Effect of a CrossMab cotargeting CD20 and HLA-DR in non-Hodgkin lymphoma

Abstract

**Objectives:** To evaluate the anti-tumor activities of CD20/HLA-DR CrossMab through cell and animal models.

**Methods:** Based on “knobs-into-holes” and “crossover” technology, CrossMab, targeting CD20 and HLA-DR, was constructed. A binding assay and a competitive inhibition assay were performed to confirm its specificity. The effects of CrossMab on antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity were measured. Cell apoptosis, lysosome-mediated cell death, and lysosomal permeability were quantified. In addition, the effects of CrossMab on peripheral blood leukocytes were tested. The pharmacokinetics were determined with a non-compartmental analysis model. Human malignant lymphoma xenograft models in CB17-SCID mice were established for an in-vivo efficacy study.

**Results:** The antitumor activities of CrossMab were shown both in vitro and in vivo. CrossMab exhibited strong binding to CD20 and HLA-DR at the same time in Raji cells. CrossMab also demonstrated antilymphoma effects by inducing antibody-dependent cellular cytotoxicity and complement-dependent cytotoxicity. Furthermore, CrossMab altered the lysosomal membrane permeability. The toxicity of CrossMab on normal peripheral blood lymphocytes (PBLs) was specific to B cells. A mouse xenograft model demonstrated the antitumor activities of CrossMab in vivo.

**Conclusions:** CrossMab exhibited an enhanced antigen recognition ability and antitumor activities in lymphoma without evident toxicity. CrossMab could be an effective immunotherapeutic strategy for non-Hodgkin lymphoma.

**Keywords:** cathepsin B; CD20; CrossMab; HLA-DR; lymphoma; lysosome-mediated apoptosis

Introduction

B-cell non-Hodgkin lymphoma (NHL), a type of heterogenous tumor derived from the lymphatic system, is prevalent among children, adolescents, and young adults [1, 2]. The progression and clinical outcomes of NHL patients largely depend on the subtype (indolent or aggressive) [3]. The patients diagnosed with aggressive NHL usually have a dismal prognosis, with an estimated survival duration of less than 6 months without treatment [4, 5]. Due to the crucial roles of lymphocytes in the etiology of NHL, immunotherapy has been employed as the first-line treatment for patients with NHL. To date, several immunoantibody drugs, including rituximab, brentuximab vedotin, and ibritumomab tiuxetan, have been approved for clinical use [6–8].

Rituximab, an anti-CD20 antibody, was the first monoclonal antibody (mAb) approved for lymphoma by the US Food and Drug Administration [9]. Its use has significantly improved the outcomes of lymphoma patients at either high or low risk, with acceptable side effects [10]. However, resistance against rituximab was observed among a subgroup of patients who only received limited clinical benefits from the treatment [11]. Therefore, it is necessary to develop novel therapeutic strategies to reduce drug resistance and to improve treatment outcomes.

An emerging candidate for antibody-based lymphoma treatment, HLA-DR, has been demonstrated to be highly expressed in hematological malignancies [12]. In addition, HL243y1, a humanized IgG1 anti-HLA-DR mAb, has been reported to induce cell death in Daudi, Raji, and Ramos cells [13]. Moreover, the excessive expression of CD20 and HLA-DR antigens, with physical and functional interactions, has been observed in a variety of B-cell lymphomas [14, 15]. Importantly, the combination of anti-HLA-DR mAb and rituximab displayed stronger efficacy than rituximab alone in Raji and SU-DHL4 cells. This finding suggests the promising future of combination therapy in patients with NHL, especially for those with resistance to rituximab [16, 17].

Bispecific antibody (BsAb) refers to an antibody that can bind to two different antigens at the same time. In 2008,
blinatumomab, the first BsAb for lymphoma treatment, was reported by Bargou et al. [18, 19]. A plethora of BsAbs have been constructed with genetic engineering techniques [20]. CrossMab technology can be combined with approaches enabling correct heavy chain association such as “knobs-into-holes” and “crossover” technology. This technology has been proven to be very versatile, allowing the generation of various bispecific antibody formats. Several academic investigators have used the CrossMab technology to successfully generate BsAbs [21]. Crosssmab has been widely developed, tested, and evaluated CrossMab to improve the clinical outcomes of NHL patients. [26]. Therefore, it is of great importance to develop, test, and evaluate CrossMab to improve the clinical outcomes of NHL patients.

