against hematologic malignancies (1–7). However, mAb treatment alone is not curative, and emerging resistance remains a problem (8, 9). A key example is resistance to anti-CD20, rituximab, thought to be caused by multiple mechanisms including downregulation of CD20 (10). Thus, there is an urgent need to develop new therapies against B-cell malignancies.

One alternative target on B-cell tumors is B-cell–activating factor receptor (BAFF-R/TNFRSF13C), a TNF receptor superfamily member specifically involved in B lymphocyte development and mature B-cell survival (11, 12). BAFF-R is expressed almost exclusively on B cells, and its surface expression has been documented on various human B-cell lymphomas (13–17). Although much of the biology of the BAFF/BAFF-R axis is known (18–21), its initial promise as a target for cancer therapy has not been met; however, it remains an attractive target for B-cell lymphomas, especially drug-resistant tumors.

Conventional recombinant immunogen proteins produced in bacteria for developing mAbs lack posttranslational modifications and are simplistically folded because compared with eukaryotes, prokaryotes lack chaperone proteins and oxidizing environments. As a result, such proteins may differ in conformational structure from the corresponding plasma membrane–anchored native proteins. Furthermore, antibodies may be raised against off-target domains, such as transmembrane or intracellular domains of the target protein. Here, we applied a strategy of generating mAbs against a natively folded, glycosylated immunogen expressed on eukaryotic cells (22, 23). Specifically, we expressed human BAFF-R as a native protein on mouse fibroblast cells and used the engineered cell clone as an immunogen in mice. We report on the generation of novel mAbs that specifically bound and lysed human malignant B-cell lines and primary lymphomas *in vitro* and inhibited growth of drug-resistant lymphoma in xenogenic tumor models *in vivo*.

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Translational Relevance

Developing novel strategies against drug-resistant B-cell tumors addresses the urgent need of patients experiencing relapse or resistance following first-line therapies. BAFF-R is a suitable alternative target for therapeutic antibody development because of its nearly exclusive expression on the surface of B cells and notable prominent expression on malignant B cells. Although previous attempts were made to target BAFF-R, our antibodies have greater potential to be translated into the clinic. We demonstrated the antibodies elicited potent antitumor effects against a broad range of B-cell tumors. Moreover, the pronounced therapeutic efficacy seen in drug-resistant human lymphoma xenograft models further supports translation of our antibodies to meet this urgent need.

Materials and Methods

Animals, cell lines, and primary human tumor samples

Mice. BALB/c mice for antibody development and NOD *scid* gamma (NSG) breeding pairs were purchased from The Jackson Laboratory. The NSG breeding colony was maintained by the Animal Resource Center at City of Hope (Duarte, CA). Mice were housed in a pathogen-free animal facility according to institutional guidelines. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC: 15020).

Tumor lines. Malignant, human hematologic cell lines, including JeKo-1, Mino, REC-1, JVM-13, SU-DHL-6, Raji, OCI-LY3, RL, RS4;11, MEC-1, SKNO-1, Jurkat, and U266, were purchased from either ATCC or DSMZ. Z-138 line was provided by Dr. Michael Wang (MD Anderson Cancer Center, Houston, TX). Ibrutinib-resistant SP49-IR line was developed and provided by Dr. Jianguo Tao (University of South Florida, Tampa, FL; ref. 24). Ibrutinib-resistant SP49 cell lines (SP49-IR) were established by treating cells with escalating doses of ibrutinib. IC_{50} was 5 nmol/L for parental SP49 compared with >100 nmol/L for SP49-IR. At 100 nmol/L ibrutinib, approximately 5% of SP49 cells were viable compared with >90% of SP49-IR cells. Human NK-92 176V cells were obtained from Conkwest Inc.

Human blood and tumor samples. Noncultured, primary human lymphomas were obtained as cryopreserved, viable single-cell suspensions in 10% DMSO from the Lymphoma Satellite Tissue Bank at MD Anderson Cancer Center under an Institutional Review Board–approved protocol (IRB: 2005-0656). Primary patient samples included leukapheresis or blood from patients with mantle cell lymphoma (MCL) or chronic lymphocytic leukemia (CLL), and excised lymph nodes from patients with diffuse large B-cell lymphoma (DLBCL) or follicular lymphoma. Tumor cells in each sample ranged from 80% to 98% for leukapheresis or blood, and from 50% to 60% for lymph node biopsies. Peripheral blood mononuclear cells (PBMC) were provided by the Michael Amini Transfusion Medicine Center at City of Hope (IRB: 15283).

Generation of human BAFF-R–expressing mouse fibroblast cells

Human *(h)BAFF-R* cDNA was from human B cells and cloned in-frame with *GFP* gene on pEGFP-N1 vector (Takara/Clontech). *(h)BAFF-R* cDNA sequence was confirmed against the NCBI gene sequence database (gene ID: 115650). The cDNA encoding *(h)BAFF-R-GFP* fusion was subsequently cloned into a lentivirus gene delivery system (pLenti6/V5-DEST Gateway Vector Kit, Life Technologies) to produce (h)BAFF-R-GFP fusion proteins when transduced into mouse fibroblast (L) cells. Single-cell clones were established from sorted GFP-positive L cells, and (h)BAFF-R-GFP–expressing L-cell clone D2C was used in further studies.

Antibody-producing hybridomas

Two 6-week-old BALB/c mice were immunized with live, untreated mouse fibroblast (L) cell clone (D2C) engineered to express human BAFF-R on the cell membrane by five subcutaneous injections at the footpad once every 3 days. Blood samples were obtained from both mice to confirm serum antibodies against D2C by ELISA. Splenic tissue from the immunized mice was harvested on day 20. Harvested splenocytes were fused with Sp2/0 myeloma to establish hybridomas and ELISA screened for antibodies against the antigen using D2C or parental L cell–coated plates. Immunization and hybridoma procedures were conducted at the Antibody Core Facility at MD Anderson Cancer Center.

