Introduction

Non-Hodgkin lymphoma (NHL) is a heterogeneous group of malignancies that represents approximately 4% of all cancers. More than 90% of NHLs have a B-cell phenotype, and almost all express cell surface CD20, a B cell-specific member of the MS4A gene family.1,2 Rituximab, the first monoclonal antibody (mAb) approved for cancer treatment, has revolutionized the management and treatment of B-cell malignancies, increasing the median overall survival of patients with many of these diseases.3,4 Despite widespread use of rituximab, the efficacy remains variable and often modest, and the pursuit of improved agents to replace rituximab is intense, with several candidates currently under clinical evaluation.5,6 Most have been selected and engineered to provide a range of potential advantages, including increased binding avidity, reduced immunogenicity, enhanced direct cell death as mediated by type II CD20 antibodies and improved antibody-dependent cell-mediated cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC).3,7-10 However, complex diseases are often multifactorial in nature, and involve redundant or synergistic action of disease mediators or upregulation of different receptors, including crosstalk between their signaling networks.11,12 Thus, simultaneous blockade of these effector molecules is likely to provide better clinical efficacy and/or reach a broader patient population than inhibition of a single target.13 Previous studies indicated that blockade of multiple targets, which can be achieved by the combination of several mAbs or bispecific antibodies generated through biochemical or genetic approaches, results in improved therapeutic efficacy.14,15 However, combination therapy of several mAbs increases healthcare costs and the financial burden to families and societies, and the option of using several approved mAbs for combination therapy is limited because of the small number of therapeutic mAbs currently on the market. These data show the urgent need to design bispecific antibody with potent anti-tumor activities against NHL.

Acquired therapy resistance is one of the prime obstacles for successful cancer treatment. Resistance is often acquired already during an early phase of tumor development when genetic changes cause defects in caspase-dependent apoptosis...
pathways and provide transformed cells with higher growth and survival potential. Additionally, cancers treated with chemotherapeutic drugs often acquire the ability to efflux drugs by increasing the expression of multidrug resistance (MDR) proteins, P-glycoproteins of the ATP-binding cassette transporter family. Thus, alternative cell death pathways capable of killing apoptosis- and therapy-resistant cancer cells have attracted vast interest among cancer researchers. Growing evidence indicated that lysosomes can be considered as an “Achilles heel” for selectively destroying cancer cells, which has been demonstrated as an effective way to kill apoptosis-resistant cancer cells and re-sensitize MDR cells to classical chemotherapy. Recently, Ivanov and colleagues have revealed that, although lysosome-mediated cell death can be elicited by both type II CD20 mAbs and HLA-DR antibody L243, HLA-DR antibody L243 could induce more potent lysosome-mediated cell death than type II CD20 mAbs, suggesting that HLA-DR can be used as an ideal target for induction of lysosome-mediated cell death against lymphoma. Previous studies demonstrated that, although HLA-DR is expressed at high levels on a range of hematologic malignancies, it is constitutively expressed on normal B cells, monocytes/macrophages and dendritic cells. Due to the fact that the antigen is expressed on normal as well as tumor cells, safety concerns have been raised regarding the clinical use of HLA-DR-directed antibodies. To reduce reliance on intact immunologic systems in the patient and effector mechanism-related toxicity, Goldenberg and his colleagues have replaced the Fc region of hL243γ1, the humanized IgG1 anti-HLA-DR mAb L243, with the IgG4 isotope (IMMU-114) to abrogate the effector cell functions of the antibody (ADCC and CDC). Their data indicated that anti-HLA-DR antibody hL243γ1 could not only induce potent lysosome-mediated cell death against lymphoma and leukemia cells, but also significantly increase anti-proliferative activity after combination with rituximab. While acknowledging that HLA-DR is not a tumor-specific antigen, HLA-DR remains an attractive molecule with potential as a target for a CD20-HLA-DR bispecific antibody.

To our knowledge, the classical IgG architecture, as it was selected during evolution, has many advantages for the therapeutic application of bispecific antibodies. The Fc part is identical to that of a conventional IgG antibody, resulting in IgG-like pharmacokinetic properties and retained effector functions such as the mediation of ADCC through FcγRIIIa binding. IgG-like size and molecular weight are expected to result in IgG-like diffusion, tumor penetration, and accumulation in comparison with bispecific tetravalent antibodies of higher molecular weight. Considering these benefits, we converted the CD20 antibody rituximab and HLA-DR antibody hL243γ1 into an IgG-like bispecific antibody (CD20–243 CrossMab) by using CrossMab technology. Our results indicated that CD20–243 CrossMab induces significantly high levels of CDC, ADCC and cell death in NHL, and has potent anti-tumor capacities against both B-lymphoma and rituximab-resistant (RR) B-lymphoma cells. More importantly, although HLA-DR is not specifically expressed on malignant cells, CD20–243 CrossMab exhibits specific anti-tumor activities against both CD20 and HLA-DR positive malignant cells.