The present study aimed to construct CD20/HLA-DR CrossMab\textsuperscript{CH1-CL} (abbreviated as CrossMab), a BsAb targeting CD20 and HLA-DR in lymphoma, and to test its efficacy in vitro and in vivo. Furthermore, the cytotoxicity of CrossMab against certain cell types related to B-cell lymphoma was evaluated in order to determine whether CrossMab can be used to treat NHL patients in the future.

Materials and methods

Cell lines and culture conditions

Burkitt human lymphoma lines, Raji (Cat. CCL-86) and Daudi (Cat. CCL-213) were purchased from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in RPMI 1640 medium (Gibco, Cat. 11875093) with 10% heat-inactivated fetal bovine serum (Gibco, Cat. 10100147), 2 mM L-glutamine (Gibco, Cat. A2916801, Grand Island, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Cat. 15070063, Grand Island, NY, USA). The cells were incubated at 37°C, 5% CO\textsubscript{2}. FreeStyle\textsuperscript{TM} 293-F cells (Gibco, Cat. R79007, Carlsbad, CA, USA) were cultured in FreeStyle 293 Expression Medium (Gibco, Cat. 12338018, Paisley, PA4 9RF, UK).

BsAb construction

Based on the “knobs-into-holes” and “crossover” technology [27], we designed the structure of a CrossMab against CD20 and HLA-DR, and constructed or modified four chains of the BsAb [28]. The monoclonal antibody rituximab against CD20 and the monoclonal antibody hL243y1 against HLA-DR were employed as the parent antibodies, then four chains were designed. On one side, an original heavy chain of rituximab was designed as a knob structure, and the T389W mutation was introduced to Fc; the original light chain of rituximab remained unchanged. On the other side, we took hL243y1 as the parent antibody, and a new modified heavy chain and a new modified light chain were designed. The heavy chain was composed of the C-end to the N-end of the hL243y1 original heavy chain Fc, the hL243y1 original light chain CL, and the hL243y1 original heavy chain VH. The new light chain was composed from the C-end to the N-end of the hL243y1 original heavy chain CH1 and the hL243y1 original light chain VL. In addition, two cysteine residues were introduced: the S377C mutation was introduced into the heavy chain of rituximab with a knob mutation, and the Y376C mutation was introduced into the heavy chain of hL243y1 with a hole mutation. These two cysteine residues formed two stable disulfide bonds. The BsAb was produced by transient expression of the heavy and light chain-expressing vectors in FreeStyle\textsuperscript{TM} 293-F cells with a HiSpeed Plasmid Maxi Kit (Qiagen, Cat. 12663, Germantown, MD, USA). CrossMab was purified by affinity chromatography on protein A-Sepharose (Amersham Biosciences, Cat. 17-5280-01, Slough, Buckinghamshire, UK) from the serum-free culture supernatants. The purified antibody was separated by sodium dodecyl sulfate–polyacrylamide gel (SDS-PAGE) electrophoresis on 13% gels under reducing conditions and on 7.5% SDS-PAGE under nonreducing conditions. The gels were visualized after staining with Coomassie Brilliant Blue (Sigma, Cat. B0770, Cleveland, Oh, USA).

Binding assay

The binding assay method used in this study was the same as that reported in our previous study [15]. The parental antibodies rituximab and hL243y1 were adopted as positive controls (produced by our lab), while anti-HER2 mAb (produced by our lab) was used as a negative control. Briefly, Raji cells (1 × 10\textsuperscript{6}/well) were incubated in a 96-well plate at 4°C for 45 min. The cells were collected through centrifugation and stained with Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1:1000, Invitrogen, Cat. A-11013, Austin, TX, USA) at 4°C for 30 min in the dark. The staining density was tested by flow cytometry (Beckman Coulter, FC500, Indianapolis, IN, USA). In order to evaluate the binding affinity of the antibodies, serial dilution was carried out to reconstitute final concentrations of 10 μg/mL to 0.0625 μg/mL. Every test was performed in triplicate.