Chimeric antibody production

cDNA from selected hybridomas encoding the variable regions of antibody light and heavy chains was engineered onto expression vectors containing respective human IgG1 constant regions. Vectors were cotransfected into the FreeStyle 293 Expression System (Life Technologies) according to the manufacturer's directions. Antibodies in culture supernatant were purified by HiTrap Protein A affinity chromatography columns (GE Healthcare) according to the manufacturer's directions.

Cytotoxicity assays

Target cells (L cells, human tumor lines, primary patient samples) were labeled with chromium-51 (^{51}Cr, PerkinElmer) for a ^{51}Cr release assay. Briefly, antibodies and effectors [natural killer (NK) cells or complement serum standard (Sigma Aldrich)], were added to labeled target cells and incubated up to 18 hours. NK cells were enriched from PBMCs (NK Cell Enrichment Kit, Stemcell Technologies). Controls included effector cells only or effectors cells + control IgG (Human IgG Isotype Control, Invitrogen). Counts per minute (CPM) of ^{51}Cr released into supernatant was detected with a Wizard Automatic Gamma Counter (PerkinElmer). Percent lysis was calculated by $\text{Lysis}\ (\%) = \frac{CPM-\overline{SR}}{\overline{MR}-\overline{SR}} \times 100\%$ | SR: CPM of spontaneous release; MR: CPM of maximum release.

Generation of JeKo-1-CD20-KO

FACS-sorted, stable JeKo-1-CD20-KO cells were generated using CD20-CRISPR/Cas9 and HDR Plasmid Systems (Santa Cruz Biotechnology) according to the manufacturer's directions. *CD20* knock-out (KO) was verified by flow cytometry and Western blots.

In vivo studies

Tumor models. Stable, luciferase-expressing tumor lines were established for bioluminescent imaging in mouse models. Briefly, a luciferase gene was introduced into tumor lines by a lentivirus gene delivery system (pLenti7.3/V5-DEST Gateway Vector Kit, Life Technologies). The minimum lethal dose was determined for each tumor cell line by dose titration (1×10^6 JeKo-1, 5×10^5

RS4;11, 5×10^5 JeKo-1-CD20-KO, or 2.5×10^4 Z-138 cells). Tumor cells were injected intravenously, and tumor development was continuously monitored by *in vivo* bioluminescence imaging.

Bioluminescent imaging. Mice were anesthetized with isoflurane and administered 150 mg/kg D-luciferin (Life Technologies) via intraperitoneal injection 10 minutes prior to imaging. Imaging was performed on an AmiX imaging system (Spectral Instruments Imaging).

Antibody studies. Mice ($n = 5$/group) were tumor challenged intravenously 3 days prior to four treatments once every 5 days. Treatments were 300 µL intravenous injections: 200 µg treatment antibody, 10×10^6 effector human NK-92-176V cells, and 5×10^4 IU IL2 (Prometheus Laboratories). Control groups received the same volume injections with saline or NK cells alone. Bioluminescent imaging was performed weekly up to 80 days. Survival was tracked up to 100 days after tumor challenge.

Results

Generation of mAbs against human BAFF-R

To generate a therapeutic antibody to a biologically relevant epitope of BAFF-R, we used a eukaryotic cell surface expression system in which endogenous cell surface proteins are presented in their native conformation with appropriate posttranslational modifications. We engineered a mouse fibroblast (L) cell clone expressing cell surface GFP-tagged, human BAFF-R. BAFF-R–