**Results**

**Design and characterization of CD20–243 CrossMab**

Based on CrossMab technology recently reported, we designed an IgG-like bispecific CrossMab (CD20–243 CrossMab) that deviates only minimally from the naturally occurring CD20 antibody rituximab and HLA-DR antibody hL243γ1. As shown in the right of Figure 1A, the constant heavy 1 (CH1) of hL243γ1 was replaced with the antibody constant light (CL), generating a polypeptide chain made of hL243 HV-CL-Hinge-CH2 and CH3. To generate CD20–243 CrossMab, exchange of CH1 and CL domains of hL243γ1 is essential for correct association of the light chain and the cognate heavy chain of the half IgG of rituximab in CD20–243 CrossMab. Hetero-dimerization of the heavy chain of rituximab and hL243γ1 was achieved by using the “knobs into holes” (KiH) method. The resulting

**Figure 1.** Characterization of CD20–243 CrossMab. (A) Schematic diagram of the Fab domain exchange resulting in the generation of CD20–243 bispecific antibody when combined with the KiH technology. (B and C) Binding of 125I-labeled CD20–243 CrossMab, rituximab Fab, hL243γ1 Fab and IMMU-114 Fab to CHo-CD20 cells (B) or Nalmalwa (C). 125I-labeled CD20–243 CrossMab is approximately 10 μg/ml, which is comparable to rituximab. The cell-bound and free 125I-labeled CrossMab or mAbs were then separated by centrifugation through phthalate oils and the cell pellets together with bound antibody counted for radioactivity. Data from saturation binding experiments were analyzed by nonlinear least-squares regression for curve-fitting and KD estimation. Data are mean ± SD (n = 3). (D) Dissociation of 125I-labeled CD20–243 CrossMab, rituximab, hL243γ1 and IMMU-114 from Raji cells. Cells were incubated with 125I-labeled CD20–243 CrossMab, rituximab or hL243γ1 and IMMU-114 (10 μg/ml) at 37 °C for 1 h, washed twice, and resuspended. Samples of cells were taken at time 0, 1, 2 and 4 h and then washed and analyzed. Shown are means and SD of at least three experiments.
highly purified CD20–243 CrossMab was assessed on SDS/PAGE (Fig. S1A) and size-exclusion HPLC (Fig. S1B). Then, we used CHO-CD20 (high expression of CD20), Namalwa (high expression of HLA-DR) and Raji (high expression of both CD20 and HLA-DR) cells to determine the binding activity of CrossMab. The binding affinity for CD20 and HLA-DR of the CD20–243 CrossMab was determined by analyzing direct cell surface saturation binding to CHO-CD20 cells (Fig. 1B) and Namalwa cells (Fig. 1C), respectively. The dissociation constant of rituximab (4.83 ± 0.32 nM) is quantitatively consistent with a previous report by Reff et al.\textsuperscript{29} It can be seen in Figure 1B that the CD20–243 CrossMab binding affinity for CD20 (4.79 ± 0.28 nM) is similar to the affinity of rituximab. In addition, the binding affinity of hL243γ1 and CD20–243 CrossMab for HLA-DR is very similar (Fig. 1C). As shown in Figure S1C, CrossMab could bind to CD20 and HLA-DR positive Raji cell with similar high level as that of IMMU-114 and hL243γ1. Binding “off-rate” experiments using \textsuperscript{125}I-labeled IgG were performed to compare the dissociation of rituximab and CD20–243 CrossMab from Raji cells. As shown in Figure 1D, the data showed a slight difference in the off-rate between CD20–243 CrossMab and rituximab. Approximately 50% of the rituximab, and more than 40% of CD20–243 CrossMab, remained bound to the cells after 4 h. In addition, similar results were achieved with F(ab\textsuperscript{1})\textsubscript{2}, which excluded an interaction with FcγR on target cells influencing mAb dissociation (data not shown). These data indicated that CD20–243 CrossMab nearly kept the intact affinity bound to CD20 and HLA-DR, and exhibited similar binding avidity against B-cell lymphoma.

CD20–243 CrossMab with specific CDC and ADCC activities efficiently eradicate B-cell lymphoma comparable to rituximab