Competitive inhibition assay

Raji cells (1 × 10\textsuperscript{6}/well) were incubated in a 12-well plate with 1 mL of PBS (Gibco, Cat. 2001027, Paisley PA4 9RF, UK) or a series of diluted purified (anti-HER2 mAb, rituximab, hL243y1, rituximab+hL243y1, and Cross-Mab) antibodies (2 μg/mL, 0.5 μg/mL, 0.1 μg/mL, and 0.02 μg/mL) at 4°C for 10 min in the dark. Then 1 μg of fluorescently labeled antibody (FITC-rituximab or PE-hL243y1, synthesized by Bioss, Beijing, China) was added. After another 30 min incubation in the dark, the cells were resuspended in 500 μL of PBS and analyzed by flow cytometry. The mean fluorescence intensity was obtained. The competitive inhibition rate was calculated based on the following formula:

\[
\text{%CIR} = \left( 1 - \frac{\text{MFIA} - \text{MFIB}}{\text{MFIP} - \text{MFIB}} \right) \times 100
\]

Where, %CIR is the percentage of the competitive inhibition rate; MFIA is the mean fluorescence intensity of the antibody-treated group; MFIB is the mean fluorescence intensity of the blank control group; and MFIP is the mean fluorescence intensity of the PBS-treated control group.
Cytotoxicity assay

For the antibody-dependent cellular cytotoxicity (ADCC) assays, 1 × 10^6 Raji cells were incubated with antibodies (diluted from 0.00002 to 2 μg/mL in a 10-fold gradient) for 30 min and then resuspended in PBS (final density of 1 × 10^6 cells/mL). Calcein-AM (Invitrogen, Cat. C3099, Austin, TX, USA) was added to a final concentration of 1 μM, and the cells were incubated for 30 min. Then, peripheral blood mononuclear cells were added based on an effector:target ratio of 15:1. After incubation at 37°C for 4 h, 100 μL of TritonX-100 (SGSB-BIO, Cat. ZLI-9308, Beijing, China) was added to the complete cell group. The mixture was incubated at 37°C for 1 h and then centrifuged at 2,000 rpm for 5 min. A 100 μL aliquot of supernatant was collected. The fluorescence value was detected with a multi-mode microplate reader (SpectraMax Paradigm, Thermo Fisher Scientific, Cat. 1410101, Waltham, MA, USA), with excitation at 490 nm and emission at 515 nm.

Complement-dependent cell cytotoxicity (CDC) was measured by a lactate dehydrogenase (LDH) release assay using a CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega, Cat. G1780, Madison, WI, USA). Raji cells were incubated with various antibodies (diluted from 2 to 0.00064 μg/mL in a 5-fold gradient) in a 96-well plate at 37°C and 5% CO₂ for 30 min. Next, 100 μL of 10% normal human serum complement was added per well. The plate was incubated at 37°C for 4 h. Then 40 μL of LDH release reagent was added, and the plate was incubated for 1 h. After centrifugation at 2,000 rpm for 5 min, 80 μL of supernatant was collected and incubated with 40 μL of LDH in the dark at room temperature for 30 min. The absorbance at 492 and 630 nm was measured at a microplate reader (Molecular Devices, SpectraMax Paradigm, Thermo Fisher Scientific, Cat. 1410101, Waltham, MA, USA). The percentage of specific lysis was calculated according to the following formula:

\[
\%\text{ lysis} = \left( \frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \right) \times 100
\]

Apoptosis assay

Raji lymphoma cells at a density of 2.5–3 × 10^5/mL were incubated in a 48-well plate with 10 μg/mL antibody (300 μL of medium/well) at 37°C and 5% CO₂ for 24 h. At the end of the incubation, apoptosis was evaluated with Annexin V-FITC/PI staining (US Everbright, Cat. F6012, San Jose, CA, USA) and detected by flow cytometry (BD FACSCalibur, San Jose, CA, USA).