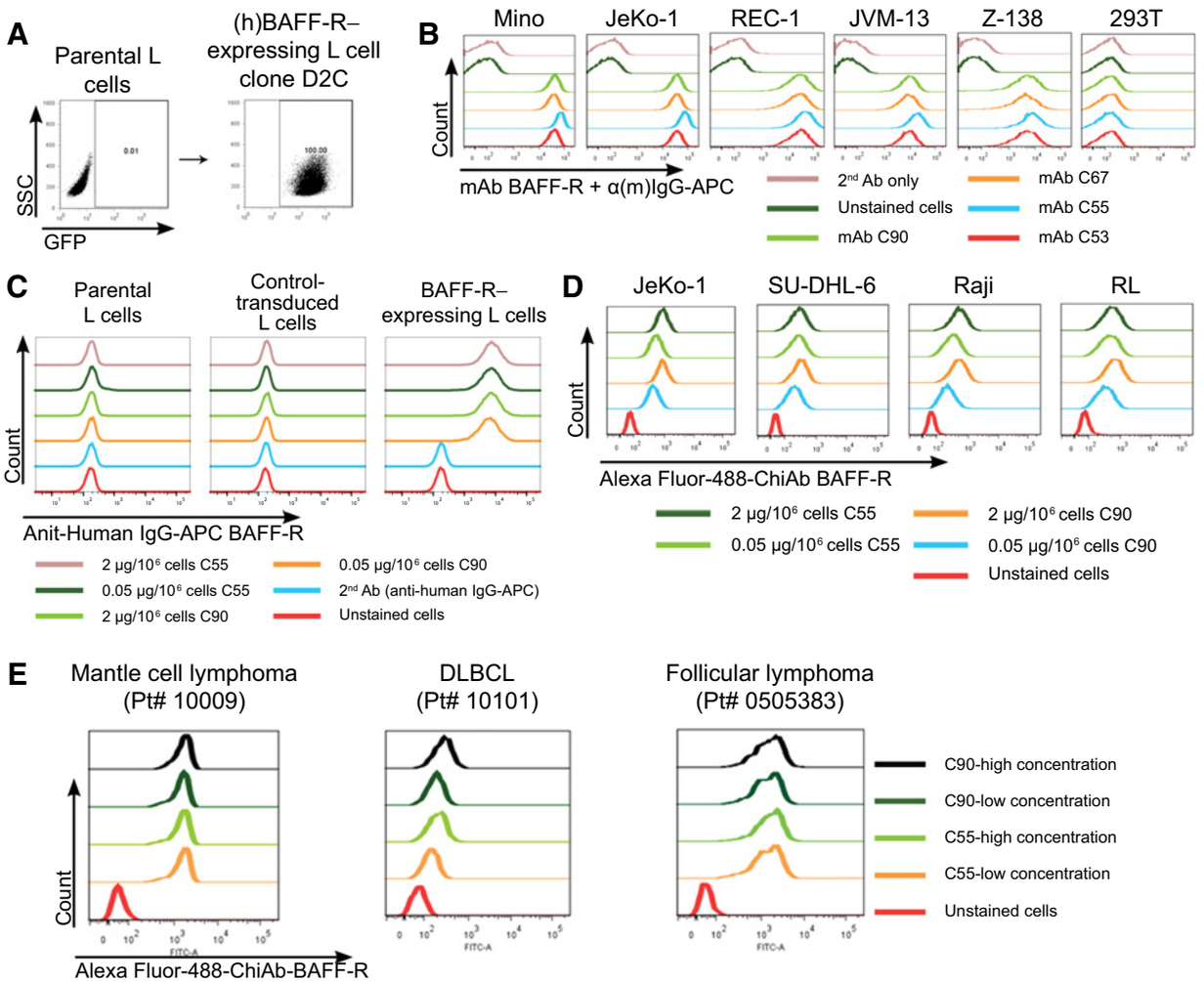

Figure 1.

Generation and specificity of novel mAbs against human BAFF-R. **A,** FACS analysis of cell surface expression of (h)BAFF-R-GFP fusion protein in mouse fibroblast L cells. Gated on GFP-positive cells, engineered L-cell clone (right plot) is compared with parental L cells (left plot). Clone D2C was selected for further studies. **B–E,** FACS histograms of anti–BAFF-R mAbs binding cell lines and patient samples: **B,** Affinity-purified hybridoma mAb (C90, C67, C55, and C53) binding BAFF-R–positive, human MCL lines including Mino, JeKo-1, REC-1, JVM-13, and Z-138 at a concentration of 0.05 µg mAb/10^6 cells. BAFF-R-negative 293T embryonic kidney cell line was used as a control. **C,** Chimeric antibodies C55 and C90 at high and low concentration binding (h)BAFF-R–expressing L cells. Parental L cells, control-transduced L cells, and secondary anti–(h)IgG-APC antibodies only were used as controls. **D,** Alexa Fluor 488–conjugated chimeric antibodies binding a panel of NHL cell lines. **E,** Chimeric antibodies binding three types of NHL primary patient samples. The data are representative of three independent experiments.

expressing L-cell clones were generated and characterized for GFP expression (Fig. 1A). Clone D2C was expanded and successfully used to immunize BALB/c mice according to Materials and Methods and immunization schedule in Supplementary Fig. S1A.

After generating and screening hybridoma clones, we identified four clones (53, 55, 67, and 90) as producing antibodies that specifically bound BAFF-R–expressing, but not parental, L cells (Supplementary Fig. S1B). Supernatants of all four clones contained antibodies that bound BAFF-R–expressing

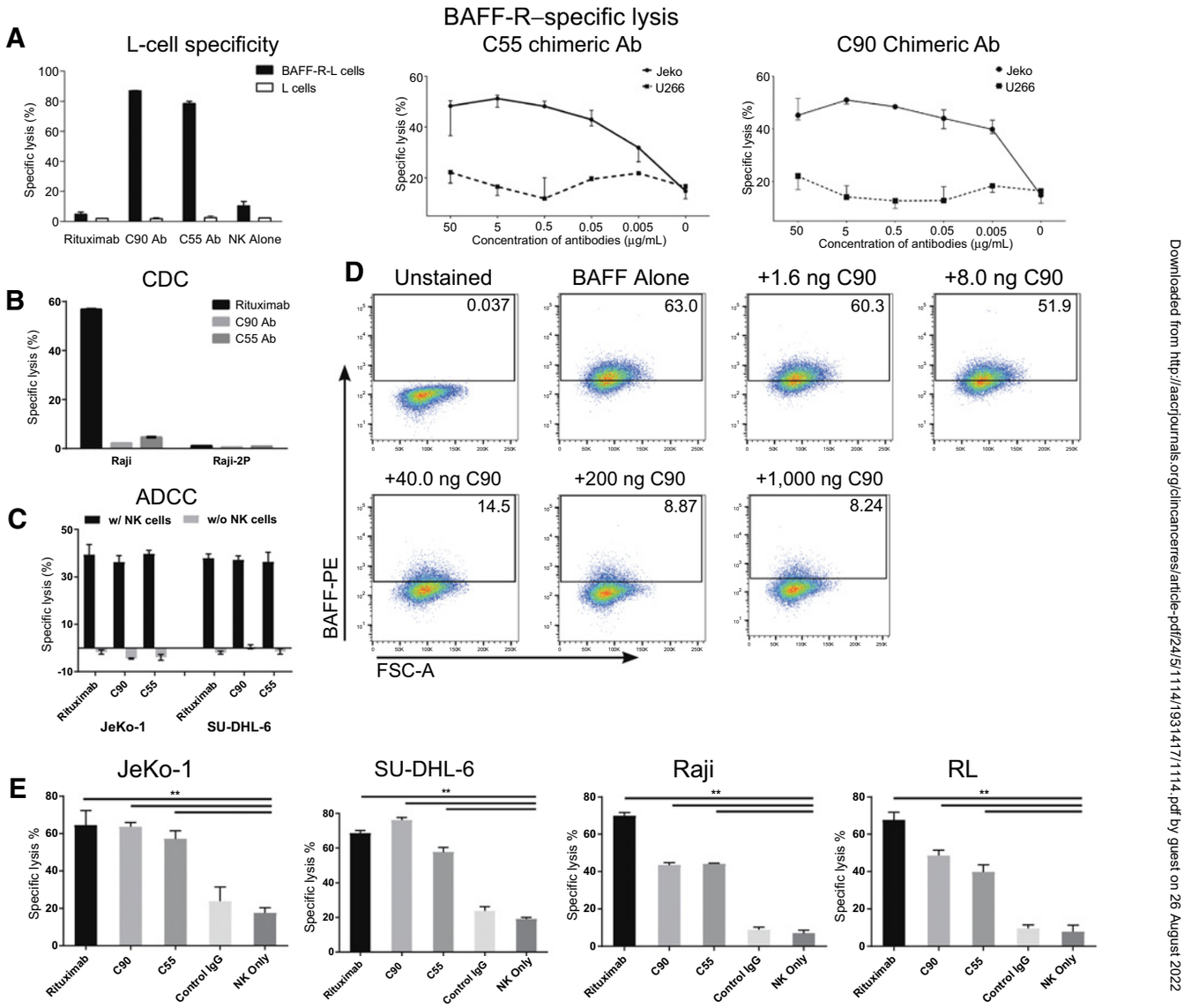

Figure 2.