The cytotoxic activities of CD20–243 CrossMab were first assessed against Raji cells. In line with our expectations, CrossMab displayed approximately the same high level of CDC activity as rituximab (Fig. 2A). To further investigate the specific cytotoxic activities of CrossMab, CHO-CD20 cells or Namalwa cells that expressed a high level of CD20 or HLA-DR proteins, respectively, were used in our further studies. Compared with rituximab or hL243γ1, the cytotoxic activities of CD20–243 CrossMab on CHO-CD20 cells and Namalwa cells were significantly decreased, separately (Fig. 2B and C). These results suggested that CD20–243 CrossMab could trigger specific cytotoxic activities against B-cell lymphoma. Then, a standard LDH assay was performed to determine the CrossMab-mediated ADCC by human peripheral blood mononuclear cells (PBMCs). Purified human PBMCs from healthy donors were used as effector cells and Daudi cells were used as the target. Assays were conducted at effector:target (E:T) cell ratios of 25:1 using antibody concentrations ranging from 0.003 to 10 μg/ml (Fig. 2D–F). Our data showed that approximately 2 μg/ml of rituximab or CrossMab was required to achieve the 50% targeted lysis levels at E:T ratios of 25:1. Furthermore, ADCC activity of CD20–243 CrossMab on CHO-CD20 cells and Namalwa cells were analyzed (Fig. 2E and F). In line with our expectation, significant decreases of ADCC activity against CHO-CD20 cells and Namalwa cells were observed after treatment with CrossMab. These data indicated that CD20–243 CrossMab with specific anti-tumor activities can induce remarkable CDC and ADCC against both CD20 and HLA-DR positive lymphoma cells.

Unusually potent antiproliferative activity of CD20–243 CrossMab

In clinical studies, the facts that (1) responding patients generally displayed progressive tumor mass reduction, and (2) complete response is usually achieved several weeks after completion of therapy, suggest that, in vivo, cell growth inhibition could play an important role in cancer treatment.\textsuperscript{30} Thus, the effect of CD20–243 CrossMab on cellular proliferation was assessed using the \textsuperscript{3}H-thymidine uptake assay on Ramos, CHO-CD20, and Namalwa cells, separately. Unexpectedly, our data showed that, after treatment with CD20–243 CrossMab, the significant inhibition of proliferation was observed (Fig. 3A). Although hL243γ1 or the combination of rituximab and hL243γ1 exhibited potent anti-proliferative capacity after cross-linking with second antibody, CD20–243 CrossMab still displayed high level of anti-proliferative activity even without...
cross-linking (Fig. 3A). Then, the anti-proliferative activity against CHO-CD20 or Namalwa cells was explored. Our data clearly showed that, even with very low expression of CD20 on Namalwa cells, CD20–243 CrossMab showed potent anti-proliferative activity against malignancies (Fig. 3B and C).

Induction of Lysosome-mediated cell death by CD20–243 CrossMab

Growing evidence has revealed that lysosomes are excellent pharmacological targets for selectively destroying cancer cells, and this approach recently emerged as an effective way to kill apoptosis resistant cancer cells and re-sensitize MDR cells to classical chemotherapy. To investigate the cell death induced by CD20–243 CrossMab, pan-caspase inhibitor was first used in our experiments. As indicated in Figure 4A, although rituximab triggered a low level of cell death (<15%) in Raji cells at the concentration of 10 μg/mL, CD20–243 CrossMab induced a substantially high level of cell death, which is comparable with that evoked by IMMU-114 and hL243γ1. Then, the cell-permeable caspase inhibitor ZVAD-FMK was used, and our experimental results revealed that ZVAD-FMK over a range of concentrations from 0.5 to 50 μM was unable to prevent the CrossMab-induced cell death (Fig. 4A). Because ZVAD-FMK was a poor inhibitor of caspase-2, we also assessed the efficacy of a specific caspase-2 inhibitor (VDVAD) in Raji cells, and similar results were observed (Data not shown). The specific cell death triggered by CD20–243 CrossMab was also evaluated (Fig. S2). In agreement with the CDC and ADCC activity of CrossMab mentioned above, CD20–243 CrossMab could induce significant cell death against both CD20 and HLA-DR positive malignant cells.

We subsequently investigated the involvement of lysosomes in the cell death initiated by CD20–243 CrossMab. As shown in Figure 4B, CrossMab could substantially induce the enlargement of the lysosomal compartment and consequent LMP (Fig. 4B and C). To determine whether lysosomes were releasing their contents in the cell, we performed fluorescent immunohistochemical staining for cathepsin B, a classical lysosomal component. Our experiments revealed a substantial increase in cathepsin B (Fig. 4C) staining throughout the cytosol after CrossMab treatment for 16 h. The importance of cathepsins in the cell death process was subsequently confirmed by using a specific cathepsin inhibitor that virtually ablated CrossMab-induced cell death in a dose-dependent manner (Fig. 4D).

The CD20–243 CrossMab with potent anti-tumor activity against RR lymphoma cells

To investigate the anti-tumor capacity of CD20–243 CrossMab against RR lymphoma, two RR cell lines established in our previous studies were used. In agreement with our previous reports, the 2 RR cell lines failed to respond to rituximab-mediated CDC (Fig. 5A). Similar to rituximab, the CDC activity of the CrossMab was not detectable in the 2 RR cell lines (Fig. 5A). However, the CrossMab exhibited a similar high level of ADCC activity in the 2 RR cell lines in vitro as rituximab (Raji-R, 25.9% ± 2.79% vs 23.3% ± 4.54%; Daudi-R, 33% ± 2.78% vs 32.9% ± 6.04%; Fig. 5B). More importantly, the CrossMab could trigger a significant high level of cell death against Raji-R (49.7% ± 3.85%) and Daudi-R (52.9% ± 1.79%) cells, whereas rituximab was ineffective in inducing cell death (Fig. 5C). Our data showed that CD20–243 CrossMab could efficiently induce potent cell death and ADCC in RR lymphoma cells, suggesting that it might be a promising therapeutic agent for B-cell lymphomas.