Detection of lysosome-mediated cell death, lysosomal permeability, and cathepsin B release

To assess the total lysosomal volume, cells were first labeled with LysoRed (KeyGen Biotech, Cat. KGMP006, Nanjing, China) for 1 h. For double LysoRed/annexin V (US Everbright, Cat. F6012, SAN RAMON, CA, USA) staining, Raji cells were incubated with LysoRed, then resuspended in binding buffer containing FITC-Annexin V, and analyzed by flow cytometry (BD FACSCalibur, San Jose, CA, USA).

The lysosomal membrane permeabilization was estimated with acridine orange (AO) (Sigma, Cat. A6014, Paisley P44 9RF, UK) staining, as reported previously [29, 30]. AO reagent is a fluorescent dye with lysosomal heterochromacity. In an intact lysosome, it is in the form of a protonated oligomer and shows red fluorescence. While in the cytoplasm, it is in the form of a deprotonated monomer and shows green fluorescence. In brief, Raji cells were labeled with 5 μM AO for 15 min at 37°C, washed, and treated with antibodies. Then, Raji cells were imaged using a Nikon Ds-Ri1-U3 microscope equipped with Nikon NIS-Elements AR 4.3 (Tokyo, Japan).

For cathepsin B release analysis, Raji cells were plated in 24-well plates (1 × 10^5 cells/well) and incubated with antibodies for 48 h. Then, double staining was carried out with MAGiC RED (Magic Red Cathepsin B Assay Kit, Immunochrome Technologies, Cat. 957) and Hoechst 33,342 (Thermo Scientific, Cat. 62249, Waltham, MA, USA). The results were observed and photographed with a Nikon Ds-Ri1-U3 microscope equipped with Nikon NIS-Elements AR 4.3.

**Effects of CrossMab on peripheral blood lymphocytes (PBLs) from healthy blood donors**

The effects of mAbs on PBLs were evaluated ex vivo with flow cytometry. Blood specimens were collected from healthy volunteers under a protocol approved by the Affiliated Hospital Institutional Ethics Committee of the Military Academy of Medical Sciences (The former name of the Military Academy of Medical Sciences) (approval number: ky-2018-2-3). Heparinized whole blood (150 μL) was incubated with FITC-anti-CD19 (Beckman Coulter, Cat. B36354, Indianapolis, IN, USA), PC5-anti-CD14 (Beckman Coulter, Cat. A07765, Indianapolis, IN, USA), PC7-anti-CD3 (Beckman Coulter, Cat. 737657, Indianapolis, IN, USA), or APC-conjugated mouse IgG1 isotype control (BD Biosciences, Cat. 30442, Bedford, MA, USA). Normal B and T cells are CD19+ and CD3+ cells, respectively, in the lymphocyte gate. Monocytes are CD14+ cells in the monocyte gate.

**Pharmacokinetic (PK) analysis**

Six specific-pathogen-free-grade BALB/c mice (3 males and 3 females; Charles River, Cat. 401), aged 6–8 weeks and weighing 20–26 g, were randomly divided into two groups. CrossMab was injected into the tail vein at 2 mg/kg. The day of injection was day 0. A 500 μL sample of whole blood was collected from the orbit at the following time points: 10 min, 3 h, 24 h, 48 h, 72 h, 96 h, 7 d, 9 d, 11 d, 14 d, 21 d, and 28 d. The blood CrossMab concentration was quantified by an enzyme-linked immunosorbent assay. The PK parameters were determined with a non-compartmental analysis model using WinNonlin. The area under the curve (AUC) was calculated using the following formula:

\[
AUC = \sum_{i=0}^{n-1} \left( x_i - x_{i+1} \right) \left( y_{i+1} + y_i \right) / 2
\]

**Animal experiment**

A suspension of 1 × 10^7 Daudi cells in PBS was mixed with Matrigel (Corning, Cat. 35462, Bedford, MA, USA) in a ratio of 1:1 and was inoculated subcutaneously on the backs of CB17-SCID mice (3 males and 3 females, Vital River, Cat. 404, Beijing, China). The day of tumor inoculation was day 0. On day 14, when the average tumor size reached 151.2 mm^3, the mice were randomly divided into two groups (CrossMab and control groups). The mice in the CrossMab group were injected with...
100 μg of CrossMab in 160 μL of PBS through the caudal vein on day 14, day 17, and day 23. The mice in the control group received the same volume of PBS at the same time points. The tumor volumes and mouse body weights were measured twice a week. In addition, the tumor weights were measured at the end of the experiment.