BAFF-R mAbs exhibited specific *in vitro* cytotoxicity against B-cell tumor lines. (**A–D**) Antibody BAFF-R specificity was determined by specific lysis and ligand-blocking assays. ADCC was measured by ^{51}Cr release assay, and specific lysis of target cells was calculated as described in Materials and Methods. Antibodies (C55, C90, or rituximab) and effectors (NK cells or complement-containing serum) were incubated with ^{51}Cr-labeled target cells. **A,** Specific and dose response lysis are show for various target cells. BAFF-R–expressing cells included BAFF-R L cells and JeKo-1, and BAFF-R–negative controls include parental L cells and U266 human multiple myeloma cell line. NK cells were included in an effector-to-target ratio (E:T) of 20:1. **B,** CDC-specific lysis is show for CDC-sensitive Raji and CDC-resistant Raji-2P. Active complement-containing human serum was incubated with target cells at 1:3 dilution. **C,** ADCC-specific lysis is shown for JeKo-1 and SU-DHL-6. Target cells and antibodies were incubated with or without effector NK cells (E:T = 20:1). **D,** FACS plot gates show percentage of BAFF/BAFF-R binding signal in the presence of C90 mAb. BAFF-R–expressing D2C L-cell clones were incubated with C90 (0–1,000 ng/10^6 cells) at 4°C for 45 minutes, followed by incubation with recombinant BAFF ligand (0.5 μg/10^6 cells) at 4°C for 90 minutes. Flow cytometry was performed and gated for anti–BAFF-PE. **E,** ADCC effects were measured on a panel of NHLs. ^{51}Cr-labeled NHL lines were incubated with antibodies rituximab, C90, C55, or control IgG and effector NK cells (E:T = 20:1). NK cell only (without antibodies) was an additional control. Calculated specific lysis is shown as the mean ± SD of triplicate samples. **, $P < 0.05$ compared with NK cell only and control IgG by two-tailed Student *t* test.

Mino cells (mantle cell lymphoma) in a dose-dependent manner. No antibody binding was detected in BAFF-R–negative control cell line, 293T (Supplementary Fig. S2).

Antibodies from the four hybridoma supernatants were purified by protein A affinity chromatography. Purified antibodies bound Mino cells in a dose-dependent manner (Supplementary Fig. S3), as well as other human MCL lines, including JeKo-1, REC-1, and ibrutinib-resistant JVM-13 and Z-138 (Fig. 1B).

An analysis of the complementarity determining regions on the four antibodies revealed that clones 53, 55, and 67 had nearly identical sequences, whereas clone 90 was unique. Therefore, we selected clones 55 and 90 for further investigation. Both clones 55 and 90 effectively bound JeKo-1 (MCL), SU-DHL-6 (DLBCL), Raji (Burkitt lymphoma), and RL (follicular lymphoma) at both high (2 μg/10^6 cells) and low (0.05 μg/10^6 cells) concentrations (Supplementary Fig. S4).

Chimeric mAb against human BAFF-R induced antitumor effects both *in vitro* and *in vivo*

Clones 55 and 90 were further developed into their respective chimeric mAbs containing human IgG1 constant regions (termed C55 and C90). The chimeric antibodies retained specific dose-dependent binding to BAFF-R–expressing L cells but not parental or control-transduced L cells (Fig. 1C). C55 and C90 were conjugated to Alexa Fluor 488 and exhibited direct binding to non-Hodgkin lymphoma (NHL) lines JeKo-1, SU-DHL-6, Raji, and RL (Fig. 1D). Importantly, both fluorochrome-labeled chimeric mAbs (Fig. 1E) and original mouse mAbs (Supplementary Fig. S5) readily bound MCL, DLBCL, and follicular lymphoma patient primary tumor samples. Binding of our mAbs correlated with the expression of BAFF-R on human NHL cell lines (Supplementary Fig. S6A) and primary tumors (Supplementary Fig. S6B), further supporting antigen specificity.

C55 and C90 elicited antibody-dependent cell-mediated cytotoxicity (ADCC) specifically against BAFF-R–expressing L cells and JeKo-1, but not BAFF-R–negative parental L cells nor the BAFF-R–negative human multiple myeloma line (U266; Fig. 2A). In contrast, antibodies did not elicit *in vitro* complement-dependent cytotoxicity (CDC, Fig. 2B). Cytotoxicity required the addition of NK cells, as shown for various types of human NHL cell lines (Fig. 2C; Supplementary Fig. S7), suggesting ADCC as a principal mechanism of antibody-mediated cytotoxicity. We found the