Effects of CD20–243 CrossMab on PBCs from healthy blood donors

The toxicity of CD20–243 CrossMab on normal PBCs was examined using an ex vivo flow cytometry assay on whole blood from healthy volunteer donors. Data shown in Figure 6 are representative of those obtained from experiments with 6 healthy volunteer blood donors. Two-day incubation of heparinized whole blood with IMMU-114 or rituximab yielded significant decreases in B cells relative to control incubations in all cases (P < 0.05). More than 70% of B cells were depleted after treatment with CD20–243 CrossMab, which is comparable to the results with rituximab. None of the treatments decreased T cells significantly. Intriguingly, although hL243γ1 and IMMU-114, but not rituximab, significantly reduced the number of monocytes (40–50% reduction vs control mAb), CD20–243 CrossMab yielded a slightly decrease in monocytes (<20% reduction vs control mAb), exhibiting similar high level of specificity on B cells as rituximab.

Figure 3. Anti-proliferative effects of CD20–243 CrossMab. Effects of CrossMab on proliferation of Raji (A), CHO-CD20 (B) and Namalwa (C) cells were determined by 3H-thymidine uptake assays. Cells were cultured with CrossMab or other mAbs with or without a second antibody for cross-linking. Error bars represent SD of triplicates.
Therapeutic efficacy of CD20–243 CrossMab in vivo

The therapeutic efficacy of the CrossMab and rituximab was evaluated in both Daudi and Daudi-R lymphoma-bearing SCID mice (SCID/Daudi and SCID/Daudi-R). The survival curves were plotted according to the Kaplan-Meier method and compared using the log-rank test. Although both rituximab and the CD20–243 CrossMab, after administration to mice at a dose of 100 μg/mouse, were shown to significantly improve the survival of SCID mice bearing disseminated Daudi tumor cells (P < 0.001 for each compared with the PBS control), a significant difference in survival was observed between rituximab and the CrossMab treatment groups (P < 0.01), and the CrossMab exhibited better anti-tumor activities (Fig. 7A). To further evaluate the therapeutic efficacy of CD20–243 CrossMab, SCID mice bearing disseminated Daudi tumor cells were treated with antibodies at a dose of 30 μg/mouse. Remarkably, the CrossMab still exhibited in vivo therapeutic effects, which has significantly prolonged the survival of animals compared with animals receiving saline or rituximab (Fig. 7B).

We then evaluated the in vivo therapeutic effects of CrossMab against RR lymphoma. As shown in Figure 7C, no statistical difference in survival was observed between the PBS- and rituximab-treated SCID/Daudi-R mice. Although rituximab-treated SCID/Daudi-R mice had a median survival time of 30 d after tumor inoculation, the median survival in the CrossMab treatment group was extended to 82 d, with statistically significant survival extension by log-rank analysis (P < 0.005 compared with the rituximab treatment group).

Discussion

Although the use of mAbs for cancer therapy has recently achieved remarkable clinical success, patient tumor-response data show the urgent need to enhance the efficacy of the current...
generation of anticancer antibodies. As we now know, cancer is usually multifactorial in nature, involving a redundancy of disease-mediating ligands and receptors, as well as crosstalk between signal cascades. A targeted therapeutic agent inhibiting one crucial pathway in a tumor may not completely shut off a hallmark capability, allowing some cancer cells to survive with residual function until they or their progeny eventually adapt to the selective pressure imposed by the therapy being applied. Therefore, blockade of multiple, different pathological factors and pathways may result in improved therapeutic efficacy, which can be achieved by use of the dual targeting strategies applying bispecific antibodies that have emerged as an alternative to combination therapy. In the present study, we successfully converted the CD20–targeting antibody rituximab and HLA-DR antibody hL243γ1 into an IgG-like bispecific antibody (CD20–243 CrossMab) using CrossMab technology as previously described. The IgG-like bispecific CrossMab with the classical IgG architecture has many advantages. For example, it can be efficiently produced by conventional mammalian expression systems as a single species for easy manufacturing and purification, while maintaining the affinities and potencies of the two parental mAbs; and the complete human Fc region of CrossMab retains potentially desired effector functions (CDC and ADCC). As expected, the bispecific CD20–243 CrossMab could bind CD20 and HLA-DR with affinity similar to that of rituximab and hL243γ1. Further study revealed that CD20–243 CrossMab could not only trigger similar high levels of CDC and ADCC as rituximab against lymphoma, but also induce significant anti-proliferation and lysosome-mediated cell death. More importantly, although HLA-DR is expressed on normal and tumor cells, CD20–243 CrossMab could specifically eradicate CD20 and HLA-DR positive lymphoma through marked CDC, ADCC and cell death, exhibiting remarkably specific anti-tumor activities. These findings have been further validated by evaluation of the effects of CD20–243 CrossMab on PBCs from healthy blood donors. We found that the CrossMab with highly tumor specificity yielded a slightly decrease in monocytes, although it could significantly decrease B cells with results comparable to rituximab.