**Statistical analysis**

SPSS 16.0 (IBM, Armonk, NY, USA) was used for statistical analysis. The data were expressed as the mean ± standard deviation. The least significant difference method of one-way analysis of variance was used to compare independent groups. The difference was statistically significant when the p-value was less than 0.05.

**Results**

**Structure and characteristics of CrossMab**

CrossMab was designed according to the DNA fragments for rituximab and hL243γ1 [31] (Figure 1A). Under nonreducing conditions, the molecular weight of intact CrossMab protein was 150–200 kDa (Figure 1B, Band 1). Under reducing conditions with dithiothreitol or 2-mercaptoethanol, CrossMab protein was divided into two heavy chains with molecular masses of ∼50 kDa (Figure 1B, Band 2) and two light chains with molecular masses of ∼25 kDa (Figure 1B, Bands 3 and 4). The difference between reducing and nonreducing conditions is that a reducing agent (such as dithiothreitol or 2-mercaptoethanol) was added or not during sample processing, respectively. The reducing agent can break the disulfide bond. The natural active structure of some proteins is a dimer or trimer. If β-mercaptoethanol was added, it reduced the disulfide bonds between and within the polyprotein structure. In this way, the electrophoretic samples were completely reduced to the monomeric forms, and the samples without the addition of reducing agent remained in the polymeric form.

One important parameter to measure the functions of BsAbs is the binding affinity [32]. The binding affinity can predict the efficacy and systemic toxicity of BsAbs by providing information on how efficient the BsAbs can bind to target cells and avoid normal cells. Here, we chose Raji cells to test the binding affinity of CrossMab. The results showed that CrossMab’s binding to Raji cells was significantly greater than that of rituximab and anti-Her2 mAb (Figure 2). Also, hL243γ1’s binding to Raji cells was significantly greater than that of rituximab and anti-Her2 mAb. However, there was no significant difference between CrossMab's binding affinity and hL243γ1’s binding affinity to Raji cells. Moreover, the binding ability of CrossMab to Raji cells increased with concentration (Figure 2). These data indicate that CrossMab retained the antigen-binding activity of both parental mAbs.

Importantly, CrossMab was shown to competitively reduce the binding of either anti-CD20 or anti-HLA-DR antibody to Raji cells, indicating the stronger binding affinity of CrossMab to Raji cells compared to that of the two mAbs. As shown in Figure 3A, at a concentration of 0.02 μg/mL, all BsAbs were shown to exert similar effects on inhibiting the binding of anti-CD20 to Raji cells. When the drug concentration reached 0.1 μg/mL, the binding rate of anti-CD20 antibody to Raji cells in the presence of CrossMab was evidently reduced. At a drug concentration of 0.5 μg/mL, the binding rates of anti-CD20 antibody in the presence of
rituximab (86.2%) or CrossMab (88.3%) were similar, and both were significantly greater than that in the presence of rituximab + hL243γ1 (61.7%). When the drug concentration reached 2 μg/mL, the binding rate of anti-CD20 antibody was inhibited in the presence of rituximab, rituximab + hL243γ1, or CrossMab, to a saturable level of about 96% (Figure 3A). Similarly, CrossMab disturbed the binding of anti-HLA-DR antibody to Raji cells. When the drug concentration was less than 0.5 μg/mL, CrossMab was found to mildly inhibit the binding of anti-HLA-DR to Raji cells (Figure 3B). Meanwhile, hL243γ1 was found to have a much greater inhibitory effect (74.4%) on the binding rate of anti-HLA-DR to Raji cells than any other drug. At a drug concentration of 2 μg/mL, CrossMab inhibited the binding of anti-HLA-DR to Raji cells to 56.4%, which is much less than those of hL243γ1 and rituximab + hL243γ1 (over 90%) (Figure 3B).