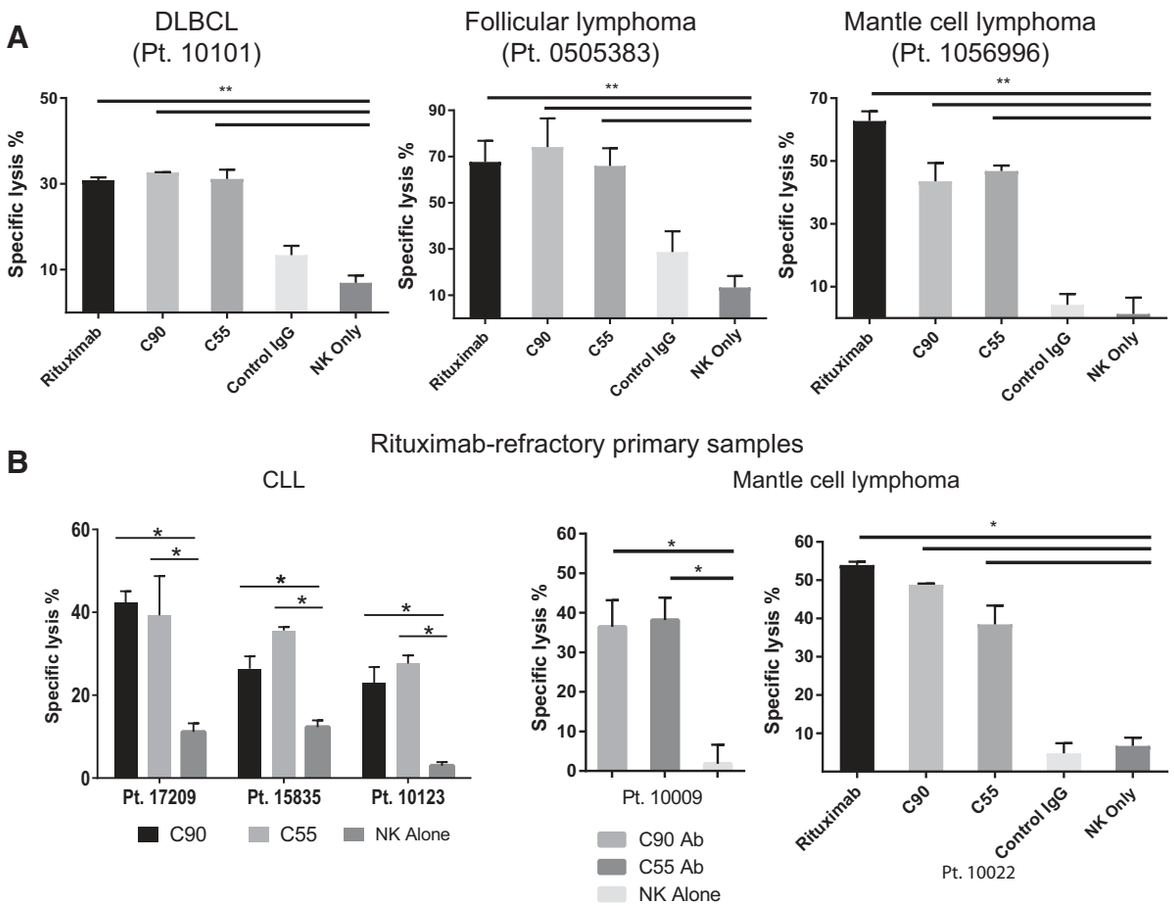

Figure 3.

BAFF-R mAbs induced *in vitro* ADCC against primary B-cell tumors. ADCC effects were measured by ^{51}Cr release after incubation with rituximab, C90, C55, control IgG, and NK cells only at E:T = 20:1. Results were calculated as percentage of cell-specific lysis of target cells: **A,** NHL patient samples **, $P < 0.05$ compared with NK cells only and control IgG by two-tailed Student *t* test; **B,** Primary CLL and MCL samples from rituximab-treated, refractory patients. Data are shown as the mean ± SD of triplicate samples. *, $P < 0.05$ compared with controls by two-tailed Student *t* test.

antibodies inhibited BAFF/BAFF-R binding in a dose-dependent manner (Fig. 2D), suggesting potential disruption of BAFF/BAFF-R interaction in tumor cells and further support for their specificity. Furthermore, C55 and C90 exhibited limited internalization upon binding BAFF-R (Supplementary Fig. S8). *In vitro* ADCC was confirmed in our panel of malignant, human B-cell lines, including JeKo-1, SU-DHL-6, Raji, RL (Fig. 2E), RS4;11, and MEC-1 (Supplementary Fig. S9A). No cytotoxicity was observed in BAFF-R–negative lines, including U266, T-cell leukemia (Jurkat), and acute myeloid leukemia (SKNO-1, Supplementary Fig. S9B). Importantly, chimeric antibodies elicited ADCC against primary patient tumor samples (Fig. 3A; Supplementary Fig. S10).

In vivo, we challenged NSG mice with luciferase knock-in JeKo-1 MCL cell line followed by antibody treatments. Treatment followed the schedule in Fig. 4A. Mice receiving either C55 or C90 demonstrated significant retardation of tumor growth, compared with PBS or NK cells alone control groups (Fig. 4B). Similarly, C55 and C90 also markedly retarded tumor growth in RS4;11 [acute lymphoblastic leukemia (ALL)] challenged NSG mice, compared with no inhibition by rituximab or controls (Fig. 4C).

Chimeric mAb induced potent antitumor effects against drug-resistant lymphomas *in vitro* and *in vivo*

We further tested our antibodies against primary CLL ($n = 3$) and MCL ($n = 2$) samples from relapsed patients who had been previously treated with rituximab. All five primary samples were sensitive to killing by ADCC with C55 and C90, suggesting their