Previous study has revealed that IMMU-114, a humanized IgG4 anti-HLA-DR antibody with direct anti-lymphoma activity could significantly increase the potency of rituximab. Targeting both CD20 and HLA-DR has been demonstrated to be highly toxic to human lymphoma and multiple myeloma cells. However, although anti-HLA-DR antibodies have been demonstrated to have the ability to efficiently eradicate lymphoma cells, HLA-DR is not a tumor-specific antigen, which may limit its clinical applications. Based on the advantages of CrossMab technology, we designed CD20–243 CrossMab with the benefits of classical IgG architecture, which exhibited high specificity against both CD20 and HLA-DR positive malignant cells. These data may suggested that CD20–243 CrossMab could be considered as a potential reagent for clinical use.

Lysosome, a separate compartment that confines highly destructive hydrolyses for the demolition and reutilization of cellular substituents has been regarded as an “Achilles heel” for cancer treatment. Numerous studies showed that it is an effective way to kill apoptosis-resistant cancer cells and also re-sensitize multidrug-resistant cells to classical chemotherapy. Recently, Ivanov and colleagues have revealed that type II CD20 mAbs and HLA-DR antibody L243 could induce lysosome-mediated cell death against human lymphoma and leukemia cells. Further studies showed that anti-HLA-DR antibody L243 could elicit more potent lysosome-mediated cell death than type II CD20 mAbs, exhibiting the potential therapeutic benefits to combat human lymphoma and leukemia cells. Based on the CrossMab technology, we constructed a IgG-like bispecific antibody targeting both CD20 and HLA-DR. Our data showed that CD20–243 CrossMab with specific anti-tumor activities...
could induce potent CDC, ADCC and lysosome-mediated cell death against both B-lymphoma cells and RR B-lymphoma cells, which subsequently prolonged the survival of SCID mice bearing lymphoma cells or rituximab-resistant lymphoma cells. Our data suggest that induction of lysosome-mediated cell death could be effective in treating hematological malignancies.

In conclusion, the bispecific IgG-like CD20–243 CrossMab with specific anti-tumor activity could efficiently eliminate both rituximab-sensitive and -resistant lymphoma cells in vitro and in vivo, suggesting that it may serve as a promising therapeutic agent for the treatment of human B-cell lymphoproliferative disorders.

Materials and Methods

Cell lines, antibodies, and animals

Three human Burkitt lymphoma cell lines, Raji, Daudi, Ramos and Namalwa were obtained from the American Type Culture Collection (ATCC). Rituximab-resistant Raji (Raji-R) cells and rituximab-resistant Daudi (Daudi-R) cells were generated as previously described and maintained in our laboratory. CHO-CD20 cells were produced by transfecting CHO cells with vector expressing human CD20 proteins. Human CD20+ cells were purified through sorting on a FACSorter (BD Biosciences). CD20 expression levels were measured using FITC-labeled rituximab. Rituximab, Flex-Ig, and CD20-Flex BiFP were labeled with FITC to produce FITC-conjugated proteins, respectively. Eight-week-old female SCID mice were housed in specific pathogen-free conditions and were treated in accordance with the guidelines of the Committee on Animals of the Second Military Medical University. The study using PBMCs and Peripheral blood cells (HPCs) from the donors was approved by the Institutional Review Board of the Second Military Medical University.

Construction, expression, and purification of CD20–243 CrossMab

The heavy and light variable region genes of rituximab were fused in frame to the 5′ terminus of the human IgG1 constant region gene (CH1, hinge, CH2, and CH3 domains) and 5′ end of the human kappa chain constant region gene, respectively. The rituximab heavy and light genes were respectively inserted into the pcDNA3.1(+) vector, generating the expression vectors. The rituximab heavy and light genes with SYTOX® Red (Life technologies) for 15 min and analyzed by flow cytometry (FCM). The dissociation constants (Kd) were determined by nonlinear least-squares regression analyses.

Off-rate Measurements

To determine the off-rate of CD20–243 CrossMab from Raji cells, cells were pelleted and resuspended in medium containing 10 μg/mL 125I-labeled CrossMab. Cells were incubated for 1 h at room temperature, pelleted, and resuspended in medium containing 1 mg/mL of the unlabeled IgG. After different time intervals, the samples were taken, washed, and analyzed to determine the percentage of the remaining cells that were still stained.