In addition to the binding affinity to targets, PK parameters including the maximum concentration (Cmax), AUC, and half-life are important indicators to evaluate the clinical potential of CrossMab. We measured these PK parameters of CrossMab in a noncompartment model. The plasma concentration of CrossMab reached its peak immediately after the tail vein injection, and then its blood concentration decreased rapidly with time. The day of CrossMab administration was recorded as day 0. At day 11, the CrossMab concentration reached the minimum detection limit (3.125 ng/mL) (Figure 4). The Cmax of CrossMab was 10.6 ± 3.8 μg/mL, and the half-life of CrossMab in mice was 18.3 ± 4.0 h. The AUC, which indicated systemic exposure to CrossMab, was 343.3 ± 59.7 h μg/mL. There was no significant difference in the PK parameters, including Cmax and AUC, between male and female mice (Table 1).

**CrossMab induces cytotoxicity and cell death in Raji cells**

To test the efficacy of CrossMab in vitro, we performed CDC, ADCC, apoptosis, and lysosome-related assays in Raji cells. Treatment with rituximab, hL243γ1, or rituximab + hL243γ1 resulted in a similar CDC activity. Our results showed that CrossMab killed Raji cells through the induction of CDC in a
concentration-dependent manner. At a concentration of 4 μg/mL, the CDC activity of CrossMab was greater than those of the other antibodies or control group. The CDC activities were similar among rituximab, hL243γ1, and rituximab+hL243γ1 (Figure 5A). Similarly, CrossMab induced cytotoxicity in Raji cells though ADCC in a concentration-dependent manner. Of note, the ADCC activity of CrossMab was indistinguishable from those of rituximab and hL243γ1 (Figure 5B). Furthermore, we evaluated whether CrossMab could induce overall cell death. Our results showed that CrossMab induced the apoptotic rate of Raji cells up to 38.9 %, similar to that of hL243γ1 (38.45 %) and slightly greater than those of rituximab and rituximab + hL243γ1 (34.05 %) (Figure 6A and B).

Apoptotic stimuli could trigger changes in the lysosomal membrane permeability, and the integrity of the lysosomal membrane is another crucial marker for cell fate [33]. As shown in Figure 7, comparisons of the AO green fluorescence intensity indicated that the lysosomal permeabilities among the different treatment groups were as follows: hL243γ1 > CrossMab > rituximab + hL243γ1 > rituximab. These results demonstrated that CrossMab treatment might cause cell death by changing the lysosomal permeability. In line with the above results, CrossMab induced a significantly higher lysosome-mediated apoptosis rate (22.8 %) than those of the rituximab + hL243γ1 (mixed mAbs) group (17.5 %), hL243γ1 group (17.1 %), rituximab group (4.85 %), and anti-HER2 mAb group (2.71 %) (Figure 6C and D). These findings further confirmed the potential clinical advantage of CrossMab for the treatment of NHL.

Cathepsin B, one of the most abundant proteases in lysosomes, has been reported to promote cell apoptosis [34]. Compared to hL243γ1 or rituximab + hL243γ1, CrossMab induced a lower level of cathepsin B release. In addition, compared to rituximab, anti-HER2, or control (blank), CrossMab induced a higher level of cathepsin B release (Figure 8). These results reveal that CrossMab treatment induced changes on cathepsin B release, likely in a differential manner on CD20 and HLA-DR when compared to the other antibodies.

Overall, CrossMab was found to possess a clear efficacy to kill Raji cells in vitro, via diverse mechanisms including the induction of CDC, ADCC, and lysosomal permeability. Thus, there is strong evidence to initiate clinical trials with CrossMab against NHL.

**CrossMab exhibits differential toxicity on normal PBLs**

Normal tissue toxicity has been a limiting factor for the clinical application of BsAbs. Therefore, the determination of the toxicity of CrossMab on normal PBLs is critical to evaluate its clinical applicability. To test its toxicity on normal PBLs, PBLs were first incubated with CrossMab or other drugs, including rituximab and hL243γ1, for 48 h. Rituximab and hL243γ1 each induced the depletion of more than 50 % of B cells, while CrossMab induced the depletion of approximately 40 % of B cells (Figure 9). Importantly, compared to the PBS group, rituximab, hL243γ1, or CrossMab did not induce significant changes in the numbers of T cells or monocytes (Figure 9). These findings suggest that the toxicity of CrossMab on PBLs was comparable to that of rituximab or hL243γ1, further providing a rationale for the clinical application of CrossMab.