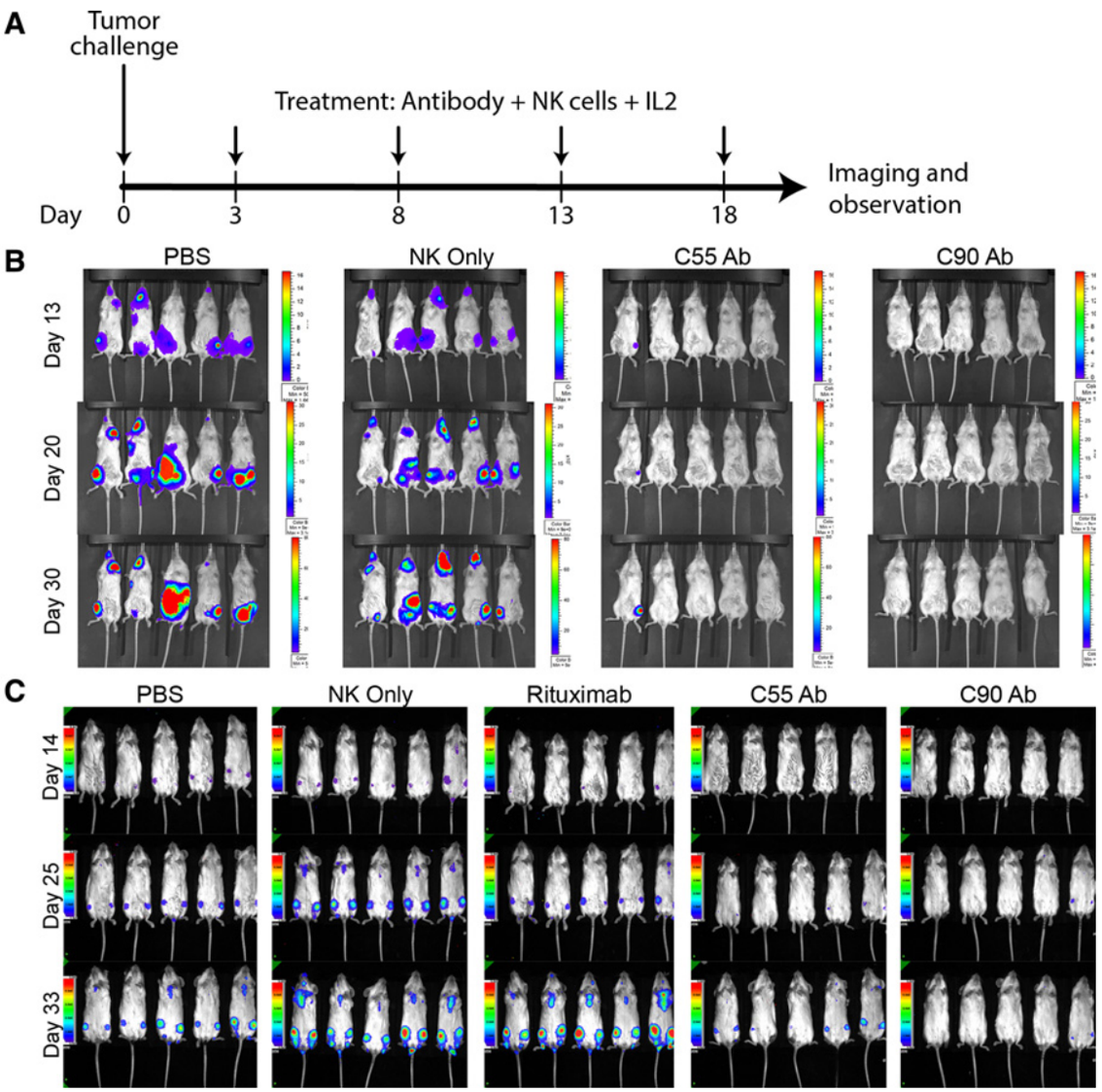

Figure 4.

Chimeric antibodies targeting human BAFF-R elicited *in vivo* therapeutic effects against B-cell tumors. **A,** Treatment schedule following day 0 tumor challenge with minimum lethal dose of tumors. Antibody treatments were given by intravenous tail vein injections: 200 μg treatment antibody, 10×10^6 effector human NK-92-176V cells, and 5×10^4 IU IL2. Bioluminescence imaging monitored mice challenged with luciferase-expressing tumors: **B** and **C,** JeKo-1 (**B**; MCL) or RS4;11 (**C**; ALL). Experimental groups received treatment of chimeric BAFF-R mAbs (C55 or C90, as indicated). Control group mice received PBS, NK cells alone, or rituximab on the same schedule. Data are representative of three independent experiments.

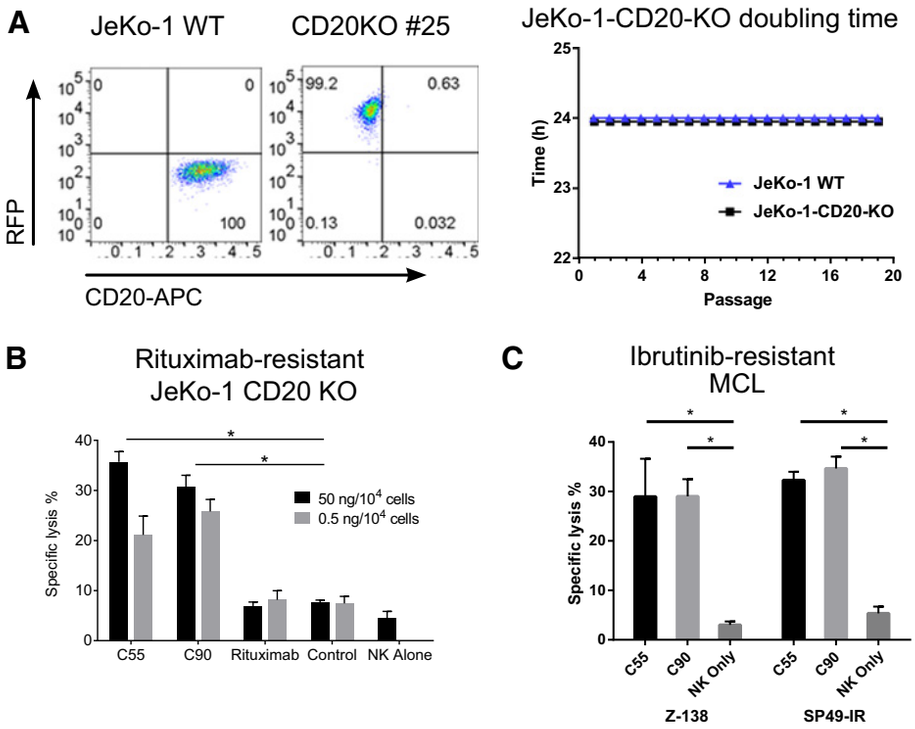

Figure 5.
Chimeric BAFF-R antibodies induced ADCC on drug-insensitive/resistant lymphoma models *in vitro*. **A,** Verification of JeKo-1 cells following CRISPR/HDR knock-out of *CD20* gene compared with JeKo-1 wild type (WT). FACS analysis showed CD20 binding on selected clone and WT. Viability and doubling time of JeKo-1-CD20-KO cells were monitored over 20 passages. ADCC effects measured by ^{51}Cr release after incubation with C55, C90, or rituximab and effectors NK cells (E:T = 20:1). Percentage of cell-specific lysis of target cells: **B** and **C,** Rituximab-resistant JeKo-1-CD20-KO (**B**) and ibrutinib-resistant Z-138 and SP49-IR (**C**). All data are representative of two or more identical experiments. Data are shown as the mean ± SD of triplicate samples. *, $P < 0.05$ compared with NK cells by two-tailed Student *t* test.

effectiveness against tumors, which progressed clinically after exposure to rituximab (Fig. 3B).