Cytotoxicity assays

CDC and ADCC assays were performed as described previously. Briefly, the cells were incubated with antibodies for 1 h in phenol red-free DMEM culture medium in a 5% CO2 incubator at 37 °C, followed by the addition of either normal human serum (NHS, 10% vol/vol) as a source of complement (for CDC assay) or human PBMCs as effector cells (for ADCC assay). After an additional incubation for 4 h at 37 °C, the cell lysis was determined by measuring the amount of LDH released into the culture supernatant. Maximum LDH release was determined by lysis in 0.2% Triton X-100.

Cell death assay

The cells were incubated with different concentrations of CrossMab at 37 °C for 48 h. After washing, cells were treated with SYTOX® Red (Life technologies) for 15 min and analyzed by flow cytometry (FCM), F(ab′)2 fragment of goat anti-human IgM (anti-IgM; Jackson Immuno Research Laboratories) was used as a positive control for the induction of cell death.

In vitro cell proliferation assay

CrossMab effects on proliferation were determined by measuring 3H-thymidine incorporation in the NHL cell lines with and without the presence of a cross-linking second antibody, essentially as described by Shan et al. All tests were performed in triplicate.

Lysosomal permeability assessment

To assess lysosomal permeability, cells were labeled with 200 nM Lyso-Tracker probe (Invitrogen) at 37 °C after treatment with CD20–243 CrossMab for 16 h. Fluorescence of Lyso-Tracker labeled cells was assessed by confocal microscopy. Unlabeled cells were used as a background control.

Ex vivo assessment of CrossMab effects on PBCs

The effects of mAbs on PBCs from healthy volunteers were evaluated ex vivo using flow cytometry. Blood specimens were
collected from healthy volunteers under a protocol approved by the Second Military Medical University. Heparinized whole blood (150 μL) was incubated with CrossMab for 2 d at 37 °C and 5% CO₂. Then, subsequently stained with FITC anti-CD19, FITC anti-CD14, APC anti-CD3, or APC mouse IgG1 isotype control mAb (BD Biosciences) and analyzed by flow cytometry using FCM. Normal B and T cells are CD19+ and CD3+ cells, respectively, in the lymphocyte gate. Monocytes are CD14+ cells in the monocyte gate.

Immunotherapy

Groups of 10 8-wk-old female SCID mice were injected via the tail vein with 3.5 × 10⁶ Daudi or Daudi-R cells on day 0, followed 7 d later by the intravenous injection of CD20–243 CrossMab (100 μg/mouse or 30 μg/mouse). The mice were observed daily and killed at the onset of hind-leg paralysis.

Statistical analysis

Statistical analysis was performed by Student unpaired t test to identify significant differences unless otherwise indicated. Differences were considered significant at a P value of less than 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Author contributions

YG, LZ designed research, analyzed data and wrote the manuscript; and FX, HL, YC, WQ, SD, JZ, ZZ, BL, DZ, JZ, JD, HW, SH performed experiments and analyzed data.

Supplemental Materials

Supplemental materials may be found here: www.landesbioscience.com/journals/mabs/article/28613/1

References

1. Shankland KR, Armitage JO, Hancock BW. Non-Hodgkin lymphoma. Lancet 2012; 380:848-57; PMID:22835603; http://dx.doi.org/10.1016/S0140-6736(12)60605-9

2. Hennessy BT, Hanrahan EO, Daly PA. Non-Hodgkin lymphoma: an update. Lancet Oncol 2004; 5:341-53; PMID:15172354; http://dx.doi.org/10.1016/S1470-2045(04)01490-1

3. Glennie MJ, French RR, Cragg MS, Taylor RP. Mechanisms of killing by anti-CD20 monoclonal antibodies. Mol Immunol 2007; 44:3823-37; PMID:17768108; http://dx.doi.org/10.1016/j.molimm.2007.06.151

4. Calcagno A, Rostagno R, Di Perri G. Anti-CD20 antibody therapy for B-cell lymphomas. N Engl J Med 2012; 367:877-8; PMID:22931340

5. Ollaguro E, Audouy IP. Evolution of anti-CD20 monoclonal antibodies in oncology. Mabs 2010; 2:14-9; PMID:20081379; http://dx.doi.org/10.4161/mabs.2.1.10789

6. Lim SH, Beers SA, French RR, Johnson PWM, Glennie MJ, Cragg MS. Anti-CD20 monoclonal antibodies: historical and future perspectives. Haematologica 2010; 95:135-43; PMID:19773256; http://dx.doi.org/10.3324/haematol.2008.001628

7. Carron G, Winter H, Golay J, Solal-Celigny P. From the bench to the bedside: ways to improve rituximab efficacy. Blood 2004; 104:2635-42; PMID:15833821

8. Fehrenbacher LD, Jäättelä M. Lysosomes as targets of therapeutic interest. Blood 2012; 119:3767-78; PMID:22271448; http://dx.doi.org/10.1182/blood-2011-09-381988