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**Table 1:** The pharmacokinetic parameters of CrossMab in BALB/c mice.

<table>
<thead>
<tr>
<th>Sex</th>
<th>T_{1/2}, h</th>
<th>T_{max}, h</th>
<th>C_{max}, μg/mL</th>
<th>AUC_{last} (h·μg/mL)</th>
<th>V_{z,obs}, mL/kg</th>
<th>Cl_{obs}, mL/h/kg</th>
<th>MRT_{last}, h</th>
</tr>
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<tr>
<td>Female</td>
<td>20.0 ± 3.7</td>
<td>0.167</td>
<td>11.0 ± 3.5</td>
<td>364.5 ± 63.0</td>
<td>0.16 ± 0.06</td>
<td>0.006 ± 0.01</td>
<td>39.8 ± 2.3</td>
</tr>
<tr>
<td>Male</td>
<td>16.5 ± 4.2</td>
<td>0.167</td>
<td>10.2 ± 4.9</td>
<td>322.1 ± 59.9</td>
<td>0.15 ± 0.05</td>
<td>0.006 ± 0.01</td>
<td>37.9 ± 4.4</td>
</tr>
<tr>
<td>Total</td>
<td>18.3 ± 4.0</td>
<td>0.167</td>
<td>10.6 ± 3.8</td>
<td>343.3 ± 59.7</td>
<td>0.16 ± 0.05</td>
<td>0.006 ± 0.01</td>
<td>38.9 ± 3.3</td>
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Figure 5: Induction of complement-dependent cytotoxicity (CDC) and antibody-dependent cell-mediated cytotoxicity (ADCC) by CD20/HLA-DR CrossMab\textsuperscript{CDR-CL} on Raji cells. (A) The potential CDC of CD20/HLA-DR CrossMab\textsuperscript{CDR-CL} on Raji cells. (B) The potential ADCC of CD20/HLA-DR CrossMab\textsuperscript{CDR-CL} on Raji cells. p>0.05, (n=3).

Figure 6: Induction of apoptosis and lysosome-mediated cell death by CD20/HLA-DR CrossMab\textsuperscript{CDR-CL}. (A) Cell apoptosis was induced by different antibodies; the antibody concentration was 10 μg/mL. (B) Quantitative analyses on CD20/HLA-DR CrossMab\textsuperscript{CDR-CL}'s induction of apoptosis on Raji cells. (C) Flow cytometry was used to measure lysosome-mediated cell death induced by different types of antibodies; the antibody concentration was 10 μg/mL. (D) Quantitative analyses of the death rate induced by different antibodies. *p<0.05 (n=3).
CrossMab exerts antitumor activity in a CB17-SCID xenograft model

The antitumor efficacy of CrossMab was evaluated in a CB17-SCID xenograft model with subcutaneous inoculation of Daudi cells. CB17-SCID mice are immunodecient mice. Tumors inoculated into these mice survive, and their autoimmune system is prevented from killing the inoculated tumors. The tumor weights of the mice in the CrossMab group were significantly less than those in the control group (Figure 10A). Moreover, compared to the control group, CrossMab treatment slowed down the tumor growth (Figure 10B). There was no significant difference in the body weights of the mice (Figure 10C).

Discussion

NHL is a malignant disorder caused by dysregulation of lymphocyte cells. Importantly, antibody-based immunotherapy has been proven to be effective for patients with NHL [8]. Rituximab, the first mAb approved to treat NHL, binds to CD20 on B cells and kills B cell lymphoma [9]. Meta-analyses have demonstrated that rituximab alone or combined with chemotherapy signiﬁcantly improves the clinical outcomes of patients with lymphomas, with acceptable levels of toxicity [35–37]. In addition to CD20, other antibodies such as CD19, CD22, and HLA-DR have been used to treat NHL [38]. For instance, anti-CD19 CAR-T cells have been shown to exhibit therapeutic potential to treat relapsed/...
refractory aggressive B-cell lymphomas [39]. Despite the clinical advantages of mAb treatments, certain groups of patients have experienced tumor recurrence [11]. The monospecific configuration of mAbs restricts their overall therapeutic efficacy [40]. Therefore, the introduction of a bi- or multi-specific antibody is necessary to improve NHL treatment.