We sought to model drug resistance due to the loss of target and developed a model to simulate CD20 downregulation and antigen-shaving, phenomena sometimes observed clinically following rituximab treatment (25). Accordingly, we generated a rituximab-insensitive, stable CD20 KO clone of JeKo-1 using a CRISPR/HDR system. CD20-KO clones were confirmed for absence of CD20 surface expression with unaffected viability and growth rate (Fig. 5A; Supplementary Fig. S11A–S11C) and presence of BAFF-R surface expression by flow cytometry (Supplementary Fig. S11D). JeKo-1-CD20-KO clone 25, selected for further studies, retained sensitivity to C55- and C90-mediated ADCC, but became insensitive to cytotoxicity mediated by anti-CD20 rituximab (Fig. 5B).

As a second model of drug-resistant lymphomas, we tested our chimeric BAFF-R mAb for ADCC against the naturally ibrutinib-resistant human MCL line, Z-138 (26), and the induced ibrutinib-resistant MCL line, SP49-IR, which had been induced *in vitro* for resistance to ibrutinib (24). Significant *in vitro* ADCC was observed with the antibodies against both ibrutinib-resistant lines (Fig. 5C). Z-138 was further confirmed to be ibrutinib resistant *in vivo* using ibrutinib-sensitive JeKo-1 as a control (Supplementary Fig. S12A and S12B).

Finally, 3 days following intravenous challenge with JeKo-1-CD20-KO tumor cells *in vivo*, NSG mice ($n = 5$/group) received BAFF-R antibody treatments (C55 or C90) or rituximab as described in Materials and Methods and according to the schedule in Fig. 4A. Bioluminescent imaging on day 20 revealed substantial tumor burden in controls and rituximab-treated mice, but no visible tumors in BAFF-R antibody treatment groups (Fig. 6A). Monitoring tumor-free and long-term overall survival confirmed the significant antitumor effects of both BAFF-R antibodies, but not rituximab (Fig. 6C). Similarly, significant effects were observed following treatment of ibrutinib-resistant Z-138 tumor-bearing mice with either BAFF-R antibody, compared with controls (PBS, NK only, or ibrutinib only; Fig. 6B and C; Supplementary Fig. S13). Specifically, although progressive tumor growth and death were observed in all controls, tumor growth was delayed significantly in mice treated with BAFF-R antibodies, and some of these developing tumors even regressed subsequently. In all *in vivo* studies, mice were closely monitored daily for toxicities, weight loss, and general signs of distress. No toxicities were observed in antibody-treated mice.

BAFF-R mAbs also bind normal B cells

When tested against normal PBMCs, anti–BAFF-R antibody C90 exhibited specific binding (Supplementary Fig. S14A and S14B) and lysing (Supplementary Fig. S15) of B cells, as expected, without staining any T cells, NK cells, granulocytes, or monocytes. The positive staining results were verified on purified B cells (Supplementary Fig. S14C). Again, purified T cells, NK cells, and gated myeloid cells showed no binding.

Discussion

BAFF-R protein is expressed almost exclusively on the surface of B cells and is particularly upregulated on B-cell tumors, making it an attractive target for immunotherapies. The BAFF/BAFF-R axis has been targeted successfully for autoimmune diseases, particularly with mAbs against the BAFF ligand (27, 28); however, the initial promise for B-cell tumor therapy has not yet been realized. Specifically, at least one mAb against the receptor has also been developed to deplete autoreactive B cells (29). The antibody was examined for its ability to deplete normal B cells, but no information on activity against malignant B cells was provided.