9. Niederfellner G, Lammens A, Mundigl O, Puissant A, Dufies M, Raynaud S, Cassuto J-P, Georges GJ, Schaefer W, Schwaiger M, Franke A, Puissant A, Dufies M, Raynaud S, Cassuto J-P, Georges GJ, Schaefer W, Schwaiger M, Franke A, Auberger P. Targeting lysosomes to eradicate imatinib-resistant chronic myelogenous leukemia cells. Leukemia 2010; 24:1099-101; PMID:20376083; http://dx.doi.org/10.1038/leu.2009.241

10. Missero E, Brunker P, Mose S, Puntenner U, Schmidt C, Herrera R, Grau R, Gardes C, Noppa A, van Puijenboek E, et al. Increasing the efficacy of CD20 antibody therapy through the engineering of a new type II anti-CD20 antibody with enhanced direct and immune effector cell-mediated B-cell cytotoxicity. Blood 2010; 115:4393-402; PMID:20194898; http://dx.doi.org/10.1182/blood-2009-06-225979

11. Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. Cell 2011; 144:646-74; PMID:21376230; http://dx.doi.org/10.1016/j.cell.2011.02.013

12. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000; 100:57-70; PMID:10647931; http://dx.doi.org/10.1016/S1092-8674(00)81683-9

13. Kontermann R. Dual targeting strategies with bispecific antibodies. MAbs 2012; 4; (Forthcoming); http://dx.doi.org/10.4161/mabs.4.2.19000

14. Wu C, Ying H, Grinnell C, Bryant S, Miller R, Cartron G, Watier H, Golay J, Solal-Celigny P. Dual-targeting immunotherapy of lymphoma: potent cytotoxicity of anti-CD20/CD74 bispecific antibodies in mantle cell and other lymphomas. Blood 2011; 117:3767-78; PMID:22271448; http://dx.doi.org/10.1182/blood-2011-09-381988

15. Gupta P, Goldenberg DM, Rossi EA, Cardillo TM, Byrd JC, Furman RR, Chang C-H. Directed to CD20 and HLA-DR can elicit homotypic adhesion followed by lysosome-mediated cell death in human lymphoma and leukemia cells. J Clin Invest 2009; 119:2143-59; PMID:19620786

16. Dechant M, Buerke J, Valerius T. HLA class II antibodies in the treatment of hematologic malignancies. Semin Oncol 2003; 30:465-75; PMID:12939715; http://dx.doi.org/10.1053/j.semonc.2003.04.011

17. Stein R, Qu Z, Chen S, Solis D, Hansen HJ, Goldenberg DM. Characterization of a humanized IgG4 anti-HLA-DR monoclonal antibody that lacks effector cell functions but retains direct antilymphoma activity and increases the potency of rituximab. Blood 2006; 108:2736-44; PMID:16778139; http://dx.doi.org/10.1182/blood-2006-04-017921

18. Siwadowski MX, Millman I. Antibody therapeutics in cancer. Science 2015; 341:1192-8; PMID:24031011; http://dx.doi.org/10.1126/science.1241145

19. Carter PJ. Potent antibody therapeutics by design. Nat Rev Immunol 2006; 6:343-57; PMID:16622479; http://dx.doi.org/10.1038/nri1837

20. Schaefer W, Regelia JTT, Bähner M, Schanzer J, Croasdale R, Durr H, Gassner C, Georges G, Kertenberger H, Ihimof-Jung S, et al. Immunoglobulin domain crossover as a generic approach for the production of bispecific IgG antibodies. Proc Natl Acad Sci U S A 2011; 108:11187-92; PMID:21690412; http://dx.doi.org/10.1073/pnas.1010987109

21. Arwell S, Ridgway JB, Wells JAJ, Carter P. Stable heterodimers from remodeling the domain interface of a homodimer using a phage display library. J Mol Biol 1997; 270:26-35; PMID:9231898; http://dx.doi.org/10.1006/jmbi.1997.1116

22. Ridgway JB, Presta LG, Carter P. ‘Knobs-into-holes’ engineering of antibody CH3 domains for heavy chain heterodimerization. Protein Eng 1996; 9:617-21; PMID:8844834; http://dx.doi.org/10.1038/protein/9.7.617