CD20 and HLA-DR have been demonstrated to be simultaneously expressed on B-cell lymphoma, with close physical and functional interactions with each other [13–15]. In addition, the combination of a CD20-specific mAb and an HLA-DR-specific mAb has been shown to enhance lysosomemediated death in Raji and SU-DHL4 cells, via a mechanism neither related to caspases nor Bcl-2 [41]. An additional study has revealed that the combination treatment leads to partial and selective release of cathepsins, such as cathepsins B and D, which initiate a cascade of cell signaling events leading to cell death [42]. In cells with resistance to CD20 antibody, the combined treatment with anti-CD20 and anti-HLA-DR mAbs contributed to a 27-fold increase in cell death in vitro compared to single treatment with rituximab or chLym-1, an anti-HLA-DR Mab [17]. Altogether, these results indicate the enhanced therapeutic potential of cotargeting both CD20 and HLA-DR in NHL. Intriguingly, one animal experiment has demonstrated that single-antibody conjugates improved survival compared to the combined mAb treatment [43]. Of note, the safety and efficacy of the combination of different mAbs have not been fully evaluated. Therefore, the design and testing of BsAbs for their efficacy and toxicity in NHL treatment are urgently needed.

Figure 8: Cathepsin B release induced by different antibodies. The blank was employed as a negative control, while H2O2 acted as a positive control (n=3).
Our results revealed that CrossMab possessed antitumor activities through the induction of ADCC, CDC, and lysosome membrane permeability. CrossMab changed the lysosomal membrane permeability, albeit without a significant influence on the release of cathepsin B. Furthermore, among a variety of PBLs, CrossMab was found to effectively deplete B cells to an extent comparable to that of rituximab or hL243y1. In addition, CrossMab exerted mild toxicity on normal T cells and monocytes. Notably, mouse models confirmed the antitumor efficacy of CrossMab, indicating its promising potential application in NHL treatment.

Of note, there are some limitations to our study. First, the study period was short, which restricted the evaluation of long-term survival benefits and toxicity of CrossMab. Second, the comparisons of efficacy and safety were not carried out among CrossMab and several other bispecific antibodies that target CD20 and HLA-DR, such as dual variable domain-immunoglobulin (DVD-Ig) [15, 31]. Further studies should be conducted to compare CrossMab with other BsAbs to advance the related technology. Third, we did not study the mechanisms by which CrossMab could be used to target NHL. Further experiments such as SDS-PAGE and western blot assays will be performed to investigate the molecular mechanisms. Finally, we did not analyze multiple animal models. Preclinical studies in other animal models might be valuable to evaluate the stability, efficacy, and safety of CrossMab to further its clinical application.

Taken together, CrossMab exhibited enhanced antitumor activity compared to mAbs against CD20 or HLA-DR. Moreover, CrossMab showed mild toxicity on PBLs, including T cells and monocytes. Thus, our findings indicate that CrossMab has potential for the treatment of NHL.

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![Figure 9: Toxicity of CD20/HLA-DR CrossMabCH1−CL on peripheral blood lymphocytes from healthy individuals (p>0.05, n=3).](image)

![Figure 10: Antitumor activity of CD20/HLA-DR CrossMabCH1−CL in Daudi xenograft models. (A) The tumor volumes at the end of the experiment. (B) The tumor volumes were measured over time (*p<0.05). (C) The body weights of the mice were measured over time (p>0.05).](image)
curation and Formal analysis; YY: Data curation and Methodology; YF: Conceptualization; Writing – review & editing. All authors reviewed the results and approved the final version of the manuscript.

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Ethics approval: This study was approved by the Affiliated Hospital Institutional Ethics Committee of the Military Academy of Medical Sciences (The former name of the Fifth Medical Center, Chinese PLA General Hospital) (approval number: ky-2018-2-3) and was conducted in accordance with the standards of the Declaration of Helsinki.

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

References


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