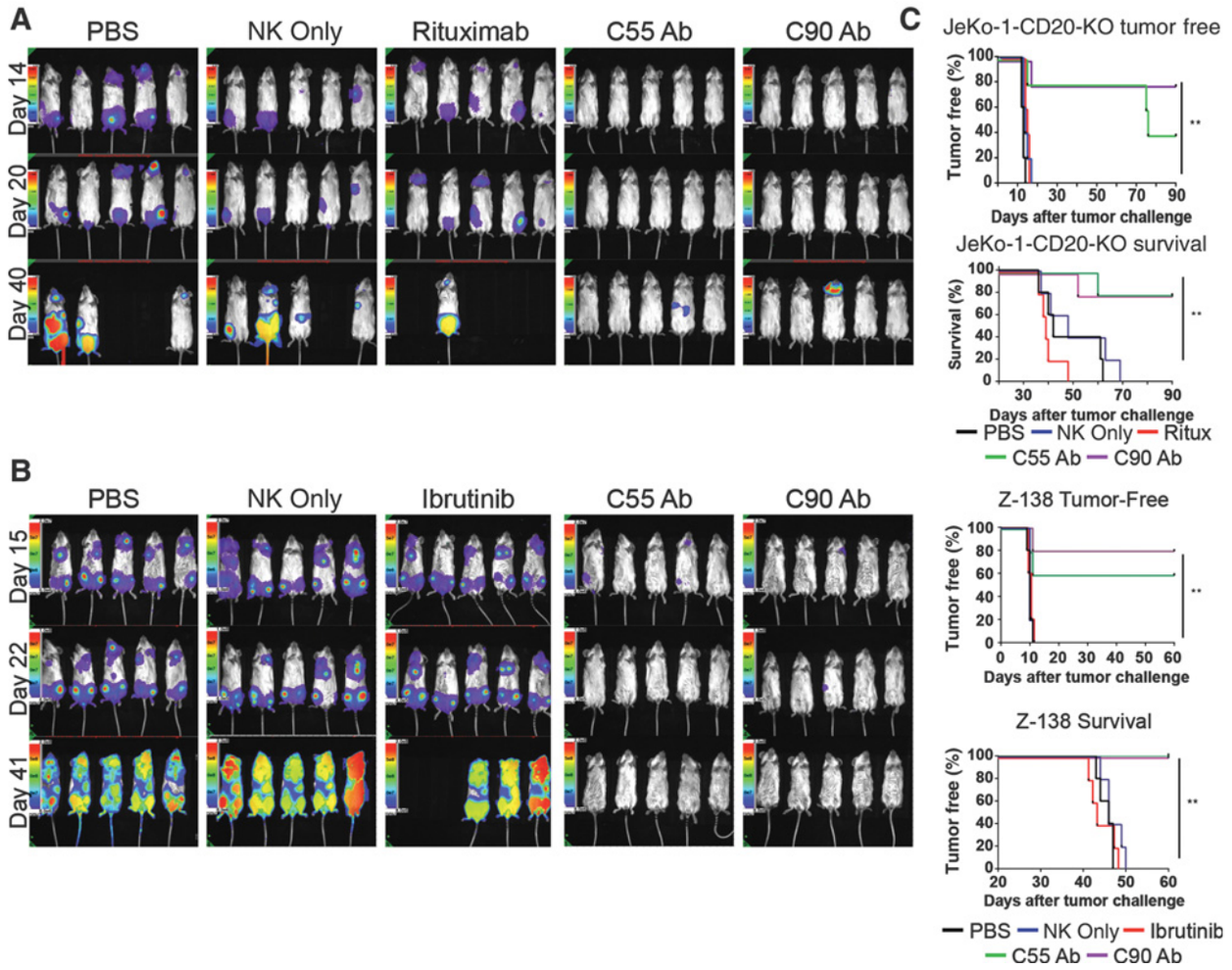

Figure 6.

Chimeric antibodies targeting human BAFF-R elicited *in vivo* therapeutic effects against drug-resistant B-cell tumors. Bioluminescence images of mice challenged with luciferase-expressing tumors (**A**), rituximab-insensitive JeKo-1-CD20-KO cells, or ibrutinib-resistant Z-138 cells (**B**), followed by antibody treatments as in Figure 4. Control group mice received PBS, NK cells alone, or rituximab (**A**; following antibody treatment in Figure 4A) or ibrutinib (**B**; 25 mg/kg i.p. every other day on days 3–17). **C,** Tumor-free and overall survival curves of the mice shown in **A** and **B**, respectively. Tumor-free rate and survival differences between experimental and all control groups were analyzed by log-rank test (**, $P < 0.001$). Data are representative of three independent experiments.

More recently, a BAFF-R antibody was developed and tested in Philadelphia-positive pre-B ALL models (30). This antibody demonstrated *in vitro* cytotoxicity against drug-sensitive tumor cells, but was not tested against drug-resistant ALL lines. Importantly, it yielded modest single-agent antitumor effects in comparison with nilotinib, and the combination of antibody with this tyrosine kinase inhibitor did not confer increased efficacy. Our BAFF-R mAbs, in contrast, elicited robust *in vivo* antitumor effects as a single agent against multiple B-cell tumor types, including NHL, CLL, and ALL. Furthermore, our antibodies eradicated established tumors, which led to long-term, tumor-free survival *in vivo*.

The distinctive features of our BAFF-R mAbs may be due to the approach we used to generate them. In contrast to prior antibodies that were generated using recombinant immunogen, our approach was to express human BAFFF-R as a native surface protein on mouse fibroblast cells for immunization, increasing the likelihood of presenting a natively folded, glycosylated immunogen. Therefore, it is very likely that our antibodies are binding an accessible human BAFF-R epitope distinct from the other antibodies described. Outside of the scope of the current study, X-ray crystallography studies are planned to identify the precise binding epitopes of our antibodies on BAFF-R.

Thus, we demonstrated a technical strategy for generating mAbs against a natively folded, eukaryotically glycosylated human BAFF-R that is able to specifically bind, lyse, and inhibit B-cell tumors *in vivo*. Our results suggest the main antitumor mechanism of our mAbs is ADCC, as NK cells were required in addition to mAbs for *in vitro* activity (Fig. 2); we observed no evidence of CDC. The antibodies were able to competitively inhibit BAFF ligand binding to BAFF-R (Fig. 2D). Although an earlier report showed blocking BAFF/BAFF-R survival signal is not sufficient to cause complete B-cell depletion (31), potential signal blockade by C55 and C90 merits further investigation.

The current portfolio of B-cell lymphoma treatments has benefitted from the availability of several FDA-approved agents, including rituximab, and the Bruton tyrosine kinase inhibitor, ibrutinib (32). Although these treatments are clinically effective, many patients will develop drug resistance, especially to monotherapies (33–35). Thus, there remains an urgent need for additional new therapeutic agents. One clinically relevant mechanism of resistance to rituximab is down-regulation of CD20 (10). We modeled this phenomenon of drug resistance with a CRISPR-edited MCL line, JeKo-1, which is deficient in CD20. The significant *in vivo* antitumor effects of C55 or C90, but not rituximab treatment, against this line and similarly against the naturally ibrutinib-resistant Z-138 MCL suggests efficacy against drug-resistant lymphomas (Fig. 5). Taken together with the *in vitro* cytotoxicity of these antibodies against primary tumors from lymphoma patients who were previously treated with, and progressed in response to rituximab, these data suggest C55 and C90 as a potential treatment strategy to overcome drug resistance (Fig. 3).

Finally, these results strongly support further development of these BAFF-R antibodies for clinical application against lymphomas, including humanization and IND-enabling studies. Future studies should also investigate the combination of our agents with other targeted therapies, such as rituximab and ibrutinib and efficacy against CD19 antigen-loss variants (ALL and lymphomas) arising in patients relapsing from CD19-directed chimeric antigen receptor T-cell and bispecific antibody therapies.

Disclosure of Potential Conflicts of Interest

H. Qin and L.W. Kwak hold ownership interest (including patents) in and are consultant/advisory board members for Innolifes Inc. and PeproMene Bio Inc. S.S. Neelapu is a consultant/advisory board member for Celgene, Kite Pharma, Merck, and Novartis. No potential conflicts of interest were disclosed by the other authors.

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