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29. Reff ME, Carner K, Chambers KS, Chinn PC, Leonard JE, Raab R, Newman RA, Hanna N, Anderson DR. Depletion of B cells in vivo by a chimeric mouse human monoclonal antibody to CD20. Blood 1994; 83:435-45; PMID:7506951
30. Bezombes C, Graziade S, Carrer C, Fabre C, Quillet-Mary A, Muller S, Jaffrezou JP, Laurent G. Rituximab antiproliferative effect in B-lymphoma cells is associated with acid-sphingomyelinase activation in raft microdomains. Blood 2004; 104:1166-73; PMID:15126316; http://dx.doi.org/10.1182/blood-2004-01-0277
31. Li B, Zhao L, Guo H, Wang C, Zhang X, Wu L, Chen L, Tong Q, Qian W, Wang H, et al. Characterization of a rituximab variant with potent antitumor activity against rituximab-resistant B-cell lymphoma. Blood 2009; 114:5007-35; PMID:19828699; http://dx.doi.org/10.1182/blood-2009-06-225474
32. Bland JM, Altman DG. Survival probabilities (the Kaplan-Meier method). BMJ 1998; 317:1572; PMID:9836663; http://dx.doi.org/10.1136/bmj.317.7172.1572
33. Scott AM, Wolchok JD, Old LJ. Antibody therapy of cancer. Nat Rev Cancer 2012; 12:278-87; PMID:22437872; http://dx.doi.org/10.1038/nrc3236
34. Carter P. Improving the efficacy of antibody-based cancer therapies. Nat Rev Cancer 2001; 1:118-29; PMID:11905803; http://dx.doi.org/10.1038/35101072
35. Alinari L, Yu B, Christian BA, Yan F, Shin J, Lapalombella R, Herrlein E, Lustberg ME, Quinioin C, Zhang X, et al. Combination anti-CD74 (mirtatumab) and anti-CD20 (rituximab) monoclonal antibody therapy has in vitro and in vivo activity in mantle cell lymphoma. Blood 2011; 117:4350-41; PMID:21228331; http://dx.doi.org/10.1182/blood-2010-08-303594
36. Rossi EA, Rossi DL, Stein R, Goldenberg DM, Chang C-H. A bispecific antibody-IFNα2b immunocytokine targeting CD20 and HLA-DR is highly toxic to human lymphoma and multiple myeloma cells. Cancer Res 2010; 70:7608-9; PMID:20876805; http://dx.doi.org/10.1158/0008-5472.CAN-10-2126
37. Petersen NHT, Olsen OD, Groth-Pedersen L, Ellegaard A-M, Bilgin M, Redmer S, Ostenfeld MS, Ulaner D, Dovmark TH, Lanzbarg A, et al. Transformation-associated changes in sphingolipid metabolism sensitize cells to lysosomal cell death induced by inhibitors of acid sphingomyelinase. Cancer Cell 2013; 24:379-93; PMID:24029234; http://dx.doi.org/10.1016/j.ccr.2013.08.003
38. Groth-Pedersen L, Jaärrela M. Combating apoptosis and multidrug resistant cancers by targeting lysosomes. Cancer Lett 2013; 352:265-74; PMID:20598437; http://dx.doi.org/10.1016/j.canlet.2010.05.021
39. Stein R, Gupta P, Chen X, Cardillo TM, Furman RR, Chen S, Chang C-H, Goldenberg DM. Therapy of B-cell malignancies by anti-HLA-DR humanized monoclonal antibody, IMMU-114, is mediated through hyperactivation of ERK and JNK MAP kinase signaling pathways. Blood 2010; 115:5180-90; PMID:20101022; http://dx.doi.org/10.1182/blood-2009-06-228288
40. Gingrich RD, Dahle CE, HoskinsKF, Sennet MJ. Identification and characterization of a new surface membrane antigen found predominantly on malignant B lymphocytes. Blood 1990; 75:2375-87; PMID:1693529
41. Czuczman MS, Olejniczak S, Gowda A, Kotowski A, Binder A, Kaur H, Knight J, Starostik P, Deans J, Hernandez-Izaliturri FJ. Acquisition of rituximab resistance in lymphoma cell lines is associated with both global CD20 gene and protein down-regulation regulated at the prerranscriptional and posttranscriptional levels. Clin Cancer Res 2008; 14:1561-70; PMID:18316581; http://dx.doi.org/10.1158/1078-0432.CCR-07-1254
42. Jazirehi AR, Vega MJ, Bonavida B. Development of rituximab-resistant lymphoma clones with altered cell signaling and cross-resistance to chemotherapy. Cancer Res 2007; 67:1270-81; PMID:17283164; http://dx.doi.org/10.1158/0008-5472.CAN-06-2184
43. Carter P. Bispecific human IgG by design. J Immunol Methods 2001; 248:7-15; PMID:11223065; http://dx.doi.org/10.1016/S0022-1759(00)00339-2
44. Cragg MS, Morgan SM, Chan HT, Morgan BP, Filatov AV, Johnson PWM, French RR, Glennie MJ. Complement-mediated lysis by anti-CD20 mAb correlates with segregation into lipid rafts. Blood 2003; 101:1045-52; PMID:12393541; http://dx.doi.org/10.1182/blood-2002-06-1761
45. Shan D, Ledbetter JA, Press OW. Apoptosis of malignant human B cells by ligation of CD20 with monoclonal antibodies. Blood 1998; 91:1644-52; PMID:9473230